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MODULATION OF HIGH AFFINITY HORMONE BINDING TO LH/CG RECEPTOR

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ABSTRACT OF THESIS

MODULATION OF HIGH AFFINITY HORMONE BINDING TO LH/CG RECEPTOR

Precise control of physiological phenomena is performed by various kinds of receptor mediated signaling. The vast majority of receptors belong to the superfamily of G protein coupled receptors (GPCRs), which form one of the largest protein families. In the classical model of GPCR signaling, stimulation of seven transmembrane spanning GPCR leads to the activation of heterotrimeric G proteins, which dissociate into $\alpha$ and $\beta\gamma$ subunits. The subunits activate effector molecules, which include second messenger generating systems, giving rise to various kinds of cellular responses. The LH/CG receptor is a member of the glycoprotein hormone receptor family along with the FSH and TSH receptors, which belongs to the GPCR superfamily. Human chorionic gonadotropin (hCG) binds to the exodomain of LH/CG receptor and the resulting hCG-exodomain complex is thought to interact with the endodomain of the receptor to bring about hormone signal. Unfortunately, little evidence is available for the precise hormone contact points in the exo domain and endo domain of the receptor. The affinity of hormone binding to the exodomain was enhanced when the endodomain was truncated. This suggests that the endodomain modulates the hormone binding to the exodomain of the receptor. To understand this, the role of exoloop 2 on the modulation of high affinity hormone binding to the exodomain was studied using photoaffinity labeling technique.

Meena Sundaramoorthy
20 May 20, 2002
MODULATION OF HIGH AFFINITY HORMONE BINDING TO LH/CG RECEPTOR

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THESIS

Meena Sundaramoorthy

The Graduate School
University of Kentucky
2002
MODULATION OF HIGH AFFINITY HORMONE BINDING TO LH/CG RECEPTOR

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at the University of Kentucky

By
Meena Sundaramoorthy
Lexington, Kentucky

Director: Professor of Chemistry
Lexington, Kentucky
2002
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Meena Sundaramoorthy

2002
This thesis work is dedicated to the love and understanding of my wonderful mom Mrs. Uma and sweet little sister Ms. Vidhya.
ACKNOWLEDGEMENTS

I would like to thank Prof. Tae H. Ji for being my philosopher and guide throughout the preparation of this thesis. I am thankful for his patience and all the wonderful opportunities he provided me. Many members of “THE JI LAB” made me feel at home – Myoungkun Jeoung, Chang Woo Lee, Johann Sohn, Becky and Gail – I am glad that I had the opportunity to know them all and work with them. My special thanks to Dr. Inhae Ji for her kind and encouraging words at the time of need.

I would like to thank the other members of my advisory committee, Profs. Allan D. Butterfield and Boyd E. Haley for all their efforts towards making me understand myself better.

I would also like to acknowledge the commitment and support of my husband Rajagopal Srirangam, without him, this would not have been possible.

Finally, I owe my mom Uma and sister Vidhya, a great big thank you for a lifetime of support and encouragement in all of my undertakings, academic and otherwise that has meant the world of difference.
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Chapter I
Introduction

G protein coupled receptors (GPCRs) constitute the largest and the most diverse membrane proteins involved in signal transduction. Approximately 1% of the human genome encodes for these G protein coupled receptors (1). The Luteinizing hormone receptor belongs to a subfamily of glycoprotein hormone receptors within the GPCR family that include the receptors for FSH, TSH and hCG. hCG is structurally very similar to LH and binds with high affinity to the LHR and hence the receptor is referred to as LH/CGR. Both the LH/CGR and the FSH-R play crucial role in the reproduction of humans. Human reproduction has been a major worldwide concern. There are countries that suffer from the population explosion whereas other countries wherein families suffer from infertility and mutations of the receptors result in hereditary reproductive disorders (2). Understanding the interaction of the LH and hCG with the LH/CGR would help in the development of drugs that can be used to solve such problems. GPCRs account for ~ 40% of all therapeutic intervention and major GPCR research projects are found in the pharmaceutical industry(2,3).

Glycoprotein hormones LH, FSH, TSH and hCG are the most complex agonists for GPCRs. The LH and hCG both bind to the LH receptor while the FSH and TSH have their own cognate receptors. GPCRs are activated by a wide variety of ligands including odorant molecules, light, peptide and nonpeptide neurotransmitters, hormones and growth factors. These glycoprotein hormones are heterodimers with a common α subunit and a hormone specific β subunit(4). LH and FSH act together to regulate gonadal functions. They are involved in the growth and functions of the testis and ovaries and regulation of gametogenesis and steroidogenesis(5).

GPCRs have an extracellular N-terminal domain and a membrane associated C-terminal domain that consists of seven transmembrane helices connected by three exoloops, three cytoloops and a cytoplasmic tail (fig 1).
Figure 1. Schematic representation of GPCR

The general structure of the seven transmembrane GPCR.

They are coupled to heterotrimeric G proteins and hence are referred to as G protein coupled receptors. Upon hormone binding, the hormone/receptor complex undergoes a conformational change and activates G protein to replace GDP with GTP in the Gα subunit. This brings about the activation of adenylyl cyclase and phospholipase C that results in the formation of the second messengers, cAMP and IP₃, respectively. These second messengers then bring about a lot of other cellular responses.

The LH/CGR consists of two equal halves, a 341 amino acids long extracellular N terminal half and a 334 amino acids long membrane associated C terminal half, which includes the seven transmembrane domains connected by three exoloops and three cytoloops(6,7). In addition, it has a signal peptide of 26 amino acids. The molecular weight of mature, lacking the signal peptide, LH/CG-R polypeptide is calculated to be ~75,000 Daltons. However, the actual molecular
weight is ~85,000 Daltons due to N-glycosylation at 6 Asn residues in the exodomain. The endodomain is palmitoylated and phosphorylated and their roles are not fully defined.

The exodomain alone is capable of high affinity hormone binding, which is modulated by the endodomain(8,9). This suggests that the exodomain, in particular the hormone contact sites, and the endodomain are intimately related to each other during the initial and secondary interactions. The exodomain, endodomain and the hormone ternary complex is essential for signal generation (10). However the precise hormone contact points in the exodomain and the endodomain are unclear. The hormone binding to the exodomain was improved when the endodomain was truncated (11-13). This suggests that the endodomain influences the hormone binding to the exodomain. Furthermore, the hCG appears to interact with the exoloop 2 of the LH/CGR (14). Ala scanning of the 20 amino acids of the exoloop 2 showed that the exoloop 2 indeed influences the binding of the hormone to the exodomain (9). The aim in the present work was to gain insight into the molecular basis of hCG binding to its receptor and how the binding is modulated by the exoloop 2, and whether hCG binds to the exoloop 2 using photoaffinity labeling.

