



University of Kentucky  
UKnowledge

---

University of Kentucky Doctoral Dissertations

Graduate School

---

2003

## CIS- AND TRANS-ACTIVATION OF HORMONE RECEPTORS: THE LH RECEPTOR

ChangWoo Lee

*University of Kentucky*, [cwlee0@uky.edu](mailto:cwlee0@uky.edu)

[Right click to open a feedback form in a new tab to let us know how this document benefits you.](#)

---

### Recommended Citation

Lee, ChangWoo, "CIS- AND TRANS-ACTIVATION OF HORMONE RECEPTORS: THE LH RECEPTOR" (2003).  
*University of Kentucky Doctoral Dissertations*. 263.  
[https://uknowledge.uky.edu/gradschool\\_diss/263](https://uknowledge.uky.edu/gradschool_diss/263)

This Dissertation is brought to you for free and open access by the Graduate School at UKnowledge. It has been accepted for inclusion in University of Kentucky Doctoral Dissertations by an authorized administrator of UKnowledge. For more information, please contact [UKnowledge@lsv.uky.edu](mailto:UKnowledge@lsv.uky.edu).

ABSTRACT OF DISSERTATION

ChangWoo Lee

The Graduate School

University of Kentucky

2003

**CIS- AND TRANS-ACTIVATION OF HORMONE RECEPTORS:  
THE LH RECEPTOR**

---

ABSTRACT OF DISSERTATION

---

A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Arts and Sciences  
at the University of Kentucky

By

ChangWoo Lee

Lexington, Kentucky

Director: Dr. Tae H. Ji, Professor of Chemistry

Lexington, Kentucky

2003

## ABSTRACT OF DISSERTATION

### **CIS- AND TRANS-ACTIVATION OF HORMONE RECEPTORS: THE LH RECEPTOR**

The Luteinizing hormone receptor (LHR) belongs to the G protein-coupled receptor family, as do the other glycoprotein hormone receptors for FSH, TSH, and CG. The LHR comprises two halves of ~350 amino acids: an extracellular hormone binding exodomain and a seven transmembrane-spanning endodomain responsible for signal generation. Hormone binds to the exodomain with high affinity, and the resulting conformational changes in the hormone/exodomain complex modulate the endodomain to generate hormone signals. Hormone binding to an LHR produces hormonal signals (cis-activation), but it is not known whether a liganded LHR could activate other unoccupied LHRs (trans-activation). The LHR activates both adenylyl cyclase and phospholipase C $\beta$ . This dissertation shows that trans-activation of the LHR leads to the activation of adenylyl cyclase to induce cAMP but not to the activation of phospholipase C $\beta$  to induce the inositol phosphate signaling. Trans-activation offers a mechanism of signal amplification at the receptor level and also provides a mechanism of multiple signal generation for a liganded LHR to cis-activate phospholipase C $\beta$  and trans-activate adenylyl cyclase. Also coexpression of G $_{i2}$  with a constitutively activating LHR (Asp<sup>578</sup>Gly), the most common mutation of male-limited precocious puberty, shows that G $_{i2}$  could completely inhibit cAMP induction by the LHR mutant. Experiments using the carboxyl terminal region of G protein  $\alpha$  subunits demonstrate that LHR has overlapping binding sites for G $\alpha$  subunits G $_s$  and G $_{i2}$ .

KEYWORDS: LH Receptor, Receptor Activation, Cis-activation, Trans-activation

ChangWoo Lee

April 2, 2003

**CIS- AND TRANS-ACTIVATION OF HORMONE RECEPTORS:  
THE LH RECEPTOR**

By

ChangWoo Lee

Dr. Tae H. Ji  
Director of Dissertation

Dr. Peter M. Mirabito  
Director of Graduate Studies

April 2, 2003

## RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgments.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

DISSERTATION

ChangWoo Lee

The Graduate School  
University of Kentucky

2003

**CIS- AND TRANS-ACTIVATION OF HORMONE RECEPTORS:  
THE LH RECEPTOR**

---

DISSERTATION

---

A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Arts and Sciences  
at the University of Kentucky

By

ChangWoo Lee

Lexington, Kentucky

Director: Dr. Tae H. Ji, Professor of Chemistry

Lexington, Kentucky

2003





Dedicated to my Father, for his love and encouragements, who passed away from cancer  
before completion of this dissertation

## ACKNOWLEDGEMENTS

I am indebted to many people for this dissertation. First and foremost, I would like to thank my advisor, Dr. Tae H. Ji, for his direction of this dissertation, support, and encouragements. He exemplifies the high quality scholarship to which I aspire and has always been patient with me. I would also like to thank Dr. Inhae Ji. Without her guidance and encouragements, this dissertation could not have been possible.

I wish to thank my Dissertation Committee and outside examiner, respectively: Dr. Judith Lesnaw, Dr. Sheldon Steiner, Dr. Douglas Harrison, Dr. Boyd Haley, and Dr. Lee Edgerton. Each individual provided insights that guided and challenged my thinking, substantially improving the finished product.

I thank Drs. KiSung Ryu, YongSang Song, YongBum Koo, and Sohee Hong for making valuable mutant LH receptors that I used in my dissertation study. I would also like to thank the other members of the lab for their help and companionship, especially Myoungkun Jeoung, Gail Sievert, Rebecca McCaffrey, Johann Sohn, Dr. HyeSook Youn, and Dr. ChongSeong Yi.

Lastly, I thank my Mom, whose love made this possible.

## Table of Contents

<b>Acknowledgments</b> .....	iii
<b>List of Tables</b> .....	vi
<b>List of Figures</b> .....	vii
<b>List of File</b> .....	ix
<b>Chapter 1: Introduction</b> .....	1
LH and FSH.....	1
The LH Receptor.....	2
Hormone Binding and Receptor Activation.....	3
Mutations of the LHR.....	6
Goal of Dissertation.....	8
<b>Chapter 2: Trans-activation of the LHR for cAMP Signaling</b> .....	18
Introduction.....	18
Materials and Methods.....	18
Results.....	21
Discussion.....	24
<b>Chapter 3: No Trans-activation of the LHR for IP Signaling</b> .....	36
Introduction.....	36
Materials and Methods.....	38
Results.....	39
Discussion.....	41
<b>Chapter 4: Overlapping G protein Binding Sites of the LHR</b> .....	53
Introduction.....	53

Materials and Methods.....	55
Results.....	55
Discussion.....	57
<b>References.....</b>	<b>62</b>
<b>Vita.....</b>	<b>73</b>

## List of Tables

Chapter 2	Table 2.1 Surface Expression of Flag-LHRs.....	27
Chapter 3	Table 3.1 Surface Expression of Flag-LHRs.....	43
Chapter 4	Table 4.1 Effect of G <sub>s</sub> , G <sub>i2</sub> , and their carboxyl terminal peptides on the cAMP induction of LHR <sup>WT</sup> and LHR <sup>D578G</sup> .....	59

## List of Figures

### Chapter 1

Figure 1.1	Crystal structure of hCG.....	10
Figure 1.2	Schematic diagram of the LHR.....	11
Figure 1.3	Important regions of the LHR for hormone binding.....	12
Figure 1.4	Side and top view of the LHR endodomain.....	13
Figure 1.5	Receptor activation and signal generation of the LHR.....	14
Figure 1.6	Mutations of the LHR.....	15
Figure 1.7	Defind-function mutants in the LHR.....	16
Figure 1.8	Cis-activation and trans-activation of LHRs.....	17

### Chapter 2

Figure 2.1	Activity of mutant receptors.....	28
Figure 2.2	Coexpression of LHR <sup>-hCG</sup> and LHR <sup>+hCG/-cAMP</sup> .....	29
Figure 2.3	Coexpression of Flag-LHR <sup>C22A</sup> and LHR <sup>K583R</sup> .....	30
Figure 2.4	Specificity of cAMP rescue.....	31
Figure 2.5	Receptor concentration-dependent cAMP rescue.....	32
Figure 2.6	Coexpression of stable LHR <sup>-hCG</sup> and transient LHR <sup>+hCG/-cAMP</sup> .....	33
Figure 2.7	cAMP rescue is dependent on the location of mutation in LHR <sup>-hCG</sup> .....	34
Figure 2.8	Coexpression of LHR <sup>WT</sup> and FSHR <sup>WT</sup> .....	35

### Chapter 3

Figure 3.1	Will there be trans-activation of LHR for inositol phosphate signaling?.....	44
Figure 3.2	Activity of mutant receptors.....	45
Figure 3.3	Induction of inositol phosphates from LHR <sup>WT</sup> .....	46
Figure 3.4	Coexpression of LHR <sup>K583R</sup> and LHR <sup>-hCG</sup> .....	47
Figure 3.5	Coexpression of ExoCD and LHR <sup>-hCG</sup> .....	48

Figure 3.6 Coexpression of stable LHR and transient LHR.....	49
Figure 3.7 Coexpression of stable ExoCD and stable LHR <sup>-hCG</sup> .....	50
Figure 3.8 Coexpression of LHRK583R and TM4 LHR <sup>-hCG</sup> .....	51
Figure 3.9 Coexpression of LHRWT and LHR <sup>-hCG</sup> and LHR <sup>+hCG/-cAMP</sup> .....	52

#### Chapter 4

Figure 4.1 Effect of GsCT and Gi2CT on cAMP induction of LHR <sup>WT</sup> and LHR <sup>D578G</sup> .....	60
Figure 4.2 Effect of Gs and Gi2 on cAMP induction of LHR <sup>WT</sup> and LHR <sup>D578G</sup> .....	61



## List of File

changwoo.pdf.....3.03 MB

## Chapter 1 Introduction

### Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH)

LH, FSH, TSH, and CG belong to the glycoprotein hormone family (1). These hormones are heterodimers of  $\alpha$  and  $\beta$  subunits; sharing a common  $\alpha$  subunit consisting of 92 amino acids (1). Hormone-specific  $\beta$  subunits vary in size; 121 amino acids are found in the LH $\beta$  while hCG $\beta$  has 145 amino acids (1) (Figure 1.1). LH, FSH, and TSH are released from the anterior pituitary, and CG originates from the placenta during pregnancy (1). LH and CG bind to the same receptor on granulosa cells, known as the LH/CG receptor or the LHR (2).

FSH elicits the primary signal for starting preovulatory follicular development (3). It takes 10-12 days of sustained stimulation by FSH for a ~5 mm follicle to attain a full preovulatory diameter of  $\geq 20$  mm; during this time the number of its granulosa cells increases 5 or 6 fold to reach over 50 million (4), while the number of FSHRs in each granulosa cell increases 4 to 5 fold (5). FSH directly stimulates proliferation of granulosa cells and induces the LH-responsive mechanisms in cells that will sustain steroid secretion by the preovulatory follicle and, after ovulation, the corpus luteum (3). FSH binds to the FSHR in granulosa cells, inducing mRNA expression of FSH-responsive genes such as LHR, steroidogenic enzyme P450 aromatase, and regulatory peptides such as inhibin subunits and follistatin (6).

LH mediates the secretion of steroid hormones by the preovulatory follicles and corpus luteum (3). Estrogen secretion by the follicles increases during the second half of the follicular phase when the frequency of pulsatile LH discharge by the pituitary increases and FSH secretion decreases (3). During this stage of development, granulosa cells express LHR, and tonic levels of LH are able to directly stimulate steroidogenic enzyme systems preinduced by FSH (3). LH acts through the LHR on theca cells and mature granulosa cells to induce mRNA expression of LH-responsive genes such as those responsible for precursor-cholesterol uptake and sustains high steroidogenic enzyme activity (6). An ovulation-inducing dose of LH is released by the pituitary gland at mid-menstrual cycle. This terminates proliferation of granulosa cells and simultaneously initiates rupture of the ovulatory follicle and onset of luteal progesterone secretion. If pregnancy

occurs, the functional lifespan of the corpus luteum is extended by choriogonadotropin acting through the same LH/CG receptor (3).

### **The LH Receptor**

These cellular responses of LH, FSH, and CG are mediated by their cognate receptor on the cell surface. The LHR and the FSHR belong to the G protein-coupled receptor family, the largest known receptor family in the human genome (7). These receptors consist of two halves of ~350 amino acids: the N-terminal half (exodomain) and the membrane-associated C-terminal half (endodomain) (7) (Figure 1.2). The exodomain consists of 10 exons and is mainly responsible for hormone binding (8, 9). The 11th exon encodes the endodomain which generates hormone signals (8, 9). The endodomain is equivalent in size to most of the other GPCRs which do not carry a long exodomain (7). The endodomain comprises seven transmembrane (TM) helices, three extracellular connecting loops (exoloops), three intracellular connecting loops (cytoloops), and a C-terminal tail (7).

The LHRs are expressed in the Leydig cells in males, and in the theca cells and the mature granulosa cells in females. The expression of LHRs in the later developmental stage of the granulosa cells requires the continual presence of FSH (10, 11), and this induction of LHR by FSH is mediated by cAMP (12, 13).

A study of hCG binding to ovarian receptors showed the Hill coefficient of 0.9 for LH and 1.1 for hCG, suggesting binding of 1 mol of gonadotropin/mol of receptor (14). The number of LHRs expressed in Leydig cells or in ovarian granulosa cells are in the range of 6,000 – 20,000 receptor/cell (15-17). However, studies suggest that only 1-3% of receptor occupancy is enough to induce maximal steroidogenesis in Leydig cells and granulosa cells (15, 18), suggesting the most likely function of excess receptors is to take up and clean circulating gonadotropins.

## Hormone Binding and Receptor Activation

Exodomain of the LHR binds to hCG with high affinity (9, 19). Many groups have revealed important regions on the LHR for hormone binding. It is now known that there are three important regions in the exodomain of the LHR for hormone binding: the N-terminal cysteine-rich region, the LRR region, and the C-terminal cysteine-rich area (the hinge region) (Figure 1.3). A study using synthetic peptides identified four hormone binding regions of the LHR: Arg<sup>21</sup>-Pro<sup>38</sup> (near the N-terminus), Arg<sup>102</sup>-Thr<sup>115</sup> (LRR 4), Tyr<sup>253</sup>-Phe<sup>272</sup> (hinge region), and Lys<sup>573</sup>-Lys<sup>583</sup> (exoloop 3) (20). Further study on the N-terminal region of the LHR shows that crucial amino acids in the region for hormone binding are Leu<sup>20</sup>, Cys<sup>22</sup>, and Gly<sup>24</sup> (21). It was also demonstrated that both the  $\alpha$  and  $\beta$  subunits of hCG contact the N-terminal region of the LHR (22).

The Leu-rich repeat (LRR) region, crucial for hormone binding, is located in the center of the exodomain and has nine repeats, each of which are 20-28 amino acids long. Based on the crystal structure of LRR proteins (23), up to 15 LRRs form a non globular horseshoe-shaped structure with an inner lining of curved  $\beta$  strands and an outer lining of helices (24). The LRR 1-6 of the LHR were shown to be important for hormone binding (25), and our group further identified that the upstream LRR 2 and 4 and downstream LRR 7 and 8 were shown also important for hormone binding (26, 27). Affinity labeling study of the LRR 4 of the LHR showed that the N-terminal region of LRR 4 interacts with hCG, preferentially the hCG  $\alpha$  subunit, and that hCG/LRR 4 complex interacts with exoloop 2 of the endodomain (28).

In addition to the N-terminal and the LRR regions, the hinge region (Thr<sup>250</sup>-Gln<sup>268</sup>) also binds to hCG with affinity, preferentially to hCG $\alpha$  (29). The interaction between hormone and the hinge region is inhibited by exoloop 2 of the endodomain but not by exoloops 1 and 3, suggesting an intimate relationship between Thr<sup>250</sup>-Gln<sup>268</sup>, exoloop 2, and hCG (29). This hinge region is elucidated not only important for hormone binding but also for signal generation. When S<sup>255</sup> was mutated to Ala, the mutant receptor showed 4-7 fold increased basal cAMP (30).

Hormone binding occurs mainly in the exodomain of the LHR with high affinity, but there is evidence that hormone also contacts to the endodomain with low affinity and modulates hormone signals. The N-terminal exodomain-truncated LHR binds to hCG with low affinity and produces cAMP at high hormone concentrations (9, 31), suggesting that there is a secondary contact of hormone/exodomain complex to the endodomain upon hormone binding, thus generating hormone signals from the endodomain of the receptor. The inhibition of hormone binding by a synthetic peptide of the exoloop 3 (Lys<sup>573</sup>-Lys<sup>583</sup>) suggests that exoloop 3 is one of the multiple hormone contact sites in the endodomain of the LHR (20). Our group showed the first physical evidence of hormone binding to the endodomain with a photoaffinity labeling of FSH by the exoloop 3 peptide of the FSHR (32).

Hormone binding to the exodomain is also constrained by the endodomain of the LHR. Endodomain-truncated LHR showed twice as high hormone binding affinity (33). Site-directed mutagenesis studies suggest that mutations in the exoloops 2 and 3 modulate hormone binding to the exodomain (33, 34). There are also many mutations located in the TM region abolishing hormone binding (35). Our group demonstrated that hormone binding to the hinge region (Thr<sup>250</sup>-Gln<sup>268</sup>) is inhibited by the exoloop 2 peptide (29). Hsueh's group showed that when Ser<sup>255</sup> in the hinge region is mutated, the LHR shows constitutive activity (30). When the hinge region including Ser<sup>255</sup> and the exoloop 2 of the fly LGR was replaced by the human LHR counterpart, the chimeric receptor had reduced constitutive activity (36), demonstrating that the hinge region and the exoloop 2 of the human LHR keep the receptor in an inactivate conformation and that the mutation in Ser<sup>255</sup> disrupts the conformation leading to constitutive activation (30). The interaction between the hinge region and the exoloop 2 was also demonstrated in the H3 relaxin receptor, another Leu-rich repeat-containing G protein-coupled receptor (37).

It has been shown that hormone binding to the LHR is constrained by the endodomain. There is evidence in the TSH receptor that the exodomain also constrains the endodomain activity. When the exodomain of the TSH receptor was removed, the endodomain of the TSH receptor showed 4-7 fold higher basal level activity in the absence of hormone (38). Serial N-terminal truncation of the TSH receptor showed that as the truncation proceeds towards Ser<sup>281</sup> (corresponding to

Ser<sup>255</sup> in the LHR), the truncated receptor showed increased constitutive activity in the absence of hormone (39). This constitutive activity of the endodomain was also assumed for the LHR, but there is evidence that the mechanism of receptor activation of the LHR is different from that of the TSH receptor. First, no constitutive activity of the LHR endodomain has been reported (31, 40, 41). Second, studies indicate that Gly<sup>109</sup> in the LRR 4 works as an activator for signal generation (27) and the exodomain itself is also required for the constitutive activity of LHR<sup>S255A</sup> (41).

Reconstitution of a functional LHR by coexpression of its split exodomain and endodomain shows evidence of sufficient interactions between the exodomain and the endodomain responsible for receptor activation and signal generation (40). The interaction between the exodomain and the endodomain were further investigated in this dissertation with defined-function LHR mutants (42, 43).

Taken together, hormone binding to the LHR leads to conformational changes of the hormone/exodomain complex and elicits hormone signals from the endodomain of the receptor (24). Hormone signals are considered to be generated from the hormone/receptor interface and the hormone/receptor conformational changes are thought to propagate through the TM to the cytoplasmic part of the receptor (24). When signals reach cytoloop 3 and the C-terminal tail, G protein  $\alpha$  subunit is activated and released from the  $\beta\gamma$  subunits, leading to the activation of effector molecules (24). The LHR activates adenylyl cyclase and phospholipase C $\beta$  to induce cAMP and inositol phosphate signaling, respectively (17). G<sub>s</sub>, upon release from the LHR, is responsible for the activation of adenylyl cyclase. G<sub>i2</sub> or  $\beta\gamma$  subunit released from G<sub>i2</sub> is known to activate phospholipase C $\beta$  (44). cAMP binds to regulatory subunits of the protein kinase A and activates intracellular signaling pathways, including mitogen-activated kinase pathways. Diacyl glycerol and IP<sub>3</sub> generated by phospholipase C $\beta$  also activates protein kinase C pathway and Ca<sup>2+</sup> mobilization in the cell, respectively. These molecular activations of signaling molecules invoke cellular responses mediated by the LHR.

## Mutations of the LHR

Many important findings on the structure-function relationship of the LHR have been revealed with the findings of mutations in patients or through mutagenesis studies. The discovery of LHR mutation Lys<sup>583</sup> to either Arg or Ala played an important role in understanding the relationship of hormone binding, signal generation, and signal divergence. Lys<sup>583</sup> is located in the junction of TM7 and exoloop 3. Exoloop 3 is the shortest exoloop, consisting of 11 amino acids (exoloop 1 has 21 amino acids, and exoloop 2 has 20 amino acids). When Lys<sup>583</sup> was mutated to Arg, the mutant LHR<sup>K583R</sup> maintained normal cell surface expression and hormone binding affinity but failed to produce cAMP and inositol phosphate signaling (45, 46). Though it was suggested a decade earlier that receptor activation is separable from hormone binding (9), LHR<sup>K583R</sup> is the first mutation found in the LHR which is crucial for receptor activation but not for hormone binding (45). Because of the short exoloop 3 size, mutation in the exoloop 3, including the LHR<sup>K583R</sup>, is considered to be more sensitive compared to mutations in exoloops 1 and 2.

When Lys<sup>583</sup> was mutated to Ala, the mutant receptor did not produce cAMP as LHR<sup>K583R</sup> did not, but it produced inositol phosphate signaling similar to wild type LHR (46). This suggests that there are two distinct transmembrane signal conductors for cAMP and inositol phosphate signals in the LHR (46). The selectivity of hormone signals by the mutation of Lys<sup>583</sup> to Arg or Ala further suggests that hormone signals generated from conformational changes in the hormone/exodomain complex are diverged in the exoloop areas.

Mutations in the LHR or the FSHR cause disorders related to reproduction and secondary sexual characteristics including infertility and precocious puberty (35, 47). 15-16 percent of the human population are infertile, affecting 80 million people worldwide with 5 million in the United States alone (48, 49). While there are other factors affecting infertility, mutations in the LHR and FSHR cause infertility in men and women, and mutations in the LHR have been found more frequently than those in FSHRs. Inactivating mutations of the LHR cause Leydig cell hypoplasia with complete female external genitalia in the male, whereas they cause menstrual disorders, cystic ovaries, and infertility in the females. Activating mutations of the LHR have only been found in males causing precocious puberty with onset at the age of 3 to 5, thus termed male-limited

precocious puberty. In females, however, activating mutations of the LHRs are asymptomatic and do not cause any phenotypes.

Inactivating mutations are found both in the exo- and endodomain of the LHR, whereas activating mutations are mainly found in the endodomain (35). Several inactivating mutations were found in the exodomain, including an insertion of 11 amino acids in the N-terminal region, deletion of the exon 8, and point mutations at Cys<sup>133</sup> and Asn<sup>291</sup> (35, 50). These do not result in production of cAMP due to their failure to bind hormone. Inactivating mutations in the endodomain mostly located in the TM5 to 7 also prevent hormone binding (35). There is no direct evidence yet as to how TM mutations affect hormone binding, but these endodomain mutations further support the evidence that hormone binding is constrained by the endodomain (33, 34). Most inactivating mutations are either from non-hormone binding or from reduced cell surface expression, and there is no report yet on a clinical mutation with normal hormone binding affinity but with impaired signaling like that of LHR<sup>K583R</sup>.

Activating LHR mutations were found in every TM except TM3 and 7, with the majority of activating mutations located in TM6, known as the “hot spot” of activating LHR mutations (Figure 1.6). Upon ligand binding to the rhodopsin and the  $\beta_2$ -adrenergic receptors, there is a movement of the cytoplasmic end of TM6 away from TM3 leading to conformational changes in TMs and cytoloops (51). Mutations in the TM6 hot spot area of the LHR cause constitutive receptor activation, not only by the disruption of interhelical bonds stabilizing the inactivate state, but also by the formation of new bonds that can stabilize an active state of the receptor (52). It was shown that the Asp<sup>578</sup>Gly mutation, the most frequently found activating LHR mutation, serves as a hydrogen bond acceptor. It is this, rather than a hydrogen bond donor, that is important for stabilizing the inactivate state of the LHR (53).

