# ACTIVATION OF THE RAT LUTEINIZING HORMONE FOLLOWING SUBSTITUTION OF TREONINE-413 WITH SELECTIVE RESIDUES.

By

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ABSTRACT OF THE THESIS

ACTIVATION OF THE RAT LUTEINIZING HORMONE FOLLOWING SUBSTITUTION OF TREONINE-413 WITH SELECTIVE RESIDUES.

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The glycoprotein hormones lutropin (LH), follitropin (FSH) and thyrotropin (TSH) control the many functions of the ovaries, testes and thyroid (27). Humans have two lutropins, (hLH) and chorionic gonadotropin (hCG). hLH is secreted from the pituitary gland while hCG is a placental protein (27). It has been known since 1989 that the receptor for these lutropins is a G protein-coupled receptor (GPCR), which constitute the largest most-conserved superfamily of signaling molecules (42).

High affinity hormone binding results from interactions between residues in the curved portion of the extracellular domain of the receptor and the groove in the hormone formed by the apposition of the second α-subunit loop and the first and third β-subunit loops. Most of the remainder of the hormone is found in the large space between the arms of the extracellular domain and makes few, if any, additional specific contacts with the receptor needed for high affinity binding. Signal transduction is caused by steric or other influences of the hormone on the distance between the arms of the extracellular domain, an effect augmented by the oligosaccharides. Because the extracellular domain is coupled

at multiple sites to the transmembrane domain, the change in conformation of the extracellular domain is relayed to the transmembrane domain and subsequently to the cytoplasmic surface of the plasma membrane.

Here I will discuss several mutations made of the LHR amino acid Threonine 413 (T413). These six mutations were analyzed by hCG binding and cAMP assays. Since hCG is capable of stimulating steriodogenesis and cyclic AMP formation in the rat testicular tissues (28), it was important that we looked at not only the ability of the mutants created to bind hCG but also their effects on cAMP activation. It is important to study this particular amino acid because it is highly conserved over many species and the unique nature of this residue has not been investigated to our knowledge.

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## INTRODUCTION

The glycoprotein hormones lutropin (LH), follitropin (FSH) and thyrotropin (TSH) control the many functions of the ovaries, testes and thyroid (27). Humans have two lutropins, (hLH) and chorionic gonadotropin (hCG). hLH is secreted from the pituitary gland while hCG is a placental protein (27). It has been known since 1989 that the receptor for these lutropins is a G protein-coupled receptor (GPCR), which constitute the largest most-conserved superfamily of signaling molecules (42).

Structurally the glycoprotein hormones are members of the superfamily of cysteine-knot growth factors including, nerve growth factor and transforming growth factor β. Glycoprotein hormones associate with their receptors as heterodimers. The α-subunits for each of the individual hormones have the same amino acid sequence, come from the same gene, and differ only in their glycosylation patterns (36), while the β-subunits are all dissimilar which creates the specificity of the individual hormone LHR belongs to the G protein-coupled receptor (GPCR) subclass that includes the receptors for the pituitary and placental glycoprotein hormones LH, FSH, TSH and hCG. FSH and LH induce expression of LHR, where growth hormone alone or in conjunction with others support receptor expression and prevent loss of the receptor (16). GPCRs represent the largest family of membrane proteins in the human genome and the richest source of targets for the pharmaceutical industry (21). Some GPCRs may also signal through a G protein independent pathway (21).

GPCRs transduce signals by activating one or more members of the small family of highly homologous heterotrimeric G proteins (21). These cell surface-associated proteins mediate the action of a variety of extracellular signals into intracellular second messengers, therefore regulating many physiological processes (35).

More specifically, the receptors of interest trigger an intracellular G protein to exchange one bound GDP for GTP. The GTP causes a conformational change which allows the interaction of the G protein with downstream cAMP (35).

Here I will discuss several mutations made of the LHR amino acid Threonine 413 (T413). These six mutations were analyzed by hCG binding and cAMP assays. Since hCG is capable of stimulating steriodogenesis and cyclic AMP formation in the rat testicular tissues (28), it was important that we looked at not only the ability of the mutants created to bind hCG but also their effects on cAMP activation. It is important to study this particular amino acid because it is highly conserved over many species and the unique nature of this residue has not been investigated to our knowledge.

**BACKGROUND**

The pulse like release of GnRH feeds the pituitary gonadotropin hormones; luteinizing hormone (LH) and follicle stimulating hormone (FSH) together coordinate mammalian reproduction by regulating gonadal function (16). LH receptors and FSH receptors mediate the actions of hCG and FSH on the gonads (26, 32a). Regulation includes the stimulation, maturation and function of testis and ovary as well as that of gametogenesis and steriodogenesis by LH and FSH (16). In the testis specifically, LH regulates function via the plasma membrane receptors on the Leydig cells to maintain general metabolic processes, steriodogenic enzymes, and the production and secretion of androgens. LH receptors (LHR) of the testis are expressed frequently throughout life beginning with the fetus and continuing through out adult life (16). In the female, an acute rise or surge of LH triggers ovulation and corpus luteum development. The LHR is necessary for follicular maturation and ovulation, as well as luteal development via the interaction with LH.

hCG is secreted by the syncytiotrophoblastic cells of the placenta during the early weeks of pregnancy to stimulate progesterone synthesized by the ovarian corpus luteum until the placenta assumes this function (25a, 40, 46). hCG levels can be detected during the first trimester in the urine to determine pregnancy since excess hCG is secreted during this period.

**Physiological Significance**

FSH regulates and promotes the maturation of follicular cells. Expression of the LHR is induced by FSH (16, 23). A surge of LH causes ovulation by promoting the preovulatory follicle to rupture and release of the ovum (16). At this point, hCG is synthesized and secreted by blastocyst cells of the placenta as early as eight days after the LH peak (15), to maintain the secretions of estrogen and progesterone by the corpus luteum during pregnancy. Throughout fetal development, the rLHR (rat LHR) is not detectable in the fetal ovary but expression is found in early neonatal life in the ovary and testis at day 12.5 (16, 35b). hCG production begins at the time of implantation in the primate.

The steriodogenic actions of LH are primarily exerted via cAMP-mediated events in the gonads, with support from the phosphoinositide (PI) signaling pathway also functioning in the ovary. Secretions of LH or hCG maintain LHR levels and steriodogenic enzymes in the adult Leydig cell (16). However, major increases in LH levels, or a single pharmacological dose of LH or hCG can cause down-regulation of the LHR and desensitization to the hormonal signal. Interestingly, the fetal and immature testes are more resilient to desensitization and treatment with LH or hCG causes up-regulation and is discussed further (16).

**Location of mRNA for LHR**

LHR expression has been studied widely in Leydig and ovarian cells as well as membrane preparations from several species; including rat, mouse, pig, human and stable murine cell lines. The LHR cDNA was cloned from pig testis and rat, mouse and human

ovarian libraries, and the structure has been defined in rat, human and mouse. LH bound to hCG receptors has been found in several human tissues that are not considered classical target tissues; Including the nonpregnant uterus, placenta, fallopian tubes, uterine vessels, umbilical cord, brain and lymphocytes. There have been studies shown with LH and hCG bound to *Candida albicans* blastophores and CG-like proteins in yeast. They induce the transition of the yeast from blastophores to hyphal forms, which may control the pathogenicity of the organisms (16).

**HORMONE**

Pituitary and placental glycoprotein hormones LH, hCG, FSH and TSH are all heterodimers containing an α-subunit and a β-subunit, the α- subunit of hCG contains 92 amino acids while the β-subunit contains 145 (32a, 46).

Each subunit is divided into three long antiparallel loops, α1, α2, α3; β1, β2, β3 by a cysteine knot as shown in Figure 2 (27). In the complex heterodimer the subunits are "head to toe" so that α2 is adjacent to β1, β3 and β2 is adjacent to α1, α3 (27, 45). Once the α-subunits combine to form heterodimers; the specificity is controlled by the β-subunit. Both of the subunits show similar topology in that three of the disulfides in each subunit form a cysteine knot core that results in an elongated protein containing two paired loops at one end and a single loop at the other (32a, 52). The β-subunits C-terminus 20 amino acids become wrapped around the α-subunit loop like a seatbelt and latch to β-subunit loop 1 by a disulfide (52).

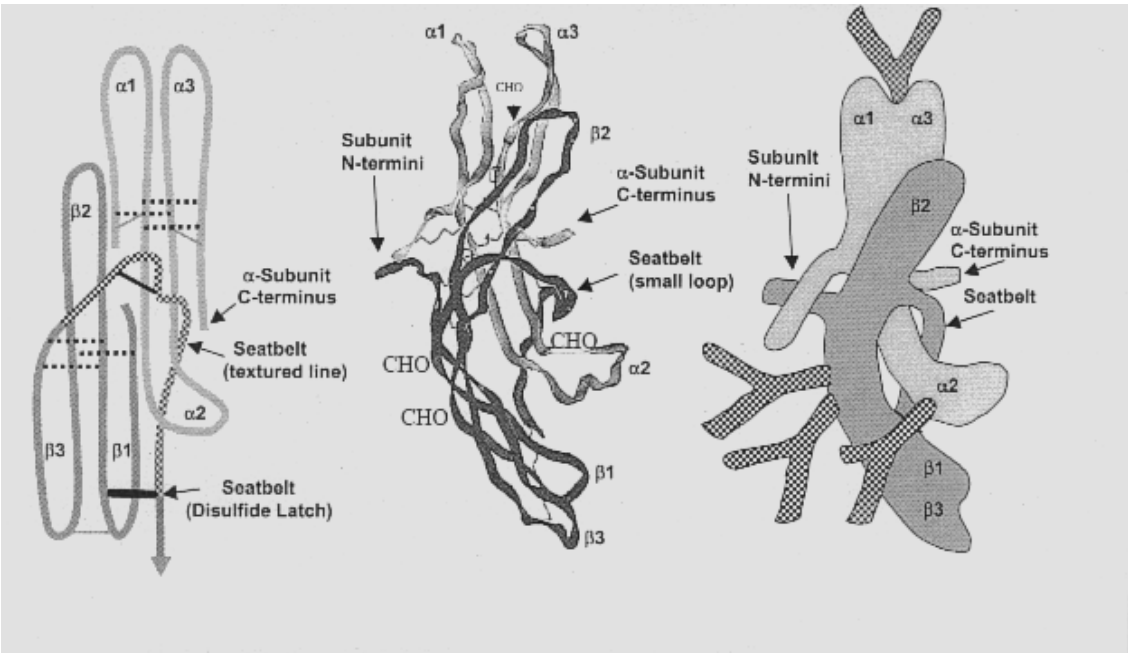



Figure 1. Provided by William R. Moyle, depicts three representations of the structure of hCG. Each shows the three loops of the α- and β-subunits. Loops α1, α2, and α3 are shown in light grey while the β-subunit loops 1β, 2β and 3β are shown in a darker shade of grey. The "seatbelt", part of the β-subunit is best seen in the first figure as a textured line, which holds the subunits together. The oligosaccharides (CHO) are best shown in the middle ribbon diagram and in the third figure as upside-down Y-shapes and textured. In vitro when the seatbelt is latched the α2 oligosaccharide has to pass through the latched seatbelt opening.

Most assembly of hCG, hFSH and hTSH heterodimers occur in a threading fashion, indicative that thread-folding may be an important part of protein folding and assembly in the endoplasmic reticulum (49). Stabilizing hCG is done so by a strand of its β-subunit, its "seatbelt" is called so because it surrounds α-subunit loop 2 and its end is "latched" by an intrasubunit disulfide bond to the β-subunit core (51). Studies have shown that the seatbelt contributes to the stability of the docked complex and the opposite is true when the seatbelt is unlatched (49, 50, 51). Most hCG is assembled by threading the glycosylated end of α-subunit loop 2 beneath the latched seatbelt rather than by wrapping the unlatched seatbelt around this loop (the wraparound pathway) (51). Primary

assembly appears to take place in the ER by threading and that the alternate wraparound pathway is used very little if at all; the wraparound pathway may be a salvage pathway, the seatbelt and its latch determines the assembly pathway used (49, 50).

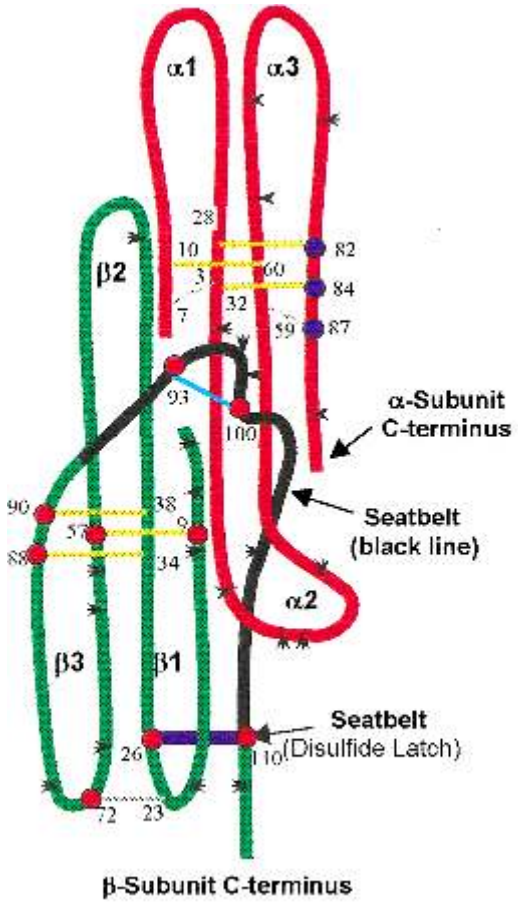



Figure 2. This figure depicts a simplified diagram depicting the relationship between the α-subunit(shown in green) and β subunit(shown in red) of the glycoprotein hormones. This diagram is based on their relationship and the crystal structure of hCG. The Seatbelt is shown in black, and the disulfide latch of the seatbelt is shown in purple. The yellow lines depict the cysteine-knot disulfide bonds that create the loops shown in both subunits (27).

The α-subunits for each of the individual hormones have the same amino acid sequence, come from the same gene, and differ only in their glycosylation patterns (36), while the β-subunits are all dissimilar which creates the specificity of the individual hormone. The seatbelt makes an important contribution to the stability of the heterodimer, even though

it often seems to be impeding if not obstructing subunit combination during threading (25, 52). With the seatbelt latched, heterodimer combination would occur only if α2 and its associated oligosaccharide are threaded through the small opening between the seatbelt and β1 (threading pathway); this would be unnecessary if the seatbelt were to open before heterodimer formation (wraparound pathway) (52).

Lin et al. suggested that through their influence on α-subunit loop 2, mutations on the seatbelt would exert an indirect influence on the binding of ligands to the glycoprotein hormones (25). Studies done by Lin et al. found that the seatbelt does not participate in key receptor contacts directly (25), as suggested by Jiang et. al (20); instead, the seatbelt seems to alter the conformation of the α-subunit loop 2, a region on the hormone, which may participate in receptor contacts (25).

Accelerating subunit combination in vitro can be done by the use of protein isomerase and small amounts of reducing agents, possibly by breaking the seatbelt latch disulfide (38, 52) Following this and other similar studies performed, there is reasoning to suggest that the unlatched seatbelt is wrapped around α2 during the combination process, as seen in the crystal structure (48, 52). Moyle et al, found that Cys110 was not labeled with alkylating agents showing it was not bonded during subunit combination, a process that occurs in oxidizing conditions efficiently in vitro; therefore suggesting that the unlatching of the seatbelt is not required for subunit combination (52). All this lead to the creation of a model of heterodimer formation in which movement of the seatbelt and α2 allow this

loop to pass through or thread itself through the small hole between the seatbelt and the β-subunit (52).

While the wraparound pathway is much less efficient than the threading pathway, it may have facilitated evolutionary experimentation with the seatbelt region during the co-evolution of glycoprotein hormones and receptors (51, 52). With the use of several salmon FSH-hCG chimeras that can be readily monitored with antibodies to hCG, Xing et al. found that hCG analogs with the teFSH fold could not be assembled by a threading pathway and were therefore formed by a wraparound pathway (51). The tendency of the hCG seatbelt for being latched prior to subunit docking is suggestive that it may become latched prior to the start of assembly; therefore it can interfere with the threading pathway when attached to a cysteine in the N-terminus (51).

The wraparound pathway therefore, allows for the formation of heterodimers that cannot be assembled by the threading pathway; this would have greatly facilitated natural experimental evolution with the seatbelt region aiding in ensuring reproduction when the threading mechanism fails or is blocked (51). Not only does the docking occur via two different pathways, it also appears to be reversible before or after the seatbelt is latched, therefore favoring the threading mechanism which would explain why threading is the most common pathway used (52).

