

CHARACTERIZATION OF PAQR PROTEINS USING *Saccharomyces cerevisiae*:  
THE HUMAN MEMBRANE PROGESTIN RECEPTORS

By

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To my family. Thank you for your support.

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## LIST OF ABBREVIATIONS

17,21-diOH PG:	17,21-dihydroxyprogesterone
DTR	Dual-topology reporter (3xHA-Suc2-His4C)
<i>FET3</i>	gene encoding Fet3p
Fet3p	Ferrous transport protein
GPCR	G-protein coupled receptor
HlyIII	Hemolysin III
17-OH PG:	17 $\alpha$ -hydroxyprogesterone
21-OH PG:	21-hydroxyprogesterone
<i>IZH</i>	Implicated in Zinc Homeostasis gene
Izhp	Implicated in Zinc Homeostasis protein
<i>lacZ</i>	gene encoding the $\beta$ -galactosidase enzyme
mPR	membrane progestin receptor
PAQR	Progestin and adipoQ Receptor
PG	Progesterone
TS	Testosterone
7TM	Seven transmembrane

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Some studies indicate that a few members of the Progesterone and AdipoQ Receptor (PAQR) family (PAQR5, PAQR7, and PAQR8) act as membrane progesterone receptors (mPRs) and signal in a G-protein dependent manner, but these studies have been disputed. To clarify the role of human PAQRs in progesterone signaling, the yeast *Saccharomyces cerevisiae* was used as a simple model organism.

When PAQRs are expressed at low levels, repression of the yeast promoter *FET3* is observed in a *FET3-lacZ* promoter-reporter assay only when certain progesterones are present. To explore the molecular mechanism by which the *FET3* promoter is affected, a genetic mutational analysis was used. The effect of PAQRs on *FET3-lacZ* is not dependent on the presence of human or yeast G-proteins, but all human PAQRs and their yeast homologues share a common intracellular signaling mechanism in yeast involving *tpk2*, the yeast homologue of human PKA. In addition, evidence is demonstrated to support that human PAQR6 and PAQR9 act as progesterone receptors, which confirms functional predictions based on bioinformatic sequence analyses. The localization and topology of some PAQR proteins is also disputed, so studies of these characteristics were also pursued in yeast. Many problems were encountered and few conclusions could be drawn.

Initial characterizations of PAQR homologues from bacteria are also described. Although attempts were made to express these proteins in *E. coli*, there was little success. However, the bacterial proteins were also expressed in *S. cerevisiae* for the *FET3-lacZ* assay and it was demonstrated that some of the proteins tested can cause repression of *FET3*.

## CHAPTER 1 INTRODUCTION

### **The Progestin and AdipoQ Receptor (PAQR) Family**

The human progestin and adipoQ receptor (PAQR) family of proteins consists of eleven proteins predicted to have least seven transmembrane (7TM) domains (Tang et al., 2005). Homologues of PAQRs can be found in simple and complex eukaryotes as well as in prokaryotes (Lyons et al., 2004; Fernandes et al., 2005; Thomas et al., 2007). In simple eukaryotes, such as the yeast *Saccharomyces cerevisiae*, the PAQRs are involved in metal and lipid metabolism (Lyons et al., 2004; Karpichev et al., 2002). In more complex eukaryotes, such as humans, the PAQRs act as receptors for adiponectin, which stimulates glucose uptake and fatty acid oxidation (Kadowaki and Yamauchi, 2005). The human PAQRs may also act as receptors for steroid hormones (Thomas et al., 2007). In prokaryotes, such as *Bacillus cereus*, *B. anthracis*, *Bacteroides fragilis*, and *Vibrio vulnificus*, the PAQR homologues, called hemolysin III proteins, are putatively involved in causing lysis of red blood cells (Baida and Kuzmin, 1995; Klichko et al., 2003; Robertson et al., 2006; Chen et al., 2004).

Besides understanding the normal physiological roles of the PAQRs, it is also important to understand their roles in pathophysiological conditions. Thus far, the human PAQRs are known to be important in diseases such as diabetes and obesity (Kadowaki and Yamauchi, 2005) or to have potential importance in breast cancer development (Dressing and Thomas, 2007). In addition, the human PAQRs may be activated by endocrine-disrupting chemicals (EDCs), as the homologues in fish are (Tokumoto et al., 2007). This is especially interesting because EDCs are known to cause many health problems, including developmental abnormalities, reproductive problems and cancers (Newbold et al., 2006).

## Structural Features of the PAQRs

All PAQRs contain a motif, named UPF0073, which is unique to the PAQR family (Tang et al., 2005). Each PAQR has the following motifs: ExxNxxH just before transmembrane domain 1 (TM1), SxxxH at the end of TM2, and HxxxH in TM7 (Figure 1-1). Other than these conserved motifs, there is little homology between the members, but further sub-classifications can be made based on sequence homologies such that the PAQRs can be divided into three classes (Fernandes et al., 2005): Class I (PAQRs 1-4), Class II (PAQRs 5-9), and Class III (PAQRs 10 and 11) (Figure 1-2).

The PAQRs are expected to have similar orientations in the membrane. Evidence suggests that the Class I and Class III PAQRs have seven transmembrane (7TM) domains with an intracellular N-terminus and extracellular C-terminus (Yamauchi et al., 2003; Kim et al., 2006; Daley et al., 2005). For the human Class I PAQRs, immunofluorescence was used to determine that the N-terminus was inside, while the C-terminus was outside the cell (Yamauchi et al., 2003). Based on experiments using the yeast dual-topology reporter (DTR) Suc2-His4C (which is described in more detail in Chapter 3) and sequence predictions, it was determined that the yeast Class I proteins (IZHs) had an extracellular C-terminus (Kim et al., 2006). For the Class III proteins, C-terminal tagging with the alkaline phosphatase and green fluorescent protein was used to determine that the C-terminus of the *E. coli* homologue (called YqfA) was located in the periplasmic space rather than in the cytoplasm (Daley et al., 2005). This putative topology results in the clustering of the conserved motifs on the intracellular side of the membrane (Figure 1-3A), suggesting that they may be important for a conserved signaling mechanism (Tang et al., 2005).

In contrast, predictions suggest that the Class II PAQRs have either 7TM domains like the other PAQRs (Zhu et al., 2003a; Thomas et al., 2007) or eight transmembrane domains (Lyons et

al. 2004; Fernandes et al., 2005) (Figure 1-3C). Some topological analyses even suggest that the Class II PAQRs have an orientation in the membrane that is opposite to that of the Class I and Class III PAQRs (Thomas et al., 2007). According to immunocytochemical analyses of cells expressing the human PAQR7, N-terminally directed antibodies were able to bind without permeabilization of cells, but C-terminally directed antibodies were not, suggesting that PAQR7 has an extracellular N-terminus and an intracellular C-terminus (Thomas et al., 2007). With this topology, the Class II PAQRs would not have all of the conserved PAQR family motifs on the same side of the cell as the Class I and Class III PAQRs (Figure 1-3B), which could mean that the Class II PAQRs signal via a different mechanism. In fact, some experimental evidence suggests that the Class II PAQRs function as G-protein coupled receptors (GPCRs), which have a topology with the N-terminus outside the cell and the C-terminus inside the cell (Thomas et al., 2007; Karteris et al., 2006).

### **History of the PAQRs: From Prokaryotes to Eukaryotes**

Findings from studies conducted on members of the different classes of PAQRs reveal that these proteins vary widely in function. The PAQR family has a common eubacterial origin, as homologues to the Class III proteins have been characterized as eubacterial hemolysin III proteins (Tang et al., 2005).

#### **PAQRs in Prokaryotes**

The first PAQR to be studied was the *Bacillus cereus* hemolysin III (HlyIII) protein (Baida and Kuzmin, 1995). Homologues of HlyIII from *B. cereus* (Baida and Kuzmin, 1996), *Vibrio vulnificus* (Chen et al., 2004), *Bacillus anthracis* (Klichko et al., 2003), and *Bacteroides fragilis* (Robertson et al., 2006) have all been purported to have hemolytic activity towards human red blood cells (RBC) by forming pores in RBC membranes. However, the hemolytic activity of HlyIIIs has only been characterized with whole bacterial cells or cell extracts, rather than

purified HlyIIIs (Baida and Kuzmin, 1996; Chen et al., 2004; Klichko et al., 2003; Robertson et al., 2006). The possibility remains that the 7TM HlyIIIs are not hemolytic themselves, but instead act as membrane proteins that initiate a signaling cascade to activate or increase expression of a different protein that has hemolytic activity. More on these proteins will be discussed in Chapter 4.

### **PAQRs in Eukaryotes**

Members of the eukaryotic PAQRs were first described when a study identified the gene for PAQR11 (also named monocyte-to-macrophage differentiation factor 1, or MMD1) in mRNA differential display studies (Rehli et al., 1995). The studies were aimed at identifying genes with enhanced expression in mature macrophages versus monocytes (Rehli et al., 1995). Much later, PAQR10, the closest human homologue of PAQR11, (also called macrophage/microglia activation factor) was identified in differential display studies of genes involved in microglia activation after brain trauma (Brauer et al., 2004). Both studies predicted that these proteins have seven transmembrane domains (Rehli et al., 1995; Brauer et al., 2004), but this has not been confirmed. The human Class III proteins have been proposed to act as ion channels or receptors (Rehli et al., 1995), although ligands for these proteins have not been identified.

In 2002, the first eukaryotic Class I PAQR was described in studies of a *Saccharomyces cerevisiae* protein called YOL002c (Cherry et al., 1998; Karpichev et al., 2002). YOL002c was originally proposed to have a role in lipid and phosphate metabolism (Karpichev et al., 2002). Later, YOL002c as well as three closely related homologues in *S. cerevisiae*, were shown to be involved in metal metabolism (Lyons et al., 2004). These proteins were named Izh1p, Izh2p, Izh3p, and Izh4p (Izh, or *Implicated in Zinc Homeostasis*). In addition, Izh2p acts as a receptor

for the plant defensin Osmotin, which is structurally similar to human adiponectin (Narasimhan et al., 2005).

The human Class I proteins (PAQRs 1-4) were first described in studies that identified them as receptors for adiponectin (also known as adipoQ) (Yamauchi et al., 2003). These proteins, called AdipoR1 (PAQR1) and AdipoR2 (PAQR2), act as receptors for adiponectin, a protein which induces fatty acid oxidation and glucose uptake (Yamauchi et al., 2003). PAQR1 and PAQR2 are plasma membrane localized, they are predicted to have 7TM domains, they have a topology with an intracellular N-terminus and extracellular C-terminus (Yamauchi et al., 2003), and they have been shown to signal via the scaffolding protein APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain and leucine zipper motif) (Mao et al., 2006). PAQR3 was recently reported to be a golgi-localized protein that sequesters the kinase Raf-1 to this organelle, resulting in extracellular signal regulated kinase (ERK) pathway inhibition (Feng et al., 2007). PAQR3 was thus renamed Raf Kinase Trapping to Golgi (RKTG) (Feng et al., 2007). PAQR4 has not been characterized yet.

Members of the Class II PAQRs (PAQRs 5-9) were first described when studies identified genes that encode membrane proteins that putatively mediate the nongenomic effects of progestins (Zhu et al., 2003a; Zhu et al., 2003b). Originally identified in fish, the Class II PAQRs were shown to play a role in oocyte meiotic maturation induced by progestins (Zhu et al., 2003a; Zhu et al., 2003b). Because they have been proposed to act as membrane progestin receptors (mPRs), PAQR5, PAQR7, and PAQR8 are also known as mPR $\gamma$ , mPR $\alpha$ , and mPR $\beta$  (Zhu et al., 2003a; Zhu et al., 2003b).

Both fish and human Class II PAQRs have been shown to cause decreases in cAMP levels that could be inhibited by the pertussis toxin, which ADP-ribosylates and inactivates G-proteins

(Zhu et al., 2003a; Thomas et al., 2007). This suggests that the Class II PAQRs function as G-protein coupled receptors (GPCRs) to affect cAMP levels (Thomas et al., 2007; Karteris et al., 2006). Experiments also demonstrated that PAQR7 and inhibitory G-proteins co-immunoprecipitate, suggesting that these proteins directly interact (Thomas et al., 2007). In contrast, results from one study show that human Class II PAQRs localize intracellularly to the endoplasmic reticulum, do not bind progesterone, and do not activate GPCR-related signaling cascades (Krietsch et al., 2006). Other studies have shown that PAQR7 localizes to an “intracellular tubuloreticular network” (Fernandes et al., 2005) and that PAQR8 is associated with lysosomes (Suzuki et al., 2001).

Most of the studies on the Class II PAQRs have focused on PAQR5, PAQR7, and/or PAQR8. Further studies of the Class II PAQRs, including PAQR6 and PAQR9, are needed to clarify the functions of this group of proteins.

### ***Saccharomyces cerevisiae* as a Model Organism for PAQR Characterizations**

The yeast *S. cerevisiae* is a unicellular eukaryotic organism that is useful as a simple model system for the study of biomolecules and biological processes of more complex organisms. Many of the genes encoded by the yeast genome have homologues in humans and many of the mechanisms involved in different cell processes, such as signal transduction, are conserved (Sturgeon et al., 2006).

*S. cerevisiae* can be used for functional expression of eukaryotic proteins, including those from humans. *S. cerevisiae* serves as a good model organism to study human proteins because, unlike mammalian cells, yeast cells are easily to handle in molecular biological procedures and cellular components needed for human protein function are often conserved in yeast (Mentesana et al., 2002). In addition, simple phenotypic screens of mutant receptors can be easily performed to identify amino acid residues involved in the signaling mechanisms (Ladds et al., 2005).

Thus far, yeast has proven to be an effective tool for the simplified study of many human proteins. For example, promoter-reporter assays have been developed for the study of G-protein coupled receptors (GPCRs) (Mentesana et al., 2002) and nuclear steroids receptors (McEwan, 2001). The successful use of yeast for characterizations of GPCRs and steroid receptors makes this system particularly attractive for the characterization of the Class II PAQRs, which are debated to be GPCRs and are putative receptors for progestin steroids (Krietch et al., 2006; Thomas et al., 2007).

For the characterization of GPCRs, the signal transduction pathway of the yeast  $\alpha$ -factor mating pheromone receptor (Ste2p) is often used (Mentesana et al., 2002). Ste2 is a GPCR which couples to a heterotrimeric G-protein consisting of Gpa1p ( $G\alpha$ ), Ste4p ( $G\beta$ ), and Ste18p ( $G\gamma$ ) (Mentesana et al., 2002). Upon ligand activation of Ste2,  $G\alpha$  is activated, the  $G\beta\gamma$  dimer is released and activates a mitogen activated protein kinase (MAPK) cascade to activate the transcription factor Ste12p and ultimately lead to increased transcription of the *FUS1* gene (Figure 1-4) (Mentesana et al., 2002). When the promoter of *FUS1* is fused to a reporter gene, such as *lacZ*, activation of the  $\alpha$ -pheromone mating pathway can be detected by measuring increases in transcription levels of *FUS1* by a simple colorimetric  $\beta$ -galactosidase assay (Mentesana et al., 2002).

Because the yeast G-protein alpha subunit Gpa1p is highly homologous to the mammalian inhibitory G-protein  $G_{i\alpha}$  (Dowell and Brown, 2002), it is able to couple to many human GPCRs (Ladds et al., 2005). Successful coupling of Gpa1p to human GPCRs allows for the characterization of various human GPCRs with a simple colorimetric *FUS1-lacZ* promoter-reporter assay. Importantly, this simple assay has been used to screen for novel synthetic

agonists and antagonists (Pausch, 1997) and for simplified structure-function studies (Beukers and IJzerman, 2005) of GPCRs.

For the characterization of nuclear steroid receptors (nSRs), yeast is an especially attractive organism to use because yeast cells do not have endogenous steroid receptors that could interfere with analyses of individual heterologous receptors (McEwan, 2001). A promoter with a steroid response element (SRE), which is a DNA sequence recognized by a nSR, in combination with a reporter gene, such as *lacZ*, is used (Figure 1-5) (McEwan, 2001). With this system, the expressed nuclear receptor binds directly to the SRE to activate transcription, the amount of which can be measured in a colorimetric assay (McEwan, 2001). Receptors for steroids such as progesterone, estrogen and glucocorticoids have all been characterized with promoter-reporter systems in yeast and these types of studies have been useful for structure-function studies as well as identifying signaling pathway components (McEwan, 2001).

In addition to human GPCRs and nuclear receptors, other PAQRs have been successfully characterized with promoter-reporter assays in yeast as well (Figure 1-6) (Kupchak et al., 2007). Signal transduction pathways activated by the Class I PAQRs from yeast (IZH proteins) and humans (PAQR1 and PAQR2) have been characterized with the *FET3-lacZ* promoter-reporter. Fet3p is involved in high-affinity iron uptake (Askwith et al., 1994). Typically, under low iron conditions, *FET3* transcription is upregulated by Aft1p (Yamaguchi-Iwai et al., 1995); however, when the yeast or human Class I PAQRs are overexpressed, *FET3* transcription is repressed in a manner which is not dependent on Aft1p (Kupchak et al., 2007).

The mechanism by which yeast or human Class I PAQRs repress *FET3-lacZ* was explored and it was found that this effect is dependent on the presence of cAMP-dependent kinase (called protein kinase A, PKA) and AMP-dependent protein kinase (AMPK) (Kupchak et al., 2007).

Interestingly, the involvement of AMPK in PAQR1 and PAQR2 signaling has previously been reported (Yamauchi et al., 2003). These findings suggest that at least all Class I PAQRs have an intracellular signaling mechanism that activates signal transduction pathways likely to be conserved from yeast to humans (Kupchak et al., 2007).

### **Summation**

Thus far, the Class II PAQRs have been proposed to act as GPCRs and to act as receptors for progestin steroids (Thomas et al., 2007); however, both of these ideas have been disputed (Tang et al., 2005; Krietsch et al., 2006). Because yeast has been successfully used to study many human proteins, including GPCRs, nuclear steroid receptors, and some PAQRs, we have chosen to use this organism to characterize the human Class II PAQRs. Our results confirm that the human Class II PAQRs mediate cell signaling in response to progestin steroids.

Interestingly, we show that the cell signaling in yeast is not dependent on the presence of human or yeast G-proteins, but is dependent on PKA similarly to the Class I PAQRs. We also attempted to localize these proteins in yeast and to determine their topology in the cell membrane. In addition, we show that overexpression of Class III PAQRs from bacteria causes cell signaling in yeast.

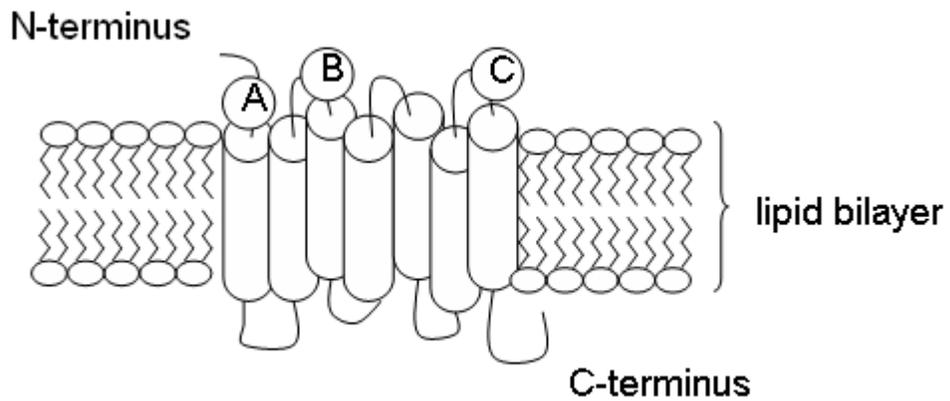


Figure 1-1. The conserved motifs of the PAQR family of proteins. The location of the motifs are indicated by letters. Motif A (ExxNxxH) is located just before TM1, motif B (SxxxH) is located at the end of TM2, and motif C (HxxxH) is located just before TM7.

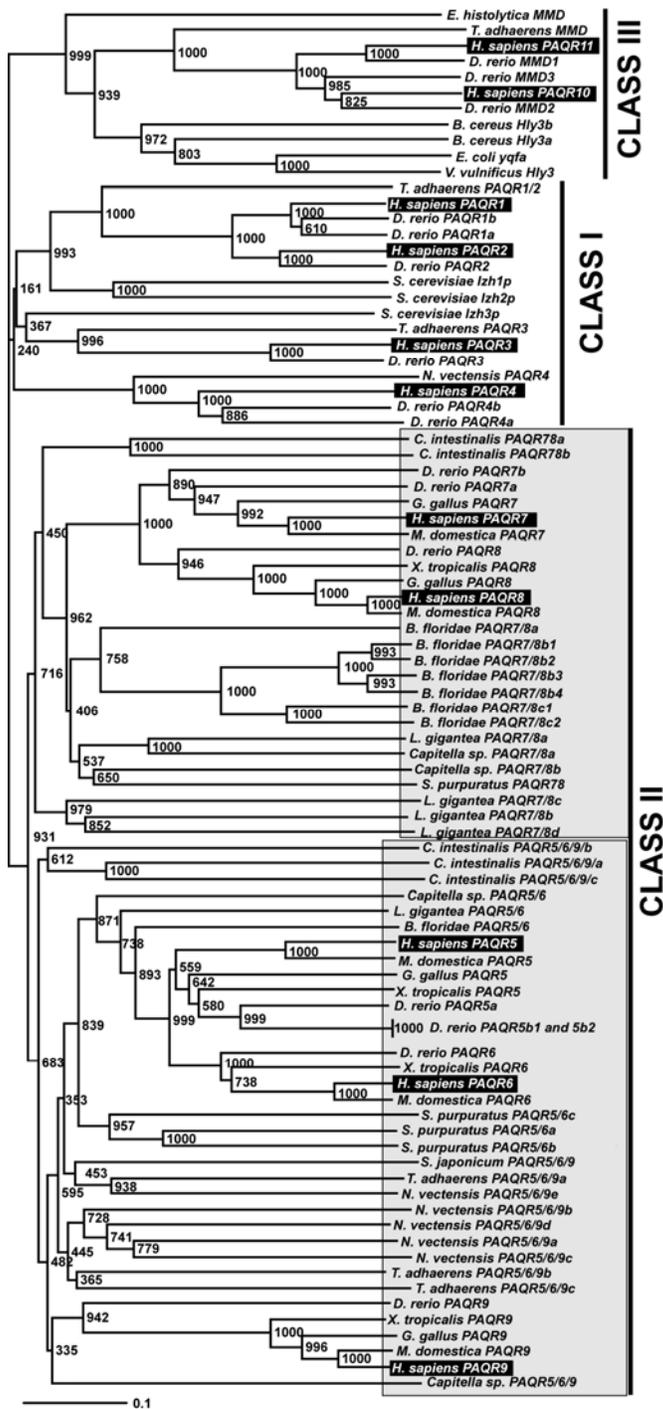


Figure 1-2. Phylogenetic tree of PAQR proteins. The three classes of PAQRs are shown: Class I (PAQRs 1-4), Class II (PAQRs 5-9), and Class III (PAQRs 10-11). The tree is rooted with the distantly related Class III PAQRs. The branch lengths represent the distance between sequences with 0.1 substitutions per site according to the scale bar. Numbers at the nodes refer to the probability that a particular grouping is made per 1000 trees drawn. This figure was generated by Tom Lyons with the ClustalX program.

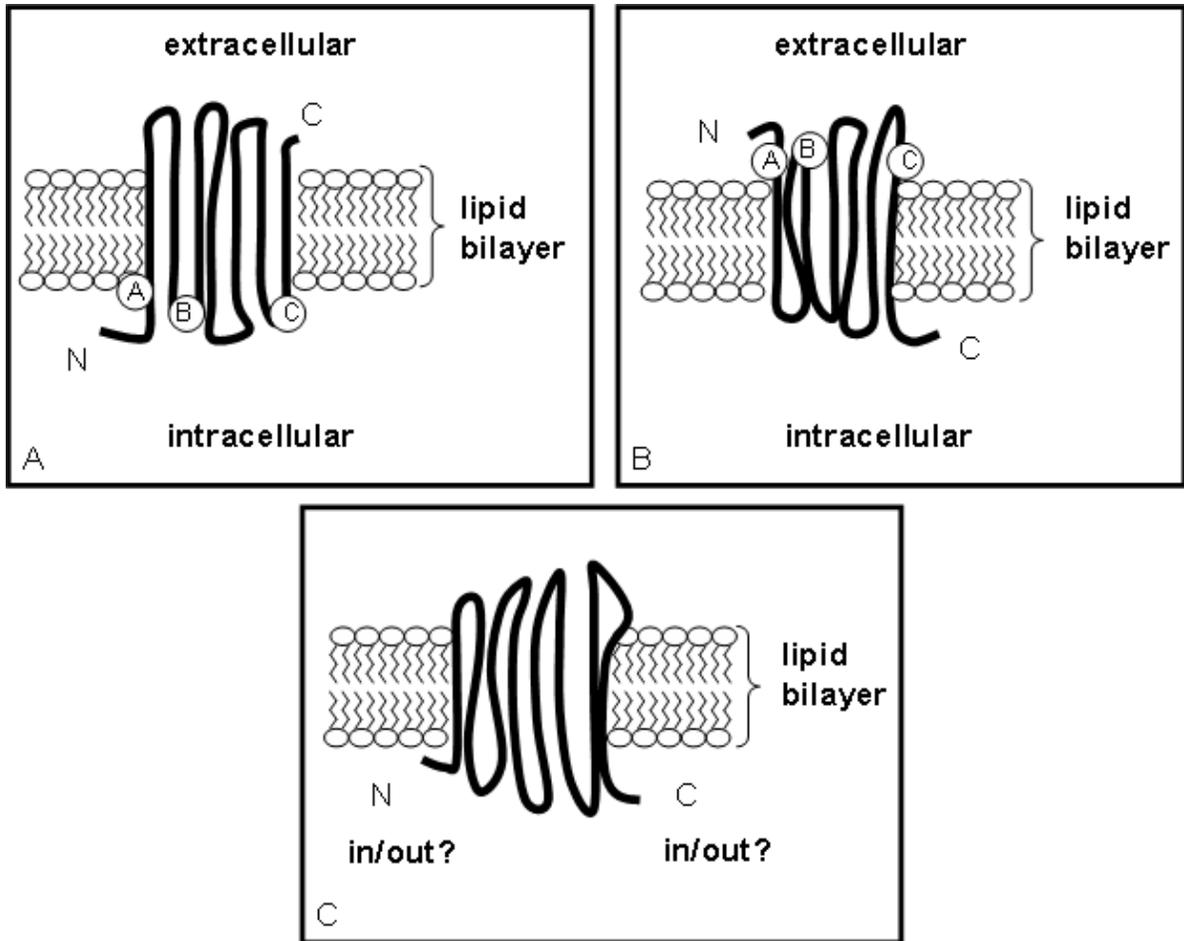


Figure 1-3. Topology models for the PAQR proteins. In panel A, the Class I and Class III PAQRs have the N-terminus located inside the cell, while the C-terminus is located outside the cell. This topology would result in the presence of the conserved motifs (represented by the encircled letters A, B, or C) on the intracellular side of the membrane. Motif A is ExxNxxH, motif B is SxxxH, and motif C is HxxxH. In panel B, the Class II PAQRs have been suggested to have a topology similar to the GPCRs, with an extracellular N-terminus and an intracellular C-terminus. While all three conserved motifs are depicted here as being on the outside surface, Zhu et al. (2003a) have proposed an alternative topology in which the first two conserved motifs would be outside and the last one would be inside. In panel C, the Class II PAQRs have also been suggested to have eight transmembrane domains.

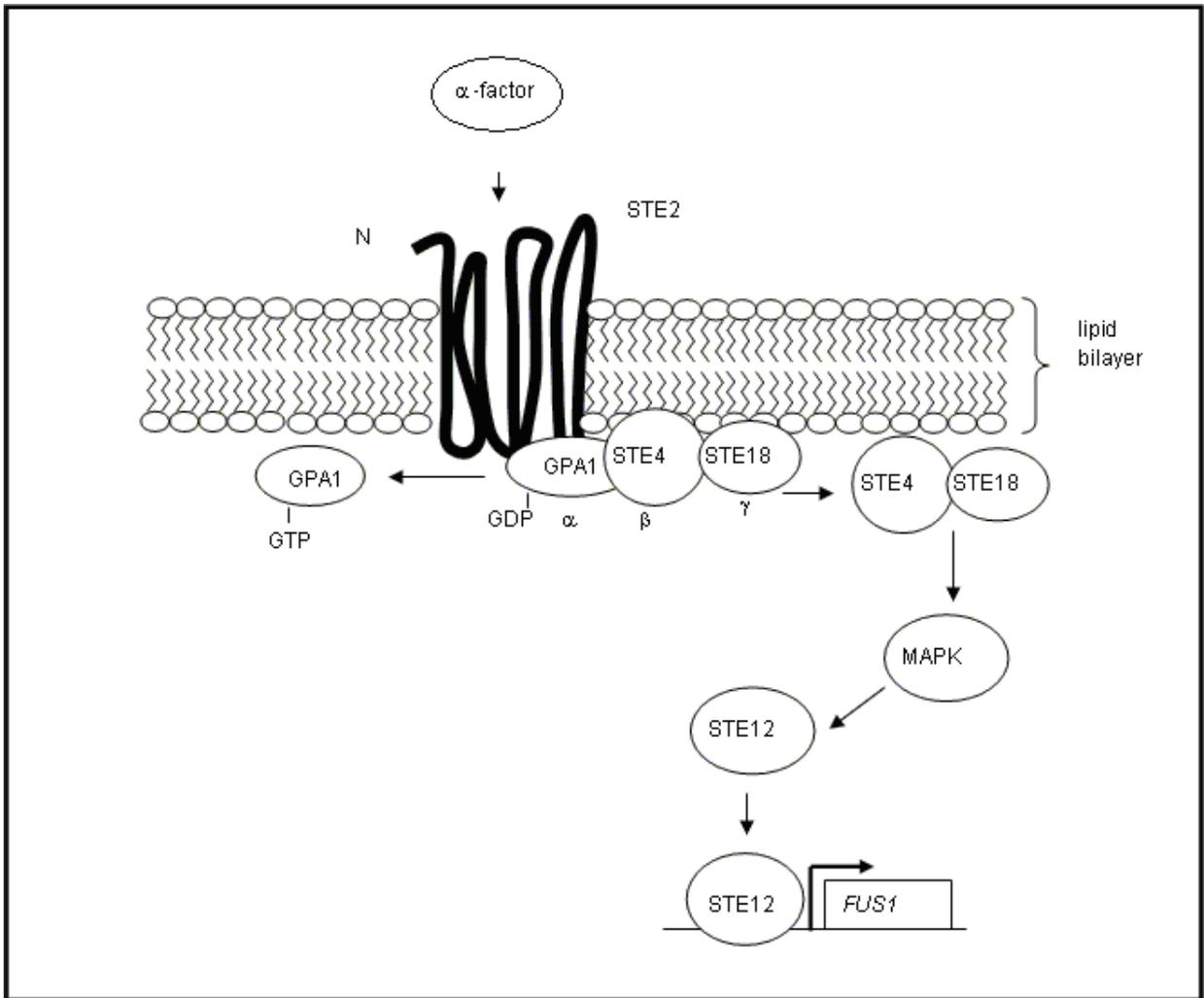


Figure 1-4. The yeast pheromone signal transduction pathway. Upon activation of Ste2p by the pheromone  $\alpha$ -factor,  $G\alpha$  is activated, the  $G\beta\gamma$  dimer is released and activates a mitogen activated protein kinase (MAPK) cascade that leads to activation of the transcription factor Ste12p and ultimately increased transcription of the *FUS1* gene. [This figure was modified from Mentasana PE, Dosil M and Konopka JB (2002) Functional assays for mammalian G-protein-coupled receptors in yeast. *Methods Enzymol* **344**:92-111 with permission from Elsevier (Figure 2, page 96).]