Each human cell contains two copies of each chromosome, one inherited from the mother and one from the father, thus called homologous chromosomes. Thus, even if there is a mutation in a gene in one allele, the other allele of the same gene can complement the function of the mutated allele. However, in some patients both copies of a gene were found to be mutated. Three cases of mutations of the LHR gene in both alleles have been reported (54-56). The finding of these



compound heterozygotes provided the grounds of this dissertation to study the relationship of two defective heterozygous LHRs, especially the relationship between the exodomain of one receptor and the endodomain of the other receptor. It is of particular interest because some patients may have compound heterozygous LHR mutations, but the patients may not be infertile due to successful rescuing of signaling by complementation of two defective functions: LHR<sup>-hCG</sup> and LHR<sup>+hCG/-cAMP</sup> (Figure 1.8). The LHR gene is located on the chromosome 2 spanning 2p21-26 (57).

The first compound heterozygote of an LHR was reported from a patient with Leydig cell hypoplasia (54). The patient had three mutations: deletion of exon 8 (exodomain, LHR) in one allele and Asn<sup>291</sup>Ser (exodomain) and Ser<sup>616</sup>Tyr (TM7) in the other allele. Hormone binding assays with the exon 8 deletion showed failure of cell surface expression of the receptor. Transfection studies with the Asn<sup>291</sup>Ser/Ser<sup>616</sup>Tyr double mutations showed reduced cell surface expression and signal generation. Another case of compound heterozygous mutation was found in a family with two 46, XY children affected with Leydig cell hypoplasia (55). The first mutation, Cys<sup>545</sup>Stop in the TM5, had impaired cell surface expression and cAMP generation, and the second mutation, insertion of 11 amino acids in the exon 1, had reduced cAMP induction. The third case of compound heterozygote resulting in complete Leydig cell hypoplasia was reported with two mutations of Cys<sup>343</sup>Ser in the hinge region of the exodomain and Cys<sup>543</sup>Arg in the TM5 (56). Both mutant LHRs had normal protein level in the cytoplasm by Western blotting study, but cell surface expression of the mutant receptors was completely impaired, implicating that translocation is halted at the endoplasmic reticulum level blocking further processing to the cell surface. In addition to the LHR mutations, three cases of compound heterozygous FSHR mutations have been reported (58-60).

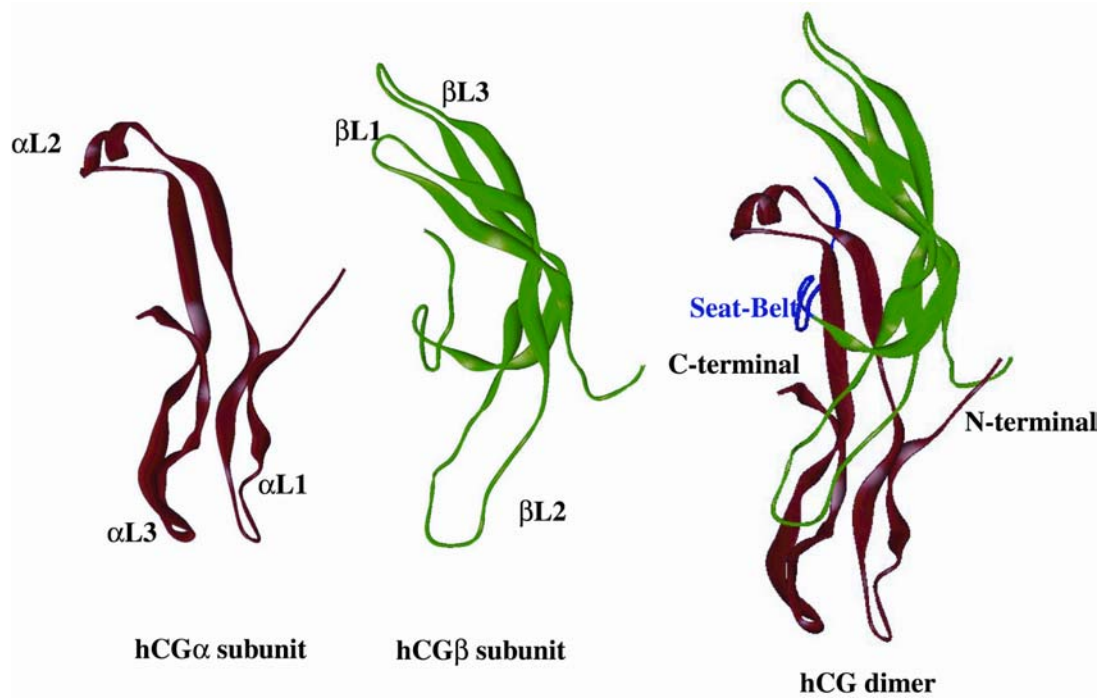
### **Goal of Dissertation**

This dissertation has three goals. The first goal is to elucidate whether the LHR, upon hormone binding, activates other unoccupied LHRs inter-molecularly for the rescue of cAMP signaling (trans-activation) (Figure 1.8). This is particularly important considering that there are patients

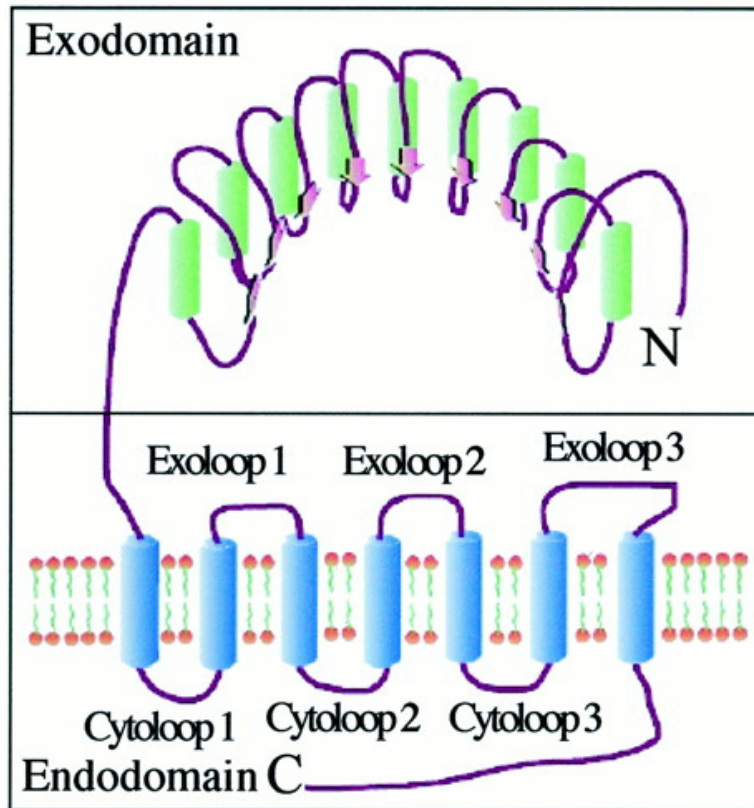
with compound heterozygotes of LHRs (54-56) or FSHRs (58-60), and the relationship between the compound heterozygous LHR or FSHR is unknown.

Second, I want to find whether or not the LHR also activates other unoccupied LHRs for inositol phosphate signaling as well as cAMP signaling. This is of interest because the LHR activates both cAMP and inositol phosphate signaling, but the activation requires different hormone concentrations and mechanisms.

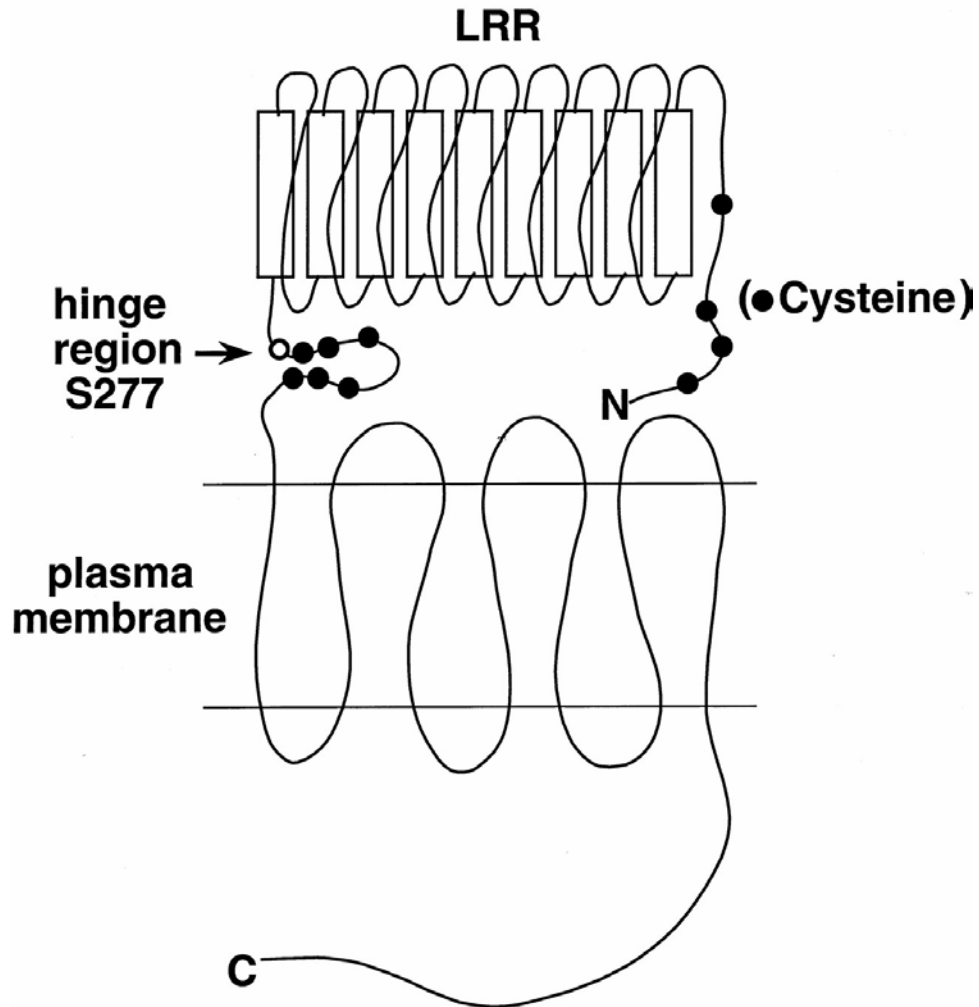
Finally, this dissertation will discuss whether or not a single LHR activates both  $G_s$  and  $G_{i2}$  molecules simultaneously or individually. Although the LHR generates both cAMP and inositol phosphate signaling, their temporal relationship has not yet been studied.



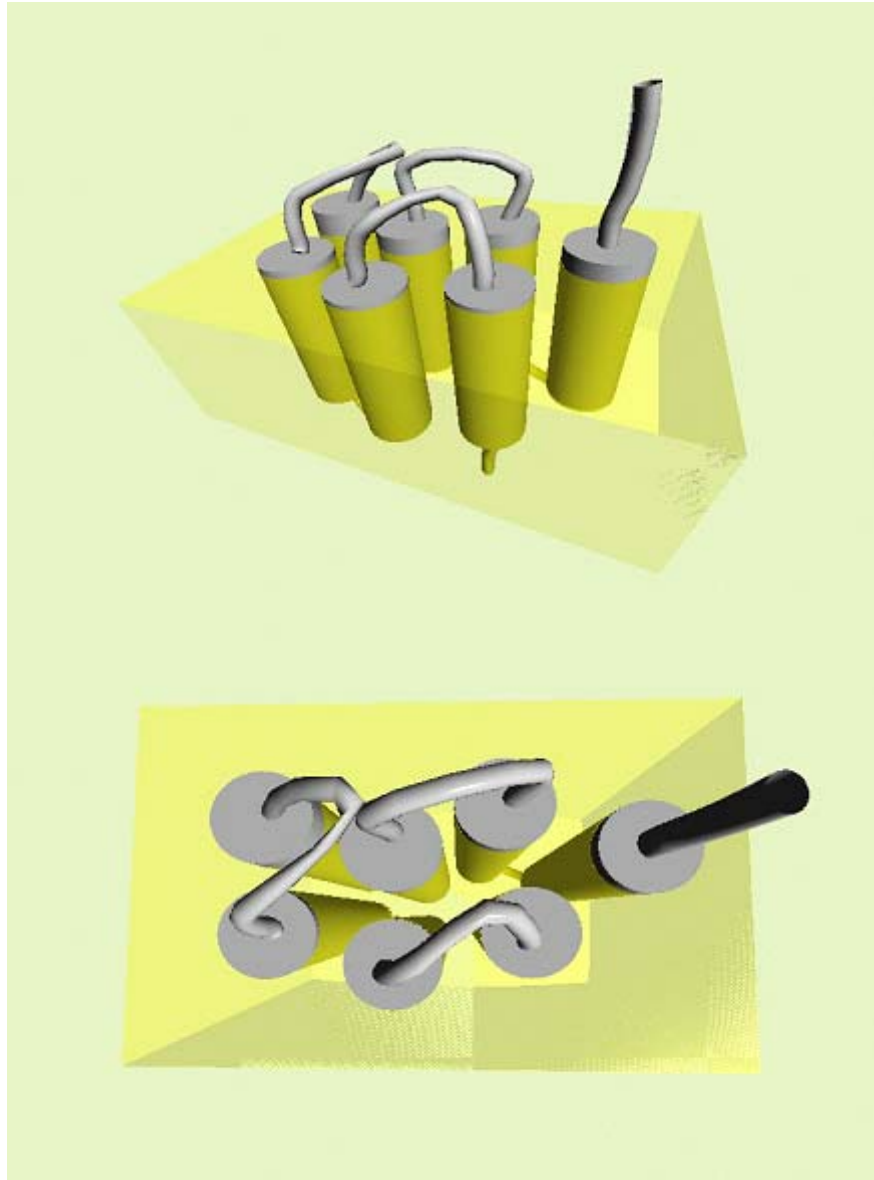
**Figure 1.1 Crystal structure of hCG.** hCG  $\alpha$  (92 amino acids) is shown in red, and hCG  $\beta$  (145 amino acids) in green. The seat belt region of hCG  $\beta$  is shown in blue, wrapping around the  $\alpha$  subunit stabilizing the heterodimers.



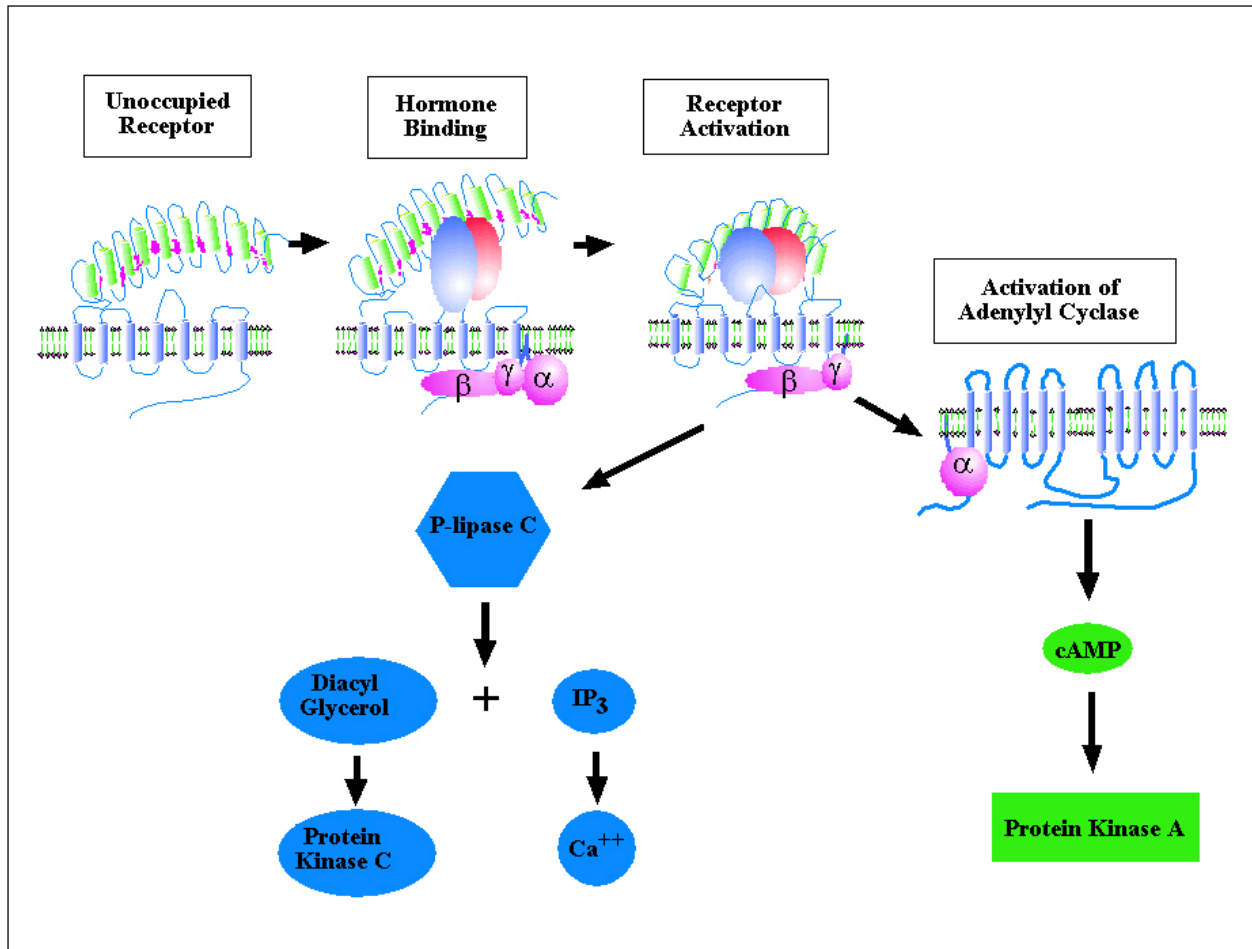
**Figure 1.2 Schematic diagram of the LHR.** The N-terminal exodomain consists of ~350 amino acids and binds to hormone with high affinity. An equal-sized endodomain is membrane-associated and is responsible for signal generation.



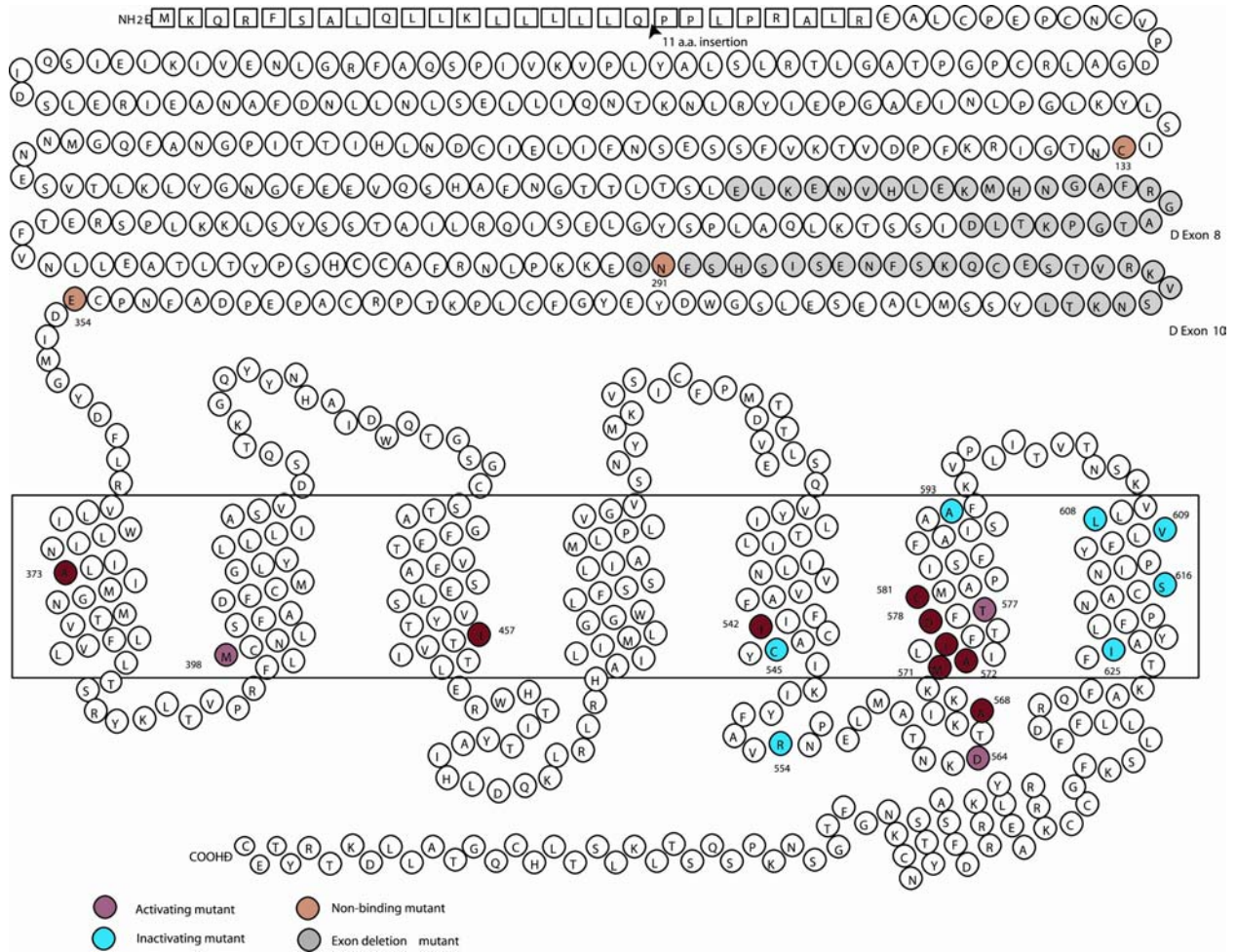
**Figure 1.3 Important regions of the LHR for hormone binding.** Hormone binds to the N-terminal cysteine-rich region, Leu-rich repeat (LRR) region, and the C-terminal hinge region. Adapted from Nakabayashi *et al.* (2000) *J Biol Chem* 275, 30264-71 (30). S<sup>277</sup> in the human LHR corresponds to S<sup>255</sup> in the rat LHR.



**Figure 1.4 Side and top view of the LHR endodomain.** TMs are located counterclockwise and connected by exoloops and cytoloops. Cytoloops are not shown in the figure.

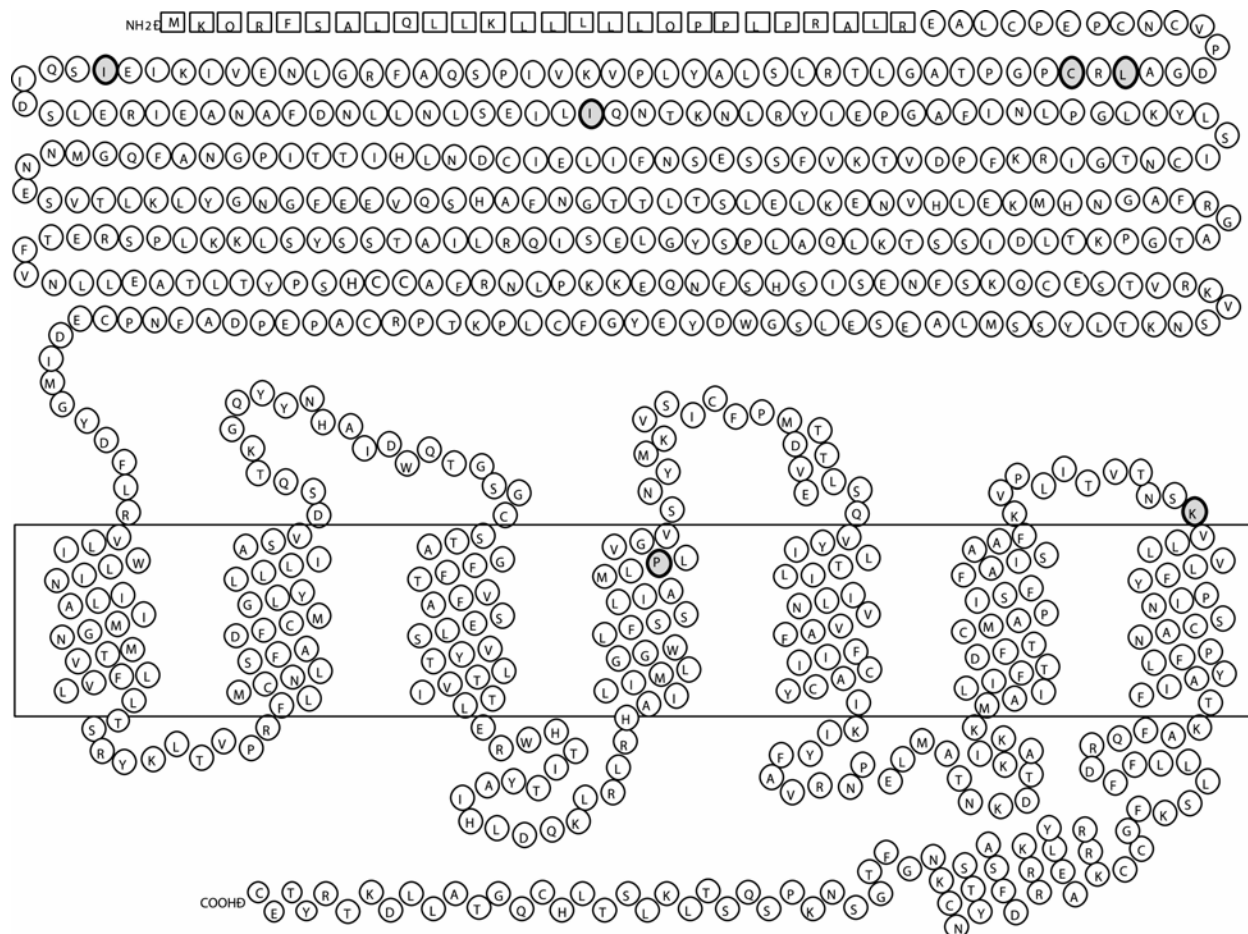


**Figure 1.5 Receptor activation and signal generation of the LHR.** Hormone binding to the exodomain undergoes conformational changes in the hormone/exodomain complex and modulates the endodomain to generate signals. The LHR activates adenylyl cyclase and phospholipase C $\beta$  to induce cAMP and inositol phosphates, respectively.

