IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR

Myoungkun Jeoung
University of Kentucky, mkjeou0@uky.edu

Recommended Citation
https://uknowledge.uky.edu/gradschool_diss/264
IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND LUTEINIZING HORMONE/CHORIOGONADOTROPIN 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
Myoungkun Jeoung
Lexington, Kentucky

Director: Dr. Tae H. Ji, Professor of Chemistry
Lexington, Kentucky
2003

Copyright © Myoungkun Jeoung 2003
ABSTRACT OF DISSERTATION

IDENTIFICATION AND CHARACTERIZATION OF THE CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR

The luteinizing hormone receptor (LHR) belongs to the G protein-coupled receptor family. It consists of two distinct domains; the N-terminal extracellular exodomain and the membrane associated endodomain which includes 7 transmembrane domains, 3 exoloops, 3 cytoloops and a C-terminal tail. Sequence alignment and computer modeling suggest the presence of Leu Rich Repeat (LRR) motifs in the exodomain. Although their structural similarity is high, each LRR is not equally important for hormone binding. Ala-scanning and truncation studies performed in our laboratory suggest that LRR2 and LRR4 appear to be the most crucial. The Ala-scanning data suggest that Leu103 and Ile105 in LRR4 are important for hormone binding. However, it is not clear whether these two residues make direct contact with human chorionic gonadotropin (hCG) or if they are necessary for the overall structural integrity of LRR4. In this work, the LHR peptide mimics of LRR4 were used for photoaffinity labeling to determine whether Leu103 and Ile105 directly interact with hormone. Furthermore, LRR4 peptides containing the photoactivable benzoylphenylalanine (Bpa) were used to determine whether the LRR structure really exists in the LHR exodomain, whether LRR4 interact with hCG, and which residues of LRR4 interact with hCG. Bpa was directly incorporated into different positions of the LRR4 peptide sequence to examine the labeling ability of individual amino acids. The results suggest that LRR4, in particular the sequence of Lys101-Cys106, makes direct contact with hCG. However Leu103 and Ile105 do not interact with hCG but may form the hydrophobic core of the LRR4 loop, which appears to be crucial for the LRR structure.
Existing data suggest that glycoprotein hormones initially bind the exodomain. The hormone/exodomain complex undergoes conformational adjustments and stimulates the endodomain of the receptor to generate hormone signals. The exoloops modulate hormone binding and signaling; however, little is known about whether the hormone/exodomain complex contacts the endodomain. To address this issue, we investigated whether the exoloops interact with the hormone. First, we examined exoloop 3 that connects transmembrane domains 6 and 7 which are important for signal generation. We present the first physical evidence that LHR exoloop 3 interacts with hCG.

**KEYWORDS:** LHR, Leu Rich Repeat, exoloop 3, Bpa, photoaffinity labeling
IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR

By

Myoungkun Jeoung

Dr. Tae H. Ji
Director of Dissertation

Dr. Peter Mirabito
Director of Graduate Studies

April 24, 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 requires also the consent of the Dean of the Graduate School of the University of Kentucky.
DISSERTATION

Myoungkun Jeoung

The Graduate School
University of Kentucky
2003
IDENTIFICATION AND CHARACTERIZATION OF THE CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND LUTEINIZING HORMONE/CHORIOGONADOTROPIN 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

Myoungkun Jeoung

Lexington, Kentucky

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

Lexington, Kentucky

2003

Copyright © Myoungkun Jeoung 2003
ACKNOWLEDGMENTS

I would first like to acknowledge and thank my parents and family for their loving support, assistance, and encouragement. Without them, neither my life nor my academic career, thus far, could have been nearly as enjoyable or beneficial.

The completion of this dissertation has been possible due to the guidance and patience of Dr. Tae H. Ji. His inputs, advise, supervision, and direction in the past five years have been invaluable. He has been a tremendous advisor, and I cannot thank him enough. I thank Dr. Inhae Ji for your encouragement to ensure that I could finish this dissertation. Your support carried me through the worst of it.

Additionally, I would like to thank the members of my Committee, Dr. Sheldon Steiner, Dr. Doug Harrison, Dr. Robin L. Cooper, and Dr. Boyd E. Haley, for their comments, revisions, advice and support through this process.

The lab members of Dr. Tae H. Ji’s lab were also a source of support and encouragement. I would especially like to thank Dr. ChangWoo Lee for his willingness to discuss research ideas with me for the past five years. I also thank Gail Sievert for being my other sister. She has a very warm heart. I thank the previous lab members, Rebecca McCaffrey, Dr. TzuLip. Pang, Dr. YongSang Song, Dr. ChongSeong Yi, Dr. YongBum Koo, Dr. Chemyong Ko, for their encouragement and advice. I also thank the current lab members, Dr. HyeSook Youn, Johann Sohn, and Katie Gieski for their support.

Finally, my wife, Jaekeum Nam, provided the emotional support to see this dissertation through to the end. I am a very fortunate husband to have your wonderful support.
### TABLE OF CONTENTS

Acknowledgments ........................................................................................................................ iii

List of Figures.................................................................................................................................. vi

List of Files.................................................................................................................................... viii

**Chapter One: Introduction** ........................................................................................................... 1

1. Structure of Glycoprotein Hormones ........................................................................ 1

2. Biological Functions of Glycoprotein Hormones ...................................................... 3

3. LH/CG Receptor Structure ....................................................................................... 3

4. Hormone-Receptor Interaction ................................................................................... 8

5. Modulation of Hormone Binding and Signaling by the Endodomain of Glycoprotein Hormone Receptor .............................. 11

6. Receptor Activation and Signal Generation .............................................................. 12

7. Photoaffinity Labeling and Affinity Cross-Linking .................................................. 13

8. Significances and Goal of Dissertation ...................................................................... 15

**Materials** .................................................................................................................................. 19

**Methods** .................................................................................................................................. 19

1. Radioiodination of hCG or Deglycosylated hCG ..................................................... 19

2. Derivatization and Radioiodination of Peptides ....................................................... 19

3. Affinity Cross-Linking of $^{125}$I-LHR$^{96-115}$ to hCG .............................................. 20

4. Photoaffinity labeling of hCG ................................................................................. 20

5. Competitive Inhibition of Affinity and photoaffinity Labeling of hCG ....................... 21

6. Inhibition of $^{125}$I-hCG Binding to LHR ................................................................. 21

**Chapter Two: hLHR peptide 96-115 labeling hCG** .................................................................. 22

A. Introduction ......................................................................................................................... 22

B. Results ................................................................................................................................... 23

1. Photoaffinity Labeling of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ to hCG ................................. 23

2. Labeling Specificity .......................................................................................................... 25

3. Biological Specificity of Photoaffinity Labeling ......................................................... 28

4. Photoaffinity Labeling Scanning on β Strand of LRR4 .................................................. 28
5. Photoaffinity Scanning
   on Proximal Region of β Strand of LRR4 .........................................................31
6. The Displacement Test with the Peptides from Other Regions of the
   Exodomain and the Endodomain of LHR..........................................................34
C. Discussion .............................................................................................................38
Chapter Three: hLHR peptide labeling of exoloop 3 ..............................................42
A. Introduction...........................................................................................................42
B. Results ...................................................................................................................43
   1. Exoloop 3 Plays Crucial and Differential Roles
      in cAMP and IP Induction .................................................................43
   2. Photoaffinity Labeling of hCG with Exoloop 3 Peptide Mimic ..................43
   3. Specificity of Exoloop 3 Labeling ...............................................................45
   4. Relationship with Exoloops 1, 2, and Other Regions of Exodomain ..........53
C. Discussion .............................................................................................................53
Appendices .................................................................................................................59
   Abbreviation ...........................................................................................................59
References ..................................................................................................................60
Vita ..............................................................................................................................66
LIST OF FIGURES

Chapter One

Figure 1-1: Human chorionic gonadotropin (hCG) crystal structure...............................2
Figure 1-2: Structure of the gonadotropin receptors......................................................4
Figure 1-3: Crystal structure of porcine ribonuclease inhibitor .....................................6
Figure 1-4: Model for the interaction of the LH/CG receptor exodomain with hCG........7
Figure 1-5: Computer model of the LH/CG receptor endodomain.................................9
Figure 1-6: LH/CG receptor activation and signal transduction.....................................10
Figure 1-7: General scheme of photoaffinity labeling..................................................14
Figure 1-8: Structure and photochemistry of 4-benzoyl-L-phenylalanine(Bpa) .............16

Chapter Two

Figure 2-1: Model of LRR4, the structure of benzoyl-L-phenylalanine (Bpa),
and the sequence alignment of wild type and Bpa analog peptides.........................24
Figure 2-2: Autoradiograph of photoaffinity labeled hCG subunits...............................26
Figure 2-3: Specificity of photoaffinity labeling ..........................................................27
Figure 2-4: Photoaffinity labeling of denatured hCG
and inhibition of $^{125}$I-hCG binding to the receptor by LHR$^{96-115}$..................29
Figure 2-5: Photoaffinity labeling of hCG with $^{125}$I-Bpa-LHR$^{96-115}$ peptides ..........30
Figure 2-6: Inhibition of $^{125}$I-Bpa-LHR$^{96-115}$ photoaffinity labeling.........................32
Figure 2-7: Inhibition of $^{125}$I-hCG binding to the receptor
by $^{125}$I-Bpa-LHR$^{96-115}$ peptides ...............................................................33
Figure 2-8: Photoaffinity labeling of hCG with $^{125}$I-Bpa-LHR$^{96-115}$ peptides ..........35
Figure 2-9: Inhibition of $^{125}$I-hCG binding to the receptor
by $^{125}$I-Bpa-LHR$^{96-115}$ peptides ...............................................................36
Figure 2-10: Inhibition of $^{125}$I-hCG photoaffinity labeling
with different region LHR peptides ...............................................................37
Figure 2-11: Model of the hCG, exodomain and endodomain of LH receptor ..........40
Chapter Three

Figure 3-1: Differential effects of Ala substitutions on hormone binding, cAMP and IP ..................................................44
Figure 3-2: Autoradiograph of photoaffinity labeled hCG subunits.................................................................46
Figure 3-3: Photoaffinity labeling of denatured hCG .....................................................................................48
Figure 3-4: Fractionation of denatured hCG ......................................................................................................49
Figure 3-5: Inhibition of $^{125}$I-hCG binding to the receptor by LHR$^{exo3}$ ..............................................50
Figure 3-6: Photoaffinity labeling of LH .............................................................................................................51
Figure 3-7: Specificity of photoaffinity labeling .................................................................................................52
Figure 3-8: Photoaffinity labeling of FSH .........................................................................................................54
Figure 3-9: Effects of other LHR peptides on photoaffinity labeling of hCG ...........................................55
LIST OF FILES

MJ.pdf............................................................................................................................ 3.7 MB
Chapter One

Introduction

1. Structure of Glycoprotein Hormones

Human chorionic gonadotropin (hCG) and luteinizing hormone (LH) belong to the glycoprotein hormone family, which also includes follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). They consist of two noncovalently associated protein subunits, a ~15 kD \( \alpha \)-subunit and a ~23 kD \( \beta \)-subunit (1). For each mammalian species, the common \( \alpha \) subunits are encoded by a single gene and have the identical amino acid sequence (2). In contrast, the \( \beta \) subunits differ for each glycoprotein hormone and are encoded by distinct genes (2, 3). The \( \alpha \)-subunit consists of 92 amino acids as compared to 145 amino acids in the \( \beta \)-subunit of hCG, the LH \( \beta \)-subunit has 121 amino acids. The sequences of hCG \( \beta \)- and LH \( \beta \)-subunits are similar except for 16 residues and the extra 24 amino acids at the hCG C-terminal region. Both hormones have similar structure and bind to the same receptor (LH/CG receptor). In 1994, the crystal structure of deglycosylated hCG was reported (4) (Figure 1-1). hCG subunits are tightly, non-covalently associated with each other. Even with no sequence homology, both subunits share similar polypeptide folds. The two individual subunits are stabilized by three intra-chain disulfides for each subunit (5). Each chain is folded into 3 loops: \( \alpha L1 \), \( \alpha L2 \), and \( \alpha L3 \) for the \( \alpha \)-subunit and \( \beta L1 \), \( \beta L2 \) and \( \beta L3 \) for \( \beta \)-subunit. In addition, a segment of the \( \beta \)-subunit wraps around the \( \alpha \)-subunit. This loop is formed by a Cys\(^{90}\)-Cys\(^{110}\) disulfide bond that is known as the seat belt region. It plays an important role in receptor binding and maintaining the structural integrity of the heterodimer (4, 6, 7). FSH also has this seat-belt loop structure (8).

