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

Rebecca McCaffrey
University of Kentucky, rmcca0@uky.edu

Recommended Citation
McCaffrey, Rebecca, "IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND THE AMINO TERMINAL REGION OF THE LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR" (2002). University of Kentucky Master's Theses. 204.
https://uknowledge.uky.edu/gradschool_theses/204
IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND THE AMINO TERMINAL REGION OF THE LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR

The luteinizing hormone / choriogonadotropin receptor (LH/CG-R) is a member of the G protein-coupled receptor family. The LH/CG-R has seven transmembrane helices, three exoloops, three cytoloops, a C-terminal tail, and an extensive N-terminal exodomain. The exodomain is capable of binding hormone with high affinity without hormone action. Previous studies have shown that the amino-terminal region of the LH/CG receptor contacts both subunits of human chorionic gonadotropin (hCG). In particular, three residues (Leu\textsuperscript{20}, Cys\textsuperscript{22}, and Gly\textsuperscript{24}) were found to be crucial for hormone binding. In this thesis work, benzoylphenylalanine (Bpa), a photoactivatable reagent, was used to continue investigating the interactions of the N-terminal region of the LH/CG-R with hCG. Bpa has been directly incorporated at a defined position into peptides representing amino acids 17-36 of the LH/CG-R. These peptides were radiolabeled with \textsuperscript{125}I and used in photoaffinity labeling studies to identify and characterize the contact site(s) between the N-terminal region of the LH/CG-R and hCG. Results suggest that Cys\textsuperscript{22} is the primary contact residue in this region. Peptide and hormone concentration dependent as well as UV duration dependent photoaffinity labeling experiments confirm that the photolabeling of hCG by hLHR\textsuperscript{17-36(C22Bpa)} is specific. Competition of labeling studies indicate that the hLHR\textsuperscript{17-36(C22Bpa)} peptide is a good mimic of the wild type N-terminal portion of the receptor. In-gel digestions of photolabeled hCG\textgreek{a} and photolabeled hCG\textgreek{b} with CNBr indicate that the N-terminal regions of both hCG\textgreek{a} and hCG\textgreek{b} were photoaffinity labeled by hLHR\textsuperscript{17-36(C22Bpa)}. Based on the fact that the N-terminal regions of each subunit are located on the convex side of the heterodimer, these results provide evidence that the N-terminal portion of the receptor wraps around the back of hCG, contacting the convex face of the hormone.

KEYWORDS: hCG, LHR, GPCR, Bpa, photoaffinity labeling

Rebecca McCaffrey
July 16, 2002

Copyright © Rebecca L. McCaffrey 2002
IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND THE AMINO TERMINAL REGION OF THE LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR

By

Rebecca Louise McCaffrey

Dr. Tae H. Ji
Director of Thesis

Dr. Peter Mirabito
Director of Graduate Studies

July 16, 2002
RULES FOR THE USE OF THESES

Unpublished theses submitted for the Master’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 thesis in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.
THESIS

Rebecca Louise McCaffrey

The Graduate School
University of Kentucky
2002
IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN CHORIONIC GONADOTROPIN AND THE AMINO TERMINAL REGION OF THE LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR

THESIS

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

By

Rebecca Louise McCaffrey

Lexington, Kentucky

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

Lexington, Kentucky

2002

Copyright © Rebecca L. McCaffrey 2002
To my Mom, Cindy, whose love and encouragement made this possible.
ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Tae Ji. I met Dr. Ji several years ago while attending the University of Wyoming. My plans at the time were to continue my education by pursuing a master’s degree in Anthropology. Dr. Ji’s enthusiasm for science is so contagious that I changed my plans, and decided to move to Kentucky with his lab and study Molecular Biology instead. I have never looked back. Also, in addition to their invaluable advice, Dr. Ji and his wife Dr. Inhae Ji, have provided encouragement and support. They have taught me that with hard work and persistence anything in life is possible.

Special thanks are owed to the members of my thesis committee, Dr. Pete Mirabito and Dr. Doug Harrison, for their helpful comments and support. I would also like to thank the other members of the lab. ChangWoo Lee, Myoungkun Jeoung, and Gail Sievert in particular have selflessly offered their time and expertise whenever I have needed assistance. More than just coworkers, you are my friends and I will miss all of you.

My mother, my brother, and my grandparents are my source of inspiration. Encouragement and love have always only been just a phone call away. Thank you.
# TABLE OF CONTENTS

Acknowledgments ............................................................................................................ iii
List of Tables ................................................................................................................ vi
List of Figures ................................................................................................................ vii
List of Files ................................................................................................................... ix

**Chapter One: Introduction and Background** ............................................................... 1

A. Glycoprotein Hormones
   1. Biological Function ................................................................................. 1
   2. Structure .................................................................................................. 2

B. The Luteinizing Hormone/Choriogonadotropin (LH/CG) Receptor
   1. Expression and Structure ........................................................................ 4
   2. Hormone/Receptor Contact Sites ............................................................ 5
   3. Signal Generation and Transduction ....................................................... 7

C. Goal of Thesis .................................................................................................... 8

**Chapter Two: Photoaffinity Labeling Studies** .......................................................... 16

A. Introduction ...................................................................................................... 16

B. Materials and Methods ..................................................................................... 17
   1. Radioiodination of Peptides .................................................................. 17
   2. Phoaffinity Labeling of hCG ................................................................ 17

C. Results .............................................................................................................. 19
   1. Photoaffinity Scanning .......................................................................... 19
   2. hCG Concentration Dependent, UV Duration Dependent, and
      hLHR_{17-36}(C22Bpa) Concentration Dependent Photoaffinity Labeling .... 19
   4. Photoaffinity Labeling of Deglycosylated hCG ................................... 21

D. Conclusions ...................................................................................................... 22

**Chapter Three: Enzymatic and Chemical Digestion Studies** ................................. 45

A. Introduction ...................................................................................................... 45

B. Materials and Methods ..................................................................................... 45
   1. Radioiodination of hCG or deglycosylated hCG .................................... 45
LIST OF TABLES

Chapter Two

Table 2-1: IC_{50} values of competitor peptides ......................................................24
Table 2-2: IC_{50} values of competitor peptides ..........................................................25
LIST OF FIGURES

Chapter One

Figure 1-1: Computer model of hCG ................................................................. 9
Figure 1-2: The LH Receptor ........................................................................ 10
Figure 1-3: Computer model of the LH/CG receptor exodomain ................. 11
Figure 1-4: Computer model of the LH/CG receptor endodomain .......... 12
Figure 1-5: N-terminal region residues of the LH/CG receptor crucial for hCG Binding ................................................................. 13
Figure 1-6: Model for the interaction of the LH/CG receptor exodomain with hCG ................................................................. 14
Figure 1-7: LH/CG receptor activation and signal transduction .............. 15

Chapter Two

Figure 2-1: Structure of 4-benzoyl-L-phenylalanine (Bpa) ....................... 26
Figure 2-2: Photochemistry of Benzophenone Photophore ....................... 27
Figure 2-3: Bpa substituted peptides .............................................................. 28
Figure 2-4: Photoaffinity scanning data ....................................................... 29
Figure 2-5: Photoaffinity scanning summary .............................................. 31
Figure 2-6: hCG concentration dependent, \(^{125}\text{I}-\text{hLHR}_{17-36}(\text{C22Bpa})\) concentration dependent, and UV duration dependent photoaffinity labeling ........................................... 32
Figure 2-7: Inhibition of photoaffinity labeling by LHR peptides ............. 34
Figure 2-8: Inhibition of photoaffinity labeling by LHR peptides .......... 36
Figure 2-9: Graphical representation of \(^{125}\text{I}-\text{hLHR}_{17-36}(\text{C22Bpa})\) photoaffinity labeling inhibition experiments ........................................... 37
Figure 2-10: Inhibition of photoaffinity labeling by LHR peptides .......... 38
Figure 2-11: Inhibition of photoaffinity labeling by LHR peptides .......... 40
Figure 2-12: Graphical representation of \(^{125}\text{I}-\text{hLHR}_{17-36}(\text{L20Bpa})\) photoaffinity labeling inhibition experiments ........................................... 41
Chapter Three

Figure 3-1: Enzymatic digestion of photoaffinity labeled hCG by Endoproteinase Glu C ....................................................................................................................54
Figure 3-2: Enzymatic digestion of photoaffinity labeled deglycosylated hCG by Endoproteinase Glu C ....................................................................................................................55
Figure 3-3: Chemical Cleavage of Photoaffinity Labeled hCG by CNBr ............56
Figure 3-4: Enzymatic deglycosylation of $^{125}$I-hCG and $^{125}$I-hCG $\beta$ ..............57
Figure 3-5: CNBr and Glu C digestion of photolabeled hCG $\alpha$ and photolabeled hCG $\beta$ ....................................................................................................................59
Figure 3-6: CNBr and Endoproteinase Glu C cleavage sites in hCG $\alpha$ and hCG $\beta$ .................................................................................................................................60
Figure 3-7: hCG fragments photoaffinity labeled by $^{125}$I-hLHR$^{17-36(C22Bpa)}$ ........61
LIST OF FILES

RLMthes.pdf.............................................................................................................. 42.9MB
CHAPTER ONE: INTRODUCTION AND BACKGROUND

A. Glycoprotein Hormones

1. Biological Function

Chorionic gonadotropin (CG), luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) are members of the glycoprotein hormone family. LH, FSH, and TSH are secreted by the anterior pituitary gland. 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. In contrast to LH, FSH, and TSH, CG is of placental origin [1]. CG acts like pituitary LH to maintain progesterone and estrogen secretion by the ovarian corpus luteum through the first trimester of pregnancy [2].