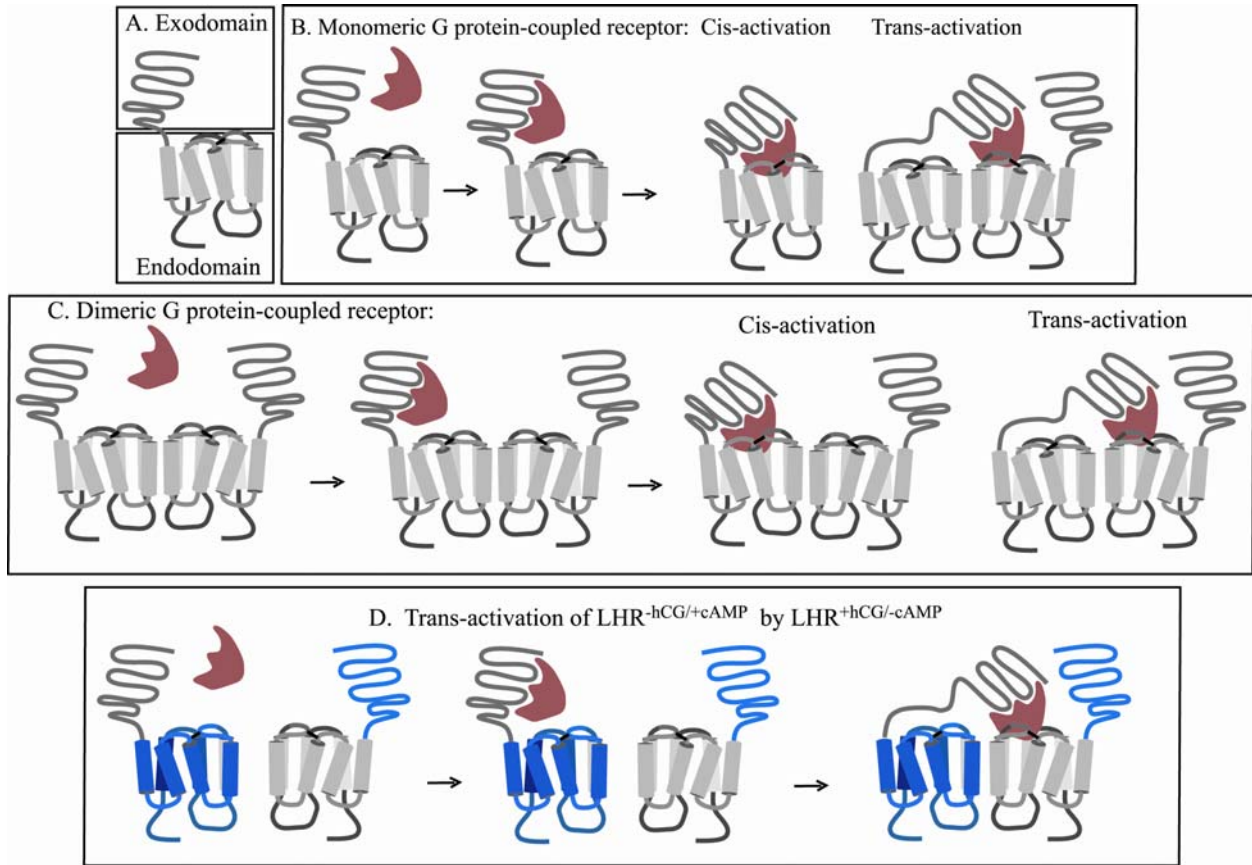


**Figure 1.6 Mutations of the human LHR.** Amino acids relevant for hormone binding, inactivating, and activating mutations are denoted with different colors. Due to a different numbering custom between human and rat LHR, subtract 22 to find the corresponding amino acid in the rat LHR from the above sequence.





**Figure 1.7 Defined-function mutants in the LHR used in this dissertation to access receptor-receptor interactions.** L<sup>20</sup>A, C<sup>22</sup>A, I<sup>55</sup>A, and I<sup>80</sup>A (LHR<sup>-hCG</sup>) are located in the exodomain. P<sup>479</sup>A and P<sup>479</sup>G (LHR<sup>-hCG</sup>) are located in the TM4. K<sup>583</sup>R is located in the junction of exoloop 3 and the TM7 (LHR<sup>+hCG/-cAMP</sup>).



**Figure 1.8 Cis-activation and trans-activation of LHRs.** **A.** Domain structure of LHR showing the exodomain where the ligand binds and the endodomain where the hormone signal is generated. **B.** Cis- and trans-activation of monomeric LHRs. The ligand is shown in red. **C.** Cis- and trans-activation of dimeric LHRs. **D.** Trans-activation of a mutant LHR that is defective in ligand binding in the exodomain (blue) but is capable of generating a hormone signal in the endodomain (gray) (LHR<sup>-hCG/+cAMP</sup>) by a mutant LHR that is capable of binding ligand in the exodomain (gray) but incapable of generating a hormone signal in the endodomain (blue) (LHR<sup>+hCG/-cAMP</sup>).

## **Chapter 2 Trans-activation of the LHR for cAMP Signaling**

### **Introduction**

The luteinizing hormone receptor (LHR) plays a crucial role in the development of the gonads in both sexes and ovulation in females. Defective mutations of the receptor often cause infertility (61). Gain of function mutations are generally dominant, whereas loss of function mutations are recessive. The genetic prediction of mutations is not straightforward, because the effects of some mutations are partial and some patients are defective heterozygotes. For example, there are patients with two defective heterozygous LHR mutations (54-56) and the precise relationship of two mutant receptors in a patient is unclear. This is particularly relevant for LHR, which has two distinct domains, one for hormone binding and the other for signal generation (7, 62, 63).

We wondered about the relationship between the two alleles as to whether they would be dependent on or independent of each other. Particularly, there is the intriguing possibility that two heterozygous mutants, one defective in hormone binding and the other with normal hormone binding but defective signal generation, might interact with each other to rescue hormone action (Figure 1.8). Obviously, this would require the novel intermolecular interaction of the exodomain of one LHR with the endodomain of another LHR. Although it has never been described, it would have significant impact on the interpretation of receptor genetics and provide new insights into clinical treatments. To test this hypothesis, various heterozygous defective LHRs were paired and tested for their functional rescue were coexpressed.

### **Materials and Methods**

#### **Mutagenesis**

Mutant rat LHR cDNAs were prepared in a pSELECT vector using the non-polymerase chain reaction-based Altered Sites Mutagenesis System (Promega). The multiple cloning site of the vector was cut with XbaI and BamHI, and then uni-directionally ligated with full-length rat LHR cDNA. Single strand DNA was prepared, and mutagenesis was performed according to the

manufacturer's instruction. Oligonucleotides were purchased from Biosource. Vectors containing mutant LHR constructs were selected by ampicillin screening. After confirming mutations by sequencing, the mutant cDNAs were subcloned into the BamHI and XbaI sites of pcDNA3/neomycin (Invitrogen), a eukaryotic expression vector.

### **Plasmid Purification**

Wild type or mutant LHR cDNAs prepared through mutagenesis was transformed into TOP10 competent cells (Invitrogen) using Gene Pulser II electroporator (Bio-Rad) and plated onto LB/ampicillin (125 µg/ml) plates. LHR plasmid DNAs were purified by CsCl/ethidium bromide equilibrium centrifugation or by large-scale plasmid kits (QIAGEN and Bio-Rad).

### **Transfection**

Varying concentrations of plasmids were transfected into human embryonic kidney (HEK) 293 cells by the calcium phosphate method (64). Briefly, 6-24 µg of plasmid DNA was added into 1.7 ml tube containing 250 µl of 0.25 M CaCl<sub>2</sub> followed by 250 µl of 2X BES. The mixture was vortexed shortly, incubated for 8-12 min at room temperature, and transferred into T<sub>25</sub> flasks (Corning) of cultured cells drop by drop. Transiently transfected cells were assayed 60-72 h after transfection. Stable cell lines were established in minimum essential medium (MEM) containing 8% horse serum and 500 µg/ml G-418. All assays were carried out in duplicate and repeated three to four times (n = 6-8). Means and standard deviations were calculated.

### **cAMP Assay**

Cells were washed twice with MEM and incubated in the medium containing isobutylmethylxanthine (0.1 mg/ml) for 15 min. Increasing concentrations of hCG were then added, and the incubation was continued for 45 min at 37 °C. After the medium was removed, the cells were rinsed once with fresh medium without isobutylmethylxanthine, lysed in 70% ethanol, freeze-thawed in liquid nitrogen, and scraped. After pelleting cell debris at 16,000 × g for 10 min at 4 °C, the supernatant was collected, dried under vacuum, and resuspended in 10 µl

of the cAMP assay buffer provided by the  $^{125}\text{I}$ -cAMP assay kit (Amersham Biosciences). cAMP concentrations were determined following the manufacturer's instruction and validated for use in our laboratory.

### **Iodination of Hormones and Antibodies**

hCG (CR-127) and human FSH (AFP-7298A), provided by the National Hormone and Pituitary Program, were radio-iodinated as described previously (65). Briefly, 50  $\mu\text{l}$  of phosphate-buffered saline (pH 7.4) was added to 3 to 8  $\mu\text{g}$  of hCG. To this solution were added 7  $\mu\text{l}$  of chloramine-T (0.33 mg/ml) and 1 mCi (1 Ci =  $3.7 \times 10^{10}$  becquerels) of sodium [ $^{125}\text{I}$ ]iodide in 10  $\mu\text{l}$  of 0.1 M NaOH; 20 sec later, iodination was stopped by addition of 7  $\mu\text{l}$  of sodium metabisulfite (0.66 mg/ml). The iodinated hCG ( $^{125}\text{I}$ -hCG) was immediately fractionated on a Sephadex G-50 (superfine) column (0.6  $\times$  15 cm). Radio-activity of each fraction was measured in a gamma counter (Beckman) and the fraction of highest radio-activity was kept at 4  $^{\circ}\text{C}$  for hormone binding assays. Specific activity of  $^{125}\text{I}$ -hCG was in the range of 60,000 - 80,000 cpm/ng of hCG.

### **Hormone Binding Assay**

Cells were washed twice with medium, MEM with 20 mM of HEPES, pH 7.4 and 0.1 g/100 ml of bovine serum albumin. Cells were assayed for  $^{125}\text{I}$ -hormone (150,000 cpm) binding in the presence of increasing concentrations of non-radioactive hormone for 90 min at 37  $^{\circ}\text{C}$ . Cells were washed twice with medium, solubilized with 1 N NaOH, and collected in glass culture tube followed by measurement in a gamma counter (PerkinElmer) for 10 min. Non-specific binding (usually less than 1 % of specific binding) was determined by replacing  $^{125}\text{I}$ -hormone with excessively higher concentrations of hormone.  $K_d$  values and number of receptors were determined by Scatchard plots.

### **Radioimmunoassay for Flag-LHR**

Flag-LHR was prepared by inserting the Flag epitope, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (5-GAC TAC AAG GAC GAT GAC GAT AAG-3), between the C terminus of the signal sequence

and the N terminus of mature receptors. Mouse anti-Flag monoclonal M2 antibody (Sigma) was iodinated with  $^{125}\text{I}$  according to the published procedure for radio-iodination of hCG (65), and  $^{125}\text{I}$ -anti-Flag antibodies were purified on a Sephadex G-150 column. Binding of  $^{125}\text{I}$ -anti-Flag (150,000 cpm) to HEK 293 cells expressing Flag-LHRs was carried out in the presence of increasing concentrations of non-radioactive anti-Flag antibody in MEM containing 3 mg/ml of bovine serum albumin for 8-10 h at 4 °C.  $^{125}\text{I}$ -anti-Flag antibody (65) was used to determine the concentrations of receptors incapable of binding the hormone, in comparison with  $^{125}\text{I}$ -hCG used to determine the concentration of receptors capable of binding the hormone.

## Results

To investigate the interaction of heterozygous mutant LHRs, we chose the K<sup>583</sup>R mutant (LHR<sup>K583R</sup>) in which Lys<sup>583</sup> was substituted with Arg. This mutant receptor is normally processed and targeted to the cell surface and is capable of binding hCG but incapable of inducing cAMP production (45). The mutant receptor is referred to as LHR<sup>+hCG/-cAMP</sup>. In addition to the LHR<sup>+hCG/-cAMP</sup>, other mutant LHRs were selected that were expressed on the cell surface but were incapable of binding hCG (LHR<sup>-hCG</sup>). They are L<sup>20</sup>A, C<sup>22</sup>A, P<sup>479</sup>A, and P<sup>479</sup>G mutants (21, 66). HEK 293 cells transiently transfected with the LHR<sup>K583R</sup> plasmid showed hCG binding with the wild type affinity but did not produce cAMP in response to increasing doses of hCG (Figure 2.1). Cells transiently transfected with the plasmid for LHR<sup>L20A</sup>, LHR<sup>C22A</sup> LHR<sup>P479A</sup>, or LHR<sup>P479G</sup> did not show hCG binding or cAMP induction, consistent with previous reports (21, 66). The cells transfected with the blank plasmid, pcDNA3, failed to bind hCG and produce cAMP, indicating that the vector itself was not involved in hCG binding.

Next, cells were co-transfected with a pair of LHR<sup>+hCG/-cAMP</sup> and LHR<sup>-hCG</sup>, for example, K<sup>583</sup>R and L<sup>20</sup>A mutants or K<sup>583</sup>R and C<sup>22</sup>A mutants. The cells that were co-transfected with either LHR<sup>K583R</sup> and LHR<sup>L20A</sup> or LHR<sup>K583R</sup> and LHR<sup>C22A</sup> were capable of binding hCG, and the  $K_d$  values were similar to the wild type value (Figure 2.2, A and B). In addition, these cells induced cAMP production in an hCG dose-dependent manner (Figure 2.2, C). The maximal levels of cAMP were approximately one-third of the wild type value, and their EC<sub>50</sub> values were 15-25-

fold higher than the wild type value (Figure 2.2, *table*), suggesting the rescue of cAMP induction with lower potency.

The data shown in Figure 2.2 suggest that the non-binding receptors were expressed on the cell surface, as rigorously demonstrated by different methods in previous reports (21, 66). However, to validate the surface expression, cells were transfected with the mutant receptors that carry the Flag epitope at the N terminus (Flag-LHR<sup>C22A</sup>) and assayed for binding of anti-Flag monoclonal antibody as described previously (21, 26). The cells were incubated with <sup>125</sup>I-anti-Flag monoclonal antibody with increasing concentrations of non-radioactive antibody. The cells showed specific binding of the <sup>125</sup>I-antibody, which was gradually displaced by non-radioactive antibody, as did the cells transfected with Flag-LHR<sup>WT</sup> (Table 2.2). However, there was no specific antibody binding to the cells transfected with the LHR<sup>WT</sup>, LHR<sup>C22A</sup>, or LHR<sup>L20A</sup> plasmid as previously reported (21). These results show that the Flag-LHRs were indeed expressed on the cell surface in these experiments. To determine whether Flag-LHR<sup>C22A</sup> can rescue cAMP induction, it was coexpressed with LHR<sup>K583R</sup>. As expected, the cells bound hCG and produced cAMP (Figure 2.3).

It is unclear whether cAMP was induced by accidental collisions between the endodomains of two different mutant receptors. To test this possibility, several pairs of two different LHR<sup>-hCG</sup> mutants were coexpressed. As shown in Figure 2.4, none of the coexpressed pairs (LHR<sup>C22A</sup> and LHR<sup>L20A</sup>, LHR<sup>C22A</sup> and LHR<sup>P479A</sup>, LHR<sup>C22A</sup> and LHR<sup>P479G</sup>, LHR<sup>L20A</sup> and LHR<sup>P479A</sup>, and LHR<sup>L20A</sup> and LHR<sup>P479G</sup>) was capable of inducing cAMP or binding hCG. These results show that one of the mutant pairs has to be capable of binding hCG to rescue cAMP induction.

To test the dependence of the rescue on hCG binding, cells were co-transfected with varying concentrations (6, 12, and 18 μg) of the LHR<sup>K583R</sup> plasmid and a constant amount (6 μg) of the LHR<sup>L20A</sup> or LHR<sup>C22A</sup> plasmid. The cells were assayed first for hCG binding to determine the relationship of the surface concentration of LHR<sup>K583R</sup> with the plasmid concentration (Figure 2.5). The results show that the surface concentration of LHR<sup>K583R</sup> increased in parallel to the plasmid concentration used for transfection. The range of the LHR<sup>K583R</sup> concentration was 5,000-21,000 receptors/cell, which compares favorably with the *in vivo* LHR concentration on porcine

granulosa cells and rat Leydig cells, several thousand per cell (15-17). In addition, the variation in the receptor concentration does not appear to impact the hormone binding affinity. However, the maximum cAMP levels show an interesting trend. When 6  $\mu\text{g}$  of the LHR<sup>L20A</sup> plasmid was co-transfected with 6, 12, or 18  $\mu\text{g}$  of the LHR<sup>K583R</sup> plasmid, the maximum cAMP levels were 52.4, 71.1, and 36.5 fmol/1000 cells, respectively. The differences among the three values are statistically significant with *p* values of <0.05 to <0.001. Therefore, the maximum cAMP level increased by 36% at 12  $\mu\text{g}$  and then decreased by 30% at 18  $\mu\text{g}$  as compared with the cAMP level at 6  $\mu\text{g}$  of the plasmid. The result was similar when 6  $\mu\text{g}$  of the LHR<sup>C22A</sup> plasmid was co-transfected with 6, 12, or 18  $\mu\text{g}$  of the LHR<sup>K583R</sup> plasmid. These observations suggest that the cAMP rescue requires LHR<sup>K583R</sup> and is dependent on the concentration of this mutant receptor.

One may question whether the surface expression levels of the LHR<sup>hCG</sup> mutants shown in Figure 2.5 were constant, although 6  $\mu\text{g}$  of the plasmids was used for transfection of the cells throughout the experiment. To address this problem, we took another approach to keep the expression level of LHR<sup>hCG</sup> mutants constant. Cell lines were established after stably transfecting them with the LHR<sup>L20A</sup> plasmid or LHR<sup>C22A</sup> plasmid. These cell lines were transfected again with varying concentrations (6, 12, and 18  $\mu\text{g}$ ) of the LHR<sup>K583R</sup> plasmid. The doubly transfected cells showed increasing concentrations of LHR<sup>K583R</sup> (Figure 2.6). Transfection with 12  $\mu\text{g}$  of the LHR<sup>K583R</sup> plasmid increased the maximum cAMP level by 29-58% over that of the cells transfected with 6  $\mu\text{g}$  of the plasmid. Transfection with 18  $\mu\text{g}$  of the plasmid resulted in a 3-fold increase in the EC<sub>50</sub> value for the cAMP rescue, although the maximum cAMP levels remained high. The observations described in Figures 2.6 and 2.7 indicate that the cAMP rescue is dependent on the LHR<sup>K583R</sup> concentration. However, there is a notable difference in the results of Figures 2.6 and 2.7. In Figure 2.5 the maximum levels of cAMP peaked as the LHR<sup>K583R</sup> concentration increased, whereas it plateaued in Figure 2.6. The difference in the two experiments was LHR<sup>hCG</sup>, which was transiently expressed in the Figure 2.5 experiment and stably expressed in the Figure 2.6 experiment. A molecule is expressed in stable cell lines generally more than in transiently expressing cells because of the associated antibiotic selection. Therefore, another experiment was performed using the stable cell line expressing Flag-LHR<sup>C22A</sup>, which appears to express less than 12,800 receptors/cell. It was transiently transfected with increasing concentrations of the LHR<sup>K583R</sup> plasmid from 3 to 18  $\mu\text{g}$ . The cells produced cAMP in response to hCG, and the



maximum cAMP levels peaked (Figure 2.3). These results taken together with the data shown in Figures 2.6 and 2.7 show that there are optimal concentrations of LHR<sup>K583R</sup> to pair with LHR<sup>-hCG</sup> and rescue cAMP induction. They indicate the importance of the number of the hCG binding receptor and/or the ratio of the hCG binding receptor to the non-binding receptor.

The non-binding receptors tested so far have mutations in the exodomain that impair hormone binding. In addition to these non-binding receptors with a defective exodomain, there are non-binding receptors that have a normal exodomain but mutation in the endodomain, such as P<sup>479</sup>A and P<sup>479</sup>G of the transmembrane helix 4 (66). These mutations in the endodomain block hCG binding to the exodomain by constraining the exodomain although the exodomain itself is intact (33, 34). To test whether these mutants could pair with LHR<sup>K583R</sup> and induce cAMP production, LHR<sup>K583R</sup> was coexpressed with LHR<sup>P479A</sup> or LHR<sup>P479A</sup>. The cells co-expressing LHR<sup>K583R</sup> and LHR<sup>P479A</sup> or LHR<sup>K583R</sup> and LHR<sup>P479A</sup> failed to induce cAMP production although they were capable of binding hCG (Figure 2.6). These results indicate that not all of the mutant pairs of LHR<sup>-hCG</sup> and LHR<sup>+hCG/-cAMP</sup> are capable of rescuing the hCG dependent cAMP induction, suggesting a specificity for pairing. Furthermore, these results suggest that LHR<sup>-hCG</sup> with a mutation in the exodomain, but not in the endodomain, could be rescued.

In addition, we tested the affect of another receptor species on the activity of wild type LHR. When LHR<sup>WT</sup> was coexpressed with FSHR<sup>WT</sup>, the functional FSHR did not impact the hCG binding affinity or the EC<sub>50</sub> value and maximum level of cAMP induction by LHR<sup>WT</sup> (Figure 2.8). These results show that the cAMP induction by LHR<sup>-hCG</sup> and LHR<sup>+hCG/-cAMP</sup> was not rescued by accidental collisions between them or with different hormone receptor species.

## Discussion

The observations described in this dissertation show that cells co-expressing a pair of two differently defective mutants, one defective in hCG binding at the exodomain (LHR<sup>-hCG</sup>) and the other defective in signal generation at the endodomain (LHR<sup>+hCG/-cAMP</sup>), can induce cAMP production. This successful rescue of cAMP induction requires both types of mutant receptors. However, not all LHR<sup>-hCG</sup> were capable of pairing with LHR<sup>K583R</sup>, an LHR<sup>+hCG/-cAMP</sup>, and

rescuing cAMP induction. Rescue is observed when hormone binding of an LHR<sup>-hCG</sup> is impaired by a mutation in the exodomain but not by mutations in the endodomain. These results suggest specificity for the rescue of cAMP induction. For example, the rescue is dependent on hCG dose, the surface concentration of the mutant receptors, and the amino acid positions of the mutations. Furthermore, random collisions among mutant receptors are not involved in the rescue.