**Biology of hCG and Glycoprotein hormones**

Gonadotropins comprise of Follicle Stimulating hormone (FSH), Luteinizing hormone (LH), human Chorionic gonadotropin (hCG) and Thyroid Stimulating hormone (TSH). FSH, LH and TSH are secreted by the anterior pituitary gland, whereas the trophoblast cells of the human placenta produce hCG. hCG prevents the deterioration of the corpus luteum and thus is essential for the maintenance of pregnancy. These hormones are heterodimers with a common α subunit and a hormone specific β subunit (4). These two subunits are non-covalently associated and are encoded by distinct genes (15). High-resolution structures of hCG have been reported (16-18) and are considered as paradigms for the entire glycoprotein hormone family. Each subunit in these heterodimers is folded into three loops (α1,α2,α3; β1,β2, β3) that project from a cystine knot located in the center. The unique β subunit of hCG has a seatbelt structure formed by an intramolecular disulphide bridge (Cys26-Cys110) that embraces the α2 (fig 2).
There have been numerous studies on which subunits of the hormones are involved in receptor binding and biological activities. Photoaffinity labeling studies and immunological experiments have demonstrated that both of the $\alpha$ and $\beta$ subunits interact with the receptors. When hCG radioiodinated at either the $\alpha$ or $\beta$ subunit were incubated with the porcine granulose cells, it was shown that a complex formed with the LH/CG receptor regardless of whether the hormone was labeled in the $\alpha$ or $\beta$ subunit after photoaffinity labeling (19). Monoclonal antibodies against the $\alpha$ or the $\beta$ subunit blocked the formation of the LH/CG R complex, suggesting that both subunits of hCG are involved in binding to the receptor. Both of the gonadotropins subunits are highly glycosylated with asparagines-linked (N-linked) and/or serine-linked (O-linked) oligosaccharides (20-23). The common $\alpha$ subunits are N-glycosylated at two conserved sites in FSH and CG, and one in LH. Unlike other gonadotropins, the hCG $\beta$ subunit has four additional O-glycosylation sites (22). The importance of carbohydrates on the glycoprotein hormones have
been implicated in the hormonal stability, secretion, assembly, receptor binding and steroidogenesis. When carbohydrates are removed from hCG by anhydrous hydrogen fluoride (HF), the resulting dg-hCG fully maintained its receptor binding and immunological activities but lost its cAMP and progesterone inducibilities (24-26).

Function of Gonadotropins:

In females gonadotropins control the development of the ovarian follicles, the release of oocytes from the follicles and the transformation of the ovulated follicles into the corpus lutea (27-29). Follicles consist of an outer layer of theca interna cells, an inner layer of granulose cells and an oocyte-cumulus complex (fig 3). Diverse communications between these follicular cells and the pituitary gland, via gonadotropins, steroidogenesis and production of gonadal peptides, maintain the continuous development of follicles in a cyclic pattern.

Figure 3. Graafian follicle
During the normal cycles of follicular development, ovulation occurs in two phases—Follicular phase and Luteal phase (fig 4). The early follicular phase is characterized by high levels of FSH and low levels of LH, estradiol and progesterone, which are related to the pulsative secretion of hypothalamic gonadotropin releasing hormone (GnRH) (29-31). FSH activates its receptor, which is present in granulosa cells of the ovarian follicles, causing an elevated level of cAMP in the cytosol (32). This signal is largely responsible for stimulating follicular growth, controlling up- and down- regulation of FSH receptor levels, stimulating the activity of aromatases to synthesis estradiol and inducing LH/CGR receptor expression (32,33). The LH/CGR is present on both theca and granulose cells. Theca cells produce androstenedione, an androgen precursor, from cytoplasmic cholesterol, which is regulated by LH and cAMP levels (27). Androstenedione diffuses into granulose cells and is aromatized into estrogens. The aromatase activity in the granulose cells is regulated by FSH in immature follicle, and by both LH and FSH in mature follicles (34). Both cells contain progesterone which is secreted or used as the precursor of estrogens.

By the mid to the late follicular phase, estradiol secretion is increased and FSH secretion is suppressed. The increased estradiol and progesterone levels in plasma results in markedly increased secretion of LH. This LH surge triggers granulose cell disaggregation and subsequent release of the ovum. After ovulation both the granulosa and theca cells are luteinized to form the corpus luteum. The corpus luteum is rich in LH/CGR and produce mainly progesterone in a LH dependent manner (28). In normal menstrual cycle, the corpus luteum functions from 12 to 16 days and then regresses.

In a fertile cycle, hCG secreted from the syncytiotrophoblast maintains the corpus luteum function by sustaining the plasma levels of estradiol and progesterone through cAMP mediated protein kinases (35, 36). The hormone is detected a few days after implantation (37). The hCG levels reach their highest level in plasma at about 10-12 weeks (100,000mIU/mL) and decrease during the second and third trimesters (38). hCG also induces relaxin secretion from corpus luteum in the early stages of pregnancy, which may play an important role in maintaining pregnancy.
When fertilization does not occur, the corpus luteum produces a lower amount of estradiol and progesterone and becomes regressed during the late luteal phase. This decline in ovarian steroids leads to an increase in GnRH. GnRH stimulates FSH release, which initiates the next cycle of follicular maturation.

**Figure 4. Menstrual Cycle**

[Image: http://www.wisc.edu/ansci_repro/lec/lec_11/lec11fig.html#figure 5]

**Receptors**

G protein coupled receptors:

Nearly 2000 GPCRs have been reported since bovine opsin was cloned in 1983 (39). They are classified into over 100 subfamilies according to the sequence homology, ligand structure and receptor function. All GPCRs contain seven transmembrane spanning regions with their N-terminal segment on the exoplasmic side and their C-terminal segment facing the cytosolic side of the plasma-membrane. Binding of a ligand to a GPCR causes a conformational change that
allows the receptor to bind to specific G proteins. The activated G protein stimulates enzymes like adenylyl cyclase, which produces second messengers that bring about cellular responses. GPCRs are involved in the recognition and transduction of messages as diverse as photons, Ca^{2+}, neurotransmitters, biogenic amines and small molecules including amino acids, peptides, nucleotides as well as proteins. The size of the ligands ranges from 35kD to less than a few 100 daltons. Interestingly, a weak correlation exits such that the larger the ligand, the longer the N-terminal extension of the receptor. For example, the N-terminal extensions of the glycoprotein hormone receptor are the largest with approximately 300 amino acids, in comparison to the 11-30 amino acids long N-terminal extensions of adrenergic receptors (40).