FSH plays a crucial role in the production of the female gamete. FSH selects the dominant follicle, the follicle destined for ovulation, and stimulates follicular development. It also promotes antrum formation and induces the expression of LH receptors on the surface of granulosa cells, preparing these cells for the midcycle LH surge. The absence of FSH in the female results in infertility [3].

FSH has been believed to be essential for the initiation of spermatogenesis. However, mice lacking the FSH β subunit gene remain fertile. In contrast to their wild-type littermates, the testes of these mice are small and produce less sperm, most likely reflecting the effect of FSH on Sertoli cell number and function. The possibility does exist that FSH plays a more important role in male primate fertility [3].

LH initiates steroidogenesis in the thecal cells of the follicle and the Leydig cells of the testis. This glycoprotein hormone also promotes the ovulation and luteinization of fully developed follicles. LH and FSH have synergistic effects on estradiol synthesis in the ovary, due to the fact that FSH promotes the production of estradiol from androgens. LH may also help maintain corpus luteum progesterone production, although this role appears to vary greatly among species. In males, LH triggers the synthesis of androgens needed for spermatogenesis and the development of secondary sexual characteristics [3].
CG is closely related in structure and activity to LH. CG maintains pregnancy in primates, horses, donkeys, and zebras but few other animals. Secretion of hCG, human chorionic gonadotropin, begins at about the time of implantation and reaches peak levels during the first trimester [3]. hCG serves as a signal to the ovary to maintain the corpus luteum thus prolonging luteal progesterone synthesis. hCG binds to LH/CGR receptors expressed on the surface of luteal cells and granulosa cells, resulting in the activation of adenylyl cyclase and production of intracellular cAMP. Other intracellular messengers could also be activated by hCG [4]. In the absence of hCG, the decline in maternal LH due to inhibition of the pituitary by progesterone would result in menstruation and spontaneous abortion of the embryo [2].

LH/CGR receptors have also been found in the endometrium, myometrium, and the placenta. This suggests that hCG could be playing a broader role throughout pregnancy than previously assumed. hCG actions within the placenta direct the differentiation of cytotrophoblastic cells into the syncytiotrophoblast. In the developing fetus, hCG is thought to regulate testosterone production in the testes, which in turn propels internal and external genitalia differentiation [4].

In comparison with other animals, relatively high quantities of the gonadotropins (LH, CG, and FSH) are excreted in the urine of humans [3]. In fact, hCG detection in urine or serum serves as the basis of the pregnancy test. Elevated levels of maternal serum and urine hCG are also used in the prediction of adverse pregnancy outcomes and in screening for fetal Down syndrome. Interestingly, more than one-fourth of ovarian tumors secrete hCG. Among testicular tumors, about 66% of nonseminomatous germ cell tumors and 5% of seminomas secrete the \( \beta \) subunit of hCG [4]. Fragments of the \( \beta \) subunit of hCG have also been detected in urine and serum. Expression of the \( \beta \) core fragment, a version of the hCG \( \beta \) subunit which lacks N-terminal residues 1-5, C-terminal residues 93-145, and loop 2 (residues 41-54), has been linked to cervical carcinoma and could therefore potentially be used as a tumor marker [3].

2. Structure

Each of the glycoprotein hormones is comprised of two subunits: a common alpha subunit, and a hormone-specific beta subunit. The alpha subunit consists of 92 amino acids and the length of the beta subunit varies from hormone to hormone. The glycoprotein beta subunits
all share considerable structural homology, and are thought to have evolved from a common precursor. Interestingly, the hCG beta subunit has a carboxy-terminal extension which other members of the group lack. When compared with hLH beta, the hCG beta subunit has 24 additional residues resulting in a complete beta subunit length of 145 amino acids. The carboxyl terminal segment of hCG beta is rich in proline and serine residues [1]. Four of the latter have O-linked carbohydrate chains [5].

The alpha and beta subunits associate non-covalently to form the hormone dimer. Specific interactions between the two subunits are crucial for hormonal activity, with receptor specificity dictated by the hormone beta subunit. When hybrid hormones are prepared, for example the beta subunit of bovine TSH plus the alpha subunit of bovine LH, the beta subunit used always determines hormonal specificity. Additional studies have shown that hybrid LH preparations bind largely but not solely to receptors based on the identity of the hormone beta subunit [1].

The common alpha subunit of human glycoprotein hormones includes ten cysteine residues which form five intrasubunit disulfide linkages. The glycoprotein beta subunits, while varying in size, contain twelve cysteines which form six disulfide bonds. The crystal structure of hCG shows that the alpha and beta subunits share a similar topology, with three of the disulfide bonds forming an unusual knot (Figure 1-1). This cysteine-knot motif forms the framework of each subunit, and is found in some growth factors [6].

One region of the hCG beta subunit plays an extraordinary part in the association of alpha and beta to form the heterodimer. In heterodimeric hCG, the loop from Cys$^{90}$ to Cys$^{110}$ in the beta subunit wraps around the alpha subunit like a seatbelt. The beta disulfide linkage Cys$^{26}$-Cys$^{110}$ is completed after the association of alpha and beta, indicating that the seatbelt plays an important role in maintaining the structural integrity of the heterodimer. This seatbelt arrangement is thought to exist in all of the glycoprotein hormones [6]. The recent crystallization of FSH confirms that this hormone also has a seatbelt loop [7].

Approximately 34% of the weight of native hCG can be attributed to complex carbohydrates [6]. hCG has four N-linked carbohydrates, two in the alpha subunit at Asn$^{52}$ and Asn$^{78}$ and two in the beta subunit at Asn$^{13}$ and Asn$^{30}$ [8]. Four additional O-linked carbohydrate moieties at Ser$^{121, 127, 132, 138}$ make hCG the most heavily glycosylated member of the glycoprotein hormone family [5] [6].
Glycosylation is not necessary for receptor binding. When the bulk of the carbohydrates are removed from hCG by treatment with anhydrous hydrofluoric acid, the resulting deglycosylated hormone binds receptors with slightly increased affinity but with a loss of hormone action [9] [10] [11]. Specifically, the N-linked carbohydrates have been shown to be involved in signal transduction. Site-directed mutagenesis studies indicate that carbohydrates in the alpha subunit, particularly the carbohydrate chain at Asn\(^52\), play a critical role in hCG signal transduction [12,13]. Additional studies have shown that glycans impact the rate of clearance of the hormone from the body [1].

B. The Luteinizing Hormone/Choriogonadotropin (LH/CG) Receptor

1. Expression and Structure

LH and CG interact with the LH/CG receptor to bring about hormonal actions. The majority of LH/CG receptors are located in the gonads. Receptor expression has been reported in other tissues including the rat prostate gland, and in the human non-pregnant uterus, placenta, fallopian tubes, umbilical cord, uterine vessels, lymphocytes, and brain [3] [14]. LH/CG receptors have also been detected in epithelial cells of the normal mammary gland, in benign breast lesions, malignant breast tumors, and in several cancer cell lines [14].

The LH/CG receptor is a member of the superfamily of G protein-coupled receptors (GPCRs). Approximately 2000 GPCRs have been cloned. All GPCRs have an extracellular N-terminal segment, seven transmembrane domains, three exoloops, three cytoloops, and a C-terminal segment. N-terminal segments, exoloops, cytoloops, and C-terminal segments vary greatly in size, indicative of the diverse functions of GPCR family members. Interestingly, the N-terminal portion of the LH/CG receptor is structurally equivalent to the entire molecule of many other GPCRs [15].

The LH/CG receptor cDNA encodes a 674 amino acid glycoprotein (Figure 1-2) [16] [17]. An N-terminal cleavable signal sequence, 22 amino acids in humans, directs insertion of the receptor into the endoplasmic reticulum processing pathway [14]. The molecular weight of the receptor is calculated to be 75 kDa. Glycosylation of the LH/CG receptor results in a molecular weight of 85 to 92 kDa. The sequence of the LH/CG receptor indicates that it is
comprised of two approximately equal halves: the extracellular amino terminal half, and the membrane-associated carboxyl terminal half [18].

The extracellular amino terminal portion of the receptor, also known as the exodomain, consists of 341 amino acids and is responsible for binding hormone with high affinity [19] [20] [21]. The exodomain contains eight to nine copies of a motif known as the leucine-rich repeat (LRR) [16]. An LRR is approximately 25 amino acids long and is comprised of an $\alpha$ helix, a turn, a $\beta$ sheet, and another turn. LRRs are typically stacked in parallel with helices next to helices and sheets next to sheets forming a horseshoe-shaped structure (Figure 1-3). The LRR motif was first identified in ribonuclease inhibitor [22].

The LH/CG receptor exodomain has six consensus N-linked glycosylation sites [18]. Enzymatic deglycosylation of the receptor does not hinder hormone binding, suggesting that the removed carbohydrates are not necessary for hormone binding [23]. There is no evidence for O-linked glycosylation [18].