It is known that LHR binds hCG first at the exodomain (9, 19, 67), and the resulting hCG-exodomain complex undergoes conformational changes (68-70) and modulates the endodomain (29, 30). This secondary interaction is responsible for signal generation and receptor activation (7, 62, 63). Based on these observations and the results described in this dissertation, the cooperation between the two types of mutant LHRs includes the exodomain of LHR<sup>K583R</sup> and the endodomain of LHR<sup>L20A</sup> or LHR<sup>C22A</sup>. Furthermore, the two domains most likely interact with each other. Therefore, the results suggest an intermolecular interaction (trans-activation) between the exodomain of one receptor and the endodomain of another receptor and implicate at least partial substitution of the hCG-functional exodomain complex of a receptor for the defective exodomain of another receptor. This is appealing if LHR form dimmers. In a dimeric situation, a liganded LHR could cis-activate itself and then, trans-activate its partner LHR in the dimer. However, the existing crosslinking and immunological evidence do not support oligomerization of LHRs.

The intermolecular exodomain-endodomain interaction is also consistent with the dependence of the rescue on receptor concentrations and the existence of optimal concentrations. The observation that too few or too many LHR<sup>+hCG/-cAMP</sup> can interfere with the collaboration between LHR<sup>+hCG/-cAMP</sup> and LHR<sup>-hCG</sup> is of interest and reminiscent of the antibody and antigen interaction. Too many LHR<sup>+hCG/-cAMP</sup> would nonproductively compete for a limited number of LHR<sup>-hCG</sup>, which could lead to less effective induction of cAMP.

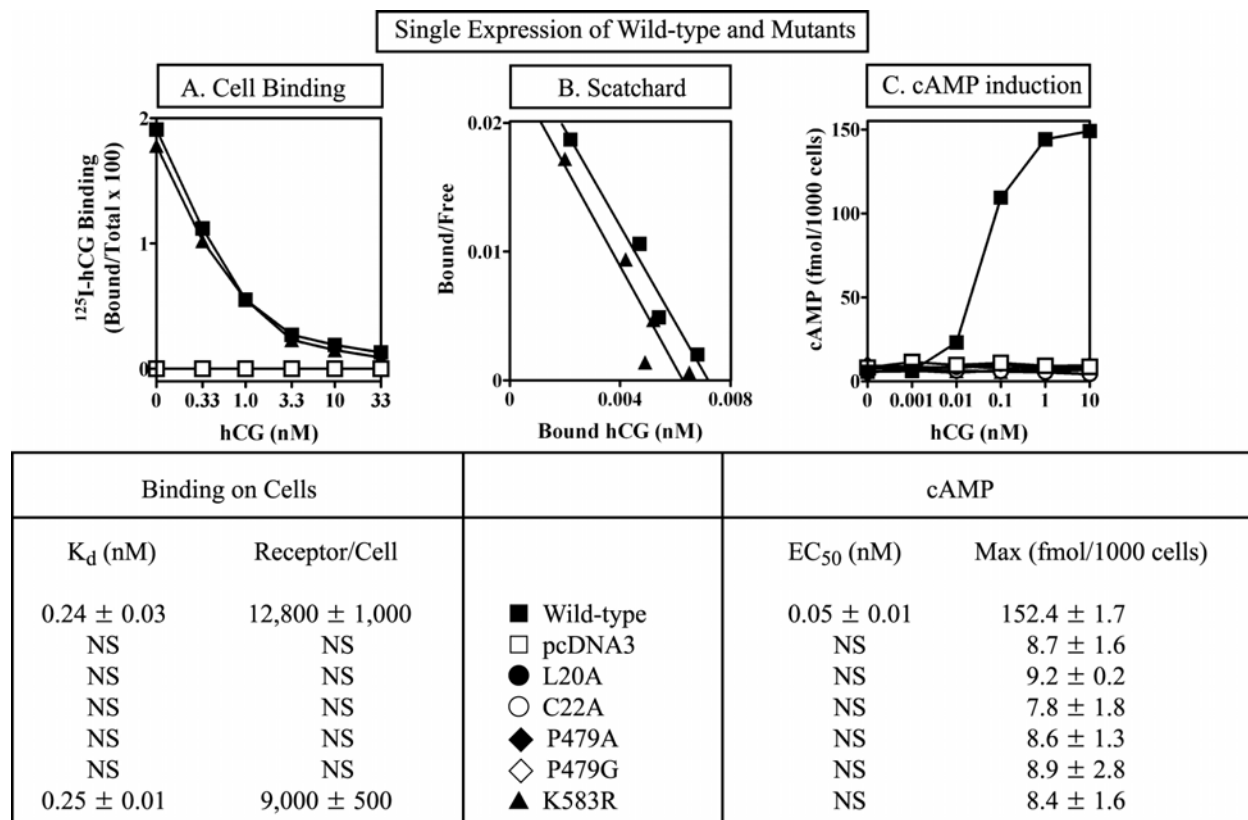
Previous studies on the dose-response relationship between hCG binding and hCG-stimulated cAMP and steroidogenesis indicate that cellular responses, such as testosterone synthesis in Leydig cells, reach their maximum at very low hormone concentrations and had no effect upon cAMP production (15, 71, 72). Hormone occupancy of only 1-3% of the receptor sites was enough to induce maximum steroidogenesis, and specific hormone binding was undetectable at

these low hormone concentrations (1-2 pM) (15). The  $EC_{50}$  of cAMP induction (50 pM) was ten times lower than the  $EC_{50}$  of hCG bound (500-600 pM) (15). The presence of these > 97% “unbound” receptors are referred to as "spare receptors". The term does not imply that these receptors are not utilized; rather, a maximum biological response is achieved when 1-3% of receptors are occupied. The importance of these spare receptors is that they compensate for low concentrations of hormone in physiological conditions and remove excess hormones soon after hormone action, a necessary step. When the spare receptor number decreases, the  $EC_{50}$  for cAMP induction increases, requiring more hormone for the same maximal cAMP induction (73). The difference of hCG concentrations required for half-maximal cAMP production and half-maximal occupancy of receptor sites, along with the absence of direct evidence of increased adenylyl cyclase activity during stimulation of steroidogenesis by low hCG concentrations have raised the possibility that other membrane-associated responses may be involved during the initial phase of gonadotropin-induced steroidogenesis in the Leydig cell (15, 74). The data from this dissertation suggests the intriguing possibility of intermolecular trans-activation of other unoccupied LHRs by a liganded LHR, in addition to intramolecular cis-activation of its own endodomain. It is tempting to speculate that pleiotropic trans-activation of the LHRs might lead to maximum steroidogenesis with hormone occupancy on 1% of the receptor sites.

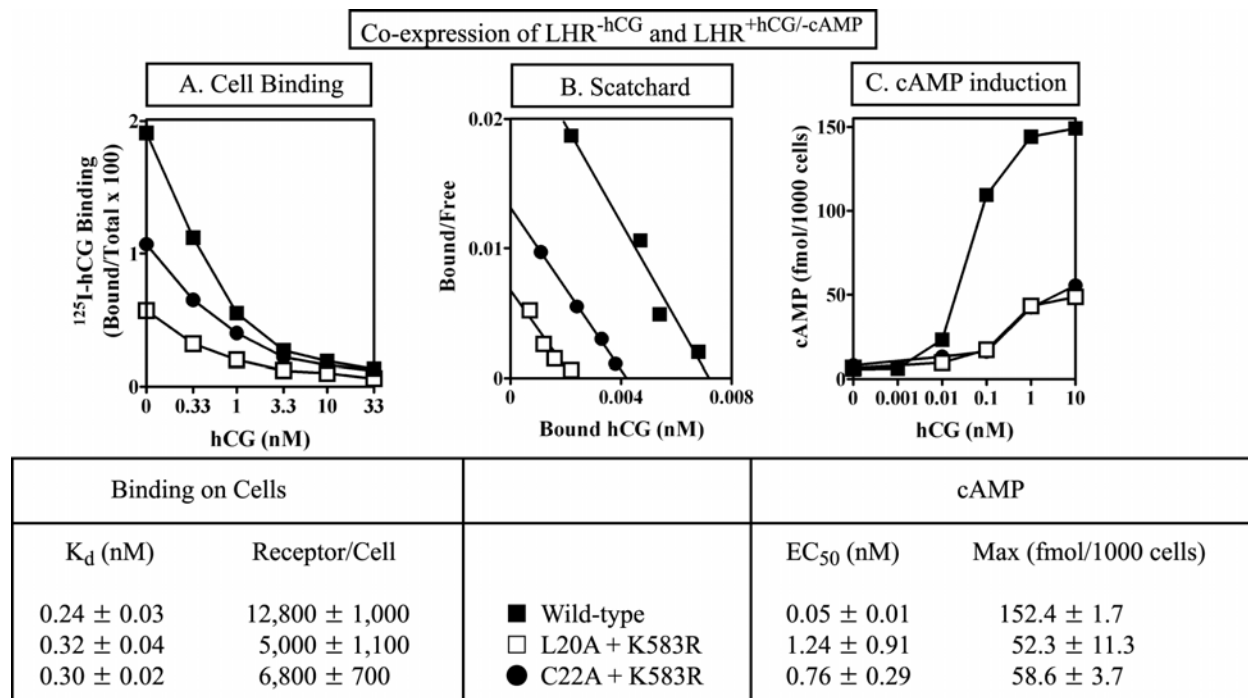
**Table 2.1 Surface expression of Flag-LHRs**

Cells were transfected with various Flag-LHR plasmids and assayed for  $^{125}\text{I}$ -monoclonal anti Flag-antibody binding in the presence of increasing concentrations of unlabeled antibody as described under "Materials and Methods." Specific antibody bindings were determined and compared with the specific binding of the Flag-LHR<sup>WT</sup>.

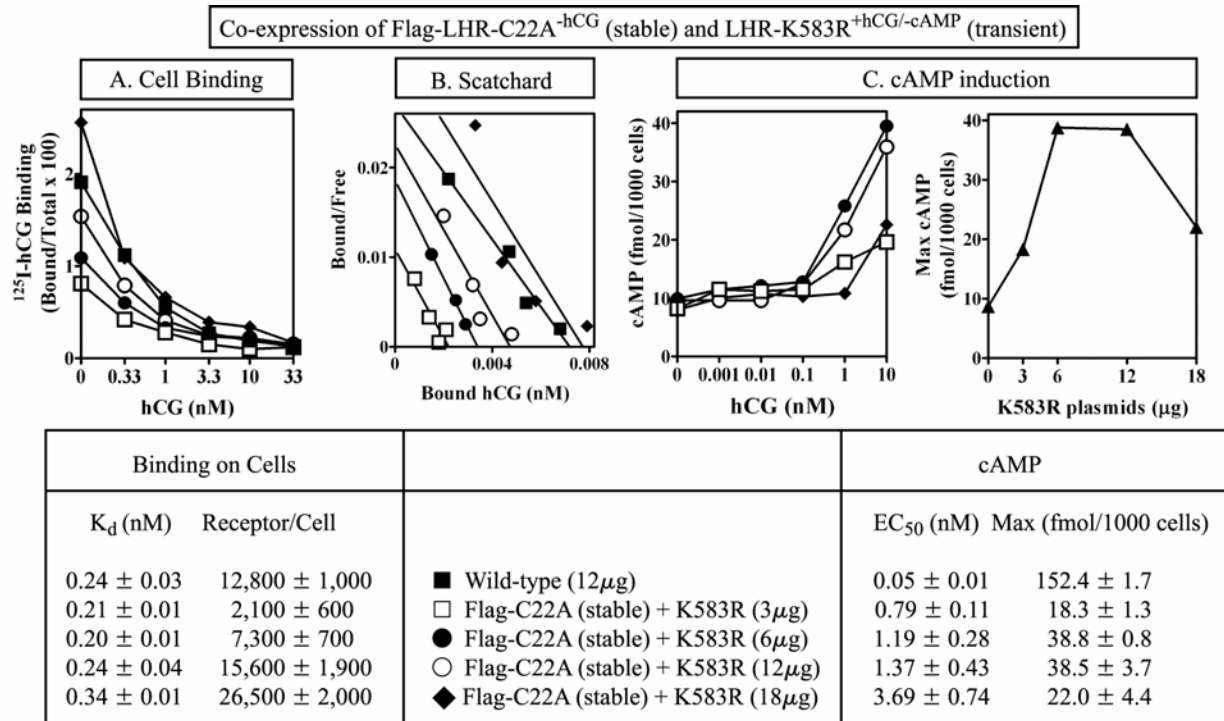
	$^{125}\text{I}$ -anti-Flag mAb
	Binding (%)
LHR <sup>WT</sup>	5 ± 3
Flag-LHR <sup>WT</sup>	100 ± 9
Flag-LHR <sup>L20A</sup>	91 ± 17
Flag-LHR <sup>C22A</sup>	60 ± 15



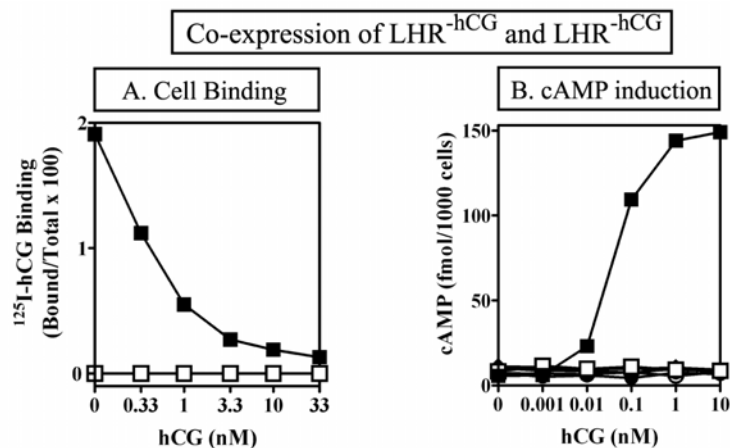
**Figure 2.1 Activity of mutant receptors.** HEK 293 cells were transiently transfected with pcDNA3, a eukaryotic expression vector, or the vector with the wild-type LHR and various mutants. The cells were assayed for <sup>125</sup>I-hCG binding in the presence of increasing concentrations of unlabeled hCG (A). The results were converted to Scatchard plots (B). In addition, the cells were treated with increasing concentrations of unlabeled hCG, and intracellular cAMP was measured (C) as described under "Materials and Methods." The experiments were performed in duplicate and repeated three to four time (n=6-8). The means and standard deviations are presented in the table (below panels A-C). NS, not significant.



**Figure 2.2** Coexpression of LHR<sup>-hCG</sup> and LHR<sup>+hCG/-cAMP</sup>. HEK 293 cells were transiently coexpressed with an LHR defective in hCG binding and an LHR defective in cAMP induction and were assayed for hCG binding and cAMP induction as described in the legend for Figure 2.1.



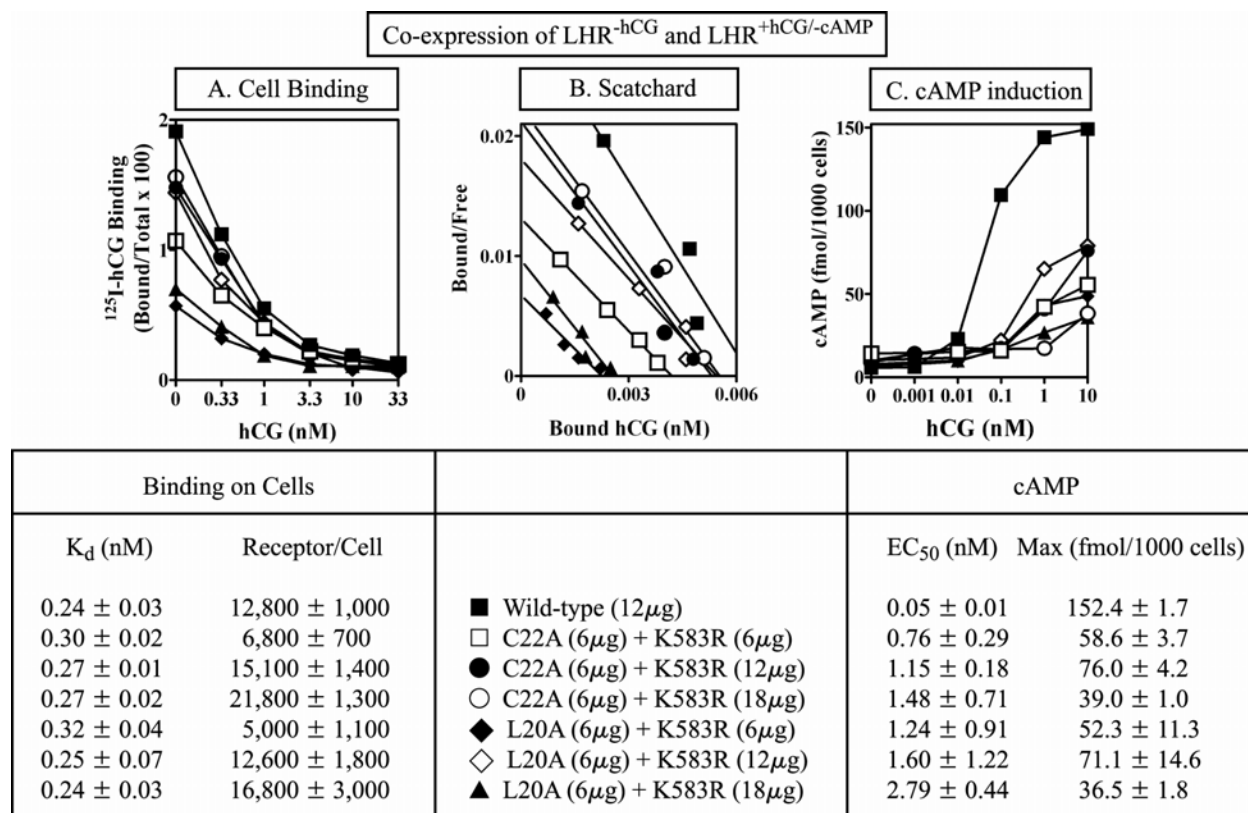
**Figure 2.3** Coexpression of Flag-LHR<sup>C22A</sup> and LHR<sup>K583R</sup>. Cells stably expressing Flag-LHR<sup>C22A</sup> were transfected with increasing concentrations of the LHR<sup>K583R</sup> plasmid. The cells were assayed for hormone binding and cAMP induction as described in the legend for Figure 2.1.



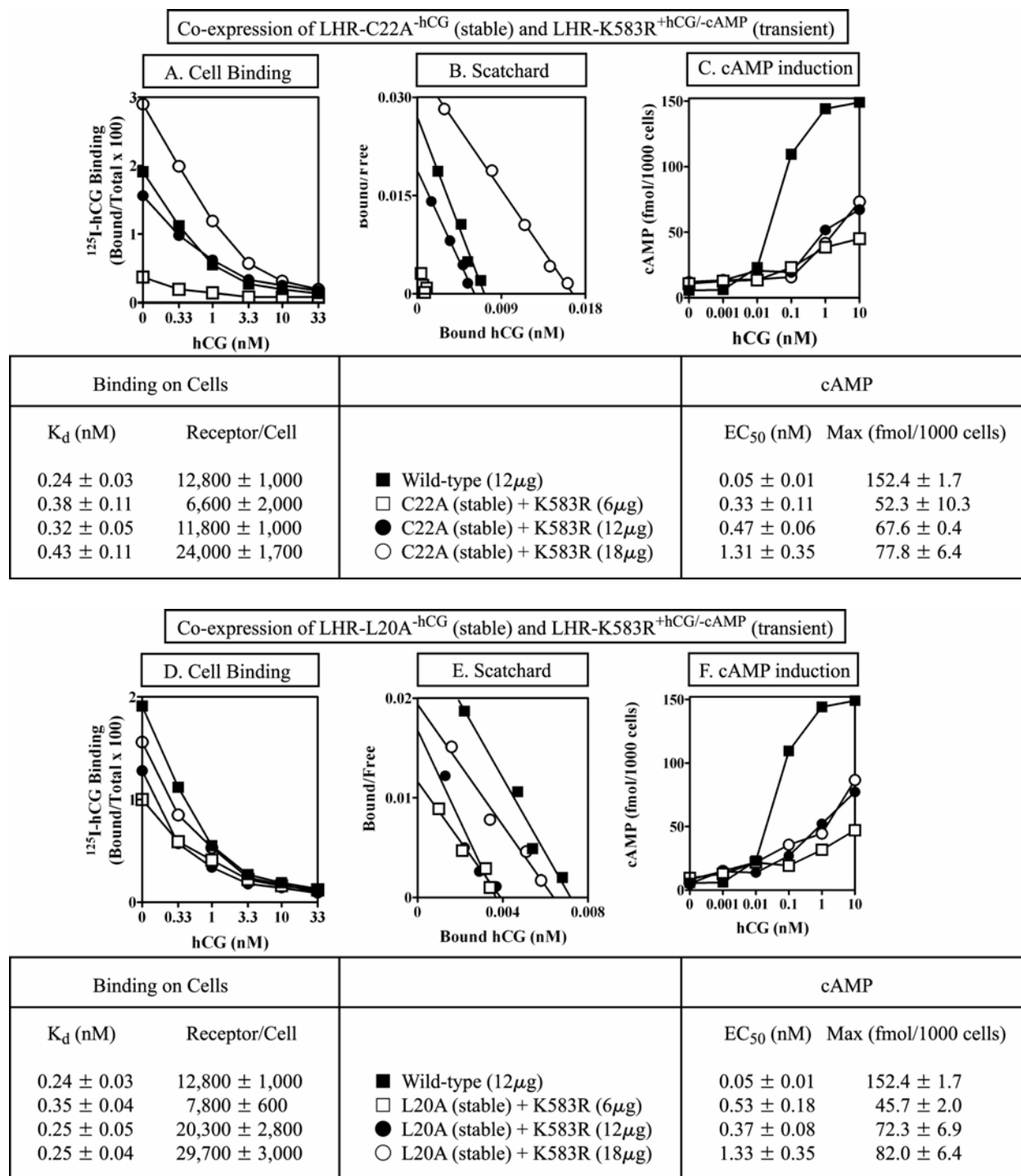
Binding on Cells			cAMP	
K <sub>d</sub> (nM)	Receptor/Cell		EC <sub>50</sub> (nM)	Max (fmol/1000 cells)
0.24 ± 0.03	12,800 ± 1,000	■ Wild-type	0.05 ± 0.01	152.4 ± 1.7
NS	NS	□ pcDNA3	NS	8.7 ± 1.6
NS	NS	● L20A + C22A	NS	8.6 ± 2.0
NS	NS	○ L20A + P479A	NS	7.2 ± 0.8
NS	NS	◆ L20A + P479G	NS	8.3 ± 1.6
NS	NS	◇ C22A + P479A	NS	10.3 ± 1.3
NS	NS	▲ C22A + P479G	NS	8.9 ± 0.4

**Figure 2.4 Specificity of cAMP rescue.** Cells were transiently coexpressed with various combinations of LHR<sup>-hCG</sup> and assayed for hormone binding and cAMP induction as described in the legend for Figure 2.1.