A vast number of biological receptors that are coupled to G proteins have been cloned and characterized (table I). All these receptors have in common a central core of seven transmembrane helices connected by three exoloops and three cytoloops. Two cysteine residues (one in exoloop 1 and other in exoloop 2) are conserved in most GPCRs, form a disulfide bond, which might be involved in the stabilization of a conformation of the domains that is required for its activity. Each of the seven transmembrane helices is generally composed of 20-27 amino acids, while the N-terminal segment (7-595 amino acids), loops (5-230 amino acids) and C-terminal segment (12-359 amino acids) vary in size indicating their diverse structures and functions.

Glycoprotein hormone receptors:

The glycoprotein hormone receptors consists of two roughly equal halves, a 350-400 amino acid N-terminal segment and an approximately 300 amino acid membrane associated segment. The N-terminal half alone is capable of high affinity hormone binding, whereas the membrane associated half is the site of receptor activation and signal generation (8). The N-terminal domain has 8-9 Leu-rich repeats. These Leu-rich repeats are thought to form a crescent with the concave inner surface consisting of β sheets, which may bind ligands (41, 42). In addition to the Leu-rich crescent, the N-terminal region of the N-terminal segment contacts the hormone (43). Although the N-terminal segment alone is capable of high affinity ligand binding, this is modulated by exoloops of the receptor (44).
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<td>Unknown-UL33</td>
<td>Viral</td>
<td></td>
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<tr>
<td>Bombesin family</td>
<td>amphibian</td>
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<tr>
<td>Bradykinins</td>
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<tr>
<td>Receptor Subtype</td>
<td>Species</td>
<td>G-proteins</td>
<td>Effector</td>
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<tr>
<td>------------------</td>
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</tr>
<tr>
<td>Tachykinins</td>
<td>amphibian</td>
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</tr>
</tbody>
</table>

* h: human, r: rat, m: mouse, b: bovine, p: procine, ham: hamster, c: canine

** AC: adenylate cyclase, PDE: cGMP phosphodiesterase, PLC: phospholipase C, PI: phosphatidyl inositol
Existing evidence suggests that glycoprotein hormones initially bind to the exodomain of the receptor with high affinity and hormone specificity and then the hormone-receptor complex is thought to interact with the endodomain of the receptor which then brings about signal generation.

LH/CG receptor:

![Figure 5. LH/CG Receptor](image)

LH/CG-R is encoded by a single copy gene in rats (45) and two genes in humans (46). It is found mainly in the gonads but also in other organs and tissues like brain, thyroid, prostate, uterus, placenta, fallopian tubes, fetal membranes and lymphocytes (47). LH/CG-R was also observed in benign breast region lesions, malignant breast tumors and other cancer cells. Unlike most G protein coupled receptors, it is comprised of an extremely long extracellular N-terminal half of 341 amino acids and a membrane associated C-terminal half of 334 amino acids (6,7) (fig 5). The endodomain is palmitoylated and phosphorylated and their roles are not fully defined. Using photoaffinity labeling studies with receptor peptides, it has been shown that both halves of the receptor have hormone contact points. The N-terminal half alone is capable of affinity hormone binding (11-13) with no hormone action (13,48). In contrast, the C-terminal half is capable of low affinity hormone contact with cAMP induction (13,48).
The exodomain binds the hormone with high affinity without hormone action but cannot generate hormone signal. Growing evidence suggests that glycoprotein hormone initially bind to the exodomain and the resulting exodomain-hormone complex undergoes conformational change (49) and interacts with the endodomain of the receptor (fig 6.a.). This secondary interaction is thought to generate signal (8,50-51). However the high affinity hormone binding at the exodomain is modulated by the endodomain (52). The LH/CG-R generates two distinct signals, one to activate adenylyl cyclase and produce cAMP and the other to activate Phospholipase C and produce diacylglycerol and inositol phosphates (Fig 6.b.). Despite the importance, only limited information is available concerning the precise hormone contact residues and sites in the exodomain.
Studies involving truncation, deletion and site-directed mutagenesis indicate that several regions of the exodomain are crucial for hormone binding. Sequence comparison and modeling suggest that the exodomain contains Leu/Ile rich repeats (LRRs) (6), which are flanked by short N-terminal and C-terminal regions (fig7). LRRs are a structural motif in proteins like ribonuclease inhibitor and also common in a large family of proteins. Based on the structure of the ribonuclease inhibitors, an issue was raised that the inner lining of the LRRs in LH/CG-R and FSHR might contact the respective hormones (42,53-55), in particular, with the concave side of the hormone (16). It was shown that the β strand Leu and Ile residues in all LRRs in the human LHR and FSHR are crucial contact sites (56), especially the LRR2 and LRR4 are important for hormone binding. Affinity labeling studies showed that the N-terminal region of the LRR4...
interacts with hCG, preferentially the \( \alpha \) subunit and the hCG/LRR4 complex interacts with the exoloop 2 of the endodomain (67).

The seven to nine Leu-rich repeats in the exodomain, are generally thought to form 1/3 donut-like structure and interact with the hCG. The resulting hCG–exodomain complex then interacts with the endodomain (exoloop 2) which is results in the signal generation.

But it was unclear whether this alone could be sufficient for the hormone action. To address this, first residues in the exon 9-10 junction of LHR, junction sequences of FSHR and TSHR were aligned. A homology was found from Tyr\(^{253}\) to Phe\(^{260}\). In addition, the sequence corresponding to LHR Arg\(^{261}\) to Gln\(^{268}\) is highly conserved within each receptor type among species. So, the exodomain was Ala scanned and receptor peptides were used for photoaffinity labeling and affinity cross-linking.

![Figure 7. Leucine rich repeat](image)

*Adapted from Jeoung, M., Phang, T., Song, Y., S., Ji, I and Ji T., H (2001) J. Biol. Chem. 276, 3443-3450*

It was shown that the C-flanking sequence (the hinge region), Thr\(^{250}\) – Gln\(^{268}\) (58) of the LRRs specifically interacts with hCG, preferentially hCG\(\alpha\). This interaction was shown to be inhibited by exoloop 2 and not by exoloops 1 and 3. This suggested the importance of the ternary complex that exists between the exodomain of the receptor, hCG and the endodomain of receptor (59). Thus the endodomain was seen to modulate the hormone binding to the exodomain. Further a
defined epitope on the hCG was shown to interact with the exoloop 2 of the LH/CG-R (14). Ala scanning of the 20 amino acids of the exoloop 2 showed that the exoloop 2 indeed influences the binding of the hormone to the exodomain (52).