The carboxyl terminal portion of the receptor, also known as the endodomain, consists of seven transmembrane domains, three extracellular loops, three cytoplasmic loops, and a carboxyl terminal tail. Cysteine residues in the first and second exoloops are thought to form an intramolecular disulfide bond that stabilizes the endodomain structure, as is the case with rhodopsin. Two adjacent cysteines (Cys$^{621, 622}$) are palmitoylated and embedded in the cytoplasmic face of the cell membrane, creating a fourth cytoloop. Palmitoylated cysteine residues are not required for coupling of the LH/CG receptor to G proteins [14].

Analyses of similar G protein-coupled receptor transmembrane domains suggest that these helices are arranged in a counterclockwise direction when viewed extracellularly (Figure 1-4) [24] [25].

2. Hormone / Receptor Contact Sites

Photoaffinity labeling studies provided the first evidence that both subunits of hCG interact directly with the LH/CG receptor [26]. However, key hormone residues participating in receptor contact remain largely unidentified [3].

Many studies suggest that the C-terminus of the hCG $\alpha$ subunit and the $\beta$ seatbelt loop play crucial roles in receptor binding and signal generation. These segments are present in the
concave face of hCG, which is believed to be the receptor binding face [27]. Photoaffinity labeling studies using a peptide mimic of the C-terminus of hCG α have provided the only evidence of direct interaction between this region of the hormone and the receptor [27] [28]. Interestingly, hCG homodimers lacking the β seatbelt or the C-terminal region of the α subunit have significant biological activity [29] [3].

αL1, αL3, βL1, and βL3 have also been implicated in receptor binding [27]. In addition, synthetic peptides representing the β2 loop of hCG bind to and activate the receptor [30]. But hCG constructs containing hFSH β2 bind LH/CG receptors, not FSH receptors [31]. It is possible that the β2 loop of hCG is important for the overall structure of the hormone, which would account for it’s influence on receptor binding [3].

Many studies have been devoted to identifying areas of the LH/CG receptor responsible for hormone binding. Photoaffinity labeling experiments and studies using peptides representing different portions of the LH/CG receptor indicate that multiple hormone contact sites exist in the receptor. The synthetic peptide study evaluated the ability of different regions of the LH/CG receptor to competitively inhibit binding of 125I-hCG to full-length membrane receptor. Four regions of the receptor capable of interacting with hCG were identified: LH/CG-R^21^-^38^, LH/CG-R^102^-^115^, LH/CG-R^253^-^272^, and LH/CG-R^573^-^583^. These regions represent the N-terminal region of the receptor, LRR4, the hinge region of the receptor, and exoloop 3, respectively [32].

Early photoaffinity labeling data pointed to the existence of at least three contact sites dispersed throughout the receptor: one in the endodomain, and two in the exodomain. Each of these three putative contact sites has a Lys residue to which hCG is affinity labeled [18]. Mutagenesis studies coupled with photoaffinity labeling have yielded a wealth of information in regard to LH/CG receptor hormone contact sites.

The LH/CG receptor exodomain binds hCG with high affinity [19] [20] [21] and contains eight to nine putative LRR sequences [16]. The beta-strand Leu/Ile-X-Leu/Ile motif is thought to make contact with hCG, based on structural similarity to ribonuclease inhibitors. Alanine-scanning mutagenesis of the beta-strand Leu/Ile residues in all LRRs of the human LH/CG receptor found that LRR2 and LRR4 are crucial for hCG binding [33]. Photoaffinity labeling results indicate that the N-terminal region of LRR4 interacts with hCG, preferentially the hCG α subunit. The hCG/LRR4 complex interacts with exoloop 2, possibly generating the hormone signal [34].
The C-terminal flanking portion of the LRRs, known as the hinge region (Thr$^{250}$-Gln$^{268}$), also modulates signal generation in the receptor endodomain. Several mutations in the hinge region result in a constitutively active LH/CG receptor, suggesting that the exodomain of the receptor constrains the endodomain [35] [36]. Photoaffinity labeling studies indicate that the hinge region specifically interacts with hCG, preferentially hCG α. This interaction is inhibited by exoloop 2, pointing to the existence of an intimate relationship between the hinge region of the exodomain, exoloop 2 of the receptor endodomain, and hCG [36].

Alanine-scanning of the N-terminal region of the LH/CG receptor identified three residues (Leu$^{20}$, Cys$^{22}$, and Gly$^{24}$) crucial for hCG binding. Substitution with alanine for any one of these residues abolished hormone binding [37]. A peptide mimic corresponding to Gly$^{18}$-Tyr$^{36}$ of the receptor N-terminal region specifically photoaffinity labels both subunits of hCG, preferentially the α subunit. These results indicate that the N-terminal region of the exodomain interacts with hCG. Leu$^{20}$, Cys$^{22}$, and Gly$^{24}$ in particular are crucial for hormone binding (Figure 1-5) [38].

Based on the results of the mutagenesis studies, photoaffinity labeling experiments, and chimeric receptor analyses, a model of hCG’s interaction with the LH/CG receptor can be proposed. The concave, front side of hCG interacts with the inner lining of the horseshoe or 1/3 donut structure formed by the exodomain LRRs (Figure 1-6). The hormone particularly interacts with LRR2 and LRR4 [36]. The N-terminal region of the receptor could potentially wrap around the hormone dimer, making contact with the convex, back surface of hCG [38]. In this model the exodomain of the LH/CG receptor traps the hormone, almost hugging hCG, in a manner that would certainly increase the binding affinity [36]. Experimental evidence suggests that the α subunit of hCG displaces the hinge region of the receptor from exoloop 2, “relaxing” the endodomain. Ligand-induced conformational changes trigger signal transduction [39].

3. Signal Generation and Transduction

Hormone action is induced when hormone binds to a receptor on the cell surface (Figure 1-7). However, only a fraction of occupied receptors actually generate a signal. In some cases, the majority of hormone-bound receptors are endocytosed by the cell, and hormonal action is induced by occupied receptors remaining on the surface of the cell [18].
Sequence homology of the LH/CG receptor endodomain with other G protein-coupled receptors suggests that this receptor also interacts with G proteins. Signaling of the LH/CG receptor is mediated by $G_s$, a G protein which is capable of stimulating adenylyl cyclase. Activated adenylyl cyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [18] [3].

LH/CG receptor activation also results in the production of inositol phosphates and an increase in the intracellular concentration of calcium ions [40]. Phospholipase C hydrolyzes phosphatidyl inositol resulting in the generation of inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol. IP$_3$ stimulates the release of calcium from the endoplasmic reticulum increasing the concentration of cytoplasmic calcium, and diacylglycerol activates protein kinase C [18].

Both the adenylyl cylcase and phospholipase C pathways are activated within minutes of LH/CG receptor activation. Yet experimental evidence suggests that the two pathways operate independently of one another [18]. For example, mutant LH/CG receptors in which Lys$^{583}$ is substituted with a variety of amino acids are no longer able to generate cAMP. In contrast, Lys$^{583}$ does not appear to play an essential role in the induction of inositol phosphates [41]. Also, the concentration of hCG which induces half of the maximum production (EC$_{50}$) of inositol phosphates is 20 to 30 times higher than the EC$_{50}$ value for cAMP synthesis. Therefore, two distinct signaling pathways are activated by the LH/CG receptor [18].

C. **Goal of Thesis**

The goal of this thesis work is to further describe the contact sites between the N-terminal region of the LH/CG receptor and hCG. Photoaffinity labeling studies examine the contribution of individual residues in this region to hormone binding. Enzymatic digestion experiments shed light on the regions of hCG that make contact with the N-terminal portion of the receptor. These results provide insight into the mechanism of hormone/exodomain interaction.

Approximately 16% of couples in the world suffer from infertility [27]. The receptor and hormone could potentially be used as fertility drugs as well as contraceptives. Further understanding of hCG/receptor interactions is a crucial first step for the future design of pharmaceuticals.
Figure 1-1: Computer model of hCG. hCG α is shown in green, and hCG β in pink. The seatbelt region of hCG β is shown in blue, wrapping around the α subunit stabilizing the heterodimer.
Figure 1-2: The LH Receptor. The amino acid sequence of the rat LHR is shown. Vertical lines indicate exon divisions, hexagons enclose signal peptide amino acids, and amino acids comprising the mature peptide are within circles (Dufau, 1998) [14].
**Figure 1-3**: Computer model of the LH/CG receptor exodomain. The LH/CG receptor exodomain contains eight to nine copies of a motif known as the leucine-rich repeat. Each LRR consists of an α helix (red), a turn (green), a β sheet (yellow), and another turn.
Figure 1-4: Computer model of the LH/CG receptor endodomain. The seven transmembrane helices, three exoloops, and three cytoloops of the receptor endodomain are shown from the top looking down (upper panel) and from the side (lower panel).
Figure 1-5: N-terminal region residues of the LH/CG receptor crucial for hCG binding.
Receptor residues Leu$^{20}$, Cys$^{22}$, and Gly$^{24}$ are essential for hCG binding. The N-terminal region of the receptor could wrap around the back of hCG, making contact with the convex face of the hormone heterodimer (Hong, 1998)[37], (Phang, 1998) [38].
Figure 1-6: Model for the interaction of the LH/CG receptor exodomain with hCG. The concave, front side of hCG is thought to interact with the inner lining of the 1/3 donut structure formed by the exodomain LRRs.
Figure 1-7: LH/CG receptor activation and signal transduction. The receptor endodomain modulates hormone binding to the exodomain. The hormone, exodomain, and endodomain complex undergo conformational adjustment activating the receptor and transducing the hormone signal. Activation of adenylyl cyclase results in the production of cAMP. Activation of PLC results in the generation of IP₃ and diacyl glycerol.
CHAPTER Two: PHOTOAffinity LABELING STUDIES

A. Introduction

Since its introduction forty years ago [42], photoaffinity labeling has been an important technique for the characterization of binding sites including protein-protein interactions in biological systems. In photoaffinity labeling experiments, a light-sensitive moiety is appended to a natural ligand. Upon activation with UV light, the modified ligand forms a very short-lived and highly reactive intermediate capable of reaction with residues of nearby molecules. The covalent bond formed between the modified ligand and the neighboring molecule is resistant to cleavage under the denaturing conditions of SDS-PAGE.