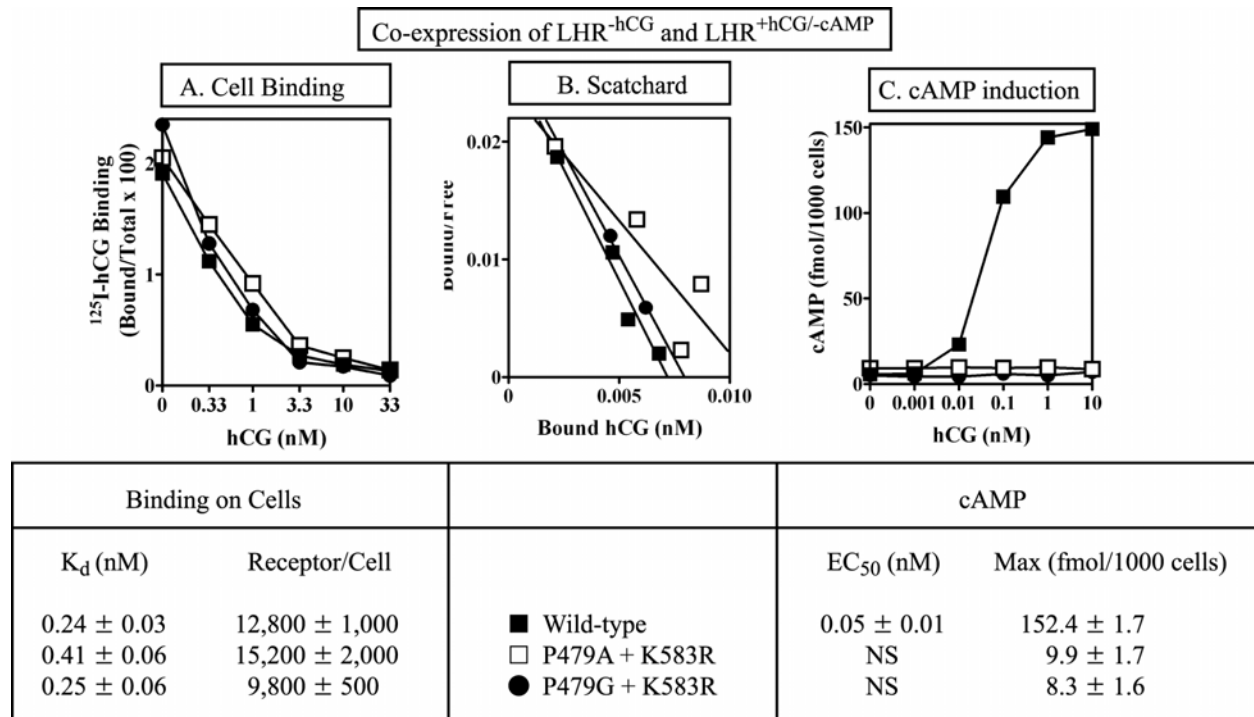




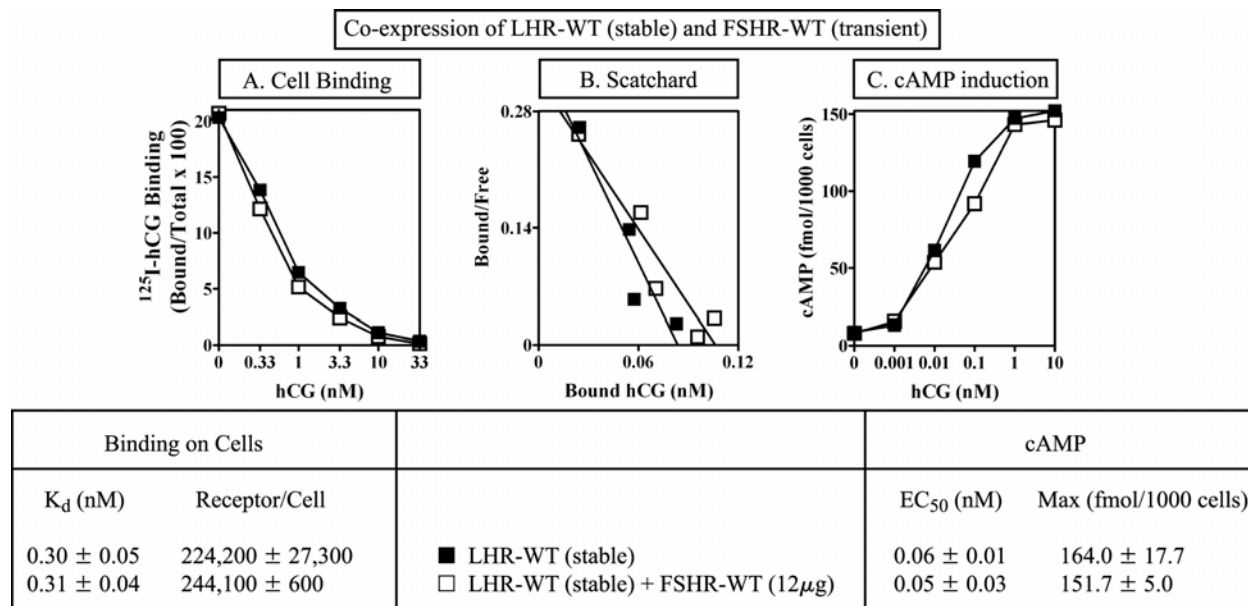
**Figure 2.5 Receptor concentration-dependent cAMP rescue.** Cells were transiently cotransfected with varying concentrations of the LHR<sup>K583R</sup> plasmid and 6 μg of the LHR<sup>C22A</sup> or LHR<sup>L20A</sup> plasmid. The cells were assayed for hormone binding and cAMP production as described in the legend for Figure 2.1.



**Figure 2.6 Coexpression of stable LHR<sup>-hCG</sup> and transient LHR<sup>+hCG/-cAMP</sup>.** Cells stably expressing LHR<sup>-hCG</sup> were transiently transfected with increasing concentrations of the LHR<sup>K583R</sup> plasmid. The cells were assayed for hormone binding and cAMP production as described in the legend for Figure 2.1.



**Figure 2.7** cAMP rescue is dependent on the location of mutation in LHR<sup>-hCG</sup>. Cells were transiently coexpressed with LHR<sup>K583R</sup> and LHR<sup>P479A</sup> or LHR<sup>K583R</sup> and LHR<sup>P479G</sup>. The cells were assayed for hormone binding and cAMP production as described in the legend for Figure 2.1.



**Figure 2.8** Coexpression of LHR<sup>WT</sup> and FSHR<sup>WT</sup>. 12  $\mu\text{g}$  of FSH receptor plasmid was transiently transfected into HEK 293 cells stably expressing LHRs. The cells were assayed for hormone binding and cAMP production as described in the legend for Figure 2.1.

## Chapter 3 No Trans-activation of the LHR for IP Signaling

### Introduction

Many hormone receptors, including those for glycoprotein hormones, activate multiple intracellular signals such as cAMP, inositol phosphate, and  $\text{Ca}^{2+}$  through their promiscuous couplings to diverse G proteins. It has been known since the 1970s that cAMP is released by rat Leydig cells during gonadotropin stimulation (71, 75), and thereafter cAMP has been recognized as a primary second messenger of LH action in the theca and granulosa cells and in the corpus luteum (52). A decade later it became known that the LH also affects phosphoinositide metabolism in rat granulosa cells (76). The LHR was reported to be dually coupled to adenylyl cyclase and phospholipase  $\text{C}\beta$  (77).  $\text{G}_s$  and  $\text{G}_i$  were revealed to activate adenylyl cyclase and phospholipase  $\text{C}\beta$ , respectively, upon hormone stimulation (78, 79). It was further identified that the LHR activates phospholipase  $\text{C}\beta$  via preferential coupling to  $\text{G}_{i2}$  (44), although phospholipase  $\text{C}\beta$  stimulation is considered to generally be activated by the  $\beta\gamma$  subunit (80).

Though the cellular responses by the LHR are mediated by both adenylyl cyclase and phospholipase  $\text{C}\beta$ , the LHR preferentially activates adenylyl cyclase with 100-fold lower  $\text{EC}_{50}$  than the activation of phospholipase  $\text{C}\beta$  (17). LH is responsible for the final maturation of the ovarian follicles and estrogen secretion (81). It is also responsible for ovulation, the initial formation of the corpus luteum, and secretion of progesterone (81). Intracellular cAMP is known to be mainly responsible for the expression of specific genes involved in the LH action (81).

The physiological role of the phospholipase  $\text{C}\beta$  pathway is not clearly defined yet. The activation of phospholipase  $\text{C}\beta$  by the LHR requires high hormone concentrations ( $\text{EC}_{50}$ : 1-10 nM) and large receptor numbers (17, 46, 82) compared with the activation of adenylyl cyclase. This suggests that the phospholipase  $\text{C}\beta$  pathway exists in granulosa cells in females. However, one wonders whether it is active in Leydig cells, because the hCG/LH concentrations are generally below the  $\text{EC}_{50}$  values. A study on the impairment of ovulation in rats with PKC inhibitors supports the role of the phospholipase  $\text{C}\beta$  pathway in ovulation (83). These three

signaling pathways, cAMP, inositol phosphate, and protein kinase C, stimulated by the LHR have been reported to be independent of each other. Pretreatment with a physiological activator of protein kinase C, PGF2 $\alpha$ , had no effect on LH-stimulated inositol phosphate accumulation (84), and neither forskolin nor prostaglandin E1, which both increase cAMP levels in L cells, had an effect on inositol phosphate accumulation and Ca<sup>2+</sup> elevation in L cells (17).

Another clue in the role of the inositol phosphate signaling from the LHR comes from a clinical LHR mutant (Asp<sup>578</sup>His) causing Leydig cell tumors (85). This mutation is located in the TM6 (Figure 1.6). Normally, hormone-mediated activation of the LHR in Leydig cells does not result in the stimulation of the phospholipase C $\beta$  pathway due to the absence of high concentrations of LH, unlike in granulosa cells (5). The main feature that distinguishes the Asp<sup>578</sup>His mutation from other LHR Asp<sup>578</sup> mutations associated with male-limited precocious puberty is its ability to elevate both the basal cAMP and IP levels in the absence of hormone (85). Other LHR mutants such as Asp<sup>578</sup> to Gly, Leu, Pro, and Tyr, constitutively activate only cAMP signaling pathway (47). The different clinical phenotype of Leydig cell adenoma by the LHR Asp<sup>578</sup>His mutation suggests distinct physiological functions of adenylyl cyclase and phospholipase C $\beta$  pathways in LH signaling and also suggests the role of the phospholipase C $\beta$  signal.

It has been demonstrated that trans-activation of LHRs lead to the activation of adenylyl cyclase to induce cAMP in the previous studies (42, 43). We wondered whether the trans-activation of the LHR also leads to the activation of phospholipase C $\beta$ . It is an intriguing question in regard to multiple hormonal signal generation because the LHR activates both adenylyl cyclase and phospholipase C $\beta$ , but they require different hormone concentrations.

The LHR<sup>-hCG</sup> and LHR<sup>+hCG/-IP</sup> mutants were utilized to elucidate the possibility of inositol phosphate signaling rescue by trans-activation of the LHR. A strategy of transient expression of two defective LHRs in HEK 293 cells, which at the same condition successfully rescued cAMP signaling, was used with high hCG concentrations of up to 1-10  $\mu$ M. Also HEK 293 cells stably expressing both defective LHRs were established to facilitate high cell surface expression and assayed for the rescue of inositol phosphate signaling.

## Materials and Methods

*Inositol Phosphate Assay*-- Stable cells were plated in 12-well plates and grown in inositol-free Dulbecco's modified Eagle's medium (Atlanta Biologicals) supplemented with 8% heat-inactivated horse serum and 2  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]inositol (PerkinElmer Life Sciences) for 48 h to 40-50% confluency. After removing the medium, the cells were incubated in 1 ml of fresh wash buffer consisting of Dulbecco's modified Eagle's medium without inositol and 15 mM HEPES (pH 7.3) for 1 h at 37 °C. This medium was removed, and 0.3 ml of wash buffer containing 20 mM LiCl was added and incubated for 15 min at 37 °C. After the cells were stimulated with increasing concentrations of hormone for 30 min at 37 °C, the incubation was terminated by the removal of medium and the addition of 0.25 ml of 0.6 N HCl to each well. The cells were scraped and transferred into microcentrifuge tubes, and the wells were again washed with 0.25 ml of 0.6 N HCl. The combined washes were treated with 0.9 ml of a mixture of chloroform:methanol (2:1), vortexed, and centrifuged at  $1000 \times g$  for 5 min at room temperature. The top aqueous layer, which was free of phospholipids, was removed, and the remaining chloroform layer was treated with 0.2 ml of methanol:water (1:1), vortexed, and centrifuged, as above. This aqueous layer was added to the previous aqueous layer, and the samples were dried in a vacuum concentrator. The dried samples were redissolved in 0.5 ml of 50 mM Tris-HCl, pH 8, and applied to Dowex AG 1-X8 formate (Bio-Rad) columns. The microcentrifuge tubes were washed twice with 0.5 ml of the same buffer, and the washes were applied to the columns for a total of 1.5 ml. The columns were sequentially washed with 4.5 ml of  $\text{H}_2\text{O}$  and 4.5 ml of 60 mM ammonium formate and 5 mM sodium tetraborate to elute the free inositol and the glycerol phosphatidylinositol, respectively.  $\text{IP}_1$ ,  $\text{IP}_2$ , and  $\text{IP}_3$  were sequentially eluted with 4 ml of 0.1 N formic acid in 0.2, 0.75, and 1.1 M ammonium formate, respectively, and collected in 1-ml fractions. Aliquots of 200  $\mu\text{l}$  were counted for radioactivity in 1.5 ml of Ultima AF scintillation fluid (Packard). Peak radioactivities were used for the data analysis.

Hormone binding assay and intracellular cAMP assay procedures are same as described in "Materials and Methods" of the Chapter 2.

## Results

Mutant LHRs used to successfully rescue cAMP induction in Chapter 2 and in our previous publications (42, 43) were selected to determine whether or not the rescuing of cAMP signaling by trans-activation of LHRs would also activate phospholipase C $\beta$  and IP signaling. The non-binding LHRs were L<sup>20</sup>A, C<sup>22</sup>A, I<sup>55</sup>A, and I<sup>80</sup>A (LHR<sup>-hCG</sup>). LHRs defective in signaling were K<sup>583</sup>R and ExoCD (LHR<sup>+hCG/-cAMP/-IP</sup>). The ExoCD is a chimera with a functional LHR exodomain linked to the non-functional CD 8 transmembrane and cytoplasmic domain (86) (Figure 3.1). HEK 293 cells stably expressing these mutant LHRs were established, and the induction of inositol phosphate was measured in the presence of hCG (Figure 3.2). None of these mutant LHRs showed any elevated basal level. IP subspecies (IP<sub>1</sub>, IP<sub>2</sub>, and IP<sub>3</sub>) were also measured but failed to show any increase.

Studies on IP induction by the LHR have revealed that two conditions are required: high hCG concentrations and large receptor numbers (17, 46). To check this, HEK 293 cells stably expressing wild type LHRs were established. Transfection studies showed that IP induction required high receptor concentrations on the cell surface (Figure 3.3). Cells stably expressing receptors usually show high receptor expression due to antibiotic selection (> 140,000 receptor/cell in this study). However, cells transiently transfected with 12  $\mu$ g of LHR plasmid show about 12,000 receptors per cell. The data in the Figure 3.3 shows that IP induction is also dependent on receptor concentrations. Net cpm increase of IP induction in stable cells is 4-5 fold higher than that in cells transiently expressing the receptors. To study whether those mutant LHR combinations which successfully rescued cAMP induction in the previous chapter, in physiological range of receptor numbers (6,000 – 20,000 receptors/cell), also rescues IP induction, the same transient transfection method was utilized. Also HEK 293 cells stably expressing both mutant receptors were selected and assayed for IP induction.

First, 12  $\mu$ g of LHR<sup>K583R</sup> were transiently transfected into HEK 293 cells with 6  $\mu$ g each of LHR<sup>L20A</sup>, LHR<sup>C22A</sup>, LHR<sup>I55A</sup>, and LHR<sup>I80A</sup>. Trans-activation between the two receptors rescued cAMP induction up to 27-41% of the max cAMP induction by wild type LHR but failed to induce IP production (Figure 3.4). IP subspecies (IP<sub>1</sub>, IP<sub>2</sub>, and IP<sub>3</sub>) induction was also measured



but none was found. Our group showed that ExoCD had slightly higher cAMP induction than LHR<sup>K583R</sup> when coexpressed with non-binding LHRs (43). Thus, ExoCD was coexpressed with LHR<sup>L20A</sup>, LHR<sup>C22A</sup>, LHR<sup>I55A</sup>, or LHR<sup>I80A</sup> transiently in HEK 293 cells (Figure 3.5). Up to 10  $\mu$ M of hCG was added to see whether this extremely high concentration of hormone stimulates LHRs for IP induction. However, no induction of IP signaling including IP<sub>1</sub>, IP<sub>2</sub>, and IP<sub>3</sub> was seen.

Next, HEK 293 cells stably expressing LHR<sup>L20A</sup> or LHR<sup>C22A</sup> were transiently transfected with increasing concentrations of LHR<sup>K583R</sup> plasmids (6 to 18  $\mu$ g) (Figure 3.6), but there was no IP induction, contrary to the successful cAMP induction. HEK 293 cells stably expressing LHR<sup>K583R</sup> were also established, and LHR<sup>L20A</sup> or LHR<sup>C22A</sup> was transiently transfected, but there was no IP induction.

To make sure two defective LHRs are expressed in HEK 293 cells in high concentrations, HEK 293 cells stably expressing ExoCD and LHR<sup>I55A</sup> were established and selected by G-418. hCG binding to cell surface ExoCD showed more than 130,000 receptors/cell, but there was no IP induction (Figure 3.7). In addition to this, another HEK 293 cell line stably expressing ExoCD and LHR<sup>I80A</sup> was also established and assayed for IP signaling but failed to induce any IP production. These HEK 293 cells had two mutant LHRs in the same G-418 resistance vector. Though they rescued cAMP signaling very successfully in the previous chapter, there was a chance that these cells used for IP rescuing might not have had both receptors on the cell surface. To ensure the expression of both mutant receptors, another stable HEK 293 cell line expressing both receptors with different antibiotic selection was established. Even with ExoCD in the hygromycin B resistance vector and LHR<sup>I55A</sup> or LHR<sup>I80A</sup> in the G-418 resistance vector, there was again no IP induction (data not shown).

LHR<sup>P479A</sup> or LHR<sup>P479G</sup> did not induce cAMP in the previous chapter when they were coexpressed with LHR<sup>K583R</sup>. However, it is still possible that they may rescue IP induction by a different trans-activation mechanism. To test this possibility, LHR<sup>P479A</sup> or LHR<sup>P479G</sup> was coexpressed with LHR<sup>K583R</sup> transiently in HEK 293 cells and assayed for IP induction. No IP induction was observed up to 1  $\mu$ M of hCG concentration (Figure 3.8).

The absence of IP induction from the stably and transiently coexpressed pairs of LHRs which successfully rescued cAMP signaling suggest the possibility that mutant LHRs might have impaired the G proteins associated with the IP signaling. LHR<sup>WT</sup> was coexpressed with LHR<sup>I80A</sup> and with LHR<sup>I80A</sup> and LHR<sup>K583R</sup>, but the coexpression of mutant LHR(s) did not affect IP induction of the wild type LHR (Figure 3.9), suggesting the absence of IP induction is not due to the impairment of G proteins by mutant LHRs.

## Discussion

The results described in this chapter show that a liganded LHR exodomain was capable of trans-activating the endodomain of some unliganded LHRs and stimulating adenylyl cyclase but not for phospholipase C $\beta$ . These observations suggest that cis-activation of LHR stimulates both adenylyl cyclase and phospholipase C $\beta$ , whereas trans-activation of LHR is limited to adenylyl cyclase. Therefore, the modulation of the endodomain by the exodomain during trans-activation is likely limited. Our group showed that mutations at downstream of the N-terminal region, L<sup>103</sup>A, I<sup>105</sup>A, L<sup>179</sup>A, and L<sup>202</sup>A do not allow trans-activation (43). A simple explanation is that the location of non-binding mutations plays a role in trans-activation. The non-binding mutations in the N-terminal region are permissible for trans-activation, whereas the non-binding mutations downstream of N-terminal region are not. Interestingly, some amino acid residues in this non-permissible region are involved in modulating the endodomain and the signal generation. S<sup>255</sup> of LHR suppresses the endodomain (30) and G<sup>91</sup> of LHR activates it (26). These residues are conserved in the FSH, LH, and TSH receptor. Furthermore, there is evidence that these regions make contacts with the endodomain (28, 36). Since the hormones also interact with the exodomain and endodomain, it is likely that the interactions among the hormone, exodomain, and the endodomain differ in trans-activation and cis-activation. The interactions appear to be more restricted in trans-activation.

The absence of IP induction by LHR trans-activation provides a clue to our understanding of 100-fold higher EC<sub>50</sub> of IP production compared to cAMP production. Trans-activation provides a mechanism of hormone signal amplification at the receptor level as was demonstrated by cAMP rescuing, thus the LHR could achieve maximal cAMP induction at low hormone

concentrations, but this was not seen with IP signaling. The IP signaling pathway only seems to be mediated by cis-activation, which requires more hormones for phospholipase C $\beta$  activation.

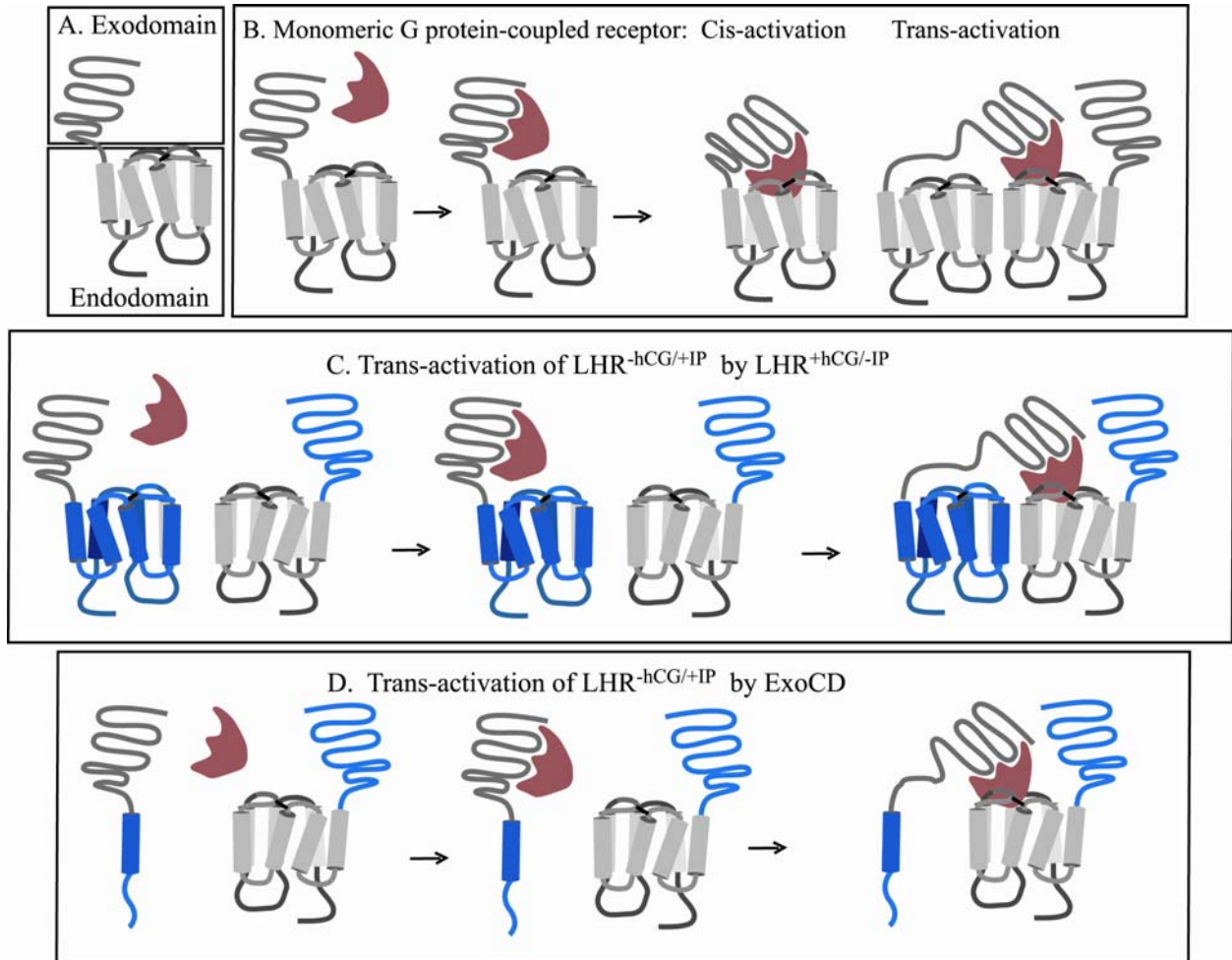
Another possibility for the lack of IP induction by the trans-activation of the LHR might be due to low affinity of G $_{12}$  to the LHR compared to that of G $_s$ . It has not been determined how much G $_s$  and G $_{12}$  are endogenously expressed in HEK 293 cells. Future study including overexpression of G $_{12}$  in HEK 293 cells with coexpression of two defective heterozygous LHRs will help to elucidate whether the lack of IP induction is due to more restriction of hormone-receptor contacts in trans-activation than in cAMP induction or to low affinities of G $_{12}$  to the LHR.