The N-terminus of the β-subunit has no role in LH receptor interaction, an observation by Slaughter et al. (38, 46). This region may be necessary for the synthesis of hormones

with lutropin activity, this region makes several contacts with the α-subunit, aiding in docking before the formation of disulfide bonds latching the seatbelt to the β-subunit (38). Before "latching" occurs, stability is attained by several means; forces including van der Waals contacts, hydrophobic interaction, hydrogen bonding and salt bridges, as well as steric hindrance, aid in stabilizing the interaction prior to seatbelt disulfide bonding (20a, 25, 38).

**Hormone Glycosylation**

LH, FSH, TSH and CG are heavily glycosylated, and their oligosaccharides are not only important for structure but also for physiological function (46). The structure of the α-subunit is consistent in all vertebrate species, including the position of oligosaccharides which can also be found consistently on homologous locations in the β-subunits as well (3). The Asn-linked oligosaccharides project away from the surface of the hormone. These Asn-linked oligosaccharides on LH contain N-acetylgalactosamine (GalNAc), this is unlike the other glycoprotein hormones, and it also does not contain sialic acid or galactose like the others. The oligosaccharides on LH are therefore, both biochemically and structurally different than those on other glycoprotein hormones (3). Without the N-linked oligosaccharides the glycoprotein hormones would be quickly removed from circulation (3).

The N-linked oligosaccharides on hCG and hFSH α-subunit loop 2 have a dominant influence on hormone efficacy (7). This influential phenomenon is in line with current

signal transduction models in which the α2 oligosaccharide functions by increasing the distance between the LRR and the signaling specificity domain of the LH receptor (7, 29). Previous work by Moyle et al. has shown that oligosaccharides of hCG and/or all of the glycoprotein hormones may have important roles in signal transduction. In addition to results detailed by Bernard et al., complete deglycosylation is not required to produce glycoprotein hormones having low efficacies; therefore making it easier to construct analytical hCG analogs with this knowledge (7). Bifunctional antagonists, such as a hormone analog that interacts with LH and FSH receptor to block the actions of LH and FSH, could be useful for aiding in the removal of the cystic follicles associated with polycystic ovary syndrome (7).

An excellent example in the potential of how an antagonist relationship could be useful is in the possible treatment of polycystic ovary syndrome. Polycystic ovary syndrome (PCOS) is characterized by hyper secretion of LH (22). Bifunctional antagonists could work by blocking LH and FSH directly or by delivering apoptosis inducing agents to these cells (7). If this were possible, there could be a reduced need for invasive surgical procedures. The importance of bifunctional antagonists has been supported by the observations that the hCG seatbelt is able to distinguish between LH, FSH and TSH receptors (7, 11, 26).

**Hormone Specificity**

It is important to study the agonists and antagonists of hCG for their potential in the treatment of infertility (46). hCG binds with high affinity to LHR on the surface of target tissues due to the fact that LH and hCG are structurally similar (16, 22a, 46).

In principle, hormone-specific receptors can bind a given hormone, but not others. All agonists and antagonists of this hormone can also bind to the receptor. This can be quite complex and interesting for example, on studies using bovine LH (bLH) by Moyle et. al. bLH and hCG are very similar however, 1,000-fold more bLH is needed to inhibit binding of radiolabeled hCG to the human LHR than to the rat LHR (8, 13). These studies helped determine regions of the extracellular domain that make contact with the lutropins by chimeras. Previous studies with truncated receptors and LHR/FSHR chimeras identified most regions to the N-terminal 3/5$^{ths}$ of the rat LHR extracellular domain (8). To add to these findings, using labeled hCG to rat/human LHR chimeras to the same N-terminal region of the hLHR and the rLHR, it was found that 10-fold more bLH was needed to inhibit binding of the hLHR vs. the rLHR.

When the C-terminal 1/5$^{ths}$ portion of the hLHR and the rLHR domains were tested, 100-fold more bLH was needed to inhibit labeled hCG to bind these chimeras. Therefore, the C-terminal portion of hLHR is inhibiting bLH binding lead the team to suggest that this region also contacts the ligand even though it is not necessary for ligand binding (8).

**RECEPTOR**

Details regarding G protein-coupled receptor signaling via intracellular mediators are becoming well-characterized; however, specific membrane events involved in signaling are less understood (23). Receptor interactions with other membrane proteins have been almost disguised by large molecular weight clusters in which the receptor becomes a part of, following hormone binding (23). These large clusters appear to form as a result of aggregated receptors cis-activated by ligand or liganded receptors that have trans-activated those nearby that are unliganded (23). Lei et al. employed various studies to determine two events which appear to be the minimum necessary for signaling by LHR. The first is that a hormone occupied receptor appears to involve receptor to receptor interactions that aid in producing high positive energy transfer efficiencies; the second involves the migration of large clusters or rafts that may aid in signaling as well (23).

Intracellular loops appear to form contacts with the sites for interaction with glycoproteins and sometimes with the C-terminal cytoplasmic domain (16). Receptor interaction specificity depends on the configuration of the intracellular surface of the receptor. Coupling of the glycoprotein with the activated receptor occurs with significant changes in binding affinity. The affinity of the LHR for LH and hCG however, is not affected by GTP or its nonhydrolyzable analogues (16).

Once activated by a hormone or by a mutation, LHR interacts with a heterotrimeric G protein. The G protein contains three subunits the $\alpha$, $\beta$, and $\gamma$ subunits play an important

role in signal transduction. This will be detailed further in a later section, however it is important to note that generally once a ligand binds the GPCR triggers an intracellular G protein to exchange one GDP for one GTP; therefore leading to increased intracellular biosynthesis of cAMP via secondary signaling of G protein to cAMP via signal transduction (2, 35). This is best shown in the simplified figure below.

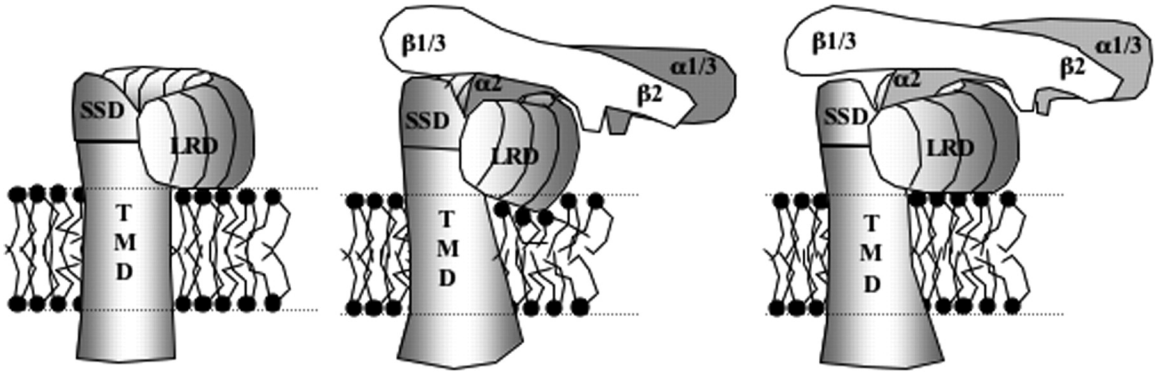
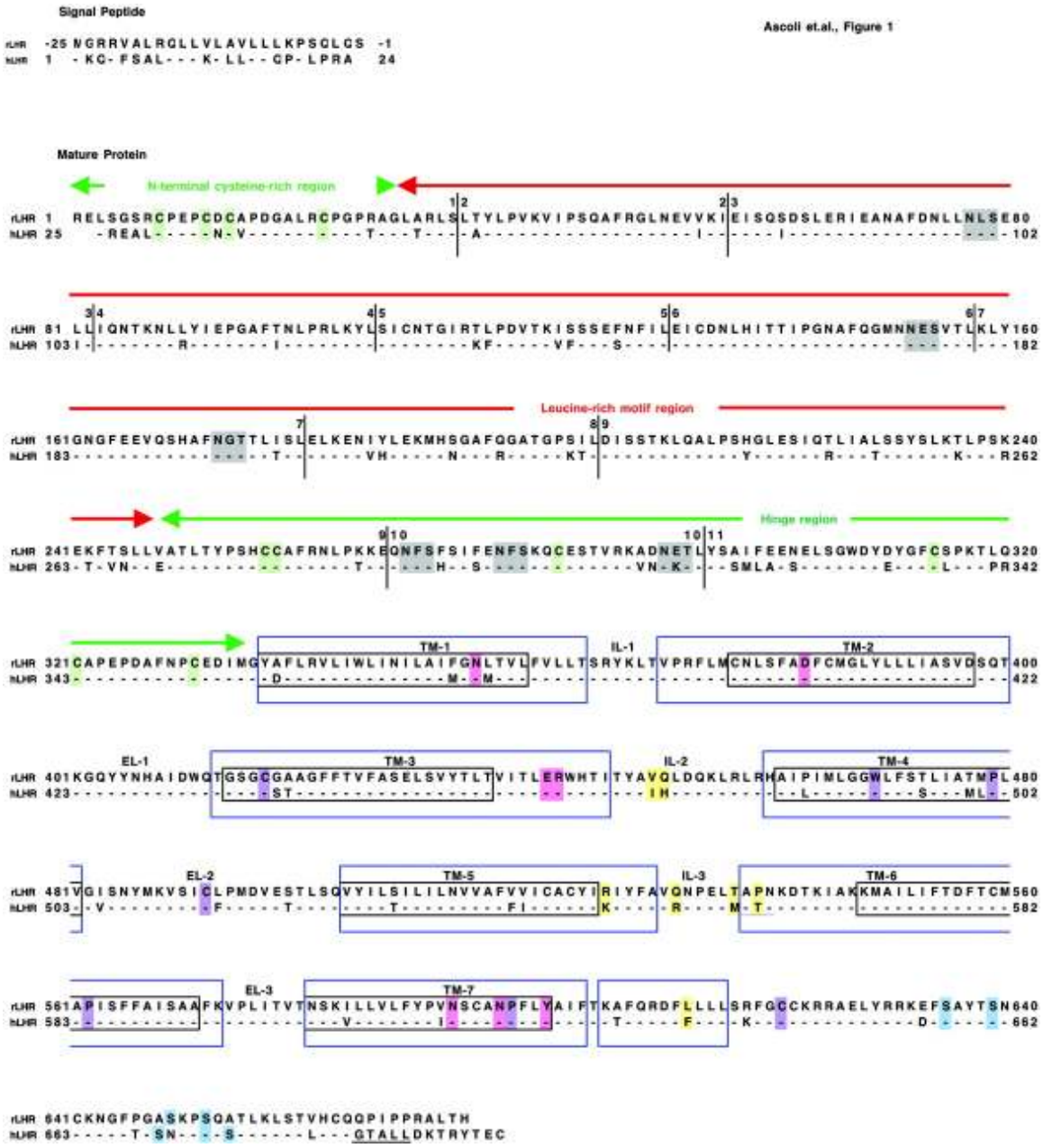



Figure 3. This figure best depicts the Moyle et al. proposed model of signal transduction. The SSD is shown extracellular to the TMD with the LRD near the SSD-TMD complex, as seen in the first panel. Most glycoprotein hormones appear to dock with their receptors in an orientation that is approximately 90º which leads to signal transduction by increasing the distance between the top of the SSD and the top of the LRD. The rotation of the LRD, shown in the second panel, and the movement of the LRD, shown in the third panel, or both occurs in combination causing a rearrangement of the TMD needed for signaling (29).

**Structure of the Receptor**

Most of the studies performed have been done with the rat LHR (rLHR) and the human LHR (hLHR) to date, an alignment of these two receptors is shown in figure 3 (2, 15). This alignment shows both receptors and where their similarities and differences exist, this is also an important tool to show how some amino acids are identically located for example and of interest as the amino acid mutated for this paper, Threonine 413(T413) on the rLHR corresponds to T439 on the human LHR (hLHR). The amino acid

sequences of the rLHR and the hLHR have similarity of approximately 85 to 88% in the extracellular domain, approximately 92% in the serpentine region, and approximately 69% in the C-terminal cytoplasmic tail (2, 8). The α-C-terminus is known to be essential for the activities of most glycoprotein hormones (6, 32a). The GPCRs have the greatest shared homology within the transmembrane domains with the most variable regions located at the C-terminus, the intracellular loop spanning TM5 and TM6 and the amino terminus where the greatest diversity is observed (21).




**Ascoli, M. et. al.**

Figure 4. This amino acid sequence alignment shows the rLHR above the hLHR, the dashes depict identical residues. The three regions shown in green, red and green are the extracellular domain regions respectively, N-terminal cysteine-rich region, leucine-rich repeat region and the hinge region. The black boxes show the seven transmembrane domains, and the blue wider boxes predict helical segments based on the rhodopsin crystal structure, and based on work by Ascoli, M. et. al. Threonine 413 of interest, which will be discussed in detail further, is located in the 6$^{th}$ row of the figure, just before the 3$^{rd}$ transmembrane domain.

**RECEPTOR DOMAINS**

The receptor has functional regions, the extracellular hormone-binding domain and the seven-membrane transmembrane/ cytoplasmic region. The N-terminal extracellular domain is characterized by its sequence similarity to the polypeptide-binding Leucine-rich repeat (LRR) protein carboxypeptidase N and to insulin-like growth factor-binding proteins (15, 16). The LRR is the several repeating structural motifs of about 25 residues in length that is rich in hydrophobic amino acids, recent studies suggest that there are 8 or 9 repeats (2).

The extracellular domain can be divided into three regions: an N-terminal cysteine-rich region followed by 8 or 9 LRRs and a C-terminal cysteine-rich region, also know as the hinge region (2, 30). LRRs have been shown to be involved in protein-protein interactions along with findings that the extracellular domain of the LHR is mostly responsible for the recognition and high affinity binding of its ligands (2). Although the LRRs of the extracellular domain in LHR are shorter than those found in the porcine ribonuclease inhibitor, the crystal structure of this protein has provided the basis for modeling of the LRRs of the LHR and for three-dimensional structural studies of the extracellular domains (2, 25b, 44). Models for the LRRs and the extracellular domains are shown in figure 4, along with the known hCG three-dimensional structure, these together are a great tool for theoretical and experimental studies into the understanding of how LH and CG interact with the LHR (2).

The intracellular C-terminus is composed of 69 amino acids containing one consensus site in the rat and two in the pig for protein kinase C phosphorylation, and one potential site for tyrosine kinase phosphorylation. The C-terminal sequence also contains several serines and threonines that can be phosphorylated by protein kinase A, as well as palmitoylated cysteines that help to provide membrane anchorage (16).

The cytoplasmic region is the anchoring unit that transduces signals initiated in the extracellular domain and couples them to G proteins (16, 30). 341 amino acids compose the large extracellular domain which binds LH and hCG with high affinity. The N-terminus contains a cleavable signal sequence of 26 amino acids in the rat, and 22 amino acids in the human, both of which direct the insertion of the LHR into the endoplasmic reticulum processing pathway (12, 16).

The extracellular domain of the LHR dictates processing efficiency, this has been seen in binding and recycling studies (12, 33); the quite large rLHR extracellular domain determines if the receptor is fit to reach the cell surface. While both rat and human LHR have been shown to work with chaperones in the ER, it may be that the hLHR utilizes them more efficiently (12).

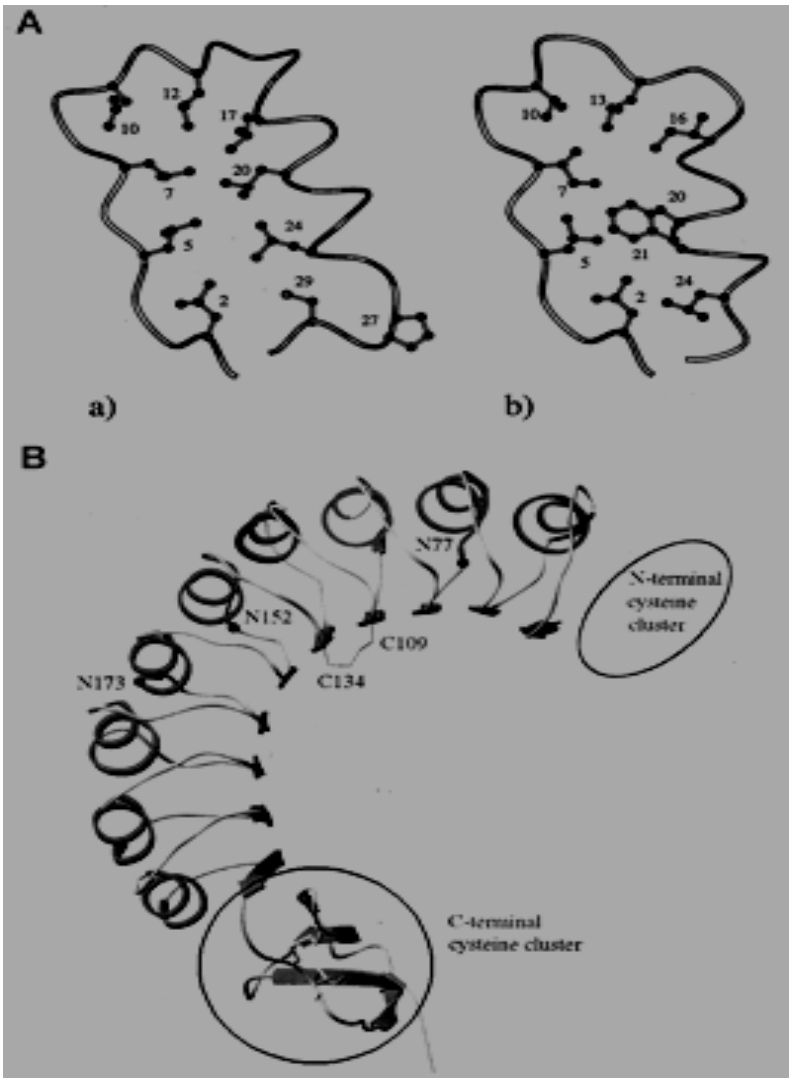
With the orientation and overall topology of other GPCRs known, there are three distinct recognizable domains in the LHR; a larger N-terminal domain that contains 340 residues and is considered to be extracellular, a serpentine region containing seven transmembrane (TM) regions connected by three extracellular loops (EL) and three intracellular loops

(IL) as well a C-terminal tail that is predicted to be intracellular (2, 20). The extracellular location of the N-terminal region of the rLHR and the intracellular location of the C-terminal region have been confirmed in rat luteal cells using site-specific antibodies to synthetic peptides derived from these two regions of the rLHR and in human kidney 293 cells expressing recombinant rLHR using antibodies directed against FLAG Epitope added to the N-terminal of the rLHR (2).