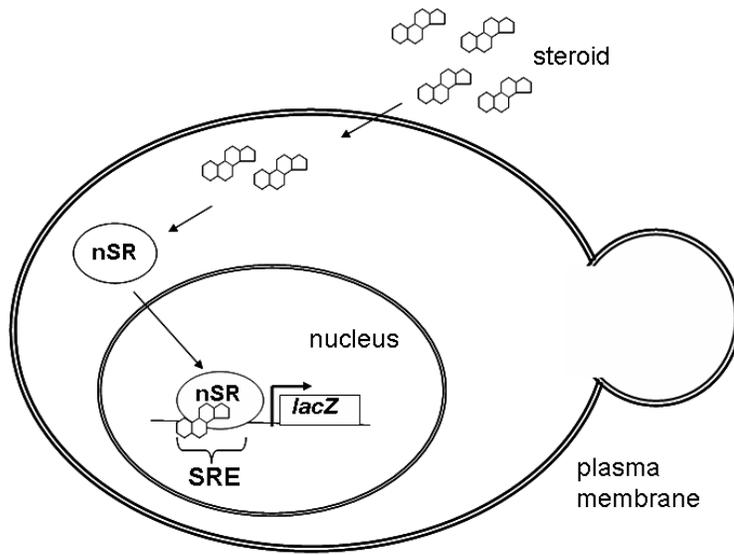
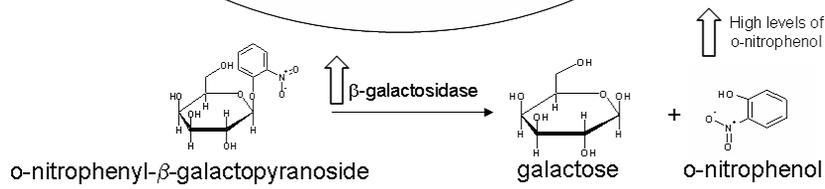
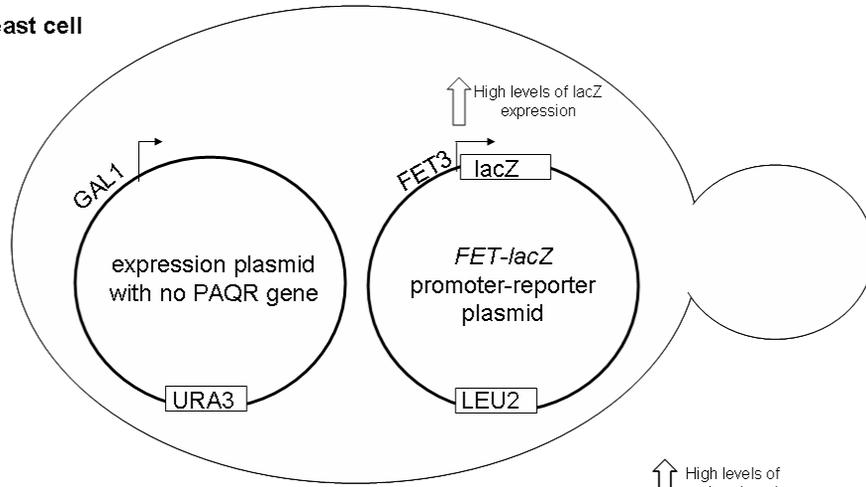
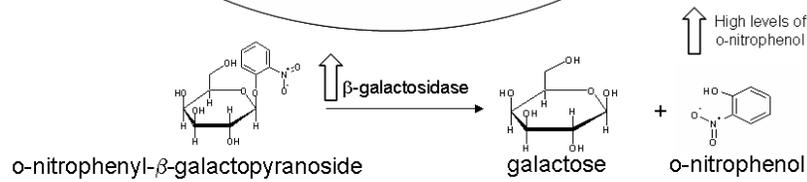
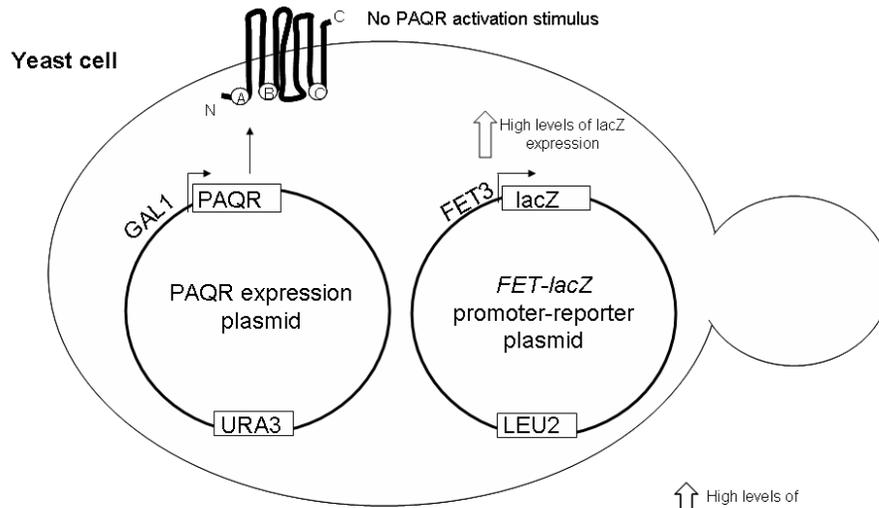


Figure 1-5. Yeast promoter-reporter assay for nuclear steroid receptor (nSR) analysis. Upon diffusion of the steroid into the cell, the steroid binds the nSR to activate it. The nSR recognizes and binds to a DNA sequence (steroid response element, SRE). This causes transcription of *lacZ*, which can be measured via the  $\beta$ -galactosidase assay.

Yeast cell



A



B

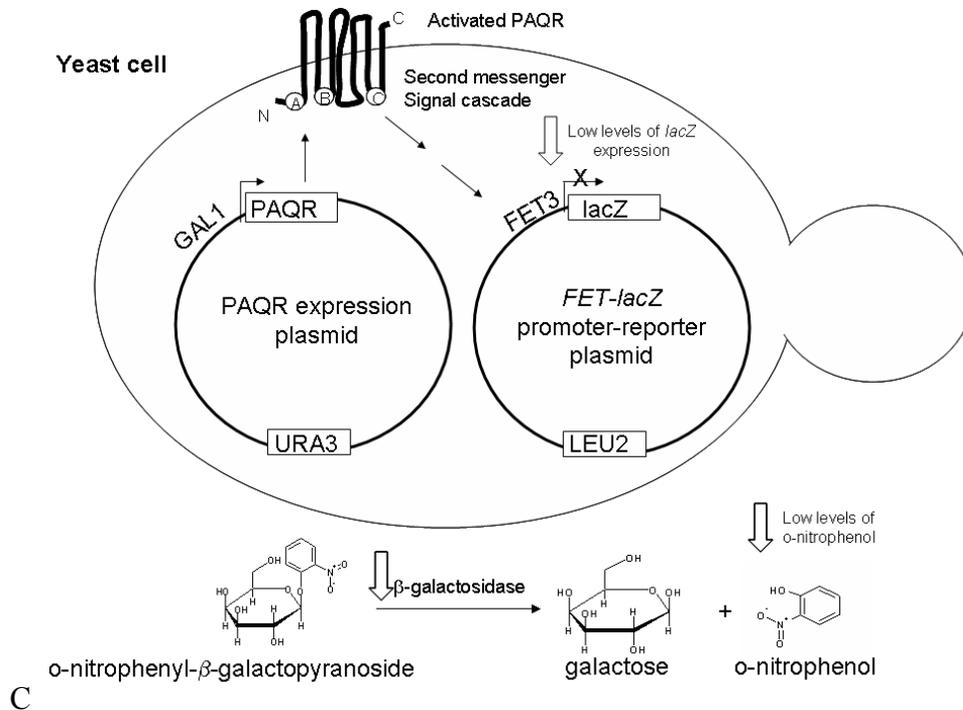


Figure 1-6. Yeast promoter-reporter assays of PAQR activation. Yeast cells are doubly transformed with the *pFET3-lacZ* and *GAL1-PAQR* vectors. The cells are grown in LIM containing galactose as an inducer of PAQR expression. In panel A, when no PAQRs are present, the *FET3* promoter is activated causing high levels of *lacZ* transcription, and thus high levels of  $\beta$ -galactosidase as determined by a colorimetric assay of ONPG hydrolysis. In panel B, when a PAQR is overexpressed but not activated, PAQR initiated signal transduction does not occur and the *FET3* promoter is activated as described above. In panel C, when a PAQR is overexpressed and activated, either constitutively or via a ligand, signal transduction occurs, resulting in repression of *FET3-lacZ*. This results in decreased  $\beta$ -galactosidase activity, as determined by decreased hydrolysis of ONPG measured in a colorimetric assay.

## CHAPTER 2 CLASS II PAQRS: MEMBRANE PROGESTIN RECEPTORS

### **Introduction**

Steroid hormones are known to affect cell physiology by at least two processes. The most studied physiological effects are known as the classic steroid effects, while the other effects are called the nonclassic steroid effects.

#### **Classic Steroid Effects**

Upon diffusion of the steroid across the plasma membrane, the steroid binds to a nuclear steroid receptor (nSR) (Chen and Farese, 1999). The activated receptor translocates to the nucleus where it binds steroid response elements (SRE) in the DNA sequence and initiates transcription of target genes (Figure 2-1) (Falkenstein et al., 2000). The steroid-dependent effect on the genome is sensitive to inhibitors of transcription and translation (Falkenstein et al., 2000). The alterations in gene transcription occur within hours to days, and so the classical steroid effects are considered to be nonrapid (Farach-Carson and Davis, 2003; Losel and Wehling, 2003).

#### **Nonclassic Steroid Effects**

In addition to the classic effects of steroids at the genomic level, it has long been recognized that steroids can also have rapid, nongenomic effects (Falkenstein et al., 2000), which do not absolutely require alterations in gene transcription (Losel and Wehling, 2003; Thomas et al., 2002). These effects are referred to as nonclassical steroid effects (Losel and Wehling, 2003; Thomas et al., 2002) and are not blocked by transcription inhibitors (Falkenstein et al., 2000; Losel and Wehling, 2003).

Instead of involving soluble nuclear receptors, rapid steroid effects are thought to be initiated at the plasma membrane by membrane-bound steroid receptors (Falkenstein and

Wehling, 2000). Activation of the nonclassical steroid receptors alters production of second messengers, such as cAMP, free intracellular calcium, and phosphoinositides, and causes protein kinase cascade activation (Falkenstein et al., 2000). While the nonclassical effects do not absolutely require transcription, the downstream effects of altered signaling pathways could ultimately affect transcription levels of some genes (Falkenstein et al., 2000; Losel and Wehling, 2003).

The mechanism by which the classical genomic steroid effects occur is attributed to the nuclear steroid receptors; however, many points of contention surround the issue of nonclassical steroid effects. Although it is widely accepted that steroid hormones can have rapid, nongenomic effects, the existence of novel, membrane-bound steroid receptors is contended for several reasons. First, some studies suggest that the classic nuclear steroid receptors can be membrane localized and may mediate at least some of the nonclassical steroid effects (Falkenstein et al., 2000). Also, reproducibility problems exist for studies that have demonstrated certain proteins to have characteristics of membrane steroid receptors (Wehling et al., 2007). More details on these problems will be discussed later in this chapter. Clearly, the mechanisms of nonclassical steroid effects are not established, so further studies of candidate membrane steroid receptors are needed.

### **Membrane Progestin Receptors (mPRs)**

The first steroid for which rapid effects were observed was progesterone (Falkenstein et al., 2000). Progestins, particularly progesterone, are important steroids that are involved in many different biological processes, including oocyte maturation, maintenance of pregnancy, stimulation and inhibition of cell proliferation, prevention of bone mass loss, spermiogenesis, modulation of sexual behavior, and alterations in immune system response (Graham and Clarke, 1997; Oettel and Mukhopadhyay, 2004; Dosiou et al., 2008). Importantly, progestins have also

been used as therapeutics for many reproductive disorders, such as endometrial cancers, because they limit the growth and proliferation of such cancerous cells (Zhou et al., 2007). Progestins and antiprogestins have also been used to treat abnormal bleeding in the uterus, prevention of miscarriage, and prevention of premature labor (Fernandes et al., 2007).

Like other steroids, progestins exert their effects via either classical genomic mechanisms or rapid, nongenomic mechanisms. Classic genomic progestin effects are mediated by either isoform (A or B) of the nuclear progestin receptors (nPR), which bind to progestin response elements (PREs) in the promoters of target genes, to activate gene transcription (Figure 2-1) (Graham and Clarke, 1997).

Nonclassical effects of progestins include activation of phosphatidylinositol 3-kinase (PI3K), activation of MAPK, and influx of  $\text{Ca}^{2+}$  (Norman et al., 2004). The mechanism by which these nonclassical effects occur is controversial but is thought to be initiated at the plasma membrane (Falkenstein et al., 2000) (Figure 2-1). The existence of novel, nonclassical progestin receptors is debated, but there is much evidence to support their existence. For instance, the rapid action of progesterone on amphibian oocyte maturation can occur in enucleated oocytes, suggesting that oocyte maturation is a nongenomic effect of the steroid (Morrill and Kostellow, 1999). Also, rapid progesterone effects on sperm cells can not be inhibited by the potent nPR antagonist RU-486 (Baldi et al., 1991). Furthermore, the nPRs are not found to be expressed at detectable levels in human sperm (Castilla et al., 1995). Likewise, while progesterone is well known to affect cytokine production in lymphocytes, the presence of nPRs in lymphocytes is often undetectable (Dosiou et al., 2008).

As for other steroids, the existence of membrane-bound nongenomic progestin receptors is debated, especially because classical nPRs can be membrane-associated (Martinez et al., 2007)

and capable of mediating many of the rapid nongenomic effects (Evaul et al., 2007). Also, some proteins that were originally proposed to act as membrane-bound progestin receptors (mPRs) could have a completely unrelated function (Wehling et al., 2007) or were not found to localize to the plasma membrane and to have an unknown function (Krietsch et al., 2006; Fernandes et al., 2007).

One group of candidate mPRs is part of the Progestin and AdipoQ Receptor (PAQR) family (Tang et al., 2005) and have been proposed to initiate some of the nonclassical effects of progestin steroids, such as activating mitogen-activated protein kinases (MAPKs) and inhibiting adenylyl cyclase to cause decreases in cyclic adenosine monophosphate (cAMP) levels (Zhu et al., 2003a; Zhu et al., 2003b). Some studies show that the mPRs that are part of the PAQR family, called PAQR5 (or mPR $\gamma$ ), PAQR7 (or mPR $\alpha$ ), and PAQR8 (or mPR $\beta$ ), localize to the cell membrane, display membrane binding to progestins, and function as G-protein coupled receptors (GPCRs) to affect signaling pathways in the cell (Zhu et al., 2003a; Zhu et al., 2003b; Hanna et al., 2006; Thomas et al., 2007). However, results from one study show that these proteins localize intracellularly to the endoplasmic reticulum, do not display membrane binding to progestins, and do not activate signaling cascades in response to progestins (Krietsch et al., 2006).

### **Class II PAQRs: Candidate Membrane Progestin Receptors (mPRs)**

Members of the PAQR family of proteins are characterized by having at least seven transmembrane domains (7TMs) and certain conserved amino acid sequence motifs (as described in Chapter 1). Phylogenetic sequence analyses show that the human PAQR family of proteins consists of eleven members that can be subdivided into three classes based on their sequence homology (Fernandes et al., 2005): Class I (PAQRs 1-4), Class II (PAQRs 5-9), and Class III

(PAQRs 10 and 11) (Figure 1-2). Despite some distinctions between classes, such as sequence homologies, proposed ligands, and physiological functions, it has been suggested that all of the PAQR proteins function via a conserved intracellular signaling mechanism (Tang et al., 2005), but this remains to be determined. Although the PAQRs are similar to GPCRs in that they have 7TM domains and the Class II PAQRs have been proposed to act as GPCRs (Thomas et al., 2007), it has been suggested that none of the PAQRs function via coupling to G-proteins (Tang et al., 2005). Furthermore, it has been suggested that the Class II PAQRs have an eighth potential transmembrane domain (Lyons et al., 2004; Fernandes et al., 2005).

While the Class II PAQRs from many different species have been partially characterized (Zhu et al., 2003a; Zhu et al., 2003b; Hanna et al., 2006; Karteris et al., 2006; Krietsch et al., 2006; Thomas et al., 2007; Josefsberg Ben-Yehoshua, 2007), a physiological role for the human homologues is unclear. Studies indicate that the human Class II PAQRs are differentially expressed in pregnancy tissues and may be important in regulating the onset of labor (Karteris et al., 2006; Fernandes et al., 2005). Importantly, PAQR7 may have a role in breast cancer development, as expression is increased in breast tumors versus normal tissue (Dressing and Thomas, 2007). Also, PAQR7 and PAQR8 may be involved in the effects of progesterone on immune system response, as these two proteins were detected in T-lymphocytes and Jurkat cells (Dosiou et al., 2008).

Whether the Class II PAQRs function as membrane-bound progestin receptors is debated in the literature. Two recent studies aimed at characterizing the human Class II PAQRs and used the same cell line, MDA-MB-231 human breast cancer, which does not express the nPRs (Thomas et al., 2007; Krietsch et al., 2006). However, these studies found conflicting results regarding progestin binding to and involvement of Class II PAQRs in progestin-induced cell

signaling events (Thomas et al., 2007; Krietsch et al., 2006). According to Thomas et al. (2007), membranes from MDA-MB-231 cells transfected with PAQR7 bind progesterone 2.5 times more than untransfected cells. Subcellular fractionation from these cells showed that the increased progesterone binding detected in radioligand binding assays was specific for plasma membranes rather than organelle membranes (Thomas et al., 2007). Furthermore, changes in second messenger production (cAMP) in human cells transfected with PAQR7 occurred only when progesterone was added (Thomas et al., 2007). These changes were not observed for the untransfected control (Thomas et al., 2007).

Despite the results obtained by Thomas et al. (2007), another group did not find evidence to support that the Class II PAQRs are membrane progesterin receptors. Krietsch et al. (2006) used the same cell type that Thomas et al. (2007) used (MDA-MB-231) as well as human embryonic kidney (HEK) 293 cells. These studies showed that neither exogenously expressed nor endogenously expressed Class II PAQRs were localized to the plasma membrane (Krietsch et al., 2006). Furthermore, the same group did not find evidence to support that cAMP levels changed in Class II PAQR transfected cells in response to progesterone, nor did crude membranes from these cells bind progesterone more than untransfected cells (Krietsch et al., 2006).

One problem with the approach of Krietsch et al. (2006) in trying to demonstrate specific progesterone binding to membranes of cells expressing the Class II PAQRs is that they used crude membrane preparations. (In these radioligand binding assays, the same amount of total protein was used for the vector control and the Class II PAQR samples. Specific binding was defined as the difference between the total binding, when only radiolabeled progesterone was used, and the nonspecific binding, which is binding in the presence of 1000-fold excess cold

progesterone). Because each sample had the same amount of total protein and because it is likely that the Class II PAQRs are not abundant in crude membrane preparations, it is not surprising that there were insignificant differences in specific binding for the vector versus the Class II PAQR samples. In contrast, Thomas et al. (2007) used the membranes in which the PAQRs localized and observed specific binding for the PAQR7 samples in binding reactions that were conducted in a manner similar to that which was used by Krietsch et al. (2006).

As a result of the conflicts in the literature, the involvement of the Class II PAQRs in progestin-induced cell signaling is unclear. Therefore, the use of a simple system for the characterization of the Class II PAQRs could help to clarify their role in progestin-induced cell signaling. In this chapter, the use of the simple eukaryotic organism *Saccharomyces cerevisiae* to characterize the Class II PAQRs as mediators of progesterone-induced cell signaling events is described. The justification for the use of this organism was described in Chapter 1.

### **Class II PAQRs: Novel Members of the G-protein Coupled Receptor (GPCR) Superfamily?**

Thus far, a conserved intracellular signaling mechanism for the PAQR family has not been established. There are certain amino acid sequences that are conserved in each PAQR throughout evolution (Figure 1-1). These motifs include ExxNxxH just before TM1, SxxxH at the end of TM2, and HxxxH in TM7. If these motifs are all located inside the cell, they could be involved in a conserved intracellular signaling mechanism (Tang et al., 2005). Current experimental evidence in the literature suggests that the PAQRs do not share a common signaling mechanism.

For instance, the Class I PAQR signaling mechanism requires the scaffolding protein adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif (APPL1) (Mao et al., 2006). The Class II PAQRs have been suggested to

act via coupling to inhibitory G-proteins ( $G_i$ -proteins) (Thomas et al., 2007). This claim is partly based on cAMP measurements and  $G_i$ -protein-PAQR co-immunoprecipitation experiments. First, activated  $G_i$ -proteins inhibit adenylylate cyclase, which causes decreased cAMP production. For PAQR7-transfected human cells treated with progesterone (for human PAQR7) or 17,20 $\beta$ ,21-trihydroxy-4-pregnen-3-one (for sea trout PAQR7), cAMP levels decreased (Thomas et al., 2007). While Thomas et al. (2007) demonstrated that this effect could be blocked for sea trout PAQR7 upon addition of pertussis toxin, an inhibitor of  $G_{i\alpha}$  activation (Thomas et al., 2007), similar inhibitory experiments were not conducted for the human PAQR7. Thus, it is unclear if the decrease in cAMP associated with the human proteins can be similarly inhibited by pertussis toxin.

Thomas et al. (2007) also claimed that the human PAQR7 co-immunoprecipitated with  $G_i$ -proteins, but the experimental evidence was not clear. After immunoprecipitation with an anti- $G_i$  antibody, Western blots were performed and the membranes were probed with an anti-PAQR7 antibody (Thomas et al., 2007). The band for human PAQR7 was quite faint (Thomas et al., 2007) and there was no negative control shown to demonstrate that the same faint band was not present when PAQR7 was not expressed. In addition,  $G_i$ -proteins and the PAQR7 proteins both localize to membranes. Immunoprecipitation of membrane proteins likely leads to co-immunoprecipitation of other membrane proteins, whether there is direct interaction between the two proteins or not.

Although no cell signaling mechanism has been proposed for the human Class III PAQRs, the bacterial homologues (Hemolysin IIIs) have been suggested to oligomerize and form pores in the membranes of human red blood cells to cause cell lysis (Baida and Kuzmin, 1996).

While the conserved PAQR sequence motifs would be expected to be on the same side of the plasma membrane if they are involved in a conserved signaling mechanism (Tang et al., 2005), studies in the literature suggest that the three classes of PAQRs have different orientations in the cell membrane. Two of the PAQRs (PAQR1, AdipoR1, and PAQR2, AdipoR2), two of the yeast Izh proteins (Izh1p and Izh4p) and one of the bacterial PAQR homologues (YqfA) have been determined to have a topology with an intracellular N-terminus and an extracellular C-terminus (Figure 1-3A) (Yamauchi et al., 2003; Kim et al., 2006; Daley et al., 2005), which is opposite to that of GPCRs. In contrast, the Class II PAQRs were found to have a topology that is similar to that of GPCRs (Figure 1-3B) (Thomas et al., 2007). If these topological determinations are correct, then the conserved amino acid sequence motifs of the PAQR family would not be located on the same side of the cell for all PAQRs. This does not support the involvement of the conserved motifs in a common intracellular signaling mechanism.

Much debate continues regarding Class II PAQR function, signaling mechanism, localization, and topology. To alleviate some of the controversies, we used the yeast *Saccharomyces cerevisiae* as a simple model organism. *S. cerevisiae* is a good model organism to study human proteins, such as GPCRs, because many of the cellular components needed for their function are conserved in this organism (Mentesana et al., 2002). However, to be functional in yeast, mammalian GPCRs often require chimeras of yeast/mammalian G-protein subunits (Mentesana et al., 2002). Yeast has also been useful for studying nuclear steroid receptors because, unlike mammalian cells, yeast lack nuclear steroid receptors that could interfere with analysis of individual heterologous steroid receptors (McEwan, 2001). Thus, yeast can simplify the study of the Class II PAQR role in progestin steroid signaling.

Some members of the PAQR family of proteins have been partially characterized in yeast (Lyons et al., 2004; Kupchak et al., 2007). Because we are interested in characterizing all members of the human PAQR family, we have chosen to use yeast to study the PAQRs that have been proposed to act as mPRs. Our studies of the Class II PAQRs describe the first characterizations of any of the human membrane steroid receptors in yeast. We used a yeast promoter-reporter (*FET3-lacZ*) assay that has been previously used to characterize the homologous adiponectin receptors (Kupchak et al., 2007). Our results confirm that the human Class II PAQRs are involved in progestin-mediated signaling, which has been disputed (Krietsch et al., 2006, Thomas et al., 2007). We show that the progestin-mediated effect of the Class II PAQRs on *FET3-lacZ* response is not dependent on the presence of human or yeast G-proteins. Thus, we have evidence that human Class II PAQRs are able to signal in a G-protein independent manner. We show that, like the Class I PAQRs, the human Class II PAQRs require the yeast homologue of human protein kinase A (called Tpk2p) for signaling. In addition, we provide the first experimental evidence that human PAQR6 and PAQR9 mediate cell signaling in response to progestin, which confirms functional predictions based on bioinformatic sequence analyses (Thomas et al., 2007). We also used the *FET3-lacZ* assay to perform structure-function studies and identify new agonists for the Class II PAQRs.

### **Results and Discussion**

Previous attempts have been made to establish whether the human Class II PAQRs mediate progestin signaling; however, these studies yielded conflicting results (Thomas et al., 2007; Krietsch et al., 2006). In addition, because the classical progesterone receptors seem to be capable of mediating many of the nongenomic effects that are attributed to membrane progestin receptors (Evaul et al., 2007), the existence of distinct membrane progestin receptors is debated. Thus, classification of the Class II PAQRs as membrane progestin receptors is still controversial.

To clarify the role of human Class II PAQRs in progestin signaling, we chose the simple model organism *Saccharomyces cerevisiae* and a promoter-reporter assay that has been previously used to characterize the related adiponectin receptors (Kupchak et al., 2007). With this system, we have found that expression of any of the PAQRs in the presence of their proposed ligands causes negative regulation of *FET3* gene expression under conditions (low amounts of iron in the media) that normally cause induction of this gene.

### **Overexpression of Some PAQRs Causes Ligand-Independent Repression of *FET3***

Under conditions of low iron media (LIM) and full induction of PAQR expression via the *GALI* promoter, some of the human PAQRs cause repression of the yeast high affinity iron uptake gene *FET3*. Previously published data demonstrated that all four of the PAQR homologues in yeast (Izh1p, Izh2p, Izh3p, and Izh4p) repressed *FET3-lacZ* (Kupchak et al., 2007). Control experiments showed that this effect was not due to problems with growth, transcription, translation or reporter activity; nor were these effects due to expression of membrane proteins or the reporter construct (Kupchak, 2008).

In addition, it was demonstrated that heterologously expressed human Class I PAQRs (PAQR1 and PAQR2) could repress *FET3-lacZ* in an adiponectin-dependent manner (Kupchak et al., 2007). Thus, human PAQR homologues can be functionally expressed in yeast and it was hypothesized that all of the PAQRs, including the Class II PAQRs, could cause repression of *FET3-lacZ* via a conserved signaling mechanism.

Here, we tested the effects of 6x-histidine tagged Class II and Class III PAQR overexpression (via the *GALI* promoter) on *FET3-lacZ* in LIM and found that some (PAQR5, PAQR8, and PAQR11) but not all of these proteins could cause repression of *FET3-lacZ* under full protein expression induction (2% galactose) (Figure 2-2).

### **Overexpression of Some PAQRs Causes Ligand-Dependent Repression of *FET3***

Under growth and induction conditions similar to those described above, it was demonstrated that repression of *FET3-lacZ* occurs in cells overexpressing PAQR6, PAQR7, or PAQR9 when their proposed ligand, progesterone, was included during growth (Figure 2-3). Control experiments showed that progesterone did not affect *FET3* in yeast carrying the empty expression vector indicating the effect of progesterone was not nonspecific. In addition, progesterone did not cause repression of *FET3-lacZ* in cells overexpressing the Class III PAQR10 protein (Figure 2-3), nor did it cause repression in cells overexpressing the Class I PAQR2 protein (Garitaonandia, 2008), demonstrating that overexpression of other membrane proteins does not cause repression of *FET3-lacZ* in the presence of progesterone.

### **Reduced PAQR Expression Alleviates *FET3-lacZ* Repression by Constitutively Active PAQRs**

Interestingly, within each of the classes of human PAQRs, the proteins can be divided into sub-groups based on sequence relatedness (Figure 1-2). For Class I PAQRs, PAQR1 and PAQR2 are highly similar. For Class II PAQRs, PAQR5 and PAQR6 are highly similar to one another and PAQR7 and PAQR8 are highly similar to one another, while PAQR9 is the most distant member. For Class III PAQRs, PAQR10 and PAQR11 are the only members, but are highly related. Based on previous results for Class I PAQRs (Kupchak et al., 2007) and the results presented here, it is apparent that one member of each sub-group causes repression of *FET3-lacZ* during full protein expression induction (PAQR1 (Kupchak et al., 2007), PAQR5, PAQR8, and PAQR11 (Figure 2-2), while the other member of each sub-group does not.

When some receptors are overexpressed, they can signal in the absence of their ligands and the level of this constitutive signaling is dependent on the level of expression (Tiberi and Caron, 1994; Milligan, 2003). Therefore, it was hypothesized that the level of *FET3-lacZ* repression

was dependent on the level of PAQR expression. This was tested by performing *FET3-lacZ* assays with PAQR5 cultures that had varying concentrations of inducer added (galactose) (Figure 2-4A). As expected, when less inducer was present, PAQR5-mediated repression of *FET3-lacZ* was alleviated, but with increasing amounts of inducer, the repression of *FET3-lacZ* occurred in a dose-dependent manner. Similar results were obtained with IZH2p and PAQR1 (Kupchak et al., 2007).

Interestingly, for all PAQRs tested, when *GALI*-mediated expression was not fully induced (0.05% galactose), even the constitutively active Class II PAQRs required the addition of their proposed ligands (Figure 2-4B). Control experiments showed that the distantly related Class III PAQRs (PAQR10 and PAQR11) (Figure 2-4B) and the adiponectin receptors (Class I PAQRs) did not respond to progesterone (Garitaonandia, 2008).

### **Detection of PAQR Expression in Yeast**

In addition, it was hypothesized that, when expression is fully induced (2% galactose), the constitutively active Class II sub-group members express at higher levels than the nonconstitutive members. To determine this, semi-quantitative Western blots were performed. Initial attempts to demonstrate expression of 6x-histidine tagged Class II PAQRs were unsuccessful, as PAQR5 was the only protein successfully detected (data not shown). Therefore, 7x-HA-tagged PAQRs were constructed and expression was successfully detected by Western blot at approximately their expected sizes (47-50 kDa) (Figure 2-5A). These constructs were also functional (Figure 2-5B). Our results indicate that there is not a major difference in expression levels, but PAQR5 and PAQR8 do express slightly more than PAQR6 and PAQR7. The frequency of codons present in the PAQR sequences that are rarely used in *S. cerevisiae* are given in Table 2-2. According to this, PAQR5 has fewer rarely used codons than the other Class II PAQRs, suggesting that this protein could express more readily in *S. cerevisiae*. On the other

hand, the number of rarely used codons in PAQR6, PAQR7, and PAQR8 is similar. Thus, there is no correlation between the frequency of rarely used codons in the PAQR sequences and their expression levels in *S. cerevisiae*. It has been noted that very different levels of constitutive activity can occur for closely related GPCRs, even when their expression levels are similar (Milligan, 2003).

Because the 6x-histidine tag is significantly smaller (840 Da) than the 7x-HA tag (~7600 Da) and because several attempts to clone PAQR9 into the pGREG536 vector for 7x-HA tagging were unsuccessful, most of the *FET3-lacZ* characterizations were performed with the 6x-histidine tagged protein.

### **Dose-Dependent Repression of *FET3-lacZ* by Certain Steroids**

For the Class II PAQRs, *FET3-lacZ* repression in the presence of progesterone occurred in a dose-dependent manner (Figure 2-6A). These findings suggest that PAQR receptor overexpression and activation modulate a similar signaling pathway in yeast and that some PAQRs have basal signaling in the absence of their activating ligand. To study the ligand specificity of the Class II PAQRs, we tested several compounds that are similar to progesterone, 17 $\alpha$ -hydroxyprogesterone, 21 $\alpha$ -hydroxyprogesterone, 17,21-dihydroxyprogesterone, and testosterone (Figure 2-6B), with a previously studied Class II PAQR (PAQR5) as well as the uncharacterized Class II PAQRs (PAQR6 and PAQR9) (Figure 2-6 C-F). Thomas et al., conducted competitive binding assays with membranes containing overexpressed PAQR7 and showed that some of the compounds we tested had relative binding affinities (RBA, measured as the ability of unlabeled steroid, compared to unlabeled progesterone, to displace 50% of radiolabeled progesterone in competitive binding assays) of 22.4% (testosterone), 19.7% (21-hydroxyprogesterone), and less than 1% (17 $\alpha$ -hydroxyprogesterone) (17,21-

dihydroxyprogesterone was not tested) (Thomas et al., 2007). Interestingly, like progesterone, we found that all compounds, except for testosterone, caused repression of *FET3-lacZ*. When PAQRs were expressed, similar results were obtained for PAQR7 in our lab (Brian Kupchak, personal communication). Interestingly, while Thomas et al. (2007) detected significant binding of testosterone, but not 17 $\alpha$ -hydroxyprogesterone, we observed a cellular response to 17 $\alpha$ -hydroxyprogesterone but not testosterone. It has not been reported whether testosterone causes a Class II PAQR-mediated cellular response in mammalian cells models. Experiments performed by Brian Kupchak in our lab show that the progesterone dose-response for PAQR5 shifts to the right in the presence of testosterone (Figure 2-6G), suggesting that testosterone could be having an antagonistic effect in our system.