Over the course of the past four decades, a variety of photophores have been developed. The majority of these reagents are diazo esters, aryl azides, or diazirines, which upon exposure to UV irradiation are converted to nitrenes or carbenes. Unfortunately, nitrenes and carbenes react quickly with water, resulting in low efficiency photolabeling [43]. Benzophenone photoprosbes have several key advantages over such reagents. First, benzophenones are chemically more stable than diazo esters, aryl azides, and diazirines. Benzophenones can also be manipulated in ambient light and activated at 350-360 nm, minimizing photolytic damage to proteins. In addition, the excited state of the benzophenones, a triplet biradical, reacts preferentially with C-H bonds, even in the presence of water and bulk nucleophiles [44] [43].

Development of one benzophenone photophore in particular, the amino acid analog 4-benzoyl-L-phenylalanine (Bpa) [45] (Figure 2-1), made possible a significant advance in the application of photoaffinity labeling to the study of peptide-protein interactions. Bpa can be directly incorporated at a defined position into peptides by solid-phase synthesis, allowing for the examination of the relative importance of individual amino acids in protein-protein interactions. The advantages of photolabeling with benzophenone reagents, particularly Bpa, generally outweigh the disadvantages resulting from the introduction of the bulky, hydrophobic benzophenone moiety [44]. The photochemistry of benzophenone reagents is shown in Figure 2-2.

Previous studies have shown that the amino-terminal region of the LH/CG receptor contacts both subunits of hCG. In particular, three residues (Leu20, Cys22, and Gly24) were
found to be crucial for hormone binding [37] [38]. In this chapter, Bpa was used to continue investigating the interactions of the N-terminal region of the LH/CG-R with hCG. Bpa has been directly incorporated at a defined position into peptides representing amino acids 17-36 of the LH/CG-R (Figure 2-3). These peptides are radiolabeled with \( ^{125} \text{I} \) and used in photoaffinity labeling studies.

B. Materials and Methods

Materials

The hCG and deglycosylated hCG were supplied by the National Hormone and Pituitary Program (NIDDK, National Institutes of Health). Denatured hCG was prepared by boiling hCG in 8 M urea and 0.1 M DTT for 30 minutes. Peptide mimics including wild-type, mutant, and Bpa-containing LH/CG receptor peptides were synthesized by Genemed Synthesis, Inc. (San Francisco, CA).

1. Radioiodination of Peptides

1 mCi of Na\(^{125}\)I in 10 µl of 0.1 M NaOH and 7 µl of chloramine T (1 mg/ml) in 0.1 M sodium phosphate (pH 7.5) were added to 30 µg of LHR peptide 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 introduced to terminate the iodination reaction. Radioiodinated \(^{125}\)I-LHR solution was mixed with 60 µl of 16% sucrose solution in 0.1 M sodium phosphate and fractionated on a Sephadex Superfine G-10 column (0.6 x 15 cm) using 10 mM Na\(_2\)HPO\(_4\) and 0.9% NaCl (pH 7.4) (PBS).

2. Photoaffinity Labeling of hCG

hCG Concentration Dependent Experiments
Disposable glass tubes were siliconized under dimethyldichlorosilane vapor overnight and autoclaved. The following solutions were sequentially introduced to siliconized glass tubes: 32 µl of increasing concentrations of hCG in PBS, and 8 µl (100ng) of $^{125}$I-LHR peptide in PBS. Samples were incubated at 37°C for 90 minutes, then irradiated with a Mineralight R-52 UV lamp for 1 minute as described previously [46]. Immediately following UV exposure, samples were boiled for 3 minutes in 2% sodium dodecyl sulfate, 100 mM dithiothreitol, and 8 M urea. The solubilized samples were electrophoresed on 8-12% polyacrylamide gradient gels. Gels were dried on filter paper, and exposed to PhosphorImager screens (Molecular Dynamics) overnight. The exposed PhosphorImager screen was scanned on a model 860 Storm System Optical Scanner (Molecular Dynamics), and radioactive band intensity was determined using ImageQuant software (Molecular Dynamics).

**Peptide Concentration Dependent Experiments**

The following solutions were sequentially introduced to siliconized glass tubes: 32 µl of increasing concentrations of $^{125}$I-LHR peptide in PBS, and 8 µl (100ng) of hCG in PBS. Samples were incubated at 37°C for 90 minutes, then irradiated with a Mineralight R-52 UV lamp for indicated period of time. Samples were processed as described above.

**UV Dependent Experiments**

The following solutions were sequentially introduced to siliconized glass tubes: 24 µl PBS, 100 ng of hCG in 8 µl of PBS, and 100 ng of $^{125}$I-LHR peptide in 8 µl of PBS. Samples were incubated at 37°C for 90 minutes, then irradiated with a Mineralight R-52 UV lamp for increasing periods of time. Samples were processed as described above.

**Competitive Inhibition of Photoaffinity Labeling of hCG**

Competitive inhibition experiments were carried out as described for the photoaffinity labeling experiments, except that 8 µl (200ng) of hCG in PBS, and 8 µl (100ng) $^{125}$I-LHR
peptide in PBS were incubated with 24 µl of increasing concentrations of nonradioactive LHR peptide in PBS.

C. Results

1. Photoaffinity Scanning

The ability of each radioiodinated Bpa-substituted peptide to photoaffinity label hCG was assessed and compared in photoaffinity scanning studies (Figure 2-4). Each of the five Bpa peptides was incubated with increasing concentrations of hCG and treated with UV for one minute. While all peptides were able to photoaffinity label both the α and the β subunits of hCG to some extent, hLHR^{17-36(C22Bpa)} clearly photolabeled the α subunit of hCG much more efficiently than any of the other Bpa peptides. Labeling of hCG α by hLHR^{17-36(C22Bpa)} was between 2.5-3.5 times greater than that of the other peptides.

The majority of the Bpa peptides preferentially labeled the α subunit of hCG. Of the five peptides, hLHR^{17-36(R21Bpa)} was the only one that labeled the β subunit of hCG as well or slightly better than the α subunit. Photolabeling of the β subunit of hCG by hLHR^{17-36(R21Bpa)}, while interesting, was still much less efficient than photolabeling of hCG α by hLHR^{17-36(C22Bpa)}. When combined at a molar ratio of 2:1 (peptide:hCG), hLHR^{17-36(C22Bpa)} photolabeled approximately 20% of the total hCG molecules (Figure 2-5). The relatively high efficiency photoaffinity labeling of hCG α by hLHR^{17-36(C22Bpa)} indicates that Cys^{22} could be the primary contact amino acid within the N-terminal region of the LH/CG receptor. The specificity of this photolabeling was tested by changing the concentration of hLHR^{17-36(C22Bpa)}, and by increasing the duration of UV exposure.

2. hCG Concentration Dependent, UV Duration Dependent, and hLHR^{17-36(C22Bpa)} Concentration Dependent Photoaffinity Labeling

When a constant amount of hLHR^{17-36(C22Bpa)} was incubated with increasing amounts of hCG, the intensity of the labeled hCG α and hCG β bands gradually increased then plateaued (Figure 2-4, Figure 2-6, A). The same pattern was seen when the converse experiment was
performed: a constant concentration of hCG was combined with increasing amounts of hLHR^{17-36}(C22Bpa) (Figure 2-6, C). This indicates that hCG and hLHR^{17-36}(C22Bpa) are interacting in a specific manner, dependent upon the concentration of each molecule.

As expected, in the absence of UV exposure photolabeling of hCG by hLHR^{17-36}(C22Bpa) was negligible (Figure 2-6, B). Gradually increasing the duration of UV exposure resulted in a steady increase in labeling of hCG $\alpha$ and hCG $\beta$, with a plateau in labeling seen after about 2 minutes of UV irradiation. Sustained maximum levels of labeling and preferential labeling of the $\alpha$ subunit of hCG indicate that the photoaffinity labeling is specific.

While these experiments illustrate that photoaffinity labeling of hCG by hLHR^{17-36}(C22Bpa) is specific, they do not indicate whether or not this interaction is biologically specific. To test this, a constant concentration of denatured hCG was incubated with increasing amounts of hLHR^{17-36}(C22Bpa) (Figure 2-6, D). hLHR^{17-36}(C22Bpa) did not photolabel denatured hCG at all, despite the presence of high concentrations of the Bpa peptide. This illustrates that the receptor peptide will only interact with biologically active hCG.

3. Photoaffinity Labeling in the Presence of Competitor Peptides

The ability of radioiodinated hLHR^{17-36}(C22Bpa) to photolabel hCG in the presence of peptides representing other regions of the LH/CG receptor was assessed. A constant concentration of hCG and a constant concentration of $^{125}$I-hLHR^{17-36}(C22Bpa) were incubated with increasing concentrations of non-radioactive competitor peptides. Those peptides which effectively inhibited photoaffinity labeling of hCG by $^{125}$I-hLHR^{17-36}(C22Bpa) include hLHR^{17-36}(C22Bpa), hLHR^{17-36}, rat LHR^{18-36} (rLHR^{18-36}), hLHR^{96-115}(C106Bpa), and hLHR^Exoloop 2 (Figure 2-7, Figure 2-9).