Both subunits of hCG are heavily glycosylated with two N-linked glycosylation sites on both the \( \alpha \)-and \( \beta \)-subunits and four additional O-linked glycosylation sites on the \( \beta \)-subunit. The carbohydrates on the hormone account for about 30-35% of the total mass of the hormone (9, 10). The carbohydrates on the hCG are not necessary for receptor binding. Deglycosylated hCG treated with anhydrous hydrofluoric acid can bind to its receptor with slightly increased affinity. However, deglycosylated hCG can not induce the hormone signaling (11-13). Only the dimer is capable of binding to receptors to induce biological responses (1), whereas the dissociated
Figure 1-1. Human chorionic gonadotropin (hCG) crystal structure

Deglycosylated hCG has been crystallized. hCG consists of α subunit (red) and β subunit (green). Both subunits are folded into a three-loop-structure, tightly intertwined with each other. A segment of the β subunit wraps around the α subunit like a seat belt (blue).
subunits lose high affinity binding activity (14).

2. Biological Functions of Glycoprotein Hormones

FSH, LH and TSH are secreted from the anterior pituitary gland. While hCG is produced from multinuclear syncytiotrophoblast cells in the human placenta. These hormones bind to their cognate receptors and generate signals into the target cells. In general, the signaling involves the activation of adenylyl cyclase (AC) to produce cAMP (14) and phospholipase Cβ (PLCβ) to produce inositol trisphosphate (IP3) and diacylglycerol (DAG) (15). LH and FSH stimulate ovarian and testicular functions by regulating gametogenesis and steroid hormone synthesis in the gonads. TSH regulates the release of thyroid hormones (1).

LH binds to the LH receptor (LHR) expressed in thecal and granulosa cells in the female ovary and Leydig cells in the male testis. LH initiates steroidogenesis. In the ovary, LH promotes the ovulation of fully developed Graffian follicles and luteinization (16, 17).

FSH and FSHR play crucial roles in the gonad development and gamete production in both sexes. FSHR is present in granulosa cells in the ovary and Sertoli cells in the testis (18). hCG stimulates progesterone production in the ovarian corpus luteum, which prevents the onset of the new ovulation cycle by stimulating the ovary to maintain the corpus luteum and by prolonging progesterone synthesis (17). During pregnancy, relatively high amounts of hCG can be detected in urine and serum. Elevated hCG in urine and serum is used for the pregnancy test. Intact hCG and hCG fragments have been detected in 25% of ovarian and 35% of testicular tumors (19, 20). In urine and serum of tumor patients, the fragments of the β-subunit have been detected. Based on these observations, hCG can be used for tumor detection in addition to the pregnancy test (18).

3. LH/CG Receptor Structure

The glycoprotein hormone receptors belong to the superfamily of G protein-coupled receptors (GPCRs) (21). Nearly 2000 GPCRs have been cloned. GPCRs consist of seven transmembrane domains (TM), an extracellular N-terminal segment, 3 extracellular loops (exoloops), 3 cytoplasmic loops (cytoloops), and a C-terminal tail (Figure 1-2) (22). The identified ligands of GPCRs are proteins, peptides, neurotransmitters, biogenic amines, odorants, and photons (21). There is relationship between the size of ligand and the length of exodomain.
Figure 1-2. Structure of the gonadotropin receptors

The LH/CG receptor consists of a ~350 amino-acid long extracellular domain (exodomain) and a membrane-associated domain (endodomain) of equal size. The exodomain is thought to include 8~9 Leu-rich repeats (LRRs), each consisting of a β strand (shown as arrow) and an α helix (shown as green cylinder). A schematic model of the LRRs in the exodomain and the seven transmembrane domains in the endodomain is shown. The endodomain consists of three exoloops, three cytoloops, seven transmembrane domains, and a C-terminal tail.
of its cognate receptor (23). For example, the exodomain of adrenergic receptor is 11-30 amino acids. The N-terminal halves of the glycoprotein hormone receptors are ~350 amino acids (21, 23).

The LH receptor has a molecular weight of 75 kD and comprises 674 amino acid residues in human. It consists of two equal halves, an extracellular N-terminal half (exodomain) and a membrane associated C-terminal half (endodomain) as shown in Fig 1-2 (24-28). The ~350 amino acid exodomain alone is capable of high affinity hormone binding (29-31), although this binding can not generate hormone action (31, 32). Receptor activation occurs in the endodomain which is similar in size to the entire molecule of many other GPCRs (21, 33). The crystal structure of porcine ribonuclease inhibitor shows that there are 15 Leu-Rich-Repeats (Leu/Ile-X-Leu/Ile), and the ligand of pRI binds to β inner lining (34, 35) (Figure 1-3). The glycoprotein hormone receptors have been modeled from the established Leu-Rich-Repeat (LRR) crystal structures, sequence alignment, and computer modeling (36-38). In the LH receptor, the exodomain assumed to have 8-9 LRRs, and the inner lining presumably interacts with the hormone. LRRs are 20-29-residue sequence motifs present in a number of proteins with diverse functions. The primary function of these motifs appears to provide a versatile structural framework for protein–protein interactions. Each LRR consists of a short β strand linked to an α helix in parallel to each other, and LRRs form a horseshoe-shaped structure with the inner lining of parallel β strands and the outer lining of α helices (Figure 1-3). LRR is stabilized by hydrophobic interactions among residues, particularly involving Leu and Ile. It has been suggested that hCG interacts with the inner side of the Leu-Rich Repeat structure (36) (Figure 1-4). The LRR domains, exon 2-8, of the LH, FSH, and TSH receptors share about forty three percent sequence similarities (22, 39). However, the similarity diminishes at the N- and C-terminal portion of the exodomain with amino acids insertion in the FSH and TSH receptors, suggesting that these regions may be important for hormone specificity (40-43). In the exodomain, LRRs are flanked by Cys-rich domains, which are thought to protect the first and last LRRs in the model (44). Many existing data support the importance of the N-terminal region of the glycoprotein hormone receptor for hormone binding (40, 43, 45, 46) and cell surface expression (47, 48).

The LHR exodomain has six consensus N-linked glycosylation sites with the consensus sequence
Figure 1-3. Crystal structure of porcine ribonuclease inhibitor
Porcine ribonuclease inhibitor (pRI) has been crystallized with its ligand. pRI has 15 Leu-rich repeat (LRR) motifs. Horseshoe conformation is formed from regular repeat units of β sheets (green) and α helical domains (red). Ribonuclease A (blue) binding sites are the inner lining of β turns.
Figure 1-4. Model for the interaction of the LH/CG receptor exodomain with hCG
The concave, front side of hCG (α subunit: red, β subunit: green) is thought to interact with the inner lining of the 1/3 donut structure formed by LRRs of the exodomain (blue).

hCG/exodomain complex is shown from the side (A) and from the top (B).
of Asn-xxx-Ser/Thr (6, 49, 50). Enzymatic removal of the glycans from the exodomain did not affect the hormone binding, suggesting that they are important for targeting but not for hormone binding and receptor activation (6).

Each of the seven transmembrane (TM) domains of LH receptor is generally composed of 20-27 amino acids. The seven TMs of rhodopsin and adrenergic receptors are arranged as a closed loop in the counterclockwise direction from TM 1 to TM 7 when viewed from the extracellular surface (51) (Figure 1-5). The intracellular C-terminal tail of the receptor contains phosphorylation sites by protein kinase C. This region also contains several Ser and Thr residues that can be phosphorylated by protein kinase A. Although the overall sequence homology is low among glycoprotein hormone receptors and other GPCR, the homology is high at the endodomains of the glycoprotein hormone receptors (22, 39).

4. Hormone - Receptor Interaction

Existing data suggest that glycoprotein hormones initially bind to the exodomain, and the resulting hormone/exodomain complex undergoes conformational adjustments (23) to make in secondary contacts with the endodomain (51) (Figure 1-6). The secondary interaction is thought to be responsible for signal generation (6, 51, 52). Although the crystal structure of deglycosylated hCG has been determined (4, 53) (Figure 1-1), the details are still unknown how the structurally similar glycoprotein hormones specifically interact with their cognate receptors. The high affinity hormone binding to the receptor is necessary for hormone signal induction. Glycoprotein hormones utilize the same effectors, adenylyl cyclase and phospholipase C β (15), (54), (55). Therefore, it was speculated that the hormone specific β subunit would bind the exodomain, while the common α subunit would activate the endodomain to induce the common hormonal signals (56).

Several important contact sites of the receptor-binding domains within the hCG molecule are identified by hCG crystal structure and other studies using photoaffinity labeling, chemical modification, and mutational analyses. Previous studies showed that the amino acids important for receptor binding are located at α 40-50 (57) and the C-terminal region (α88-92) (58-60) in the α-subunit, hydrophobic residues (β38-57), positive charged hydrophilic residues (β94-99), and the seatbelt region in the β-subunit (61-66) (Figure 1-1). This has been supported by the observation that the exodomain makes direct contacts with both subunits of hCG (47, 67).
Figure 1-5. Computer model of the LH/CG receptor endodomain

The seven transmembrane helices, three exoloops (orange), and three cytoloops of the receptor endodomain are shown from the side (A), from the outside of the cell (B), and the arrangement of the transmembrane domains (1-7) as viewed from the outside of the cell (C). Upon hormone binding, the movements of TM 3 (red) and 6 (blue) have been reported from rhodopsin, β2-adrenergic receptor, and dopamine receptors.
Figure 1-6. LH/CG receptor activation and signal transduction

The receptor endodomain modulates hormone binding to the exodomain. The hormone/exodomain/endodomain complex undergoes conformational adjustments which are responsible for activating the receptor and transducing the hormone signals. Activated adenylyl cyclase produces cAMP. Activated phospholipase Cβ generates IP3 and diacyl glycerol. They regulate protein kinase A activation, Ca^{2+} mobilization, and protein kinase C activation, respectively.
However, limited information is available regarding the precise hormone contact residues and sites in the exodomain. Three peptide mimics of the exodomain, peptide^{21-38} (the N-terminal region), peptide^{102-115} (LRR 4), and peptide^{253-266} (hinge region) could inhibit ^{125}I-hCG binding to membranes expressing the LH/CG receptor (40). Several reports of mutagenesis within the exodomain, as well as chimeric and deletion studies have further localized the binding regions of the receptor. A number of ionic amino acids in the exodomain are found to be important for hormone binding (68). Cys residues in the N-terminal region are important for hormone binding (6). Several distinct regions have been found to impact hormone binding from sequential truncation analysis (43). They are near the boundaries of exons 1-2, exons 4-5, exons 5-6, and exons 9-10.

5. Modulation of Hormone Binding and Signaling by the Endodomain of Glycoprotein Hormone Receptor

The exodomain alone is capable of high affinity hormone binding with almost 2 times higher affinity than the whole receptor (69). The ~ 600 amino acid size of the hormone-exodomain complex can not enter into the tight TM core, therefore, the exoloops are the reasonable contact sites for the interaction with hormone/exodomain complex since they are close to exodomain and can modulate the TM core.

Exoloop1 is the longest of the three exoloops with 21 amino acids. However, it has not been studied extensively. Asp^{397} is conserved in all glycoprotein hormone receptors and is located at the junction of exoloop 1 and TM2, and the Asp^{397}Asn mutation was found to abolish hormone binding (70). It has not been proven whether exoloop 1 makes contact with the hormone/exodomain. Exoloop 2 consists of 20 amino acids, it makes contact with the ligand in many GPCRs (71). Ala substitutions for exoloop 2 residues of the LHR did not severely impair cAMP induction but reduced hormone binding affinity at Ser^{484}, Asn^{485}, Lys^{488} and Ser^{490} (69). Therefore, exoloop 2 is important for hormone binding, not for signal generation. Recently, it has been reported that exoloop 2 and hinge region constrain fly LGR (Leu-rich repeat-containing G protein-coupled receptors) in inactive state (72). Exoloop 3 is the shortest loop, consisting of 11 amino acids, and connects TM6 and TM7. It plays a crucial role in the generation of cAMP and IP signals. All Ala substituted mutants bound hCG and induced cAMP except for Lys^{583}. Lys^{583} is located in the junction of exoloop 3 and TM 7. The Lys^{583}Ala mutation significantly
increased hormone binding affinity. Particularly, the Lys<sup>583</sup>Arg mutant has normal cell surface expression and hormone binding affinity, but the cAMP and IP signals were abolished. This suggests that the hormone binding and receptor activation are separated (73, 74).