Whether testosterone is binding to the Class II PAQRs expressed in our yeast cells is unknown, but will be the subject of future studies. Regardless, the progestin response of yeast cells expressing the Class II PAQRs strongly suggests that these receptors sense and respond to progestins and that they are, indeed, progestin receptors.

### **PAQR-Mediated Repression of *FET3* Does Not Absolutely Require G-proteins**

The human Class II PAQRs are proposed to act as G<sub>i $\alpha$</sub> -protein coupled receptors that cause decreased adenylate cyclase activity (Thomas et al., 2007; Dressing and Thomas, 2007) (Figure 2-7). Upon activation of G<sub>i</sub>-proteins, GDP bound to the G<sub>i</sub>-proteins is converted to GTP, which causes inactivation of adenylate cyclases and a subsequent decrease in cAMP. For cell membranes from PAQR7-transfected human cells treated with progesterone, immunoassay experiments demonstrated that cAMP production decreased and this effect could be blocked upon addition of pertussis toxin, an inhibitor of G<sub>i $\alpha$</sub>  activation (Thomas et al., 2007). Co-immunoprecipitation experiments with anti-G<sub>i</sub>-protein antibodies demonstrated significant

increases in membrane-bound GTP (Thomas et al., 2007). Despite this experimental evidence, it has also been previously suggested that none of the PAQRs are coupled to G-proteins (Tang et al., 2005). Therefore, it was of interest to determine the involvement of G-proteins in Class II PAQR signaling in our yeast system.

The Class II PAQR activity observed in the *FET3-lacZ* assays does not require the presence of human G-proteins, as these proteins are not expressed in our yeast system; however, yeast have two genes encoding heterotrimeric G-proteins related to human  $G_{i\alpha}$  (*GPA1* and *GPA2*) (Slessareva et al., 2006), so it is possible that the PAQRs function as Gpa1p- or Gpa2p-linked GPCRs in yeast. Thus, the Class II PAQR-mediated repression of *FET3-lacZ* in the absence of the yeast G-proteins was tested. Knock-out mutants for each of the known yeast G-proteins (Gpa1p and Gpa2p) were used in the *FET3-lacZ* assay (Figure 2-8). These results show that neither G-protein is required for the response of PAQR5 to progesterone. A double-mutant for the *GPA* genes could not be obtained because the *GPA1* single-knockout is lethal, so knockouts of *GPA1* must be in combination with a mutation in *STE7* (Henrick Dohlman, personal communication). A triple knockout in *GPA1*, *STE7*, and *GPA2* would be required, and this was not readily available. Regardless, the *GPA1* and *GPA2* genes are involved in completely separate signaling pathways and do not have overlapping functions (Slessareva and Dohlman, 2006), so knockout mutants for *GPA1* are not expected to be complemented by *GPA2* and knockout mutants for *GPA2* are not expected to be complemented by *GPA1*.

To further address the possibility that the yeast G-proteins are involved in Class II PAQR-mediated repression of *FET3-lacZ*, vectors for the overexpression of truncated PAQR5 and PAQR7 were made. The truncated proteins include the first 289 out of 330 amino acids for PAQR5 and the first 310 out of 346 amino acids for PAQR7 such that the proteins lack the final

transmembrane domain and C-terminal amino acids. These truncated PAQRs lack a domain that is homologous to a part of the spotted sea trout PAQR7 previously shown to be important for G-protein activation (Thomas et al., 2007). Our human PAQR truncations expressed similarly to the full-length proteins (Figure 2-9A) and progesterone signaling was unaffected (Figure 2-9B), suggesting that the regions of the proteins purported to activate G-proteins in sea trout PAQR7 (Thomas et al., 2007) are not needed for sensing progesterone and signaling, at least in yeast. In addition, by characterizing the Class II PAQRs in yeast in the absence of any other human proteins, such as  $G_{i\alpha}$ , it was demonstrated that the Class II PAQRs are still capable of signaling. Together, these results suggest that the capability of the Class II PAQRs to signal does not absolutely require coupling to human or yeast G-proteins.

Finally, to further study the possible involvement of G-protein activation in causing *FET3-lacZ* repression, we performed *FET3-lacZ* assays in cells transformed with plasmids that constitutively express the mutants GPA1<sup>Q323L</sup> or GPA2<sup>Q300L</sup>. These mutants lack GTPase activity and are constitutively active forms of the yeast  $G_{\alpha}$ -proteins Gpa1p (Dohlman et al., 1996) and Gpa2p (Harashima and Heitman, 2002), respectively. While GPA1<sup>Q323L</sup> was previously shown to cause transcription of the pheromone-pathway activated *FUS1* promoter even in the absence of pheromone (Guo et al., 2003), an effect of GPA1<sup>Q323L</sup> on *FET3-lacZ* in LIM containing galactose was not observed, nor was an effect of GPA2<sup>Q300L</sup> on *FET3-lacZ* (Figure 2-10). These results indicate that the repression of *FET3-lacZ* is unrelated to activation of  $G_{\alpha}$ -proteins and that the Class II PAQR signaling that leads to *FET3-lacZ* repression is unlikely to involve activation of  $G_{\alpha}$ -proteins.

## **PAQR-Mediated Repression of *FET3* Requires the Presence of the PKA Yeast Homologue**

Besides involving G-proteins, the signaling pathway of the Class II PAQRs has been proposed to involve a decrease in cAMP that leads to a decrease in cAMP-dependent kinase (PKA) activation (Figure 2-7) (Thomas et al., 2007; Dressing and Thomas, 2007). Interestingly, it was previously shown that the presence of the yeast Tpk2p isoform of human PKA was essential for Class I PAQR signaling in yeast (Kupchak et al., 2007). In addition, Tpk1p, Tpk2p, and Tpk3p happen to be upstream of several yeast proteins that undergo changes in phosphorylation in the presence of PAQR7 and progesterone (Regalla, 2007), which also suggests that these proteins are important for the signaling pathway of the PAQRs. Thus, we performed the *FET3-lacZ* assay in the knockout mutant for *TPK2*. As shown in Figure 2-11, *TPK2* is also required for the Class II PAQR signaling that leads to repression of *FET3-lacZ*. This suggests that all of the PAQRs that we have studied share the same signaling pathway.

## **Identification of Novel Agonists of Class II PAQRs**

Previously, promoter-reporter assays in yeast have been used as a convenient tool for the identification of novel agonists and antagonists of drug targets, such as human GPCRs (Pausch, 1997). Thus, we decided to test repression of *FET3-lacZ* in cells expressing the Class II PAQRs and in the presence of compounds other than progestins.

The synthetic estrogen diethylstilbestrol (DES) and the antiprogestin RU-486 were chosen (Figure 2-12A). Although DES is a potent estrogen, it was chosen because it showed increased binding to membranes from cells expressing goldfish PAQR7 and it was previously shown to induce oocyte maturation in goldfish, which is a nonclassical effect of progestins, suggesting that DES can have agonistic effects on PAQR7 (Tokumoto et al., 2007). In addition, DES is an interesting endocrine-disrupting compound (EDC) to study because it has been banned from use

due to its serious negative effects, such as carcinogenicity and teratogenicity (Greenwald et al., 1971; Herbst et al., 1971).

RU-486 was chosen because, while it is known to be a potent antagonist of the nPRs, it does not show increased binding to membranes from cells expressing human PAQR7 (Thomas et al., 2007). An effect of this compound was unexpected.

As an initial screen, assays of the repression of *FET3-lacZ* were performed during reduced expression (0.05% galactose inducer) of PAQR5, PAQR6, PAQR7, and PAQR8 in the presence of 10  $\mu$ M progesterone, 10  $\mu$ M DES or 10  $\mu$ M RU-486. A concentration of 10  $\mu$ M was chosen because this was the highest concentration of progesterone tested in the dose-response curves (Figure 2-5). Neither DES nor RU-486 caused repression of *FET3-lacZ* activity in the vector control cells, as compared to the untreated vector control cells (Figure 2-12). While a t-test analysis indicates that the increase in *FET3-lacZ* activity that is observed for the vector control cells treated with progesterone, DES, and RU-486 was significant ( $P \leq 0.05$ ) in this particular assay, vector control cells did not always have an increase in *FET3-lacZ* activity in response to progesterone (for example, see Figures 2-4, 2-6, and 2-8). The increase in *FET3-lacZ* activity was also not observed in the RU-486 dose response curves for the vector control (Figure 2-13).

Interestingly, for the Class II PAQR samples, while neither DES nor RU-486 were as effective as progesterone for causing repression of *FET3-lacZ*, both of these compounds did cause repression of *FET3-lacZ* (Figure 2-12B) for some of the PAQRs. A t-test analysis indicates that repression of *FET3-lacZ* in the presence of RU-486 was significant ( $P \leq 0.05$ ) for cells expressing PAQR5, PAQR7, and PAQR8, and that the repression of *FET3-lacZ* in the presence of DES was significant ( $P \leq 0.05$ ) only for cells expressing PAQR7. This is the first demonstration of an effect of DES on any of the human PAQRs.

This is also the first demonstration of an effect of RU-486 on the human PAQRs. So, although the Class II PAQRs seem to respond more to progestins (Figure 2-6B), some activation of these proteins in the presence of RU-486 was observed (Figure 2-12B). Because the effect of RU-486 was significant for more of the PAQRs than the effect of DES, and because an effect of RU-486 was unexpected, dose-response analysis of this compound was performed (Figure 2-13).

These results were especially interesting because we did not expect to see an agonistic effect of RU-486 on the Class II PAQRs for several reasons: 1.) RU-486 is used clinically as an antiprogestin (Gass et al., 1998), 2.) the activity of RU-486 is well-known to directly interact with the nPRs to prevent efficient interaction of nPRs with the transcriptional machinery (Beck et al., 1993), and 3.) Thomas et al. showed that RU-486 competed poorly with progesterone for binding to membranes from cells expressing PAQR7 (Thomas et al., 2007).

Despite the primary effect of RU-486 as an antagonist (Gass et al., 1998), it has been noted that in some cell models, RU-486 can act as an agonist to strengthen the effects of progesterone on inducing gene expression (Rodriguez et al., 2002). This result has been suggested to indicate that these particular progesterone effects are nonclassical in nature (Oettel and Mukhopadhyay, 2004).

Also, under some conditions, such as stimulation of cAMP signaling pathways, RU-486 can have a stimulatory effect on transcription (Beck et al., 1993). This effect is thought to result from cross-talk between the signaling pathways of second messengers and steroid receptors (Beck et al., 1993). This study monitored expression of an nPR target gene, and it was suggested that stimulation of cAMP signaling pathways alter phosphorylation of nPR or transcriptional machinery components (Beck et al., 1993). Such modifications could cause increased efficiency

of nPR-RU-486 interactions with the transcriptional machinery and thus, increased transcription (Beck et al., 1993).

No effects on nPR-target gene expression were observed with either cAMP signaling pathway stimulation or RU-486 alone (Beck et al., 1993); however, it is possible that RU-486 could stimulate cAMP signaling pathways to cause other, nonclassical progesterone-like effects on cells. These effects could occur via the Class II PAQRs. Interestingly, the activity of the Class II PAQRs alters cAMP levels (Thomas et al., 2007), which then causes altered cAMP dependent kinase (PKA) activity. We have demonstrated that the repression of *FET3-lacZ* requires the presence of the yeast Tpk2p isoform of human PKA (Figure 2-11).

## **Materials and Methods**

### **Plasmids.**

The plasmids used were pYES260 (Melcher, 2000) and pGREG536 (Jansen et al., 2005). All plasmids allowed for *GALI* promoter-driven expression of PAQR proteins. All pYES260 primer sequences were designed by Julie Russell to amplify the gene from commercially available cDNA and included 30-40 bases for homologous recombination into pYES260 (Melcher, 2000). PAQR5, PAQR6, PAQR7, and PAQR8 were sub-cloned from pYES260 constructs into pGREG536. For cloning into pGREG536, primers were designed to amplify PAQR5, PAQR6, PAQR7, and PAQR8 and included sections of DNA for homologous recombination into pGREG536 (Jansen et al., 2005) (Table 2-1). For cloning of truncated PAQR5 and PAQR7, the reverse primers were designed to anneal to the genes at the location of the desired truncation. Proteins expressed in pYES260 have an N-terminal 6x-histidine tag and proteins expressed in pGREG536 have an N-terminal 7x-HA tag. The National Center for Biotechnology Information (NCBI) accession numbers for the cDNA sequences of the Class II PAQRs are given in Table 2-2. In addition, the frequencies of codons in the PAQR sequences

that are rarely used by *S. cerevisiae* that are also given in Table 2-2 were determined with the Gene Designer program (Villalobos et al., 2006).

Empty pAD4M and pAD4M expression vectors carrying the yeast G $\alpha$ -proteins GPA1, GPA1<sup>Q323L</sup>, and GPA2<sup>Q300L</sup> were obtained from Henrick Dohlman, University of North Carolina at Chapel Hill. These constructs allow *ADHI* promoter-driven expression of GPA1, GPA1<sup>Q323L</sup>, and GPA2<sup>Q300L</sup>. Expression of genes in this vector is constitutive. The GPA1<sup>Q323L</sup>, and GPA2<sup>Q300L</sup> mutants are constitutively active forms of GPA1 (Dohlman et al., 1996) and GPA2 (Harashima and Heitman, 2002), respectively.

### **Yeast Strains and Assays.**

All yeast strains used in this study were obtained from Euroscarf unless otherwise noted. *tpk2* $\Delta$  and *gpa2* $\Delta$  mutants were in the BY4742 (Mat  $\alpha$ ) background and *gpa1* $\Delta$  *ste7* $\Delta$  was obtained from Henrick Dohlman (University of North Carolina at Chapel Hill) and was in the BY4741 (Mat a) background. The following are the manufacturer's for the carbon sources that were used: D-(+)-glucose (170080025, Acros Organics), D-(+)-galactose (150610010, Acros Organics), and D-(+)-raffinose (19567-1000, Acros Organics). All growth conditions and  $\beta$ -galactosidase assays were performed as previously described (Kupchak et al., 2007). Briefly, cells were grown in Low Iron Medium (LIM) containing 1  $\mu$ M Fe<sup>3+</sup>, conditions which normally induce the expression of *FET3* (Eide and Guarente 1992).

Galactose was used as a carbon source to induce full target protein expression (2%) or reduced expression (0.05% galactose/1.95% raffinose). Cells were harvested, washed and permeabilized prior to assay for  $\beta$ -galactosidase activity (*lacZ*).  $\beta$ -galactosidase assays were performed on permeabilized cells as described (Guarente, 1983). The substrate was o-nitrophenyl- $\beta$ -galactopyraniside (ONPG, 4 mg/mL, 128820050, Acros Organics).  $\beta$ -

galactosidase activity is presented as a percentage of activity seen in cells expressing empty expression vector treated with vehicle (ethanol). For individual experiments, each data point was done in triplicate and the error bars represent +/- 1 standard deviation. Experiments were generally performed at least three times and a representative experiment is shown. All constructs were tested for the ability to repress *FET3-lacZ* in a progesterone-dependent manner. Experiments to ensure that different tags did not affect PAQR activity were performed at least twice or experiments performed as initial screens for responsiveness to the ligands diethylstilbestrol and RU-486 were performed once or twice with further validation of responses to RU-486 as shown in the Results and Discussion.

When needed, the following compounds were added to the LIM during overnight growth: progesterone (P8783, Sigma), testosterone (T-1500, Sigma), 21-hydroxyprogesterone (D6875, Sigma), 17- $\alpha$ -hydroxyprogesterone (H5752, Sigma), 17,21-dihydroxyprogesterone (R0500, Sigma) adiponectin (RD172029100, Biovendor Laboratory medicine, Inc.), diethylstilbestrol (D4628, Sigma), and RU-486 (M8046, Sigma). These compounds were all dissolved in ethanol. The untreated control cultures contained the same concentration of ethanol as the steroid-treated cultures.

As noted in the captions of the appropriate figures, some  $\beta$ -galactosidase assay data were collected in collaboration with Brian Kupchak and Ibon Garitaonandia. Some of these data or similar trends are also presented in Kupchak (2008) and Garitaonandia (2008).

### **Expression of Hemagglutinin (HA)-Tagged Class II PAQRs.**

For Class II PAQR localization studies, overnight cultures in SD media were reinoculated to an optical density (OD) at 600 nm ( $OD_{600}$ ) equal to approximately 0.2 in LIM containing 2% galactose for full target protein expression at 30°C. After the  $OD_{600}$  reached 1, the cultures were

harvested, washed twice with 25 mL of cold water, and stored at -80°C until total membrane protein preparations were made.

### **Total Membrane Protein Extraction and Detection by Western Blot.**

After thawing, the cell pellets were resuspended in 250  $\mu$ L of membrane isolation buffer [MIB, 0.6 M D-mannitol (M-4125), 20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (P7626, Sigma), Complete, EDTA-free protease inhibitor tablet (11836170001, Roche Diagnostics), and protease inhibitor cocktail (P8215, Sigma)], similar to that described by Gitan and Eide (2000). Glass beads (G8772, Sigma) were added, and the suspension was vortexed at 4°C six times for 1 minute each, with 1 minute on ice in between. The samples were centrifuged at 3000 x g for 10 minutes, the supernatant was removed and centrifuged at 130,000 x g for 90 minutes. The supernatant was saved and glycerol was added to a final concentration of 15%. The pellets were resuspended in fresh mannitol buffer supplemented with 15% glycerol (250  $\mu$ L) and aliquots were stored at -80°C. Before SDS-PAGE analysis, protein concentration determinations were performed with the bicinchoninic acid (BCA) Protein Assay Kit (23227, Pierce). To detect expression of HA-tagged proteins, equal amounts of protein (in  $\mu$ g) were loaded onto a 10% polyacrylamide gel and electrophoresis was performed according to standard procedures (Sambrook and Russell, 2001). Dual Color Precision Plus Protein<sup>TM</sup> Standards (161-0374, Bio-Rad) were used for molecular weight markers. Western blots were performed using nitrocellulose according to standard procedures (Sambrook and Russell, 2001). Transfer buffer was Tris-glycine, pH 8.3, 10% methanol, and 0.1% SDS. The transfer was performed at 80 volts for 60 minutes. Membranes were blocked overnight at 4°C with 1% bovine serum albumin (BSA) in phosphate buffered saline (BP3994, Fisher Scientific). For HA-tagged PAQRs, the primary antibody (rabbit polyclonal IgG HA-

probe (Y-11), SC-805, Santa Cruz Biotechnology, Inc.) was diluted 1:500 in PBS containing 1% BSA. The secondary antibody used was horse radish peroxidase-conjugated goat anti-rabbit IgG-HRP (1:10000, SC-2004, Santa Cruz Biotechnology, Inc.). The blot was incubated with SuperSignal® West Pico Substrate Working Solution (Pierce) for 5 minutes, exposed to film and developed. The membrane was stripped with Restore™ Western Blot Stripping Buffer (21059, Thermo Scientific) according to the manufacturer's directions and re-probed with an anti-porin primary antibody (diluted to 0.5 µg/mL, anti-porin yeast mitochondrial mouse IgG monoclonal, A6449, Invitrogen). The secondary antibody used was goat anti-mouse IgG (H+L)-HRP (170-6516, Bio-Rad) (1:10000). Again, the blot was incubated with SuperSignal® West Pico Substrate Working Solution (Pierce) for 5 minutes, exposed to film and developed. The ImageJ software program (NIH) was used as described (<http://rsb.info.nih.gov/ij/>) to obtain values for the integrated densities of the HA-tagged PAQRs or the corresponding porin band for each sample. The density for each porin band was used to normalize the bands for the HA-tagged PAQRs so that comparisons of PAQR expression levels could be made.

### **Summation**

Here, it was demonstrated that, like the Class I PAQRs (Kupchak et al., 2007), expression of Class II PAQRs can have a physiological effect on yeast (i.e. repression of *FET3* in LIM) and this effect can be induced by the presence of progesterone. By screening different steroids, it was demonstrated that 17- $\alpha$ -hydroxyprogesterone, 21-hydroxyprogesterone, and 17,21-dihydroxyprogesterone, but not testosterone, also induce repression of *FET3* when Class II PAQRs are expressed. In addition, this heterologous system allowed the study of the role of the Class II PAQRs in sensing and responding to progesterone in the absence of other steroid receptors. Experimental evidence presented here suggests that the Class II PAQRs do not

absolutely require human or yeast G-proteins to respond to progesterone. This system was also used to begin structure/function studies on human Class II PAQRs and to identify potential new agonists for these proteins.

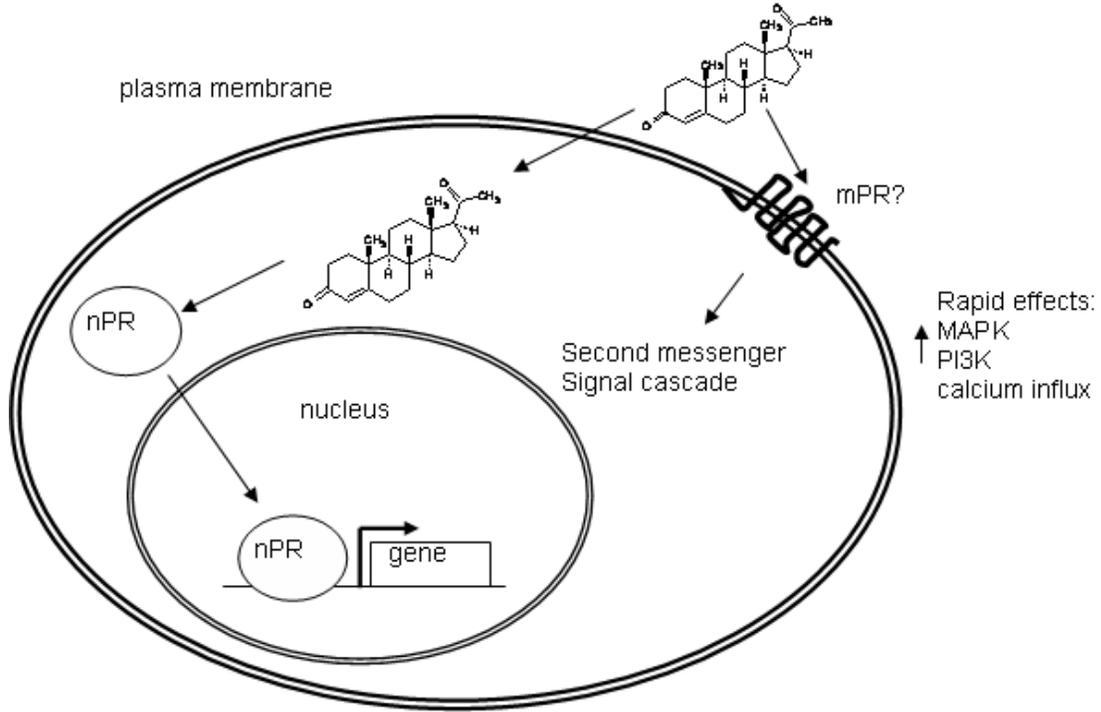


Figure 2-1. Mechanisms of progestin steroids. Progestins exert their effects via either classical genomic mechanisms or rapid, nongenomic mechanisms. Classic genomic progestin effects are mediated by the soluble nuclear progestin receptors (nPR), which bind to the promoters of target genes to activate gene transcription. Rapid, nonclassical effects of progestins are thought to occur by membrane-bound receptors, but this is debated.

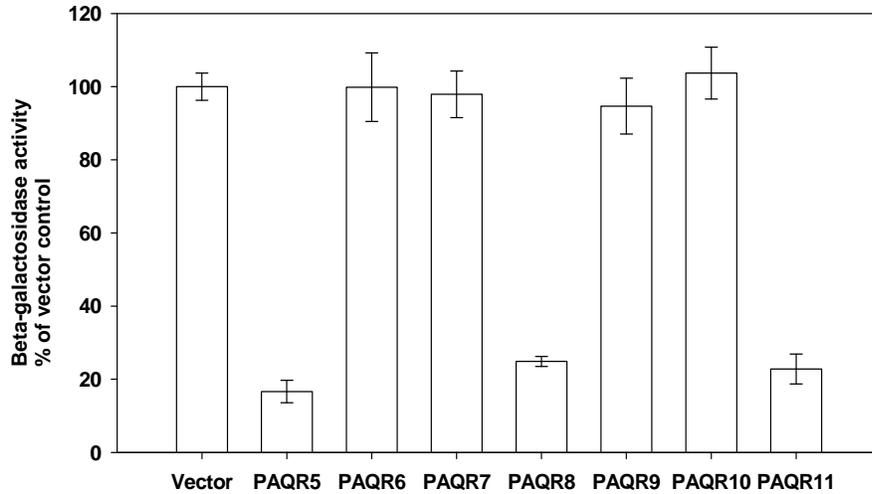


Figure 2-2. Overexpression of some PAQRs cause repression of *FET3-lacZ*. Yeast cells doubly transformed with the *pFET3-lacZ* vector and *GAL1-6x* histidine-PAQR vector were grown in LIM containing 1  $\mu\text{M}$   $\text{Fe}^{3+}$  and 2% galactose and  $\beta$ -galactosidase assays were performed as described in the Materials and Methods. PAQR5, PAQR8, and PAQR11 cause constitutive repression of *FET3-lacZ*. The data for this figure were obtained in collaboration with Brian Kupchak and Ibon Garitaonandia.

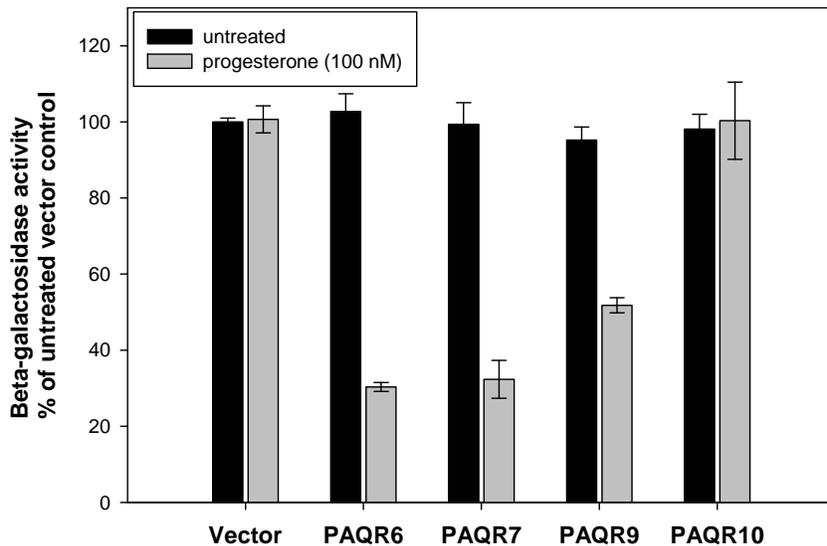


Figure 2-3. Repression of *FET3-lacZ* is ligand-dependent for some PAQRs. Yeast cells doubly transformed with the *pFET3-lacZ* vector and *GAL1-6x* histidine-PAQR vector were grown in LIM containing 1  $\mu\text{M}$   $\text{Fe}^{3+}$  and 2% galactose and  $\beta$ -galactosidase assays were performed as described in the Material and Methods. Cultures were untreated, or had progesterone (100 nM) added during the overnight growth in LIM. The data for this figure was obtained in collaboration with Brian Kupchak and Ibon Garitaonandia.

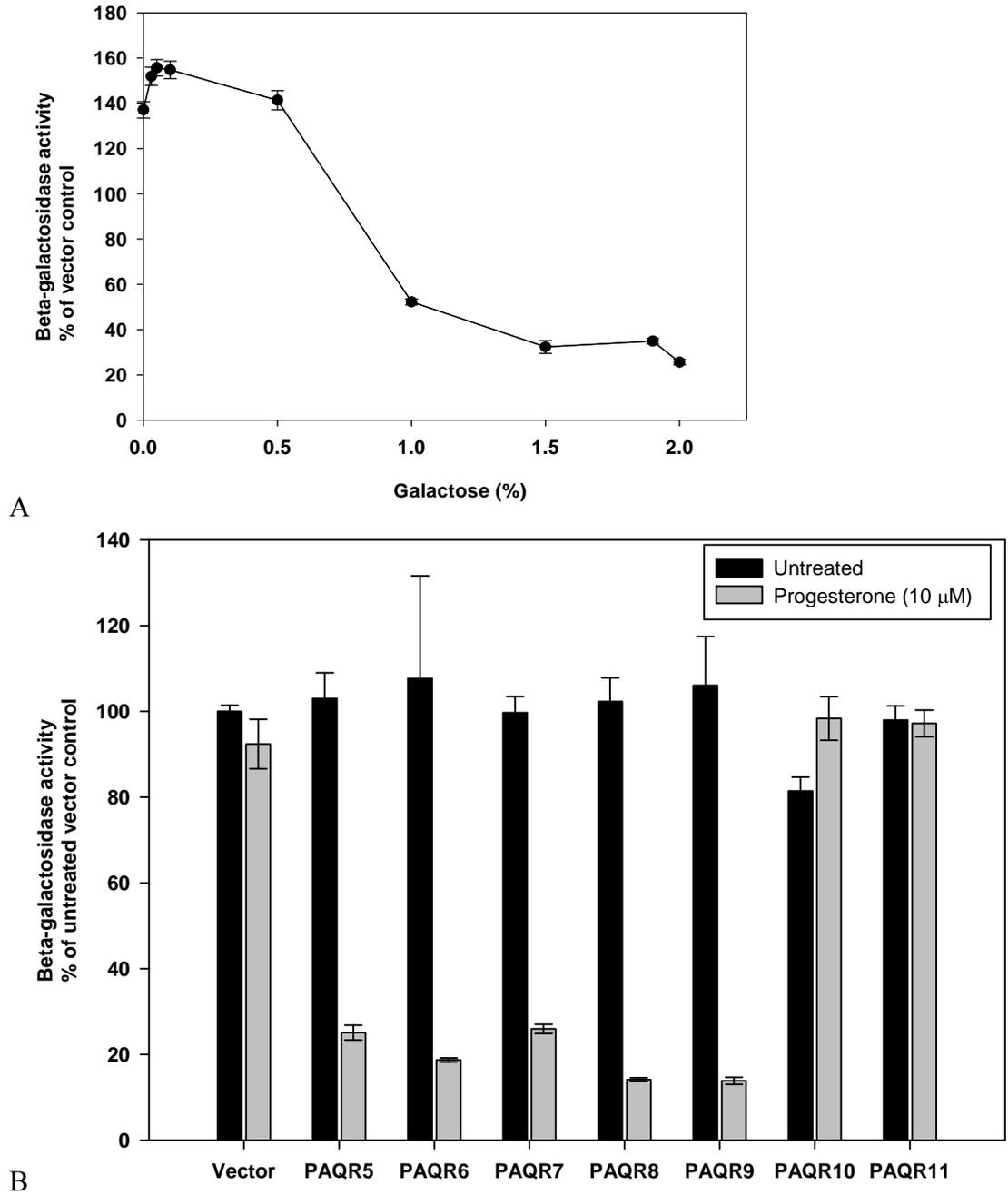
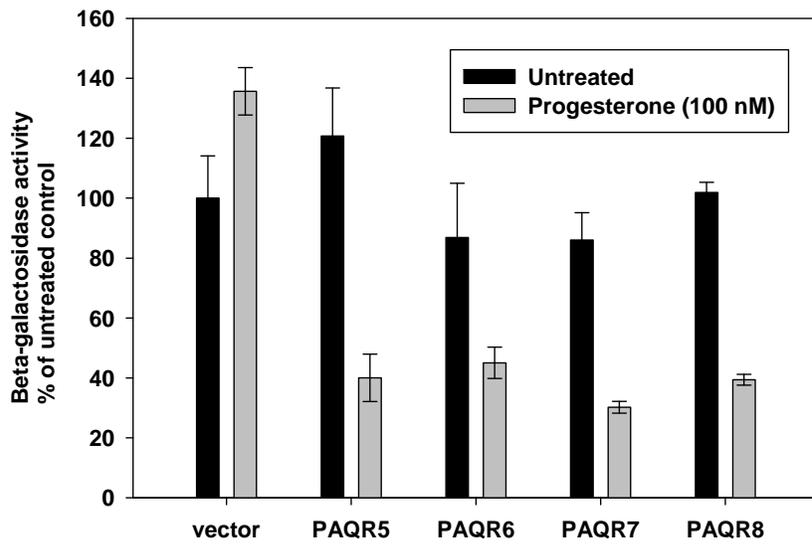
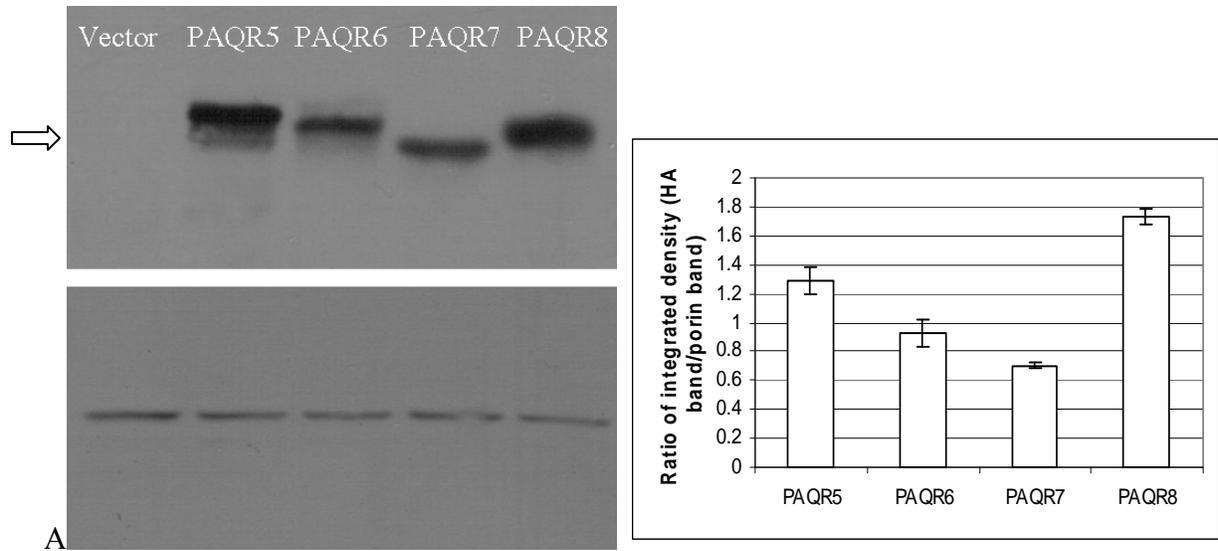
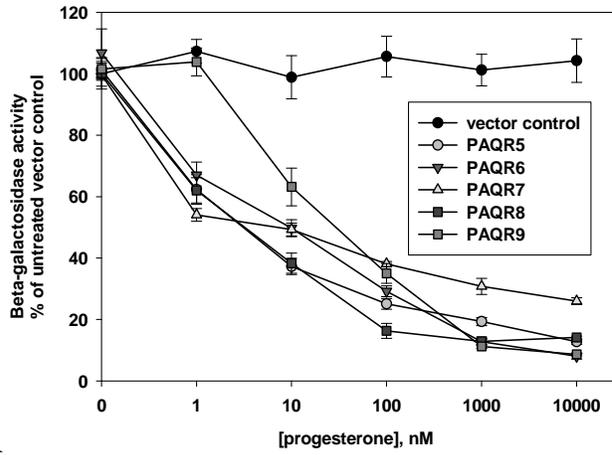


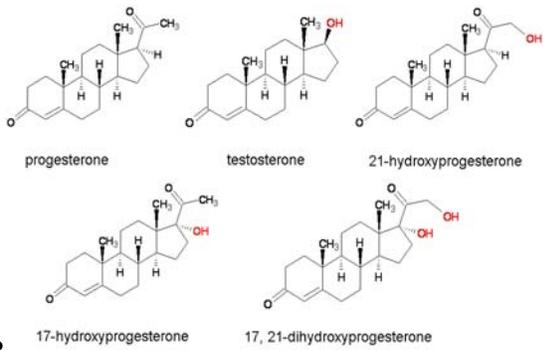
Figure 2-4. Constitutive repression of *FET3-lacZ* can be alleviated and become ligand-dependent under low expression induction conditions. In panel A, the repression of *FET3-lacZ* by PAQR5 increases with increasing amounts of expression inducer (galactose). In panel B, yeast cells doubly transformed with the p*FET3-lacZ* vector and *GAL1-6x* histidine-PAQR vector were grown in LIM containing 1 μM Fe<sup>3+</sup> and 0.05% galactose and β-galactosidase assays were performed as described in the Material and Methods. Cultures were untreated, or had progesterone (10 μM) added during the overnight growth in LIM. The data for this figure was obtained in collaboration with Brian Kupchak and Ibon Garitaonandia.