Residue 1 of the rLHR is taken conventionally as the N-terminal residue of the mature rLHR. This residue was determined by sequencing the receptor protein purified from the rat ovary, in contrast, the mature hLHR N-terminus is not known with certainty because the hLHR has yet to be purified and subjected to protein synthesis (2). Therefore, by convention, the amino acid residues of the hLHR have been numbered from the initiator methionine of the precursor sequence. For example, residue T413, which forms the major expected work discussed here, in the rLHR corresponds to its equivalent in the hLHR at T439, when other laboratories count the 26 residue loading sequence. For simplicity T413 will be referred to for both rat and human.

The extracellular domain of the rLHR and hLHR has six potential glycosylation sites and is composed of seven transmembrane helices with three extracellular and three intracellular loops. LHR contains cysteine residues in the first and second extracellular loops, as does other members of the superfamily (2, 16). The potential functional role of LHR glycosylation has been studied with hLHR and rLHR showing that the individual

mutation of any one of the six sites has no effect on hCG binding affinity of hCG-stimulated cAMP production in 293 cells expressing the mutants (2).




**Ascoli, M. et. al**

Figure 5. Figure 5Aa depicts a single LRR and figure 5B depicts the entire Leucine-Rich Repeat domain of the LHR. The two figures shown in 5A at two slightly different representations based on information derived from the three-dimensional structure of the ribonuclease inhibitor (a) and the proposed structure on the right is a depiction of the extracellular domain of LHR based on figure a (b). The numbers detail the conserved hydrophobic residues based on Kobe and Deisenhofer. Figure 5B is a ribbon diagram of the LRR region of the extracellular domain of the LHR, the arrows depict β-sheets and spirals depict α-helices (2).

The LRRs are similar in primary sequence to the LH, FSH and TSH receptors. The LRR domains of these receptors share sequence similarity of about 43% within exons 2-8, this similarity decreases at the N-terminal and C-terminal domains of the extracellular region with the presence of amino acid inserts in the FSH and TSH receptors, therefore suggesting that these regions are important for denoting hormone specificity (16). 80% similarity is shown among the glycoprotein hormone receptors within the transmembrane domain and in the extracellular and intracellular loops and intracellular C-terminal tails. It's important to note that the overall similarity of the seven-transmembrane domain of the LHR and other GPCRs is low.

The hydrophobic leucine-rich repeat region that makes up most of the binding domain is intercalated between cysteine-rich domains in exons 1 and 9. The LHR contains only three cysteine residues, Cys109, 134 and 282 that are not conserved in the extracellular domain of the FSHR or the TSHR (16).

Multiple experimental approaches have been reported of mutagenesis within the extracellular binding domain, as well as deletion and chimeric approaches to localize the binding regions of these receptors. These studies indicate many binding domains between amino acids 1 and 184, for example chimeric experiments using LHR and FSHR show that the domain from amino acids 58 to 93 of the LHR blocked FSH binding, but amino acids 95-171 are necessary for hCG binding (16).

Unlike the low similarity of the 7TMD of LHR and other GPCRs within the transmembrane, extracellular loops (ELs)1-3, intracellular loops (ILs)1-3, and C-terminal domains where many of the amino acids are conserved within the family of GPCRs (16). In line with the above data, mutations of the hormone coupling were investigated by Bernard et al. and it was proposed that a knob placed on residues other than residue 95 and 99 in the seatbelt loop causes reduced signal transduction because it may alter the conformation of the heterodimer (6). Moyle et al. suggest that glycoprotein hormones need to have two contacting portions on the extracellular domains to attain hormone-receptor signaling, which is sensitive to heterodimer conformation (6, 29).

**Serpentine Transmembrane Domain**

The serpentine domain of the LHR is characterized by the looped GPCR structure with seven transmembrane segments joined by three alternating intracellular loops and three extracellular loops. The GPCR superfamily is divided into three major subfamilies; the LHR is a member of the rhodopsin/ β2-adrenergic receptor-like subfamily. The serpentine domain is characterized by a series of highly conserved residues in this family. The availability of the rhodopsin crystal structure has aided in the study of a new model of the hLHR by comparative modeling, these molecular models of the hLHR have aided studies attempting to investigate the molecular basis of the mutation-induced activation of the receptor (2). The crystal structure of rhodopsin shows the presence of eight α-helical segments, the first seven are considered the seven transmembrane domains of the GPCRs; the eighth is completely cytoplasmic and is located between the cytoplasmic end of TM7 and the conserved intracellular cysteine residue that is palmitoylated (2).

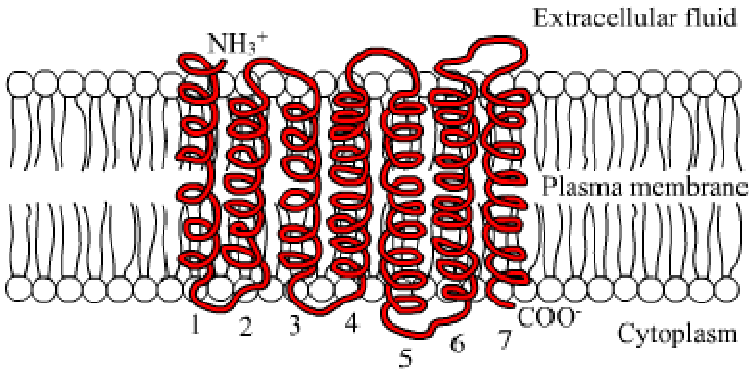



Figure 6. This cartoon representation of a truncated rat LHR showing the seven transmembrane domain, the three extracellular loops are on the top of the figure, and the three intracellular loops are shown at the bottom. Threonine 413 is found in extracellular loop 1.

The understanding of GPCR structure is predominantly based on the high-resolution structures of the inactive state of rhodopsin. Rhodopsin is best suited for structural studies over most other GPCRs because it is very stable and it is possible to obtain large quantities of enriched protein from bovine retina (21, 31a). Molecular dynamic simulations were conducted on all characterized naturally occurring and engineered gain-of-function LHR mutants based on the crystal structure of rhodopsin (35). An example of how these results have aided in the distinction between inactive and active forms is that a network of Hydrogen bonds between highly conserved amino acid residues in the cytosolic part of transmembrane helices 1, 2, 3, 6 and 7 are charge-reinforced H-bonding interactions between the cytosolic extensions of transmembrane helices 3 and 6 to stabilize LHR in the inactive state (35). Crystal structure resolution of hCG has been helpful to clarify concerns about hormone-receptor interactions and helps researchers on finding the exact receptor-binding domains in hCG (16). More recently, the amino

terminal ligand binding domain of the FSH receptor in complex with its ligand was crystallized (17b); the structure does not however, include the transmembrane domains. These all help to provide important insight into glycoprotein binding and with the use of site-directed mutagenesis and cysteine scanning mutagenesis to detect receptor-ligand interactions to verify and aid in the improvement of GPCR modeling (21).

**Hormone-Receptor Specificity**

The distinguishing ability of hCG to decide between LHR and FHSR is controlled predominantly by β-subunit residues 94-117 (20). These observations along with a model by Moyle et al. support that residues 94-96, specifically residue 95, influence LHR binding specificity through an effect on hormone structure rather than by direct contact with essential high affinity receptor contacts (20). The β-subunit region that limits binding of hCG to LHR was found between the tenth cysteine and the C-terminus; including most of the β-subunit seatbelt region discussed below (11, 20, 26). The entire sequence between the tenth cysteine and the C-terminus of the hCG β-subunit was replaced with a corresponding portion of the hFSH β-subunit. The analog became an excellently functioning FSH but lost most of its LH activity (11, 20, 25b). The antibodies used in this study are well characterized and have been described in several references (17a, 25a, 26a). Specifically antibody A113 which recognizes an epitope on the α-subunit and B105 which recognizes and epitope of the β-subunit distant from the residues 94-96 were used, this assay was therefore specific for αβ-heterodimers in media (14, 20). While the influence on FSHR binding was small, these residues had more influence on rat and human LHRs, due to the primary net charge of the residues; the least active

analogs contained negatively charged amino acids and no positively charged amino acids (20). Therefore, this study demonstrates how the LH receptors binding specificity can be modified in a reasonable fashion to make gonadotropins that have differing abilities for binding of LHR and FSHR (20). This is based on the fact that positively charged amino acids in the region studied have more influence on LH activity than they do on FSH activity; also residues between the eleventh and twelfth cysteines influence FSH activity more than LH activity (26, 20). The analogs in this study with a ratio of LH/FSH activity can have great potential for clinical application (20).

Moyle et al. showed that once rat testicular or ovarian LH receptors are bound to hCG, LH can be detected with monoclonal antibodies geared against a conserved epitope on the β-subunit of the hormones (28). This lead to a better understanding of the fact that the glycoprotein hormones are not rigid structures, even with five disulfide bonds in the α-subunit and six in the β-subunit. The conformation of hCG alters after it binds to a receptor, this is also known via the help of using monoclonal antibodies (28), as detailed in the work below by Dufau et al.

The term efficacy is used to describe the effect of a ligand as it relates to the functional properties of the receptor (20b, 21). Agonists are ligands that fully activate the receptor, partial agonists induce submaximal activation even at saturating concentrations, inverse agonists inhibit basal activity and antagonists have no effect of basal activity but compete for access to other ligands (21). The efficacy of a drug may vary depending on the signaling pathway under investigation (20c).

Between different species there are differences that are important to note in the processing of LHR. The human LHR (hLHR) is predominantly expressed as a mature 90KDa species while the rat LHR (rLHR) exists predominantly as a 70KDa precursor form (12). With such homology between rLHR and hLHR it can be suggested that their amino acid sequences lead to the different efficiencies of processing (12).

The 65-75 kDa species was revealed as a precursor of the cell-surface receptor via biosynthetic labeling of transfected cells with radioactive amino acids (2). This precursor is located intracellularly due to its insensitivity to surface proteolysis; it cannot be detected by surface biotinylation of intact cells and is insensitive to neuraminidase digestion, unlike the mature 85-95 kDa species (2). It is considered to be an immature glycoprotein localized in the endoplasmic reticulum because it was found to be readily susceptible to EndoH digestion (2).

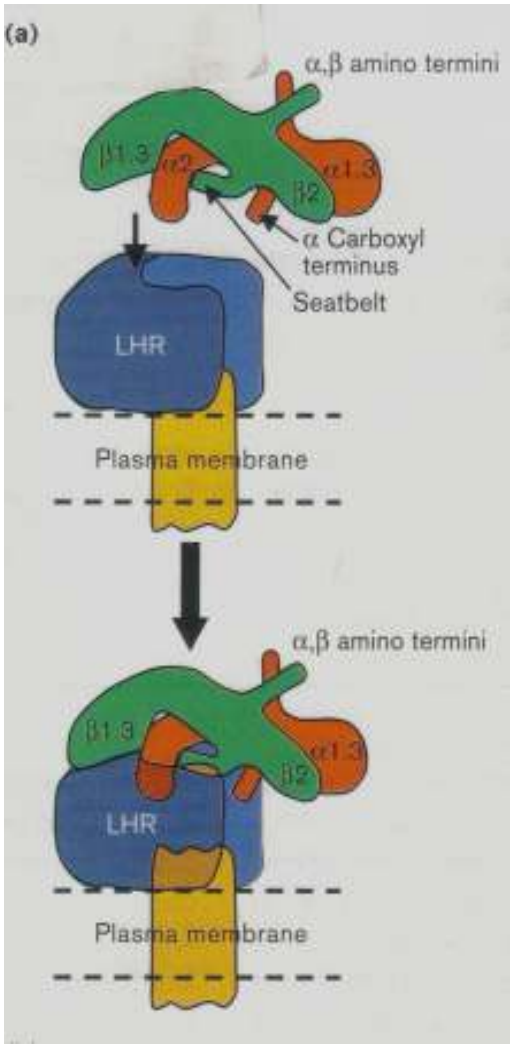
The mature form of the LHR present at the cell surface is a glycoprotein with an approximate molecular mass of 85-95 kDa that comes from the maturation and transport of a precursor glycoprotein that is localized in the endoplasmic reticulum (2, 33).

Pietila et al. examined the processing and maturation rates of LHR to determine if newly synthesized receptors are prone to premature ER-associated degradation (ERAD). These studies resulted in the finding that the LHRs that have hormone binding ability make it out of the ER and to the cell surface in the time allowed by the ER. While receptors that fold improperly are targeted for degradation in proteasomes; high LHR expression did

not induce improperly folded protein response, that is a consequence of accumulation of misfolded proteins in the ER, rather it is mediated by the proteasomes (33).

## MODELS OF GLYCOPROTEIN HORMONE-RECEPTOR INTERACTION

Two very different models have been proposed in the past to try and explain the interactions of the glycoprotein hormones with their receptors, as shown in Figure 7, these are shown for background purposes, followed by a more up-to-date model shown in figure 8. The first model shown in figure 7 by Moyle et al. depicts the groove between loops α2 and β1,β3 making the primary receptor contact with the seatbelt region controls hormone specificity through its influence on subunit interaction and the shape of the β1,α2,β3 groove (25b). The second model shown in figure 7c details the LH receptor contacts involving the α subunit C-terminus, portions of β2 and portions of the seatbelt, but not the β1,α2,β3 groove; therefore suggesting that the influence of the seatbelt on hormone specificity is mediated through its interaction with the receptor (14, 20a).



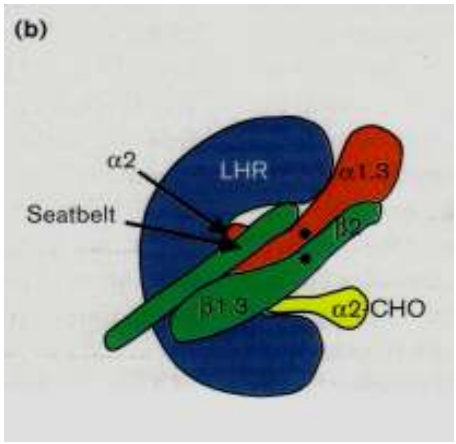




Figure 7a and figure 7b depict the β1, α2, β3 groove which is was proposed by Moyle to interact with the rim the horseshoe-shaped extracellular domain of the receptor. There is limited contact with the seatbelt

Moyle's model utilized a different orientation and alignment of the Leucine rich repeat (LRR) than other researchers. It predicted that the center of the LRR fits into the central groove of hCG and therefore a conformational change induced an interaction with the transmembrane domain beneath the extracellular domain (16).

A more current published model by Moyle et al. in 2004 is depicted in Figure 6 (29). In an effort to explain the differences seen between the crystalline and membrane forms of FSHR, Moyle et al. developed a chimera to help explain if the FSHR contained hidden ligand docking regions (24). The chimera called FSHR2 contains both FSH and LH binding sites (rat FSHR-K88N, K163G). To determine if these docking sites existed, hCG and hFSH binding was determined by antibody binding assays. Findings showed that FSHR2 bound both hCG and hFSH independently in a characteristic orientation for their respective receptors. Therefore suggesting the FSHR should contain at least one "cryptic" hCG binding site (24). Further comparisons of hFSH/FSHR contacts seen in the crystal structure with the known membrane receptor properties showed a "cryptic" FSH docking site on the extracellular domain of the FSHR. This site has the potential to recognize hCG similarly to LHR which may explain why equine lutropins bind many mammalian FSHR and why mutation in the TMD enables the FSHR to bind hCG. These sites can be responsible for the physiological regulation of receptor binding specificity (24).