6. Receptor Activation and Signal Generation

The signal transduction in response to hormones is mediated by G protein-coupled receptors. Activated GPCRs recruit intracellular heterotrimeric G proteins and stimulate GTP/GDP exchange to initiate receptor-specific signals. Most of glycoprotein hormones utilize the same effectors, adenylyl cyclase to generate cAMP and phospholipase C β to produce inositol phosphates (IPs) and diacylglycerol (DAG) (15, 54, 55). G<sub>αs</sub> is responsible for the activation of adenylyl cyclase. The Gβγ subunit is known to activate phospholipase C β (75). The common seven TM helical structure in GPCRs is essential for signal transduction. Therefore, a common molecular mechanism has been suggested for activation of GPCRs. A simple mechanism to transmit signal to the cytoplasmic surface is the modulation of transmembrane helices, which can cause structural change in the cytoplasmic domains of receptors (23). There are several ways to accomplish this: rotation, piston, pulse, pivot and shuffle of TMs (23). Upon receptor activation, the movements of TMs have been reported for different receptors showing different movements in different TMs; in TM3 and TM6 of rhodopsin, β2-adrenergic receptor, dopamine receptors, muscarinic receptors (76, 77), in TM2 and TM7 of angiotensin II (AT1) receptor (78). In both rhodopsin and the β2-adrenergic receptor, fluorescence spectroscopic studies show the movement of the TM helices. Preventing TM3-TM6 movement blocks receptor-dependent G protein activation, implying that movement of these TM helices is critical for activation.

G protein-coupled receptors are maintained in an inactive conformation by intramolecular interactions. The important conformational constraints maintain the receptor, preferentially, in an inactive conformation, and the constraints are released upon activation. For example, cleavage of the thrombin receptor's amino-terminal exodomain at the Arg<sup>41</sup>-Ser<sup>42</sup> peptide bond is necessary and sufficient for receptor activation (79). When the exodomain was removed from TSHR, the basal level of cAMP was increased 4-7 fold without hormone binding (80). From fly LGRs study, it is known that the exoloop 2 and hinge region constrain the receptor in an inactive state (81).
Agonist-induced receptor dimerization is required for signal transduction for single TM receptors, such as growth factor receptors, which are thought to be activated by oligomerization (82, 83). Several reports provide evidence that GPCRs can also form dimers (84-86). However, different mechanisms of dimer formation were observed for different receptors suggesting that receptor oligomerization is not essential for G protein activation. Some neurotransmitter receptors form a disulfide-linked dimer (87, 88). There is evidence that the exodomain of one glycoprotein hormone receptor interacts with other receptor molecules to activate the endodomains of other receptors. The coexpression of two different defective mutants receptor could rescue the hormone signaling. (89-91).

7. Photoaffinity Labeling and Affinity Cross-Linking

A number of techniques have been employed to identify the protein-protein contact sites. These techniques include X-ray crystallography, protein modification, affinity labeling, and photoaffinity labeling. The first use of photogenerated reagents was introduced forty years ago (92). Photoaffinity labeling has become a widely used tool to identify residues in the binding sites of biological macromolecules. A photolabile reagent is attached to the macromolecule by a covalent bond. The photolysis of the complex generate a highly reactive species that labels chemical groups in the immediate environment (93) (Figure 1-7). Photoaffinity labeling takes advantage of the principle that the affinity of the ligand for the receptor is the primary driving force of interaction. Thus, this interaction would result in a site-specific modification of any amino acid in the vicinity of the binding domain on the receptor, as opposed to the chemical cross-linking, which is directed by the interacting amino acid for that particular reagent (94, 95). The general considerations for designing a photoaffinity label include the size of the photoactive group, its stability and reactivity, and the ease of synthesis. Carbene, nitrene and free radical generating agents have been widely used in the study of proteins for identification, elucidation of the ligand binding domain, and study of function of the protein (95).

A heterobifunctional reagent contains two different reactive groups: one photosensitive and the other conventional N-hydroxysuccinimide (NHS) ester and imidate. The general scheme of photoaffinity labeling is shown in figure 1-7. Receptor peptides are derivatized with photoactivable reagents in the dark and radioactively labeled, and the peptide derivative is incubated with hCG to allow the specific interaction. Since the binding is directed by the affinity
Receptor peptides are derivatized with heterobifunctional photoactivable NHS-ABG, and then the radioactively labeled and derivatized peptides are incubated with hCG to allow the specific interaction. After UV irradiation, the samples are separated on SDS-PAGE gel.
of the hCG for receptor peptide, the specificity of interaction is established in this step. A short UV irradiation generates the reactive group on the photoreactive moiety. The complex forms a covalent link with any amino acid approximately in the binding sites between hCG and receptor peptides. The cross-linked complexes are slubilized and separated by SDS-polyacrylamide gel electrophoresis.

A benzophenone (Bp) unit is commonly employed for photoaffinity labeling (95). It abstracts, irreversibly, hydrogen atom from available site (Figure 1-8). Benzophenone has several advantages over other photoprobes: it is more reactive with C-H bonds than are others, also it is chemically more stable than other probes. It has low reactivity toward water and other common solvents, and can be manipulated in ambient light and activated at long wave length (350-360 nm) UV which minimizes photolytic damage to proteins (95). The amino acid analog 4-benzoyl-L-phenylalanine (Bpa) has been used to study protein-protein interactions (96). Since benzophenone substituted amino acids are stable under typical peptide synthesis conditions, Bpa can be directly incorporated at a defined position into the peptide sequence during solid-phase synthesis. It makes it possible to the examination of individual amino acids for protein-protein interactions. The advantages of Bpa as a photoprobe generally compensate the disadvantages resulting from the bulky and hydrophobic moiety (96, 97).

The photoaffinity technique has been used to study the interaction of glycoprotein hormones and their receptors for several years and have produced many data. Our lab has synthesized a number of novel photosensitive, radioiodinatable and heterobifunctional reagents. We have established the macromolecular photoaffinity labeling technology (94, 98). Our novel photoprobes have been used to identify hormone and receptor interactions. In 1977, we used the cross-linking technique to identify the receptor on the cell surface. Our photoaffinity labeling studies demonstrated the interaction of both hormone subunits to the receptor (99). This was confirmed by independent studies of antibody binding, peptide binding, mutational analyses, chemical modification, and the hCG crystal structure (53, 57, 100-104). We have studied where the contact sites are located on hormone to receptor (60, 105-107). We also have examined where the contact sites are on the hormone, the exodomain, and endodomain of receptor (43, 47, 67, 108-113).

8. Significance and Goals of Dissertation

hCG, LH and LHR play important roles in reproduction, women’s health, and some
Figure 1-8. Structure and photochemistry of 4-benzoyl-L-phenylalanine (Bpa)

(A) Bpa has an amino group and a carboxyl group, allowing for direct incorporation at a defined position into a peptide by solid-phase synthesis. (B) Absorption of a photon at approximately 350 nm results in the formation of a diradical (1). The electrophilic oxygen interacts with weak C-H bonds (2), resulting in hydrogen abstraction. The ketyl (3) and alkyl (4) radicals recombine to form a new C-C bond (5). The diradical is only able to attack geometrically accessible C-H bonds.
cancers. The hormone-receptor interactions generate signals and induce the hormone action. For example, antagonists can bind to receptors but cannot induce hormonal responses (114, 115). Therefore, the ternary hormone-exodomain-endodomain complex should make the secondary conformational changes and adjustments to generate signals. To understand the receptor activation mechanism, it is necessary to identify the amino acids responsible for the primary and secondary contacts.

hCG is important for maintaining pregnancy during the first trimester. Insufficient amounts of hCG in the early pregnancy stage can cause miscarriage. In addition, mutations on hCG and its receptor can impair the signal generation, resulting in infertility. Approximately 6.7 million couples in the United States suffer from infertility (116). Analogs of the receptor and hormones could be used as therapeutics and diagnosis methods. Current steroid contraceptives have serious side effects since steroids can affect most body cells. Since gonadotropin receptors are found mainly in the reproductive organs, the drugs based on gonadotropins will have a higher specificity and fewer side effects.

Therefore, it is essential to identify the contact points between hormone and receptor. hCG, but not its receptor, has been crystallized (4). The receptor is difficult to crystallize due to its large flexible exodomain and transmembrane domains. The secondary contact sites may be hard to find even from the crystal structure. Understanding the hormone and receptor interactions will provide new insights in designing drugs and understanding the basic mechanism of GPCR activation. Previously several regions important for hormone binding were mapped (43). Ala-scanning and truncation studies on LHR suggest that some of the LRRs of the LH receptor are crucial for hormone binding. In particular, LRR2 and LRR4 appear to be the most crucial (43, 117, 118). These data also suggest that Leu\textsuperscript{103} and Ile\textsuperscript{105} in LRR4 are important for hormone binding (118). However, it is not clear whether these two residues make contacts with LH/CG or if they are necessary for the overall structural integrity of LRR4. Furthermore, LRR structure of LHR has been suggested from mutational analysis, sequence alignment and computer modeling. There is no direct evidence for the existence of LRR structure in the LHR.

The goals of this dissertation work are to identify where the contact sides are on LRR4 with hormone. Some amino acids of exoloop 3 in the endodomain modulate the hormone binding to the exodomain. It is necessary to examine whether exoloop 3 makes direct contacts with hCG.
To accomplish these goals, I have utilized synthetic peptides, photoaffinity scanning, and binding assays to identify the contact sites between hCG and LH receptor.
Materials

The N-hydroxysuccinimide (NHS) ester of 4-azidobenzoic acid (AB) was synthesized as described previously (119). The N-hydroxysulfosuccinimide esters of ethylene glycolbis(sulfosuccinimidylsuccinate) (SES) were purchased from Pierce. The hCG CR 127 and hCG subunits were supplied by the National Hormone and Pituitary Program. Denatured hCG was prepared by boiling hCG in 8 M urea for 30 min. Receptor peptides were synthesized and N-acetylated and C-amidated by Biosynthesis (Lewisville, TX). They were purified on a Vydac C18 high performance liquid chromatography column using a solvent gradient from 100% of 0.1% trifluoroacetic acid in water to 20% of 0.1% trifluoroacetic acid in water and 80% 1-propanol. The peptide mimics include the wild type receptor peptide corresponding to the LHR sequence of Asn<sup>96</sup>-Asp<sup>115</sup> (LHR<sup>96-115</sup>), a mutant LHR<sup>96-115</sup> with Leu<sup>103</sup> Ala and Ile<sup>105</sup> Ala mutations (LHR<sup>96-115(L103A/I105A)</sup>), a mutant LHR<sup>96-115</sup> with the Lys<sup>101</sup> Ala mutation (LHR<sup>96-115(K101A)</sup>), a mutant LHR<sup>96-115</sup> with the Lys<sup>112</sup> Ala mutation (LHR<sup>96-115(K112A)</sup>). Bpa-containing LH/CG receptor peptides were synthesized, N-acetylated and C-amidated in >95% pure grade by Genemed Synthesis, Inc. (San Francisco, CA).

Methods

1. Radioiodination of hCG or deglycosylated hCG

1 mCi of Na<sup>125</sup>I in 10 µl of 0.1 M NaOH and 7 µl of chloramine T, initiating the reaction (1 mg/ml) in 0.1 M sodium phosphate (pH 7.5) were added to 5 µg hCG or deglycosylated hCG in 40 µl of 0.1 M sodium phosphate. After 20 seconds, 7 µl of sodium metabisulfite (2.5 mg/ml) in 0.1 M sodium phosphate was added to terminate the iodination reaction. Radioiodinated <sup>125</sup>I-hCG solution was mixed with 60 µl of 16% sucrose solution in 0.1 M sodium phosphate and fractionated on a Sephadex Superfine G-50 column (0.6 x 15 cm). 0.1% gelatin in 10 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.9% NaCl (pH 7.4) (PBS) was used to pre-equilibrate the column and elute the sample.