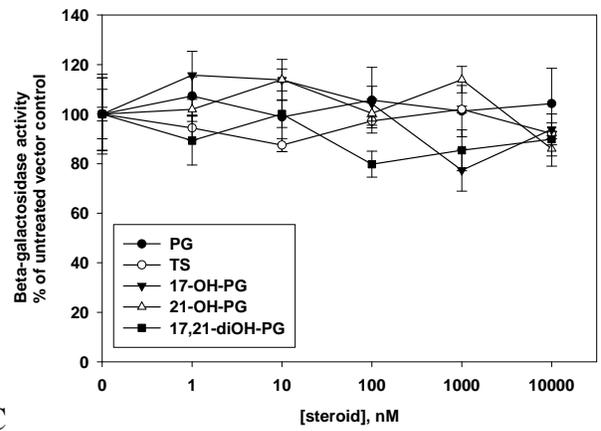
B  
 Figure 2-5. Expression of PAQRs in yeast can be detected by Western blot. In panel A, the PAQRs were re-cloned in vector such that they had N-terminal 7x-HA tags. The arrow indicates the approximate position of the 50 kDa band of the molecular weight marker. After expression of PAQRs was detected with an anti-HA antibody (top left), the membrane was stripped and re-probed with an anti-porin antibody (bottom left) for normalization (chart, right). In panel B, *FET3-lacZ* repression by the HA-tagged PAQRs was confirmed in LIM containing 0.05% galactose and progesterone.



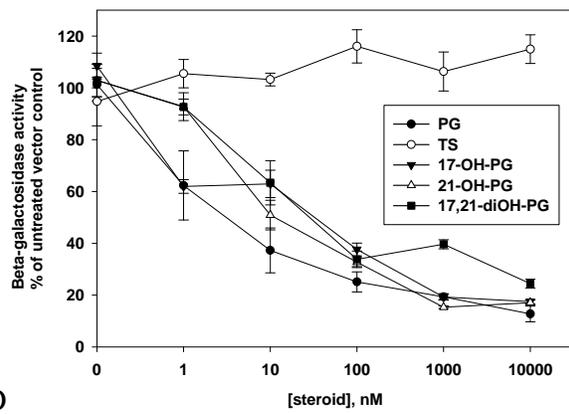
**A**



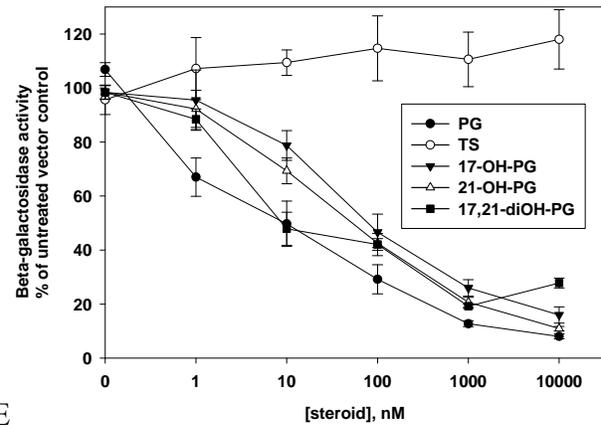
**B**



**C**



**D**



**E**

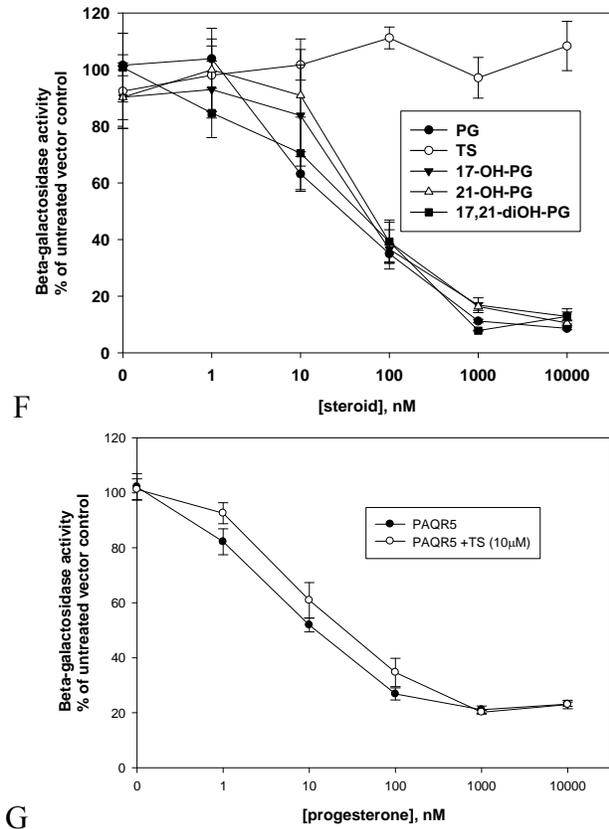


Figure 2-6. Ligand-dependent repression of *FET3-lacZ* by Class II PAQRs occurs in a dose-dependent manner and is steroid specific. In panel A, all Class II PAQRs were used in  $\beta$ -galactosidase assays and different concentrations of progesterone were included in the growth media. In panel B, different steroids were tested: progesterone (PG), testosterone (TS), 17 $\alpha$ -hydroxyprogesterone (17-OH-PG), 21-hydroxyprogesterone (21-OH-PG), or 17,21-dihydroxyprogesterone (17,21-diOH-PG) were tested with the vector control (panel C), PAQR5 (panel D), PAQR6 (panel E), and PAQR9 (panel F). In panel G, the progesterone dose-response for PAQR5 shifts to the right in the presence of testosterone (the experiment in panel G was performed by Brian Kupchak).

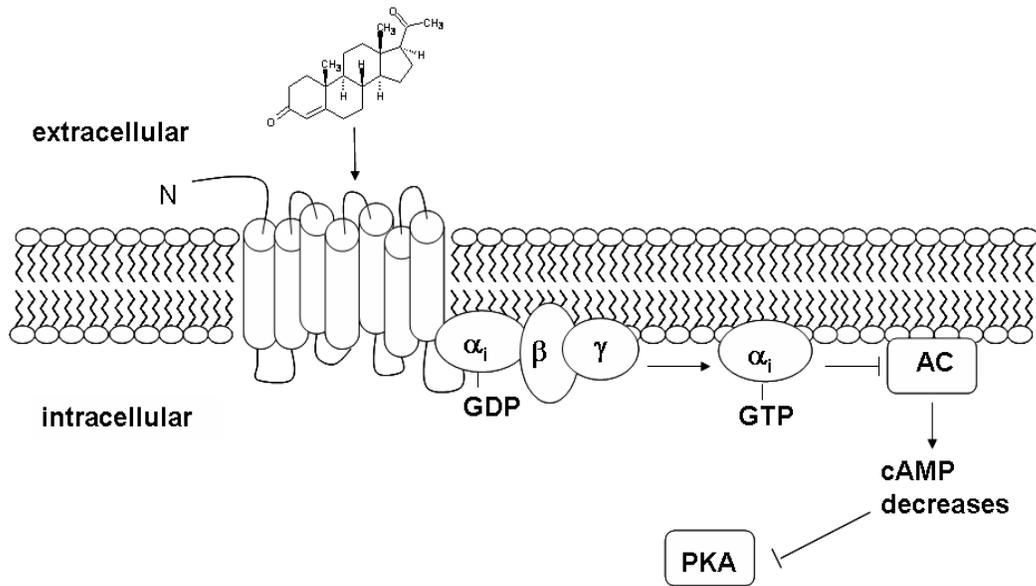
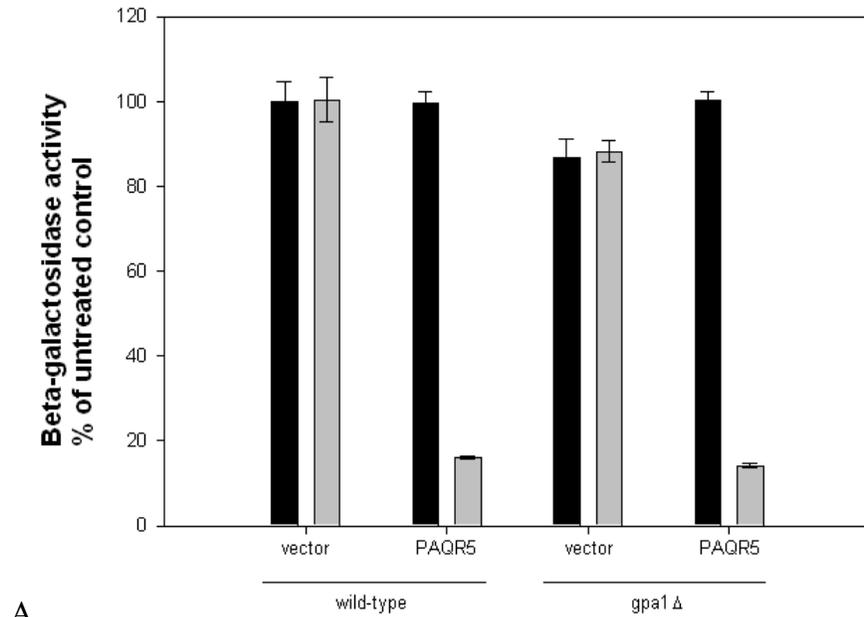
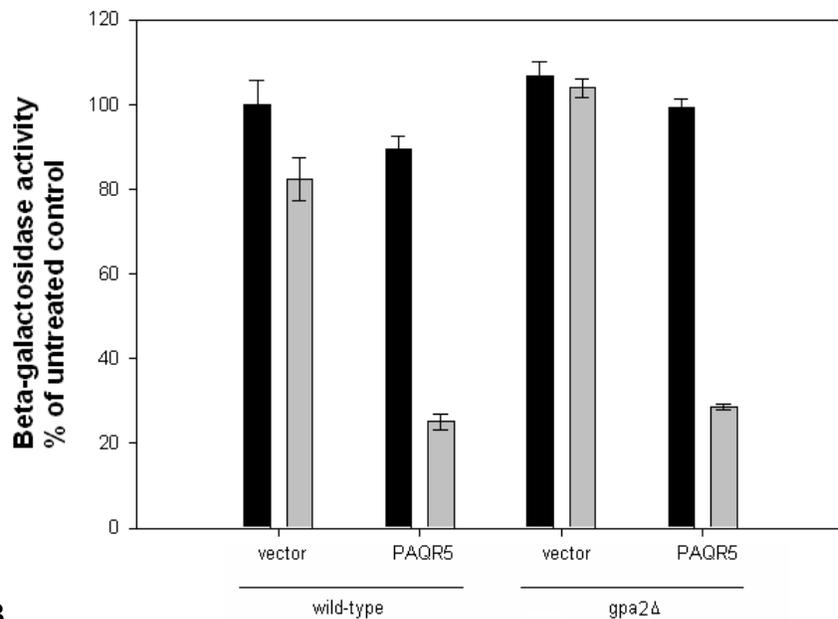


Figure 2-7. Class II PAQR signaling model. A portion of the model proposed in the literature (Thomas et al., 2007; Dressing and Thomas, 2007) is presented here. This model shows that the Class II PAQRs are localized to the plasma membrane, are activated by progesterone, and are coupled to inhibitory G-proteins. Upon activation of G<sub>i</sub>-proteins, GDP bound to the G<sub>i</sub>-proteins is converted to GTP, which causes inactivation of adenylate cyclases and a subsequent decrease in cAMP, leading to decreased PKA activity. [This figure was modified from Dressing GE and Thomas P (2007) Identification of membrane progestin receptors in human breast cancer cell lines and biopsies and their potential involvement in breast cancer. *Steroids* 72:111-116 with permission from Elsevier (Figure 3, page 114).]



A



B

Figure 2-8. Repression of *FET3-lacZ* does not require yeast  $G\alpha$ -proteins. In panel A, the repression of *FET3-lacZ* by PAQR5 still occurs in a *gpa1* mutant (*gpa1*Δ). In panel B, the repression of *FET3-lacZ* by PAQR5 still occurs in a *gpa2* mutant (*gpa2*Δ). For both panels, the black bars are for untreated cultures, and the gray bars are for cultures treated with progesterone (100 nM) in LIM containing 0.05% galactose.

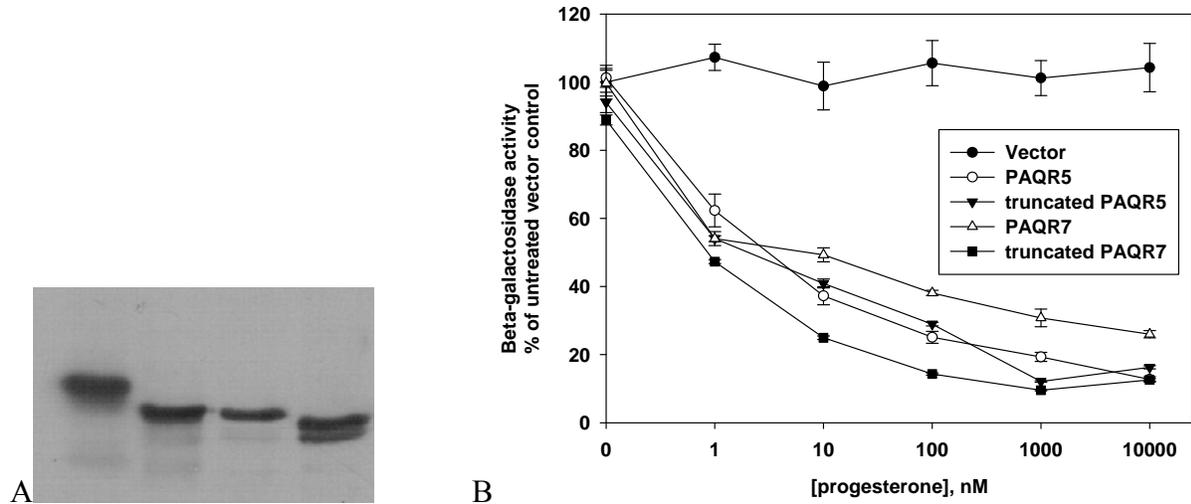


Figure 2-9. Expression of truncated human Class II PAQRs cause ligand-dependent repression of *FET3-lacZ*. In panel A, Western blots were performed on yeast total membrane preparations on cells expressing full-length PAQR5 (lane 1), truncated PAQR5 (lane 2), full-length PAQR7 (lane 3), and truncated PAQR7 (lane 4). In panel B, yeast cells doubly transformed with the *pFET3-lacZ* vector and *GAL1-7xHA-PAQR* vector were grown in LIM containing  $1 \mu\text{M Fe}^{3+}$  and 0.05% galactose.  $\beta$ -galactosidase assays were performed as described in the Materials and Methods. Without the last hydrophobic domain, the truncated PAQRs still cause ligand-dependent repression of *FET3-lacZ*.

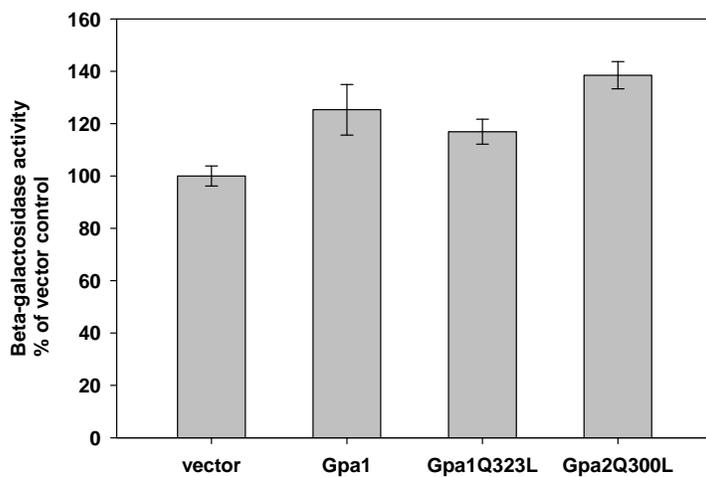


Figure 2-10. Yeast cells that express constitutively active yeast  $G_{\alpha}$  proteins do not demonstrate repression of *FET3-lacZ*. Wild-type Gpa1p, or constitutively active forms of the yeast  $G_{\alpha}$ -proteins Gpa1p (Gpa1<sup>Q323L</sup>) or Gpa2p (Gpa2<sup>Q300L</sup>) were constitutively expressed by the *ADHI* promoter. Yeast cells doubly transformed with the *pFET3-lacZ* vector and *ADHI-Gpa* vector were grown in LIM containing  $1 \mu\text{M Fe}^{3+}$  and 2% galactose and  $\beta$ -galactosidase assays were performed as described in the Material and Methods.

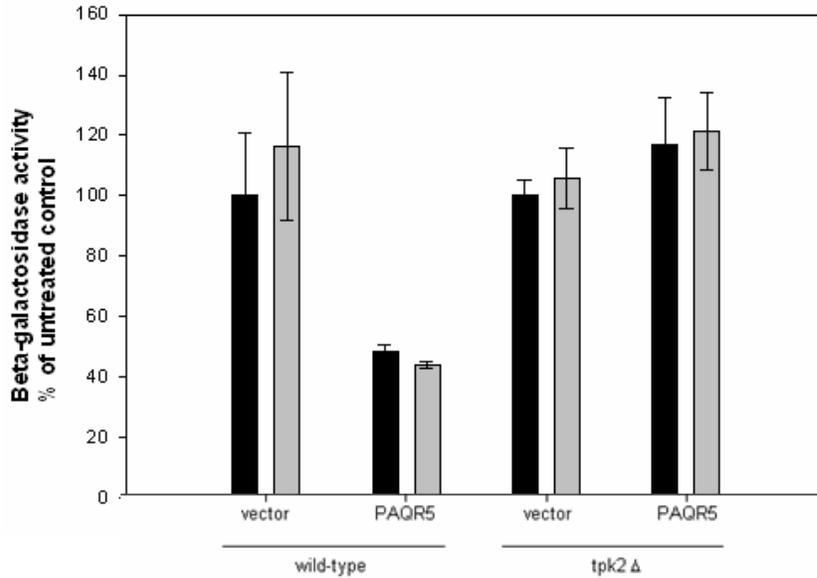


Figure 2-11. Repression of *FET3-lacZ* requires yeast Tpk2p, a subunit for the yeast homologue of human PKA. The repression of *FET3-lacZ* by PAQR5 does not occur in a *tpk2Δ* strain. The black bars are for untreated cultures, and the gray bars are for cultures treated with progesterone (100 nM) in LIM containing 2% galactose.

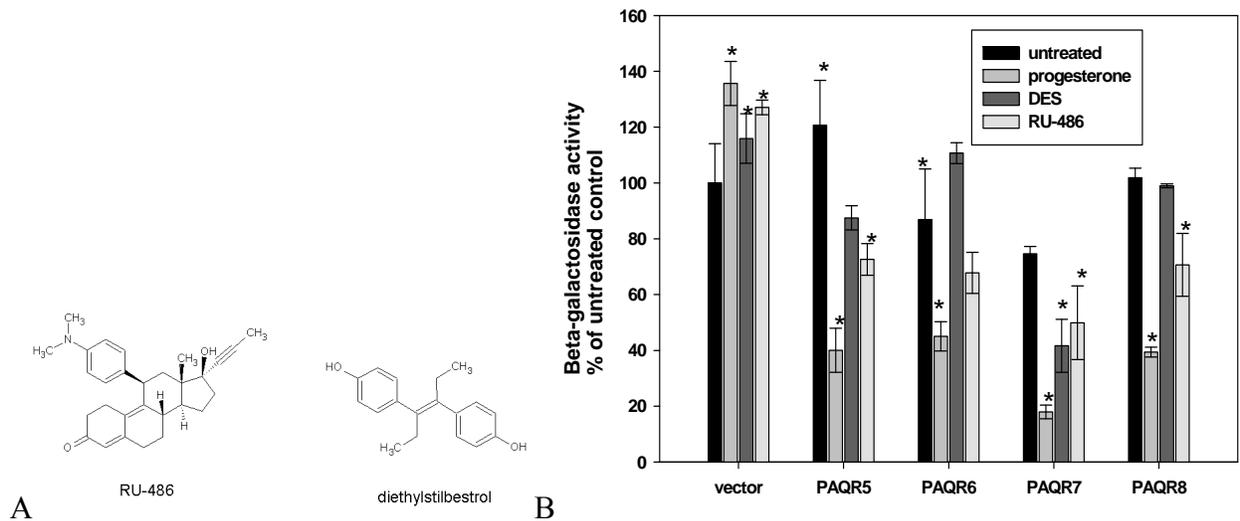


Figure 2-12. Synthetic compounds tested for activation of Class II PAQRs. In panel A, the structures of RU-486 and diethylstilbestrol are shown. In panel B, the effect of 10  $\mu$ M RU-486 or diethylstilbestrol on the Class II PAQR-mediated repression of *FET3-lacZ* is demonstrated. Yeast cells doubly transformed with the *pFET3-lacZ* vector and *GALI-7xHA-PAQR* vector were grown in LIM containing 1  $\mu$ M  $Fe^{3+}$  and 0.05% galactose.  $\beta$ -galactosidase assays were performed as described in the Materials and Methods. \*,  $P \leq 0.05$ .

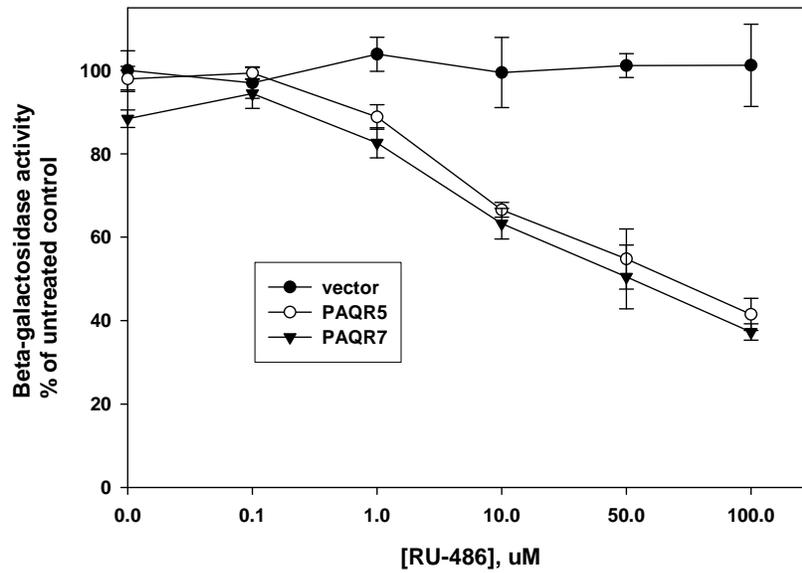


Figure 2-13. Dose response of PAQR7 to RU-486. The effect of varying concentrations of RU-486 on PAQR7-mediated repression of *FET3-lacZ* is demonstrated. Yeast cells doubly transformed with the p*FET3-lacZ* vector and *GAL1-7xHA-PAQR* vector were grown in LIM containing 1  $\mu\text{M}$   $\text{Fe}^{3+}$  and 0.05% galactose.  $\beta$ -galactosidase assays were performed as described in the Materials and Methods. These data were collected in collaboration with Brian Kupchak.

Table 2-1. Primers used for cloning PAQRs into pGREG536 (listed from 5'→3')

Primer name	Primer sequence
Fwd PAQR5	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGCTGAGCCTGAAGCT
Rev PAQR5	GCGTGACATAACTAATTACATGACTCGAGGTCGACTCATGTTTCTTTTTTATGTAAT TCTGG
Rev Trunc5	GCGTGACATAACTAATTACATGACTCGAGGTCGACTCAGGGCTTGGAGGTGGCCAG
Fwd PAQR6	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGCTCAGTCTCAAGCTGC
Rev PAQR6	GCGTGACATAACTAATTACATGACTCGAGGTCGACTTACTGTTGTTTGGCCTGGG
Fwd PAQR7	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGGCCATGGCCCAGAAA
Rev PAQR7	GCGTGACATAACTAATTACATGACTCGAGGTCGACTCACTTGGTCTTCTGATCAA
Rev Trunc7	GCGTGACATAACTAATTACATGACTCGAGGTCGACTCAGTGC GTGTGCAGAGGCTC
Fwd PAQR8	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGACGACCGCCATCTTGG
Rev PAQR8	GCGTGACATAACTAATTACATGACTCGAGGTCGACTCAGGAATCTTTCTTGGTCAG

Table 2-2. Codons in PAQR sequences that are rarely used in *S. cerevisiae*. Rare codon analysis was conducted with the Gene Designer software (Villalobos et al., 2006).

Gene name	NCBI Accession	Number of codons used with <5% frequency	Number of codons used with <10% frequency
PAQR5	Q9NXX6	2	17
PAQR6	Q6TCH4	4	30
PAQR7	Q86WK9	4	24
PAQR8	Q8TEZ7	4	25
PAQR9	Q6ZVX9	5	25

## CHAPTER 3 CLASS II PAQRs: LOCALIZATION AND TOPOLOGY

### **Introduction**

The Class II PAQRs consist of five proteins, PAQRs 5-9. PAQR5, PAQR7, and PAQR8 are debated as being G-protein coupled receptors for progesterone that localize to the plasma membrane (Thomas et al. 2007, Krietsch et al. 2006) while PAQR6 and PAQR9 have yet to be characterized. In addition to their localization, the topology of the Class II PAQRs is also uncertain.

### **Class II PAQR Localization?**

Studies which demonstrate that PAQR5, PAQR7, and PAQR8 are involved in mediating nonclassical effects of progesterone have also shown that these proteins are plasma membrane localized (Thomas et al., 2007). However, another group, which found no evidence to support that the Class II PAQRs mediate nonclassical effects of progesterone, showed that PAQR5, PAQR7, and PAQR8 localize to the endoplasmic reticulum (Krietsch et al., 2006). Other studies have shown that PAQR7 localizes to an “intracellular tubuloreticular network” (Fernandes et al., 2005) and that PAQR8 is associated with lysosomes (Suzuki et al., 2001). So the cellular location of the Class II PAQRs is unclear.

### **Class II PAQR Topology?**

Other points of contention surrounding the Class II PAQRs are their topology and whether they act as G-protein coupled receptors for progesterone. Besides evidence from second messenger and G-protein inhibitor assays, immunofluorescence studies of the human Class II PAQRs in mammalian cells (Thomas et al., 2007) suggests that these proteins have an orientation in the membrane that is similar to that of GPCRs, with an extracellular N-terminus and an intracellular C-terminus (Thomas et al., 2007).

In contrast to the Class II PAQRs, immunofluorescence studies showed that the human Class I PAQRs expressed in mammalian cell systems have the N-terminus located inside the cell and the C-terminus located outside the cell (Yamauchi et al., 2003). A global topology study of yeast membrane proteins (Kim et al., 2006) suggested that the yeast Class I PAQRs have the same topology as the human Class I PAQRs. Finally, while a topology study of the human Class III PAQRs has not been conducted, the Class III homologue from *Escherichia coli* (called YqfA) was characterized in *E. coli* and was found to have a Class I PAQR-like topology (Daley et al., 2005).

If the topological determinations reported for the PAQR family members thus far are correct, then the three classes of PAQRs do not share similar structural orientations. Instead, the Class II PAQRs may have a topology that is similar to that of the GPCR superfamily of proteins. Because the localization and topology of the Class II PAQRs is unclear, we were interested in studying each of these characteristics in the yeast model organism as well and our approaches are described in this chapter.

### **Determining Protein Localization and Topology in Yeast**

There are numerous approaches that can be used to localize proteins in cells. The techniques that are often used with yeast and that have been used in the work for this dissertation are described below.

#### **Green Fluorescent Protein**

One approach for localization of proteins in yeast is to use green fluorescent protein (GFP) to tag the proteins of interest (Huh, et al., 2003). The GFP protein, a 238 amino acid protein, has a  $\beta$ -can fold, with a  $\beta$ -sheet surrounding a  $\alpha$ -helix (Yang et al., 1996). The fluorophore in the wild-type GFP is formed by spontaneous cyclization of Ser65 and Gly67 coupled with reduction of the  $C_{\alpha}$ - $C_{\beta}$  bond of Tyr66 to form a conjugated system (Yang et al., 1996).

Many mutants of GFP have been engineered for various applications (Shaner et al., 2005). One of these mutants is the S65T mutant, which folds four times more rapidly (2 hours) than the wild-type GFP (8 hours) and has increased fluorescence and photostability (Heim et al., 1995). This mutant has been used successfully when fused to other proteins for protein localization in subcellular locations such as the yeast plasma membrane and endoplasmic reticulum (Huh et al., 2003; Jansen et al., 2005). Both of these locations have been proposed for the Class II PAQRs (Thomas et al., 2007; Krietsch et al., 2006; Fernandes et al., 2005).

### **Immunofluorescence**

Alternatively, immunofluorescence has been successfully used to localize proteins in yeast (Pringle et al., 1991) as well as determine their topology in the plasma membrane (Severance et al., 2004). For either of these methods, spheroplasts, which are yeast cells without their cell walls, must be made to allow access of the antibody to the cell (Baggett et al., 2003). For localization, conditions which permeabilize the cells are used so that the primary antibody can access the epitope, whether it is located inside or outside the cell (Baggett et al., 2003). A fluorophore-labeled secondary antibody is then used for detection of the primary antibody (Baggett et al., 2003). For topology studies, conditions which permeabilize or keep the cells unpermeabilized are used (Severance et al., 2004). So if the epitope is located inside the cell, it will only be detected under cell permeabilization conditions; however, if the epitope is located outside the cell, it can be detected whether the cells are permeabilized or not.