In contrast, hLHR^Exoloop 1, hLHR^Exoloop 3, hLHR^{246-269}, and hLHR^{17-36}(L20A-C22A-G24A) do not effectively inhibit photolabeling of hCG by $^{125}$I-hLHR^{17-36}(C22Bpa) (Figure 2-8, Figure 2-9). hLHR^{17-36}(L20A-C22A-G24A) (hLHR^Triple mutant) is a peptide representing the N-terminal region of the LH/CG receptor in which the three residues previously found to be important for hCG binding (Leu^{20}, Cys^{22}, and Gly^{24}) were substituted with Ala. Previous studies indicate that this mutant peptide does not photolabel hCG [38]. As expected, hLHR^Triple mutant is unable to inhibit photolabeling of hCG by $^{125}$I-hLHR^{17-36}(C22Bpa).
IC50 values, in this case the concentration of competitor peptide which reduces photolabeling of hCG by 50%, are a helpful tool in the analysis and comparison of competitor efficacy. IC50 values for each competitor peptide are listed in Table 2-1. A constant concentration of 1.12 µM \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(C22Bpa)}\) was used in the competition of labeling experiments. hLHR\(^{17-36}\text{(C22Bpa)}\) had an IC50 value of 1.28 µM for the \(\alpha\) subunit of hCG, meaning that when this peptide was present at a molar ratio of 1:1 (competitor: \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(C22Bpa)}\)) photolabeling of hCG by \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(C22Bpa)}\) was reduced by 50%. hLHR\(^{17-36}\), rLHR\(^{18-36}\), hLHR\text{Exoloop}^2, and hLHR\(^{96-115}\text{(C106Bpa)}\) are all also reasonably good competitors based on their IC50 values. Interestingly, hLHR\(^{246-269}\) has a relatively low IC50 value, 1.44 µM. However, despite very high concentrations, hLHR\(^{246-269}\) was unable to completely inhibit photolabeling of hCG by \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(C22Bpa)}\). hLHR\text{Exoloop}^1 was also unable to completely inhibit photolabeling of hCG, but it’s IC50 value is much lower than that of hLHR\text{Exoloop}^3. Even at the highest concentrations tested, 29.03 µM and 19.85 µM respectively, hLHR\text{Exoloop}^3 and hLHR\text{triple mutant} were unable to reduce photolabeling of hCG by 50%.

Competition of labeling experiments were also performed using a different radioiodinated Bpa peptide, \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(L20Bpa)}\) (Figure 2-10, Figure 2-11, Figure 2-12, Table 2-2). Results obtained using \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(L20Bpa)}\) corroborate the \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(C22Bpa)}\) data. hLHR\(^{17-36}\text{(L20Bpa)}\), hLHR\(^{17-36}\), rat LHR\(^{18-36}\) (rLHR\(^{18-36}\)), hLHR\(^{96-115}\text{(C106Bpa)}\), and hLHR\text{Exoloop}^2 all were all effective inhibitors of hCG photolabeling, whereas hLHR\text{Exoloop}^1, hLHR\text{Exoloop}^3, hLHR\(^{246-269}\), and hLHR\(^{17-36}\text{(L20A-C22A-G24A)}\) were not. Additionally, each of the five Bpa-substituted peptides were also able to inhibit photolabeling of hCG by \(^{125}\text{I}-\text{hLHR}^{17-36}\text{(L20Bpa)}\) (Figure 2-13). This indicates that these peptides all interact with hCG in a similar manner, and most likely share a similar structure.

4. Photoaffinity Labeling of Deglycosylated hCG

As mentioned earlier, deglycosylated hCG acts as an LH/CG receptor antagonist. Deglycosylated hCG binds the LH/CG receptor with an affinity similar to that of hCG, but is unable to elicit hormone action [9] [10] [11]. Affinity labeling studies found that deglycosylated hCG was cross-linked to a 34 kDa component of the LH/CG receptor, whereas hCG was consistently cross-linked to a 24 kDa receptor component [47]. This difference in receptor binding could be responsible for the success or failure of signal transduction [47] [11].
Studies assessing the ability of hLHR<sub>17-36(G18Bpa)</sub>, hLHR<sub>17-36(A19Bpa)</sub>, hLHR<sub>17-36(L20Bpa)</sub>, hLHR<sub>17-36(R21Bpa)</sub>, and hLHR<sub>17-36(C22Bpa)</sub> to photolabel deglycosylated hCG indicate that the N-terminal region of the LH/CG receptor interacts with deglycosylated hCG (Figure 2-14, A). The efficiency of photolabeling varied based on the position of the Bpa photophore, with hLHR<sub>17-36(C22Bpa)</sub> labeling both subunits of deglycosylated hCG better than any of the other Bpa peptides. Photolabeling experiments in which a constant concentration of radioiodinated hLHR<sub>17-36(C22Bpa)</sub> was incubated with increasing amounts of deglycosylated hCG, show saturation of labeling and therefore specificity (Figure 2-14, B). Interestingly, all of the Bpa peptides preferentially photolabeled the β subunit of deglycosylated hCG. Based on these results, the N-terminal region of the LH/CG receptor does not appear to be involved in the differential binding seen with hCG and deglycosylated hCG.

D. Conclusions

Photoaffinity scanning results clearly show that hLHR<sub>17-36(C22Bpa)</sub> labels hCG α with the highest efficiency. Interestingly, photolabeling of the α subunit increased slightly with closer proximity to the Cys<sup>22</sup> position. In other words, if the Bpa peptides were listed in order of hCG α subunit labeling ability, from lowest to highest, that list would look like this: hLHR<sub>17-36(G18Bpa)</sub>, hLHR<sub>17-36(A19Bpa)</sub>, hLHR<sub>17-36(L20Bpa)</sub>, hLHR<sub>17-36(R21Bpa)</sub>, and hLHR<sub>17-36(C22Bpa)</sub>. This pattern does not appear to hold true for photolabeling of the β subunit.

The majority of the Bpa peptides preferentially labeled the α subunit of hCG. Of the five peptides, hLHR<sub>17-36(R21Bpa)</sub> was the only one that labeled the β subunit of hCG as well or slightly better than the α subunit. The spatial arrangement of the Bpa photophore in this position could be one possible explanation. When in this position, the benzophenone moiety could be approximately the same distance from both the α subunit and the β subunit of hCG, allowing for photolabeling of both subunits with essentially the same efficiency.

The ability of hLHR<sub>17-36(C22Bpa)</sub>, hLHR<sub>17-36</sub>, rLHR<sub>18-36</sub>, hLHR<sub>96-115(C106Bpa)</sub>, and hLHR<sup>Exolooop 2</sup> to inhibit photolabeling of hCG by <sup>125</sup>I-hLHR<sub>17-36(C22Bpa)</sub> could be occurring through either of two inhibitory mechanisms: direct competition or allosteric inhibition. Receptor peptides could potentially directly compete with <sup>125</sup>I-hLHR<sub>17-36(C22Bpa)</sub> for interaction with the same site on hCG. In this scenario, the competitor peptide prevents the Bpa peptide from
labeling hCG by physically occupying the binding site. Another possibility is that the non-
radioiodinated peptide could interact with a different portion of hCG, causing a change in
conformation of the hormone which prevents $^{125}$I-hLHR$^{17-36}$(C22Bpa) from photolabeling hCG.

The results of experiments designed to determine the mechanism of inhibition by each of
these receptor peptides were inconclusive. However, it seems likely that hLHR$^{17-36}$(C22Bpa),
hLHR$^{17-36}$, and rLHR$^{18-36}$ directly inhibit $^{125}$I-hLHR$^{17-36}$(C22Bpa) labeling of hCG. The fact that
hLHR$^{17-36}$ is able to inhibit the photolabeling indicates that $^{125}$I-hLHR$^{17-36}$(C22Bpa) is a good mimic
of the wild-type N-terminal portion of the receptor. hLHR$^{\text{Exoloop } 2}$ could inhibit photolabeling by
$^{125}$I-hLHR$^{17-36}$(C22Bpa) either directly or allosterically, either mechanism is plausible. hLHR$^{96-115}$(C106Bpa)
is a Bpa peptide representing LRR 4. Computer modeling studies of the LH/CG
receptor suggest that eight to nine LRRs assume a one-third doughnut structure which lines the
inside face of the receptor exodomain, interacting with the concave face of hCG. The N-terminal
portion of the receptor is thought to wrap around the back of hCG, making contact with the
convex face of the hormone dimer [38]. While it is possible that hLHR$^{96-115}$(C106Bpa) could
directly compete with $^{125}$I-hLHR$^{17-36}$(C22Bpa) for hCG binding, the structure of the receptor
exodomain favors a scenario where the two regions of the receptor interact with opposite faces of
the hormone. Whatever the mechanism of inhibition, these results suggest that an intimate
relationship exists between the N-terminal region of the receptor, exoloop 2, and LRR 4.