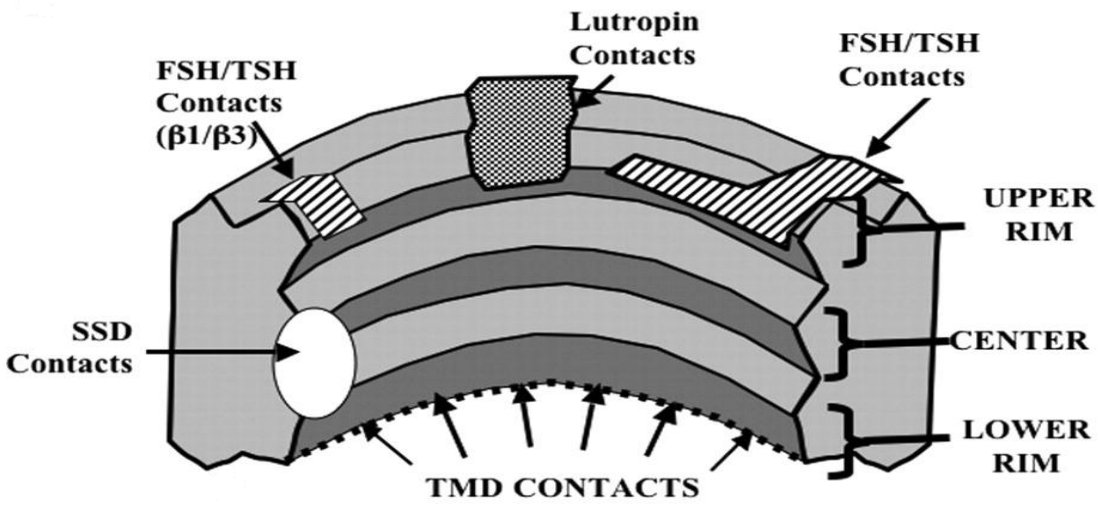



Figure 7. Shown here is the overall conformation of the Leucine Rich Repeat domain (LRD). This figure by Moyle et al. depicts the contacts of the LRD with the ligands, the SSD and TMD, for clarity the junction of the LRD and the SSD has been omitted from the right on the figure (29).

To more accurately model the LRD of the glycoprotein hormone receptors, Moyle et al. aligned the leucine-rich repeat regions of each receptor with that of ubiquitin ligase and made homology models of the amino acid sequences of the rat LHR, FSHR and TSHR (29). The regions of the LRD that are thought to contact the ligand, SSD and TMD are shown above in figure 7.

The positions of the SSD and LRD may account for contacts of hCG with the LRD and SSD with a large portion of the hormone remaining exposed when the hormone and receptor are in contact. This model shown in Figure 6 suggests also that a reasonable

amount of the surface of the hormone contacts the receptor. This would explain the high affinity of most glycoprotein hormone-receptor interactions (29). This model accounts for the fact that much of the α-subunit loop 2 may be near the receptor although few of its residues participate in essential receptor contacts (29, 46a). Additionally, the position of the hormone as shown below accounts for the finding that the C-terminal end of the α-subunit can be cross-linked by a disulfide to most portions of the seatbelt loop with not disruption to the biological activity of hCG (6, 29). As previously published residues Arg95 and Asp99 in this loop appear to contact the receptor (29).

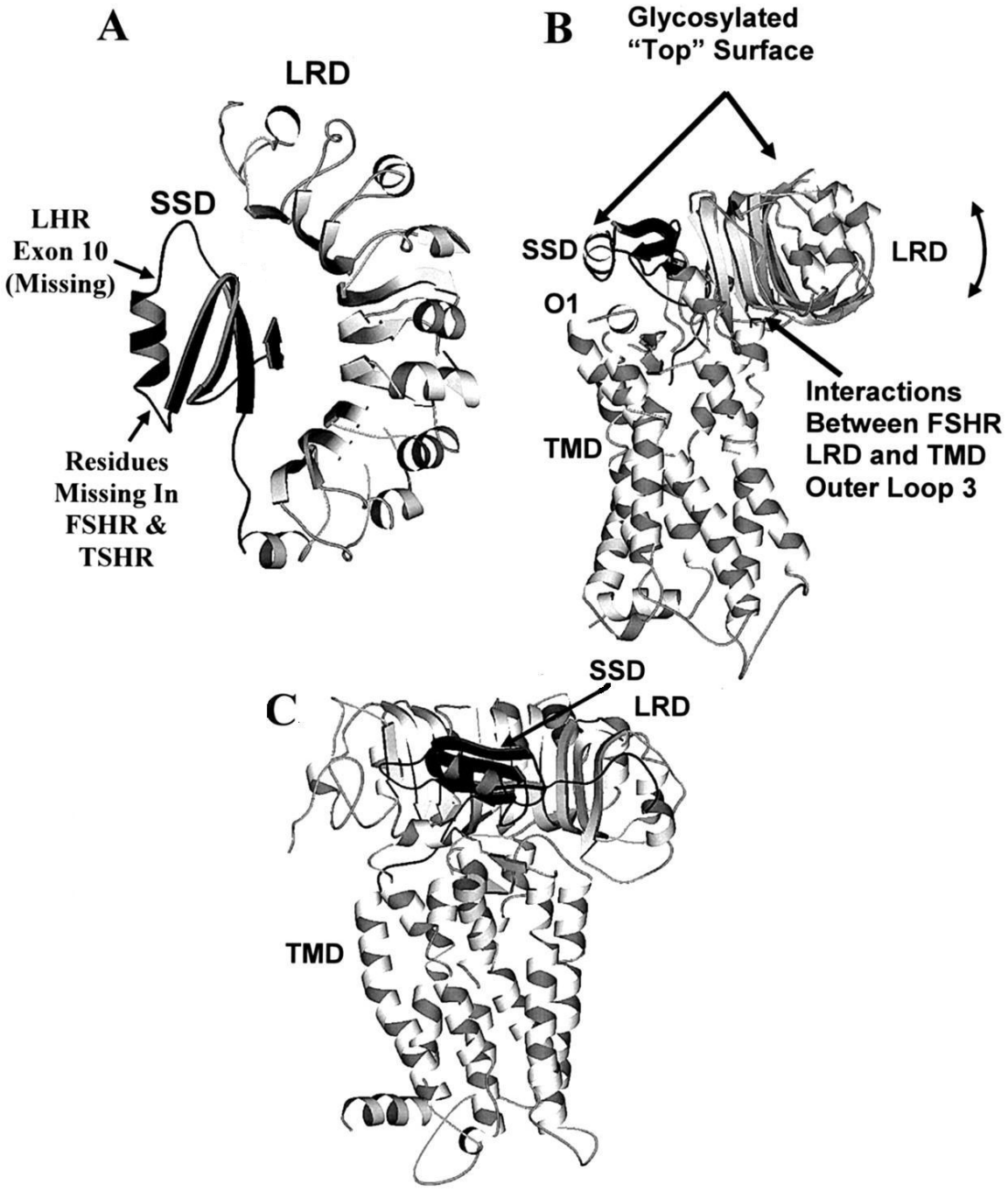



Figure 8. This figure depicts the receptor structure as shown from different perspectives by Moyle et al. Figure 8A shows the LRD and SSD as seen from looking at the cell surface. The arrows depict the regions of the SSD that are missing in the SSD of models of the LHR and FSHR. Figure 8B and Figure 8C shows the LRD and SSD as seen from the transmembrane domain, Figure C is rotated 90 degrees as compared to Figure 8B.

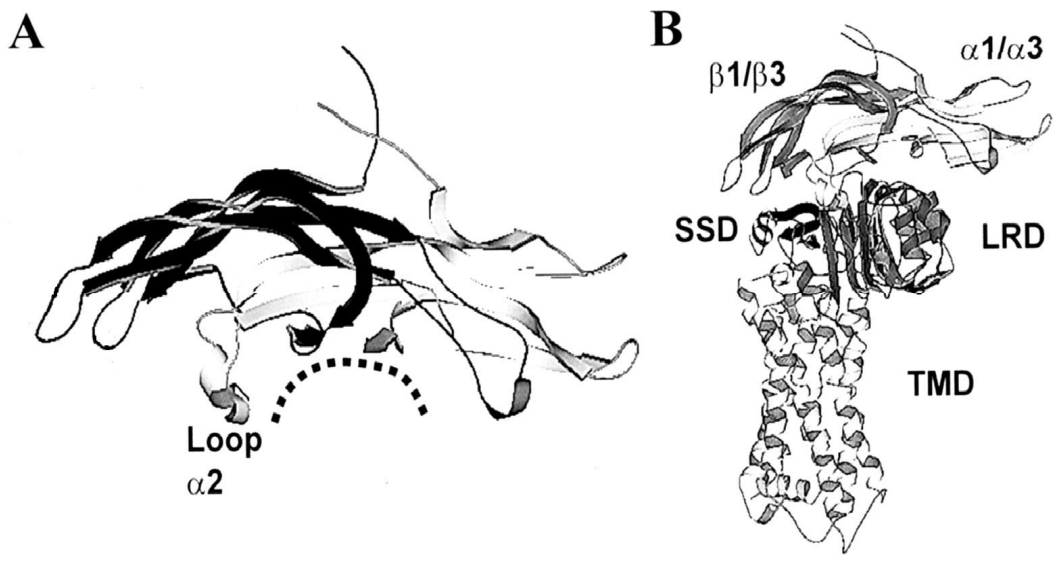



Figure 9. This figure depicts hCG and hormone receptor complexes. Figure 9A depicts the most recent published model of hCG by Moyle et al. 2004, the top surface of the LRD is shown by the dotted curved line in this figure. Figure 9B depicts the docking of hCG to the extracellular domain of the receptor.

The most current published models of hormone/receptor interaction (Moyle et al), shown in Figure 8 and Figure 9, help to explain an interaction of the SSD, LRD and TMD, that are expected to change ligand binding specificity (29). The shape of the LRD and several other leucine-rich repeat proteins are similar. Along with the observation that it is composed of repeating elements leads to the possibility that the LRD may contain several binding sites, see figure 7.

## MATERIALS & METHODS

### Solutions

All solutions were made in sterile conditions using sterile pipettes and conducted under a hood. All water used in solutions was sterile distilled water.

**Tris HCl** was made in aliquots of 10X and kept frozen at -20 ˚C until needed. A 1X stock solution of Tris was added to PBS in the ratio of 1 to 9.

**DMEM** media was made to 10 L then aliquots of 500 mL were kept refrigerated at 4 ˚C. To make 10 L of media start with 10L of $dH_20$ and add 37.9 g of Sodium Bicarbonate and 8.76 g of L-Glutamine and stir until dissolved completely, cover with parafilm while solution is being mixed and pH to 6.9 to 6.95 once complete. All media was filter-sterilized and checked for contamination before being used in tissue culture. DMEM aliquots of 500 mL were then used for tissue culture and modified based on the specific need. A Complete DMEM media would be used to maintain cells by adding 10 % Fetal Bovine Serum (FBS), 5 % Glutamate and 5 % Pen. Strep. to 500 mL of DMEM media aliquot.

**1X Trypsin** is best if relatively fresh, therefore only 50 mL were made a t a time. This was made with 5 mL 10X Trypsin stock kept frozen and 45 mL 1X PBS kept at room temperature (23 ˚C).

**2X BBS** was made to 100 mL by adding 1.066 g 50mM BES(N,N-bis(2-hydroxyethel)-2 aminoethane sulfonic acid) Anhydrous MW 213.2 g, 5.6 mL 5M NaCl, and 150 λ 1M $Na_2HPO4$ to 100 mL distilled sterile water. pH to 6.95 and filter sterilize. Store in aliquots at -20 ˚C.

**PBS** for tissue cultured was prepared to 10X and made to 1 L with 2.98 g KCl, 0.204 g $KH_2PO_4$, 81.82 g NaCl, 1.32 g $Na_2HPO_4$ and 1 L distilled water. The solution was adjusted to pH 7.3 to 7.4, autoclaved and stored at room temperature. 1 X was made with $1/10^{th}$ the amounts listed except for 1 L of distilled water as for 10X.

**KR Buffer** used in all binding and cAMP studies. KR consists of 5.75 % KCl, 6.1 % $CaCL_2$, 10.55 % $KH_2PO_4$, and 19.1 % $MgSO_4$ (7 $H_20$) in 20 mL of 0.1M Hepes.

**Luria Broth(LB) /Plates** The broth is made to 1 L with 10 g Bacto-tryptone, 5 g Yeast-extract, 5 g NaCl, 1 g Glucose and 1 L of distilled water. pH was attained with NaOH to 7.5 and autoclaved. For plates, 500 mL of broth was used with 7.5 g of Agar added and swirled then autoclaved and allowed to come down to a approximately room temperature, this was done either on the lab bench alone or in a 58°C water bath to bring the temperature down quicker. 1.5 mL Ampicillin was then added and swirled into solution and quickly poured into plates. The plates were allowed to set over night on the lab bench and then refrigerated at 4°C the next morning until needed.

**Terrific (TB)/Super Broth (SB)** was used for all over-day and over-night cultures of bacteria for all the experiments done for Mini preparations or Maxi/ Plasmid preparations. TB was made to 1 L with 4.32 g $KH_2PO_4$ (monobasic), 12.54 g $K_2HPO_4$ (dibasic), 12 g Tryptone, 24 g Yeast-extract, 4 mL Glycerol and 1 L distilled water. Then aliquots of 500 mL were autoclaved and stored at room temperature until needed.

**Charcoal suspension for cAMP assay** can be made in advance of study. 100 mM $KPO_4$, 2.5 mg / mL BSA saline pH 6.3, and 2 mg / mL Charcoal. Combine and mix with a stir bar for 10 minutes at R.T. Keep refrigerated until needed, when needed mix solution in a bath of ice.

**Plasmid Preparation/Maxi prep Solutions:**

**RNase A** was made using 10 mg/mL in 10 mM Tris HCl (pH7.5), 15 mM NaCl. RNase powder/pellet to 10mL distilled water. Swirl mixture until completely dissolved before boiling. Boil water at 100 ˚C, add RNase tube to water bath and heat at this temperature for 15 minutes. Remove water bath from heat and allow this to cool to 23 ˚C. Aliquot is necessary and store frozen at -20 ˚C.

**Cell Resuspension Solution** was made with no RNase A added, this was added as separately to the plasmid preparations. 50 mM Tris-HCl and 10 mM EDTA was added to a total volume of 1 L of $dH_20$ and stored at room temperature.

**Neutralization Buffer** was made with 4M Potassium Acetate in 500 mL of total volume distilled water, 165 mL 4M Potassium Acetate to 335 mL $dH_20$. This must be at pH 4.8 and stored at room temperature.

**Cell Lysis Solution** was made with 0.2 M NaOH and 1 % Sodium Dodecyl Sulfate electrophoresis purity agent (SDS) to a total volume of 1 L of $dH_20$. This was stored at room temperature.

**Column Wash** was made with 80 mM potassium acetate, 8.3 mM Tris-HCl, 40 mM EDTA, 578.9 mL 95% ETOH and 393 mL $dH_20$ for a 1 L total volume. This was stored at room temperature.

**Wizard DNA Resin** was ordered from Promega Corporation (608) 274-4330 Madison, W.I. U.S.A. under the proper name Wizard® Megapreps DNA Purification Resin containing 7 M Guanidine HCl. This was stored at room temperature.

**TE** was made with 10 mM Tris-HCl and 0.001 M EDTA to 500 mL total volume of $dH_20$. This solution was stored at room temperature.

**Protocols:**

**PCR with Tag DNA polymerase** was done with the following calculations, 34 λ $dH_20$, 5 λ 10X buffer, 2.5 λ dntps, 5 λ DNA (3187), 1 λ each of oligo, and 1 λ of enzyme. Hot start at 55 ˚C for 20 cycles, after labeling tubes and adding above amounts, as listed below.

1. 414-2733
2. 414-2734
3. 414-2735
4. 414-2736
5. 414-2737
6. 414-1738
7. 1315-2732 (universal control)

An addition of two drops of mineral oil is added to each tube and the PCR is begun before the enzyme is added. The first temperature is set at 95 ˚C for 1 minute, second temperature is 55 ˚C for 1 minute and the third in a cycle is set at 72 ˚C for 1 minute, there were 20 cycles total. The enzyme was added at 95 ˚C, Taq DNA polymerase, 1 λ to each tube is added below the mineral oil layer by injecting the pipette below the oil layer to the bottom and then releasing the enzyme. 20 cycles total at 3 minutes each equals 1 hour total PCR time.

**PCR with KOD Hot Start** was performed as detailed with the following calculations for each tube: 30 λ $dH_20$, 5 λ 10X buffer, 3 λ 25 mM $MgSO_4$, 5 λ dntps, 5 λ DNA (3187) and 0.5 λ each oligo and 0.5 λ 2732 to 2738 in respective tubes with KOD hot start polymerase enzyme added then 2 drops of mineral oil added last. PCR begins with 2 minutes at 95 ˚C then the cycles will run at 95 ˚C for 20 seconds, 60 ˚C for 10 seconds and 70 ˚C for 20 seconds.