### **The 3xHA-Suc2-His4C Dual Topology Reporter (DTR)**

Finally, an alternative method that has been widely used for topology studies in yeast is the 125 kDa 3xHA-Suc2-His4C dual-topology reporter (DTR) (Figure 3-1) (Sengstag, 2000). The DTR is useful for proteins which are located in the membranes of some organelles, such as the ER, or the plasma membrane (Kim et al., 2003). When this method was used in a global

topology study of yeast membrane proteins, the yeast Class I PAQRs (the IZHs) were found to have the C-terminus located outside the cytoplasm (Kim et al., 2006). The DTR has also been successfully used to determine the topology of a yeast ubiquitin ligase and its human homologue (Kreft et al., 2006).

For topology studies, the DTR is fused to the C-terminus of membrane proteins to determine whether this part of the protein is cytoplasmic or not (Sengstag, 2000). First, the *HIS4C* portion of the tag encodes the full-length yeast histidinol dehydrogenase, which can only convert the substrate histidinol to histidine if His4Cp is present in the cytoplasm. If His4Cp is located in the ER, histidinol can not be converted to histidine because polar, charged molecules such as histidinol do not penetrate the ER membrane (Deshaies and Schekman, 1987; Kim et al., 2003). The *SUC2* portion encodes a part of the enzyme invertase that has several acceptor sites for *N*-linked glycosylation, as does the His4Cp portion. These sites do not become glycosylated unless they have been exposed to the lumen of the ER during protein folding (Kim et al., 2003). The topology of the protein determined during folding and insertion in the ER membrane is maintained during transport to other cell membranes (Alberts et al., 2002). Thus, if the protein under study has a topology such that the Suc2p-His4Cp tag is located in the cytoplasm during folding in the ER membrane, the tag does not become glycosylated. This topology will be maintained such that the tag remains in the cytoplasm after the protein is sorted to its appropriate subcellular location.

To determine the topology of a DTR-tagged protein, two assays are performed. First, growth assays on histidinol-containing plates lacking histidine are performed in a yeast strain that is *his4<sup>-</sup>* (STY50) (Sengstag, 2000). The plates are typically grown at 30° C from 3-5 days and the presence of growth is determined (Sengstag, 2000). If the yeast cells grow on histidinol,

it is concluded that the C-terminus is located in the cytoplasm; however, errors in topology determinations have been made when this was the only assay performed and intermediate growth levels were observed (Sengstag, 2000). Therefore, a second assay is performed in which the glycosylation status of the expressed protein is determined with endoglycosidase H (Endo H) digestion (Sengstag, 2000; Kim et al., 2003). Western blots are performed with an anti-HA antibody to detect whether there is a mobility shift of the 3xHA-Suc2-His4C tagged protein after Endo H treatment (Sengstag, 2000; Kim et al., 2003). If the protein has evidence of glycosylation, it is concluded that the C-terminus has translocated to the ER lumen (Kim et al. 2003). Usually, the localization assignment of the C-terminus is made only if the results of the two assays are in agreement (i.e. if there is growth on histidinol, then there should be no glycosylation of the protein and the C-terminus is assigned to a cytoplasmic location. Alternatively, if there is no growth on histidinol, then the protein should be glycosylated, indicating that the C-terminus of the protein was located in the ER lumen during secretion and is considered to be extracytoplasmic) (Kim et al., 2003; Kim et al., 2006).

This chapter describes attempts to localize the Class II PAQRs with GFP fluorescence and immunofluorescence. It also describes attempts to determine the topology of the proteins by immunofluorescence and the DTR 3xHA-Suc2-His4C.

## **Results and Discussion**

Previously, experimental evidence has been published that shows plasma membrane localization for the Class II PAQRs and that these proteins function as receptors for progestin steroids (Thomas et al., 2007). However, it has also been published that the Class II PAQRs are localized to the endoplasmic reticulum and are not progestin receptors (Krietsch et al., 2006). Many attempts have been made to localize the Class II PAQRs in mammalian cells, with varying results (Thomas et al., 2007; Krietsch et al., 2006; Fernandes et al., 2005; Suzuki et al., 2001). In

addition, although data suggest that the Class II PAQRs have a topology similar to that of GPCRs (Thomas et al., 2007), other members of the PAQR family have the opposite topology (Yamauchi et al., 2003; Kim et al. 2006, Daley et al., 2005). So the cellular location and topology of the Class II PAQRs is unclear. Because our yeast system has demonstrated functional expression of the PAQR proteins, we wanted to determine the localization and topology of the Class II PAQRs in this system. The results of the attempts to do so are described below.

### **Localization of Class II PAQRs with GFP**

GFP has been used successfully to determine the locations of many yeast proteins (Huh et al., 2003). To try to determine where the Class II PAQRs were expressed in the cell, PAQR5 and PAQR7 were tagged at the N-terminus with the S65T mutant variant of GFP using the pGREG575 vector (Jansen et al., 2005). The constructs were tested in *FET3-lacZ* assays and were able to cause repression of *FET3-lacZ* in low iron media (Figure 3-2). Despite this, neither of the GFP constructs showed fluorescence as observed with an epifluorescence microscope, while expression of soluble GFP alone showed much fluorescence localized to the cytoplasm (Figure 3-3). Some of the Class I PAQRs cloned into the same vector (pGREG575) fluoresced and seemed to localize around the periphery of the cell (Garitaonandia, 2008). Western blots were performed using total membrane preparations from yeast cells expressing GFP-PAQR5 and GFP-PAQR7 and showed that neither of the constructs could be detected by Western blot (Figure 3-4).

C-terminal GFP-tagged constructs for PAQR5, PAQR6, PAQR7, and PAQR8 were made and the cells expressing these constructs were observed with a fluorescence microscope. *FET3-lacZ* assays were performed and the C-terminally GFP-tagged Class II PAQRs were able to respond to progesterone (Figure 3-5); however, fluorescence could not be observed for any of the

constructs (data not shown). Western blots were performed using total membrane preparations from yeast cells expressing the C-terminally GFP-tagged PAQRs and bands for PAQR5-GFP and PAQR6-GFP were observed (Figure 3-6), while bands for PAQR7-GFP (Figure 3-6) and PAQR8-GFP were not detected (Figure 3-4). It should be noted that the bands for PAQR5-GFP and PAQR6-GFP were located at around 50 kDa, approximately 15 kDa lower than expected. Whether this disparity is due to a lack of a full-length GFP tag is unknown, but if it is, this could be a reason for a lack of signal during fluorescence microscopy. An alternative explanation for a lack of fluorescence is that the GFP tag could be misfolded but not degraded. According to Wooding and Pelham (1998), GFP may not fold properly in the yeast ER lumen.

The cause for the lack of detection of either the N-terminally GFP-tagged PAQR5 and PAQR7 or the C-terminally GFP-tagged PAQR7 and PAQR8 by fluorescence microscopy or Western blot with chemiluminescence is unknown and there are several possible reasons. For instance, these proteins may not be expressing in sufficient quantities for observation. The addition of a GFP tag to a protein could cause the protein to misfold and lead to degradation of both the protein and the GFP tag (Pedelacq et al., 2006). Also, it is possible that the GFP tag is cleaved for some of the constructs, but not all. Regardless, all of the constructs were able to express in sufficient quantity to allow for progesterone-dependent repression of *FET3-lacZ*. This may be because PAQR-mediated repression of *FET3-lacZ* does not require much expression of the PAQR (see Figure 2-4A).

### **Protein Localization of Class II PAQRs by Immunofluorescence**

Because expression of PAQR5-GFP and PAQR6-GFP was detectable by Western blot, localization studies using immunofluorescence were continued with these constructs. In addition, HA-PAQR5 and HA-PAQR6 were also used for localization studies using immunofluorescence.

Although numerous attempts were made, only a few spheroplasts appeared in the brightfield light images that were also visible in the fluorescence images. Examples of images for these few spheroplasts are presented here. For negative control cells stained with the anti-GFP primary antibody (Figure 3-7A) or the anti-HA primary antibody (Figure 3-7E), there appears to be minimal fluorescence, indicating that the conditions used were sufficient to reduce nonspecific binding of primary and secondary antibodies. The fluorescence that was observed for the PAQR5-GFP, PAQR6-GFP, HA-PAQR5 and HA-PAQR6 appears to outline the perimeter of the cell (Figure 3-7 C, D, F, G), suggesting a possible plasma membrane localization for these proteins. For soluble GFP, the fluorescence appears to be distributed throughout the whole cell (Figure 3-7B).

### **Topology of Class II PAQRs by Immunofluorescence and the Dual Topology Reporter**

To determine the topology of the Class II PAQRs, immunofluorescence was performed with and without permeabilization of yeast spheroplasts for the N-terminally HA-tagged PAQR5 and PAQR6 and the C-terminally GFP-tagged PAQR5 and PAQR6. In similar topology studies of the PAQRs expressed in mammalian cells, immunofluorescence studies showed that the human Class I PAQRs expressed in mammalian cell systems have the N-terminus located inside the cell and the C-terminus located outside the cell (Yamauchi et al., 2003). In contrast, immunofluorescence studies in human cells transfected with human PAQR7 expression plasmids demonstrated that the N-terminus could be clearly detected without permeabilization of the cells, while detection of the C-terminus required cell permeabilization (Thomas et al., 2007). These results suggest that the human Class II PAQRs have an orientation in the membrane that is the opposite to that of the Class I PAQRs, with an extracellular N-terminus and an intracellular C-terminus. Therefore, an immunofluorescence approach to topology determination was used for the Class II PAQRs expressed in yeast.

Unfortunately, the negative control for the unpermeabilized conditions (cytoplasmic GFP) was detected during every attempt (Figure 3-8). This problem occurs because many of the steps involved in yeast spheroplast generation and manipulation cause unwanted permeabilization of the cells (Dr. Colin Macdiarmid, University of Missouri, personal communication). So, although similar experiments were conducted for PAQR5 and PAQR6 (data not shown), no conclusions could be made regarding the location of their N- or C-termini.

An alternative method for topology determination in yeast was used. To determine PAQR C-terminal locations, PAQR6 and PAQR7 were tagged with 3xHA-Suc2-His4C (Figure 3-1). These fusions were tested in *FET3-lacZ* assays to ensure that the proteins retained their ability to repress the *FET3* promoter (Figure 3-9). When histidinol growth assays were conducted with C-terminally 3xHA-Suc2-His4C tagged human PAQRs, growth at 30°C after 3 days was not apparent. However, when plates were incubated longer (5-8 days), STY50 cells expressing tagged PAQRs had intermediate levels of growth (Figure 3-10) when compared to STY50 cells that were expressing the positive control Ost4-3xHA-Suc2-His4C or the negative controls 7xHA-PAQR6 and 7xHA-PAQR7. (The negative controls show slight growth and this is likely due to the presence of residual histidine from the media used prior to the histidinol growth experiments). Although this result suggests that the C-terminus of the PAQRs is located in the cytoplasm, intermediate growth phenotypes on histidinol are not conclusive (Sengstag, 2000). Therefore, the glycosylation status of the Suc2 portion of the protein was determined (Figure 3-11). A shift in PAQR-3xHA-Suc2-His4C migration upon Endo H treatment was not observed. Prior to Endo H treatment, immunoprecipitation was performed, and the immunoprecipitated proteins were released from the agarose beads with SDS sample buffer containing 2% SDS. To make sure that the Endo H was still active in this buffer, a reaction was performed using RNase

B as a positive control. A shift in the migration of the undigested versus the digested RNase B demonstrated that the conditions used for the Endo H reaction (SDS sample buffer with 2% SDS) did not inhibit the activity of Endo H (Figure 3-12).

The positive results for the histidinol growth assay and the lack of glycosylation indicate that the C-termini of PAQR6 and PAQR7 are located in the cytoplasm of the cell. However, because histidinol growth assays are normally carried out for 3-5 days, and only intermediate levels of growth were observed for the DTR-tagged PAQR6 and PAQR7 after 7-8 days, it is possible that the assignment of the C-termini of PAQR6 and PAQR7 to the cytoplasm is incorrect. Because they express with higher yields in yeast (Figure 2-5A), DTR-tagged PAQR5 and PAQR8, might have stronger growth phenotypes on histidinol, which would be a better indicator of a cytoplasmic location for the C-termini of the Class II PAQRs. Furthermore, as was previously done with another human membrane protein (Kreft et al., 2006), a more detailed analysis of topology using the DTR tag in combination with various PAQR truncations could also lead to better conclusions about the topology of these proteins.

If the Class II PAQRs have only seven transmembrane domains, and the C-termini are located inside the cell, then the Class II PAQRs have a topology that is similar to GPCRs, but highly divergent from the other PAQRs, as has been previously reported (Thomas et al., 2007).

A few examples of closely related membrane proteins that have opposite topologies have been reported (Sääf et al., 1999; Rapp et al., 2006). Topology is largely determined by the number of lysines and arginines in the loop regions of membrane proteins, with a bias towards having more positively charged residues (“K+R bias”) in the cytoplasm (von Heijne, 1989). In fact, single-charge point mutations can alter the topology of membrane proteins that have a low K+R bias, demonstrating that even highly related proteins can have opposite topologies (Rapp et

al., 2006). So, even though the PAQRs are related to each other, there are quite a few differences in their sequences, and it is possible that the different classes of PAQRs have different topologies.

## **Materials and Methods**

### **Plasmids.**

Human PAQR5, PAQR6, PAQR7, and PAQR8 were sub-cloned from expression plasmids previously constructed by other lab members (Lisa Regalla and Julie Russell). Primer sequences were designed to amplify the gene and included bases for homologous recombination into pGREG575 for N-terminal GFP-tagging of PAQRs (Jansen et al., 2005), pGREG600 for C-terminal GFP-tagging of PAQRs (Jansen et al., 2005), or pJK90 for C-terminal 3xHA-Suc2-His4C tagging of PAQRs (Kim et al., 2003) (Table 3-1). All plasmids except for pJK90 allowed for *GALI* promoter-driven expression of PAQR proteins. The pJK90 plasmid allowed for *TPI* promoter-driven expression of proteins (Kim et al., 2003). Cloning was performed by gap repair.

### **Yeast Strains and Assays.**

All yeast strains used in this study were obtained from Euroscarf unless otherwise noted. All growth conditions and  $\beta$ -galactosidase assays were performed as previously described (Kupchak et al., 2007). Briefly, cells were grown in Low Iron Medium (LIM) (Eide and Guarente 1992) supplemented with 1  $\mu$ M  $\text{Fe}^{3+}$  to induce the expression of *FET3*.

Galactose was used as a carbon source to induce full target protein expression (2%) or reduced expression (0.05% galactose/1.95% raffinose). Cells were harvested, washed and permeabilized prior to assay for  $\beta$ -galactosidase activity (*lacZ*).  $\beta$ -galactosidase assays were performed on permeabilized cells as described (Guarente, 1983).  $\beta$ -galactosidase activity is presented as a percentage of activity seen in cells expressing empty expression vector treated

with vehicle (ethanol). For individual experiments, each data point was done in triplicate and the error bars represent +/- 1 standard deviation. Experiments were performed at least three times and a representative experiment is shown. All constructs, including the GFP-tagged PAQRs, were tested for the ability to repress *FET3-lacZ* in a progesterone-dependent manner.

### **Expression of GFP-tagged Class II PAQRs.**

For Class II PAQR localization studies, overnight cultures in SD media were reinoculated to an OD<sub>600</sub> equal to approximately 0.2 into LIM containing 2% galactose for full target protein expression at 30°C. After the OD<sub>600</sub> reached 1, the cultures (150 mL) were harvested for total membrane protein preparations, washed twice with 25 mL of cold water, stored at -80°C before total membrane extraction (see below). Alternatively, the cultures (5 mL) were used directly in fluorescence microscopy experiments (5 mL).

### **Total Membrane Protein Extraction and Detection by Western Blot.**

Total membrane protein samples were prepared as described in Chapter 2. For GFP-tagged PAQRs, the primary antibody (anti-GFP rabbit IgG fraction, A11122, Invitrogen) was diluted 1:1000 in PBS containing 1% BSA. The secondary antibody (goat anti-rabbit IgG-HRP (SC-2004, Santa Cruz Biotechnology, Inc.) was diluted 1:10000. The blot was incubated with SuperSignal® West Pico Substrate Working Solution (Pierce) for 5 minutes, exposed to film and developed. The membrane was stripped with Restore™ Western Blot Stripping Buffer (21059, Thermo Scientific) according to the manufacturer's directions and re-probed with an anti-yeast mitochondrial porin primary antibody (diluted to 0.5 µg/mL, anti-porin yeast mitochondrial mouse IgG<sub>1</sub>, monoclonal antibody, A6449, Invitrogen). The secondary antibody was diluted 1:10000 (goat anti-mouse IgG (H+L)-HRP, 170-6516, Bio-Rad). Again, the blot was incubated with SuperSignal® West Pico Substrate Working Solution (Pierce) for 5 minutes, exposed to film and developed.

### **Immunofluorescence of *S. cerevisiae* Expressing the Class II PAQRs.**

To determine the localization of overexpressed Class II PAQRs by immunofluorescence, a procedure obtained from Dr. Colin Macdiarmid (University of Missouri) was modified. Overnight cultures in SD media were reinoculated to an OD<sub>600</sub> equal to approximately 0.2 in 25 mL of LIM containing 2% galactose and incubated at 30°C. After the OD<sub>600</sub> was 0.8-1, the cells were harvested at 1000 x g and washed twice with PBS. The cells were resuspended in 9 mL of PBS and 1 mL of 37% formaldehyde (BP531, Fisher Scientific) was added. The cells were incubated at 30°C for 2 hours with agitation (230 RPM). The cells were washed twice with 10 mL of cold PBS and resuspended in 2 mL of cold PBS containing 10 mM dithiothreitol. The OD<sub>600</sub> was determined and a solution of Zymolyase 20T was added at a ratio of 2 units per OD<sub>600</sub> to remove the cell wall. The cells were incubated for 2 hours at 30°C with gentle agitation (100 RPM). The spheroplasts were collected by centrifugation (200 x g), washed twice with 10 mL of PBS, and resuspended in either 5 mL of cold methanol for permeabilization or 5 mL of PBS for unpermeabilized spheroplasts. The permeabilized spheroplasts were stored at -20°C for 1 hour, while the unpermeabilized spheroplasts were stored at 4°C for 1 hour. The spheroplasts were washed twice with 5 mL of PBS and resuspended in 100 µL of PBS. An aliquot (50 µL) of spheroplasts was applied to the well of a slide pre-treated with poly-L-lysine. The slides were incubated for 1 hour at 4°C to allow cells to adhere and the slides were washed twice with PBS to remove unbound cells. The slides were blocked for 1 hour at 4°C in PBS containing BSA (1%). For permeabilized spheroplasts, the blocking solution also contained 0.1% Tween-20. The primary antibody was diluted (1:250, anti-GFP rabbit IgG fraction, A11122, Invitrogen or 1:50, rabbit polyclonal IgG HA-probe (Y-11), SC-805, Santa Cruz Biotechnology Inc.) in the appropriate blocking buffer and incubated with the spheroplasts for 1 hour at room temperature.

The slides were washed 5 times for 5 minutes each with PBS either without or with 0.5% Tween-20 for unpermeabilized or permeabilized spheroplasts, respectively. The procedure was repeated with the fluorescently labeled secondary antibody (Alexa Fluor® 555 goat anti-rabbit IgG, A31629) diluted 1:200 in the appropriate blocking buffer. After the last wash step, 1 drop of Mowiol solution was added to the slide and a coverslip was placed over the well. Slides were allowed to harden overnight at 4°C before viewing.

### **Fluorescence Microscopy Experiments.**

For Class II PAQR localization studies, overnight cultures in SD media were reinoculated into LIM containing 2% galactose for full target protein expression at 30°C. For GFP fluorescence, after 18-24 hours of growth, a small aliquot of cells was placed on a slide coated in poly-L-lysine (7799, Lab Scientific, Inc.) and coverslip was placed on top of the cells. A Zeiss Axiovert s100 microscope with a 63X objective was used (courtesy of Dr. R.J. Cousins, University of Florida). The same microscope was used for visualization of immunostained spheroplasts or an Olympus FV500-IX81 confocal microscope with a 100X objective was used (courtesy of Dr. W. Tan, University of Florida).

### **Histidinol Growth Assays.**

Experiments were conducted essentially as described (Kim et al., 2003). STY50 transformants containing each of the 3xHA-Suc2-His4C-tagged proteins were streaked onto SD containing histidine (0.6 mM) or histidinol (6 mM) plates. Alternatively, cell pellets from liquid cultures in SD containing histidine (0.6 mM) were washed with sterile water. The optical density at 600 nm ( $OD_{600}$ ) was adjusted to 0.5, 0.1, and 0.01. Aliquots of 5  $\mu$ L of each dilution were spotted onto SD-Ura containing histidine (0.6 mM) or histidinol (6 mM) plates. All histidinol assays were incubated at 30°C and monitored for 3-8 days.

## **Glycosylation Analysis.**

Experiments were conducted essentially as described (Kim et al., 2003). Briefly, STY50 transformants were grown to OD<sub>600</sub> 0.8 to 1 in 250 ml of SD-Ura. After cells were harvested, the pellets were washed with dH<sub>2</sub>O and stored at -80°C. Frozen cells were resuspended in SDS sample buffer (1 mL per 50 mL of cells, 50 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 50 mM dithiothreitol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Roche Diagnostics, Inc.), 0.01% bromphenol blue), incubated at 60°C for 10 to 15 min and centrifuged for 10 min at 13,000 rpm in an Eppendorf microfuge. Soluble fractions were transferred to new tubes. The solution was adjusted to contain 80 mM potassium acetate, pH 5.6. Endo H (0.01 Units, product #11088726001, Roche Diagnostics, Inc.) was added. Samples were incubated at 37°C for 2 hours. Negative control samples were treated and incubated similarly but without Endo H. RNase B (P7817S, New England BioLabs, Inc.) was used as a positive control for the Endo H reaction and was separated on a 10% polyacrylamide gel that was subsequently stained with Coomassie Blue dye. Solubilized proteins from the 3xHA-Suc2-His4C samples were separated on 6.5% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and the membranes were probed with HRP-conjugated anti-HA antibody (Santa Cruz Biotechnology, Inc.). Dual Color Precision Plus Protein<sup>TM</sup> Standards (161-0374, Bio-Rad) were used for molecular weight markers. Alternatively, frozen cell pellets were lysed with glass beads in MIB buffer as described above for total membrane protein isolation. After the samples were centrifuged at 3000 x g for 10 minutes, the supernatant was removed and immunoprecipitation was performed with agarose beads conjugated to anti-HA mouse monoclonal IgG<sub>2a</sub> (HA-probe (F-7): sc7392 AC, Santa Cruz Biotechnology) at 4°C with constant shaking (800 rpm) overnight. The samples were washed three times with 1X phosphate-buffered saline (PBS) (Fisher Scientific) and resuspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 80 mM potassium

acetate, 5% glycerol, 5% SDS, 50 mM dithiothreitol, 5 mM EDTA, 0.01% bromphenol blue). The samples were treated with Endo H (0.01 Units, Roche Diagnostics, Inc.) and incubated at 37°C for 2 hours. Control samples were treated and incubated similarly but without Endo H. Solublized proteins were separated on 6.5% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with the primary antibody (rabbit polyclonal IgG HA-probe (Y-11), SC-805, Santa Cruz Biotechnology, Inc.) diluted 1:500 in PBS containing 1% BSA. The secondary antibody used was horse radish peroxidase-conjugated goat anti-rabbit IgG-HRP (1:10000, SC-2004, Santa Cruz Biotechnology, Inc.).

### **Summation**

In this chapter, different techniques were used to try to determine PAQR localization (using GFP and immunofluorescence) and PAQR topology (using immunofluorescence and the DTR). As described in the Results and Discussion, numerous problems were encountered which prevented strong conclusions from being made. For localization, these problems included a lack of GFP expression detection (by Western blot and fluorescence microscopy) and a lack of optimized conditions for immunofluorescence. For topology, based on the intermediate growth on histidinol plates and a lack of glycosylation of the DTR, it was concluded that the C-terminus of PAQR6 and PAQR7 are located in the cytoplasm, which is different from the Class I and Class III PAQRs, (Yamauchi et al., 2003; Kim et al., 2006; Daley et al., 2005).

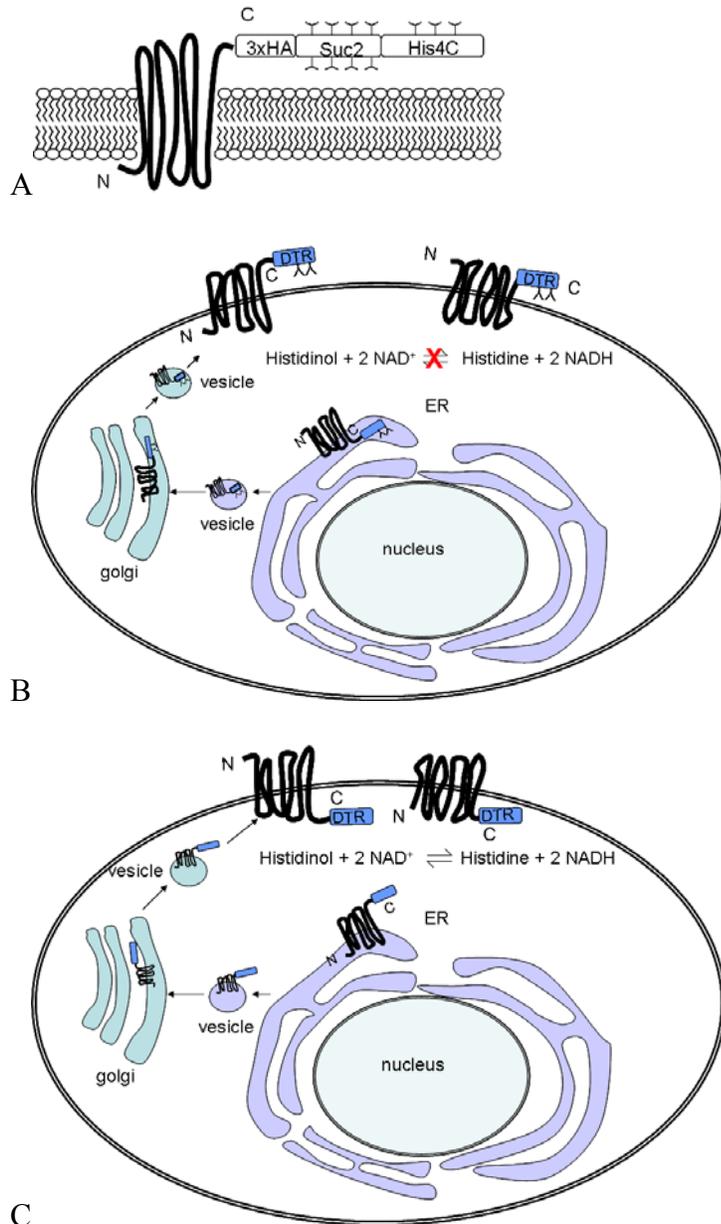


Figure 3-1. The yeast dual-topology reporter (DTR). The DTR is a tag on the C-terminal end of a membrane protein. In panel A, the DTR structure is shown with a 3x-HA tag for detection of expression via Western blotting and with glycosylation sites on the Suc2 and His4C portions indicated by Y-shaped attachments. In panels B and C, the DTR is indicated by the blue colored square. In panel B, if the DTR passes through the ER lumen during protein folding, it becomes glycosylated, but *his4-* cells can not grow on histidinol. In panel C, if the DTR passes does not pass through the ER lumen during protein folding, it will not become glycosylated, but His4C will be able to convert histidinol to histidine to allow growth on histidinol-containing plates. [The figure in panel A was modified from Sengstag C (2000) Using SUC2-HIS4C reporter domain to study topology of membrane proteins in *Saccharomyces cerevisiae*. *Methods Enzymol* **327**:175-190 with permission from Elsevier (Figure 1, page 179).]

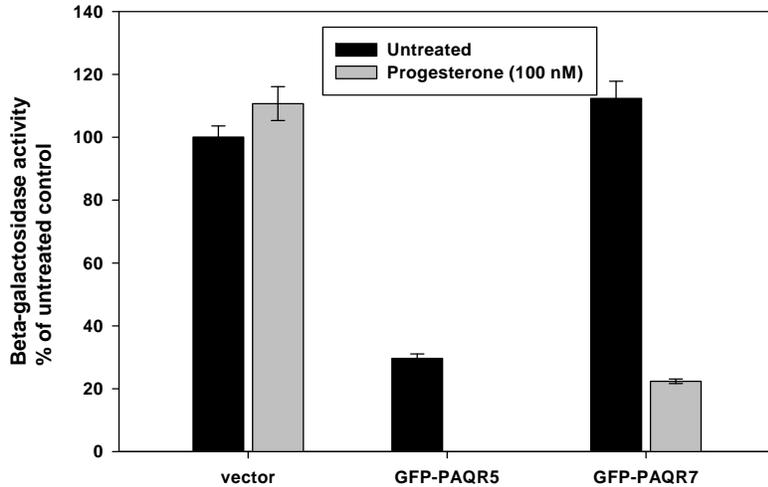


Figure 3-2. Overexpression of N-terminally GFP-tagged PAQRs causes repression of *FET3-lacZ*. Yeast cells doubly transformed with the *pFET3-lacZ* vector and *GALI-GFP-PAQR* vector and  $\beta$ -galactosidase assays were performed. As described in chapter 2, PAQR5 caused constitutive repression of *FET3-lacZ*, while PAQR7 required the addition of progesterone, indicating that the N-terminal GFP tag does not affect PAQR activity.

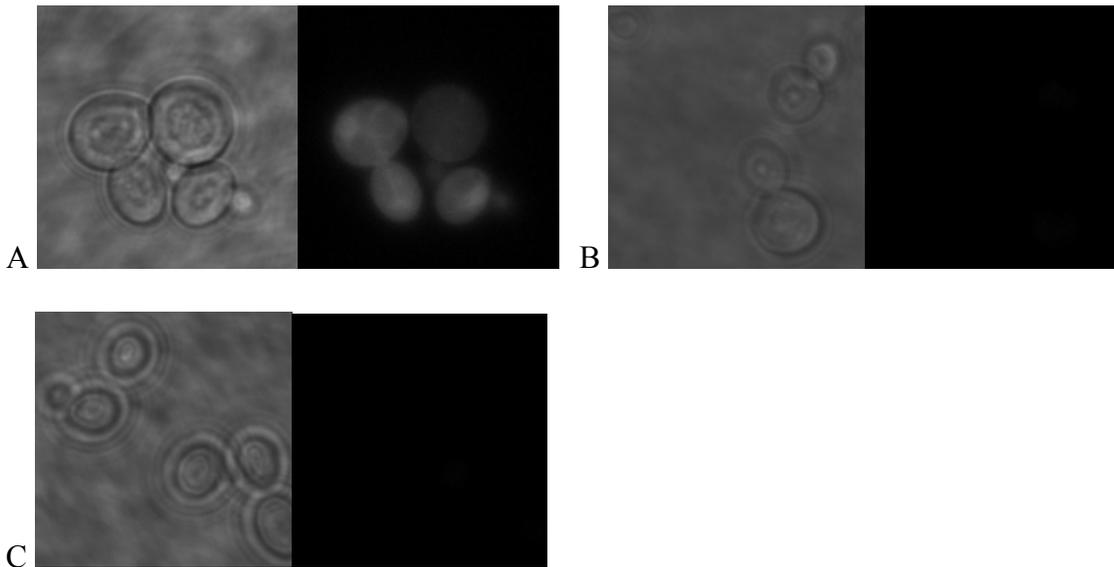


Figure 3-3. Human Class II PAQRs do not fluoresce when tagged with GFP at the N-terminus. For each panel, the image of the cells in the bright field was taken (left side of each panel) and the fluorescence image of the same cells was taken (right side of each panel). In panel A, soluble GFP was expressed and is visible throughout the cell. In panel B and panel C, cells expressing GFP-PAQR5 and GFP-PAQR7, respectively, did not fluoresce.



Figure 3-4. Western blot of GFP-tagged PAQRs expressed in yeast. An anti-GFP antibody was used. On the left, the top arrow indicates the position of the 25 kDa molecular weight marker band, and the bottom arrow indicates the position of the 20 kDa molecular weight marker band. The lanes are as follows: GFP, soluble fraction (lane 1), GFP membrane fraction (lane 2), GFP-PAQR5, soluble fraction (lane 3), GFP-PAQR5 membrane fraction (lane 4), GFP-PAQR7, soluble fraction (lane 5), GFP-PAQR7 membrane fraction (lane 6), PAQR8-GFP soluble fraction (lane 7), PAQR8-GFP membrane fraction (lane 8).