2. Derivatization and Radioiodination of Peptides

NHS-ABG was freshly dissolved in dimethyl sulfoxide to a concentration of 50 mM, and then diluted with 0.1 M sodium phosphate (pH 7.5) to make a concentration of 20 mM NHS-
ABG. This reagent solution was immediately used to derivatize receptor peptides. In the dark, 10 µl of NHS-ABG was added to 30 µg of LHR receptor peptide in 40 µl of 0.1 M sodium phosphate (pH 7.5). The mixture was incubated for 30 min for NHS-ABG at 25 °C. The following were added to the derivatization mixture: 1 mCi of Na$^{125}$I in 10 µl of 0.1 M NaOH and 7 µl of chloramine T (1 mg/ml) in 10 mM Na$_2$HPO$_4$ and 0.9% NaCl (pH 7.4) (PBS). After 20 s, 7 µl of sodium metabisulfite (2.5 mg/ml) in PBS was introduced to terminate radioiodination. Derivatized and radioiodinated AB-125I-LHR$^{96-115}$ solution was mixed with 60 µl of 16% sucrose solution in 0.1 M sodium phosphate buffer and fractionated on Sephadex Superfine G-10 column (0.6 x 15 cm) using PBS.

3. Affinity Cross-linking of 125I-LHR$^{96-115}$ to hCG

Disposable glass tubes were siliconized under dimethyldichlorosilane vapor overnight and autoclaved. In each siliconized tube, 20 µl of PBS, hCG (70 ng in 10 µl PBS), and 125I-LHR$^{96-115}$ (100 ng in 10 µl of PBS) were mixed and incubated at 37 °C for 90 min. After incubation, 3 µl of 0.1 mM of SES in DMSO was added to each tube and further incubated at 25 °C for 20 min. The cross-linking reaction was terminated by adding 3 µl of 5 mM Gly in PBS. The samples were boiled for 2 min in 2% SDS, 100 mM DTT, and 8 M urea. The solubilized samples were electrophoresed on 8-12% polyacrylamide gradient gels. Gels were dried on filter paper, and were exposed to PhosphorImager screens (Molecular Dynamics) overnight. The imaging screen was scanned on a model 860 Storm Molecular Imager System (Molecular Dynamic), and radioactive band intensity was analyzed using ImageQuant software (Molecular Dynamics). Gels were exposed to BioMax- x-ray film at –75 °C for ~4 days.

4. Photoaffinity Labeling of hCG

The following solutions were sequentially introduced to siliconized glass tubes: 20 µl of PBS, 10 µl of hCG (10 ng/µl) in PBS, and 10 µl of ABG-125I-LHR$^{96-115}$ (10 ng/µl) in PBS. The mixtures were incubated at 37 °C for 90 min in the dark, irradiated with a Mineralight R-52 UV lamp for 90 s, and solubilized in 2% SDS, 100 mM dithiothreitol, and 8 M urea. The samples were electrophoresed on 8-12% polyacrylamide gradient gels. Gels were dried on filter paper and processed as described above.
5. Competitive Inhibition of Affinity and Photoaffinity Labeling of hCG

Competitive inhibition experiments were carried out as described for the affinity cross-linking and photoaffinity labeling experiments, except that 10 µl instead of 20 µl of PBS was introduced to each tube, and the mixture was incubated with 10 µl of increasing concentrations of nonradioactive wild type or mutant LHR\textsuperscript{96-115} peptides in PBS.

6. Inhibition of \textsuperscript{125}I-hCG binding to LHR

A human embryonic kidney 293 cell line stably expressing human LHR was incubated with 100,000 cpm of \textsuperscript{125}I-hCG in the presence of increasing concentrations of nonradioactive wild type or mutant LHR\textsuperscript{96-115} peptides. After several times washing the cells, the radioactivity associated with the cells was counted, and percent bound \textsuperscript{125}I-hCG was plotted against the nonradioactive receptor peptides. The results were converted to Scatchard plot by plotting bound/free peptide \textit{versus} bound peptide. The plot was used to calculate the \textit{Ki} value following the Scatchard equation.
Chapter Two

hLHR peptide 96-115 labeling of hCG

A. Introduction

The glycoprotein hormone receptors comprise two equal halves, an extracellular N-terminal half (exodomain) and a membrane associated C-terminal half (endodomain) as shown in Figure 1-2. The exodomain of LHR alone is capable of binding hCG with high affinity (29-31). The sequence alignment shows a Leu Rich Repeat (LRR) motif (Leu/Ile-x-Leu/Ile) in the exodomain (24), (120) (Figure 1-4). In the crystal structure of ribonuclease inhibitors, the LRRs assume the nonglobular horseshoe like structure in which an individual LRR forms a loop consisting of a $\beta$ strand connected to parallel $\alpha$ helices. The glycoprotein hormone receptors have been modeled based on these established LRR crystal structures, (36), (37), (121). They show a one-third donut structure consisting of 8-9 LRRs and suggest that the inner lining interacts with the hormones (41).

The previous results suggest that both flanking regions of the LRRs of LHR make contact with both subunits of hCG (43, 110, 112). In particular, the region upstream of the LRRs has many contact sites with hCG. Also, LRRs had been examined to see whether they exist and contact hCG. Leu/Ile residues in the $\beta$ strands of the LRRs were Ala-scanned because they are thought to form the inner lining of the 1/3 donut structure, which is supposed to contact hCG like porcine ribonuclease inhibitor. The results showed that LRRs 2 and 4 are important for hormone binding since the substitutions for any of the Leu/Ile residues in the LRRs 2 and 4 resulted in the complete loss of hCG binding though the mutant receptors were expressed on the cell surface (117, 118). Next, LRR2 was examined by Ala-scanning the amino acids from Val$^{38}$ to Arg$^{63}$. Several amino acids were found to be important: Ile$^{39}$ and Phe$^{44}$ in the $\alpha$ helix and Ile$^{53}$ and Ile$^{55}$ in the $\beta$ strand and Ser$^{59}$, Leu$^{61}$ and Glu$^{62}$ in the linker. Subsequently, LRR4 was also Ala-scanned. Similarly, the Ala substitution for Leu$^{103}$ and Ile$^{105}$ in the $\beta$ strand abolished hCG binding though the mutants receptors were expressed on the cell surface (118). To characterize the side chain, Leu$^{103}$ and Ile$^{105}$ were individually substituted with a panel of different amino acids of hydrophobic, neutral, hydrophilic, or ionic nature. The substitutions of Val, Phe, and Ile/Leu did not affect hCG binding although the Kd values were increased by 2-8 fold. Deletion
and substitutions of other residues abolished hCG binding, although those mutants were expressed on the cell surface. When Leu$^{103}$ and Ile$^{105}$ were switched with each other, the double mutant's Kd increased by 5 fold over the wild type Kd. These data suggest that Leu$^{103}$ and Ile$^{105}$ have unique functions. Although mutational analysis is a powerful approach to screening potentially important contact sites of protein-protein interaction, this raises the question as to whether the LRRs directly interact with the hormone or indirectly influence the hormone/receptor interaction by affecting the global structure of the receptor exodomain due to mutation-induced conformational change.

B. Results

Previously, it was tested whether LRR4 directly interacts with hCG using the peptide mimic of LRR4 spanning Asn$^{96}$-Asp$^{115}$ (111). It was necessary to employ a tool that allows LRR4 peptide to bind tightly to the hormone. To attain efficient cross-linking of LRR4 peptide into the hormone binding site, LHR$^{96-115}$ was derivatized to produce $^{125}$I-AB-LHR$^{96-115}$. $^{125}$I-AB-LHR$^{96-115}$ labeled both subunits with slightly higher labeling of the $\alpha$ subunit. $^{125}$I-AB-LHR$^{96-115}$ labeling of hCG is saturable depending on the UV irradiation time and concentration of $^{125}$I-AB-LHR$^{96-115}$ and hCG. $^{125}$I-AB-LHR$^{96-115}$ does not label denatured hCG. The derivatization of LHR$^{96-115}$ did not affect LHR$^{96-115}$ binding to hCG. Furthermore, the derivatized peptide inhibited $^{125}$I-hCG binding to the LH receptor on intact cells. Therefore, the labeling appears to be specific (111).

The labeling could be blocked by underivatized wild type peptide but not by double mutant peptide with Leu$^{103}$Ala and Ile$^{105}$Ala substitutions (LHR$^{96-115(L103A/I105A)}$). To determine the labeling site, we synthesized LHR$^{96-115}$ analogs in which photoactivable benzoylephynylalanine (BPA) (96) replaced an amino acid at Lys$^{101}$, Tyr$^{102}$, Leu$^{103}$, Ser$^{104}$, Ile$^{105}$ and Cys$^{106}$ positions (Figure 2-1).

1. Photoaffinity Labeling of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ to hCG

In a previous study, it was shown that the photoreactive group at K$^{101}$ position of LHR$^{96-115}$ specifically labeled hCG with specificity (111). Therefore, the next amino acid of LHR$^{96-115}$ of the $\beta$ structure of LRR4 was replaced with Bpa. $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ was used to test whether Bpa analog peptide LHR$^{96-115}$ would bind and label hCG with specificity. $^{125}$I-
Figure 2-1. Model of LRR4, the structure of 4-benzoyl-L-phenylalanine (Bpa), and the sequence alignment of wild type and Bpa analog peptides

(A) β structure is formed by Y_{102}, L_{103}, S_{104}, I_{105} and C_{106}.  (B) Bpa has an amino group and a carboxyl group, allowing for direct incorporation at a defined position into a peptide by solid-phase synthesis.  (C) hLHR\textsuperscript{96-115} analog peptides have been synthesized by replacing one amino acid individually with Bpa at K\textsuperscript{101}, Y\textsuperscript{102}, L\textsuperscript{103}, S\textsuperscript{104}, I\textsuperscript{105} and C\textsuperscript{106} position. Bpa positions on LHR\textsuperscript{96-115} were marked with \textcolor{red}{B}.  The Leu-X-Ile motifs in LRR4 have been marked with gray box.
Bpa$^{102}$-LHR$^{96-115}$ was incubated with hCG and treated with UV. The samples were solubilized in SDS under the reducing condition and electrophoresed, as described in “Materials and Methods”. The phosphoimage of the gel shows that $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ labeled both the α and β subunits of hCG (Figure 2-2,A). The positions of hCG α and hCG β were determined by comparing the respective positions of $^{125}$I-hCG α and $^{125}$I-hCG β on the autoradiograph (Figure 2-2,A). $^{125}$I-Bpa$^{102}$-LHR $^{96-115}$ labeled hCG α 3 fold more than hCG β.

2. Labeling Specificity

In order to determine the specificity of photoaffinity labeling, $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ was incubated with hCG and then irradiated with UV for different durations. The labeling required UV irradiation and was dependent on the irradiation time, reaching the maximum labeling after 60–90 s irradiation. This UV dependence clearly indicates photoaffinity labeling. In addition, the preferential labeling of hCG α was consistent, suggests the labeling specificity. To further examine the specificity of photoaffinity labeling, the concentration of either hCG or the Bpa analog peptide was varied. When a constant concentration of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ was incubated with increasing concentration of hCG, the intensity of labeled hCG α and β bands gradually increased and showed saturation (Figure 2-2,B). A similar result was observed when a constant amount of hCG was incubated with increasing concentrations of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ (Figure 2-2,C). These results indicate that the photoaffinity labeling is dependent on both the Bpa analog peptide and hCG as the limiting factors. In both cases, $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ labeled hCG α more than hCG β, an indication of labeling specificity. Next, we examined the labeling specificity of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$. One way to test the specific labeling is the displacement test using wild type peptide (LHR$^{96-115}$) and a mutant peptide (LHR$^{96-115(L103A/1105A)}$). From previous data, the wild type peptide could bind to hCG with specificity, and also could inhibit the specific labeling of ABG-$^{125}$I-LHR$^{96-115}$. However, the double mutant peptide (LHR$^{96-115(L103A/1105A)}$) could not bind and label hCG(111). To test whether the wild type and double mutant peptides could inhibit photoaffinity labeling of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$, hCG was incubated with 1µM $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ in the presence of increasing concentrations of non-iodinated wild type peptide (Figure 2-3,A) and non-iodinated double mutant peptide (Figure 2-3,B). Increasing concentrations of wild type peptide inhibited photoaffinity labeling in a dose-dependent manner, and the labeling was almost completely inhibited at 4 µM of non-iodinated LHR$^{96-115}$ (Figure 2-
Figure 2-2. Autoradiograph of photoaffinity labeled hCG subunits

The peptide corresponding to the Bpa\(^{102}\) analog LHR\(^{96-115}\) sequence (Bpa\(^{102}\)-LHR\(^{96-115}\)) was synthesized and radioiodinated to produce \(^{125}\)I-Bpa\(^{102}\)-LHR\(^{96-115}\). (A) A constant concentration of \(^{125}\)I-Bpa\(^{102}\)-LHR\(^{96-115}\) was incubated with a constant amount of hCG and irradiated with UV for increasing time periods. The samples were solubilized in SDS under the reducing condition and electrophoresed as described in "Materials and Methods". After electrophoresis, the gel was dried and autoradiographed using phosphoimager. The percentage of the labeled hCG \( \alpha \) and \( \beta \) subunit bands in each lane were calculated based on the total intensity of each lane, and presented in the bar graph. (B) A constant concentration of \(^{125}\)I-Bpa\(^{102}\)-LHR\(^{96-115}\) was incubated with increasing concentrations of hCG and irradiated with UV for 60 s. (C) Increasing concentrations of \(^{125}\)I-Bpa\(^{102}\)-LHR\(^{96-115}\) were incubated with a constant amount of hCG and irradiated with UV for 60 s.
Figure 2-3. Specificity of photoaffinity labeling

hCG was incubated with 1µM $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ in the presence of increasing concentrations of nonlabeled LHR$^{96-115}$ (A), Bpa$^{102}$-LHR$^{96-115}$ (B), and double mutant peptide LHR$^{96-115}$($L103A/I105A$) (C). The samples were irradiated with UV for 60 s and processed as described in the legend of Figure 2-2.
3.A). In contrast, the inhibition by LHR$_{96-115(I103A/I105A)}$ was significantly less effective, it could not compete out $^{125}$I-Bpa$_{102}$-LHR$_{96-115}$ labeling (Figure 2-3,B). The results of the displacement test support the specificity of photoaffinity labeling of $^{125}$I-Bpa$_{102}$-LHR$_{96-115}$.