Both the LH/CG-R and FSHR play crucial role in the reproduction of humans. Human reproduction has been a major worldwide concern. Approximately 16% of the American families experience infertility problems and mutations of these receptors result in hereditary reproductive disorders. Therefore, these hormones and LH/CG-R have been implicated for the treatment of fertility and infertility disorders. GPCRs account for ~ 40% of all therapeutic intervention and major GPCR research project are found in the pharmaceutical industry (2,3)

The wealth of information gained over the last decade has substantially improved our understanding of GPCR function. However the precise identification of the regions of hormone and receptors that are capable of interaction and/or triggering transmembrane signaling remains incomplete (60). The further clarification of these mechanisms represents a daunting task coupled with efforts aimed at obtaining high-resolution X-ray crystals.

The high affinity hormone binding to the N terminal extracellular domain of the GPCR and the G proteins activated by the intracellular regions of the receptors suggests that upon hormone binding, a portion of the hormone or the receptor’s extacellular regions might interact with the exoloops of the GPCRs in ligand binding. The possibility of hormone-receptor interaction through the exoloops of GPCRs has already been reported. The exoloop 2 of the LH/CGR consisting of 20 amino acids connects the TM 4 and TM 5 domains. The sequence alignment is as shown in fig8. The 20 amino acids sequence

**Fig8. Comparison of exoloop 2 sequences among different species**

order to identify the amino acid residues that are crucial for the hormone binding and signal generation Ala scanned the 20 amino acids long exoloop 2. They found that the high affinity hormone binding at the exodomain is constrained by the amino acids Ser\(^{484}\), Asn\(^{485}\), Lys\(^{488}\), Ser\(^{490}\) and Ser\(^{499}\) (61). As a result of this, the exodomain attains maximal affinity for hormone binding when the endodomain is truncated and cAMP induction is disengaged. It also demonstrated that exoloop 2 of LH/CG R influences the hormone binding affinity, the affinity for cAMP induction and maximal cAMP production, probably by the interaction of the exoloop 2 with the exodomain and other parts of the endodomain like the TM 6 and TM 7.

However, one may raise an issue as to whether the Ala substitution effects were due to the changes in the overall structure of the receptor rather than the changes in the structure close to the substituted residues. But there is growing evidence that support local conformational changes induced by substitution of a single amino acid. The Cys residue in the exoloop 2, Cys\(^{492}\) is highly conserved among the different species and it has been implicated to form a disulphide bridge with the Cys\(^{416}\) present in exoloop 1, in other GPCRs (62). However, in LH/CG R the C492A

|        | S | N | Y | M | K | V | S | I | C | F | P | M | D | V | E | T | T | L | S | Q |
| LHR Human | S | N | Y | M | K | V | S | I | C | F | P | M | D | V | E | T | T | L | S | Q |
| LHR Mouse | * | S | * | * | * | * | * | L | * | * | * | * | * | * | * | * | * | * | * | * |
| LHR Rat | * | * | * | * | * | * | * | L | * | * | * | S | * | * | * | * | * | * | * | * |
| LHR Bovine | * | * | * | * | * | * | * | L | * | * | * | S | * | * | * | * | * | * | * | * |
| LHR Pig | * | S | * | * | * | * | * | L | * | * | * | * | * | * | * | * | * | * | * | * |
| LHR Calja | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Q14751 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| Q15996 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
substitution resulted in the loss of surface expression but not the high affinity hormone binding. This shows that the Cys residue in exoloop 2 is involved in surface expression but is not important for hormone binding. Computer modeling suggests that Ser\textsuperscript{484}, Asn\textsuperscript{485}, Lys\textsuperscript{488}, Ser\textsuperscript{490} and Ser\textsuperscript{499} may be positioned on one side of exoloop 2. Two models were constructed (fig9). In both models these residues are clustered together in the middle of the exoloop 2 suggesting their role in binding affinity.

**Figure 9. Computer models of exoloop 2**

All these results support the significance of exoloop 2 in the modulation of the hormone binding to the exodomain of the LH/CG R. The next question is whether or not the crucial exoloop 2 actually interacts with the hormone. To test if the region actually interacts with the hormone, hCG was photoaffinity labeled with a peptide mimic corresponding to Ser\textsuperscript{484} – Gln\textsuperscript{503} of the receptor.

**Chapter II**

**Materials and Methods**

Materials:
The N-hydroxysuccinimide (NHS) ester of 4-azidobenzoic acid (AB) was synthesized as described previously (63). The N-hydroxysulfosuccinimide esters of ethylene glycolbis(sulfo succinimidyl succinate) (SES) was purchased from Pierce. hCG CR127 and hCG subunits were supplied by the National Hormone and Pituitary Program. 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 484-503 (exoloop 2), 246-269 (hinge region) and receptor peptides in which one of the amino acid residue was substituted by Bpa like LHR484-503 F493Bpa, LHR96-115 C106Bpa, LHR96-115 S104Bpa and LHR96-115 G109Bpa. All these were purchased from Genemed Synthesis Inc. South San Francisco California, USA.

Radioiodination

Introduction:
Radioiodination remains one of the extensively used methods for labeling compounds of medical and biological interest. The radionuclide I$^{125}$, with its 60 days half-life, has been the radionuclide of choice for producing labeled compounds for in vitro assays (63). Among several reactions used for I$^{125}$ labeling, the Chloramine-T (N-chloro-p-toluenesulfonamide) oxidation, developed by Hunter and Greenwood in 1962 (64), has proven to be the method of choice for various compounds containing tyrosyl residues. Thus effective radioiodination can take place in many compounds and Chloramine labeling often results in biomolecules with high specific activities. The principle of labeling is based on the “in situ” oxidation of iodide to atomic iodine and its nucleophilic substitution into phenol rings in ortho position located in the hydroxyl group of tyrosyl residues of proteins. However, damage to target molecules is a matter of concern while using this radiolabeling technique.

Reagents:
Sodium iodide-125 (I\(^{125}\)) was purchased from Amersham Pharmacia Biotech, UK. Chloramine-T was purchased from Sigma (Sigma Chemical Company, St. Louis, MI, USA) and stored in a dry place at room temperature. Shortly before labeling, a stock solution was prepared at a concentration of 1mg/mL in 10mM Na\(_2\)HPO\(_4\) and 0.9% NaCl (pH 7.4) (PBS). Sodium metabisulfite stock solution was also prepared at a concentration of 2.5mg/mL in PBS that was used as a reducing agent to stop the reaction. The target peptides were dissolved in 0.1M sodium phosphate (pH 7.5) at a concentration of 30ug/40uL.

Radiolabeling and reaction conditions
1mCi of Na\(^{125}\)I in 10uL of 0.1M NaOH and 7uL of chloramine-T (1ug/\(\mu\)L) in 10mM Na\(_2\)HPO\(_4\) and 0.9% NaCl (pH 7.4) was added to 30ug of peptide in 40uL of 0.1M Sodium phosphate (pH 7.5). After 20 s, 7uL of sodium metabisulfite (2.5mg/mL) in PBS was introduced to terminate the radioiodination.