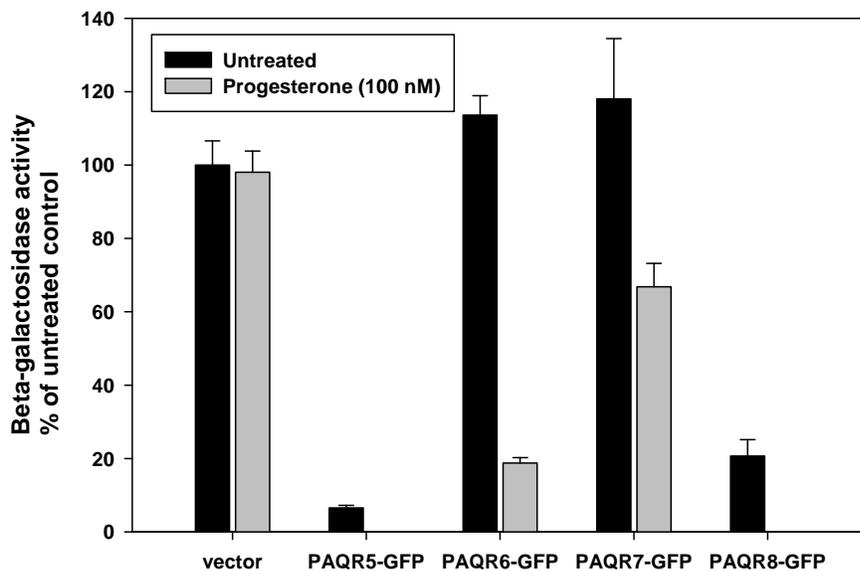


Figure 3-5. Overexpression of C-terminally GFP-tagged PAQRs causes repression of *FET3-lacZ*. Yeast cells doubly transformed with the *pFET3-lacZ* vector and *GALI-PAQR-GFP* vector were grown in LIM containing 1  $\mu\text{M}$   $\text{Fe}^{3+}$  and 2% galactose and  $\beta$ -galactosidase assays were performed as described in the Materials and Methods. As described in chapter 2, PAQR5 and PAQR8 caused constitutive repression of *FET3-lacZ*, while PAQR6 and PAQR7 required the addition of progesterone, indicating that the C-terminal GFP tag does not affect PAQR activity.

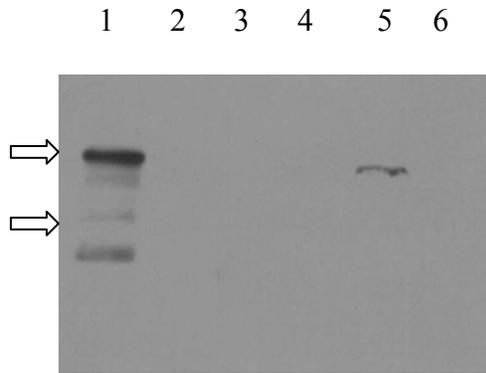


Figure 3-6. Western blot of GFP-tagged PAQRs expressed in yeast. An anti-GFP antibody was used. On the left, the top arrow indicates the position of the 50 kDa molecular weight marker band, and the bottom arrow indicates the position of the 37 kDa molecular weight marker band. The lanes are as follows: PAQR5-GFP, membrane fraction (lane 1), PAQR5-GFP, soluble fraction (lane 2), PAQR7-GFP, membrane fraction (lane 3), PAQR7-GFP, soluble fraction (lane 4), PAQR6-GFP, membrane fraction (lane 5), PAQR6-GFP, soluble fraction (lane 6).

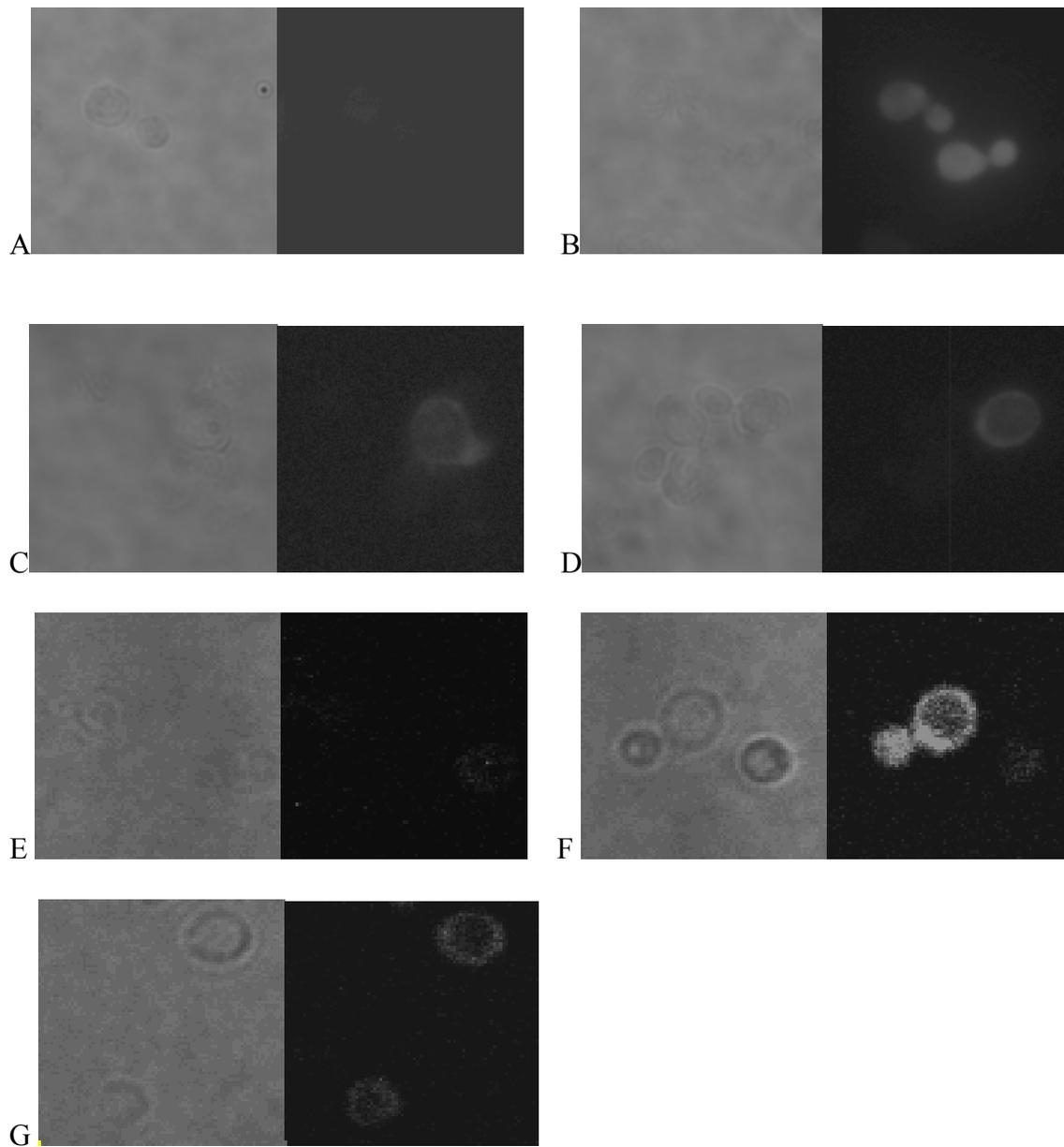


Figure 3-7. Immunofluorescence of GFP-tagged and HA-tagged PAQRs. For panels A-D, the anti-GFP antibody was used and for panels E-G, the anti-HA antibody was used. For each panel, the image of the cells in the bright field was taken (left side of each panel) and the fluorescence image of the same cells was taken (right side of each panel). For negative control cells stained with the anti-GFP primary antibody (panel A) or the anti-HA primary antibody (panel E), there appears to be minimal fluorescence. For soluble GFP, the fluorescence appears to be distributed throughout the whole cell (panel B). Only a few spheroplasts were visible for PAQR5-GFP (panel C), PAQR6-GFP (panel D), HA-PAQR5 (panel F) and HA-PAQR6 (panel G) and the fluorescence appears to outline the perimeter of the cell, suggesting a possible plasma membrane localization for these proteins.

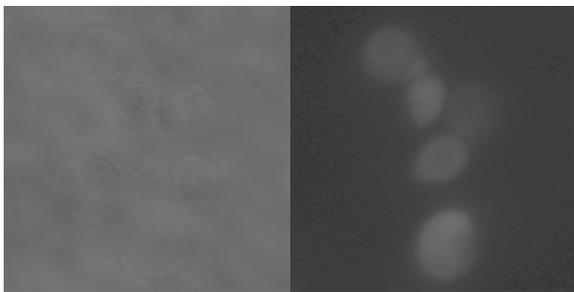


Figure 3-8. Immunofluorescence of soluble GFP in unpermeabilized spheroplasts. The image of the cells in the bright field was taken (left side) and the fluorescence image of the same cells was taken (right side). These experiments were used as a control to test the unpermeabilized spheroplasts for topology analysis. Soluble GFP was expressed in yeast cells and spheroplasts were generated. Immunofluorescence conditions were used without intentionally permeabilizing the cells; however, permeabilization still occurred leading to immunofluorescent staining of GFP inside the cell. The conditions could not be improved, so this method of topology analysis was not used.

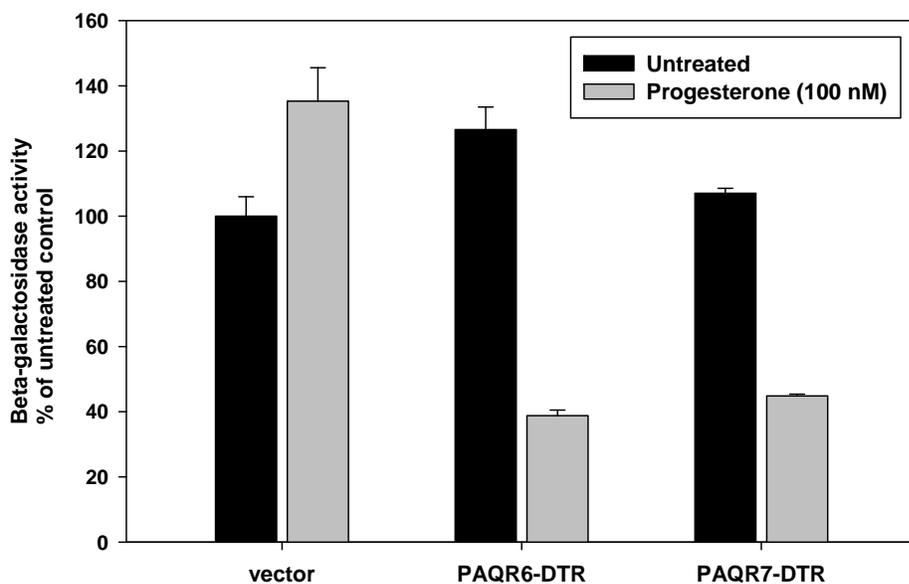


Figure 3-9. Overexpression of C-terminally DTR-tagged PAQRs causes repression of *FET3-lacZ*. Yeast cells doubly transformed with the *pFET3-lacZ* vector and *TPI-PAQR-DTR* vector were grown in LIM containing 1  $\mu\text{M}$   $\text{Fe}^{3+}$  and 2% galactose and  $\beta$ -galactosidase assays were performed as described in the Materials and Methods. The vector control here expresses an ER membrane-bound subunit of yeast oligosaccharyltransferase (*Ost4p-DTR*) (Kim et al., 2006) because an empty vector was not available. As described in chapter 2, PAQR5 and PAQR8 caused constitutive repression of *FET3-lacZ*, while PAQR6 and PAQR7 required the addition of progesterone, indicating that the C-terminal DTR tag does not affect PAQR activity.

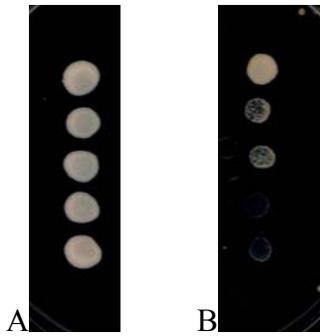


Figure 3-10. Histidinol growth assays of DTR-tagged PAQRs. For both panels, the samples are as follows from top to bottom: Ost4-DTR, PAQR6-DTR, PAQR7-DTR, PAQR6-HA, and PAQR7-HA. Spots from 5  $\mu$ L aliquots of cultures that were adjusted to an  $OD_{600}$  of 0.01 are shown here. In panel A, a plate containing histidine was used to ensure that each culture of cells was viable. In panel B, the plate containing histidinol was used to assess whether the His4C portion of the DTR was located in the cytoplasm.

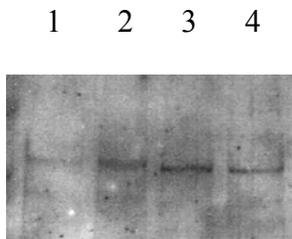


Figure 3-11. Endo H digestion of DTR-tagged PAQR6 and PAQR7. A Western blot was performed and an anti-HA antibody was used. No shift in migration occurred for undigested PAQR6-DTR (lane 1) versus digested PAQR6-DTR (lane 2) or for undigested PAQR7-DTR (lane 3) versus digested PAQR7-DTR (lane 4).

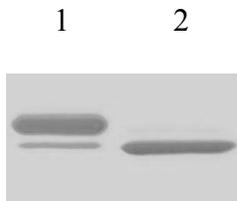


Figure 3-12. Endo H digestion of RNase B. To ensure that the conditions used for Endo H digestion were allowed for activity of Endo H, digestion of RNase B was performed and the samples were run on a polyacrylamide gel. A shift in migration occurred of the RNase B band occurred for undigested RNase B (lane 1) versus digested RNase B (lane 2). The gel was stained with Coomassie Blue dye.

Table 3-1. Primers used for cloning PAQRs into pGREG575, pGREG600, and pJK90 (listed from 5' → 3')

Primer name	Vector	Primer sequence
Fwd PAQR5	pGREG575, pGREG600	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGCTGAGCCTGA AGCT
Rev PAQR5	pGREG575	GCGTGACATAACTAATTACATGACTCGAGGTCGACTCATGTTTCTTTT TTATGTAATTCTGG
Rev PAQR5	pGREG600	GTTCTTCTCCTTTACTCATTCTCGAGGTCGATGTTTCTTTTTATGTAA TTCTGG
Fwd PAQR6	pGREG600	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGCTCAGTCTCA AGCTGC
Rev PAQR6	pGREG600	GTTCTTCTCCTTTACTCATTCTCGAGGTCGACTGTTGTTTGGCCTGGG TAC
Fwd PAQR7	pGREG575, pGREG600	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGGCCATGGCCC AGAAA
Rev PAQR7	pGREG575	GCGTGACATAACTAATTACATGACTCGAGGTCGACTCACTTGGTCTT CTGATCAA
Rev PAQR7	pGREG600	GTTCTTCTCCTTTACTCATTCTCGAGGTCGACTTGGTCTTCTGATCAA GTTT
Fwd PAQR8	pGREG600	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGACGACCGCCA TCTTGG
Rev PAQR8	pGREG600	GTTCTTCTCCTTTACTCATTCTCGAGGTCGACGGAATCTTCTTGGTC AGTCT
Fwd PAQR6/90	pJK90	GTGGTTTGTACGCATGCAAGCTTGATATCGAAATGTTCACTCTCAA GCTGCC
Rev PAQR6/90	pJK90	TGGTCTAGAGGTGTAACCACTTGAGTTCTTAGGCTGTTGTTTGGCCTG GGTAC
Fwd PAQR7/90	pJK90	GTGGTTTGTACGCATGCAAGCTTGATATCGAAATGTCCATGGCCCA GAAACT
Rev PAQR7/90	pJK90	TGGTCTAGAGGTGTAACCACTTGAGTTCTTAGGCTTGGTCTTCTGATC AAGTTT

## CHAPTER 4 HEMOLYSIN III PROTEINS: BACTERIAL HOMOLOGUES OF PAQR PROTEINS

### **Introduction to Bacterial PAQRs**

The PAQR family of proteins has evolutionary origins in eubacteria with potential bacterial virulence factors called the Hemolysin III (HlyIII) proteins (Tang et al., 2005; Thomas et al., 2007). Thus far, results of partial characterizations of HlyIII-1 from *Bacillus cereus* (Baida and Kuzmin, 1995; Baida and Kuzmin, 1996), HlyIII from *Bacillus anthracis* (called anthralysin III, or AnlIII) (Klichko et al., 2003), and HlyIII from *Vibrio vulnificus* (Chen et al., 2004) (referred to as v-HlyIII) indicate that these proteins are involved in hemolysis (lysis of human erythrocytes) (Baida and Kuzmin, 1995; Baida and Kuzmin, 1996; Klichko et al., 2003; Chen et al., 2004), and may thus be important for pathogenicity.

### **Roles in Virulence?**

Although the role of HlyIII *in vivo* during pathogenesis is unclear (Klichko et al., 2003; Chen et al., 2004), a *V. vulnificus* mutant for *hlyIII* had a 16-fold increase in the LD<sub>50</sub> (dose that is lethal to 50% of test animals) upon infection in mice, suggesting that this protein plays a role in the pathogenesis of *V. vulnificus* (Chen et al., 2004). This is especially interesting since little is understood about the virulence mechanisms of this pathogen, which can cause death within 24 hours of infection and has mortality rates as high as 50% for patients with wound infections or 75% for patients with septicemia (the presence of bacteria in the blood system) (Gulig et al., 2005).

Whether the HlyIIIs are directly or indirectly involved in hemolysis is unclear. In the case of HlyIII from *B. cereus* (called HlyIII-1), Baida and Kuzmin (1996) suggested that this protein functions as a hemolysin by forming pores in the cell membranes of erythrocytes. However, this conclusion was based on indirect evidence because crude cell extracts from recombinant

*Escherichia coli* expressing HlyIII were used in the hemolysis assays (Baida and Kuzmin, 1996). In fact, all of the hemolytic activity characterizations of the HlyIII homologues involved the use of crude cell extracts from recombinant *E. coli* expressing these proteins (Baida and Kuzmin, 1996; Klichko et al., 2003; Chen et al., 2004), thus raising the possibility that these proteins are indirectly involved in cell lysis. Hemolysis by purified HlyIIIs has yet to be demonstrated.

### **Other Possible Functions**

Interestingly, the HlyIII homologues are found in a variety of bacteria, including *E. coli* K-12, a non-hemolytic bacterium, leading to the hypothesis that these proteins may be involved in other, non-pathogenic roles in bacteria. Bioinformatic predictions of transcription factor binding sites in *E. coli* (McCue et al., 2001) have led to the hypothesis that the *E. coli* HlyIII homologue (called YqfA) is regulated by a transcription factor called FabR (for Fatty Acid Biosynthesis Regulator) and that YqfA is potentially involved in unsaturated fatty acid metabolism (McCue et al., 2001). However, DNA microarray analysis of a FabR knockout mutant strain showed no significant change in *yqfA* expression (Zhang et al., 2002), so the role of FabR in regulating *yqfA* expression is unclear.

Interestingly, the HlyIII proteins have homologues that affect lipid metabolism in *Saccharomyces cerevisiae* (Karpichev et al., 2002; Lyons et al., 2004), (called Implicated in Zinc Homeostasis protein, or Izhp) and humans (called progesterin/adipoQ receptors, or PAQRs) (Yamauchi et al., 2002; Tang et al., 2005). *IZH* transcription can be induced by different fatty acids (Karpichev et al., 2002; Lyons et al., 2004) and some Izhp proteins seem to be involved in altering sterol content (Villa, 2007). The adiponectin receptors also seem to be involved in fatty acid metabolism, as they are receptors for a protein, adiponectin, that cause fatty acid oxidation (Yamauchi et al., 2002; Yamauchi et al., 2003).

## Mechanisms of Action?

The roles of the HlyIII proteins are not well-established and neither is the mechanism by which they function. As suggested by Baida and Kuzmin (1996), these proteins may function by forming pores in cell membranes. Alternatively, the HlyIII proteins may function similarly to their human PAQR relatives by acting as membrane receptors (Tang et al., 2005). Structurally, the HlyIII proteins and the PAQRs share the same predicted seven transmembrane (7TM) architecture (Kyte and Doolittle, 1982) with an intracellular N-terminus and an extracellular C-terminus. These proteins also share four small, highly conserved amino acid sequence motifs that are predicted to cluster on the cytoplasmic face of the membrane (Lyons et al., 2004; Tang et al., 2005) and may be important for their intracellular molecular mechanism. If acting as membrane receptors, the HlyIII proteins could activate a cytoplasmic protein that is responsible for the hemolytic activity that is associated with HlyIII.

Assays conducted in *S. cerevisiae* for the human PAQRs and all of the yeast IZHs indicate that overexpression of any of these proteins results in repression of the promoter for the high affinity iron uptake gene *FET3* (Kupchak et al., 2007). Importantly, expression of the uncharacterized PAQR11, which is the closest human homologue of the HlyIIIs, responds like the other, established membrane receptor PAQRs (Figure 2-2). This response is hypothesized to result from an intracellular molecular mechanism common to all PAQRs (Kupchak et al., 2007). Because the HlyIIIs share the conserved motifs with the IZHs and PAQRs (Figure 1-1), it was hypothesized that the HlyIIIs can cause similar intracellular signaling in yeast to affect *FET3* expression.

Although HlyIII homologues can be found in a wide range of eubacterial genomes, these proteins and their human homologues have not been well characterized. Because their role in pathogenesis and potential function in lipid metabolism are not well understood, further

characterizations of the HlyIII homologues are needed to better understand this family of proteins. This work describes the cloning and expression of two HlyIII homologues from *B. cereus* (HlyIII-1, HlyIII-2) and one HlyIII homologue from *E. coli* (YqfA) in *S. cerevisiae* as well as their effect on the repression of the *S. cerevisiae* *FET3* promoter. In addition, attempts to overexpress these proteins in *E. coli* for further characterization of function were made.

### **Results and Discussion**

The HlyIII proteins are an uncharacterized group of proteins that are related to a family of proteins in humans called the PAQRs (Tang et al., 2005; Thomas et al., 2007) and related proteins in yeast called the IZHs (Lyons et al., 2004). It was previously discovered that, in low iron media, the IZHs and human PAQRs can negatively affect expression of *FET3* (Kupchak et al., 2007). This is interesting because *FET3* encodes a high affinity iron uptake protein and is typically upregulated in low iron media (Askwith et al., 1994; Yamaguchi-Iwai et al., 1995).

All PAQRs and their homologues share similar amino acid sequence motifs that are predicted to be located intracellularly (Figure 1-1). Thus, it was hypothesized that all of these proteins share the same signaling mechanism. In addition, because there is no evidence in the literature that shows direct involvement of HlyIIIs in hemolysis, it was hypothesized that the HlyIIIs do not directly cause hemolysis. Instead, like the PAQRs, the HlyIIIs may be capable of signaling, and this signaling could cause increased expression or activity of a hemolytic protein.

#### **Overexpression of some bacterial PAQRs causes repression of *FET3***

To begin examining the possibility that the HlyIII proteins are capable of causing a non-hemolytic physiological response similar to that of the PAQRs and the IZHs, the HlyIIIs we cloned and expressed in *S. cerevisiae*. Overexpression of the *E. coli* HlyIII (YqfA) protein reduced expression of *FET3-lacZ* so that the level of  $\beta$ -galactosidase activity was 20-25% of that of the vector control (Figure 4-1). This result is similar to that which was observed for the IZHs

and PAQRs (Kupchak et al., 2007), which suggests that YqfA may signal by a mechanism that is similar to that of the IZHs and PAQRs.

In addition, it was observed that overexpression of *B. cereus* HlyIII-2 consistently affected expression of *FET3-lacZ* so that  $\beta$ -galactosidase activity was reduced to 50-60% of that of the vector control (Figure 4-2). *B. cereus* HlyIII-1 did not seem to affect *FET3-lacZ* (Figure 4-1). It is possible that the *B. cereus* HlyIIIs do not properly fold or are not expressed as well as the *E. coli* YqfA protein in *S. cerevisiae*. Alternatively, it has been shown that overexpression of some of the human PAQRs can not cause repression of *FET3-lacZ* unless the ligand for the protein is added during growth in LIM (Kupchak et al., 2007). So it is possible that some HlyIII proteins require a ligand for activation to cause repression of *FET3-lacZ*. Because the human Class I and Class II PAQR proteins have known ligands, but the bacterial HlyIIIs do not, the characterization of the effect of PAQRs on *FET3* was refocused on the human Class III proteins. In these studies, progesterone was tested as a potential ligand for PAQR10 and PAQR11, but activation of these proteins did not occur (Figure 2-3 and Figure 2-4).

### **Expression of HlyIIIs in *E. coli***

In addition to expressions in *S. cerevisiae*, we attempted to express the HlyIIIs in *E. coli*. Figures 4-2 and 4-3 show the results of the protein expression for HlyIII-1 (25.5 kDa) and HlyIII-2 (26 kDa), respectively. No protein expression has been observed for YqfA (data not shown).

Figure 4-2 shows the results of a pilot expression of HlyIII-1. In Figure 4-2A, a broad, indistinct band between 20 kDa and 30 kDa is present in the induced, 4 h HlyIII-1 sample (lane 4), that was not detected in the uninduced 4 h HlyIII-1 sample (lane 3), the 0 h HlyIII-1 sample (lane 2) or the induced, 4 h pKM260 empty vector sample (lane 1). The same gel was then

stained for total protein using Coomassie Blue (Figure 4-2B), but the difference in expression of the between 20 kDa and 30 kDa was not detected.

Figure 4-3 shows the results of a pilot expression of HlyIII-2. A broad, indistinct band between 20 kDa and 30 kDa is present in the induced, 4 h HlyIII-2 sample (lane 3), but is not detected in the uninduced 4 h HlyIII-2 sample (lane 4), the 0 h HlyIII-2 sample (lane 6) or the induced, 4 h pKM260 empty vector sample (lane 1). The same gel was then stained for total protein using Coomassie Blue (Figure 4-3B), but the difference in expression of the protein at that appears between 20 kDa and 30 kDa was not detected.

Because expressions consistently yielded more protein for HlyIII-2 than HlyIII-1, further expression analyses focused on HlyIII-2. The solubility of expressed HlyIII-2, was determined as described in the Materials and Methods.

In Figure 4-4A, a comparison of the insoluble cytoplasmic sample and the soluble cytoplasmic sample for the HlyIII-2 expressions (lanes 1 and 2, respectively) shows a distinct band between 20 kDa and 30 kDa in the insoluble sample, but not in the soluble sample. This band does not appear in the insoluble cytoplasmic fraction from the negative control sample (C43(DE3) containing pKM260, lane 3).

These results indicate that a protein between 20 kDa and 30 kDa appears to be expressed in the HlyIII-2 samples as an insoluble protein. Estimation of the molecular weight of this band is 23 kDa based on the migration distances of this band and the migration distances of the 15-40 kDa Benchmark<sup>TM</sup> His-tagged Standard protein bands (the linear range of separation of 15% polyacrylamide gels is 15-43 kDa) (Sambrook and Russell, 2001). The same gel was stained for total protein using Coomassie Blue (Figure 4-4B), but the difference in expression of the protein at 23 kDa was not detected.

Estimation of the amount of protein that is present at 23 kDa in the insoluble cytoplasmic sample (lane 1, Figure 4-4A), could not be made based on the amount of protein present in each of the Benchmark<sup>TM</sup> His-tagged Standard protein bands because this marker is not recommended by the manufacturer for quantitation (Invitrogen). Because the 23 kDa protein in the insoluble cytoplasmic sample can be detected with the InVision<sup>TM</sup> His-tag In-gel Stain (lane 1, Figure 4-5A) but not Coomassie Blue staining (lane 1, Figure 4-4B), a rough estimate of the quantity of protein in this band was made based on the detection limits of the InVision<sup>TM</sup> His-tag In-gel Stain (~15 ng for a 23 kDa protein, Invitrogen) and Coomassie Blue staining (~100 ng of polypeptide in a single band) (Sambrook and Russell, 2001). Based on these differences in detection limit, the quantity of the 23 kDa protein per liter of cell culture was estimated to be between 10-60 µg.

The quantity of expressed HlyIII was very low (10-60 µg per liter) and the proteins seemed to be expressing as insoluble inclusion bodies, which makes purification and refolding extremely difficult. Thus we decided to focus our characterization of the PAQRs and their homologues on other areas, including the effects of their expression on *FET3* in *S. cerevisiae*, as described in Chapter 2.

## **Materials and Methods**

### **Cloning of the Bacterial Hemolysin III Genes**

To clone *hlyIII-1* and *hlyIII-2*, *Bacillus cereus* strain 10987 genomic DNA was obtained from the American Type Culture Collection (ATCC). To clone *yqfA*, a colony of *Escherichia coli* TOP10 (Invitrogen) was used directly in PCR reactions. For yeast expression plasmids, the amplified gene sequences were cloned into the pRS316 plasmid (Sikorski and Hieter, 1989) via homologous recombination. After PCR amplification of the genes and restriction enzyme

digestion of the pRS316 vector (BamHI, New England Biolabs R0136 and SacI, New England Biolabs R0156), the PCR product and cut vector were transformed into the wild-type *S. cerevisiae* strain BY4742 (mat  $\alpha$ ) via the standard lithium acetate protocol (Guthrie and Fink, 1991) for homologous recombination. The DNA was rescued from the cells and transformed into the TOP10 strain of *E. coli* (Invitrogen). The Wizard Plus Minipreps DNA Purification System [Promega A7500] was used to prepare the plasmid DNA from the *E. coli* cells. The DNA sequence was confirmed by the DNA Sequencing Core Facility [University of Florida]. For *E. coli* expression plasmids, *hlyIII-1*, *hlyIII-2*, and *yqfA* were cloned into pKM260 and pilot expressions were performed. The pKM260 vector, derived from the pET3b vector, has a T7 promoter and is constructed in such a way to allow N-terminal 6x-histidine tagging of expressed proteins (Melcher, 2000). A tobacco etch virus (TEV) protease cleavage site is located between the 6x-histidine tag and the N-terminus of the expressed protein. To clone *hlyIII-1* and *hlyIII-2*, *Bacillus cereus* strain 10987 genomic DNA was obtained from the American Type Culture Collection (ATCC). To clone *yqfA*, a colony of *Escherichia coli* TOP10 (Invitrogen) was used directly in PCR reactions. The amplified gene sequences were cloned into pKM260 using standard restriction enzyme and ligation methods (Sambrook and Russell, 2001). The plasmids were transformed into competent TOP10 *E. coli* cells (Invitrogen) by standard procedures (Sambrook and Russell, 2001), and the sequences of the cloned genes were confirmed by the ICBR DNA sequencing core laboratory at the University of Florida. Expression plasmids constructed with pKM260 that contain gene inserts have been named as follows: pJLS1 (*hlyIII-1*), pJLS2 (*hlyIII-2*), and pJLS3 (*yqfA*).

### **Expression of the Hemolysin III Proteins in *S. cerevisiae***

The wild-type BY4742 (mat  $\alpha$ ) strain of *S. cerevisiae* was double-transformed with pFET3-*lacZ-LEU2* and either pRS316-*GALI-URA3* or pRS316-*GALI-HlyIII-URA3*. Yeast overnight cultures were routinely grown at 30 °C in synthetic dextrose (SD) media with the appropriate selection of amino acids. Overnight cultures were reinoculated into low iron media (LIM) (Eide and Guarente 1992), containing 1  $\mu$ M FeCl<sub>3</sub> for iron-deficient media or 1 mM FeCl<sub>3</sub> for iron-replete media. Galactose (2%) was used to fully induce expression of *GALI*-driven genes.

### **$\beta$ -galactosidase Reporter Assays**

Transformants containing pFET3-397 and overexpression plasmids were used in  $\beta$ -galactosidase assays as previously described (Kupchak et al., 2007). Cells were grown to mid-log phase in LIM and  $\beta$ -galactosidase assays were performed on permeabilized cells as described (Guarente, 1983). Production of the *lacZ* gene product ( $\beta$ -galactosidase) was determined by measuring the activity of the enzyme. Upon hydrolysis of o-nitrophenyl- $\beta$ -galactopyranoside (colorless) to o-nitrophenol (yellow) and galactose, the absorbance at 420 nm was measured. Activity (in Miller Units) was determined as follows:  $(A_{420} \times 1000) / (\text{mL of culture} \times \text{reaction time (minutes)} \times A_{600})$ .

### **Expression of the HlyIII Homologues in *Escherichia coli***

For expression of each gene, the C43(DE3) strain of *E. coli* was used, as it is more amenable to membrane proteins expressions (Miroux and Walker, 1996). Also, HlyIII-1 seems to have toxic effects when expressed in *E. coli* (Baida and Kuzmin, 1995), so procedures for expression of toxic genes in pET vectors were used (Novagen 2002). An overnight culture of the transformed cells was grown at 30°C in 5 mL of LB/Amp (200  $\mu$ g/mL) and 1% glucose. The

culture was re-inoculated using a 1:100 dilution into fresh media containing ampicillin (500  $\mu\text{g}/\text{mL}$ ) and incubated at 30°C with shaking until the  $\text{OD}_{600}$  reached 0.6-0.8. The cells were collected by centrifugation and resuspended in fresh LB/Amp (500  $\mu\text{g}/\text{mL}$ ). Expression was induced with 0.4 mM IPTG for 4-16 hours at 30°C. Aliquots of cells (1 mL each) were removed at different time points between 0-16 h post-induction. The aliquots were prepared for analysis by SDS-PAGE as described above except that after sample buffer was added, the mixture was incubated at 42°C for 30 minutes. These conditions were used for all subsequent protein expressions and analyses.