**Gel Extraction** was done to extract the bands from the agarose gel, 300 Kb fragment was cut from each indicating the 414 oligo and the 700 Kb was cut indicating the 1314 oligo. All buffers are part of a Gel Extraction kit from Qiagen, the procedure went as follows:

1. 300 λ of G.C. at 50 ˚C for 10 minutes in sand heat was warmed before use.

2. Add 100 λ of Isopropyl and vortex.
3. Transfer to spin column and centrifuge for 1 minute/ 12K RPMs / 23˚ C.
4. Wash with 500 λ of G.C. again.
5. Centrifuge for 1 minute / 12K RPMs / 23 ˚C.
6. Add 750 λ PE buffer, allow soaking into column for 5 minutes.
7. Centrifuge for 1 minute / 12K RPMs / 23 ˚C.
8. Pour off bottom level and repeat spin.
9. Transfer column to new tubes and label appropriately
10. Add 30 λ of E.B. buffer, can also use $dH_20$, wait for 1 minute.
11. Centrifuge for 1 minute / 12K RPMs / 23 ˚C.

These samples are now ready for SOE Reactions as noted below are the samples and how they were sewn together, each was done with 2 λ of each column, column one was added to column two, therefore totaling 6 SOE reactions:

| | |
|---|---|
| 414-2733 | 1315-2732 |
| 414-2734 | 1315-2732 |
| 414-2735 | 1315-2732 |
| 414-2736 | 1315-2732 |
| 414-2737 | 1315-2732 |
| 414-2738 | 1315-2732 |

These were then done via a modified KOD hot start PCR in which the temperature was 70 ˚C for 30 seconds, considering the fact that the plasmids are much bigger in the SOE reactions then the first KOD hot start PCR. The protocol follows the same as noted above. After PCR, all samples were run on an agarose gel at 110 volts for 45 minutes and a picture was taken to ensure results.

**Digestion of SOEs (6 total)** was done as the procedure depicts below:

1. Take 27 λ of each sample into new tubes, if there are is not enough, add $dH_20$ to 27 λ total volume.
2. Add 3 λ of 10X buffer #3, 0.8 λ BSU, and 0.8 λ BAM-H1
3. Water bath samples at 37 ˚C for 1 to 1.5 hours.
4. Need a $1/10^{th}$ volume of NaOAc and 2.5 volumes of Ethyl Alcohol for each sample.
5. Shake and/or vortex the samples to attain precipitate to form.
6. Centrifuge 30 minutes / 14K RPMs / R.T.
7. Remove Ethyl Alcohol with out removing DNA which is seen as a white precipitate on the bottom and side of the tube.
8. Lafelize/Speed Vacuum for 15 minutes.

**Phosphatase Vector** was done as the procedure depicts below:

1. 10 λ 10X buffer #3 to 90 λ $dH_20$ and mix with yellow tip.
2. Add 1 λ Calf Intestinal Alkaline Phosphatase (CIAP) and mix.
3. Water bath for 1 hour at 37 ˚C.
4. Add 50 λ Phenol, saturated and 50 λ Chloroform
5. Vortex for 15 seconds
6. Centrifuge 5 minutes / 12K RPMs / R.T.
7. Remove top 90 λ and transfer to new tubes. Discard remaining.

8. Need 1/10$^{th}$ volume of NaOAc and 2.5 volumes of Ethyl Alcohol.
9. Vortex and Centrifuge 30 minutes / 12K RPMs / R.T.
10. Remove 200 λ and Speed vacuum for 15 minutes.

**Ligation** was done as the procedure notes, resuspend 20 λ for SOEs, 50 λ for Vectors. The chart below depicts the amounts and sample numbers for each ligation:

| Sample | Tube# | Vector | $dH_20$ | 5X Ligase buffer | Ligase | SOE |
|---|---|---|---|---|---|---|
| Control | 1 | 1 λ | 14 λ | 4 λ | 0.5 λ | None |
| 3217 | 2 | 1 λ | 7.5 λ | 5 λ | 0.5 λ | 2733 |
| 3218 | 3 | 1 λ | 7.5 λ | 5 λ | 0.5 λ | 2734 |
| 3219 | 4 | 1 λ | 7.5 λ | 5 λ | 0.5 λ | 2735 |
| 3220 | 5 | 1 λ | 7.5 λ | 5 λ | 0.5 λ | 2736 |
| 3221 | 6 | 1 λ | 7.5 λ | 5 λ | 0.5 λ | 2737 |
| 3222 | 7 | 1 λ | 7.5 λ | 5 λ | 0.5 λ | 2738 |

Transformations for each sample were done as described below:

1. 4 aliquots of 100 λ of competent cells were needed for the 7 transformations, competent cells were made and frozen in aliquots at -80 ˚C.
2. Bring cells to lab bench in an ice bucket to allow the cells to thaw for 1 hour.
3. 4 LB plates were warmed at 37 ˚C during this time.
4. 50 λ of competent cells to each sample are needed and heat shocked for 27 seconds in a 37 ˚C water bath.
5. The samples are then placed back in the ice bucket for 2 minutes.
6. Add R.T. SOS buffer at 250 λ each.
7. Shake in 55 ˚C incubator for 1 hour at 300 RPMs.
8. Plate cells, using half the plate when possible, and incubate over night at 37 ˚C.

**Minipreparation** for bacterial DNA was done with the QIAprep® Miniprep kit for purification of molecular biology grade DNA ordered from Qiagen U.S.A. (800) 426-8157. The kit includes all buffers needed and was performed to protocol given, the overnight cultures were grown in 3 mL of TB in a shaker at 37°C.

1. Tubes were labeled accordingly and each sample was transferred to theses new tubes and centrifuged for 17 seconds/12K RPM/ R.T.
2. Super was vacuumed off and the pelleted bacterial cells were resuspended in 250 λ Buffer P1 and vortexed until in solution.
3. 250 λ Buffer P2 was added and mixed by inverting the tubes several times.
4. 350 λ Buffer P3 was added and inverted again.
5. Centrifuged for 10 minutes/ 12K RPMs /R.T.
6. Super was transferred to QIAprep spin columns provided and spun again for 1 minute/ 12K RPMs / R.T. with the lower tube emptied after this spin.
7. 750 λ of PE buffer was added and allowed to sit for approximately 1 hour.
8. Centrifuged for 1 minute/ 12K RPMs / R.T.
9. Lower level removed and process repeated a second time.
10. The filter on the spin columns were placed in new tubes and labeled accordingly,
11. 65 λ of $dH_20$ was added to each and centrifuged for 1 minute/ 12K RPMs / R.T. The filters were discarded and the tubes were then stored at -20 °C.
12. Screen minipreps using BspE1 and BAM-H1 for a 780bp fragment.

**Sequencing** all positive samples were sent out of the laboratory for sequencing by that department. All tubes contained 3 λ $dH_20$, 2.5 λ DNA and 0.5 λ oligo to each. Each sample, 3217 to 3222 was sequenced in duplicate, one tube with oligo 414 and the other with oligo 2731.

**Restriction Digest** was done by the following calculations per tube, 2 λ of 10X buffer #3, 13 λ $dH_20$, 0.8 λ BSU, 0.8 λ BAM-H1, 5 λ miniprep DNA, an approximate total volume of 20 λ. Water bath at 37 ˚C for 1 to 1.5 hours to allow the enzyme to cut the fragments. Agarose gel and buffer were now made and once time has past, the samples were run on the gel at 110 volts for approximately 45 minutes.

**Agarose Gel** was prepared with Seakem agarose 1.75 g to 110 mL distilled water and 5 mL 10X TBE. Solution was heated until boiling and then removed from heat for 10 minutes. The well was then constructed and all seems were coated lightly with this solution, after 10 minutes Ethidium Bromide 1 λ was added as solution was being swirled. This was then poured into well with combs added last and allowed to set for over an hour. Buffer needed was made from 950 mL of $dH_20$ and 50 mL 10X TBE, this was poured over wells and reservoirs after the gel has set and samples have been inserted into each desired well. All gels were run at 110 volts for approximately 25 to 45 minutes depending on the movement of the marker.

**Plasmid /Maxi Preparation** for bacterial DNA. Over night cultures are grown in 250 mL TB with 385 λ Ampicillin, by shaking at 55 ˚C from over day cultures grown from one colony of interest in 2 mL TB with the same percent of AMP added.

1. Cultures are transferred to a 500 mL flask for centrifugation.
2. Centrifuge 10 minutes / 3K RPMs / R.T.
3. Discard super
4. Add 15 mL Resuspension buffer and 150 λ RNase A.
5. Shaker for 10 minutes / 110 RPMs / R.T.
6. Transfer to new tubes (1059) and label.
7. Add 15 mL Cell Lysis solution and invert to mix 10 times.
8. Add 15 mL Neutralization buffer and invert to mix 10 times.
9. Centrifuge 30 minutes / 2K RPMs / R.T.
10. Filter super through cheese cloth into new 1059 tubes.
11. Add ½ volumes of Isopropyl alcohol and invert to mix 10 times.
12. Centrifuge 30 minutes / 2K RPMs / R.T.
13. Discard super
14. Dissolve pellet in 4 mL TE and vortex until in solution.
15. Add 20 mL Wizard Resin and invert to mix 30 times.
16. Vacuum columns should be set up in advance, pour into columns and vacuum until column forms on top of the tubes column.
17. Do not discard 1059 tubes until they are washed with 20 mL Column Wash solution and transferred to column.

18. Discard 1059 tubes and add 20 mL Column Wash solution directly to vacuum tubes.
19. Add 10 mL 80 % Etoh, and allow all liquid to be vacuumed through before removing from the vacuum.
20. Remove column tubes and place in new 1059 tubes.
21. Centrifuge for 20 minutes / 2K RPMs / R.T. to dry columns.
22. Vacuum column tubes for an additional 5 minutes.
23. Add 1 mL 70 ˚C $dH_20$, wait 1 minute.
24. Repeat step #23.
25. Centrifuge for 5 minutes / 2K RPMs / R.T.
26. Check optical density and store at -20 ˚C.

**RNasing & Phenol-Chloroform extraction on Plasmid Prep DNA.** All samples were treated with RNase by the following procedure to further purify the sample and confirmed by running on an agarose gel. It is important to have equal amounts of samples, so some samples were split into 2 tubes to make the procedure run efficiently.

1. Insert 2 λ (for a 500 λ DNA sample) of RNase A to each labeled tube, start a timer after the first insertion and wait 10 minutes at R.T.
2. Stop RNase A activity in order of insertion with Phenol (1/2 volume) 250 λ
3. Shake vigorously with each addition of Phenol.
4. Add Chloroform (1/2 volume) 250 λ and shake vigorously, a white / cloudy precipitate should form.
5. Vortex 15 seconds to break down the protein.

6. Centrifuge for 5 minutes / 12K RPMs / R.T.
7. Set up new tubes for each.
8. Carefully remove samples.
9. Remove the top layer, approximately 400 λ maximum.
10. Top layer is transferred to new tubes, bottom layer is discarded in Chloroform waste container.
11. Add $1/10^{th}$ volume of 3 M NaOAc (sodium acetate).
12. Add 2.5 volumes of Ethanol.
13. Gently mix by inversion.
14. Centrifuge for 30 minutes/ 14K RPMs / R.T.
15. Pour off ethanol and dab tubes on a paper towel to assist in drying.
16. Leave upside-down on paper towel for 30 minutes.
17. If one tube per sample add 800 λ $dH_20$, if two tubes add 400 λ $dH_20$ to each.
18. Water bath for 15 minutes at 37 ˚C.
19. The pellet may go into solution by then, if not use a nutator.
20. O.D. the samples and store at -20 ˚C.

**Retransformation of DNA** was done by first pre-warming an LA plate in the incubator at 37 ˚C for approximately 45 minutes to an hour. This allows for a slight heat-shock of the cells upon retransformation. The competent cells were kept at -80 ˚C and were put on ice for 20 minutes to allow the cells to defrost. The DNA samples of interest, given the numbers 3217 to 3222 were also put on ice from the -20 ˚C freezer. For each sample 5 λ

of competent cells were added to new tubes with the respective 1 λ of DNA added to each appropriately labeled tube. These samples were now put back on ice for another 20 minutes. The samples were streaked onto the plates in a fashion that covered the area of the plate while diluting the sample with each stroke. The plates were allowed to dry at room temperature (23 ˚C) and then placed upside-down in the 37 ˚C incubator overnight.

**Defrosting Cells for Tissue Culture** was needed because when cells arrive to the lab they are quickly frozen in liquid Nitrogen until they are thawed for experimentation, to avoid the rise of damage or death that can occur at higher temperatures. To thaw the cells, vessels were set up in advance with pre-warmed media to allow for a smooth and easy transfer. Cryovials should be removed from liquid Nitrogen and quickly thawed in a 37 ˚C water bath until small ice crystals remain. The cells were removed and placed in a T-75 flask with 15 mL of media. 1mL of this flask was then transferred to a T-25 flask with 5 mL of media and allowed to incubate and later frozen in liquid Nitrogen, approximately two weeks later. Incubation for tissue culture was a consistent 37 ˚C 5 % (v/v) Carbon Dioxide. The media was changed the next day to ensure any remaining DMSO was removed.

**Counting Cells for tissue culture** was done on the basis of needing $5 \times 10^5$ cells per plate for a 100 mM plate and $2 \times 10^2$ for a 35 mM plate. Media was removed from the flask and it was washed with PBS which was also then removed for the addition of trypsin (2 mL for a T-75 flask). Once cells are loosened, 10 mL media with no pen. strep. was added and the cells were flushed off the surface to allow for a small sample to

be counted. A counting slide with a 9 grid layout was used with 50 λ of cells were added to the slide, under the cover slip and counted accordingly. The regions counted are shown below under 100X magnification and the calculations for determining the amount of cells in the sample was determined by the 5 regions total cell count x 2 x $10^3$.

| | | |
|---|---|---|
| 1 | | 2 |
| | 3 | |
| 4 | | 5 |

**Transient Transfection of COS-7 cells,** all transfections and tissue culture techniques were conducted under strict sterile conditions under the hood. Pre-warmed DMEM and 1X trypsin were set under the hood and a flask of approximately 70% confluent cells was set as well. The media was removed with a sterile pipette and the cells were rinsed with 5 mL 1X PBS to remove remaining media. The addition of 2 mL 1X Trypsin to the flask and rotating to cover the cells was done to aid in the removal of the cells from the flask. Store with cap slightly loosened in the 37 ˚C incubator for approximately 3 minutes.

Add DMEM complete to the flask to the volume needed for approximately 1 mL for each plate needed, plates used were 35 mM. With 7 samples (including a control) I used 35 plates in order to have 5 for each sample. The exact amount of cells needs to be calculated as described above. Each plate should have 9 mL of media already set aside so the 1ml of cells added will make a 10 mL total volume per plate. Swirl plate slightly to disperse cells around the plate, and place in incubator for transfection the next day, given cells are 30 to 60% confluent. pH changes occur if the plates are left out for too long, therefore only 3 to 5 plates were done at a time.

The next day cells were checked to ensure confluency of 30-60%. 2X BBS and 2M $CaCl_2$ were defrosted at room temperature (this can take up to an hour). Calculations were made based on the O.D. for each DNA sample to determine the amount needed for each plate. 25 λ / (O.D. of sample in γ/λ) x number of plates per sample = the amount of DNA needed for the transfection solution. This DNA was added to $dH_2O$ (amount determined by subtracting DNA, 2 M $CaCl_2$ and BBS from mL total volume) to 312.5 λ 2M Ca $Cl_2$ and 2.5 mL 2X BBS drop wise to ensure pH stability. The tube should be tapped lightly as 2X BBS is added so that a precipitate does not form, the solution should remain clear through out. These calculations were determined on a 5 plate per sample scenario which was the case for the first experiments conducted, these change based on the amount of plates used and or the size the plate used. Solutions were allowed to sit at room temperature for 15 minutes, then 1 mL of each were added to the respective labeled plates.

| **Transfections** | **2M $CaCl_2$** | **2X BBS** |
|---|---|---|
| 1 | 62.5 λ | 0.5 mL |
| 2 | 125 λ | 1.0 mL |
| 5 | 312.5 λ | 2.5 mL |
| 10 | 625 λ | 5.0 mL |

A precipitate was seen under 10X magnification after transfection solutions were added to each plate, this sometimes took up to 30 minutes on incubation before the precipitate was seen. Incubate plates for 16 to 20 hours and change media after this time to Complete DMEM with out FBS added this was harvested for 3 to 4 days.

**Stable Transfection with 293 cells** was done in EMEM media made complete as DMEM was made complete. This transfection was performed on 6-well plates requiring only 0.2 mL media per plate with no pen. step. added, approximately 1 mL of cells depending on the cell count was added to each plate to give an approximate 3 mL total volume per plate.