Gel permeation of labeled molecules:
Gel permeation was done to remove unbound I\(^{125}\). For this purpose radioiodinated peptide solution was mixed with 60uL of 16% sucrose solution in PBS and fractionated on Sephadex Superfine G-10 column (0.6 * 15 cm) previously saturated with elution buffer (PBS). Elution was done with PBS and the fractions were counted for radioactivity. The specific activity was calculated.

Derivatization with NHS-AB or SES was done by freshly dissolving the the crosslinking reagent in dimethyl sulfoxide to a concentration of 50mM in 0.1M sodium phosphate (pH 7.5) to a concentration of 20mM. This reagent solution was immediately used to derivatize receptor peptides. In the dark, 10uL of NHS-AB or SES was added to the 30ug of peptide in 40uL of 0.1 sodium phosphate (pH 7.5). The mixture was incubated for 30 min with the cross-linking reagent.

Radioiodination of hCG:
The following protocol is for the radioiodination of hormone only, NOT peptide. It is set up stoichiometrically such that one I\(^{125}\) molecule will react with one hormone molecule.
Reagents:
Sodium iodide-125 (I^{125}) was purchased from Amersham Pharmacia Biotech, UK.
Chloramine-T was purchased from Sigma (Sigma Chemical Company, St. Louis, MI, USA) and stored in a dry place at room temperature. Shortly before labeling, a stock solution was prepared at a concentration of 0.3mg/mL in 10mM Na_{2}HPO4 and 0.9% NaCl (pH 7.4) (PBS). Sodium metabisulfite stock solution was also prepared at a concentration of 0.66mg/mL in PBS that was used as a reducing agent to stop the reaction. The target hormone was dissolved in 0.1M sodium phosphate (pH 7.5) at a concentration of 3ug/40uL.

Radiolabeling and reaction conditions are similar to radioiodination of peptide and to purify the iodinated hormone, gel permeation was carried out as described above but on a Sephadex G-50 superfine column.

Storage:
I^{125} labeled peptides and hormone were stored in 4° C with the tubes containing photosensitive peptides wrapped with aluminium foil.

Special Considerations while working safely with I^{125} (Amersham pharmacia biotech):

1. Freezing or acidification of solutions containing iodide ions can lead to the formation of volatile elemental iodine.
2. Opening a vial of high radioactive concentration of I^{125} can produce active aerosols.
3. Some iodo-compounds can penetrate surgical rubber gloves; two pairs or polythene alternatives are recommended.
4. In the event of suspected contamination of personnel, block the thyroid by the administration of potassium iodate (170mg) or potassium iodide (130mg).
5. Spills of I^{125} should be stabilized with alkaline sodium thiosulphate solution before commencing decontamination.

Although the method is considered easy and reliable, the damage to target molecules due to excess of oxidant is of major concern. Loss of immunological activity in proteins is
thought to be due to Chloramine-T action on the disulphide bridges and sulphhydryl groups, often giving rise to macro molecular protein complexes (64,65). In summary, radioiodination of biomolecules by the Chloramine-T method is the best method if excessive use of oxidizing agent is avoided.

B. Photoaffinity labeling:

Photoaffinity labeling is a technique that makes use of capacity of certain compounds to make covalent bonds after UV irradiation and it overcomes the non-specificity of using homobifunctional reagents for crosslinking by eliminating random collisional cross-links. The general protocol for photoaffinity labeling is as shown in the figure 10.

Figure 10. Diagramatic representation of Photoaffinity Labeling
Method I:

Photoaffinity labeling using N-hydroxysuccinimide ester (NHS):

To photoaffinity label hCG with $^{125}$I-LHR$^{484-503}$, the receptor peptide was derivatized with N-hydroxysuccinimide ester of 4-azidobenzoic acid, (NHS-AB) to produce NHS-AB-$^{125}$I-LHR$^{484-503}$ as described earlier. Disposable glass tubes were siliconized under dimethyldichlorosilane vapor for 4 hours and autoclaved. In each of the tubes, 20uL of PBS, 10uL of hCG in PBS (7ng/uL) and 10uL of AB-$^{125}$I-LHR$^{484-503}$ (10ng/uL) in PBS were mixed and incubated at 37°C for 90 min in the dark, irradiated with Mineralight R-52 UV lamp for 1 min as described previously (66) and solubilized in 2% SDS, 100mM dithiothreitol and 8M urea to cleave disulfides and dissociate interacting components of complexes. The samples were electrophoresed on 8-12% polyacrylamide gradient gels. Gels were dried on Model 583 gel dryer (Bio-Rad), which was exposed to a molecular imaging screen (Bio-Rad) overnight. The imaging screen was scanned on a Model GS-525 Molecular Imager System Scanner (Bio-Rad).

Method II:
Photoaffinity labeling with Bpa as the photoactive group:

A radioiodinatable peptide, LHR\textsuperscript{484-503}, that had a photolabile para-benzoyl-L-phenylalanine (Bpa) residue at 493 position, instead of the phenylalanine originally present, was used to photoaffinity label hCG. For this purpose, the Bpa peptide was radioiodinated as described previously.

Advantages of using Bpa as a photoactivable reagent:

– Excited state is inert towards water
– Chemical stability
– Can be incorporated at a defined position of peptides during synthesis

![Structure of Bpa](image)

**Figure 12. Structure of Bpa**
Adapted from Gyorgy Dorman and Glenn D. Prestwich (1994) Biochemistry, 33,5661-5671

Labeling and reaction conditions:

The following solutions were sequentially introduced into siliconized tubes: 30\textmu L of radioiodinated Bpa peptide and 10\textmu L of hCG. The mixture was incubated for 90 min at 37\degree C in the dark, irradiated with Mineralight R-52 UV lamp for 1 min and solubilized with 2\% SDS, 100mM dithiothreitol and 8M urea to cleave disulfides and dissociate interacting components of complexes. The samples were electrophoresed on 8-12\% polyacrylamide gradient gels. Gels were dried on Model 583 gel dryer (Bio-Rad), which was exposed to a molecular imaging screen (Bio-Rad) from 24-36 hours depending on the radioactivity present in the gel. The imaging screen was scanned on a Model GS-525 Molecular Imager System Scanner (Bio-Rad). The photoaffinity group of choice is p-benzoylphenylalanine (Bpa), activated at 300-350 nm. However it has to be taken into account that Bpa is a bulky molecule that can alter the binding of the peptide to receptor. To make a replacement of other
aminoacid to Bpa, it is better to replace the hydrophobic amino acid in order to try to preserve the activity.