These experimental findings suggest that of the three residues previously found to be
crucial for hCG binding, Cys$^{22}$ may be the primary contact amino acid. Photoaffinity labeling of
hCG by hLHR$^{17-36}$(C22Bpa) was shown to be saturable and specific. hLHR$^{17-36}$(C22Bpa) did not
photolabel denatured hCG, indicating that the interaction is also biologically specific.
Photolabeling of hCG by hLHR$^{17-36}$(C22Bpa) was inhibited by hLHR$^{17-36}$, indicating that the Bpa-
substituted peptide is a good mimic of the wild-type N-terminal region of the receptor.

The next set of experiments were designed to determine where the corresponding contact
site(s) in hCG are located.
Table 2-1: IC\textsubscript{50} values of competitor peptides. The concentration of each competitor peptide required to inhibit \(^{125}\text{I}-\text{hLHR}^{\text{17-36(C22Bpa)}}\) photoaffinity labeling of hCG by 50% was calculated. Even at the highest concentrations tested, 29.03 μM and 19.85 μM respectively, hLHR\textsuperscript{Exoloop3} and hLHR\textsuperscript{17-36(L20A-C22A-G24A)} \textsuperscript{(hLHR triple mutant)} were unable to reduce photolabeling of hCG by 50%.

<table>
<thead>
<tr>
<th>Cold Peptide</th>
<th>α subunit IC\textsubscript{50} (μM)</th>
<th>β subunit IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hLHR\textsuperscript{17-36(C22Bpa)}</td>
<td>1.28</td>
<td>1.1</td>
</tr>
<tr>
<td>hLHR\textsuperscript{17-36}</td>
<td>2.07</td>
<td>2.04</td>
</tr>
<tr>
<td>rLHR\textsuperscript{18-36}</td>
<td>2.16</td>
<td>1.99</td>
</tr>
<tr>
<td>hLHR\textsuperscript{96-115(C106Bpa)}</td>
<td>2.48</td>
<td>2.48</td>
</tr>
<tr>
<td>hLHR\textsuperscript{Exoloop2}</td>
<td>1.32</td>
<td>1.19</td>
</tr>
<tr>
<td>hLHR\textsuperscript{Exoloop1}</td>
<td>2.95</td>
<td>3.8</td>
</tr>
<tr>
<td>hLHR\textsuperscript{Exoloop3}</td>
<td>&gt; 29.03</td>
<td>&gt; 29.03</td>
</tr>
<tr>
<td>hLHR\textsuperscript{246-269}</td>
<td>1.44</td>
<td>2.17</td>
</tr>
<tr>
<td>hLHR\textsuperscript{triple mutant}</td>
<td>&gt; 19.85</td>
<td>&gt; 19.85</td>
</tr>
</tbody>
</table>
Table 2-2: IC$_{50}$ values of competitor peptides. The concentration of each competitor peptide required to inhibit $^{125}$I-hLHR$^{17-36}$ (L20Bpa) photoaffinity labeling of hCG by 50% was calculated. Even at the highest concentration tested (29.03 μM) hLHR$^{\text{Exoloop 3}}$ was unable to reduce photolabeling of hCG by 50%. Inhibition of photolabeling by hLHR$^{246-269}$ was not maintained at a level under 50%, therefore no IC$_{50}$ value could be calculated.

<table>
<thead>
<tr>
<th>Cold Peptide</th>
<th>α subunit IC$_{50}$ (μM)</th>
<th>β subunit IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hLHR$^{17-36}$ (L20Bpa)</td>
<td>2.1</td>
<td>1.67</td>
</tr>
<tr>
<td>hLHR$^{17-36}$</td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td>rLHR$^{18-36}$</td>
<td>1.08</td>
<td>1.64</td>
</tr>
<tr>
<td>hLHR$^{96-115}$ (C106Bpa)</td>
<td>2.5</td>
<td>1.26</td>
</tr>
<tr>
<td>hLHR$^{\text{Exoloop 2}}$</td>
<td>2.12</td>
<td>1.99</td>
</tr>
<tr>
<td>hLHR$^{\text{Exoloop 1}}$</td>
<td>2.95</td>
<td>2.28</td>
</tr>
<tr>
<td>hLHR$^{\text{Exoloop 3}}$</td>
<td>&gt; 29.03</td>
<td>&gt; 29.03</td>
</tr>
<tr>
<td>hLHR$^{246-269}$</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hLHR$^{\text{triple mutant}}$</td>
<td>13.52</td>
<td>17.59</td>
</tr>
</tbody>
</table>
Figure 2-1: Structure of 4-benzoyl-L-phenylalanine (Bpa). Bpa has an amino group and a carboxyl group, allowing for direct incorporation at a defined position into a peptide by solid-phase synthesis [44].
Figure 2-2: Photochemistry of Benzophenone Photophore. 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 [44].
Figure 2-3: Bpa substituted peptides. Peptides corresponding to the N-terminal region of the LH/CG-R were synthesized in which 5 amino acids were individually replaced with Bpa, a photoactivatable reagent. This allows for the analysis of each individual amino acid’s interaction with hCG.
<table>
<thead>
<tr>
<th>Sample</th>
<th>% Labeling</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-G18Bpa(μM)</td>
<td>1.10</td>
<td>1.10</td>
<td>1.10</td>
<td>1.10</td>
<td>1.10</td>
<td>1.10</td>
<td>1.10</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td>hCG (μM)</td>
<td>0</td>
<td>0.03</td>
<td>0.07</td>
<td>0.13</td>
<td>0.26</td>
<td>0.53</td>
<td>1.05</td>
<td>2.11</td>
<td>1.10</td>
</tr>
<tr>
<td>I-A19Bpa(μM)</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
</tr>
<tr>
<td>hCG (μM)</td>
<td>0</td>
<td>0.03</td>
<td>0.07</td>
<td>0.13</td>
<td>0.26</td>
<td>0.53</td>
<td>1.05</td>
<td>2.11</td>
<td>1.11</td>
</tr>
<tr>
<td>I-R21Bpa(μM)</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
<td>1.15</td>
</tr>
<tr>
<td>hCG (μM)</td>
<td>0</td>
<td>0.03</td>
<td>0.07</td>
<td>0.13</td>
<td>0.26</td>
<td>0.53</td>
<td>1.05</td>
<td>2.11</td>
<td>1.15</td>
</tr>
<tr>
<td>I-C22Bpa(μM)</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
</tr>
<tr>
<td>hCG (μM)</td>
<td>0.015</td>
<td>0.03</td>
<td>0.07</td>
<td>0.13</td>
<td>0.26</td>
<td>0.53</td>
<td>1.05</td>
<td>2.11</td>
<td>1.12</td>
</tr>
<tr>
<td>I-L20Bpa(μM)</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
<td>1.13</td>
</tr>
<tr>
<td>hCG (μM)</td>
<td>0</td>
<td>0.03</td>
<td>0.07</td>
<td>0.13</td>
<td>0.26</td>
<td>0.53</td>
<td>1.05</td>
<td>2.11</td>
<td>1.13</td>
</tr>
</tbody>
</table>

**Notes:**
- % Labeling refers to the percentage of labeling in the experiment.
- Concentrations are given in μM.
- hCG concentrations are provided in μM for reference.
Figure 2-4: Photoaffinity scanning data. Each of the five Bpa-substituted peptides was incubated with increasing concentrations of hCG and treated with UV for 1 minute as described in “Materials and Methods”. Following electrophoresis, gels were dried and exposed to PhosphorImager screens (Molecular Dynamics) overnight. The imaging screens were scanned on a model 860 Storm System Optical Scanner (Molecular Dynamics), and radioactive band intensity was measured using ImageQuant software (Molecular Dynamics). The bar graphs show the percent radioactivity of the α band and the β band in a gel lane. These results indicate that hLHR^17-36(C22Bpa) photolabels hCG much more efficiently than any of the other Bpa peptides.
**Figure 2-5: Photoaffinity scanning summary.** The maximum percent of hCG that was photolabeled by each Bpa peptide was calculated using the molar ratio of peptide to hormone in Lane 6 of Figure 2-4. hLHR$^{17-36(Bpa)}$ labels the $\alpha$ subunit of hCG much more efficiently than any of the other 4 Bpa-substituted peptides.
Table: hCG and I-C22Bpa concentrations in different UV exposure times

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG (μM)</td>
<td>0.015</td>
<td>0.03</td>
<td>0.07</td>
<td>0.13</td>
<td>0.26</td>
<td>0.53</td>
<td>1.05</td>
<td>2.11</td>
</tr>
<tr>
<td>I-C22Bpa (μM)</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>UV (minutes)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table: hCG and I-C22Bpa concentrations in different UV exposure times

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG (μM)</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>I-C22Bpa (μM)</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
<td>1.12</td>
</tr>
<tr>
<td>UV (minutes)</td>
<td>0</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>2.5</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Table: Denatured hCG and I-C22Bpa concentrations in different UV exposure times

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG (μM)</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>I-C22Bpa (μM)</td>
<td>0.14</td>
<td>0.28</td>
<td>0.56</td>
<td>1.12</td>
<td>2.25</td>
<td>3.37</td>
<td>4.50</td>
<td>6.75</td>
</tr>
<tr>
<td>UV (minutes)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table: Denatured hCG and I-C22Bpa concentrations in different UV exposure times