The next day Solutions A and B were made to and combined to form the transfection solution. Solution A was made from 2 λ of DNA (dependant on O.D.) 8 λ PSV2Neo (diluted 1 λ/300 λ) 90 λ Optimem to 100 λ total volume of sterile distilled water. Solution B was made from 6 λ Lipofectamine and 94 λ Optimem. This was made per plate per sample and combined for 15 minutes, after this time 800 λ of Optimem was added to each combined AB solution. The media was removed from the well and washed with 2 mL Optimem and removed. The AB solution was then added to each plate and labeled accordingly, precipitate was noted and plates were incubated for 5 hours. At this time 2 mL of complete EMEM and 100 λ FBS was added to each well and allowed to incubate for 24 hours before media was changed again to complete EMEM and then allowed to incubate for 48 more hours.

After 48 hours new 10 cm plates were set up, in this instance 3 plates were made for each sample with 10 mL complete EMEM and 50 λ G418. Media was removed from the 6-well plates, washed with PBS and trypsinized before fresh media was added and like-wells were added together to be transferred to the larger plates previously prepared (one-

third to each new plate). Cells were checked everyday and took approximately 7 to 10 days before there were colonies ready to be picked.

Picking colonies was done under the microscope at 100X magnification under the hood. A solution of 100mL complete EMEM and 500 λ G418 which was aliquoted into a 48-well plate at 0.5 mL per well. The colony was gently removed with a yellow-tip and added to the well, one colony per well was added and labeled. Each plate that was picked from had an additional 1 mL of fresh complete EMEM with G418 added to each. Colonies were picked as they formed and were done so over several days. All colonies and plates selected from were kept in the 37 °C with 5% (v/v) $CO_2$ incubator.

**Competitive hCG Binding Assay.** Was performed as follows, using control DNA 1664. The first steps involved preparing the cells from tissue culture plates.

1. Put on ice enough tubes for all samples, including controls.
2. Remove plates from incubator and scrap each plate with approximately 10 mL of media also from the plate into respective labeled tubes.
3. Balance with PBS if needed and centrifuge for 10 minutes / 2K RPMs / R.T.
4. Put tubes back on ice, also put KR buffer on ice.
5. 4 glass tubes will be needed for each sample, labeled and kept on ice.
6. Defrost and vortex cold hCG, mini-centrifuge for 5 seconds at R.T. and keep on ice.

7. A stock solution can be made to aliquot into 2 of the 4 tubes for each sample, 1 microgram / 50 λ KR buffer per tube is needed, and once aliquots are distributed hCG should be stored at -20 ˚C.
8. The other 2 tubes are to have only 50 λ KR buffer added.
9. Hot hCG needs to be quantified in a sample of KR to determine radioactivity and therefore determine the amount needed for each tube. The desired level is 100,000 counts per minute.
10. 5 λ hCG* added to 250 λ KR buffer was used to check radioactivity and either diluted or further concentrated to desired concentration. Keep on ice.
11. The tubes containing cells need to have the super removed in order to count the cell volume, approximately 300 λ is sufficient.
12. Transfer cells, $1/4^{th}$ to each respective tube and add aliquots of hCG* needed.
13. Cover tubes in rack and shake in 37 ˚C water bath for 1 hour.
14. Add 2 mL BSA Saline into each tube. BSA is kept refrigerated until needed, and should be cold when aliquots added.
15. Centrifuge for 10 minutes / 2K RPMs / R.T.
16. Tubes go back on ice and super are aspirated under a radiation hood.
17. Pellet is counted to determine radiation levels with a Scintillation counter.

The tubes containing hCG* and receptor signify the amount of receptor bound to ligand, the tubes containing both cold hCG and hCG* and receptor signify a competitive binding concentration between the two for the receptor.

**cAMP Assay.** Requires 2 days to complete, it is best to do both binding and cAMP day 1 together so that the cells are harvested and used at their best. Therefore 4 tubes are needed per sample per experiment, 8 tubes total. The procedure above for binding can be altered to allow for cAMP studies by dividing the 300 λ of cells to 8 tubes. The following is for cAMP samples only:

1. All 4 tubes contain aliquots of cells, 2 tubes will then only contain 50 λ KR buffer and the other 2 will contain 1 λ of cold hCG to 50 λ KR buffer.
2. Place in water bath shaker for 20 minutes / 37 ˚C.
3. Quickly transfer rack of tubes to a second water bath set at 75 ˚C to release the cAMP.
4. Refrigerate the rack until binding assay is complete.
5. 18 tubes are needed to set up cAMP standards.
6. Serial dilutions were made from 3,000 fM sample, starting with 70 λ in 210 λ KR buffer to equal first 1,000cAMP dilution. From that dilutions were made to 300, 100, 30, 10, 3, 1, 0.3 and 2 tubes were kept blank for 0.
7. 50 λ of each dilution went into 2 tubes, for a total of 18 tubes, including the blanks.
8. cAMP* was calculated and added to each tube, all dilutions and samples.
9. 10 λ of antibody was added to each tube was vortexed and then refrigerated overnight.
10. Place rack in ice bucket, make sure charcoal is mixing in ice bath.
11. Using an injection pipetter, place 1 mL of suspended cold charcoal to each tube.
12. Vortex samples, place back in rack on ice.

13. Centrifuge for 13 minutes / 2K RPMs / 8 ˚C.
14. Pour off super to new glass tubes, careful to keep charcoal in old ones.
15. Count super in liquid Scintillation machine. A lower count in solution equals more cAMP produced by the sample.

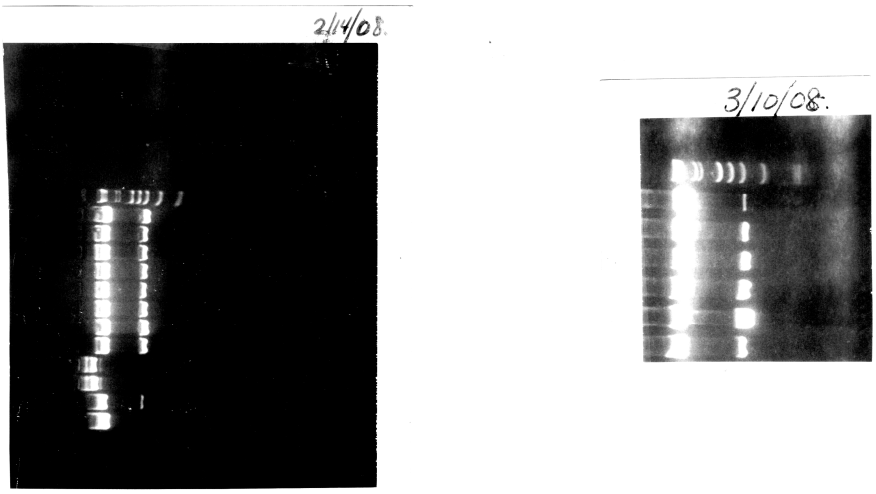
**Freezing cells in liquid Nitrogen** was done at the close of the experiments to ensure the integrity of the cells for further experimentation if needed.

1. Take a 100 % confluent flask and remove the media.
2. Rinse the flask with 10 mL 1X PBS.
3. Remove the PBS and add 1 mL Trypsin and incubate for 5 minutes.
4. Tap sides of the flask gently to see if cells are easily moving for the flask.
5. Add 9 mL complete media and rinse the bottom of the flask well, all cells should be rinsed to the bottom and there should be no clumps.
6. Remove media with cells to a 15 mL tube.
7. Centrifuge for 10 minutes / 1K RPM / R.T.
8. During this time, mix 2.7 mL complete media with 300 λ DMSO (dimethylsulfoxide) to make a 10 % solution. Keep on ice.
9. Remove super from cells, place 2 mL of media with the cold DMSO into the tube with pelleted cells and resuspend the cells.
10. Transfer 1 mL into 2 separate cryovials, and keep on ice.
11. Transfer tubes to -80 ˚C freezer for 1 to 2 days before placing in liquid Nitrogen.
12. Transfer tubes to liquid Nitrogen and log in book the cell type, media used, tank, cane and any other pertinent information.

**RESULTS**

PCR results were first inconclusive, considering that the bands seen in the Agarose gel photograph were not very clear. In order to see intensified bands, the PCR was done at a slightly higher temperature, from 55 ˚C to 60 ˚C, and changing from Taq DNA polymerase enzyme to KOD Hot Start enzyme. The bands first seen at 300 and 700 base pairs of oligo 414 and 1315 respectively were shown to be intensified with the second PCR. The successful results of an agarose done on the 6 SOE reactions done from the previous gel extraction. Note that these pictures are not included due to the clarity of the photograph, all were confirmed under ultraviolet light.

The first round of Mini preparations done with the mutants were not entirely successful, Figure 10 below shows the agarose gel and the lanes at the bottom reflect 4 samples that were retransformed and made again to see if lab error was to blame or if the transforms were to blame. There must have been lab error involved; given the new Mini preps were equal to the other mutants shown at the top of the gel, the second picture shown in Figure 10 details the positive plasmid preps after RNasing.




| Left Agarose Gel | Right Agarose Gel |
|---|---|
| Marker | Marker |
| 3222-2 | 3222 |
| 3222-1 | 3221 |
| 3221-2 | 3220 |
| 3221-1 | 3219 |
| 3220-2 | 3218 |
| 3220-1 | 3217 |
| 3219-2 | |
| 3219-1 | |
| 3218-2 | |
| 3218-1 | |
| 3217-2 | |
| 3217-1 | |

Figure 10. These two agarose gels depict the mutants made in mini and plasmid reparations respectively. It can be clearly seen that the bottom 4 rows in the first gel were not obviously successful, while the entire gel on the right is clearly more consistent. The gels are numbered as follows above, and align with the pictures above. The dimensions of the gels above were measured at 15cm in length, 13 cm in width and 1cm in height after gel is allowed to cool for 45 minutes.

All mutants were sent to the sequencing laboratory and upon return, plasmid preparations were made and the optical densities were analyzed and are listed below, all were run on an agarose gel to further confirm these results.

| DNA Sample | O.D. |
|---|---|
| 3217 LHR T413V | 2.079 γ/λ |
| 3218 LHR T413S | 3.145 γ/λ |
| 3219 LHR T413A | 2.440 γ/λ |
| 3220 LHR T413N | 2.632 γ/λ |
| 3221 LHR T413D | 2.000 γ/λ |
| 3222 LHR T413K | 3.954 γ/λ |

Figure 11 . Optical densities for the 6 mutants made of LHR Threonine 413.

Transfections were performed using CHO cells, HEK 293 and finally 293T cells before if was found that 293T cells were the only successful way to transiently transfect the mutants to the confluency needed for binding and cAMP assays. The chart below details the data attained from a particular binding assay and cAMP assay. To ensure the quality and control of the experiments, the assays I performed were completed in stages, using only one sample and control until all worked successfully. Once the entire set of mutations were completed together, and after several repeated experiments, the results were verified by Dr. Win Lin in the laboratory who repeated all transfection, binding and cAMP studies discussed.

| DNA Sample | Radiation Level (cpm) |
|---|---|
| 1664 rLHR-T413 (wild type) | 15,183 |
| 1664 rLHR-T413 | 17,964 |
| 1664 rLHR-T413 w/ cold hCG | 2,200 |
| 1664 rLHR-T413 w/ cold hCG | 2,194 |
| 3217 rLHR-T413V | 7,540 |
| 3217 rLHR-T413V | 7,480 |
| 3217 rLHR-T413V w/ cold hCG | 1,106 |
| 3217 rLHR-T413V w/ cold hCG | 1,283 |
| 3218 rLHR-T413S | 20,020 |
| 3218 rLHR-T413S | 16,148 |
| 3218 rLHR-T413S w/ cold hCG | 2,268 |
| 3218 rLHR-T413S w/ cold hCG | 1,473 |
| 3219 rLHR-T413A | 10,763 |
| 3219 rLHR-T413A | 10,762 |
| 3219 rLHR-T413A w/ cold hCG | 1,310 |
| 3219 rLHR-T413A w/ cold hCG | 1,358 |
| 3220 rLHR-T413N | 15,259 |
| 3220 rLHR-T413N | 13,984 |
| 3220 rLHR-T413N w/ cold hCG | 1,682 |
| 3220 rLHR-T413N w/ cold hCG | 2,029 |
| 3221 rLHR-T413D | 2,539 |
| 3221 rLHR-T413D | 2,389 |
| 3221 rLHR-T413D w/ cold hCG | 1,189 |
| 3221 rLHR-T413D w/ cold hCG | 1,303 |
| 3222 rLHR-T413K | 10,866 |
| 3222 rLHR-T413K | 12,110 |
| 3222 rLHR-T413K w/ cold hCG | 1,471 |
| 3222 rLHR-T413K w/ cold hCG | 1,560 |

Figure 12. hCG competitive binding assay. This chart shows the mutants made of Threonine 413 including the wild type labeled number 1664. All are shown in quadruplets, because 2 samples were tested with out the addition of cold hCG and 2 samples were tested with cold hCG as labeled above. All samples contained $^{125}$I-hCG.

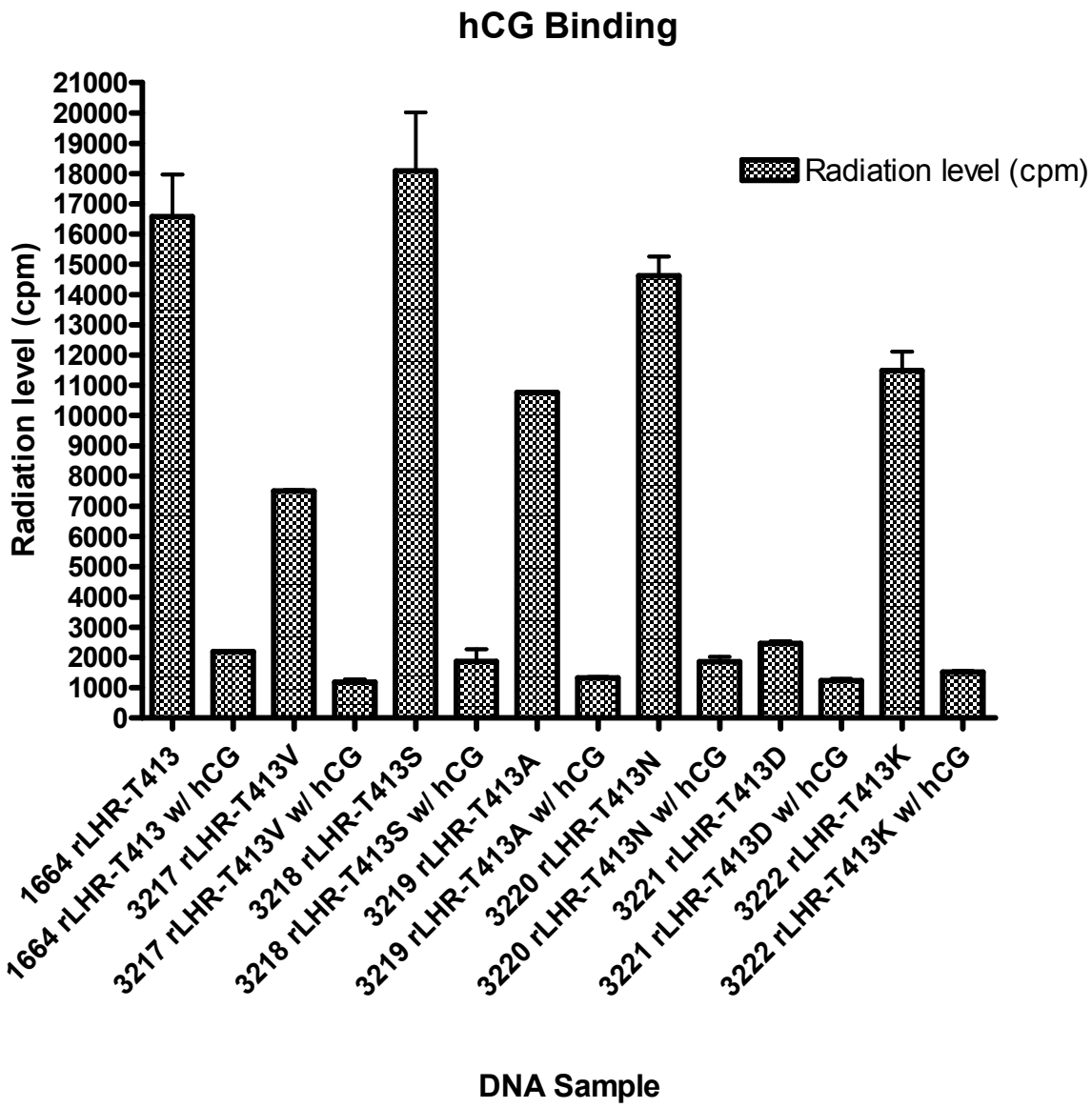



Figure 13. hCG competitive binding assay. The y-axis depicts increasing radiation levels in counts per minute (cpm). The x-axis depicts the control and 6 mutants as either with out cold hCG or with cold hCG respectively.