It has been an enigma as to how a hormone receptor can generate two or more signals, such as LHR being capable of activating two enzymes, adenylyl cyclase and phospholipase C $\beta$ . Particularly, it is unclear whether one receptor molecule can generate only one signal or two distinct signals at a time. If the former is the case, cis- and trans-activation provide a mechanism for generation of two signals by one receptor. For example, a liganded LHR could cis-activate itself to stimulate phospholipase C $\beta$  and trans-activate another LHR to stimulate adenylyl cyclase. This mechanism would allow one liganded receptor to generate multiple signals without a receptor simultaneously interacting with multiple G proteins.

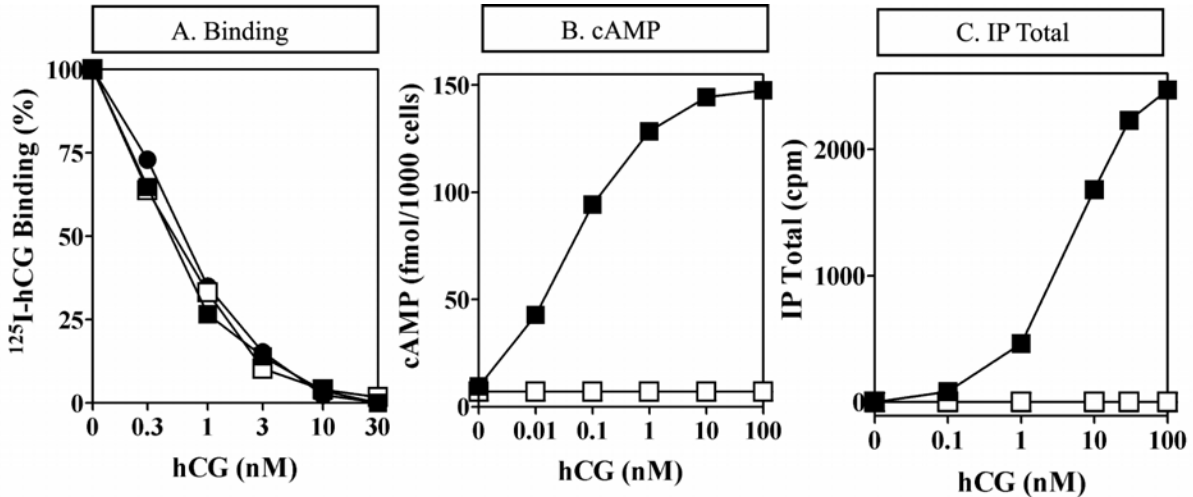
**Table 3.1 Surface expression of Flag-LHRs**

Cells were transfected with various Flag-LHR plasmids and assayed for  $^{125}\text{I}$ -monoclonal anti Flag-antibody binding in the presence of increasing concentrations of unlabeled antibody as described under "Materials and Methods." Specific antibody bindings were determined and compared with the specific binding of the Flag-LHR<sup>WT</sup>.

	$^{125}\text{I}$ -anti-Flag mAb
	Binding (%)
LHR <sup>WT</sup>	5 ± 3
Flag-LHR <sup>WT</sup>	100 ± 9
Flag-LHR <sup>C22A</sup>	25 ± 3
Flag-LHR <sup>I55A</sup>	33 ± 5
Flag-LHR <sup>I80A</sup>	30 ± 5

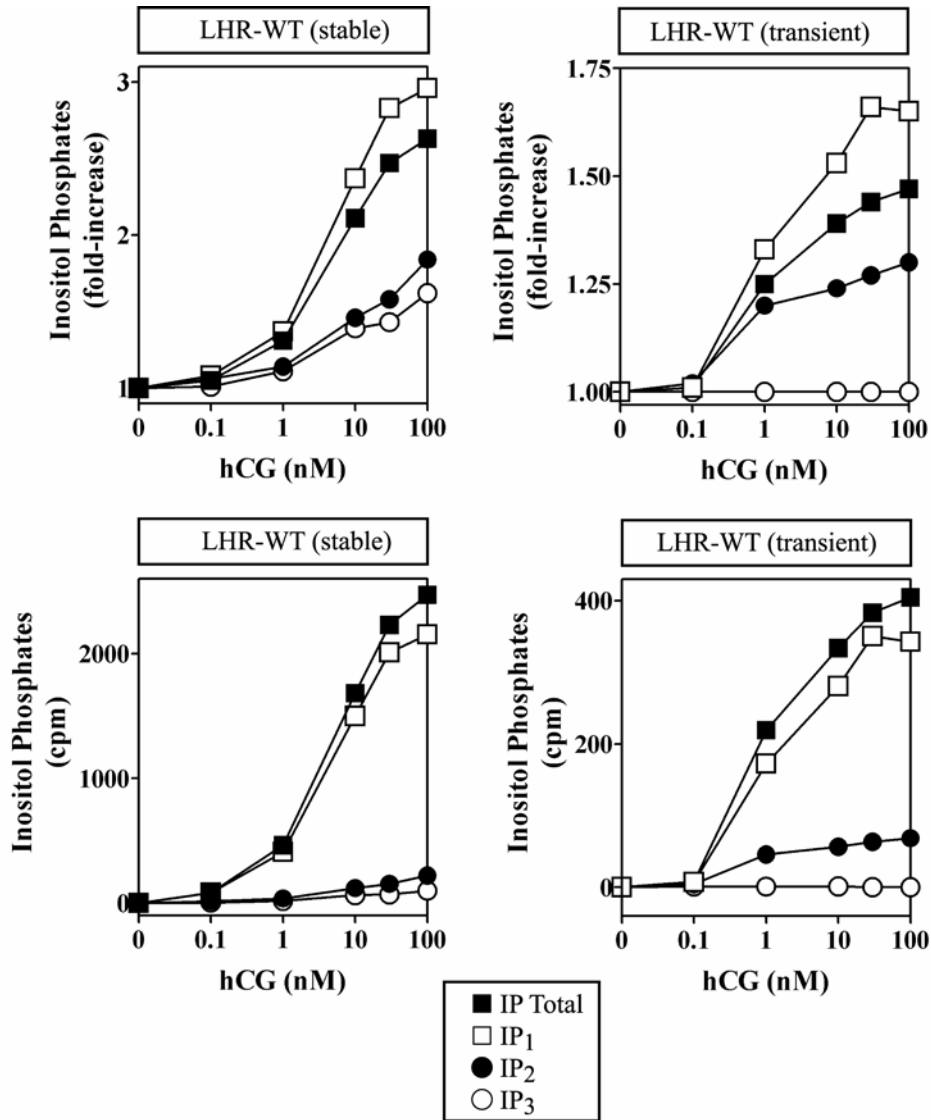


**Figure 3.1 Will there be trans-activation of LHR for inositol phosphate signaling?** **A.** Domain structure of LHR showing the exodomain where the ligand binds and the endodomain where the hormone signal is generated. **B.** Cis- and trans-activation of monomeric LHRs. Trans-activation of the LHR generates cAMP signaling. The ligand is shown in red. **C.** Hypothesis of trans-activation of a non-binding mutant LHR (LHR<sup>-hCG/+IP</sup>) by a mutant LHR that is capable of hormone binding but defective in signal generation (LHR<sup>+hCG/-IP</sup>). Normal parts are shown in gray and defective parts are shown in blue. **D.** Hypothesis of trans-activation of a mutant LHR (LHR<sup>-hCG/+IP</sup>) by a chimera with a functional LHR exodomain linked to the non-functional CD 8 transmembrane and cytoplasmic domain (ExoCD). Normal parts are shown in gray and defective parts are shown in blue.

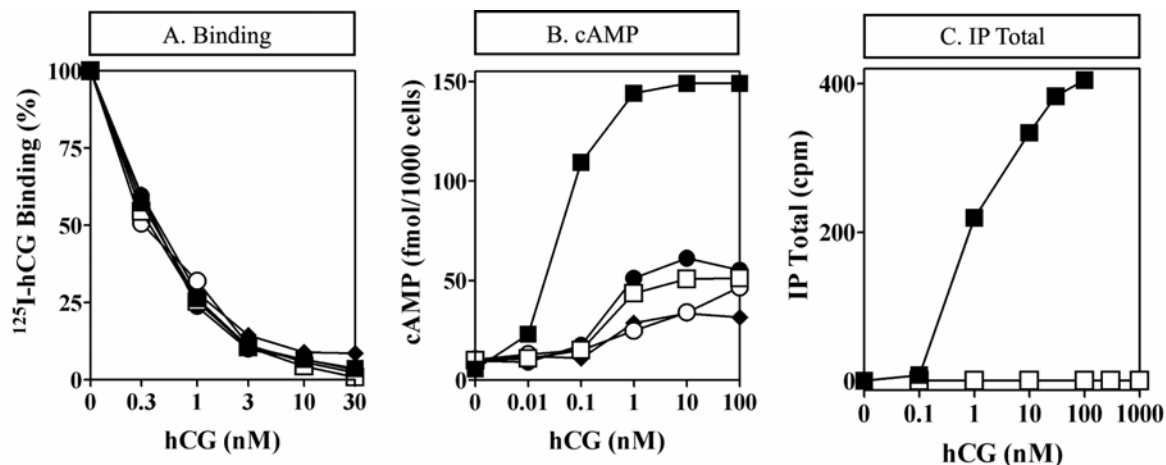


Cell Binding			cAMP	IP Total
$K_d$ (nM)	Receptor/Cell		Max (fmol/1000 cells)	Max (cpm)
$0.43 \pm 0.02$	$160,400 \pm 12,100$	■ LHR-WT	$149.1 \pm 3.5$	$2,670 \pm 280$
$0.38 \pm 0.03$	$212,800 \pm 8,600$	□ $K^{583}R$	NS	NS
$0.39 \pm 0.01$	$141,900 \pm 1,700$	● ExoCD	NS	NS
NS	NS	○ $L^{20}A$	NS	NS
NS	NS	◆ $C^{22}A$	NS	NS
NS	NS	◇ $I^{55}A$	NS	NS
NS	NS	▲ $I^{80}A$	NS	NS

**Figure 3.2 Activity of mutant receptors.** HEK 293 cells stably expressing wild-type or mutant LHRs were established by transfecting each with 12  $\mu$ g of receptor plasmid followed by G-418 selection. The cells were assayed for  $^{125}I$ -hCG binding in the presence of increasing concentrations of unlabeled hCG (A). In addition, the cells were treated with increasing concentrations of unlabeled hCG, and intracellular cAMP (B) and inositol phosphates including inositol mono- (IP<sub>1</sub>), bis- (IP<sub>2</sub>), and tris-phosphates (IP<sub>3</sub>) (C) were measured as described under "Materials and Methods." The experiments were performed in duplicate and repeated three to four time (n=6-8). The means and standard deviations are presented in the table (below panels A-C). NS, not significant.



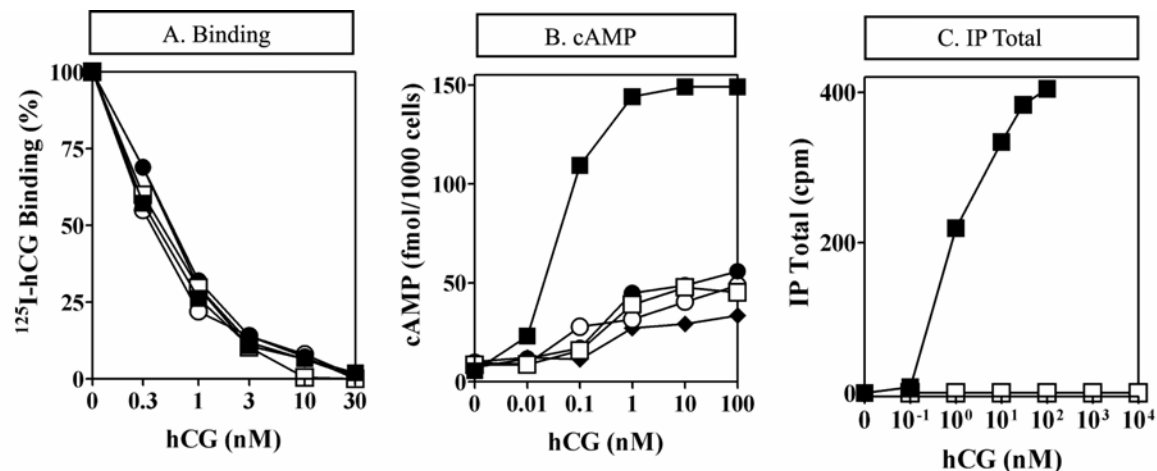
**Figure 3.3 Induction of inositol phosphates from LHR<sup>WT</sup>.** HEK 293 cells either stably or transiently expressing LHR<sup>WT</sup> were established. The cells were assayed for intracellular inositol phosphates (IP<sub>1</sub>, IP<sub>2</sub>, and IP<sub>3</sub>) induction in response to hCG as described in the legend for Figure 3.2.



Cell Binding			cAMP		IP Total	
K <sub>d</sub> (nM)	Receptor/Cell		EC <sub>50</sub> (nM)	Max (fmol/1000 cells)	EC <sub>50</sub> (nM)	Max (cpm)
0.29 ± 0.05	14,800 ± 1,800	■ LHR-WT	0.06 ± 0.01	149.1 ± 3.5	3.2 ± 2.5	390 ± 21
0.30 ± 0.01	10,600 ± 500	□ K <sup>583</sup> R/L <sup>20</sup> A	0.47 ± 0.17	42.7 ± 12.2	NS	NS
0.28 ± 0.02	11,300 ± 1,700	● K <sup>583</sup> R/C <sup>22</sup> A	0.52 ± 0.02	61.1 ± 0.4	NS	NS
0.34 ± 0.03	16,500 ± 1,200	○ K <sup>583</sup> R/I <sup>55</sup> A	1.09 ± 0.34	50.0 ± 8.2	NS	NS
0.33 ± 0.01	15,500 ± 4,500	◆ K <sup>583</sup> R/I <sup>80</sup> A	0.79 ± 0.05	41.0 ± 3.11	NS	NS

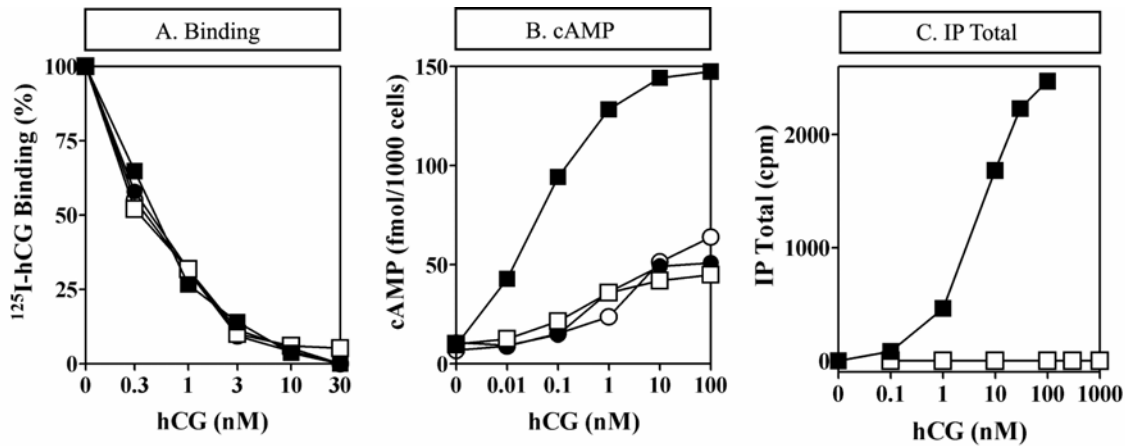
**Figure 3.4** Coexpression of LHR<sup>K583R</sup> and LHR<sup>-hCG</sup>. 12 μg of LHR<sup>K583R</sup> plasmid was cotransfected into HEK 293 cells with 6 μg of various mutant LHR plasmid, respectively. The cells were assayed for hormone binding and cAMP and IP induction in response to hCG, as described in the legend for Figure 3.2.



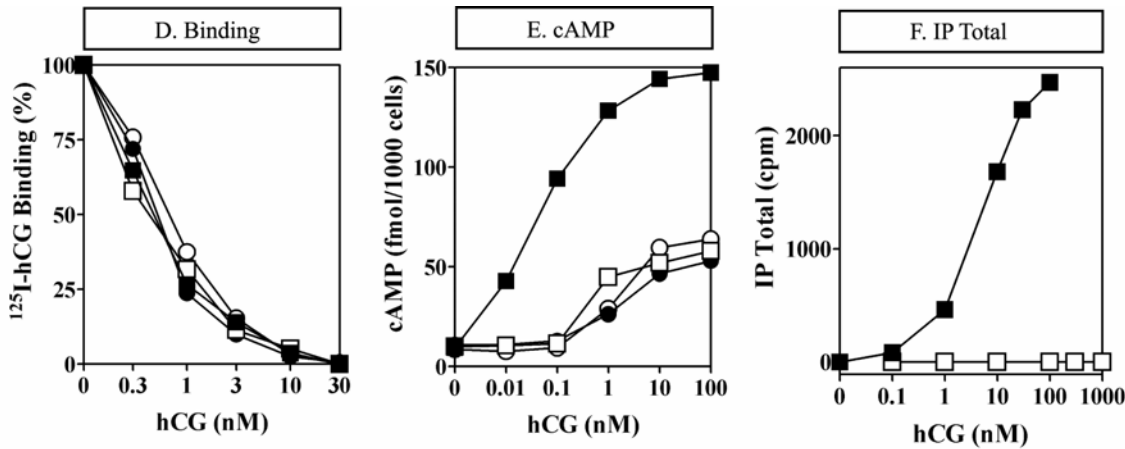


Cell Binding			cAMP		IP (Total)	
K <sub>d</sub> (nM)	Receptor/Cell		EC <sub>50</sub> (nM)	Max (fmol/1000 cells)	EC <sub>50</sub> (nM)	Max (cpm)
0.29 ± 0.05	14,800 ± 1800	■ LHR-WT	0.06 ± 0.01	149.1 ± 3.5	3.2 ± 2.5	390 ± 21
0.35 ± 0.04	10,100 ± 500	□ ExoCD/L <sup>20</sup> A	0.91 ± 0.37	46.5 ± 2.1	NS	NS
0.44 ± 0.02	8,300 ± 700	● ExoCD/C <sup>22</sup> A	1.06 ± 0.69	53.7 ± 3.1	NS	NS
0.38 ± 0.06	10,300 ± 1,400	○ ExoCD/I <sup>55</sup> A	1.70 ± 0.60	44.2 ± 10.7	NS	NS
0.33 ± 0.04	9,400 ± 600	◆ ExoCD/I <sup>80</sup> A	0.82 ± 0.25	37.0 ± 10.2	NS	NS

**Figure 3.5 Coexpression of ExoCD and LHR<sup>-hCG</sup>.** 12 μg of ExoCD plasmid was cotransfected into HEK 293 cells with 6 μg of various mutant LHR plasmid, respectively. The cells were assayed for hormone binding and cAMP and IP induction in response to hCG, as described in the legend for Figure 3.2.

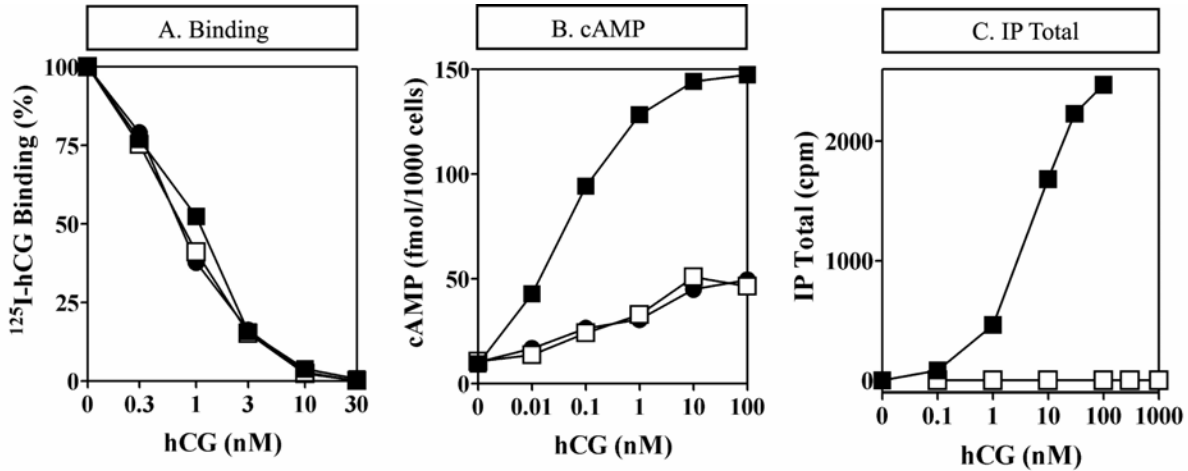


Cell Binding			cAMP		IP (Total)	
K <sub>d</sub> (nM)	Receptor/Cell		EC <sub>50</sub> (nM)	Max	EC <sub>50</sub> (nM)	Max (cpm)
0.43 ± 0.02	160,400 ± 12,100	■ LHR-WT (stable)	0.07 ± 0.01	146.5 ± 6.3	5.8 ± 1.2	2,670 ± 280
0.36 ± 0.16	6,500 ± 1,900	□ L <sup>20</sup> A(stable)/K <sup>583</sup> R(6µg)	0.52 ± 0.20	43.5 ± 2.1	NS	NS
0.32 ± 0.04	11,500 ± 600	● L <sup>20</sup> A(stable)/K <sup>583</sup> R(12µg)	0.53 ± 0.18	52.6 ± 4.8	NS	NS
0.28 ± 0.03	15,500 ± 2,100	○ L <sup>20</sup> A(stable)/K <sup>583</sup> R(18µg)	2.02 ± 1.04	65.3 ± 4.7	NS	NS



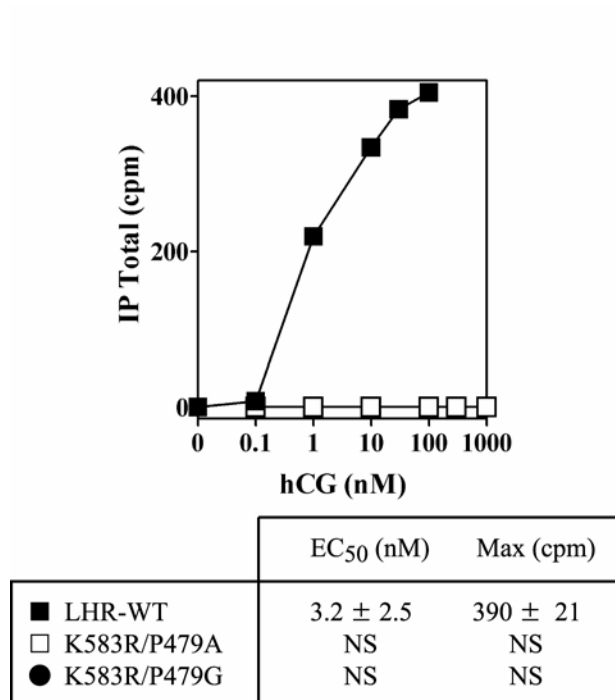
Cell Binding			cAMP		IP (Total)	
K <sub>d</sub> (nM)	Receptor/Cell		EC <sub>50</sub> (nM)	Max	EC <sub>50</sub> (nM)	Max (cpm)
0.43 ± 0.02	160,400 ± 12,100	■ LHR-WT (stable)	0.07 ± 0.01	146.5 ± 6.3	5.8 ± 1.2	2,670 ± 280
0.32 ± 0.04	11,800 ± 900	□ C <sup>22</sup> A(stable)/K <sup>583</sup> R(12µg)	0.83 ± 0.08	55.0 ± 9.2	NS	NS
0.36 ± 0.05	215,800 ± 10,500	● K <sup>583</sup> R(stable)/C <sup>22</sup> A(12µg)	2.98 ± 1.73	54.1 ± 10.5	NS	NS
0.39 ± 0.03	227,300 ± 5,200	○ K <sup>583</sup> R(stable)/L <sup>20</sup> A(12µg)	1.55 ± 0.78	65.1 ± 1.6	NS	NS

**Figure 3.6 Coexpression of stable LHR and transient LHR.** HEK 293 cells expressing stable LHR<sup>+hCG/-cAMP</sup> and transient LHR<sup>-hCG</sup>, or vice versa, were assayed for hormone binding and intracellular cAMP and IP induction in response to hCG, as described in the legend for Figure 3.2.