C. Affinity crosslinking:

Introduction:
In affinity crosslinking, radioactively labeled ligands are allowed to bind to the receptor and the resultant ligand-receptor complex, along with all other protein molecules present in the system, is subject to covalent crosslinks by the introduction of homobifunctional reagents called crosslinkers. Example of a crosslinker is ethylene glycolbis(sulfosuccinimidylsuccinate) (SES) also abbreviated as (Sulfo EGS).

![Figure 13. Structure of SES](Adapted from www.usalchemy.com)

Properties:
1. M.W. 660.5
2. Primary amine reactive
3. Hydroxylamine cleavable
4. Water soluble

Method:
In siliconized tubes, 20uL of PBS, 10uL of hCG and 10uL of \(^{125}\) I-LHR \(^{484-503}\) were mixed and incubated at 37\(^\circ\) C for 90 min. After incubation, 5uL of 0.3mM SES in dimethyl sulfoxide was added to each tube and further incubated at 25\(^\circ\) C for 20 min. The samples were boiled for 2 min in 2\% SDS, 100mM dithiothreitol and 8M urea to cleave disulfides and
dissociate interacting components of complexes. The solubilized samples were electrophoresed on 8-12% polyacrylamide gradient gels. The gels were dried on a Model 583 Gel Dryer (Bio-Rad) and exposed to molecular imaging screen (Bio-Rad). The imaging screen was scanned on a model GS-525 Molecular Imager System Scanner (Bio-Rad).

Advantages and Disadvantages of photoaffinity labeling and affinity crosslinking:

The primary advantage of photoaffinity labeling over affinity crosslinking is that formation of a covalently crosslinked complex is limited to the ligand and the receptor in photoaffinity labeling in other words is highly specific, whereas in affinity crosslinking formation of crosslinked complexes is random and non-specific. However, one major advantage of affinity crosslinking is the complex is not light sensitive and hence reaction need not be carried out in the dark.

D. Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Introduction:

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique.

SDS-PAGE:

A technique for analyzing mixtures of proteins or for estimating protein molecular weights, involving electrophoresis in a polyacrylamide gel in the presence of the anionic detergent, sodium dodecyl sulphate (SDS). The SDS binds to the proteins, denaturing them and conferring on them a uniform density of negative charges. The denatured protein / SDS
complexes show a constant relationship between mobility and log (MW). The molecular weight of a particular protein can be assessed by comparison of its migration with those of a series of marker proteins of known sizes.

The pore size of the gel may be varied to produce different molecular seiving effects for separating proteins of different sizes. In this way, the percentage of polyacrylamide can be controlled in a given gel. By controlling the percentage (from 3% to 30%), pore sizes can be modulated, usually to resolve molecules of 5 to 2,000 kdal. This is the ideal range for gene sequencing, protein, polypeptide, and enzyme analysis. Polyacrylamide gels can be cast in non gradient or with varying gradients. Gradient gels provide continuous decrease in pore size from the top to the bottom of the gel, resulting in better defined bands. Because of this banding effect, detailed genetic and molecular analysis can be performed on gradient polyacrylamide gels. Polyacrylamide gels offer greater flexibility and more sharply defined banding than agarose gels.

![Figure 14. Structure of SDS](image)

**Figure 14. Structure of SDS**
Figure 15. General Schematic Representation of SDS-PAGE

Method:
The solubilized samples were loaded onto a 8-12% polyacrylamide gradient gel. The electrophoresis was carried out initially at 60V for 2 hours and then at 100V for 8 hours. The gel was fixed in 33% methanol for 45 min and dried using Model 583 Gel dryer (Bio-Rad).

E. Deglycosylation and Glu C digestion of hCG:
1. Deglycosylation of iodinated hCG:

Introduction:
Glycosylation of glycoprotein hormones begins in the rough endoplasmic reticulum (RER). The figure 16 below shows the glycosylation sites on the human glycoprotein hormone α- and β-subunits. The subunit proteins are indicated by the horizontal bars and the N-linked oligosaccharides by the branch-like structures. The lollipop indicates O-linked glycosylation sites in CGβ. The amino acid residue to which the oligosaccharide is attached is indicated by the numbers under each glycosylation site.

![Diagram of glycosylation sites](image)

**Figure 16. Glycosylation sites in Glycoprotein hormones**

Oligosaccharide structures of glycoprotein hormones play an essential role in many of the functional characteristics of the molecule. They are important not only for the folding, assembly, conformational maturation and posttranslational processing of the subunits but also for heterodimer secretion, metabolic fate, interaction with cognate receptor and signal transduction. Chemical or enzymatic treatments to remove the N-glycans on hCG reveal that removal of the carbohydrate moieties has little effect on receptor binding. So, to check the binding of hCG without the sugars to LH/CG R, deglycosylation of iodinated hCG was carried out.
Materials and Method:

Peptide:N-glycosidase F (PNGase F) was bought from New England BioLabs Inc and O-Glycosidase from Roche Diagnostics.
PNGase is an amidase with an apparent molecular weight of 36,000 Daltons. It cleaves between the innermost GlcNAc and Asn residue of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins.

O-Glycosidase is used to release the Gal\(\beta\) (1-3) GalNAc from O-glycans. Bindings to serine as well as to threonine are hydrolysed.

![PNGase F hydrolyzes nearly all types of N-glycan chains from glycopeptides/proteins. \(x = H\) or sugar(s)](image)

To 10uL iodinated hCG the following was added, 5uL of 40mM DTT and 10uL of 1M Urea. The mixture was boiled for 4 min, cooled and then added 2uL of PNGase F and 1uL of O-Glycosidase. Incubated the samples for 18 hours at 37\(^\circ\) C. The samples were loaded onto 15% polyacrylamide single concentration gel. Gel was dried on a Model 583 gel dryer and exposed to molecular imaging screen (Bio-Rad) overnight. The imaging screen was scanned on a Model GS-525 Molecular Imager System Scanner (Bio-Rad).

2. Deglycosylation and Glu-C digestion of iodinated hCG photoaffinity labeled with peptide:

Introduction:
Endoproteinase Glu-C cleaves at Glutamic acid – X and Aspartic acid – X where X is any other amino acid residue. By treating the hCG photoaffinity labeled with peptide for deglycosylation and further to Glu C digestion, the fragments obtained can be analyzed by Mass Spectrometry to identify labeled amino acid residues.