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured hCG (μM)</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>I-C22Bpa (μM)</td>
<td>0.14</td>
<td>0.28</td>
<td>0.56</td>
<td>1.12</td>
<td>2.25</td>
<td>3.37</td>
<td>4.50</td>
<td>6.75</td>
</tr>
<tr>
<td>UV (minutes)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Figure 2-6: hCG concentration dependent, $^{125}$I-hLHR$^{17-36}$\textsubscript{(C22Bpa)} concentration dependent, and UV duration dependent photoaffinity labeling. A) A constant concentration of $^{125}$I-hLHR$^{17-36}$\textsubscript{(C22Bpa)} was incubated with increasing concentrations of hCG and exposed to UV irradiation as indicated. B) Constant concentrations of hCG and $^{125}$I-hLHR$^{17-36}$\textsubscript{(C22Bpa)} were incubated and irradiated with UV for increasing periods of time. C) A constant concentration of hCG was incubated with increasing concentrations of $^{125}$I-hLHR$^{17-36}$\textsubscript{(C22Bpa)} and exposed to UV irradiation as indicated. D) A constant concentration of denatured hCG was incubated with increasing concentrations of $^{125}$I-hLHR$^{17-36}$\textsubscript{(C22Bpa)} and exposed to UV irradiation as indicated. All gels were processed as described in the legend to Figure 2-4.
hLHR\textsuperscript{17-36(C22Bpa)}

hLHR\textsuperscript{17-36}

rLHR\textsuperscript{18-36}

hLHR\textsuperscript{96-115(C106Bpa)}

hLHR\textsuperscript{Exoloop 2}

Unlabeled Peptide (ng)
Figure 2-7: Inhibition of photoaffinity labeling by LHR peptides. A constant concentration of hCG (0.13 µM) and a constant concentration of $^{125}$I-hLHR$^{17-36}$(C22Bpa) (1.12 µM) were incubated with increasing concentrations of non-radioactive competitor peptides representing various regions of the LH/CG receptor and exposed to UV irradiation for 2 minutes. Gels were processed as described in the legend to Figure 2-4. Competitor peptides included in this figure effectively inhibited photoaffinity labeling of hCG by $^{125}$I-hLHR$^{17-36}$(C22Bpa).
Figure 2-8: Inhibition of photoaffinity labeling by LHR peptides. A constant concentration of hCG (0.13 μM) and a constant concentration of $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$ (1.12 μM) were incubated with increasing concentrations of non-radioactive competitor peptides and exposed to UV irradiation for 2 minutes. Gels were processed as described in the legend to Figure 2-4. Competitor peptides included in this figure do not effectively inhibit photoaffinity labeling of hCG by $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$. 

Figure 2-8: Inhibition of photoaffinity labeling by LHR peptides. A constant concentration of hCG (0.13 μM) and a constant concentration of $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$ (1.12 μM) were incubated with increasing concentrations of non-radioactive competitor peptides and exposed to UV irradiation for 2 minutes. Gels were processed as described in the legend to Figure 2-4. Competitor peptides included in this figure do not effectively inhibit photoaffinity labeling of hCG by $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$. 

Figure 2-8: Inhibition of photoaffinity labeling by LHR peptides. A constant concentration of hCG (0.13 μM) and a constant concentration of $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$ (1.12 μM) were incubated with increasing concentrations of non-radioactive competitor peptides and exposed to UV irradiation for 2 minutes. Gels were processed as described in the legend to Figure 2-4. Competitor peptides included in this figure do not effectively inhibit photoaffinity labeling of hCG by $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$. 

Figure 2-8: Inhibition of photoaffinity labeling by LHR peptides. A constant concentration of hCG (0.13 μM) and a constant concentration of $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$ (1.12 μM) were incubated with increasing concentrations of non-radioactive competitor peptides and exposed to UV irradiation for 2 minutes. Gels were processed as described in the legend to Figure 2-4. Competitor peptides included in this figure do not effectively inhibit photoaffinity labeling of hCG by $^{125}\text{I}\text{-hLHR}^{17-36}(\text{C22Bpa})$.
Figure 2-9: Graphical representation of $^{125}$I-hLHR$^{17-36}$ (C22Bpa) photoaffinity labeling inhibition experiments. The percent labeling of the $\alpha$ and $\beta$ subunits were plotted against increasing concentrations of LHR peptides.
**Figure 2-10: Inhibition of photoaffinity labeling by LHR peptides.** A constant concentration of hCG (0.13 µM) and a constant concentration of $^{125}$I-hLHR$_{17-36(L20Bpa)}$ (1.13 µM) were incubated with increasing concentrations of non-radioactive competitor peptides representing various regions of the LH/CG receptor and exposed to UV irradiation for 1.5 minutes. Gels were processed as described in the legend to Figure 2-4. Competitor peptides included in this figure effectively inhibited photoaffinity labeling of hCG by $^{125}$I-hLHR$_{17-36(L20Bpa)}$. 
Figure 2-11: Inhibition of photoaffinity labeling by LHR peptides. A constant concentration of hCG (0.13 μM) and a constant concentration of $^{125}$I-hLHR$^{17-36}$ (1.13 μM) were incubated with increasing concentrations of non-radioactive competitor peptides and exposed to UV irradiation for 1.5 minutes. Gels were processed as described in the legend to Figure 2-4. Competitor peptides included in this figure do not effectively inhibit photoaffinity labeling of hCG by $^{125}$I-hLHR$^{17-36}$. 

40
Figure 2-12: Graphical representation of $^{125}\text{I-hLHR}^{17-36(\text{L20Bpa})}$ photoaffinity labeling inhibition experiments. The percent labeling of the $\alpha$ and $\beta$ subunits were plotted against increasing concentrations of LHR peptides.
Unlabeled Peptide (ng)

hLHR\(^{17-36}(G18Bpa)\)

hLHR\(^{17-36}(A19Bpa)\)

hLHR\(^{17-36}(L20Bpa)\)

hLHR\(^{17-36}(R21Bpa)\)

hLHR\(^{17-36}(C22Bpa)\)
Figure 2-13: Inhibition of photoaffinity labeling by Bpa peptides. A constant concentration of hCG (0.13 µM) and a constant concentration of $^{125}\text{I-hLHR}_{17-36(L20Bpa)}$ (1.13 µM) were incubated with increasing concentrations of non-radioactive Bpa peptides and exposed to UV irradiation for 1.5 minutes. Gels were processed as described in the legend to Figure 2-4. All 5 Bpa peptides effectively inhibit photoaffinity labeling of hCG by $^{125}\text{I-hLHR}_{17-36(L20Bpa)}$. 
Figure 2-14: Photoaffinity labeling of deglycosylated hCG. A) Each of the 5 Bpa substituted peptides was incubated with deglycosylated hCG and treated with UV for 2 minutes. B) A constant concentration of $^{125}$I-hLHR$^{17-36}$(C22Bpa) was incubated with increasing concentrations of deglycosylated hCG and exposed to UV irradiation for 2 minutes. Gels were processed as described in the legend to Figure 2-4. All 5 of the Bpa peptides photoaffinity labeled deglycosylated hCG, with varying degrees of efficiency. Photoaffinity labeling of deglycosylated hormone by $^{125}$I-hLHR$^{17-36}$(C22Bpa) is saturable.
CHAPTER THREE: ENZYMATIC AND CHEMICAL DIGESTION STUDIES

A. Introduction

Results of photoaffinity labeling studies suggest that Cys^{22} may be the primary contact amino acid in the N-terminal region of the LH/CG receptor. The next series of experiments were designed to determine which fragment of hCG is photoaffinity labeled by the hLHR^{17-36(C22Bpa)} peptide. Enzymatic digestion and chemical cleavage of the hCG/ hLHR^{17-36(C22Bpa)} complex were the techniques used to answer this question.

B. Materials and Methods

Materials

The hCG, hCG β, and deglycosylated hCG were supplied by the National Hormone and Pituitary Program (NIDDK, National Institutes of Health). Bpa substituted LH/CG receptor peptides were synthesized by Genemed Synthesis, Inc. (San Francisco, CA). Endoproteinase Glu-C, neuraminidase, and O-glycosidase were purchased from Roche. Cyanogen bromide (CNBr) was purchased from Sigma. PNGase F was purchased from New England Biolabs.

1. Radioiodination of hCG or deglycosylated hCG

1 mCi of Na^{125}I in 10 µl of 0.1 M NaOH and 7 µl of chloramine T (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 introduced to terminate the iodination reaction. Radioiodinated ^{125}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_{2}HPO_{4} and 0.9% NaCl (pH 7.4) (PBS) was used to pre-equilibrate the column and elute the sample.
Iodination of hCG \( \beta \) was carried out as described for radioiodination of hCG or deglycosylated hCG, except that a Sephadex Superfine G-25 column was used for sample fractionation.

3. Enzymatic Digestion with Endoproteinase Glu C

The following solutions were sequentially introduced to siliconized glass tubes: 25 \( \mu l \) of 10mM PBS, 800 ng of hCG or 700 ng of deglycosylated hCG in 10 \( \mu l \) 10mM PBS, and 50 ng of \( ^{125}I \)-hLHR\(^{17-36(Bpa)} \) in 5 \( \mu l \) 10mM PBS. Samples were incubated at 37°C for 90 minutes, then irradiated with a Mineralight R-52 UV lamp for 1.5 minutes. Samples were boiled for 3 minutes in 0.1M urea and 5mM DTT. After cooling to room temperature, samples were transferred to siliconized 0.65 ml Eppendorf tubes. 6% Endoproteinase Glu C was added as indicated. Following an 18 hour incubation at 25°C, individual sample volumes were reduced to approximately 12 \( \mu l \) in a vacuum centrifuge on medium heat setting (43°C). Samples were boiled for 3 minutes in 2% sodium dodecyl sulfate, 100mM dithiothreitol, and 8M urea. The solubilized samples were electrophoresed on 15% polyacrylamide single gradient gels. Gels were dried on filter paper, and exposed to PhosphorImager screens (Molecular Dynamics) overnight. The exposed PhosphorImager screen was scanned on a model 860 Storm System Optical Scanner (Molecular Dynamics), and radioactive band intensity was determined using ImageQuant software (Molecular Dynamics).