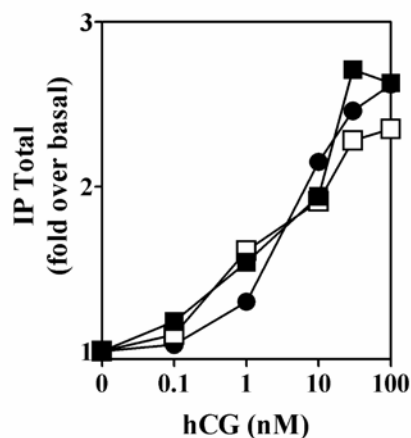


Cell Binding			cAMP		Inositol Phosphates	
K <sub>d</sub> (nM)	Receptor/Cell		EC <sub>50</sub> (nM)	Max (fmol/1000 cells)	EC <sub>50</sub> (nM)	Max (cpm)
0.43 ± 0.02	160,400 ± 12,100	■ LHR-WT	0.07 ± 0.01	146.5 ± 6.3	5.8 ± 1.2	2,670 ± 280
0.38 ± 0.01	131,300 ± 4,200	□ ExoCD/I <sup>55</sup> A	0.50 ± 0.39	48.7 ± 3.11	NS	NS
0.39 ± 0.03	148,200 ± 14,000	● ExoCD/I <sup>80</sup> A	0.79 ± 0.05	50.0 ± 5.6	NS	NS

**Figure 3.7 Coexpression of stable ExoCD and stable LHR<sup>-hCG</sup>.** 6 μg of ExoCD plasmid and 6 μg of various LHR<sup>-hCG</sup> plasmid was transfected into HEK 293 cells followed by G-418 selection. The cells were assayed for hormone binding and cAMP and IP induction in response to hCG, as described in the legend for Figure 3.2.



**Figure 3.8 Coexpression of LHR<sup>K583R</sup> and TM4 LHR<sup>-hCG</sup>.** 12 µg of LHR<sup>K583R</sup> plasmid was co-transfected with 6 µg of LHR<sup>P479A</sup> or LHR<sup>P479G</sup> plasmid in HEK 293 cells, respectively. The cells were assayed for hormone binding and cAMP and IP induction in response to hCG, as described in the legend for Figure 3.2.



	EC <sub>50</sub> (nM)	Max (fold over basal)
■ WT (stable)/pcDNA3	6.2 ± 2.0	2.6 ± 0.2
□ WT (stable)/I <sup>80A</sup> /pcDNA3	5.1 ± 3.1	2.3 ± 0.3
● WT (stable)/I <sup>80A</sup> /K <sup>583R</sup>	6.4 ± 3.4	2.6 ± 0.3

**Figure 3.9** Coexpression of LHR<sup>WT</sup> with LHR<sup>-hCG</sup> and LHR<sup>+hCG/-cAMP</sup>. LHR<sup>I80A</sup> with pcDNA3 vector and LHR<sup>I80A</sup> with LHR<sup>K583R</sup> plasmids were transiently transfected into HEK 293 cells stably expressing LHR<sup>WT</sup>. The cells were assayed for IP induction in response to hCG, as described in the legend for Figure 3.2.

## Chapter 4 Overlapping G Protein Binding Sites of the LHR

### Introduction

Ligand/hormone binding to its cognate receptor leads to receptor activation and signal transduction. Receptors for LH, FSH, and TSH belong to the G protein-coupled receptor (GPCR) family and are unique in structure, possessing two halves of ~350 amino acids; the N-terminal extracellular hormone binding exodomain and the C-terminal half endodomain including three exoloops, seven transmembrane helices, and three cytoloops, responsible for signal generation (7). Hormone binding to the high affinity exodomain leads to conformational changes in the hormone/exodomain complex and modulates hormone signals from endodomain of the receptor, especially through the exoloops (24). The LHR activates both adenylyl cyclase and phospholipase C $\beta$  to induce cAMP and inositol phosphate, respectively (17, 77). Our group showed that certain amino acids in exoloops 2 and 3 of the LHR and FSHRs are critical for hormone binding and signal generation (33, 34, 45), and the exoloop 3 of the FSHR physically contacts with the exodomain (32, 46). Multiple hormonal signals of the LHR and FSHR are distinct and considered to be generated at the exoloops (32, 46). Gudermann's group and Hunzicker-Dunn's group showed the involvement of both G<sub>s</sub> and G<sub>i</sub> with the LHR in membranes of bovine corpus luteum and L cells (78) and in porcine ovarian follicular membranes (87), respectively. Gudermann's group further identified that the LHR activates phospholipase C via preferential coupling to G<sub>i2</sub> (44).

There are a small number of receptors that couple to G proteins for the activation of both adenylyl cyclase and phospholipase C $\beta$ . These include receptors for the LH (17), FSH (88), TSH (89), GnRH (90), PACAP (91, 92), PTH (93), and calcitonin (94). Interestingly, all of these receptors belong to a subfamily of GPCRs which recognize peptide hormones as their ligands. An exception to this case is the P2Y<sub>11</sub> receptor which has high affinity to the small nucleotide ATP. The P2Y<sub>11</sub> receptor is, however, more homologous to G protein-coupled peptide hormone receptors than to receptors for structurally related small nucleotide ligands (95), and it also preferentially couples to phospholipase C $\beta$  compared to adenylyl cyclase (96), whereas other peptide hormone receptors have preferential coupling to adenylyl cyclase. In addition to the G

protein-coupled peptide hormone receptor subfamily, histamine receptor (97) and  $\alpha_{2A}$ -adrenergic receptor (98) also activate both adenylyl cyclase and phospholipase C $\beta$ .

Usually amino acids in the exoloops affect differential ligand signaling (46, 99), but certain mutations in the transmembrane domain and cytoloop areas have been shown to affect multiple signaling of receptors. Tyr<sup>601</sup> (TM5) of the TSH receptor (100, 101) and Ser<sup>232</sup> (cytoloop 3) of the  $\alpha_{2A}$ -adrenergic receptor (98) serve as molecular switches to transduce cAMP and inositol phosphate signaling selectively. Due to the location of these mutations near the receptor-G protein coupling site, cytoloop 3, Tyr<sup>601</sup> and Ser<sup>232</sup> mutations are considered to affect structural requirements of the receptors for proper G protein-coupling.

This dual and differential cAMP and inositol phosphate signaling of these G protein-coupled receptors requires sophisticated cellular machinery along with G proteins, such as G<sub>s</sub>, Gi, and/or G<sub>q</sub>, and effector molecules. For example, wild type H<sub>1</sub> and H<sub>2</sub> histamine receptors, when expressed in human breast epithelial cells, produced inositol phosphate but failed to produce cAMP, though both signaling pathways are functional in those cells (102).

Studies using carboxyl terminal minigene peptides of G proteins indicate that different G proteins may have overlapping binding sites on the thrombin receptor (103) and the  $\alpha_{1B}$ - and  $\alpha_{2A}$ -adrenergic receptors (104), but it has not yet been elucidated with the LHR. We studied this dual G protein-coupling mechanism of the LHR using transient expressions of G<sub>s</sub>, Gi, and their carboxyl terminal peptides with wild type and mutant LHRs. A constitutively activating TM6 mutant LHR, LHR<sup>D578G</sup>, provides a valuable tool to study G protein interactions with the LHR due to its elevated cAMP level in the absence of hormone (105). The D<sup>578</sup>G mutation is the most common cause of male-limited precocious puberty (106).

The data presented in this dissertation demonstrate that coexpression of the G<sub>i2</sub> carboxyl terminal region (G<sub>i2</sub>CT) surprisingly enhanced cAMP induction from the mutant LHR<sup>D578G</sup>, whereas the G<sub>i2</sub>CT inhibited cAMP induction from the wild type LHR. Coexpression of G<sub>i2</sub> whole molecule with LHR<sup>D578G</sup> completely inhibited elevated cAMP level of the LHR<sup>D578G</sup> in the absence of hormone to the basal level of LHR<sup>WT</sup>, suggesting its application to the development of future

therapeutics for the treatment of patients with Asp<sup>578</sup> to Gly mutation. Coexpression of G<sub>q</sub> or G<sub>12</sub> with the LHR<sup>D578G</sup> did not affect agonist-dependent cAMP induction. The data suggests that the LHR has overlapping binding sites for G<sub>s</sub> and G<sub>12</sub>.

## Materials and Methods

*Mutagenesis and Functional Expression of Receptors and G proteins*-- Mutant human LHR<sup>D578G</sup> cDNA was prepared in a pSELECT vector using the non-polymerase chain reaction-based Altered Sites Mutagenesis System (Promega), sequenced, and subcloned into pcDNA3 (Invitrogen) as described previously (107). After subcloning into pcDNA3, the mutant cDNA was sequenced again. Human G proteins were purchased from Guthrie (Sayre, PA). The carboxyl terminal G protein peptides were constructed using PCR. cDNAs encoding amino acids 288-380 (93 a.a.) of G<sub>s</sub> (G<sub>s</sub>CT) and 280-355 (76 a.a.) of G<sub>12</sub> (G<sub>12</sub>CT) were amplified from G protein cDNAs in pcDNA3.1 and subcloned into BamHI/XbaI sites of a pcDNA3 containing haemagglutinin epitope. 1:1 ratio of receptor (6 µg) and G protein plasmids (6 µg) were transfected into human embryonic kidney (HEK) 293 cells by the calcium phosphate method (64). Transiently transfected cells were assayed 60-72 h after transfection. All assays were carried out in duplicate and repeated three to four times (n=6-8). Means and standard deviations were calculated.

cAMP assay procedure is same as described in the "Materials and Methods" of the Chapter 2.

## Results

*Competition of G<sub>s</sub>CT with endogenous G<sub>s</sub>* -- To investigate the interaction of G proteins and the LHR, first we transfected 6 µg of 93-amino acid G<sub>s</sub>CT plasmid with 6 µg of the wild type and mutant LHR plasmids, respectively, in HEK 293 cells (Fig. 1). Our previous study on transient expressions of the LHR in HEK 293 cells shows that 5,000-7,000 receptor/cell are expressed with transfection of 6 µg of the LHR cDNA plasmid (42), which is consistent with LHR numbers in physiological conditions (15, 16). Coexpression of G<sub>s</sub>CT competitively inhibited cAMP induction from both LHR<sup>WT</sup> and LHR<sup>D578G</sup>, but this inhibition of cAMP by G<sub>s</sub>CT was not



complete (Figure 4.1). This data suggest that the 93-amino acid G<sub>s</sub>CT competes for binding to the LHR with endogenous G<sub>s</sub> molecules.

*Enhancement of cAMP induction by G<sub>i2</sub>CT* -- Next, we transfected 76-amino acid G<sub>i2</sub>CT plasmid with LHR<sup>WT</sup> and LHR<sup>D578G</sup>, respectively, in HEK 293 cells (Figure 4.1). Coexpression of G<sub>i2</sub>CT inhibited cAMP induction from LHR<sup>WT</sup>, but when G<sub>i2</sub>CT was coexpressed with LHR<sup>D578G</sup>, it surprisingly increased already elevated cAMP level two fold and, plus enhanced hormone-induced cAMP induction. These data suggest that upon G<sub>i2</sub>CT binding to the cytoloop 3 and other G protein-coupling sites on the LHR, there were structural changes, either allosterically or non-allosterically, which facilitated G<sub>s</sub>-coupling to the LHR<sup>D578G</sup>. The enhancement of cAMP induction by coexpression of 76-amino acid G<sub>i2</sub>CT with LHR<sup>D578G</sup> also suggests that the LHR has different or overlapping binding sites for G<sub>s</sub> and G<sub>i2</sub>.

*G<sub>s</sub> increases basal cAMP level* -- Although the carboxyl terminal region of G<sub>s</sub> and G<sub>i2</sub> seems to have overlapping binding sites on the LHR, it is unclear whether G<sub>s</sub> or G<sub>i2</sub> whole molecules can compete for binding to the same LHR. First, G<sub>s</sub> was coexpressed with LHR<sup>WT</sup> and LHR<sup>D578G</sup>, respectively, in HEK 293 cells (Figure 4.2). G<sub>s</sub> facilitated basal cAMP level from both LHR<sup>WT</sup> and LHR<sup>D578G</sup>, but as wild type and mutant LHRs are activated by hormone, both receptors restored their normal cAMP induction curve, and the effect of G<sub>s</sub> was minimal.

*Inverse Agonism of G<sub>i2</sub> on LHR<sup>D578G</sup>* -- We coexpressed the G<sub>i2</sub> whole molecule with LHR<sup>WT</sup> and LHR<sup>D578G</sup>, respectively (Figure 4.2). When G<sub>i2</sub> was coexpressed with LHR<sup>D578G</sup>, it completely inhibited its elevated basal and hormone-induced cAMP level down to the basal level of LHR<sup>WT</sup>. As was suggested with enhancement of cAMP induction with G<sub>i2</sub>CT, these data suggest that LHR<sup>D578G</sup> has more exposed G protein binding sites than LHR<sup>WT</sup>, thus promoting G<sub>i2</sub> binding. Also, G<sub>i2</sub> inhibited hormone-induced cAMP induction from LHR<sup>WT</sup> but did not affect the basal cAMP level of LHR<sup>WT</sup>, suggesting that LHR<sup>WT</sup> keeps its basal cAMP level low.

We also transiently expressed G<sub>s</sub> and G<sub>i2</sub>, respectively, in HEK 293 cells stably expressing wild type LHRs (> 100,000 receptors/cell). The effect of transient expression of G<sub>s</sub> or G<sub>i2</sub> was small compared to transient expression of both receptor and G proteins (data not shown). This

indicates that there is a stoichiometric relationship of interactions between the LHR and G proteins.

## Discussion

The Asp<sup>578</sup>Gly mutation is the most common cause of male-limited precocious puberty. This mutant LHR shows 4-7 fold increased cAMP basal level in the absence of hormone, but the maximal cAMP induction reaches 70-80% of wild type LHR. Substitutions of Glu, Tyr, and His for Asp<sup>578</sup> were also found in patients. Inhibition of cAMP induction by coexpression of G<sub>i2</sub> suggests that G<sub>i2</sub> can work as an inverse agonist for the constitutively activating Asp<sup>578</sup>Gly mutant LHR. In addition to the frequent mutations of Asp<sup>578</sup> in the LHR, an activating mutation of the FSHR bearing Asp<sup>567</sup>Gly mutation (TM6) was found in a hypophysectomized man (108). This activating FSHR mutation has so far been the only case of activating mutation among FSHR mutations, and like the activating mutations of the LHR with the corresponding amino acid Asp<sup>578</sup>Gly, it had elevated cAMP level when transfected into COS-7 cells in the absence of hormone. The findings from this study may be helpful to the development of future therapeutics for the treatment of patients with activating mutations of the LH or FSHR.

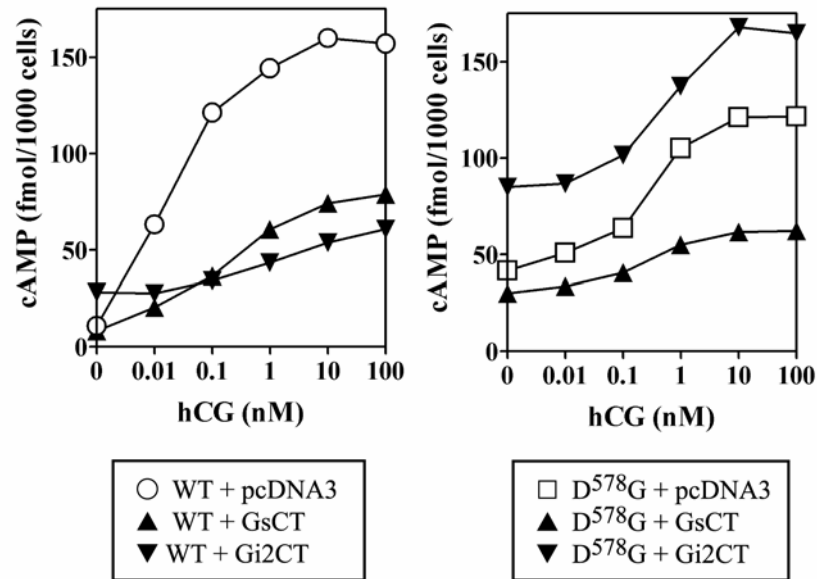
Studies using the carboxyl terminal minigene peptides of G proteins suggest that G proteins may have overlapping binding sites on the receptor. Hamm's group showed that 13-amino acid minigene peptides of different G protein carboxyl termini specifically inhibited the thrombin receptor-G protein interactions (103). Luttrell's group showed that an 83-amino acid peptide of G<sub>s</sub> carboxyl terminal region selectively inhibited G<sub>s</sub> coupling without affecting G<sub>i</sub> or G<sub>q</sub>-coupled phosphoinositide hydrolysis from the  $\alpha_{1B}$ - and  $\alpha_{2A}$ -adrenergic receptors (104). The use of this relatively large 83-amino acid peptide suggests that G<sub>s</sub> binding sites on the receptor may be different or not too much overlapped with G<sub>i</sub> or G<sub>q</sub> binding sites (104). This observation is also consistent with our data that a 73-amino acid G<sub>i2</sub>CT facilitates G<sub>s</sub> binding to the LHR enhancing cAMP induction. Berlot showed that coexpression of a dominant-negative G<sub>s</sub> with the calcitonin receptor inhibited both cAMP and inositol phosphate signaling, suggesting G<sub>s</sub> and G<sub>q</sub> compete for binding to the same receptor (109).

Trans-activation of the LHRs lead to the activation of adenylyl cyclase to induce cAMP, but failed to activate phospholipase C $\beta$  to induce inositol phosphate. This raises an intriguing possibility that a liganded LHR could cis-activate phospholipase C $\beta$  and trans-activate adenylyl cyclase simultaneously. Trans-activation of the LHR and the results on overlapping G protein binding sites on the LHR from this study suggest a mechanism of simultaneous multiple G protein activations, with activation one G protein at a time from a single LHR.

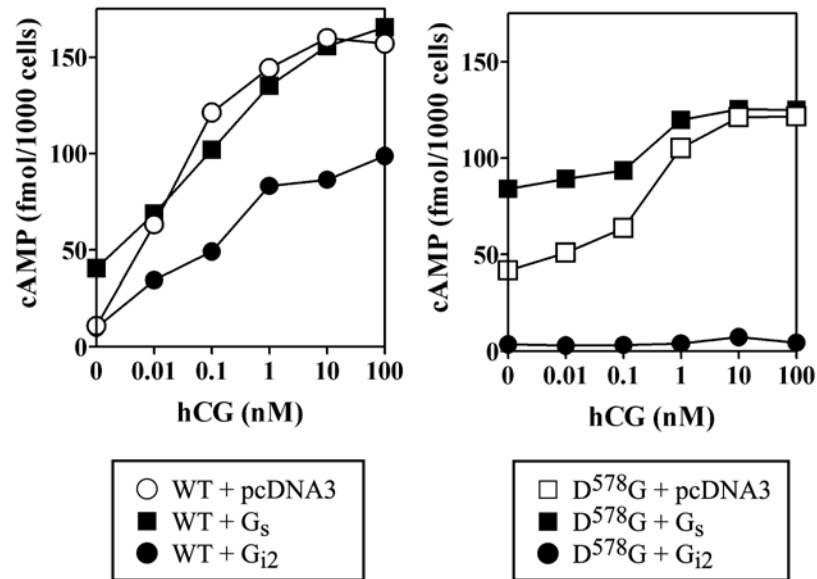
**Table 4.1 Effect of G<sub>s</sub>, G<sub>i2</sub>, and their carboxyl terminal peptides on the cAMP induction of LHR<sup>WT</sup> and LHR<sup>D578G</sup>**

	cAMP induction		
	EC <sub>50</sub> (nM)	Basal (fmol/1000 cells)	Max (fmol/1000 cells)
LHR <sup>WT</sup> + pcDNA3	0.21 ± 0.02	10.3 ± 1.1	157.2 ± 0.2
LHR <sup>WT</sup> + G <sub>s</sub> CT	0.32 ± 0.06	8.6 ± 0.4	79.6 ± 1.1
LHR <sup>WT</sup> + G <sub>i2</sub> CT	0.72 ± 0.36	26.3 ± 2.8	60.5 ± 0.4
LHR <sup>WT</sup> + G <sub>s</sub>	0.30 ± 0.24	42.7 ± 2.9	156.5 ± 12.9
LHR <sup>WT</sup> + G <sub>i2</sub>	0.68 ± 0.22	10.9 ± 1.5	79.3 ± 16.8
LHR <sup>D578G</sup> + pcDNA3	0.48 ± 0.01	46.4 ± 6.6	122.5 ± 1.2
LHR <sup>D578G</sup> + G <sub>s</sub> CT	0.38 ± 0.18	33.5 ± 1.0	65.1 ± 2.5
LHR <sup>D578G</sup> + G <sub>i2</sub> CT	0.88 ± 0.13	86.0 ± 1.3	167.3 ± 11.8
LHR <sup>D578G</sup> + G <sub>s</sub>	0.42 ± 0.08	79.0 ± 7.3	125.0 ± 0.1
LHR <sup>D578G</sup> + G <sub>i2</sub>	<i>NS</i>	7.2 ± 0.6	7.9 ± 0.8

(*NS: Not Significant*)



**Figure 4.1** Effect of G<sub>s</sub>CT and G<sub>i2</sub>CT on cAMP induction of LHR<sup>WT</sup> and LHR<sup>D<sup>578</sup>G</sup>. 6 μg of LHR plasmid and 6 μg of G<sub>s</sub>CT or G<sub>i2</sub>CT minigene plasmid were transfected into HEK 293 cells, respectively. 60-72 hours after transfection, cells were assayed for cAMP induction.



**Figure 4.2** Effect of G<sub>s</sub> and G<sub>i2</sub> on cAMP induction of LHR<sup>WT</sup> and LHR<sup>D578G</sup>. 6 μg of LHR plasmid and 6 μg of G<sub>s</sub> or G<sub>i2</sub> plasmid were transfected into HEK 293 cells, respectively. 60-72 hours after transfection, cells were assayed for cAMP induction.