Materials and Method:

Glu C was purchased from Roche Diagnostics. The iodinated hCG was incubated with wild type peptide (LHR\textsuperscript{96-115} G 109 Bpa) for 90 min at 37\,\degree C. The samples were irradiated with Mineralight R-52 UV lamp for 1 min. Added 10\,\mu L of 1M urea and 5\,\mu L of 20mM DTT to the above mixture and boiled for 4 min. The sample was cooled down and 8\% Glu-C was added and incubated at room temperature for 16 hours. The samples were solubilized in 2\% SDS, 100mM dithiothreitol and 8M urea to cleave disulfides and dissociate interacting components of complexes and electrophoresed on 15\% polyacrylamide single concentration gels. Gels were dried on Model 583 gel dryer (Bio-Rad), which was exposed to a molecular imaging screen (Bio-Rad) overnight. The imaging screen was scanned on a Model GS-525 Molecular Imager System Scanner (Bio-Rad).

Drawbacks of the enzymatics treatments:
1. The treatments may damage the protein backbone
2. Removal of the oligosaccharides may be incomplete.

Chapter III

Results and Conclusion

A.1. Radioiodination of peptide:

Radioiodination of compounds by the Chloramine-T method results in biomolecules with high specific activities. The following figure shows the radioiodiation of the LHR\textsuperscript{484-503} F 493 Bpa peptide. The \textsuperscript{125}I is incorporated on the Tyrosine, 10 fractions were collected. A sharp peak
which corresponds to the hottest fraction is number 3 with 408,477 cpm/2uL. The specific activity is 10,667.2 cpm/ng. The fraction number 3 was stored in a glass tube wrapped with aluminium foil to prevent exposure to light until use.

<table>
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<tr>
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<tr>
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<tr>
<td>10</td>
<td>82867</td>
</tr>
<tr>
<td>Total</td>
<td>1391370</td>
</tr>
</tbody>
</table>

Total cpm = 1391370 * 230

**Specific activity = 10667.2 cpm/ng**

Fraction 3 has 408477 * 230 = 93949710 cpm

Therefore ng of peptide present in fraction 3 is 8807.3 ng

100ng of peptide is present in 2.6uL of fraction 3

**Figure 17. Iodination of LHR\textsuperscript{484-503} F493Bpa**

For photoaffinity labeling using NHS-AB, the derivatized peptide is iodinated.
CT(0.38umol)=1mg/1mL spb
SM(0.92umol)=2.5mg/1mL spb
SU(16%)= 0.024g/150uL spb
NHS-AB = 0.00065g/50uL
peptide = 30ug/40uL
Flow rate=200uL/4 drops

Total cpm = 2040232 * 200
= 408050000

Specific Activity = total cpm/ng of peptide used
= 408050000/30000
= 13601.5cpm/ng

Specific Activity = 13601.5cpm/ng
Fraction 2 has 730474 cpm
Therefore ng of peptide present is 730474 * 200/ 13601.5

10741ng of peptide is present in fraction 2
100ng of peptide is present in 1.9 uL of fraction 2
100 ng of peptide has 1360160 cpm

Figure 18. Radioiodination of NHS-AB Exoloop 2 Peptide

A.2. Radioiodination of hCG:

Radioiodination of hormone was designed such that only one $^{125}$I molecule reacts with one hormone molecule. Below is the figure showing the results of iodination of hCG. 6 fractions
were collected. There was a peak with 478016 cpm/2uL of fraction 2. This fraction was stored in a glass tube for further use.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1500</td>
</tr>
<tr>
<td>2</td>
<td>478016</td>
</tr>
<tr>
<td>3</td>
<td>263100</td>
</tr>
<tr>
<td>4</td>
<td>25200</td>
</tr>
<tr>
<td>5</td>
<td>12488</td>
</tr>
<tr>
<td>6</td>
<td>3103</td>
</tr>
</tbody>
</table>

Total cpm = 783407 * 230

Specific activity = \( \frac{783407 \times 230}{3,000} = 60,061.2 \text{ cpm/ng} \)

Fraction 2 has 478016 * 230 cpm = 109,943,680 cpm

Therefore ng of hCG present in fraction 2 is 1830.5ng

**Figure 19 Iodination of hCG**

B. Exoloop 2 modulates hormone binding to LH/CG receptor:

The LHR\(^{484-503}\) F493Bpa was radioiodinated on Tyr residue in position 486 using Na\(^ {125}\)I and fractionated on a sephadex G-10 column. The peptide was incubated with hCG, exposed to UV light, solubilized and then separated on SDS-Polyacrylamide gel. Dried gels were analyzed on a phosphoimager. The hormone concentration dependent, peptide concentration dependent and the UV duration dependent assays were carried out.
Figure 20. Photoaffinity Labeling of Exoloop 2 Bpa peptide

hCG was incubated with $^{125}$I-LHR$^{484-503}$ F493Bpa and irradiated with UV. In this series, UV irradiation time (C), $^{125}$I-LHR$^{484-503}$ F493Bpa concentration (B), or hCG concentration (A) was varied, while the other two factors were kept constant. After electrophoresis of the samples, gels were dried on Model 583 gel dryer and exposed to a molecular imaging screen (Bio-Rad). The imaging screen was scanned on a model GS-525 Molecular Imager System Scanner (Bio-Rad).

One major limitation of the Bpa peptide for these studies is that it is photolysable. To determine the photolysis and overcome this problem the UV duration dependent study was performed, shown in the figure 20. A striking feature observed in all the experiments performed with the exoloop 2 Bpa peptide was that in the second lane with no hormone in hormone concentration dependent assay there is labeling seen. One possible explanation is contamination during loading the samples onto the gel. For all these assays the first lane is $^{125}$I-hCG used as marker. To check if this was due to some technical error, photoaffinity labeling of S104Bpa and C106Bpa of LHR$^{96-115}$ was carried out (fig21). From the figure it is obvious that the $\alpha$ subunit of the hCG was labeled more than the $\beta$ subunit. With increasing concentration of hormone, the intensity of labeling also increased.
Figure 21. Photoaffinity Labeling of LHR<sup>96-115</sup> S104Bpa and C106Bpa peptides

hCG was incubated with <sup>125</sup>I-LHR<sup>96-115</sup> C106Bpa (A) and <sup>125</sup>I-LHR<sup>96-115</sup> S104Bpa (B) and irradiated with UV. hCG concentration was varied while the concentration of the peptide and UV irradiation time were kept constant. After electrophoresis of the samples, gels were dried on Model 583 gel dryer and exposed to a molecular imaging screen (Bio-Rad). The imaging screen was scanned on a model GS-525 Molecular Imager System Scanner (Bio-Rad).

The bands seen in experiments with exoloop 2 Bpa peptide may represent oxidation products that overshadow the actual labeled α subunit. There are residues in the exoloop 2 that can possibly be oxidized like methionine and cysteine. One way to eliminate this potential oxidative damage is to replace these residues with some other amino acid like isoleucine that are resistant to oxidation. However we cannot exclude the possibility of these amino acid substitutions not affecting the global structure of the protein or the peptide. Another possible explanation for the diffuse bands is that the exoloop2 Bpa peptide might dimerize by itself. Compounding this problem is the Bpa being a highly hydrophobic bulky group. A homobifunctional crosslinker SES was used to crosslink the radioactively labeled Exoloop 2 Bpa peptide to hCG (diagram not shown). However since the crosslinking is random, formation of hormone-hormone complexes and peptide-peptide complexes is also possible.