This competitive binding assay shows how the receptor binding to ligand is affected by the mutations made compared to the wild type receptor, basically it is important to see how binding has changed, either for the better, for the worse, or no change at all. Binding and cAMP studies were done in tandem encompassing two day and using the same cells from transfection to cAMP completion. Studies varied depending on the

health of the cells, which all were transfected at approximately 70% confluency, and the concentration of the $^{125}$I-hCG at the time of each study, which was factored into consideration for each experiment. The chart below in Figure 14 depicts the cAMP assay beginning with standard.

| Standards | Radiation Level (cpm) |
|---|---|
| cAMP 1,000 | 1,381 |
| cAMP 1,000 | 1,396 |
| cAMP 300 | 1,232 |
| cAMP 300 | 1,276 |
| cAMP 100 | 1,275 |
| cAMP 100 | 1,273 |
| cAMP 30 | 1,425 |
| cAMP 30 | 1,480 |
| cAMP 10 | 1,793 |
| cAMP 10 | 1,741 |
| cAMP 3 | 2,254 |
| cAMP 3 | 2,192 |
| cAMP 1.0 | 3,072 |
| cAMP 1.0 | 2,947 |
| cAMP 0.3 | 4,236 |
| cAMP 0.3 | 4,146 |
| cAMP 0 | 4,6184,618 |
| cAMP 0 | 5,017 |
| **DNA sample** | **Radiation Level (cpm)** |
| 1664 rLHR-T413 wild type | 4,448 |
| 1664 rLHR-T413 wild type | 4,200 |
| 1664 rLHR-T413 w/cold hCG | 1,338 |
| 1664 rLHR-T413 w/cold hCG | 1,302 |
| 3217 rLHR-T413V | 4,623 |
| 3217 rLHR-T413V | 4,367 |
| 3217 rLHR-T413V w/cold hCG | 1,402 |
| 3217 rLHR-T413V w/cold hCG | 1,379 |
| 3218 rLHR-T413S | 3,790 |
| 3218 rLHR-T413S | 3,550 |
| 3218 rLHR-T413S w/cold hCG | 1,347 |
| 3218 rLHR-T413S w/cold hCG | 1,419 |
| 3219 rLHR-T413A | 3,206 |
| 3219 rLHR-T413A | 2,967 |
| 3219 rLHR-T413A w/cold hCG | 1,320 |
| 3219 rLHR-T413A w/cold hCG | 1,327 |
| 3220 rLHR-T413N | 3,904 |
| 3220 rLHR-T413N | 3,687 |
| 3220 rLHR-T413N w/cold hCG | 1,495 |
| 3220 rLHR-T413N w/cold hCG | 1,492 |
| 3221 rLHR-T413D | 4,410 |
| 3221 rLHR-T413D | 4,565 |
| 3221 rLHR-T413D w/cold hCG | 1,337 |
| 3221 rLHR-T413D w/cold hCG | 1,451 |
| 3222 rLHR-T413K | 3,478 |
| 3222 rLHR-T413K | 3,646 |
| 3222 rLHR-T413K w/cold hCG | 1,309 |
| 3222 rLHR-T413K w/cold hCG | 1,356 |

Figure 14. The cAMP study results are listed above, and shown in the following graphs.

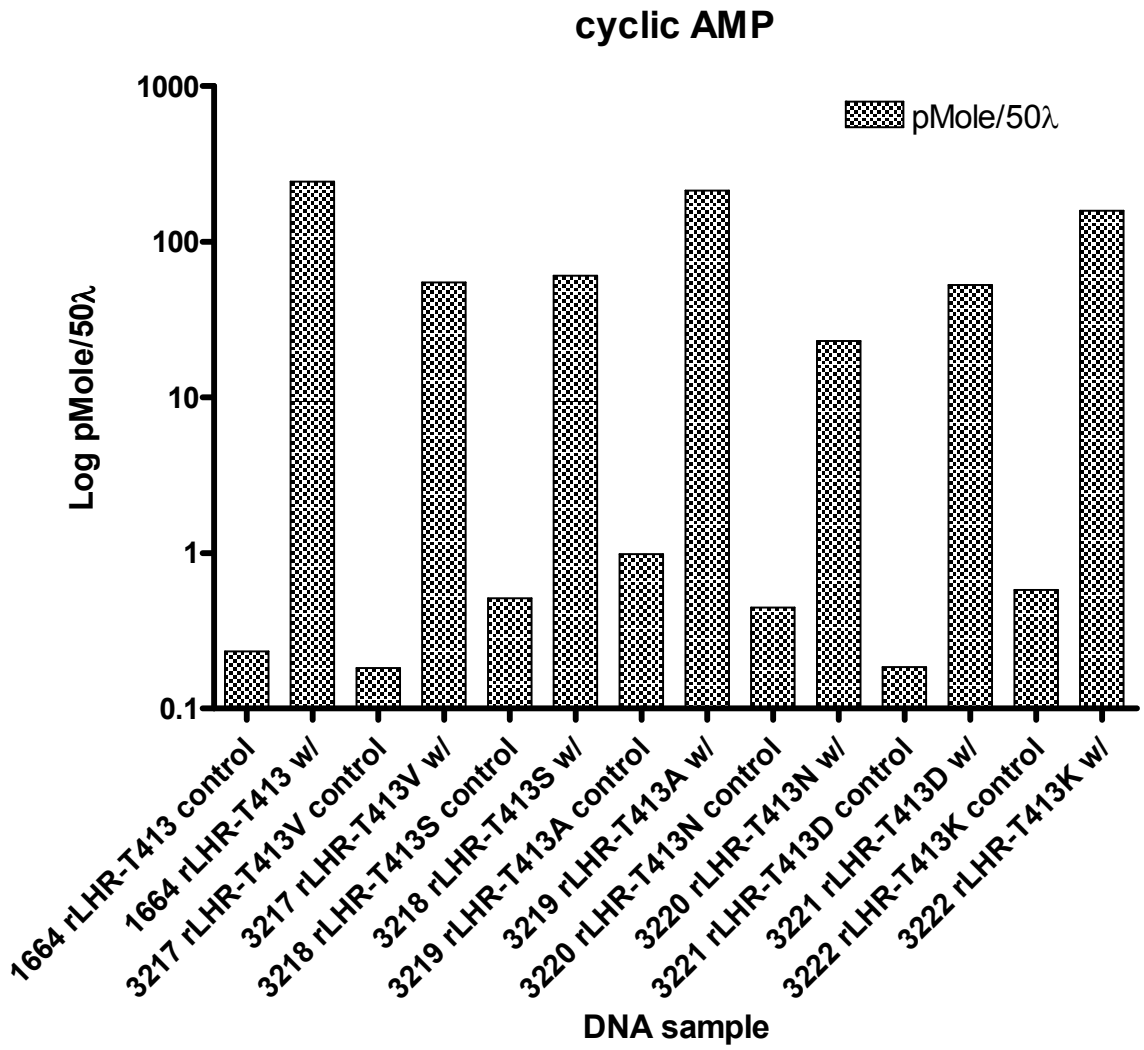



Figure 15. Cyclic AMP accumulation assay. The x-axis depicts the wildtype control #1664 rLHR-T413, without and with the addition of cold hCG, followed by each mutation made. The y-axis depicts cAMP accumulation levels as noted in log pMole/50λ.

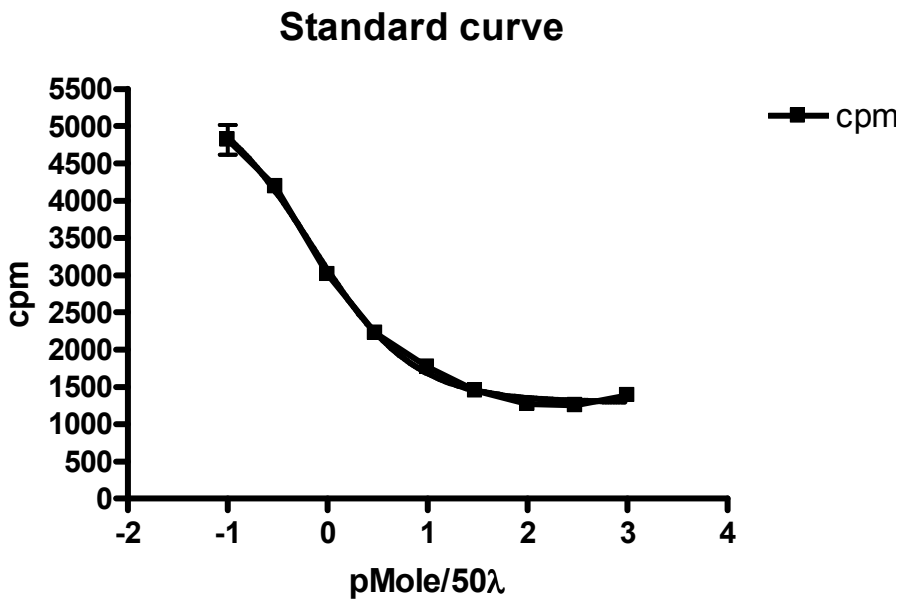



Figure 16. This graph depicts the standard curve for the cAMP results depicted above. The thicker areas of the curve denote the closely related radiation amounts found in (cpm)s after transfection of the mutants followed by the cAMP assay described in Materials & Methods section.

## DISCUSSION

The importance of cytoplasmic loops in glycoprotein activation has been studied extensively by mutational analysis and by competition studies using synthetic peptides containing amino acid sequences of domains within these loops (16). One finding is the importance of the N-terminal and C-terminal domains of the third intracellular loop, regions of the cytoplasmic tail and extracellular loops in G protein coupling. The choice of residue Threonine 413 to mutate is that this residue is highly conserved among most vertebrates. Figure 17 depicts an extensive species listing of the genomic sequence of the Outer Loop 1 of the LH receptor as compared with the residue of interest in the rat with these other species and their respective glycoprotein hormone equivalent. In an effort to determine why this residue is so highly conserved, the following mutations were made and analyzed as follows in this study. Figure 17 represents this highly conserved residue with a few exceptions. The Tilapia FSH has a methionine in the position of 413 instead of threonine, this may be a sequencing error. The difference between the codons for threonine and methionine is ACG and AUG respectively. The bottom four species in the figure sea urchin, sea anemones and drosophila are so far removed genetically, that it is no wonder why not only threonine 413 is not conserved through out the entire listing.

| Species | Outer loop 1 Genomic Sequence |
|---|---|
| Rat-LH | DSQTKGQYYNHAIDWQ-T-GSGCG |
| Pig-LH | DAQTKGQYYNHAIDWQ-T-GNGCS |
| Human-LH | DSQTKGQYYNHAIDWQ-T-GSGCS |
| Bovine-LH | DAQTKGQYYNHAIDWQ-T-GSGCS |
| Marmoset-LH | DSQTKGQYYNHAIDWQ-T-GSGCN |
| Chicken-LH | DAQTSGQYYNHAIDWQ-T-GSGCS |
| Turkey-LH | DAQTSGQYYNHAIYWQ-T-GSGCS |
| Xenopus-LH | DSQTRSQYYNHAIDWQ-T-GSGCS |
| Zebrafish-LH | DLRTRGHYSHHAIEWQ-T-GAGCD |
| A. Catfish-LH | DLRTRGYYSQHAIEWQ-T-GVGCN |
| C. Catfish-LH | DYRSRQEYYNHATDWQ-T-GMGCG |
| Tilapia-LH | DYHSHHEYYNHATDWQ-T-GPGCG |
| Salmon-LH | DLHTRGHYSEHAIDWQ-T-GAGCS |
| Trout1-LH | DLHTRGHYSEHAIDWQ-T-GAGCS |
| Trout2-LH | DLHTRGHYSEHAIDWQ-T-GAGCS |
| Rat-FSH | DIHTKSQYHNYAIDWQ-T-GAGCD |
| Human-FSH | DIHTKSQYHNYAIDWQ-T-GAGCD |
| Monkey-FSH | DIHTHSQYHNYAIDWQ-T-GAGCD |
| Horse-FSH | DIHTHSQYHNYAIDWQ-T-GAGCD |
| Donkey-FSH | DIHTHSQYHNYAIDWQ-T-GAGCD |
| Sheep-FSH | DVHTHSQYHNYAIDWQ-T-GAGCD |
| Chick-FSH | DIQTKSRYYNYAIDWQ-T-GAGCN |
| Snake-FSH | DMQSRTQYYNYAIDWQ-T-GAGCN |
| Lizard-FSH | DIQTKSQYYNYAIDWQ-T-GAGCN |
| Newt-FSH | DIKTKSQYYNHAIDWQ-T-GSGCA |
| A. Catfish-FSH | DLLTQSRYYNHGIEWQ-T-GPGCG |
| C. Catfish-FSH | DLQTRSHYYNYGIEWQ-T-GVGCG |
| Tilapia-FSH | DMLTRGRYYNYAIDWQ-M-GLGCN |
| Salmon-FSH | DVRTRGLYYNHAISWQ-T-GAGCD |
| Trout-1-FSH | DVRTRGLYYNHAISWQ-T-GAGCD |
| Trout-2-FSH | DVRTRGLYYNHAISWQ-T-GAGCD |
| Zebrafish-FSH | DIHTQSRYYNYGIDWQ-T-GAGCH |
| Lamprey-R | DVYTRGEYHNHAIDWQ-T-GLGCR |
| Human-TSH | DLYTHSEYYNHAIDWQ-T-GPGCN |
| Dog-TSH | DLYTHSEYYNHAIDWQ-T-GPGCN |
| Rat-TSH | DLYTHTEYYNHAIDWQ-T-GPGCN |
| Bass-TSH | DLHTRAEYFNFAIDWQ-T-GPGCG |
| Tilapia-TSH | DLHTRAEYFNHAIDWQ-T-GPGCG |
| Salmon-TSH | DLHTKMEYYNHAIEWQ-T-GPGCR |
| C. elegans | DAKMSDEYYRHAVWWQ-T-GWGCR |
| Sea Urchin | DVHTAGEYFNYSIQWQ-Y-GAGCS |
| Sea Anemones | SAVTRGDYHNYVQQWQ-N-GAGCK |
| Drosophila | DAHSMGEYFNFAYDWQ-Y-GLGCK |

Figure 17. The sequences of the Outer Loop 1 for the corresponding glycoprotein hormones of the species listed to the left of the figure. The Threonine shown within dash marks is T413, the amino acid of interest.

**Residue Selection**

Threonine 413 of the rat LHR is the basis for this study in which several amino acids were substituted in order to better understand why this threonine has been evolutionarily conserved in all vertebrates. Alanine Scanning Mutagenesis while important to use for a region or entire protein to determine significance, it is not efficient to determine specific interactions that aid in binding. To that end, we have decided to look at several amino acid substitutions to get the best possible picture for the threonine of interest. Also, since the sequence of the gene is known, site-directed mutagenesis is ideal for looking at this specific residue in the Leucine Rich Region of the rLHR. It is ideal so that each interaction can be analyzed, while some interactions between amino acids may have no effect on binding efficiency, some mutations may be effected to varying degrees based on the amino acids in close proximity. Figure 18 below depicts the truncated rat LHR where the seven transmembrane domains can clearly be represented. T413 can be found in the extracellular loop 1. This Threonine of interest is the second Threonine in loop 1 from left to right.

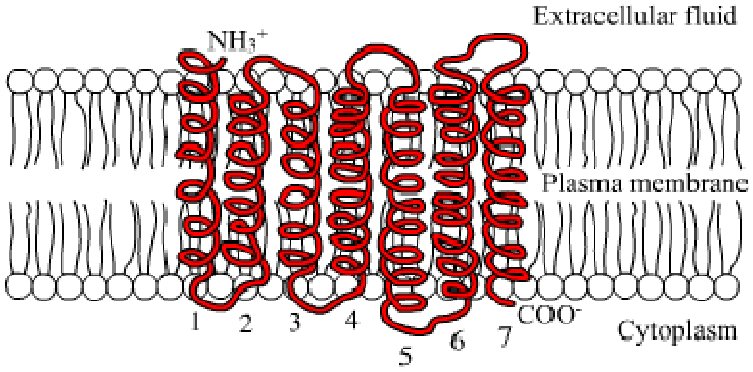



Figure 18. This cartoon representation, also shown in figure 5, of a truncated rat LHR showing the seven transmembrane domain, the three extracellular loops are on the top of the figure, and the three intracellular loops are shown at the bottom. Threonine 413 is found in extracellular loop 1.

We chose, as substitutes for threonine (T), the amino acids: alanine (A), asparagine (N), aspartate (D), lysine (K), serine (S) and valine (V). These amino acids were chosen based on homology to threonine and the differing qualities of some of these residues. Alanine is an aliphatic nonpolar hydrophobic residue which can be found on the surface or within a protein molecule. The hydroxyl group on threonine makes it a polar hydrophilic molecule; like serine, both of which are sites for glycosylation or phosphorylation and both hydrogen bond with other polar molecules. Carbohydrates attached to either residue are O-linked oligosaccharides. They differ by having a methyl substituted in place of the hydrogen on the β carbon. Threonine like serine, can both donate and accept hydrogen bonds, but because of threonines additional methyl group, it is slightly more hydrophobic than serine.