3. Biological Specificity of Photoaffinity Labeling

Next, I wanted to demonstrate the biological activity of $^{125}$I-Bpa$_{102}$-LHR$_{96-115}$. It was checked by the photoaffinity labeling experiment with denatured hCG and the inhibition study on intact cells. Denatured hCG lost its original conformation and could not bind to LHR expressed on the cell surface (111). To test whether $^{125}$I-Bpa$_{102}$-LHR$_{96-115}$ could label denatured hCG, the increasing concentrations of $^{125}$I-Bpa$_{102}$-LHR$_{96-115}$ were incubated with a constant amount of denatured hCG and treated with UV (Figure 2-4,A). $^{125}$I-Bpa$_{102}$-LHR$_{96-115}$ did not label the biologically inactive denatured hCG even at the highest concentration. If the Bpa peptide analog has a similar or the same structure as the native region of hCG and the corresponding region is involved in receptor binding, Bpa$_{102}$-LHR$_{96-115}$ could inhibit $^{125}$I-hCG binding to receptor on the cell surface. The iodination of hCG did not affect hormone binding to receptors (111). $^{125}$I-hCG was incubated with 293 cells stably expressing wt LHR in the presence of increasing concentrations of the wild type LHR$_{96-115}$ or Bpa$_{102}$-LHR$_{96-115}$ (Figure 2-4,B). The wild type LHR$_{96-115}$ inhibited $^{125}$I-hCG binding to the receptor with an inhibition constant ($K_i$) value of 30 µM, suggesting its binding to the receptor with a reasonable affinity for the peptide (40, 110). Similary, the $K_i$ value of Bpa$_{102}$-LHR$_{96-115}$ was 38 µM. These results show that the structures of LHR$_{96-115}$ and Bpa$_{102}$-LHR$_{96-115}$ were similar enough to inhibit hCG binding to receptor on cell surface with similar $K_is$.

4. Photoaffinity Scanning of β Strand of LRR4

To determine the labeling residues in LRR 4, individual amino acids on LHR$_{96-115}$ were substituted with photoreactive Bpa (Figure 2-1). The Bpa peptides were tested in the same manner as Bpa$_{102}$-LHR$_{96-115}$ to determine which Bpa peptides label hCG effectively. Some Bpa peptides could label hCG more efficiently than others (Figure 2-5). These results indicate that the photoaffinity labeling is dependent on the Bpa position. The peptides with Bpa at the Lys$_{101}$, Tyr$_{102}$, Ser$_{104}$ and Cys$_{106}$ position, labeled hCG efficiently, and their labeling was saturable depending on the UV irradiation time and the concentrations of $^{125}$I-Bpa peptides and hCG.
Figure 2-4. Photoaffinity labeling of denatured hCG and inhibition of $^{125}$I-hCG binding to the receptor by LHR$^{96-115}$

(A) A constant amount of denatured hCG was incubated with increasing concentrations of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ and irradiated with UV for 60 s. The samples were processed as described in the legend of Figure 2-2. (B) $^{125}$I-hCG and intact 293 cells expressing LHR were incubated with increasing concentrations of unlabeled hCG, LHR$^{95-115}$, Bpa$^{102}$-LHR$^{96-115}$, and LHR$^{96-115(L103A/I105A)}$ peptides separately. The results were plotted against the concentration of unlabeled hormone and peptides.
Bpa-LHR\textsuperscript{96-115} analog peptides were synthesized with a substitution of Bpa at K\textsuperscript{101}, Y\textsuperscript{102}, L\textsuperscript{103}, S\textsuperscript{104}, I\textsuperscript{105}, and C\textsuperscript{106}. 100 nM hCG was incubated with 1 µM \textsuperscript{125}I-Bpa-LHR\textsuperscript{96-115} and irradiated with UV for 60s. The samples were processed as described in the legend of Figure 2-2.
These Bpa peptides could not label denatured hCG. To test whether Bpa substitution impacted the Bpa peptide structure, the displacement tests were performed by incubation of hCG and $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ in the presence of increasing concentrations of other Bpa-LHR$^{96-115}$ peptides. The data are shown in Figure 2-6. $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ labeling was inhibited by other nonradiolabeled Bpa-LHR$^{96-115}$ peptides with $K_i$ values of 1.8, 1.0, 1.4, 0.7, 0.9 and 1.1 µM for Bpa$^{101}$, Bpa$^{102}$, Bpa$^{103}$, Bpa$^{104}$, Bpa$^{105}$, and Bpa$^{106}$, respectively. Furthermore, $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ labeling of hCG was blocked by wild type peptide but not by double substituted LHR$^{96-115}$ (L103A/I105A) peptide. Bpa$^{104}$-LHR$^{96-115}$ peptide had Bpa in the middle of the β strand and showed the lowest $K_i$ value of 0.7 µM. The results suggest that the structures of Bpa analog peptides are similar to each other and can compete with wild type LHR$^{96-115}$ peptide for hormone binding. Also, the biological significance of these Bpa analog peptides was tested by inhibition of $^{125}$I-hCG binding to intact cells cells (Figure 2-7). The $K_i$ values are reasonable, as peptide concentration ranged from 35.3 to 50 µM. Bpa$^{104}$-LHR$^{96-115}$ showed the lowest $K_i$. This is consistent with the result in Figure 2-6. This means Bpa$^{104}$-LHR$^{96-115}$ could inhibit $^{125}$I-hCG binding to receptor on cell surface the most efficiently among Bpa-LHR$^{96-115}$ peptides. Bpa$^{102}$-LHR$^{96-115}$ labeled with the highest percentage at labeling hCG 58% of the time, but Bpa$^{104}$-LHR$^{96-115}$ inhibited $^{125}$I-hCG binding to receptor the most efficiently. In contrast, Bpa$^{103}$-LHR$^{96-115}$ and Bpa$^{105}$-LHR$^{96-115}$ could not label hCG efficiently, but they could inhibit $^{125}$I-hCG binding to receptor on cell surface with $K_i$ values of 44.3 and 43.3 µM respectively. These results indicate that the photoaffinity labeling is dependent on the position of the Bpa group, but Bpa substitutions in LHR$^{96-115}$ did not affect the Bpa-LHR$^{96-115}$ binding to hCG (Figure 2-7). These results suggest that Lys$^{101}$, Tyr$^{102}$, Ser$^{104}$ and Cys$^{106}$ face hCG, whereas Leu$^{103}$ and Ile$^{105}$ do not, consistent with the model in Figure 2-1. Furthermore, the results are consistent with the view that Ser$^{104}$, in the center of the β strand inner lining, is most effective in inhibition of $^{125}$I-hCG binding to receptors on cell surface. In contrast, Leu$^{103}$ and Ile$^{105}$ appear to form the hydrophobic core of the LRR4 loop and to be crucial for the LRR structure. The $K_i$ values of inhibition by the Bpa analog LHR$^{96-115}$ peptides were similar to each other: 50, 38, 44.3, 35.3, 43.3 and 36.5 µM for LHR$^{96-115}$ with Bpa$^{101}$, Bpa$^{102}$, Bpa$^{103}$, Bpa$^{104}$, Bpa$^{105}$ and Bpa$^{106}$, respectively. This suggests that Bpa substitutions were tolerable, and their $K_i$ values are reasonable as peptides. However, the labeling is dependent on the Bpa position.

5. Photoaffinity Scanning on Proximal Region of β Strand of LRR4
Figure 2-6. Inhibition of $^{125}\text{I}-\text{Bpa}^{102}-\text{LHR}^{96-115}$ photoaffinity labeling

(A) hCG was incubated with 1µM $^{125}\text{I}-\text{Bpa}^{102}-\text{LHR}^{96-115}$ in the presence of increasing concentrations of nonlabeled LHR$^{96-115}$, LHR$^{96-115(L103A/I105A)}$, and Bpa-LHR$^{96-115}$ peptides. The samples were irradiated with UV for 60 s and processed as described in the legend of Figure 2-2.

(B) The inhibition constants (Ki) were calculated.

<table>
<thead>
<tr>
<th>Inhibitor peptides</th>
<th>Ki (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHR$^{96-115}$</td>
<td>0.7</td>
</tr>
<tr>
<td>LHR$^{96-115(L103A/I105A)}$</td>
<td>NA</td>
</tr>
<tr>
<td>Bpa$^{101}$-LHR$^{96-115}$</td>
<td>1.8</td>
</tr>
<tr>
<td>Bpa$^{102}$-LHR$^{96-115}$</td>
<td>1.0</td>
</tr>
<tr>
<td>Bpa$^{103}$-LHR$^{96-115}$</td>
<td>1.4</td>
</tr>
<tr>
<td>Bpa$^{104}$-LHR$^{96-115}$</td>
<td>0.7</td>
</tr>
<tr>
<td>Bpa$^{105}$-LHR$^{96-115}$</td>
<td>0.9</td>
</tr>
<tr>
<td>Bpa$^{106}$-LHR$^{96-115}$</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Figure 2-7. Inhibition of $^{125}$I-hCG binding to the receptor by Bpa-LHR$^{96-115}$ peptides

$^{125}$I-hCG was incubated with intact 293 cells expressing LHR in the presence of increasing concentrations of unlabeled Bpa-LHR$^{96-115}$ analog peptides with Bpa at $K^{101}$, $Y^{102}$, $L^{103}$, $S^{104}$, $I^{105}$, and $C^{106}$ positions as described in "Materials and Methods". The results were plotted against the concentration of unlabeled peptides. The inhibition constants ($K_i$) were calculated by scatchard plot.
The β structure in the LHR\textsuperscript{96-115} seems to consist of Ser\textsuperscript{104} in the middle and Leu\textsuperscript{103} and Ile\textsuperscript{105} facing the inside of the loop structure (Figure 2-1). To confirm this β structure in LHR\textsuperscript{96-115}, more Bpa analog peptides in LHR\textsuperscript{96-115} were synthesized and used for photoaffinity scanning. The locations of Bpa substitution were Asn\textsuperscript{96}, Leu\textsuperscript{97}, Arg\textsuperscript{99}, Asn\textsuperscript{107}, Thr\textsuperscript{108}, Gly\textsuperscript{109}, Ile\textsuperscript{110} and Asp\textsuperscript{115}. Proline was not substituted since many Proline residues are important for protein structures. Lys\textsuperscript{112} was not substituted either since it was already tested (111). These Bpa peptides were subjected to photoaffinity labeling, inhibition study with wild type LHR\textsuperscript{96-115} peptide, and \textsuperscript{125}I-hCG binding to receptor on cell surface. The percentages of photoaffinity labeling data are shown in Figure 2-8 and the inhibition of \textsuperscript{125}I-hCG binding data in Figure 2-9, A-C. Although the percentages of labeling of Bpa analog peptides were diminished when Bpa was located away from β structure, the \textit{Ki} values of Bpa analog peptides at Leu\textsuperscript{97}, Arg\textsuperscript{99}, Asn\textsuperscript{107}, Thr\textsuperscript{108}, Gly\textsuperscript{109}, Ile\textsuperscript{110} and Asp\textsuperscript{115} were similar to each other, except for Asn\textsuperscript{96}, and to those of previous Bpa analog peptides at Lys\textsuperscript{101}, Tyr\textsuperscript{102}, Ser\textsuperscript{104} and Cys\textsuperscript{106}. This result suggests that most of the Bpa analog peptides at LHR\textsuperscript{96-115} have a similar structure, and are competing with each other for the same binding site on hCG. However, none of the Bpa analog peptides at Asn\textsuperscript{96}, Leu\textsuperscript{97}, Arg\textsuperscript{99}, Asn\textsuperscript{107}, Thr\textsuperscript{108}, Gly\textsuperscript{109}, Ile\textsuperscript{110} and Asp\textsuperscript{115} could label hCG more efficiently than at those of Lys\textsuperscript{101}, Tyr\textsuperscript{102}, Ser\textsuperscript{104} and Cys\textsuperscript{106}. These results suggest that the photoaffinity labeling is dependent on the position of the Bpa group. When A Bpa was closer to the putative β strand could label hCG much more efficiently than a Bpa at a position distant from the β strand.