NHS-AB can reach and label target molecules up to 7° A. <sup>125</sup>I-LHR<sup>484-503</sup> derivatized with NHS-AB bind to hCG and when irradiated with UV, crosslinking is restricted between <sup>125</sup>I-LHR<sup>484-503</sup> and hCGα or between <sup>125</sup>I-LHR<sup>484-503</sup> and hCGβ. The reagent however will not be able to crosslink one hCG subunit to another. As shown in the figure 22, NHS-AB – <sup>125</sup>I-LHR<sup>484-503</sup> was
capable of photoaffinity labeling either hCG α or hCGβ but not both subunits at the same time to produce hCGαβ complex. In addition hCG α was more preferentially labeled than hCG β. One possible explanation for this is that the N-terminus of the LHR\textsuperscript{484-503} derivatives is < 7° A from the hCG α and β subunits and the peptide derivatives are bound closer to the α than β subunit.

**Figure 22. Photoaffinity Labeling of NHS-AB exoloop 2 peptide**

hCG was incubated with AB\textsuperscript{125}I-LHR\textsuperscript{484-503} and irradiated with UV. UV irradiation time (C), AB\textsuperscript{125}I-LHR\textsuperscript{484-503} concentration ( B ), or hCG concentration (A) was varied, while the other two factors were kept constant. In (D), denatured hCG was incubated with increasing concentration of AB\textsuperscript{125}I-LHR\textsuperscript{484-503} and irradiated with UV for 2 min. After electrophoresis of the samples, gels were dried on Model 583 gel dryer and exposed to a molecular imaging screen (Bio-Rad). The imaging screen was scanned on a model GS-525 Molecular Imager System Scanner (Bio-Rad).

Concentration effect of hCG and the peptide:
When a constant amount of hCG was incubated with increasing concentrations of the NHS-AB\textsuperscript{125}I-LHR\textsuperscript{484-503}, the intensity of the labeled hCG α and β bands increased gradually and plateaued as shown in the fig22. A similar result was obtained when a constant amount of the peptide was incubated with increasing concentrations of hCG. These results show that the concentrations of
both of the peptide and hCG are the limiting factors for the photoaffinity labeling. The labeling was dependent on the hCG concentration, derivatized peptide and UV exposure and in all cases the hCG was preferentially labeled. In conclusion, although the NHS-AB derivatized peptide was shown to label the hCG preferentially the α subunit than the β subunit, the exoloop 2 Bpa peptide was definitely a tough one to work with and had lots of problems hard to define.

Deglycosylation of iodinated hCG was carried out. Figure 23 shows this. The α band is shifted much lower when sample source treated with a glycosidase and PNGase F, which is in agreement to the removal of sugar molecules. Glu C digestion of the iodinated hCG after deglycosylation was also carried out (fig 24). It can be seen that the deglycosylation and the digestion both are incomplete.

Figure 23. Deglycosylation of $^{125}$hCG

125 I-hCG was solubilized with DTT and urea, treated with PNGase F and O-Glycosidase and incubated. After electrophoresis of the samples, gels were dried on Model 583 gel dryer and exposed to a molecular imaging screen (Bio-Rad). The imaging screen was scanned on a model GS-525 Molecular Imager System Scanner (Bio-Rad).
Figure 24. Glu-C digestion of deglycosylated hCG

125 I-hCG was solubilized with DTT and urea and in lane 2 treated with PNGase F and O-Glycosidase and incubated while in lane 3 treated with PNGase F and O-Glycosidase and Glu-C and incubated. After electrophoresis of the samples, gels were dried on Model 583 gel dryer and exposed to a molecular imaging screen (Bio-Rad). The imaging screen was scanned on a model GS-525 Molecular Imager System Scanner (Bio-Rad).
Figure 25. Glu-C digestion of hormone incubated with peptide

hCG was incubated with $^{125}$I-LHR$^{96-115}$ G109Bpa (lanes 5 and 6) and $^{125}$I-LHR$^{484-503}$ F493Bpa (lanes 3 and 4) and irradiated with UV. Samples were solubilized with DTT and urea and incubated with Glu-C. After electrophoresis of the samples, gels were dried on Model 583 gel dryer and exposed to a molecular imaging screen (Bio-Rad). The imaging screen was scanned on a model GS-525 Molecular Imager System Scanner (Bio-Rad).

hCG photoaffinity labeled with peptide LHR$^{96-115}$ G109Bpa or LHR$^{484-503}$ F493Bpa that was deglycosylated and digested with Glu-C digestion is shown in figure 25. The first lane is just the iodinated hCG and second lane shows hCG deglycosylated and then digested with 8% Glu-C. In the lanes 3 to 6 the $^{125}$I hCG labeled with the peptides, and lanes 4 and 6 are the samples treated with glycosidase, PNGase F and Glu-C. The lanes 1, 3 and 5 look the same because the peptide molecular weight is in the order of 2000 Daltons which is negligible when compared to that of the hCG. The same is the case with lanes 2, 4 and 6, and additional the Glu-C digestion did not work very well.

Since the deglycosylation and Glu-C digestion work, it will be interesting to analyze the fragments by Mass Spectrometry.
Appendix: Abbreviations and Symbols

GPCR    G protein coupled receptor
hCG     human choriogonadotropin
FSH     Follicle Stimulating Hormone
TSH     Thyroid Stimulating Hormone
LH      Luteinizing hormone
GnRH    Gonadotropin releasing hormone
LH/CGR  Luteinizing hormone/ Chorionic gonadotropin hormone receptor
FSHR    Follicle Stimulating Hormone Receptor
GTP     Guanine Triphosphate
GDP     Guanine diphosphate
dg-hCG  Deglycosylated human chorionic gonadotropin
cAMP    cyclic Adenosine mono phosphate
Ca$^{2+}$ Calcium ions
Asn     Asparagine
Leu     Leucine
LRR     Leucine rich repeats
NHS-AB  N-hydroxysuccinimide ester of amino benzoic acid
Bpa     4-benzoyl phenylalanine
SES     ethylene glycolbis(sulfosuccinimidylsuccinate)
PBS     Phosphate buffered saline
SDS     Sodium dodecyl sulphate
DTT     Dithiothrietol
NaCl    Sodium chloride
Na$_2$HPO$_4$ Sodium Phosphate Dibasic
NaOH    Sodium Hydroxide
UV      Ultra violet
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