Each gel was first stained using the InVision™ His-tag In-gel Stain (Invitrogen) according to the manufacturer's protocol. This stain consists of a fluorescent dye conjugated to  $\text{Ni}^{2+}$ :nitriloacetic acid (NTA) complex. The same gel was then stained for total protein using Coomassie Blue.

To determine the solubility of expressed HlyIII-2, procedures outlined in the manufacturer's pET System Manual (Novagen 2002) were followed for soluble versus insoluble (inclusion body) cytoplasmic fractions. After 4 hour expressions, pellets from 40 mL cultures were lysed using buffer A (4 mL) [50 mM Tris-HCl (pH 8), 5% glycerol, 100 mM NaCl, 10 mM 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM Ethylenediamine Tetraacetic Acid (EDTA), 1 mM Phenylmethylsulfonyl fluoride (PMSF) and egg white lysozyme (0.2 mg/mL)]. The mixtures were incubated at 37°C for 20 minutes and then placed on ice while sonication was performed. Samples were centrifuged at 3000 rpm to remove unlysed cells, and the supernatants were centrifuged at 16,000 x g (14,500 rpm) for 30 minutes at 4°C to isolate inclusion bodies in the pellet. The inclusion body pellet was washed with buffer A twice, then resuspended in 0.25 mL of 1% SDS with heating (42°C for 30 minutes)

and mixing (1400 rpm). The supernatant was saved for subsequent analysis by SDS-PAGE. SDS-PAGE samples were prepared by mixing a 10  $\mu$ L aliquot of the soluble protein fraction or solubilized inclusion bodies with an equal aliquot of 2X sample buffer. The mixture was incubated at 42°C for 30 minutes prior to SDS-PAGE analysis.

### **Summation**

The HlyIII proteins have been proposed as pore-forming toxins that cause lysis of red blood cells (Baida and Kuzmin, 1995; Baida and Kuzmin, 1996; Klichko et al., 2003; Chen et al., 2004). Alternatively, the HlyIII proteins may function similarly to their human PAQR relatives by acting as membrane receptors (Tang et al., 2005). Here, we showed that, like the human and yeast PAQRs, the HlyIII proteins can cause repression of *FET3-lacZ* when expressed in yeast. Whether this occurs because of activation of a common signaling pathway remains to be determined. Further characterizations of the HlyIIIs were attempted by expressing these proteins in *E. coli*, but only low levels of expression could be obtained.

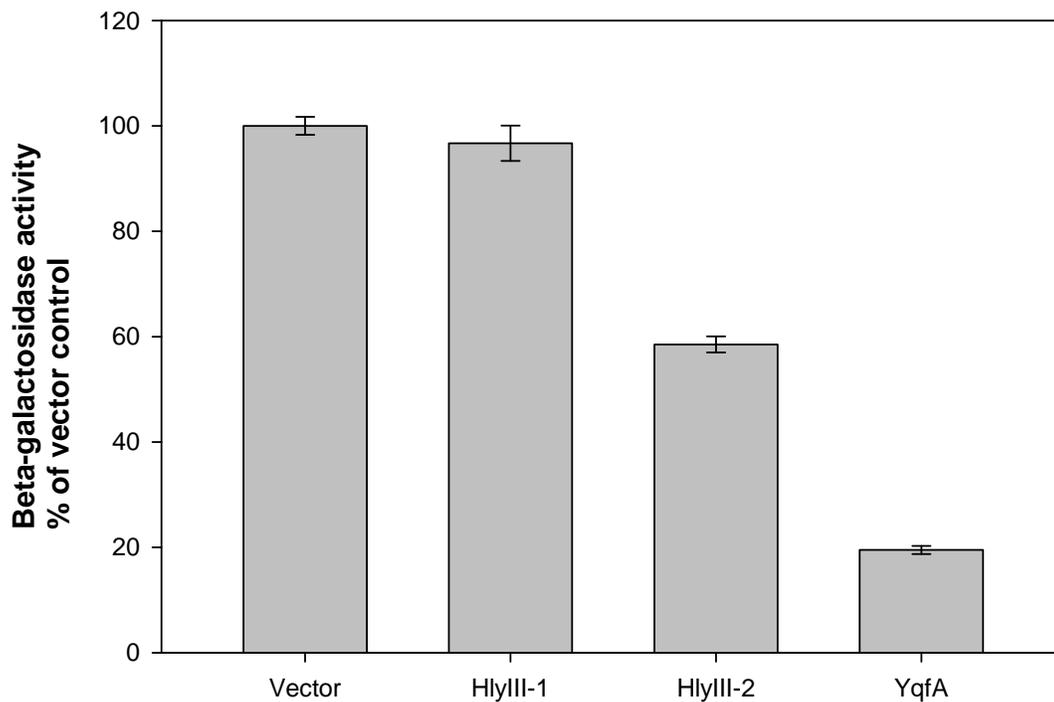


Figure 4-1. Overexpression of some bacterial PAQR homologues causes repression of *FET3-lacZ*. HlyIII-1 and HlyIII-2 from *B. cereus* and YqfA from *E. coli* were tested. Yeast cells doubly transformed with the *pFET3-lacZ* vector and protein expression vector were grown in LIM containing 1  $\mu\text{M}$   $\text{Fe}^{3+}$  and 2% galactose and  $\beta$ -galactosidase assays were performed as described in the Materials and Methods. The proteins do not have epitope tags and antibodies to the proteins do not exist, so expression was not confirmed. Also, there are no known ligands for the bacterial PAQRs that could be tested for ligand-dependent repression of *FET3-lacZ*.

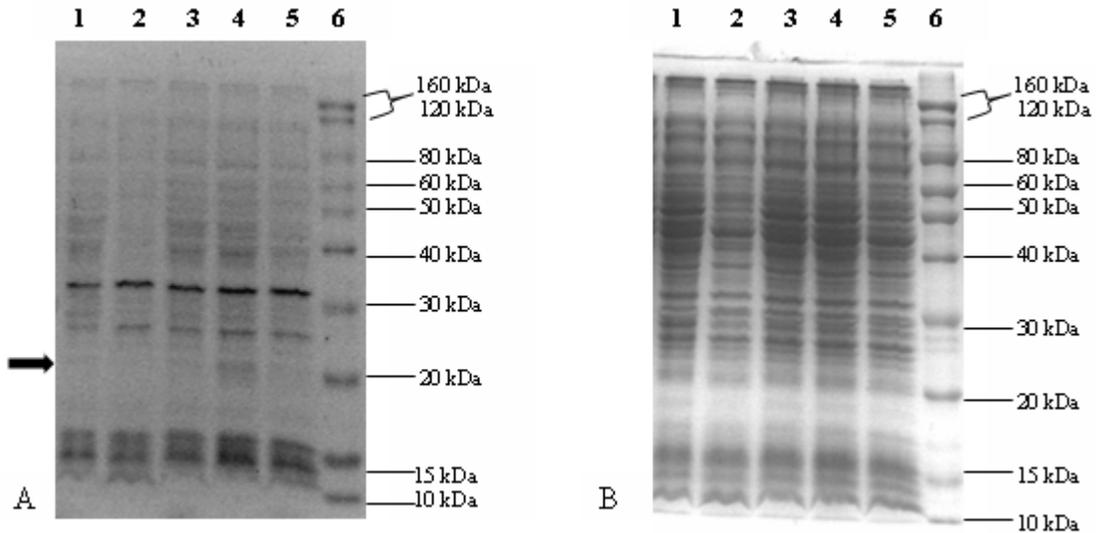


Figure 4-2. SDS-PAGE of HlyIII-1 expressions from the pKM260 vector. The same gel was stained with the InVision™ His-tag In-gel Stain (Invitrogen) (A), and then Coomassie Blue stain (B). The contents of the lanes are as follows: pKM260 (lane 1), 4 h induced; HlyIII-1, 0 h time point (lane 2); HlyIII-1, uninduced 4 hour time point (lane 3); HlyIII-1, induced 4 hour time point (lane 4); HlyIII-1, induced 2 hour time point (lane 5); Benchmark™ His-tagged Standard (lane 6, Invitrogen). This was a 12% polyacrylamide gel.

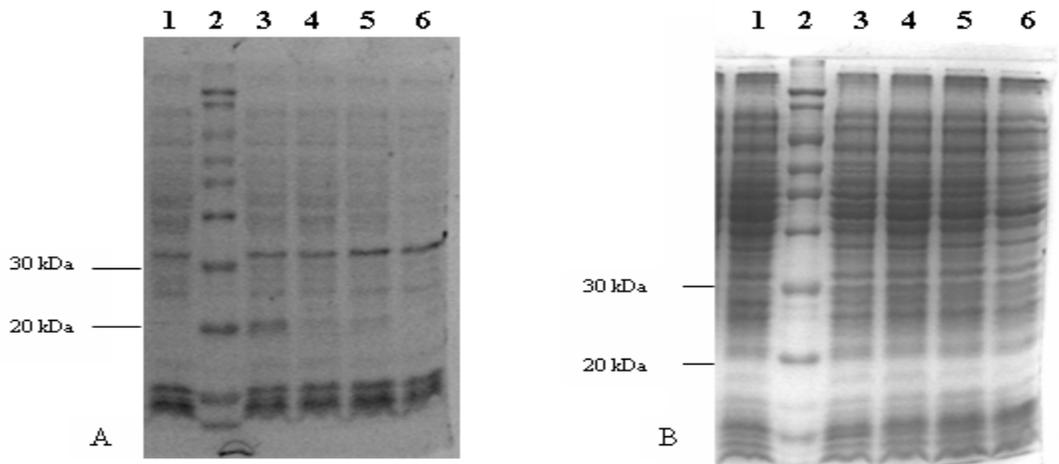


Figure 4-3. SDS-PAGE of HlyIII-2 expressions using the pKM260 vector. The same gel was stained with the InVision™ His-tag In-gel Stain (Invitrogen) (A), and then Coomassie Blue stain (B). The contents of the lanes are as follows: pKM260 (lane 1), 4 h induced; Benchmark™ His-tagged Standard (lane 2); HlyIII-2, induced 4 h time point (lane 3); HlyIII-2, uninduced 4 hour time point (lane 4); HlyIII-2, induced 2 h time point (lane 5); HlyIII-2, 0 h time point (lane 6). This was a 12% polyacrylamide gel.

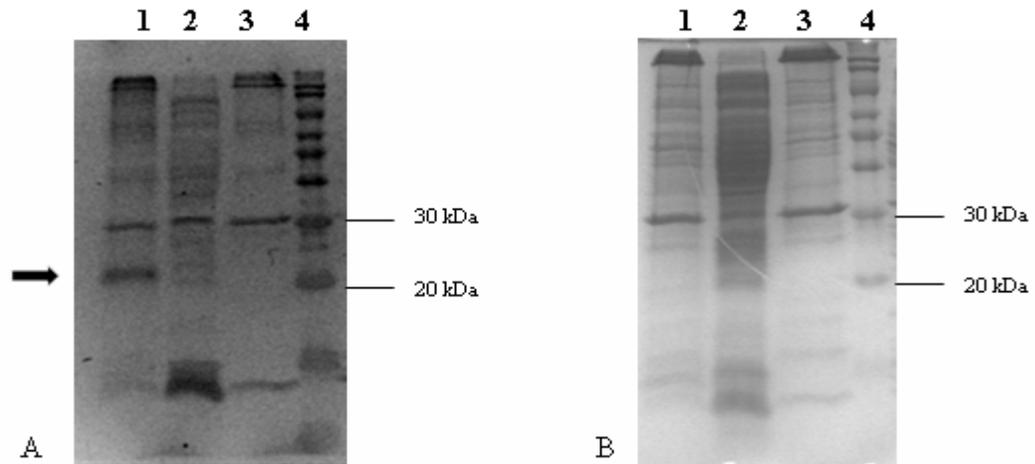


Figure 4-4. SDS-PAGE of soluble cytoplasmic samples and insoluble cytoplasmic samples for HlyIII-2 in pKM260. Expressions were at 4 h at 30°C. The same gel was stained with the InVision™ His-tag In-gel Stain (Invitrogen) (A), and then Coomassie Blue stain (B). The contents of the lanes are as follows: HlyIII-2 insoluble cytoplasmic sample (lane 1, the band for HlyIII-2 is indicated with an arrow); HlyIII-2 soluble cytoplasmic sample (lane 2); pKM260 insoluble cytoplasmic sample (lane 3); Benchmark™ His-tagged Standard (lane 4).

Table 4-1. Primers used for cloning HlyIII<sub>s</sub> (listed from 5'→3')

Primer name	Vector	Primer sequence
Fwd yqfA	pRS316	TACTTCTTATTCTCTACCGGATCCCGCTCGAGGTCGACATGGTTCAGAA GCCCTC
Rev yqfA	pRS316	TGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGAGCTCTTACGC CTGCCCAATATAC
Fwd hlyIII-1	pRS316	TACTTCTTATTCTCTACCGGATCCCGCTCGAGGTCGACATGACACAATT TGTGAAAGAA
Rev hlyIII-1	pRS316	TGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGAGCTCTTATGA TGCTGTAGGTAGG
Fwd hlyIII-2	pRS316	TACTTCTTATTCTCTACCGGATCCCGCTCGAGGTCGACATGAATGCTTA TGTAAGGGA
Rev hlyIII-2	pRS316	TGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGAGCTCTTAAAT TACGTAACAATATACA
Fwd yqfA	pKM260	CACCATCACCATGCTAGCGAGAATCTTTATTTTCAGGGCGCCATGGTTCA GAAGCCCCTC
Rev yqfA	pKM260	ATATCTGCAGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCTTAC GCCTGCCCAATATAC
Fwd hlyIII-1	pKM260	CACCATCACCATGCTAGCGAGAATCTTTATTTTCAGGGCGTCATGACACA ATTTGTGAAAGAA
Rev hlyIII-1	pKM260	ATATCTGCAGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCTTAT GATGCTGTAGGTAGGA
Fwd hlyIII-2	pKM260	CACCATCACCATGCTAGCGAGAATCTTTATTTTCAGGGCGCCATGGATGC TTATGTAAGGGAA
Rev hlyIII-2	pKM260	ATATCTGCAGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCTTAA ATTACGTAACAATATACAC

## CHAPTER 5 FUTURE DIRECTIONS FOR CLASS II PAQR CHARACTERIZATIONS

### **Introduction**

This dissertation has described the characterization of the Class II PAQRs in yeast, including the use of a promoter-reporter assay to study signaling and structure/function studies. These studies indicate that the Class II PAQRs, including the uncharacterized proteins PAQR6 and PAQR9, are able to sense and respond to progestins and do not require the presence of G-proteins (Chapter 2). In addition, attempts to determine the localization and topology of these proteins when expressed in yeast were also performed, but were generally inconclusive (Chapter 3). The characterization of the bacterial Class III PAQRs was performed in yeast and indicates that some of these proteins are able to transduce signals in this organism as well (Chapter 4). Still, much work remains in order to understand the PAQR family of proteins.

In this Chapter, ideas for future Class II PAQR studies are described, including identification of novel protein-protein interactions between PAQRs and other proteins and study of a possible intrinsic enzymatic activity of the PAQRs. In addition, some preliminary work for other future studies is described, including the establishment of an expression system using Sf9 insect cells and ligand binding assays conducted with membranes from yeast expressing the PAQRs.

### **Identification of Novel Interactions Between PAQRs and Other Proteins**

While we were able to demonstrate that the presence of progestins causes a physiological effect in cells that express the Class II PAQRs, several questions remain regarding the mechanism of signaling initiated by the Class II PAQRs and whether this mechanism is conserved for all PAQR family members.

By expressing the Class II PAQRs in yeast in the absence of any other human proteins, we have demonstrated that human G-proteins are not necessary for signaling. In addition, we showed that the Class II PAQRs can signal in the absence of yeast G-proteins as well (Chapter 2). Thus, the previous characterization of the human Class II PAQRs as GPCRs (Thomas et al., 2007) should be re-evaluated.

First, the evidence for the human Class II PAQRs as GPCRs includes bioinformatic predictions of structural similarity, with seven predicted transmembrane domains (Zhu et al., 2003a); however, this evidence is not concrete. Other analyses have predicted that the Class II PAQRs have eight transmembrane domains (Lyons et al., 2004; Fernandes et al., 2005). Also, no researchers have performed a detailed experimental analysis of the number of transmembrane domains or the topology of the proteins in the membrane.

Other evidence for the Class II PAQRs as GPCRs includes an increase in [<sup>35</sup>S]GTPγ-S bound to membranes of cells transfected with sea trout PAQR7 or human PAQR7 in the presence of 17,20-β,21-trihydroxy-4-pregnen-3-one (20β-S) or progesterone, respectively (Thomas et al., 2007). In an incomplete characterization of these two proteins, Thomas et al. (2007) demonstrated that increased [<sup>35</sup>S]GTPγ-S binding can be abolished for a truncated sea trout PAQR7 protein, but a truncated human PAQR7 was not tested.

Finally, Thomas et al. (2007) claim that PAQR7 co-immunoprecipitated with G-proteins when an anti-G<sub>i/o</sub> antibody was used for immunoprecipitation, although the evidence presented is unclear, as the Western blot bands for PAQR7 proteins were faint and no control was shown for untransfected cells or similar co-immunoprecipitation experiments with a membrane protein that is not a GPCR.

It is possible that the Class II PAQRs are coupled to G-proteins; however, it is also possible that their ability to sense and respond to the presence of progestins can occur independently of G-proteins. If the Class II PAQRs are able to function independently of G-proteins, then how are they able to initiate a signaling response in the presence of progesterone?

One approach to try to answer this question lies in the continued use of yeast to characterize these proteins. Previously, Mao et al. (2006) used the cytoplasmic domain of PAQR1 in the yeast two-hybrid system to screen a human cDNA library to identify interactions that take place between PAQR1 and other human proteins. In this study, an interaction between PAQR1 and APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain and leucine zipper motif) was identified and this interaction was verified in mammalian cells (Mao et al., 2006). Mao et al. (2006) also observed that APPL1 and a small GTPase (Rab5) interacted more upon treatment of cells with adiponectin, leading to translocation of the glucose transport 4 (GLUT4) protein to the membrane.

It is possible that the Class II PAQRs also interact with the APPL1 protein to lead to downstream signaling effects. Interestingly, the yeast homologue of APPL1, is required for Izh2p-, PAQR1-, and PAQR2-dependent repression of *FET3* (Kupchak et al., 2007). Amongst other proteins, Kupchak et al. (2007) also determined that the yeast and human Class I PAQRs required the yeast AMPK subunits *SIP1* and *SNF4* for *FET3-lacZ* repression. Interestingly, in human cells, it has been demonstrated that AMPK is involved in PAQR1 and PAQR2 signaling (Yamauchi et al., 2003). By using a genetic mutational approach similar to that described in Chapter 2 and by Kupchak et al. (2007), the *FET3-lacZ* assay could be used in combination with several mutants to determine if the Class II PAQRs require the same yeast proteins as the yeast and human Class I PAQRs for PAQR-dependent *FET3-lacZ* repression.

Similarly to Mao et al. (2006), an approach using the yeast two-hybrid system could be used to identify novel interactions between the Class II PAQRs and other human proteins. Because the locations (cytoplasmic or extracytoplasmic) of different portions of the Class II PAQRs are unclear, a yeast two-hybrid approach in which the whole PAQR protein is used could be better than just using a soluble portion of the protein, as Mao et al. (2006) did. One such system that can be used for identification of membrane protein-protein interactions is the split-ubiquitin membrane yeast two-hybrid system (MbYTH) (Iyer et al., 2005).

For the MbYTH, the Class II PAQRs would be fused at either their N- or C- terminus to the C-terminal half of ubiquitin (Cub) and a transcription factor (Cub-TF). The Class II PAQRs would serve as the “bait” while a library of human cDNAs encoding fusions between human proteins and the N-terminal half of ubiquitin (Nub) would serve as the “prey”. Upon interaction of a PAQR with a bait protein, the two halves of ubiquitin would be reconstituted and recognized by ubiquitin-specific proteases, leading to cleavage of the transcription factor (Iyer et al., 2005). The transcription factor then activates transcription of a reporter gene (Iyer et al., 2005). The prey protein from clones which express the reporter can be identified and the interaction between the PAQR and the prey protein can be confirmed by another method, such as co-immunoprecipitation.

Because the Cub-TF must be located in the cytoplasm for interactions to be detected (Iyer et al., 2005) and the topology of the human Class II PAQRs is debated (Thomas et al., 2007; Tang et al. 2005), the ability to identify proteins that interact with the N- or C-termini using this system could help in topology determination; however, difficulty identifying protein-protein interactions could be due to a topology that results in the extracytoplasmic localization of Cub-

TF. In this case, truncated Class II PAQRs that are still functional could be used. Functionality could be tested by the *FET3-lacZ* assay described in Chapter 2.

### **Possible Intrinsic Enzymatic Activity of PAQR Proteins**

In addition to identifying potential protein-protein interactions involved in PAQR signaling, another possibility is that the PAQRs have an intrinsic enzymatic activity that leads to a signaling cascade and eventually repression of *FET3-lacZ*. PSI-BLAST searches indicated that the PAQR proteins are distantly related to the alkaline ceramidase family of proteins (Lyons lab, unpublished). Alkaline ceramidases in yeast are Ypc1p and Ydc1p and catalyze the hydrolysis of yeast ceramides to produce sphingosine and fatty acids, with the catalytic activity of Ypc1p being reversible (Mao et al., 2000a; Mao et al., 2000b). Ceramide and sphingosine serve as important signaling molecules (Mao et al., 2000a; Mao et al., 2000b).

So how might sphingolipids affect *FET3*? Based on experimental evidence, Kupchak et al. (2007) proposed that Izh2p overexpression leads to repression of *FET3-lacZ* by activating the Nrg1p/Nrg2p repressors via AMPK and/or inactivating the Msn2p/Msn4p activators via PKA. If the PAQRs are able to alter levels of sphingolipids, signaling events may occur, such as activation of AMPK or PKA, that ultimately lead to repression of *FET3-lacZ*. Some experimental evidence from yeast for this hypothesis includes alleviation of Izh mediated *FET3-lacZ* repression in the presence of *D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol* (Kupchak, 2008), a known alkaline ceramidase inhibitor (Bielawska et al., 1996).

Further evidence that sphingolipid metabolism is affected by PAQRs was described by Villa (2007) in experiments in which the levels of sphingoid bases increased when yeast PAQRs were overexpressed. Interestingly, in a global study of signal transduction pathways affected in yeast by PAQR7 overexpression, the yeast proteins Pil1p and Lsp1p were identified as having increased phosphorylation (Regalla, 2007). Pil1p (phosphorylation is inhibited by long chain

bases) and Lsp1p (long chain bases stimulate phosphorylation) are regulated by the sphingolipid long-chain base-sensing kinases Pkh1p and Pkh2p (Zhang et al., 2004). It was determined that PAQR-mediated repression of *FET3-lacZ* is dependent on the presence of Pkh1p and Pkh2p (Kupchak, 2008). Although direct enzymatic activity assays for isolated PAQRs still need to be carried out and optimized, the evidence thus far supports that the PAQRs could affect sphingolipid metabolism.

Similar experiments in yeast could be conducted for the Class II PAQRs to examine whether or not they too may be involved in sphingolipid metabolism. Of course, the results would need to be verified in mammalian cells before claims of this nature could be made.

### **Expression of Class II PAQRs in Sf9 Insect Cells**

Because there is much conflict in the literature regarding the existence of membrane-bound steroid receptors (see Chapter 2), a model organism that is simpler than mammalian cells could be used to clarify the role of certain proteins in steroid signaling. While the majority of this thesis has focused on the use of yeast to characterize the Class II PAQRs as steroid receptors, cells from the insect *Spodoptera frugiperda* (or Sf9 insect cells) are also an attractive option for Class II PAQR studies for several reasons.

Sf9 insect cells have been useful for the functional expression of many eukaryotic proteins (Altmann et al., 1999), including multi-transmembrane domain proteins such as GPCRs (McIntire et al., 2002) and the human PAQR1 and PAQR2 proteins (Dr. Chasta Parker, Winthrop University, personal communication). Expression of PAQR1 and PAQR2 in insect cells caused activation of AMPK in the presence of adiponectin (Dr. Chasta Parker, Winthrop University, personal communication). Because there has been success with the functional expression of some PAQRs, it was expected that Sf9 insect cells could also be used for characterizations of the Class II PAQRs. Also, Sf9 insect cells are an especially attractive

organism to use for Class II PAQR characterizations because insects do not have Class II PAQR homologues (Thomas et al., 2007) and may not have nuclear progesterone receptors (Maglich et al., 2001) that could interfere with individual human Class II receptor characterizations.

### **Ligand Binding Experiments**

While our yeast system for studying the Class II PAQR response to progestins seems to indicate that these proteins sense and respond to these steroids, ligand binding assays to membranes from cells expressing the PAQRs should be performed to further support that the PAQRs are receptors for progestins. So far, different groups have obtained conflicting results for progesterone binding to membranes from bacterial or mammalian cells expressing PAQR7 (Zhu et al., 2003a; Krietsch et al., 2006; Thomas et al., 2007).

Most of the methods used to demonstrate progesterone binding to Class II PAQRs involved radiolabeled progesterone. In these assays, crude membrane preparations or purified plasma membrane preparations were used (Krietsch et al., 2006; Thomas et al., 2007). Membrane samples were incubated with tritiated progesterone and then the samples were filtered to remove unbound progesterone. Thus, we conducted some preliminary ligand binding experiments with cell membranes isolated from yeast cells overexpressing the PAQRs. While our experiments were limited by the lack of appropriate equipment, the attempts to demonstrate binding are presented in this chapter with recommendations to improve similar experiments in the future.

### **Results and Discussion**

This chapter, as well as Chapter 2, describes the use of non-mammalian eukaryotic model organisms (yeast and Sf9 insect cells) for the expression of the Class II PAQRs. In this chapter, ideas for future studies of the Class II PAQRs are described and preliminary results for some of

these ideas, including the use of insect cells for expression and ligand binding assays, are presented.

Like yeast, insect cells do not have endogenous Class II PAQRs (Thomas et al., 2007) and may not have nuclear progesterone receptors (Maglich et al., 2001). These features make these two organisms attractive for the study of these proteins because interference from endogenous progesterone receptors does not exist. While the majority of our characterizations of the Class II PAQRs were performed in yeast, expression of these proteins in Sf9 insect cells were initiated as described in this chapter. In addition, attempts to demonstrate binding to membranes from yeast cells expressing the Class II PAQRs are also described.

### **Expression of Class II PAQRs in Insect Cells**

Initial attempts to detect the expression of PAQR5 and PAQR6 were not successful. However, one attempt yielded successful expression of a protein that is close to the expected size of PAQR5 and PAQR6 (~42 kDa) (Figure 5-1). Because detectable expression of the PAQRs was inconsistent and we had some success with yeast expressions of Class II PAQRs, efforts were focused on the use of yeast.

Because some success was achieved with expressing the Class II PAQRs in insect cells, this system can be considered for future studies, including topology studies. In fact, insect cells may be more amenable than yeast for topology studies by immunofluorescence because insect cells do not have cell walls that need to be removed. As described in Chapter 3, the procedure for removal of yeast cell walls (called spheroplasting) causes permeabilization of the cells. Permeabilization of the cells allows access of the antibody to the epitope, whether it is located inside or outside the cell, making topology determination difficult.

In addition to topology studies, the Sf9 insect cells can be used to further explore the possibility that these proteins are coupled to G-proteins, as this system has been used for the characterization of GPCRs (McIntire et al., 2002).

In addition, because preliminary work with the Class I PAQRs indicates that these proteins could be expressed sufficiently in the Sf9 insect cell system for purification (Dr. Chasta Parker, Winthrop University, personal communication), it is possible that the Class II PAQRs could also be expressed in this system and purified for future biochemical characterizations, including ligand binding, signaling pathway studies, and structural studies.

### **Ligand Binding Experiments**

We wanted to demonstrate that progesterone binds to membranes containing the PAQRs. Because our expressions in yeast were consistently more successful than our insect cell expressions, we used yeast to isolate membranes containing the PAQRs. Because of equipment limitations, we chose to pursue a single-point binding assay to try to demonstrate higher specific binding to membranes from cells expressing PAQRs versus vector control cells. The vacuum manifold to which we had access was capable of filtering twelve samples per time point and did not have a pressure regulator. With this system, the nonspecific binding (NSB) was high compared to total binding (TB), and we found a rather high error when trying to determine total binding and nonspecific binding for triplicate samples. The results for PAQR5 and PAQR6 are presented in Figure 5-2. Previous single point binding assays conducted by Thomas et al. (2007) demonstrated that progesterone bound to plasma membranes of transfected human cells expressing PAQR7 about 2.5 times more than plasma membranes from untransfected cells. In our case, triplicate samples (for PAQR5, Figure 5-2) and duplicate samples (for PAQR7, data not shown) did not seem to indicate that there was significant binding of progesterone to cells

expressing either of these proteins (data not shown). For PAQR6, when specific binding was calculated, there was a high error (Figure 5-3).

There are several possible reasons that we are observing poor specific binding and poor reproducibility of progesterone to the membranes of cells expressing the PAQRs. First, steroids are considered to be “very sticky”, which can cause them to bind to various proteins and other biological structures, such as membranes (Wehling et al., 2007). This stickiness would cause high nonspecific binding.

In addition, according to Thomas et al. (2002), the Class II PAQRs have rapid association and dissociation rates. This is especially important because rapidly dissociating ligand-receptor complexes require a method for removal of unbound ligand that minimizes dissociation, such as rapid separation times and wash steps (Qume, 1999). With our system, the filtration was not uniform for the twelve different sample holders as some sample holders seemed to allow more rapid filtration than others. Variations in sample filtering times could cause variation in dissociation of specifically bound progesterone during washes. Also, with the system we used, the vacuum pressure could not be regulated, so the pressure could have fluctuated between filtration of samples and during the washes. Fluctuations in vacuum pressure can cause fluctuations in the amount of time that it takes to filter samples and the amount of dissociated ligand that can be washed away. These problems could be improved by using a 96-well cell harvester that allows for simultaneous filtering and washing of binding reactions at controlled vacuum pressures.

Finally, it is possible that membrane solubilization causes loss of ligand binding activity. It has been noted that different preparation procedures have caused loss of ligand binding activity of some receptors (Thomas et al., 2002). Optimization of parameters such as protein

concentration may also improve the results. If the protein concentration is too low, specific binding would be lower. If the protein concentration is too high, nonspecific binding to non-PAQR proteins and membrane components could increase. Until a better sample filtering system can be used, it is difficult to assess these possibilities.

## **Materials and Methods**

### **Plasmids.**

For Class II PAQR expression in insect cells, the genes for these proteins were cloned into pIEx-4 (Novagen) in collaboration with Dr. Chasta Parker (Winthrop University). This plasmid has an *Autographa californica* nucleopolyhedrovirus (AcNPV) derived hr5 enhancer and immediate early promoter (IE1) and allows for C-terminal 6x-histidine tagging of proteins. Primers were designed to have an Nco I or Pci I site at the beginning of the gene and a Not I site at the end of the gene (Table 5-1) for cloning into the pIEx-4 vector.

### **Expression of PAQR5 and PAQR6 in Sf9 Insect Cells**

Sf9 insect cells (71104-3, Novagen) were passaged and expressions were performed according to the manufacturer's instructions using serum-free BacVector® Insect Cell Medium (70590-3, Novagen). Rapidly growing cells ( $1 \times 10^7$ ) were used in 10 mL suspension cultures. Approximately 20 µg of plasmid DNA was diluted with 1 mL of serum-free medium. Separately, 100 µL of Insect GeneJuice® Transfection Reagent (71259-4, Novagen) was diluted with 1 mL of serum-free medium and the DNA was added to this mixture dropwise. The transfection mixture was added to the cells in a 125 mL Erlenmeyer flask. The cells were grown at 28°C with shaking for 48 hours. The cells were harvested and the pellets were stored at -80°C.

To detect PAQR protein expression, the Insect PopCulture™ Reagent (71187-3, Novagen) was used according to the manufacturer's instructions. Briefly, 0.05 culture volume of Insect PopCulture™ Reagent and 100 Units of RQ1 RNase-Free DNase (M610A, Promega) were

added to thawed cell pellets. The sample was mixed by inversion and incubated for 15 minutes at room temperature.