6. The Displacement Test with the Peptides from Other Regions of the Exodomain and the Endodomain of LHR

The existing data suggest multiple contact sites for the hCG/receptor interaction. Several regions have been found by our group. They are the N-terminal region (43, 110), Leu Rich Repeat 4 (111, 118), and the hinge region (112). In addition, exoloop 2 is involved in the interaction of the exodomain and endodomain (72, 112). To extend the study on the relationship between the exodomain and the endodomain, hCG was photoaffinity labeled with 1 \textmu M \textsuperscript{125}I-Bpa\textsuperscript{102-LHR\textsuperscript{exo3}} in the presence of increasing concentrations of nonradioactive N-terminal peptide (LHR17-36), Leu Rich Repeat 4 peptide (LHR\textsuperscript{96-115}), the hinge region peptide (LHR\textsuperscript{246-269}), or the exoloop peptides (LHR\textsuperscript{exo1}, LHR\textsuperscript{exo2} and LHR\textsuperscript{exo3}). Nonradiolabeled LHR\textsuperscript{96-115} and LHR\textsuperscript{exo2} blocked the labeling (Figure 2-10, A), but the inhibition by LHR\textsuperscript{exo1} was
Figure 2-8. Photoaffinity labeling of hCG with $^{125}$I-Bpa-LHR$^{96-115}$ peptides

More Bpa-LHR$^{96-115}$ analog peptides were synthesized with Bpa at Asn$^96$, Leu$^97$, Arg$^99$, Asn$^{107}$, Thr$^{108}$, Gly$^{109}$, Ile$^{110}$ and Asp$^{115}$ positions. 100 nM hCG was incubated with 1 µM $^{125}$I-Bpa-LHR$^{96-115}$ and irradiated with UV 60 s. The samples were solubilized in SDS under the reducing condition and electrophoresed. After electrophoresis, the gel was dried and autoradiographed using phosphoimager. The data has been analyzed as described in the legend of Figure 2-2.
Figure 2-9. Inhibition of $^{125}$I-hCG binding to the receptor by Bpa-LHR$^{96-115}$ peptides

$^{125}$I-hCG was incubated with intact 293 cells expressing LHR in the presence of increasing concentrations of unlabeled Bpa-LHR$^{96-115}$ peptides: (A) N-terminal, (B) $\beta$ strand, and (C) C-terminal regions of LHR$^{96-115}$. The results were plotted against the concentration of unlabeled peptides. The inhibition constants ($K_i$) were calculated by scatchard plot.
Figure 2-10. Inhibition of $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ photoaffinity labeling with different region LHR peptides

(A) hCG was incubated with 1µM $^{125}$I-Bpa$^{102}$-LHR$^{96-115}$ in the presence of increasing concentrations of nonlabeled N-terminal (LHR$^{17-36}$), LRR4 (LHR$^{96-115}$), hinge region (LHR$^{246-269}$), exoloop 1 (LHR$^{exo1}$), exoloop 2 (LHR$^{exo2}$), and exoloop 3 (LHR$^{exo3}$) peptides. The samples were irradiated with UV for 60 s and processed as described in the legend of Figure 2-2. (B) Schematic model for interaction of hCG and the LHR exodomain. (C) 3D model of the LHR endodomain.
considerably weaker. These results suggest the binding sites of LHR$^{96-115}$ and LHR$^{exo2}$ are close enough to compete with each other. This gives an idea as to how hCG and the exodomain contact with each other (Figure 2-10, B) and also how the hCG/exodomain complex makes contact the endodomain, which is likely to be the exoloops (Figure 2-10, C).

**C. Discussion**

Our observations on the exodomain and hCG suggest, for the first time, the existence of functional LRRs, particularly LRR 4, in the LHR exodomain. The β strand of LRR4 consists of Tyr$^{102}$, Leu$^{103}$, Ser$^{104}$, Ile$^{105}$ and Cys$^{106}$ (Figure 2-1). $^{125}$I-Bpa-LHR$^{96-115}$ with Bpa at the side of Lys$^{101}$, Tyr$^{102}$, Ser$^{104}$ and Cys$^{106}$ could label hCG much more efficiently than those with Bpa at the Leu$^{103}$ and Ile$^{105}$ position. This result suggests that hCG binds at the side of Lys$^{101}$, Tyr$^{102}$, Ser$^{104}$ and Cys$^{106}$. In contrast, Leu$^{103}$ and Ile$^{105}$ could not label hCG efficiently suggesting that Leu/Ile-X-Leu/Ile motives appear to be crucial for forming the hydrophobic core of the LRR loops and providing the structural framework of the 1/3 donut structure. They do not face and interact with hCG. The labeling of Bpa peptides at Lys$^{101}$, Tyr$^{102}$, Ser$^{104}$ and Cys$^{106}$ are saturable and dependent on the hCG concentration, $^{125}$I-Bpa-LHR$^{96-115}$ concentration, and UV irradiation time. $^{125}$I-Bpa-LHR$^{96-115}$ peptides label bioactive hCG but not denatured hCG. The photoaffinity labeling of $^{125}$I-Bpa-LHR$^{96-115}$ blocked by non-iodinated wild type LHR$^{96-115}$ but not by double-substituted mutant LHR$^{96-115(L103A/I105A)}$. Both α and β subunits are labeled, the α subunit preferentially, suggesting that photoreactive Bpa can reach both. This is possible since two subunits are closely entangled in its crystal structure (53, 104) (Figure 1-1). Considering the length of Bpa, the physical proximity of Bpa and hCG α could be less than 5 Å. Although the length of Bpa is quite similar to those of long side chain amino acids like Lys or Arg, the possible interference of the photoactivable Bpa group on binding of $^{125}$I-Bpa-LHR$^{96-115}$ to hCG tested by affinity cross-linking experiments with wild type $^{125}$I-LHR$^{96-115}$ with SES (Ethylene glycolbis-[sulfosuccinimidylsuccinate]) (111). The data from affinity-cross-linking by SES and photoaffinity labeling by Bpa are similar, suggesting that the Bpa substitution does not affect the peptide structure and interfere with hCG binding and the subsequent labeling. Furthermore, the biological significance of Bpa-LHR$^{96-115}$ has been shown by the fact that Bpa-LHR$^{96-115}$ peptides could inhibit $^{125}$I-hCG binding to receptor on intact cell surface with similar $K_i$ values to wild type LHR$^{96-115}$. Although the $K_i$
values of peptides with Bpa at Leu\textsuperscript{97}, Arg\textsuperscript{99}, Asn\textsuperscript{107}, Thr\textsuperscript{108}, Gly\textsuperscript{109}, Ile\textsuperscript{110} and Asp\textsuperscript{115} are similar to each other and to those of previous Bpa analog peptides at Lys\textsuperscript{101}, Tyr\textsuperscript{102}, Ser\textsuperscript{104} and Cys\textsuperscript{106}, the labeling percentages of Bpa peptides at Asn\textsuperscript{96}, Leu\textsuperscript{97}, Arg\textsuperscript{99}, Asn\textsuperscript{107}, Thr\textsuperscript{108}, Gly\textsuperscript{109}, Ile\textsuperscript{110} and Asp\textsuperscript{115} were diminished when Bpa was located away from the putative $\beta$ structure. When the percentages of labeling are compared between at the N-terminus and C-terminus of LHR\textsuperscript{96-115} peptide, Bpa from the C-terminus of LHR\textsuperscript{96-115} could label hCG more than from the N-terminus of LHR\textsuperscript{96-115}. From previous studies (43, 112), the N-terminus (LHR\textsuperscript{17-36}) and the hinge (LHR\textsuperscript{246-269}) regions can label N-terminus of hCG, the exodomain would wrap around hCG and LRR4 can bind to the concave side of hCG, and the N-terminal region of LHR\textsuperscript{96-115} is closer to hCG than the C-terminal region of LHR\textsuperscript{96-115} (Figure 2-10,B).

The glycoprotein hormones share the common $\alpha$ subunit and common effectors. It is logical to speculate that the $\alpha$ subunit interacts with the endodomain and activates it since hormone activation is taken place in the endodomain. The contact sites between the glycoprotein hormones and their cognate receptors are likely the exoloops, which could modulate transmembrane helices which causes an allosteric structural change in the cytoplasmic domains.

The crystal structure of hCG has facilitated the investigations to localize the potential receptor-binding regions (104). The important regions are $\alpha$L2 (40-50), $\alpha$ C-terminus (88-92), $\beta$L2 (38-57) and the $\beta$ seatbelt region (94-99). However, the glycoprotein hormone receptors have not been crystallized probably due to the flexible N-terminal region, the glycans, their exodomain size which is similar to other whole GPCRs (21), and their membrane associated region. The exodomain of glycoprotein hormone receptors has been modeled based on the sequence alignment, porcine ribonuclease inhibitor’s crystal structure, and homology modeling (36, 122). There are eight to nine LRRs providing the primary contact site for the cognate ligands. The $\beta$ sheet inner lining is thought to be the ligand contact site (36, 56, 123), perhaps interacting with the putative receptor binding C terminus and seat belt of hCG (4). The importance of LRRs for hormone binding was proved by the deletion study (124). LRR 1-6 are involved in hormone binding in LHR. However, there is little experimental evidence for the hCG and receptor contact sites. Recently, several reports described the LRR structure of LHR and the direct interaction of
Figure 2-11. Model of the hCG, exodomain and endodomain of LH receptor

Deglycosylated hCG has been crystalized. hCG consists of α subunit (red) and β subunit (green). Two subunits are intertwined with each other. Exodomain (blue) of LHR has the Leu-rich-repeat motifs (LRR)s. Endodomain was modeled based on the rhodopsin transmembrane domain (gray). It has three extracellular loops (orange), three cytoloops, 7 transmembrane domain, and a C-terminal tail.
the LRR4 β structure with hCG (111, 117, 118). The imperfect LRRs are flanked by the short N- and C-terminal hinge regions. These two flanking regions do not have homology among the glycoprotein hormone receptors; they are ascribed to the hormone specificity (43) and /or protecting the LRR structure (48). It has been reported that the N-terminal region is important for receptor surface expression (48) and hormone binding (43, 124). The C-terminal (hinge) region of the exodomain is important for receptor constraining in hTHSR (81) and also makes a contact with the hormone after the release of the receptor constraining (112).

This work has been confirmed that the LRR structure exists in the exodomain of LHR and also proves that the LRR4 contacts with hCG at Lys\textsuperscript{101}, Tyr\textsuperscript{102}, Ser\textsuperscript{104} and Cys\textsuperscript{106} side of the β structure. The information on exodomain/endodomain interaction could provide a general mechanism for the mutual modulation of the two distinct exodomain and endodomain and signal generation on GPCR.
Chapter Three

hLHR peptide labeling of exoloop 3

A. Introduction

The gonadotropin and other glycoprotein hormone (FSH, hCG, LH and TSH) receptors consist of a ~350 amino acid long N-terminal extracellular exodomain and a C-terminal membrane associated endodomain of similar size (24, 25, 28). Glycoprotein hormones initially bind to the exodomain, and the resulting hormone/exodomain complex modulates the endodomain (23). The ternary interactions among the hormone, exodomain, and endodomain are crucial for activation of adenylyl cyclase to generate cAMP and phospholipase C β to produce inositol phosphate and diacylglycerol (6, 23, 51) (Figure 1-6).