Asparagine is an uncharged molecule, but with its polar side chain containing an amide group it is the third amino acid that can be glycosylated; a carbohydrate attached to asparagine is considered an N-linked oligosaccharide and promote proper protein folding. This residue has extensive hydrogen bonding capabilities, and can be found on the surface, or buried within proteins. This neutral residue is the amide of aspartic acid which has no charge in biologically relevant pH conditions. The amide can be easily hydrolyzed converting asparagines to aspartic acid.

Aspartic acid is negatively charged and is important as a general acid in enzyme active centers, as well as in maintaining the solubility and ionic character of proteins.

Valine is structurally very similar to threonine, they both have roughly the same shape and volume, but chemically they are very different. By replacing the β-hydroxyl of threonine with a methyl group, the resulting amino acid with two methyl substituent groups on the β-carbon is now a valine. It is one of three branched chain amino acids that are highly hydrophobic, which makes it mainly found buried deep within globular proteins. It can be difficult even in a high resolution structure to distinguish one from the other.

Lysine is a basic polar positively charged residue due to the positive charge on its primary amine. Lysine is an alanine but with a propylamine on the β-carbon. The amino group is highly reactive and often participates in reactions at the active centers of enzymes. The side chain has three methylene groups, so that even though the terminal amino group will be charged under physiological conditions, the side chain has significant hydrophobic character. Therefore, lysines are mostly found buried within a protein with only the terminal amino group exposed to the surface. Asparagine, aspartic acid and lysine are all larger in mass then threonine while alanine, serine and valine are smaller.

Glycoproteins contain small diverse oligosaccharides that are linked to amino acids of a protein via O- or N-glycosidic bonds; these information rich carbohydrates commonly act as recognition sites for enzymes or receptors or for the targeting of a protein to a specific cellular location.

It was important to choose amino acids with positive, negative and neutral charge to see if the charge of the residue affected the receptor. It is known that the electrostatic potential of hCG is positive on the side implicated in receptor binding, this is a great example of how charge can aid in low affinity binding. Mutagenesis has shown that at least one positively charged residue should be present between residues 93-100 for tight binding affinity and the introduction of negatively charged residues gave the lowest affinity for the LH/CG receptor, accounting, at least in part, for the low affinity of hFSH for the LH/CG receptor (26).

**$^{125}$I-hCG Competitive Binding**

To monitor receptor binding, we compared the ability of our control/wildtype (1664) and the mutants constructed to inhibit the binding of $^{125}$I-hCG to cells expressing hCG. As shown in the Results section, the binding data showed some significant points of interest. In comparison to the wild type rLHR receptor labeled number 1664, threonine is shown not be the absolute best residue for binding in this study. With this highly conserved residue it is surprising to see that serine, asparagine and alanine show a very similar binding affinity when substituted in this position. With serine being slightly smaller than threonine, considering it is the same amino acid with the loss of a carbon in serine, it is not entirely surprising to see how these two can be interchanged. Asparagine is relatively larger in size with the addition of an amino group in comparison, which does not seem to affect binding greatly. Serine and asparagine are hydrophilic, polar uncharged residues like threonine which may aid in their ability to bind similarly. Alanine is the smallest

amino acid with only contains a methyl group, it is a threonine with out its carboxyl group.

Lysine also shows similar binding to that of the alanine mutant. While both are hydrophilic like threonine, lysine is a basic amino acid residue with a long chain of 4 methyl groups and an amino group at the end of the chain, in comparison to the 2 methyl groups of threonine, with a carboxyl group these two are not structurally similar. The last in order of effective binding is the mutants made to valine and glutamate with glutamate having the lowest binding affinity of all the residues in this position. Valine is hydrophobic, and does not contain a carboxyl group, it is also quite small which in combination must add to its poor binding in relation to those previously discussed. Aspartate is an acidic amino acid and this residue was chosen to see if this would make a big difference as was expected it is the worst in relation to binding affinity; yet both valine and aspartate do bind, and do not entirely disturb the receptor so much that it is totally lost. While these studies are interesting, they are inconclusive, binding does not translate to expression, in order to check expression levels cAMP assays were performed and discussed below.

**Cyclic AMP accumulation assay.**

The best way to characterize the influence on expression by the mutants made is to monitor signal transduction. We tested this by examining the ability of the samples to stimulate cAMP accumulation and how this compares to the wildtype rLHR-T413. In the

testis and ovary, agonist-activated LHR couples leading to the stimulation of cAMP and phosphorylation of intracellular proteins through activation of protein kinase A (16, 25a, 40). Also, agonist activation of LH/hCG receptors promotes PI hydrolysis and calcium signaling activation in the ovaries and in several cell lines transiently or stably expressing LHR (15, 16). There is no definitive evidence to suggest that activation of the phospholipase C/calcium signaling pathway by LH in Leydig cells, where androgen production is increased solely by cAMP (16). In several studies, cAMP and protein kinase C-induced phosphorylation of LHR in a stably transfected cell line and was mapped to Ser635, Ser639, Ser646 and Ser652 in the C-terminal cytoplasmic tail; also indicating that protein kinase A-induced phosphorylation cannot fully account for hCG-induced uncoupling of LHR (15).

The GPCRs activate both cAMP and phospholipase C (PI) varying on the type of cell. This may depend on the receptors density and in the subunits present in individual tissues and at specific stages of differentiation (16, 33). Leydig cells may lack stimulation of the PI pathway due to specific inhibitors, deficient receptor coupling or activation, or competition for overlapping effectors sites of other G proteins (16).

The cytoplasmic C-terminal domain and intracellular loops of the LHR contain several serines and threonines and a single tyrosine residue that are amendable to phosphorylation and could participate in agonist-induced desensitization (15). Affinity-purified ovarian and testicular receptors are rapidly phosphorylated at serine and threonine residues by the catalytic subunit of protein kinase A within ten minutes (15).

Gonadotropin binding initially increases and decreases LHR phosphorylation suggesting that agonist occupancy of the receptor causes a series of conformational changes that influence its susceptibility to phosphorylation (15).

The removal of phosphorylation sites from the C-terminal tail of LHR, by truncation or mutation, produces unexpected results. Phosphorylation of residues within this domain does not appear to be essential for desensitization. Studies of the loops and of the participation of other kinase in desensitization are suggested to help unravel the mechanisms for agonist-induced uncoupling of the LHR (15).

The overall process involves the activated receptor binding Gsα and affecting a GDP-GTP exchange on Gsα, then by dissociation of α from the GPCR and from βγ, therefore allowing GTP-Gsα to bind to and activate adenylyl cyclase (35).

LHR can be constitutively activated by single base changes in its gene that result in replacement of specific amino acid residues, like other members of the GPCR superfamily (35). This was found in the experiments discussed using T413.

As seen in the Results section the rLHR-T413A mutant elicits the most constitutive activity in comparison to the wild type rLHR-T413. Some mutants, those including serine, asparagine and lysine elicit some constitutive activity in relation to the wild type. Only valine and aspartate elicit no constitutive activity and very little binding, as discussed.

In our assay, there are relatively few steps between ligand binding and the measured response, i.e., cyclic AMP accumulation (7). Therefore, this assay was ideal versus others which depend on the ability of hCG to induce the expression of a luciferase reporter gene in LHR transfected cells (7). This would require much more time and many more steps involving more expense as well.

**Evolutionary Significance**

Observations by Moyle et al. support a model in which distinct negative determinants restrict the ligand to receptor interactions; leading to an explanation of co-evolution of binding specificity in families of homologous ligands and receptors (26). It is also suggested therefore that naturally occurring or laboratory generated mutations of these determinants leads to the evolution of new and sometimes specific protein to protein interactions (26). These studies were facilitated by the fact that the high-affinity ligand-binding sites of the LH and FSH receptors are located in the large extracellular domains (ECD) and as long as they contain LH receptor residues 93-170, the chimeras retained their high affinity for hCG, while one had high affinity for both hCG and hFSH (26).

The evolution of LH, FSH and their receptors would therefore seem to be an example in "where specificity reached a maximum", per Moyle et al. Whereas, many ligand-receptor pairs are in different stages of this process based on observations involving the multiple examples of homologous ligands that bind a common receptor, homologous receptors that bind a common ligand or homologous ligands that share homologous

receptors (26). These observations show how specificity determinants can be manipulated in a rational and useful way to create analogues with novel ligand-binding properties; therefore, it should be possible to reverse the steps of divergent evolution and fully restore cross-reactive binding to other homologous ligands, like hCG and FSH, which display very high receptor-binding specificity (26). This has been helpful in the development of new analogues that stimulate ovarian development in rodents; this can be useful for treating human infertility and/or to aid in stimulating fertility in endangered species (26).

The glycoprotein hormone receptors have been proposed to have resulted as recombinant events early in evolution between the Family 1 GPCR, described above, and a member of the leucine-rich repeat family (15). Identification of LHR-like proteins in the sea anemone, the mollusk *Lymmea stagnalis*, and the arthropod *Drosophila* suggests that the LRR/GPCR recombinant event took place prior to vertebrate evolution (11, 15, 30a, 39a). The deduced amino acid sequences of these GPCRs show remarkable similarity to the mammalian LHR, FSHR and TSHR in particular between LHR exons 2 and 8 within the LRR region and in the TM/loop module (15). Intronic positions within the LHR extracellular domain are conserved in the *Drosophila* GPCR, and the intronic sites appear to be conserved at the same position within the LRR in other LRR proteins. Thus the initial recombination event may have involved an intron-containing LRR module (15).

Phylogenetic analyses by Oba et al. revealed evolutionary relationships between the fish glycoprotein hormones with those of the human, Northern blot analysis and in situ

hybridization also show parallel expression patterns between the two; which shows both genetic and functional comparability between the two species (31). This evolutionary divergence appears to have occurred before the teleost and tetrapod split, also seen in glycoprotein ligands (31). This split can be seen best in Figure 19, which depicts the neighbor joining unrooted tree to suggest the existence of three clades TSHR, FSH/GTHRI (Gonadotropin hormone receptor I) and LH/GTHRII (31). A clade signifies a grouping of organisms made on the basis of phylogenetic relationship; they are not based purely on shared features. Clades consist of a common ancestor and all its descendants, Oba et. al suggest that these three claced, as shown below are consistent with the phylogeny of vertebrate speciation.

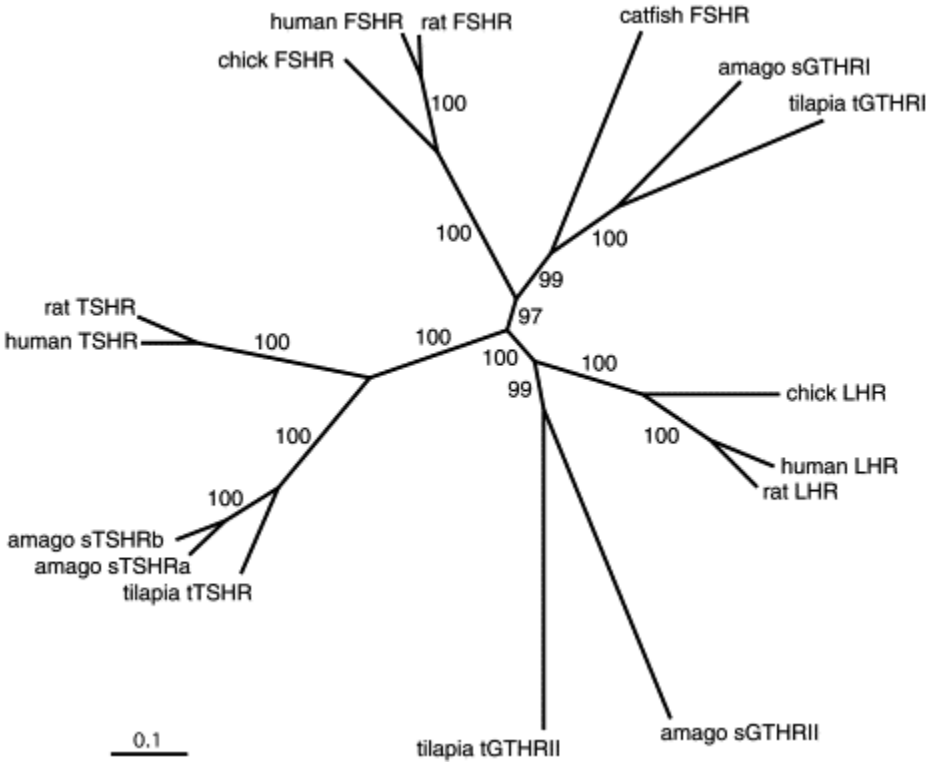



Figure 19. Unrooted phylogenetic tree of the full ORF amino acid sequences of vertebrate gonadotropin hormone receptors and TSH receptors constructed by Oba et al. using the NJ method. Bootstrap values from 100 replicates are noted by the numbers. Long lines indicate genetic distances, an estimate of the amount of genetic change that has occurred between two sequences since they diverged from the common ancestor (31).

Gene duplication is known to be a powerful tool for creating new genetic material that drives evolution (37). Once the duplicate gene is copied into the genome, it has multiple possibilities: the gene can be lost or deleted, create a novel function due to sequence divergence, become a complementary function, or attain regulatory elements that can enhance transcription levels or expand by changing its spatial and temporal expression (37). Species specific gene duplication events have occurred and have been verified multiple times during placental evolution creating a massive amount of genetic information for the advancement of evolutionary study (37). Since the placenta is a

major source of hormones as described, it is important to consider how these evolutionary changes have arisen and affected the placenta-specific hormones discussed here.

**POINT MUTATION IN THE "NATURAL" POPULATION**

A single naturally occurring loss of function mutation of the FSHR, present in the Finnish population, impairs receptor function. This impairment causes the blocking of follicular development at primary stages and presents in patients with the absence of menstruation in productive age (amenorrhea) with atrophic ovaries (4). Once the initial report was published on women of Finnish decent, many studies have followed on German, English, and French patients suggesting that this mutant is isolated evolutionarily to the Finnish population (4).

DNA from phenotypic patients of Finnish decent was analyzed depicting the presence of mutated amino acids in the extracellular domain and tin the third extracellular loop of the FSHR. This is a highly concentrated region of hydrophobic (Val or Ile) or positively charged (Arg or Lys) residues in FSHR, LHR and TSHR of different species; suggesting an important function role for these areas (4). The phenotype primarily connected to hypergonadotropic primary amenorrhea with streak ovaries including primordial follicles corresponds to the Finnish isolate. However, this phenotype also corresponds to other etiologies beyond just the FHSR defect. Patients with this phenotype have been studied in countries all over the world, with no genetic abnormality of the FSHR found in any patients outside of Finland, at the time this study was published (4).

There are many amino acids that can be found in the same corresponding positions throughout various species both vertebrate and invertebrate among the glycoprotein

hormones. With even a high level of sequence similarity Dobzhansky & Muller wondered why it is so difficult for closely related species to produce viable offspring. Their theory along with Barbash et al. suggested that hybrid failure is due to pairs of genes that interact, evolve differently after populations split (34). Therefore, eventually one pair will have evolved quite differently then the other, whereas they will no longer work together. These hybrid offspring are usually sterile if they survive at all. These mutations can accumulate to cause reproductive isolation, which is further complicated because the issues originate between two different species, a cross-species genetic problem (34).

## CONCLUSIONS

Without further investigation it is still not certain why threonine is the evolutionarily conserved residue at position 413 in the extracellular loop 1 of the transmembrane domain of LHR for so many species and their glycoprotein hormone receptors. If it were simply the size, charge, or composition of threonine that made it so conserved why is it easily replaced and manipulated with out complete loss of binding, there must be a combination of elements at work. These studies like all other point mutation studies just scratch the surface of why one residue is selected and kept over millions of years. There is only hope that a better understanding of each residue will aid in a better understanding of the whole receptor and for that there are limitless applications.

There are major obstacles that have stymied researchers from obtaining structures of other GPCRs, including the production and purification of proteins and their stability and homogeneity (21). It is now possible to generate sufficient quantities of several GPCRs for crystal screening using bacterial, yeast, insect and mammalian cell expression systems; and with the aid of robotic systems for preparing huge setups; protein production is no longer a major hindrance (21). A remaining issue is the inherent flexibility of GPCRs causing conformational heterogeneity, this flexibility may be functionally important for structural changes associated with agonist binding and activation of membrane receptors but also and obstacle for researchers (21). GPCR structure can ultimately help in the design of more selective drugs with the appropriate efficacy for the desired physiological function (21).

A more concise understanding of the function and structure of these hormones and their receptors would also greatly facilitate the development of new pharmacological agents for a plethora of diseases and disorders, as well as assist in fertility treatments (27). It may also bring us closer to understanding why certain residues are conserved in so many different species and how these residues are kept conserved.

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