To detect expression of 6x-histidine tagged PAQRs, the SuperSignal® WestHisProbe™ Kit (15168, Pierce) was used according to the manufacturer's instructions. Briefly, equal amounts of protein (in µg) were loaded onto a 10% polyacrylamide gel and electrophoresis was performed according to standard procedures (Sambrook and Russell, 2001). Western blots were performed using nitrocellulose according to standard procedures (Sambrook and Russell, 2001). Transfer buffer was Tris-glycine, pH 8.3, 10% methanol, and 0.1% SDS. The transfer was performed at 80 volts for 60 minutes. Membranes were blocked overnight at 4°C with 1% bovine serum albumin (BSA) in BupH™ Tris Buffered Saline containing 0.05% Tween®-20 (TBST). Membranes were incubated with the HisProbe™-HRP (diluted 1:5000 in TBST). The blot was incubated with SuperSignal® West Pico Substrate Working Solution (Pierce) for 5 minutes, exposed to film and developed.

### **Ligand Binding Assays for PAQRs Expressed in Yeast**

Ligand binding was tested with total membrane extracts from cells expressing the PAQRs. Briefly, yeast cells transformed with expression vectors for the HA-tagged PAQRs (described in Chapter 2) were grown in synthetic dextrose (SD) media and reinoculated into LIM containing 2% galactose. Total membrane pellets were prepared as described in Chapter 2 with membrane isolation buffer (MIB) and ultracentrifugation at 130,000 x g for 90 minutes. To detect expression of HA-tagged proteins, Western blots were performed as described in Chapter 2 with the primary antibody (rabbit polyclonal IgG HA-probe (Y-11), SC-805, Santa Cruz Biotechnology, Inc.) diluted 1:500 in PBS containing 1% BSA. The secondary antibody used was horse radish peroxidase-conjugated goat anti-rabbit IgG-HRP (1:10000, SC-2004, Santa

Cruz Biotechnology, Inc.). The blot was incubated with SuperSignal® West Pico Substrate Working Solution (Pierce) for 5 minutes, exposed to film and developed.

For binding reactions, samples for total binding were set up to have 5 nM tritiated progesterone ( $^3\text{H-PG}$ , P5050, Sigma), an amount similar to that which was previously used in Class II PAQR single-point binding assays (Karteris et al., 2006), in ethanol, while nonspecific binding samples had 1000 fold excess (5  $\mu\text{M}$ ) cold progesterone in addition to 5 nM tritiated progesterone. The ethanol concentration was adjusted to 2% when necessary. In 500  $\mu\text{L}$  of YP (yeast extract and peptone) to reduce nonspecific binding (Blumer et al., 1988), either 40  $\text{ng}/\mu\text{L}$  total protein or 110  $\text{ng}/\mu\text{L}$  total protein was used, similar to amounts described in Windh and Manning (2002) for radioligand binding assays using tritiated ligands for GPCRs. Samples were incubated at room temperature before being filtered onto GF/B filters (Whatman) that were presoaked in polyethylenimine (0.5%) (Thomas et al., 2007; Krietsch et al., 2006). A 1225 Sampling Manifold (Millipore) was used to filter the samples. The vacuum was supplied by an aspirator vacuum pump without a pressure regulator. A 100 or 200  $\mu\text{L}$  aliquot of sample was filtered and washed three times with 1 mL of ice cold YP. After the filters dried, they were transferred to scintillation vials and 20 mL of Scintisafe 30% cocktail (SX23-5, Fisher Scientific) was added to the vial. The vials were mixed vigorously and allowed to sit for a 2-3 hours before radioactivity was determined. Liquid scintillation counting was performed using in a Packard 1600 TR instrument. Each sample was counted for 5 minutes. The counts per minute (cpm) were converted to dpm as follows:  $\text{dpm} = \text{cpm}/(\text{counter efficiency})$ , where the counter efficiency is 0.6. The dpm per amount of protein filtered was calculated for the data plots. Specific binding was calculated as the difference between total binding (TB) and nonspecific binding (NSB).

## Summation

The focus of this dissertation has been on the use of *S. cerevisiae* as a model organism to study the human PAQR family of proteins. In particular, the function of the Class II PAQRs in sensing and responding to progestin steroids was examined. While we were able to demonstrate that the presence of progestins causes a physiological effect in cells that express the Class II PAQRs, several questions remain regarding the mechanism of signaling initiated by the Class II PAQRs and whether this mechanism is conserved for all PAQR family members.

Here, to try to elucidate the way by which the Class II PAQRs signal, it was proposed that novel protein-protein interactions could be explored using yeast as a tool. In addition, it was proposed that an intrinsic enzymatic activity could also be explored as a possibility. Although preliminary experiments have not been conducted for these experiments, preliminary work was performed for the expression of the Class II PAQRs in insect cells and to demonstrate binding of progesterone to these proteins.

While different attempts were made to express the Class II PAQRs in insect cells, these attempts yielded inconsistent success, perhaps due to inexperience working with this expression system. Because we saw expression of PAQR5 with this system, it could be very useful for future studies, including protein topology analysis and protein purification.

In addition, while it is important to demonstrate binding of progesterone to the PAQRs, we had difficulty doing so. Whether the problems we had can be solved with the use of a better binding reaction filtering system will have to be determined once access to such a system is available.

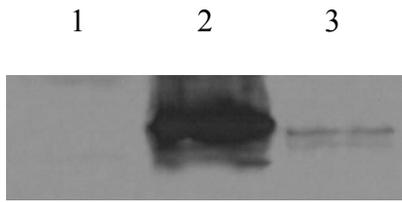


Figure 5-1. Western blot of PAQR 5 and PAQR 6 expressed in Sf9 insect cells. There is no band in the empty vector sample (lane 1), but a very strong band is visible in the sample for PAQR5 around 42 kDa (lane 2) and a faint band is present in the sample for PAQR6 (lane 3) around the same size. The cells used for this expression were maintained by Ibon Garitaonandia.

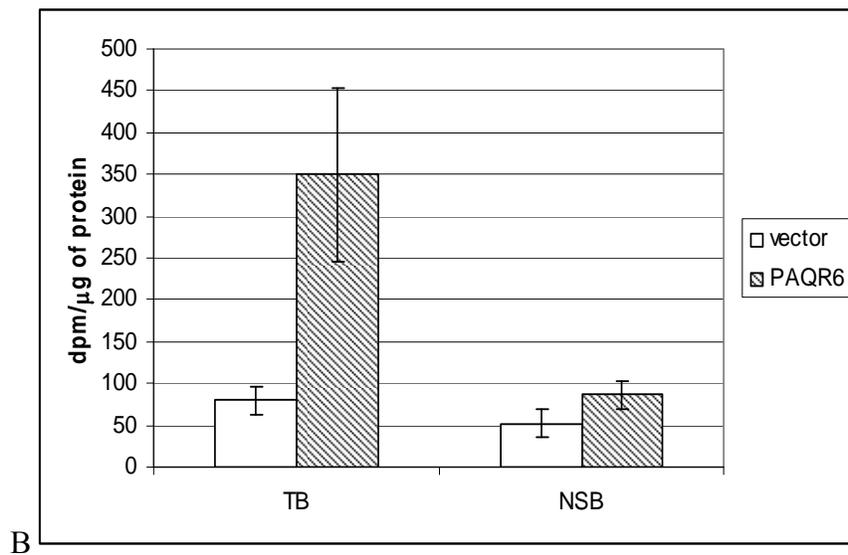
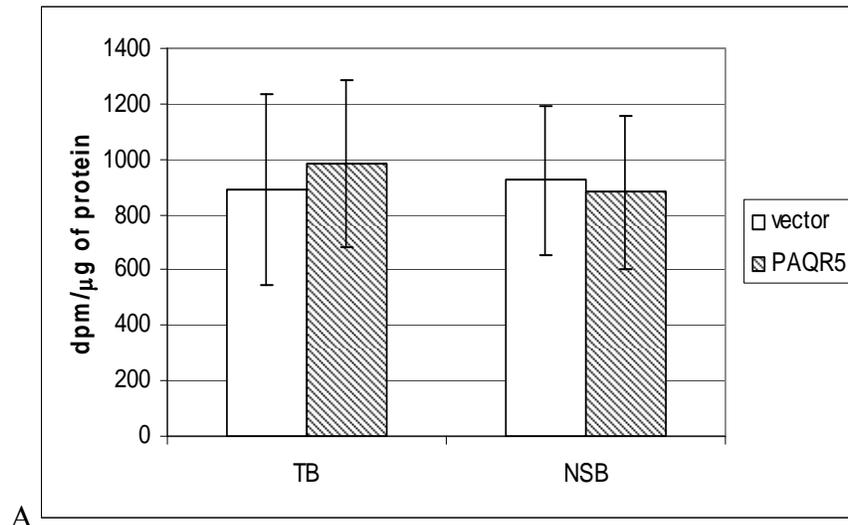


Figure 5-2. Binding of <sup>3</sup>H-PG in a single-point binding assay with total membranes isolated from yeast cells expressing PAQR5 (panel A) or PAQR6 (panel B). Specific binding was calculated as the difference between total binding (TB) and nonspecific binding (NSB), as presented in Figure 5-2. Conditions used for binding are described in the Material and Methods.

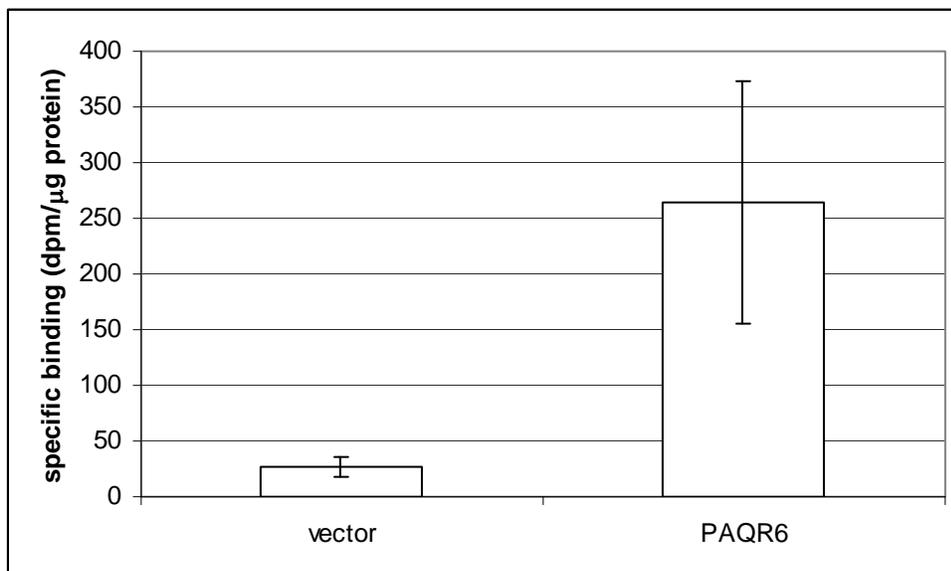


Figure 5-3. Specific binding of  $^3\text{H}$ -PG in a single-point binding assay with total membranes isolated from yeast cells expressing PAQR6. Specific binding was calculated as the difference between total binding and nonspecific binding.

Table 5-1. Primers used for cloning PAQRs into pIEx-4 (listed from 5'  $\rightarrow$  3')

Primer name	Primer sequence
Fwd PAQR5	CCAAGTGACCATGGTGAGCCTGAAGCTC
Rev PAQR5	AGAAGATGCGGCCGCTGTTTCTTTTTTATGTAATTCTG
Fwd PAQR6	CCAAGTGACATGTTTCAGTCTCAAGCTGCC
Rev PAQR6	AGAAGATGCGGCCGCTGTTGTTTGGCCTGGGTAC

## LIST OF REFERENCES

- Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P (2002) *Molecular Biology of the Cell*. Garland Science, New York, NY.
- Altmann F, Staudacher E, Wilson IB and Marz L (1999) Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj J* **16**:109-123.
- Askwith C, Eide D, Van Ho A, Bernard PS, Li L, Davis-Kaplan S, Sipe DM and Kaplan J (1994) The *FET3* gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76**:403-410.
- Baggett JJ, Shaw JD, Sciambi CJ, Watson HA and Wendland B (2003) Fluorescent labeling of yeast. *Curr Protoc Cell Biol* **Chapter 4**:Unit 4 13.
- Baida GE and Kuzmin NP (1995) Cloning and primary structure of a new hemolysin gene from *Bacillus cereus*. *Biochim Biophys Acta* **1264**:151-154.
- Baida GE and Kuzmin NP (1996) Mechanism of action of hemolysin III from *Bacillus cereus*. *Biochim Biophys Acta* **1284**:122-124.
- Baldi E, Casano R, Falsetti C, Krausz C, Maggi M and Forti G (1991) Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J Androl* **12**:323-330.
- Beck CA, Weigel NL, Moyer ML, Nordeen SK and Edwards DP (1993) The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. *Proc Natl Acad Sci U S A* **90**:4441-4445.
- Beukers MW and IJzerman AP (2005) Techniques: how to boost GPCR mutagenesis studies using yeast. *Trends Pharmacol Sci* **26**:533-539.
- Bielawska A, Greenberg MS, Perry D, Jayadev S, Shayman JA, McKay C and Hannun YA (1996) (1*S*,2*R*)-*D*-erythro-2-(*N*-Myristoylamino)-1-phenyl-1-propanol as an inhibitor of ceramidase. *J Biol Chem* **271**:12646-12654.
- Blumer KJ, Reneke JE and Thorner J (1988) The *STE2* gene product is the ligand-binding component of the  $\alpha$ -factor receptor of *Saccharomyces cerevisiae*. *J Biol Chem* **263**: 10836-10842.
- Brauer AU, Nitsch R and Savaskan NE (2004) Identification of macrophage/microglia activation factor (MAF) associated with late endosomes/lysosomes in microglial cells. *FEBS Lett* **563**:41-48.
- Castilla JA, Gil T, Molina J, Hortas ML, Rodriguez F, Torres-Munoz J, Vergara F and Herruzo AJ (1995) Undetectable expression of genomic progesterone receptor in human spermatozoa. *Hum Reprod* **10**:1757-1760.

- Chen HC and Farese RV (1999) Steroid hormones: Interactions with membrane-bound receptors. *Curr Biol* **9**:R478-481.
- Chen YC, Chang MC, Chuang YC and Jeang CL (2004) Characterization and virulence of hemolysin III from *Vibrio vulnificus*. *Curr Microbiol* **49**:175-179.
- Cherry JM, Adler C, Ball C, Chervitz SA, Dwight SS, Hester ET, Jia Y, Juvik G, Roe T, Schroeder M, Weng S and Botstein D (1998) SGD: *Saccharomyces* Genome Database. *Nucleic Acids Res* **26**:73-79.
- Daley DO, Rapp M, Granseth E, Melen K, Drew D and von Heijne G (2005) Global topology analysis of the *Escherichia coli* inner membrane proteome. *Science* **308**:1321-1323.
- Deshaies RJ and Schekman R (1987) A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J Cell Biol* **105**:633-645.
- Dohlman HG, Song J, Ma D, Courchesne WE and Thorner J (1996) Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). *Mol Cell Biol* **16**:5194-5209.
- Dosiou C, Hamilton AE, Pang Y, Overgaard MT, Tulac S, Dong J, Thomas P and Giudice LC (2008) Expression of membrane progesterone receptors on human T lymphocytes and Jurkat cells and activation of G-proteins by progesterone. *J Endocrinol* **196**:67-77.
- Dowell SJ and Brown AJ (2002) Yeast assays for G-protein-coupled receptors. *Receptors Channels* **8**:343-352.
- Dressing GE and Thomas P (2007) Identification of membrane progesterone receptors in human breast cancer cell lines and biopsies and their potential involvement in breast cancer. *Steroids* **72**:111-116.
- Eide D and Guarente L (1992) Increased dosage of a transcriptional activator gene enhances iron-limited growth of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**:347-354.
- Evaul K, Jamnongjit M, Bhagavath B and Hammes SR (2007) Testosterone and progesterone rapidly attenuate plasma membrane Gbetagamma-mediated signaling in *Xenopus laevis* oocytes by signaling through classical steroid receptors. *Mol Endocrinol* **21**:186-196.
- Falkenstein E, Tillmann HC, Christ M, Feuring M and Wehling M (2000) Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev* **52**:513-556.
- Falkenstein E and Wehling M (2000) Nongenomically initiated steroid actions. *Eur J Clin Invest* **30 Suppl 3**:51-54.
- Farach-Carson MC and Davis PJ (2003) Steroid hormone interactions with target cells: cross talk between membrane and nuclear pathways. *J Pharmacol Exp Ther* **307**:839-845.

- Feng L, Xie X, Ding Q, Luo X, He J, Fan F, Liu W, Wang Z and Chen Y (2007) Spatial regulation of Raf kinase signaling by RKTG. *Proc Natl Acad Sci U S A* **104**:14348-14353.
- Fernandes MS, Brosens JJ and Gellersen B (2007) Honey, we need to talk about the membrane progesterin receptors. *Steroids*.
- Fernandes MS, Pierron V, Michalovich D, Astle S, Thornton S, Peltoketo H, Lam EW, Gellersen B, Huhtaniemi I, Allen J and Brosens JJ (2005) Regulated expression of putative membrane progesterin receptor homologues in human endometrium and gestational tissues. *J Endocrinol* **187**:89-101.
- Garitaonandia I (2008) Progesterin and AdipoQ Receptors, Identification of PAQR3 as a New Possible Adiponectin Receptor, in *Unpublished doctoral dissertation*, University of Florida, Gainesville.
- Gass EK, Leonhardt SA, Nordeen SK and Edwards DP (1998) The antagonists RU486 and ZK98299 stimulate progesterone receptor binding to deoxyribonucleic acid in vitro and in vivo, but have distinct effects on receptor conformation. *Endocrinology* **139**:1905-1919.
- Gitan RS and Eide D (2000) Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter. *Biochem J* **346**: 329-336.
- Graham JD and Clarke CL (1997) Physiological action of progesterone in target tissues. *Endocr Rev* **18**:502-519.
- Greenwald P, Barlow JJ, Nasca PC and Burnett WS (1971) Vaginal cancer after maternal treatment with synthetic estrogens. *N Engl J Med* **285**:390-392.
- Guarente L (1983) Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. *Methods Enzymol* **101**:181-191.
- Gulig PA, Bourdage KL and Starks AM (2005) Molecular Pathogenesis of *Vibrio vulnificus*. *J Microbiol* **43 Spec No**:118-131.
- Guo M, Aston C, Burchett SA, Dyke C, Fields S, Rajarao SJ, Uetz P, Wang Y, Young K and Dohlman HG (2003) The yeast G protein alpha subunit Gpa1 transmits a signal through an RNA binding effector protein Scp160. *Mol Cell* **12**:517-524.
- Hanna R, Pang Y, Thomas P and Zhu Y (2006) Cell-surface expression, progesterin binding, and rapid nongenomic signaling of zebrafish membrane progesterin receptors alpha and beta in transfected cells. *J Endocrinol* **190**:247-260.
- Harashima T and Heitman J (2002) The Galpha protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gbeta subunits. *Mol Cell* **10**:163-173.
- Heijne GV (1986) The distribution of positively charged residues in bacterial inner membrane proteins correlates with the trans-membrane topology. *EMBO J* **5**:3021-3027.

- Heim R, Cubitt AB and Tsien RY (1995) Improved green fluorescence. *Nature* **373**:663-664.
- Herbst AL, Ulfelder H and Poskanzer DC (1971) Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med* **284**:878-881.
- Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS and O'Shea EK (2003) Global analysis of protein localization in budding yeast. *Nature* **425**:686-691.
- Iyer K, Burkle L, Auerbach D, Thaminy S, Dinkel M, Engels K and Stagljar I (2005) Utilizing the split-ubiquitin membrane yeast two-hybrid system to identify protein-protein interactions of integral membrane proteins. *Sci. STKE* **2005**: pl3.
- Jansen G, Wu C, Schade B, Thomas DY and Whiteway M (2005) Drag&Drop cloning in yeast. *Gene* **344**:43-51.
- Josefsberg Ben-Yehoshua L, Lewellyn AL, Thomas P and Maller JL (2007) The role of *Xenopus* membrane progesterone receptor beta in mediating the effect of progesterone on oocyte maturation. *Mol Endocrinol* **21**:664-673.
- Kadowaki T and Yamauchi T (2005) Adiponectin and adiponectin receptors. *Endocr Rev* **26**:439-451.
- Karpichev IV, Cornivelli L and Small GM (2002) Multiple regulatory roles of a novel *Saccharomyces cerevisiae* protein, encoded by YOL002c, in lipid and phosphate metabolism. *J Biol Chem* **277**:19609-19617.
- Karteris E, Zervou S, Pang Y, Dong J, Hillhouse EW, Randeva HS and Thomas P (2006) Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term. *Mol Endocrinol* **20**:1519-1534.
- Kim H, Melen K, Osterberg M and von Heijne G (2006) A global topology map of the *Saccharomyces cerevisiae* membrane proteome. *Proc Natl Acad Sci U S A* **103**:11142-11147.
- Kim H, Melen K and von Heijne G (2003) Topology models for 37 *Saccharomyces cerevisiae* membrane proteins based on C-terminal reporter fusions and predictions. *J Biol Chem* **278**:10208-10213.
- Klichko VI, Miller J, Wu A, Popov SG and Alibek K (2003) Anaerobic induction of *Bacillus anthracis* hemolytic activity. *Biochem Biophys Res Commun* **303**:855-862.
- Kreft SG, Wang L and Hochstrasser M (2006) Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). *J Biol Chem* **281**:4646-4653.

- Krietsch T, Fernandes MS, Kero J, Losel R, Heyens M, Lam EW, Huhtaniemi I, Brosens JJ and Gellersen B (2006) Human homologs of the putative G protein-coupled membrane progesterin receptors (mPR $\alpha$ ,  $\beta$ , and  $\gamma$ ) localize to the endoplasmic reticulum and are not activated by progesterone. *Mol Endocrinol* **20**:3146-3164.
- Kupchak BR (2008) The PAQR Family of Membrane Proteins in *Saccharomyces cerevisiae*: Their Role in the Signal Transduction Pathway of *FET3* and Flow Cytometric Analysis of AdipoR1 Binding to Adiponectin and TNF-Alpha, in *Unpublished doctoral dissertation*, University of Florida, Gainesville.
- Kupchak BR, Garitaonandia I, Villa NY, Mullen MB, Weaver MG, Regalla LM, Kendall EA and Lyons TJ (2007) Probing the mechanism of *FET3* repression by Izh2p overexpression. *Biochim Biophys Acta* **1773**:1124-1132.
- Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**:105-132.
- Ladds G, Goddard A and Davey J (2005) Functional analysis of heterologous GPCR signalling pathways in yeast. *Trends Biotechnol* **23**:367-373.
- Losel R and Wehling M (2003) Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol* **4**:46-56.
- Lyons TJ, Villa NY, Regalla LM, Kupchak BR, Vagstad A and Eide DJ (2004) Metalloregulation of yeast membrane steroid receptor homologs. *Proc Natl Acad Sci U S A* **101**:5506-5511.
- Maglich JM, Sluder A, Guan X, Shi Y, McKee DD, Carrick K, Kamdar K, Willson TM and Moore JT (2001) Comparison of complete nuclear receptor sets from the human, *Caenorhabditis elegans* and *Drosophila* genomes. *Genome Biology* **2**:0029.1-0029.7.
- Mao C, Xu R, Bielawska A and Obeid LM (2000a) Cloning of an alkaline ceramidase from *Saccharomyces cerevisiae*. An enzyme with reverse (CoA-independent) ceramide synthase activity. *J Biol Chem* **275**:6876-6884.
- Mao C, Xu R, Bielawska A, Szulc ZM and Obeid LM (2000b) Cloning and characterization of a *Saccharomyces cerevisiae* alkaline ceramidase with specificity for dihydroceramide. *J Biol Chem* **275**:31369-31378.
- Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, Liu F and Dong LQ (2006) APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat Cell Biol* **8**:516-523.
- Martinez S, Pasten P, Suarez K, Garcia A, Nualart F, Montecino M, Hinrichs MV and Olate J (2007) Classical *Xenopus laevis* progesterone receptor associates to the plasma membrane through its ligand-binding domain. *J Cell Physiol* **211**:560-567.

- McCue L, Thompson W, Carmack C, Ryan MP, Liu JS, Derbyshire V and Lawrence CE (2001) Phylogenetic footprinting of transcription factor binding sites in proteobacterial genomes. *Nucleic Acids Res* **29**:774-782.
- McEwan IJ (2001) Bakers yeast rises to the challenge: reconstitution of mammalian steroid receptor signalling in *S. cerevisiae*. *Trends Genet* **17**:239-243.
- McIntire WE, Myung CS, MacCleery G, Wang Q and Garrison JC (2002) Reconstitution of G protein-coupled receptors with recombinant G protein alpha and beta gamma subunits. *Methods Enzymol* **343**:372-393.
- Melcher K (2000) A modular set of prokaryotic and eukaryotic expression vectors. *Anal Biochem* **277**:109-120.
- Mentesana PE, Dosil M and Konopka JB (2002) Functional assays for mammalian G-protein-coupled receptors in yeast. *Methods Enzymol* **344**:92-111.
- Milligan G (2003) Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol* **64**:1271-1276.
- Miroux B and Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* **260**:289-298.
- Morrill GA and Kostellow AB (1999) Progesterone induces meiotic division in the amphibian oocyte by releasing lipid second messengers from the plasma membrane. *Steroids* **64**:157-167.
- Narasimhan ML, Coca MA, Jin J, Yamauchi T, Ito Y, Kadowaki T, Kim KK, Pardo JM, Damsz B, Hasegawa PM, Yun DJ and Bressan RA (2005) Osmotin is a homolog of mammalian adiponectin and controls apoptosis in yeast through a homolog of mammalian adiponectin receptor. *Mol Cell* **17**:171-180.
- Newbold RR, Padilla-Banks E and Jefferson WN (2006) Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology* **147**:S11-17.
- Norman AW, Mizwicki MT and Norman DP (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat Rev Drug Discov* **3**:27-41.
- Oettel M and Mukhopadhyay AK (2004) Progesterone: the forgotten hormone in men? *Aging Male* **7**:236-257.
- Pausch MH (1997) G-protein-coupled receptors in *Saccharomyces cerevisiae*: high-throughput screening assays for drug discovery. *Trends Biotechnol* **15**:487-494.
- Pedelacq JD, Cabantous S, Tran T, Terwilliger TC and Waldo GS (2006) Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotechnol* **24**:79-88.

- Pringle JR, Adams AE, Drubin DG and Haarer BK (1991) Immunofluorescence methods for yeast. *Methods Enzymol* **194**:565-602.
- Qume M (1999) Overview of ligand-receptor binding techniques. *Methods Mol Biol* **106**:3-23.
- Rapp M, Granseth E, Seppala S and von Heijne G (2006) Identification and evolution of dual-topology membrane proteins. *Nat Struct Mol Biol* **13**:112-116.
- Regalla, LM (2007) Characterization of the PAQR family of membrane proteins in *Saccharomyces cerevisiae*: examination of their role in metal and lipid metabolism and mass spectrometric-based analysis of signal transduction pathways, in *Doctoral dissertation*, University of Florida, Gainesville.
- Rehli M, Krause SW, Schwarzfischer L, Kreutz M and Andreesen R (1995) Molecular cloning of a novel macrophage maturation-associated transcript encoding a protein with several potential transmembrane domains. *Biochem Biophys Res Commun* **217**:661-667.
- Robertson KP, Smith CJ, Gough AM and Rocha ER (2006) Characterization of *Bacteroides fragilis* hemolysins and regulation and synergistic interactions of HlyA and HlyB. *Infect Immun* **74**:2304-2316.
- Rodriguez AM, Monjo M, Roca P and Palou A (2002) Opposite actions of testosterone and progesterone on UCP1 mRNA expression in cultured brown adipocytes. *Cell Mol Life Sci* **59**:1714-1723.
- Sääf A, Johansson M, Wallin E and von Heijne G (1999) Divergent evolution of membrane protein topology: the *Escherichia coli* RnfA and RnfE homologues. *Proc Natl Acad Sci U S A* **96**:8540-8544.
- Sambrook J and Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sengstag C (2000) Using SUC2-HIS4C reporter domain to study topology of membrane proteins in *Saccharomyces cerevisiae*. *Methods Enzymol* **327**:175-190.
- Severance S, Chakraborty S and Kosman DJ (2004) The Ftr1p iron permease in the yeast plasma membrane: orientation, topology and structure-function relationships. *Biochem J* **380**:487-496.
- Shaner NC, Steinbach PA and Tsien RY (2005) A guide to choosing fluorescent proteins. *Nat Methods* **2**:905-909.
- Sikorski RS and Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19-27.
- Slessareva JE and Dohlman HG (2006) G protein signaling in yeast: new components, new connections, new compartments. *Science* **314**:1412-1413.

- Sturgeon CM, Kemmer D, Anderson HJ and Roberge M (2006) Yeast as a tool to uncover the cellular targets of drugs. *Biotechnol J* **1**:289-298.
- Suzuki T, Ganesh S, Agarwala KL, Morita R, Sugimoto Y, Inazawa J, Delgado-Escueta AV and Yamakawa K (2001) A novel gene in the chromosomal region for juvenile myoclonic epilepsy on 6p12 encodes a brain-specific lysosomal membrane protein. *Biochem Biophys Res Commun* **288**:626-636.
- Tang YT, Hu T, Arterburn M, Boyle B, Bright JM, Emtage PC and Funk WD (2005) PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. *J Mol Evol* **61**:372-380.
- Thomas P, Pang Y, Dong J, Groenen P, Kelder J, de Vlieg J, Zhu Y and Tubbs C (2007) Steroid and G protein binding characteristics of the seatrout and human progesterin membrane receptor alpha subtypes and their evolutionary origins. *Endocrinology* **148**:705-718.
- Thomas P, Zhu Y and Pace M (2002) Progesterin membrane receptors involved in the meiotic maturation of teleost oocytes: a review with some new findings. *Steroids* **67**:511-517.
- Tiberi M and Caron MG (1994) High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype. *J Biol Chem* **269**:27925-27931.
- Tokumoto T, Tokumoto M and Thomas P (2007) Interactions of diethylstilbestrol (DES) and DES analogs with membrane progesterin receptor-alpha and the correlation with their nongenomic progesterin activities. *Endocrinology* **148**:3459-3467.
- Villa NY (2007) Characterization of novel yeast receptors implicated in metal and lipid metabolic pathways, in *Doctoral dissertation*, University of Florida, Gainesville.
- Villalobos A, Ness JE, Gustafsson C, Minshull J and Govindarajan S (2006) Gene Designer: a synthetic biology tool for constructing artificial DNA segments. *BMC Bioinformatics* **7**:285-292.
- von Heijne G (1989) Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. *Nature* **341**:456-458.
- Wehling M, Schultz A and Losel R (2007) To be or not to be (a receptor). *Steroids* **72**:107-110.
- Windh RT and Manning DR (2002) Expression of G protein-coupled receptors and G proteins in Sf9 cells: analysis of coupling by radioligand binding. *Methods Enzymol* **343**:417-429.
- Wooding S and Pelham HR (1998) The dynamics of golgi protein traffic visualized in living yeast cells. *Mol Biol Cell* **9**:2667-2680.
- Yamaguchi-Iwai Y, Dancis A and Klausner RD (1995) AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J* **14**:1231-1239.

- Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R and Kadowaki T (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423**:762-769.
- Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB and Kadowaki T (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8**:1288-1295.
- Yang F, Moss LG and Phillips GN, Jr. (1996) The molecular structure of green fluorescent protein. *Nat Biotechnol* **14**:1246-1251.
- Zhang X, Lester RL and Dickson RC. (2004) Pil1p and Lsp1p negatively regulate the 3-phosphoinositide-dependent protein kinase-like kinase Pkh1p and downstream signaling pathways Pkc1p and Ypk1p. *J Biol Chem* **279**:22030-22038.
- Zhang YM, Marrakchi H and Rock CO (2002) The FabR (YijC) transcription factor regulates unsaturated fatty acid biosynthesis in *Escherichia coli*. *J Biol Chem* **277**:15558-15565.
- Zhou XC, Dowdy SC, Podratz KC and Jiang SW (2007) Epigenetic considerations for endometrial cancer prevention, diagnosis and treatment. *Gynecol Oncol* **107**:143-153.
- Zhu Y, Bond J and Thomas P (2003a) Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progesterin receptor. *Proc Natl Acad Sci U S A* **100**:2237-2242.
- Zhu Y, Rice CD, Pang Y, Pace M and Thomas P (2003b) Cloning, expression, and characterization of a membrane progesterin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A* **100**:2231-2236.

## BIOGRAPHICAL SKETCH

Jessica L. Smith was born and raised in a rural area of Western Pennsylvania. While pursuing her B.S. with a double major in biology and chemistry at the Clarion University of Pennsylvania, she developed an interest in molecular biology. She attended Texas A&M University for two semesters in the biochemistry graduate program and then transferred to the Department of Chemistry at the University of Florida, where she pursued her doctorate with Dr. Thomas Lyons.

Jessica will join the research group of Dr. Vinay Pathak in the National Cancer Institute's HIV Drug Resistance Program to work as a postdoctoral fellow and to pursue her interest in studying infectious diseases at the molecular level.