Despite the crucial roles of the ternary interactions, their character has not been well defined. Only recently, some information has become available. For instance, the exodomain of the TSH receptor appears to hold the endodomain in the inactive state (80). In the case of the LH receptor, the hinge region (72, 81, 112) and Leu Rich Repeat 4 (LRR4) (111) of the exodomain interact with the endodomain. Interestingly, the hinge residues are involved in pairing with exoloop 2 and suppressing the receptor activation (72, 81, 112). In contrast, the LRR 4 residues appear to promote the activation of the endodomain (111), but the contact site is unknown. Conversely, some residues of exoloops 2 and 3 in the endodomain modulate the hormone binding to the exodomain (69, 125). These observations suggest that the three exoloops are likely involved in interactions between the exodomain and endodomain. However, the information on the function and structure of the exoloops is limited, and in particular, little is known about whether the hormone/exodomain complex contacts the exoloops.

To address this issue, we set out to investigate whether exoloops indeed interact with the hormone. As a first step towards this goal, we examined the shortest of the three exoloops in the endodomain, exoloop 3 of LHR and FSHR. Exoloop 3 consists of 11 amino acids, connects transmembrane domains 6 and 7 that are important for activation of adenylyl cyclase (126, 127), and has been implicated in cAMP signal generation (109, 125).

These results suggested the involvement of exoloop 3 in hormone binding, and raised a crucial question of whether exoloop 3 contacts the hormones. However, the results raise the
question whether or not the observed effect of mutagenesis is caused directly by the mutated amino acid or indirectly by conformational changes in exoloop 3 due to mutation-induced folding changes. One way to test its direct interaction is to use a synthetic peptide covering the mutated sequence.

B. Results

1. Exoloop 3 plays crucial and differential roles in cAMP and IP induction.

Exoloop 3 is the shortest of the three exoloops, consisting of 11 amino acids, and one of the shortest exoloops found in G protein coupled receptors (51). It is barely long enough to connect two TMs, 6 and 7, which have been implicated in signal transduction of LHR (6, 128). A slight change in exoloop 3 could modulate the two TMs allosterically. LHR exoloop 3 is known to constrain the hormone binding at the exodomain but does not play a crucial role in cAMP induction except Lys\textsuperscript{583} (109). However, little is known about the mechanisms or its role in IP induction. To address this question, individual Ala substitutions of the exoloop 3 residues were stably expressed on HEK 293 cells and assayed for inositol phosphates IP\textsubscript{1}, IP\textsubscript{2}, IP\textsubscript{3} and IP\textsubscript{t} (129) (Figure 3-1). Most of the mutant receptors were incapable of inducing any of the IP species. However, the Val\textsuperscript{574}Ala and Ser\textsuperscript{582}Ala mutants responded to hCG and induced IP. Since most of the mutants were incapable of inducing the IP species, we wondered whether the non-responding mutant receptors were expressed on the cell surface. Therefore, the cells stably expressing with the mutants were assayed for hormone binding as well as hormone-dependent cAMP production. All of the mutants bound hCG and all except LHR\textsuperscript{K583A} induced cAMP, supported by the previous report (125). The Ala substitutions differentially impacted hormone binding, cAMP induction, and IP induction. IP induction was most sensitive to the Ala substitutions, whereas hormone binding was least sensitive. Furthermore, the substituted residues impacting the three functions were diverse, suggesting distinct mechanisms and residues involved in each of the three functions. For IP induction, all residues except Val\textsuperscript{574} and Ser\textsuperscript{582} appear to be crucial, whereas Lys\textsuperscript{583} and perhaps a few others are essential for cAMP induction (129). The results clearly show the differential mechanisms for cAMP and IP induction and the role of exoloop 3 in activation of adenylyl cyclase and PLC \(\beta\).

2. Photoaffinity labeling of hCG with exoloop 3 peptide mimic
Figure 3-1. Differential effects of Ala substitutions on hormone binding, cAMP and IP induction

To easily compare the Ala substitution effects on mutant receptors, hormone binding, cAMP and IP induction, the ratios of $K_{d}^{\text{wild type}}/K_{d}^{\text{mutant}}$ ($K_{d}^{\text{wt}/\text{mut}}$), maximum cAMP level of mutant/wild type ($c\text{AMP}_{\text{mut}/\text{wt}}$) and maximum IPt level of mutant ($IP_{\text{mut}/\text{wt}}$) were displayed. Values over 1 reflect that mutants are better than wild type.
In an attempt to test whether the LHR exoloop 3 interacts with hCG, the exoloop 3 peptide mimic, LHR$^{exo3}$, was synthesized and used for affinity labeling of hCG. The peptide was derivatized with a photoactivable reagent, the N-hydroxysuccinimide of 4-azidobenzoyl glycine (ABG), and radio-iodinated to produce ABG-$^{125}$I-LHR$^{exo3}$. A constant amount of ABG-$^{125}$I-LHR$^{exo3}$ was incubated with increasing concentrations of hCG and irradiated with UV. Samples were solubilized in SDS under the reducing condition and electrophoresed. The autoradiographic phosphoimage of the gel (Figure 3-2,A) revealed the labeling of the hCG $\alpha$ and $\beta$ bands with the $\alpha$ band labeled slightly more than the $\beta$ band. The labeling increased in parallel to the concentration of hCG and then reached a plateau. The result suggests that the labeling was saturable at a certain hCG concentration and that additional hCG was not labeled. Furthermore, the maximum labeling was reached at 50 nM hCG for both of the hormone subunits, indicating that the slightly different labeling efficiencies of the subunits were independent of the hormone concentration. Next, increasing concentrations of ABG-$^{125}$I-LHR$^{exo3}$ were incubated with a constant amount of hCG, photolyzed, and processed as before. The resulting autoradiograph shows the labeling of both hCG $\alpha$ and $\beta$ subunits, and shows a labeling plateau (Figure 3-2,B).

In the next experiment, a constant amount of ABG-$^{125}$I-LHR$^{exo3}$ was incubated with a constant concentration of hCG and treated with UV for increasing time periods. The extent of the labeling increased as the UV photolysis time increased, saturating at 90 s irradiation (Figure 3-2,C). The hormone subunits were not labeled when the sample was not irradiated with UV, indicating that the labeling required UV photolysis. These results show that the labeling requires ABG-$^{125}$I-LHR$^{exo3}$, hCG and UV irradiation, and is saturable dependent on each of them. Both of the hCG $\alpha$ and $\beta$ subunits were labeled, slightly more $\alpha$ than $\beta$. The extent of labeling of the $\alpha$ subunit and $\beta$ subunit increased and saturated in parallel throughout the hCG, peptide, and UV dependent experiments (Figure 3-2,A-C). These results suggest that the hCG subunits and photoprobe were stably and specifically arranged in the ternary complex. In this spatial arrangement, the photoprobe is capable of labeling the $\alpha$ subunit slightly better than the $\beta$ subunit, suggesting new insights into the geometry and proximity of the interacting exoloop 3 and the hCG subunits.

3. Specificity of Exoloop 3 Labeling

Although the labeling was saturable, its specificity was unclear. To test the biospecificity, ABG-
Figure 3-2. Autoradiograph of photoaffinity labeled hCG subunits

The peptide corresponding to the LHR exoloop 3 sequence (LHR\textsuperscript{exo3}) was synthesized, derivatized with NHS-ABG and radioiodinated to produce ABG-\textsuperscript{125}I-LHR\textsuperscript{exo3}.

(A) Increasing concentration of hCG were incubated with a constant concentration of ABG-\textsuperscript{125}I-LHR\textsuperscript{exo3} and irradiated with UV for 90 s. (B) Increasing concentrations of ABG-\textsuperscript{125}I-LHR\textsuperscript{exo3} were incubated with a constant amount of hCG and irradiated with UV for 90 s. (C) A constant concentration of ABG-\textsuperscript{125}I-LHR\textsuperscript{exo3} was incubated with a constant amount of hCG and irradiated with UV for increasing time periods. The samples were solubilized in SDS under the reducing condition and electrophoresed as described in "Materials and Methods". After electrophoresis, the gels were dried and exposed under phosphoimager screen. The intensity of each band in each lane was measured as described in "Materials and Methods". The percentage of the labeled hCG \(\alpha\) and \(\beta\) subunit bands in each lane were calculated based on the total intensity of a gel lane, and presented in the bar graph.
was incubated with increasing concentrations of denatured hCG and irradiated with UV. Denatured hCG was not labeled at (Figure 3-3). To find out if higher concentrations of ABG-125I-LHRexo3 were needed for labeling denatured hCG, increasing concentrations of ABG-125I-LHRexo3 were incubated with a constant amount of denatured hCG. Higher concentrations of ABG-125I-LHRexo3 failed to label denatured hCG. One concern is whether denatured hCG precipitated or adhered to the test tube during boiling in 8 M urea. To test whether denatured hCG remains in solution, unlabeled hCG was mixed with radioactively labeled hCG in 8 M urea and boiled for 30 min. Three microliter of the mixture was transferred to another tube every 3 min, and the radioactivity was counted. The transfer was quantitative, indicating that denatured hCG remained in solution and was present in photoaffinity labeling tubes (Figure 3-4). The results demonstrate that the labeling requires bioactive hCG, not denatured hCG. If the biospecificity is true, hCG and the exoloop 3 peptide should inhibit 125I-hCG binding to LHR, but denatured hCG should not. To test the hypothesis, cells stably expressing wt LHR were incubated with 125I-hCG in the presence of increasing concentrations of nonradioactive hCG, nonradioactive LHRexo3 or denatured hCG (Figure 3-5). The results show that both of the nonradioactive hCG and nonradioactive LHRexo3 inhibited 125I-hCG binding to LHR, but hCG was >10,000 times more potent than the peptide mimic. However, denatured hCG failed to inhibit 125I-hCG binding to LHR.

hCG and LH bind to the same receptor and induce similar hormone actions. Therefore, LHRexo3 is expected to similarly label both hormones. To test the possibility, increasing concentrations of LH and denatured LH were photoaffinity labeled with ABG-125I-LHRexo3 (Figure 3-6). Both of the α and β subunits of LH were labeled, but denatured LH was not. Despite the biospecificity of photoaffinity labeling of hCG by LHRexo3, it was unclear whether the derivatization of the peptide with ABG impacted the peptide's specificity for hCG. To test the possibility, hCG was photoaffinity labeled with ABG-125I-LHRexo3 in the presence of increasing concentrations of unlabeled LHRexo3 and scrambled LHRexo3. The photoaffinity labeling was blocked by nonlabeled peptide but not by nonlabeled scrambled peptide (Figure 3-7,A and B). In addition, phospholipase A, phospholipase C, phospholipase D, and urokinase were incubated with ABG-125I-LHRexo3 and photolyzed. These proteins were not photoaffinity labeled (Figure 3-7,C). These results show the specificity of the hCG photoaffinity labeling. There is a remarkable difference in the photoaffinity labeling of hCG/LH and FSH.
Figure 3-3. Photoaffinity labeling of denatured hCG
(A) Increasing concentrations of denatured hCG were incubated with a constant amount of ABG-\(^{125}\text{I}\)-LHR\(^{exo3}\) and irradiated with UV for 90 s. (B) Constant amounts of denatured hCG were incubated with increasing concentrations of ABG-\(^{125}\text{I}\)-LHR\(^{exo3}\) and irradiated with UV for 90 s. The samples were processed as described in the legend of Figure 3-2.
Figure 3-4. Fractionation of denatured hCG

1 µg of $^{125}\text{I}$-hCG was denatured in 50 µl of 8 M Urea, 0.1 M DTT by boiling for 30 min. 3 µl of sample was collected every 3 min. Radioactivity was measured for 1 min using a gamma counter.
Figure 3-5. Inhibition of $^{125}$I-hCG binding to the receptor by LHR$^{exo3}$

$^{125}$I-hCG was incubated with 293 intact cells expressing LHR in the presence of increasing concentrations of unlabeled LHR$^{exo3}$ as described in "Materials and Methods". The results were plotted against the concentration of unlabeled hCG, denatured hCG, and LHR$^{exo3}$ peptide.
**Figure 3-6. Photoaffinity labeling of LH**

Increasing concentrations of human LH and denatured human LH were incubated with a constant amount of ABG-\(^{125}\text{I}-\text{LHR}^{exo}\) and irradiated with UV for 90 s. The samples were processed as described in the legend of Figure 3-2.
**Figure 3-7. Specificity of photoaffinity labeling**

hCG was photoaffinity labeled with ABG-$^{125}$I-LHR$^{exo3}$ in the presence of increasing concentrations of nonlabeled LHR$^{exo3}$ (A) and scrambled LHR$^{exo3}$ (B). (C) Various proteins (100 nM), hCG, phospholipase A (PLA), phospholipase C (PLC), phospholipase D (PLD) and urokinase (Uro) were incubated with ABG-$^{125}$I-LHR$^{exo3}$ and irradiated with UV for 90 s. The samples were processed as described in the legend of Figure 3-2.
with their respective exoloop 3 peptides. Whereas both subunits of hCG and LH were labeled by ABG-\(^{125}\)I-LHR\(^{exo3}\) (Figure 3-2, Figure 3-6), only the \(\alpha\) subunit of FSH was labeled by ABG-\(^{125}\)I-FSHR\(^{exo3}\) (Figure 3-8,A) as previously described (113). Since the \(\alpha\) and \(\beta\) subunits of FSH comigrate in gel electrophoresis, photoaffinity labeled FSH was digested with PNGase F and electrophoresed, which resolves the two subunits. The labeled band of FSH corresponded to the \(\alpha\) subunit, which is in contrast to the photoaffinity labeling of the FSH \(\beta\) subunit by the N-terminal peptide of the FSHR exodomain (unpublished).