## References

1. Pierce JG, Parson TS. 1981. Glycoprotein hormones: structure and function. *Annu Rev Biochem* 50: 465-95
2. Catt KJ, Dufau ML, Tsuruhara T. 1971. Studies on a radioligand-receptor assay system for luteinizing hormone and chorionic gonadotropin. *J Clin Endocrinol Metab* 32: 860-3
3. Hiller SG. 1994. Hormonal control of folliculogenesis and luteinization. In *Molecular Biology of the Female Reproductive System*, ed. JK Findlay, pp. 1-37. San Diego: Academic Press, Inc.
4. McNatty KP. 1981. Hormonal correlates of follicular development in the human ovary. *Aust J Biol Sci* 34: 249-68
5. Peluso JJ, Steger RW, Hafez ES. 1976. Development of gonadotrophin-binding sites in the immature rat ovary. *J Reprod Fertil* 47: 55-8
6. Richards JS. 2001. New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. *Mol Endocrinol* 15: 209-18.
7. Ji TH, Grossmann M, Ji I. 1998. G protein-coupled receptors. I. Diversity of receptor-ligand interactions. *J Biol Chem* 273: 17299-302
8. McFarland K, Sprengel R, Phillips H, Kohler M, Rosemblyt N, Nikolics K, Segaloff D, Seeburg P. 1989. Lutropin-choriogonadotropin receptor: An unusual member of the G protein-coupled receptor family. *Science* 245: 494-9
9. Ji I, Ji TH. 1991. Exons 1-10 of the rat LH receptor encode a high affinity hormone binding site and exon 11 encodes G-protein modulation and a potential second hormone binding site. *Endocrinology* 128: 2648-50
10. Nimrod A, Tsafiriri A, Lindner HR. 1977. In vitro induction of binding sites for hCG in rat granulosa cells by FSH. *Nature* 267: 632-3
11. Erickson GF, Wang C, Hsueh AJ. 1979. FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium. *Nature* 279: 336-8
12. Nimrod A. 1981. The induction of ovarian LH-receptors by FSH is mediated by cyclic AMP. *FEBS Lett* 131: 31-3

13. Segaloff DL, Limbird LE. 1983. The cAMP-Dependent induction of LH receptors in primary cultures of porcine granulosa cells is not due to the expression of an intracellular pool of LH receptors. *Endocrinology* 113: 825-7
14. Lee CY, Ryan RJ. 1973. Interaction of ovarian receptors with human luteinizing hormone and human chorionic gonadotropin. *Biochemistry* 12: 4609-15
15. Mendelson C, Dufau M, Catt K. 1975. Gonadotropin binding and stimulation of cyclic adenosine 3':5'-monophosphate and testosterone production in isolated Leydig cells. *J Biol Chem* 250: 8818-23
16. Rao MC, Richards JS, Midgley AR, Jr., Reichert LE, Jr. 1977. Regulation of gonadotropin receptors by luteinizing hormone in granulosa cells. *Endocrinology* 101: 512-23
17. Gudermann T, Birnbaumer M, Birnbaumer L. 1992. Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca<sup>2+</sup> mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. *J Biol Chem* 267: 4479-88
18. Catt KJ, Dufau ML. 1973. Spare gonadotrophin receptors in rat testis. *Nat New Biol* 244: 219-21
19. Xie YB, Wang H, Segaloff DL. 1990. Extracellular domain of lutropin/choriogonadotropin receptor expressed in transfected cells binds choriogonadotropin with high affinity. *J Biol Chem* 265: 21411-4
20. Roche PC, Ryan RJ, McCormick DJ. 1992. Identification of hormone-binding regions of the luteinizing hormone/human chorionic gonadotropin receptor using synthetic peptides. *Endocrinology* 131: 268-74
21. Hong S, Phang T, Ji I, Ji TH. 1998. The amino-terminal region of the lutropin/choriogonadotropin receptor contacts both subunits of human choriogonadotropin: I. mutational analysis. *J Biol Chem* 273: 13835-40
22. Phang T, Kundu G, Hong S, Ji I, Ji TH. 1998. The amino-terminal region of the lutropin/choriogonadotropin receptor contacts both subunits of human choriogonadotropin: II. photoaffinity labeling. *J Biol Chem* 273: 138401-13847
23. Kobe B, Deisenhofer J. 1993. Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *Nature* 366: 751-6



24. Yi CS, Song YS, Ryu KS, Sohn J, Ji I, Ji TH. 2002. Common and differential mechanisms of gonadotropin receptors. *Cell Mol Life Sci* 59: 932-40
25. Thomas D, Rozell T, Liu X, Segaloff D. 1996. Mutational analyses of the extracellular domain of the full-length lutropin/choriogonadotropin receptor suggest leucine-rich repeats 1-6 are involved in hormone binding. *Mol Endocrinol* 10: 760-8
26. Song YS, Ji I, Beauchamp J, Isaacs NW, Ji TH. 2001. Hormone interactions to Leu rich repeats in the gonadotropin receptors: I. analysis of Leu rich repeats of human luteinizing hormone/chorionic gonadotropin receptor and Follicle Stimulating Hormone Receptor. *J Biol Chem* 276: 3426-35
27. Song YS, Ji I, Beauchamp J, Isaacs NW, Ji TH. 2001. Hormone interactions to Leu rich repeats in the gonadotropin receptors: II. analysis of Leu rich repeat 4 of human luteinizing hormone/chorionic gonadotropin receptor. *J Biol Chem* 276: 3436-42
28. Jeoung M, Phang T, Song YS, Ji I, Ji TH. 2000. Hormone interactions to Leu rich repeats in the gonadotropin receptors: III. photoaffinity labeling of human chorionic gonadotropin with receptor Leu rich repeat 4 peptide. *J Biol Chem*
29. Zeng H, Phang T, Ji I, Ji TH. 2001. The role of the hinge region in the luteinizing hormone receptor in hormone interaction and signal generation. *J Biol Chem* 276: 3451-8
30. Nakabayashi K, Kudo M, Kobilka B, Hsueh AJ. 2000. Activation of the luteinizing hormone receptor following substitution of ser-277 with selective hydrophobic residues in the ectodomain hinge region. *J Biol Chem* 275: 30264-71
31. Ji I, Ji TH. 1991. Human choriogonadotropin binds to a lutropin receptor with essentially no N-terminal extension and stimulates cAMP synthesis. *J Biol Chem* 266: 13076-9
32. Sohn J, Ryu K, Sievert G, Jeoung M, Ji I, Ji TH. 2002. Follicle-stimulating hormone interacts with exoloop 3 of the receptor. *J Biol Chem* 277: 50165-75
33. Ryu K, Lee H, Kim S, Beauchamp J, Tung CS, Isaacs NW, Ji I, Ji TH. 1998. Modulation of high affinity hormone binding: human choriogonadotropin binding to the exodomain of the receptor is influenced by exoloop 2 of the receptor. *J Biol Chem* 273: 6285-91
34. Ryu K, Gilchrist RL, Tung c, Ji i, Ji TH. 1998. High affinity hormone binding to the extracellular N-terminal exodomain of the follicle-stimulating hormone receptor is critically modulated by exoloop 3. *J Biol Chem* 273: 28953-8

35. Huhtaniemi I. 2000. The Parkes Lecture - Mutations of gonadotrophin and gonadotrophin receptor genes: what do they teach us about reproductive physiology? *J Reprod Fertil* 119: 173-86
36. Nishi S, Nakabayashi K, Kobilka B, Hsueh AJ. 2002. The Ectodomain of the luteinizing hormone receptor interacts with exoloop 2 to constrain the transmembrane region. Studies using chimeric human and fly receptors. *J Biol Chem* 277: 3958-64.
37. Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RAD, Hsueh AJW. 2003. H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. *J. Biol. Chem.* 278: 7855-62
38. Zhang M, Tong KP, Fremont V, Chen J, Narayan P, Puett D, Weintraub BD, Szekudlinski MW. 2000. The extracellular domain suppresses constitutive activity of the transmembrane domain of the human TSH receptor: implications for hormone-receptor interaction and antagonist design. *Endocrinology* 141: 3514-7
39. Vlaeminck-Guillem V, Ho SC, Rodien P, Vassart G, Costagliola S. 2002. Activation of the cAMP pathway by the TSH receptor involves switching of the ectodomain from a tethered inverse agonist to an agonist. *Mol Endocrinol* 16: 736-46
40. Remy JJ, Bozon V, Couture L, Goxe B, Salesse R, Garnier J. 1993. Reconstitution of a high-affinity functional lutropin receptor by coexpression of its extracellular and membrane domains. *Biochem Biophys Res Commun* 193: 1023-30
41. Sangkuhl K, Schulz A, Schultz G, Schoneberg T. 2002. Structural requirements for mutational lutropin/choriogonadotropin receptor activation. *J Biol Chem* 277: 47748-55.
42. Lee C, Ji I, Ryu K, Song Y, Conn PM, Ji TH. 2002. Two defective heterozygous luteinizing hormone receptors can rescue hormone action. *J Biol Chem* 277: 15795-800
43. Ji I, Lee C, Song Y, Conn PM, Ji TH. 2002. Cis- and Trans-Activation of Hormone Receptors: the LH Receptor. *Mol Endocrinol* 16: 1299-308
44. Kuhn B, Gudermann T. 1999. The luteinizing hormone receptor activates phospholipase C via preferential coupling to Gi2. *Biochemistry* 38: 12490-8
45. Ryu K, Gilchrist RL, Ji I, Kim SJ, Ji TH. 1996. Exoloop 3 of the LH/CG receptor: Lys583 is essential and irreplaceable for hCG dependent receptor activation but not for high affinity hCG binding. *J Biol Chem* 271: 7301-4

46. Gilchrist RL, Ryu K, Ji I, Ji TH. 1996. The luteinizing hormone/human choriogonadotropin receptor has distinct transmembrane conductors for cAMP and inositol phosphate signals. *J Biol Chem* 271: 19283-7
47. Huhtaniemi IT. 2002. LH and FSH receptor mutations and their effects on puberty. *Horm Res* 57 Suppl 2: 35-8
48. Page H. 1989. Estimation of the prevalence and incidence of infertility in a population: a pilot study. *Fertil Steril* 51: 571-7
49. Forti G, Krausz C. 1998. Evaluation and treatment of the infertile couple. *J Clin Endocrinol Metab* 83: 4177-88
50. Latronico AC, Segaloff DL. 1999. Naturally occurring mutations of the luteinizing-hormone receptor: lessons learned about reproductive physiology and G protein-coupled receptors. *Am J Hum Genet* 65: 949-58
51. Farrens D, Altenbach C, Yang K, Khorana H. 1996. Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science* 274: 768-70
52. Ascoli M, Fanelli F, Segaloff DL. 2002. The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocr Rev* 23: 141-74.
53. Kosugi S, Mori T, Shenker A. 1996. The role of Asp578 in maintaining the inactive conformation of the human lutropin/choriogonadotropin receptor. *J Biol Chem* 271: 31813-7
54. Laue LL, Wu SM, Kudo M, Bourdony CJ, Cutler GB, Hsueh AJ, Chan WY. 1996. Compound heterozygous mutations of the luteinizing hormone receptor gene in Leydig cell hypoplasia. *Mol Endocrinol* 10: 987-97.
55. Wu SM, Hallermeier KM, Laue L, Brain C, Berry AC, Grant DB, Griffin JE, Wilson JD, Cutler GB, Chan WY. 1998. Inactivation of the luteinizing hormone/chorionic gonadotropin receptor by an insertional mutation in Leydig cell hypoplasia. *Mol Endocrinol* 12: 1651-60.
56. Martens JW, Lumbroso S, Verhoef-Post M, Georget V, Richter-Unruh A, Szarras-Czapnik M, Romer TE, Brunner HG, Themmen AP, Sultan C. 2002. Mutant luteinizing hormone receptors in a compound heterozygous patient with complete leydig cell hypoplasia: abnormal processing causes signaling deficiency. *J Clin Endocrinol Metab* 87: 2506-13

57. Rousseau-Merck M, Misrahi M, Loosfelt H, Milgrom E, Berger R. 1990. Localization of the human luteinizing hormone/choriogonadotropin receptor gene (LHCGR) to chromosome 2p21. *Cytogenet Cell Genet* 54: 77-9
58. Beau I, Touraine P, Meduri G, Gougeon A, Desroches A, Matuchansky C, Milgrom E, Kuttenn F, Misrahi M. 1998. A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor. *J Clin Invest* 102: 1352-9.
59. Touraine P, Beau I, Gougeon A, Meduri G, Desroches A, Pichard C, Detoef M, Paniel B, Prieur M, Zorn JR, Milgrom E, Kuttenn F, Misrahi M. 1999. New natural inactivating mutations of the follicle-stimulating hormone receptor: correlations between receptor function and phenotype. *Mol Endocrinol* 13: 1844-54.
60. Doherty E, Pakarinen P, Tiitinen A, Kiilavuori A, Huhtaniemi I, Forrest S, Aittomaki K. 2002. A Novel mutation in the FSH receptor inhibiting signal transduction and causing primary ovarian failure. *J Clin Endocrinol Metab* 87: 1151-5.
61. McGee EA, Hsueh AJ. 2000. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* 21: 200-14
62. Ji TH, Murdoch WJ, Ji I. 1995. Activation of membrane receptors. *Endocrine* 3: 187-94
63. Dufau ML. 1998. The luteinizing hormone receptor. *Annu. Rev. Physiol.* 60: 461-96
64. Chen C, Okayama H. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7: 2745-52
65. Ji I, Ji TH. 1980. Macromolecular photoaffinity labeling of the lutropin receptor on granulosa cells. *Proc Natl Acad Sci U S A* 77: 7167-70
66. Hong S, Ryu K-S, Oh M-O, Ji I, Ji TH. 1997. Roles of transmembrane prolines and proline-induced kinks of the lutropin/choriogonadotropin receptor. *J Biol Chem* 272: 4166-71
67. Tsai-Morris CH, Buczko E, Wang W, Dufau ML. 1990. Intronic nature of the rat luteinizing hormone receptor gene defines a soluble receptor subspecies with hormone binding activity. *J Biol Chem* 265: 19385-8
68. Ji I, Pan Y-N, Lee Y-M, Phang T, Ji TH. 1995. Receptor binding dependent structural changes in human choriogonadotropin: photochemical inter-subunit crosslinking. *Endocrine* 3: 907-11

69. Hong S, Ji I, Ji TH. 1999. The alpha-subunit of human choriogonadotropin interacts with the exodomain of the luteinizing hormone/choriogonadotropin receptor. *Endocrinology* 140: 2486-93
70. Hong SH, Ji IH, Ji TH. 1999. The beta-subunit of human choriogonadotropin interacts with the exodomain of the luteinizing hormone/choriogonadotropin receptor and changes its interaction with the alpha-subunit. *Mol Endocrinol* 13: 1285-94
71. Dufau ML, Watanabe K, Catt KJ. 1973. Stimulation of cyclic AMP production by the rat testis during incubation with hCG in vitro. *Endocrinology* 92: 6-11
72. Moyle WR, Ramachandran J. 1973. Effect of LH on steroidogenesis and cyclic AMP accumulation in rat Leydig cell preparations and mouse tumor Leydig cells. *Endocrinology* 93: 127-34
73. Mendelson CR. 2000. Mechanisms of hormone action. In *Textbook of Endocrine Physiology*, ed. JE Griffin, SR Ojeda, pp. 51-88. New York: Oxford University Press
74. Browne ES, Bhalla VK. 1991. Does gonadotropin receptor complex have an amplifying role in cAMP/testosterone production in Leydig cells? *J Androl* 12: 132-9
75. Catt KJ, Watanabe K, Dufau ML. 1972. Cyclic AMP released by rat testis during gonadotrophin stimulation in vitro. *Nature* 239: 280-1
76. Davis JS, West LA, Farese RV. 1984. Effects of luteinizing hormone on phosphoinositide metabolism in rat granulosa cells. *J Biol Chem* 259: 15028-34
77. Davis JS, Weakland LL, West LA, Farese RV. 1986. Luteinizing hormone stimulates the formation of inositol trisphosphate and cyclic AMP in rat granulosa cells. Evidence for phospholipase C generated second messengers in the action of luteinizing hormone. *Biochem J* 238: 597-604
78. Herrlich A, Kühn B, Grosse R, Schmid A, Schultz G, Gudermann T. 1996. Involvement of Gs and Gi proteins in dual coupling of the luteinizing hormone receptor to adenylyl cyclase and phospholipase C. *J Biol Chem* 271: 16764-72
79. Rajagopalan-Gupta RM, Mukherjee S, Zhu X, Ho YK, Hamm H, Birnbaumer M, Birnbaumer L, Hunzicker-Dunn M. 1999. Roles of Gi and Gq/11 in mediating desensitization of the luteinizing hormone/choriogonadotropin receptor in porcine ovarian follicular membranes. *Endocrinology* 140: 1612-21

80. Blank JL, Brattain KA, Exton JH. 1992. Activation of cytosolic phosphoinositide phospholipase C by G-protein beta gamma subunits. *J Biol Chem* 267: 23069-75
81. Richards JS, Hedin L. 1988. Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. *Annu Rev Physiol* 50: 441-63
82. Zhu X, Gilbert S, Birnbaumer M, Birnbaumer L. 1994. Dual signaling potential is common among Gs-coupled receptors and dependent on receptor density. *Mol Pharmacol* 46: 460-9.
83. Shimamoto T, Yamoto M, Nakano R. 1993. Possible involvement of protein kinase C in gonadotropin-induced ovulation in the rat ovary. *Endocrinology* 133: 2127-32
84. Davis JS. 1992. Modulation of luteinizing hormone-stimulated inositol phosphate accumulation by phorbol esters in bovine luteal cells. *Endocrinology* 131: 749-57
85. Liu G, Duranteau L, Carel JC, Monroe J, Doyle DA, Shenker A. 1999. Leydig-cell tumors caused by an activating mutation of the gene encoding the luteinizing hormone receptor. *N Engl J Med* 341: 1731-6.
86. Osuga Y, Kudo M, Kaipia A, Kobilka B, Hsueh AJ. 1997. Derivation of functional antagonists using N-terminal extracellular domain of gonadotropin and thyrotropin receptors. *Mol. Endocrinol.* 11: 1659-68
87. Rajagopalan-Gupta RM, Lamm MLG, Mukherjee S, Rasenick MM, Hunzicker-Dunn M. 1998. Luteinizing hormone/choriogonadotropin receptor-mediated activation of heterotrimeric guanine nucleotide binding proteins in ovarian follicular membranes. *Endocrinology* 137: 4547-55
88. Hirsch B, Kudo M, Naro F, Conti M, Hsueh AJ. 1996. The C-terminal third of the human luteinizing hormone (LH) receptor is important for inositol phosphate release: analysis using chimeric human LH/follicle-stimulating hormone receptors. *Mol Endocrinol* 10: 1127-37
89. Van Sande J, Raspe E, Perret J, Lejeune C, Maenhaut C, Vassart G, Dumont JE. 1990. Thyrotropin activates both the cyclic AMP and the PIP2 cascades in CHO cells expressing the human cDNA of TSH receptor. *Mol Cell Endocrinol* 74: R1-6
90. Stanislaus D, Ponder S, Ji TH, Conn PM. 1998. Gonadotropin-releasing hormone receptor couples to multiple G proteins in rat gonadotrophs and in GGH3 cells: evidence from palmitoylation and overexpression of G proteins. *Biol Reprod* 59: 579-86

91. Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D, Huang J, Jaye M, Crumley G, Schlessinger J, Lax I. 1994. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* 79: 1015-24
92. Pisegna JR, Wank SA. 1996. Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase C. *J Biol Chem* 271: 17267-74
93. Abou-Samra AB, Juppner H, Force T, Freeman MW, Kong XF, Schipani E, Urena P, Richards J, Bonventre JV, Potts J, Jr., Kronenberg HM, Segre GV. 1992. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci U S A* 89: 2732-6
94. Houssami S, Findlay DM, Brady CL, Myers DE, Martin TJ, Sexton PM. 1994. Isoforms of the rat calcitonin receptor: consequences for ligand binding and signal transduction. *Endocrinology* 135: 183-90
95. Lustig KD, Shiau AK, Brake AJ, Julius D. 1993. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci U S A* 90: 5113-7
96. Qi AD, Kennedy C, Harden TK, Nicholas RA. 2001. Differential coupling of the human P2Y(11) receptor to phospholipase C and adenylyl cyclase. *Br J Pharmacol* 132: 318-26
97. Mitsuhashi M, Mitsuhashi T, Payan DG. 1989. Multiple signaling pathways of histamine H2 receptors. Identification of an H2 receptor-dependent Ca<sup>2+</sup> mobilization pathway in human HL-60 promyelocytic leukemia cells. *J Biol Chem* 264: 18356-62
98. Liang M, Freedman NJ, Theiss CT, Liggett SB. 2001. Serine 232 of the alpha(2A)-adrenergic receptor is a protein kinase C-sensitive effector coupling switch. *Biochemistry* 40: 15031-7.
99. Qi AD, Zamboni AC, Insel PA, Nicholas RA. 2001. An arginine/glutamine difference at the juxtaposition of transmembrane domain 6 and the third extracellular loop contributes to the markedly different nucleotide selectivities of human and canine P2Y11 receptors. *Mol Pharmacol* 60: 1375-82.

100. Biebermann H, Schoneberg T, Schulz A, Krause G, Gruters A, Schultz G, Gudermann T. 1998. A conserved tyrosine residue (Y601) in transmembrane domain 5 of the human thyrotropin receptor serves as a molecular switch to determine G-protein coupling. *FASEB J* 12: 1461-71
101. Arseven OK, Wilkes WP, Jameson JL, Kopp P. 2000. Substitutions of tyrosine 601 in the human thyrotropin receptor result in increase or loss of basal activation of the cyclic adenosine monophosphate pathway and disrupt coupling to Gq/11. *Thyroid* 10: 3-10.
102. Davio C, Mladovan A, Lemos B, Monczor F, Shayo C, Rivera E, Baldi A. 2002. H1 and H2 histamine receptors mediate the production of inositol phosphates but not cAMP in human breast epithelial cells. *Inflamm Res* 51: 1-7.
103. Gilchrist A, Vanhauwe JF, Li A, Thomas TO, Voyno-Yasenetskaya T, Hamm HE. 2001. G alpha minigenes expressing C-terminal peptides serve as specific inhibitors of thrombin-mediated endothelial activation. *J Biol Chem* 276: 25672-9
104. Feldman DS, Zamah AM, Pierce KL, Miller WE, Kelly F, Rapacciuolo A, Rockman HA, Koch WJ, Luttrell LM. 2002. Selective inhibition of heterotrimeric Gs signaling. Targeting the receptor-G protein interface using a peptide minigene encoding the Galpha(s) carboxyl terminus. *J Biol Chem* 277: 28631-40
105. Shenker A, Laue L, Kosugi S, Merendino J, Jr., Minegishi T, Cutler G, Jr. 1993. A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* 365: 652-4
106. Themmen APN, Huhtaniemi IT. 2000. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. *Endocr Rev* 21: 551-83.
107. Ji I, Ji TH. 1991. Asp383 in the second transmembrane domain of the lutropin receptor is important for high affinity hormone binding and cAMP production. *J Biol Chem* 266: 14953-7
108. Gromoll J, Simoni M, Nieschlag E. 1996. An activating mutation of the follicle-stimulating hormone receptor autonomously sustains spermatogenesis in a hypophysectomized man. *J Clin Endocrinol Metab* 81: 1367-70



109. Berlot CH. 2002. A highly effective dominant negative alpha s construct containing mutations that affect distinct functions inhibits multiple Gs-coupled receptor signaling pathways. *J Biol Chem* 277: 21080-5

## VITA

Name                                ChangWoo Lee  
Date of Birth                      April 03, 1973  
Place of Birth                      Taegu, Korea

## EDUCATION

B.S.    1997    Taegu University, Kyungsan, Korea

## PUBLICATIONS

1. Lee C, Ji I, Ryu K, Song Y, Conn PM, Ji TH. 2002. Two Defective Heterozygous Luteinizing Hormone Receptors Can Rescue Hormone Action. **Journal of Biological Chemistry**. 277, 15795-800.
2. Ji I, Lee C, Song Y, Conn PM, Ji TH. 2002. Cis- and Trans-Activation of Hormone Receptors: the LH Receptor. **Molecular Endocrinology**. 16, 1299-308.
3. Lee C, Ji I, Ji T. 2002. Use of Defined-function Mutants to Access Receptor-Receptor Interactions. **Methods**. 27, 318-323.
4. Song Y, Lee C, Ji I, Ji TH. 2002. Luteinizing Hormone. **Wiley Encyclopedia of Molecular Medicine** 1962-65.

## RESEARCH EXPERIENCE

1999 - 2003    Graduate Student & Research Assistant  
                          University of Kentucky, Lexington, Kentucky  
                          Department of Biology  
                          Principal Investigator: Dr. Tae H. Ji

1997 - 1999 Graduate Student & Research Assistant  
University of Wyoming, Laramie, Wyoming  
Department of Molecular Biology  
Principal Investigator: Dr. Tae H. Ji

1993 - 1997 Undergraduate Student & Research Assistant  
Taegu University, Kyungsan, Korea  
Department of Molecular Biology  
Principal Investigator: Dr. Sei-Heon Jang

### **SCHOLARSTIC HONORS**

2002 Second Place, Poster Presentation, Predoctoral Scholar Division  
The 21st Annual University of Kentucky Symposium in Reproductive Sciences

2001 First Place, Poster Presentation, Predoctoral Scholar Division  
The 20th Annual University of Kentucky Symposium in Reproductive Sciences

1996 Taegu University Alumni Association Scholarship, Taegu University, Korea

1995-1996 College Student Scholarship, The Korea Foundation for Advanced Studies