Denatured FSH was not labeled at all (Figure 3-8, B and C). The photoaffinity labeling of the FSH \(\alpha\) subunit is remarkable because there are three potential residues, Lys\(^{580}\), Lys\(^{588}\) and Lys\(^{590}\), for ABG derivatization in FSHR\(^{exo3}\), as compared to two derivatization sites of Lys\(^{573}\) and Lys\(^{583}\) in LHR\(^{exo3}\). The results suggest notable differences in the structure and interaction of the exoloop 3s of LHR and FSHR.

4. Relationship with Exoloops 1, 2, and Other Regions of Exodomain

hCG binds the exodomain with high affinity and three regions have been identified for the interaction. They are the N-terminal region (43, 110), Leu Rich Repeat 4 (111, 118), and the hinge region (112). In addition, exoloop 2 is involved in the interaction of the exodomain and endodomain (72, 112). The relationship between these various contact points likely plays a crucial role in the signal generation and the interaction of hCG and exoloop 3. Therefore, it is necessary to determine the relationship among the various interactions. hCG was photoaffinity labeled with \(1 \mu M\) ABG-\(^{125}\)I-LHR\(^{exo3}\) in the presence of \(4 \mu M\) of nonlabeled exoloop peptides (LHR\(^{exo1}\), LHR\(^{exo2}\) and LHR\(^{exo3}\), N-terminal peptide (LHR\(^{18-36}\)), Leu Rich Repeat 4 peptide (LHR\(^{96-115}\)), and hinge region peptide (LHR\(^{246-269}\)). Nonlabeled LHR\(^{exo3}\), LHR\(^{96-115}\), and LHR\(^{246-269}\) blocked the labeling (Figure 3-9). LHR\(^{exo1}\) and LHR\(^{exo2}\) inhibited the labeling, but the inhibition by LHR\(^{exo1}\) was considerably weak. These results suggest diverse affinities of the hCG labeling with these LHR peptides. On the other hand, LHR\(^{18-36}\) failed to inhibit the labeling. These results suggest the specificity of the hCG labeling by ABG-\(^{125}\)I-LHR\(^{exo3}\), which necessitates further studies.

C. Discussion

Our results show that LHR\(^{exo3}\) specifically photoaffinity labeled both subunits of hCG/LH,
Figure 3-8. Photoaffinity labeling of FSH

(A) Human FSH was photoaffinity labeled with increasing concentrations of ABG-\(^{125}\)I-FSH, treated with PNGase F, and processed as described in "Materials and Methods". In addition, \(^{125}\)I-FSH was electrophoresed with and without digestion with PNGase F. (B) A constant amount of denatured FSH was photoaffinity labeled with increasing concentrations of ABG-\(^{125}\)I-FSHR\(^{exo3}\). (C) Increasing concentrations of denatured FSH were photoaffinity labeled with a constant amount of ABG-\(^{125}\)I-FSHR\(^{exo3}\). The samples were processed as described in the legend of Figure 3-2.
Figure 3-9. Effects of other LHR peptides on photoaffinity labeling of hCG

hCG was photoaffinity labeled with ABG-^{125}I-LHR^{Exo3} in the presence of 4 µM of nonlabeled peptides: exoloop 1 peptide (Exo1), exoloop 2 peptide (Exo2), exoloop 3 peptide (Exo3), scrambled exoloop 3 peptide (Exo3S), LHR^{18-36} (18-36), LHR^{96-115} (96-115), and LHR^{246-269} (246-269).
whereas the labeling of FSH by FHR$^{exo3}$ was restricted to the $\alpha$ subunit of FSH. These gonadotropins share the common $\alpha$ subunits as well as the common hormone signals to activate AC for cAMP production and PLC $\beta$ for production of IP and diacyl glycerol production (6, 121). Because of these common structural features and functions, the common $\alpha$ subunit has been suspected to be involved in the hormone action. Our results clearly support the possibility. In addition, they provide intriguing new insights into a pivotal question of how hCG/LH and FSH distinctly generate the common hormone action. The differential photoaffinity labeling of hCG/LH and FSH provides an explanation. Both subunits of hCG/LH are in the proximity of the C-terminal region of LHR exoloop 3, whereas the $\alpha$ subunit of FSH is close to the FSHR exoloop 3. The crystal structures of hCG (53, 104) and FSH (8) support these distinct interactions between the hormones and three exoloops. Although the overall structures are similar, there are differences in the $\beta$ subunits that may be important with respect to receptor binding specificity or signal generation (8).

Though our previous studies (125) suggest the involvement of exoloop 3 in hormone binding, it was unclear whether exoloop 3 interacts with hCG. The specific photoaffinity labeling of hCG by ABG-$^{125}$I-LHR$^{exo3}$ and the inhibition of hCG binding to LHR by the peptide indicates the direct interaction of exoloop 3 and hCG. The photoaffinity labeling is blocked by the exoloop 2 peptide, the LRR 4 peptide (LHR$^{96-115}$), and the hinge region peptide (LHR$^{246-269}$). The simplest explanation is that the competing peptides bind hCG at the same or overlapping sites. Alternatively, allosteric effects or their putative interactions with ABG-$^{125}$I-LHR$^{exo3}$ might have disrupted the photoaffinity labeling. One way to address this issue is to photoaffinity label hCG with the competing receptor peptides. Both of the LRR 4 peptide (111) and hinge peptide (112) photoaffinity label hCG, indicating their direct interactions with hCG. Therefore, the exoloop 3 peptide, LRR 4 peptide, and hinge peptide either interact with hCG at the same or overlapping sites or bind at distinct sites in hCG but allosterically impact another's binding to hCG. Consistent with the latter possibility is the fact that hCG undergoes a conformational change upon the initial binding with the receptor (130). Whether the exoloop 2 peptide interacts with hCG is unclear. In contrast, the exoloop 1 peptide and the N-terminal peptide (LHR$^{18-36}$) were less potent in the inhibition of photoaffinity labeling of hCG by ABG-$^{125}$I-LHR$^{exo3}$. Apparently, the labeling site of the exoloop 3 peptide in hCG is different from the N-terminal peptide labeling site. The endodomain is the site of signal generation, which likely
involves all three exoloops. It will be of interest to see whether the differential signal
generations for PLC β/IP induction and AC/cAMP induction are related with the distinct potency
of the exoloops 1 and 2 to inhibit the labeling of hCG by the exoloop 3 peptide. Our data show
that exoloop 3 plays roles in the activation of PLC β/IP induction and activation of AC/cAMP
induction, as well as in the affinity of hormone binding. However, these roles are not equally
important: the PLCβ activation is most crucial and hormone binding is least crucial. In fact, the
role in the PLCβ activation is so crucial that most of the exoloop 3 residues appear to be
involved. In contrast, there are fewer residues that appear crucial for the AC activation. They
are Pro575, Leu576, Val579 and Lys583. Substitution of these residues with Ala impaired the
activation of PLCβ, AC, or both. In addition, exoloop 3 constrains hormone binding at the
exodomain, and Ala substitution for the residues often improved the binding affinity. In
particular, the Lys583Ala substitution resulted in a 2 fold improvement in the binding affinity.

These residues are not in tandem in a linear sequence, suggesting a spatial orientation or
cluster. The expression level of the LHR mutants varied from 2,000-230,000 receptors/cell.
However, it does not appear to hold in the gonadotropin receptors, at least in the homologous
receptor system. Over the years, different individuals in our laboratory have consistently shown
that varying numbers of wild type receptors in the range of 8,000 - 265,000 receptors/cell
induced the similar maximum cAMP production as long as the same HEK 293 cell line was
used. In addition, the Pro574Ala mutant induced consistently the lowest maximum cAMP level,
which is 19% of the wild type value with 2,400 receptors/cell in this study and 24% with 17,000
receptors/cell in the previous study (109). This result is consistent with the classic observations
(131-133); only a small fraction of receptors expressed on the cell surface are actually involved
in receptor activation, whereas the bulk of receptors are involved in taking up excess hormones
for internalization and degradation. In conclusion, we present the first evidence that LHR
exoloop 3 interacts with hCG and is involved in the differential activation of PLC β and AC.

Although FSHR exoloop 3 interacts with FSH and differentially modulates activation of
PLC β and AC, there are striking differences in the mode of the interactions and modulation
between the two systems. LHR exoloop 3 is close to both of the hCG α and β subunits, whereas
FSHR exoloop 3 is close to the FSH α subunit. In parallel to these distinct spatial arrangements,
the tandem Leu-Ile sequence near the middle of exoloop3 is crucial for AC activation in FSHR
but not in LHR. The penultimate C-terminal residue is essential for PLC β activation in LHR but
not in FSHR. The interaction of exoloop 3 with hCG is related to the interactions of hCG with the hinge and LRR4 regions but not the N-terminal region of the exodomain. Our results provide new insights into the transition from the initial interaction of hCG with the exodomain to the subsequent interaction with the exoloops, leading to signal generation.
### Appendices

#### Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupling receptor</td>
</tr>
<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotropin</td>
</tr>
<tr>
<td>IC50</td>
<td>Effective concentration for 50% inhibition</td>
</tr>
<tr>
<td>IP</td>
<td>Inositol phosphate</td>
</tr>
<tr>
<td>IP₁</td>
<td>Inositol monophosphate</td>
</tr>
<tr>
<td>IP₂</td>
<td>Inositol bisphosphate</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IP₅</td>
<td>Total inositol phosphate</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LHR</td>
<td>LH/CG receptor</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-Rich Repeat</td>
</tr>
<tr>
<td>NHS-AB</td>
<td>N-hydroxsulfosuccinimide ester of 4-azidobenzoic acid</td>
</tr>
<tr>
<td>NHS-ABG</td>
<td>N-hydroxsulfosuccinimide ester of 4-azidobenzyol glycine</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SES</td>
<td>Ethylene glycolbis-[sulfosuccinimidylsuccinate]</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
References

VITA

Name: Myoungkun Jeoung
Date of Birth: July 29, 1966
Place of Birth: Pusan, Republic of Korea

EDUCATION

B.S. 1986-1989 Pusan National University, Pusan, Republic of Korea
Major: Molecular Biology
M.S. 1995-1997 San Diego State University, San Diego, California
Major: Biology

RESEARCH EXPERIENCE

1998-present Graduate Student
University of Kentucky, Lexington, KY
Biology Department
Principle Investigator: Dr. Tae H. Ji
1997-1995 Graduate Student
San Diego State University, San Diego, California
Biology Department
Principle Investigator: Dr. Michael Breindle
1990-1991 Graduate Student
Pusan National University, Pusan, Republic of Korea
Molecular Biology Department
Principle Investigators: Dr. Kyu-won Kim
1987-1989 Student Investigator
Pusan National University, Pusan, Republic of Korea
Molecular Biology Department
Principle Investigators: Dr. Kyu-won Kim
PUBLICATIONS