4. Chemical Cleavage with CNBr

First, the 6 samples that were photolabeled were prepared. The following solutions were sequentially introduced to siliconized 0.65 ml Eppendorf tubes: 20 \( \mu l \) PBS, 800 ng of hCG in 10 \( \mu l \) PBS, and 50 ng or 100 ng of \( ^{125}I \)-hLHR\(^{17-36(C22Bpa)} \) in 10 \( \mu l \) 10mM PBS. Samples were incubated at 37°C for 90 minutes, then irradiated with a Mineralight R-52 UV lamp for 2 minutes. Samples were completely dried in a vacuum centrifuge on medium heat setting (43°C). 50 \( \mu l \) 70% formic acid or 50 \( \mu l \) 70% TFA was added to each tube as indicated. 1 \( \mu l \) \( ^{125}I \)-hCG or \( ^{125}I \)-hCG \( \beta \) were added as indicated to tubes not containing photolabeled hCG. A small crystal
of CNBr was added to each tube, and swirled gently to dissolve. Samples were incubated at room temperature for 12 hours in the dark. Reactions were quenched by evaporating the formic acid or TFA in a vacuum centrifuge (43°C) after diluting the acid to 7% with water. Digests were dried completely in the vacuum centrifuge. 10 µl PBS was added to each tube. Samples were boiled for 3 minutes in 2% sodium dodecyl sulfate, 100mM dithiothreitol, and 8M urea. The solubilized samples were electrophoresed on a 15% polyacrylamide single gradient gel, and processed as described above.

5. Deglycosylation of hCG

19 µl 20mM sodium phosphate (pH 7.2) was added to each siliconized 0.65 ml Eppendorf tube. 1 µl 125I-hCG or 125I-hCG β were added as indicated. Samples were denatured by boiling for 10 minutes in 0.5% SDS and 1% β-mercaptoethanol. 1% NP-40 was then added to each tube. After allowing the samples to cool completely, 0.056 mU neuraminidase, 0.069 mU O-glycosidase, and 27.8 units of PNGase F were added to each tube as indicated. Samples were incubated at 37°C for 18 hours. Individual sample volumes were reduced to approximately 10 µl in a vacuum centrifuge on medium heat setting (43°C). Samples were boiled for 3 minutes in 2% sodium dodecyl sulfate, 100mM dithiothreitol, and 8M urea. The solubilized samples were electrophoresed on a 12.5% polyacrylamide single gradient gel, and processed as described above.

Alternatively, samples were prepared as described but denatured by boiling for 10 minutes in 0.1% SDS and 10mM DTT. The samples were cooled to room temperature and deglycosylation enzymes were added as indicated.

6. In-gel Digestion of Photolabeled hCG α and Photolabeled hCG β

The following solutions were sequentially introduced to siliconized 0.65 ml Eppendorf tubes: 10 µg hCG in 20 µl sodium phosphate (pH 7.2) and 1.76 µg 125I-hLHR17-36(C22Bpa) in 40 µl sodium phosphate. Samples were incubated at 37°C for 90 minutes, then irradiated with a Mineralight R-52 UV lamp for 2 minutes. Samples were denatured by boiling for 10 minutes in 0.5% SDS and 1% β-mercaptoethanol. 1% NP-40 was then added to each tube. After allowing
the samples to cool completely, 5 mU neuraminidase was added to each tube. Samples were incubated at 37°C for 12 hours. 0.1 mU O-glycosidase, and 250 units of PNGase F were added to each tube, and samples were incubated at 37°C for 28 hours. Individual sample volumes were reduced to approximately 40 µl in a vacuum centrifuge on medium heat setting (43°C). Samples were boiled for 3 minutes in 2% sodium dodecyl sulfate, 100mM dithiothreitol, and 8M urea. The solubilized samples were electrophoresed on a 12.5% polyacrylamide single gradient gel. The gel was stained with Coomassie Blue (Bio-Rad) and destained until the background level of stain was acceptable. Two bands representing photolabeled, deglycosylated hCG β were excised from the gel using a clean razorblade, chopped up into small cubes and combined in a siliconized 1.5 ml Eppendorf tube. This process was repeated for the other two photolabeled, deglycosylated hCG β bands and for both sets of photoaffinity labeled, deglycosylated hCG α bands. In-gel digestions of hCG α and hCG β were performed using CNBr on one sample set, and Glu C on the other.

Excised gel pieces were rehydrated in 100mM NH₄HCO₃. After 15 minutes, an equal volume of acetonitrile was added and tubes were vortexed for 15 minutes. All liquid was removed and acetonitrile was added to shrink the gel pieces. All liquid was removed and gel pieces were dried down in a vacuum centrifuge (43°C). The gel pieces were then incubated in 10mM dithiothreitol/100mM NH₄HCO₃ for 60 minutes at 56°C to reduce the proteins. After allowing the samples to cool to room temperature, the gel pieces were spun down and excess liquid was removed. 55mM iodoacetamide/100mM NH₄HCO₃ was used to alkylate the proteins. Samples were incubated in the iodoacetamide solution for 45 minutes in the dark. All liquid was removed, gel pieces were shrunk with acetonitrile, and gel particles were dried down in a vacuum centrifuge (43°C).

Samples designated for cleavage by CNBr were rehydrated in 70% formic acid. A small crystal of CNBr was added to each tube. Samples reserved for Glu C digestion were rehydrated in a digestion solution consisting of 25mM NH₄HCO₃ (pH 7.8) and 10% Glu C by weight. Samples were incubated overnight at room temperature, in the dark.

Next, peptides were extracted from the gel particles. Samples were incubated at 37°C for 15 minutes with shaking. Gel particles were spun down, and the supernatant was collected and saved. The extraction was repeated twice with a 5% formic acid:acetonitrile solution. All supernatants were combined and dried down in a vacuum centrifuge.
100 µl of water was added to each tube and samples were dried down again. Each digest was solubilized in 40µl 20mM sodium phosphate. Samples were boiled for 3 minutes in 2% sodium dodecyl sulfate, 100mM dithiothreitol, and 8M urea. The solubilized samples were electrophoresed on a 15% polyacrylamide single gradient gel, and processed as described above.

C. Results

1. Enzymatic Digestion of Photoaffinity Labeled Hormone by Endoproteinase Glu C

The ability of Endoproteinase Glu C to cleave photolabeled hCG was evaluated (Figure 3-1). Each of the five Bpa peptides was incubated with hCG and treated with UV for 90 seconds. Following denaturation, samples were incubated with Glu C for 18 hours. A faint band located approximately half-way between photolabeled hCG α and free 125I-Bpa peptide can be seen on the autoradiograph. This band is located in the same position for each of the Bpa peptides, suggesting that the same fragment of hCG was photolabeled by each Bpa-substituted peptide. These results further confirm the specificity of the photoaffinity labeling. However, the vast majority of the photolabeled hCG β and photolabeled hCG α molecules remain intact. This indicates that cleavage of the hCG/ hLHR17-36(Bpa) complex by Glu C under these conditions was relatively inefficient.

The ability of Endoproteinase Glu C to cleave photolabeled deglycosylated (dg) hCG was also assessed (Figure 3-2). Immediately below the band corresponding to photolabeled dghCG α, a faint band representing a photolabeled fragment of dghCG can be seen. Just above the free 125I-Bpa peptide band, a second band even fainter than the first, can also be detected. The presence of these two bands in the same position for each of the Bpa peptides indicates that the same fragments of dghCG were photolabeled by each of the five Bpa-substituted peptides. Cleavage of the dghCG/ hLHR17-36(Bpa) complex by Glu C under these conditions also appears to have been inefficient.

2. Chemical Cleavage of Photoaffinity Labeled Hormone by Cyanogen Bromide
The ability of CNBr to cleave photolabeled hCG was evaluated (Figure 3-3). $^{125}$I-hLHR$^{17-36}$ was incubated with hCG and exposed to UV irradiation for 2 minutes. Samples were dried down then solubilized in either formic acid or TFA, and incubated for 12 hours with CNBr. Two bands representing photolabeled fragments of hCG can be seen in Figure 3-3, B. The majority of photolabeled hCG $\alpha$ appears to have been cleaved by CNBr, consistent with cleavage of $^{125}$I-hCG seen in gel lanes two and three of Figure 3-3, A. Cleavage of photolabeled hCG $\beta$ and $^{125}$I-hCG $\beta$ was less efficient (Figure 3-3, B: lanes 9,10,12,13; Figure 3-3, A: lanes 5,6). Overall, these results indicate that cleavage of the hCG/hLHR$^{17-36}$ complex by CNBr was more efficient than digestion by Glu C.

3. Enzymatic Deglycosylation of Radioiodinated hCG

In an attempt to further improve enzymatic and chemical cleavage efficiency, and also to facilitate hCG fragment identification, hCG was deglycosylated. Deglycosylation enzymes used include neuraminidase, PNGase F, and O-glycosidase. Neuraminidase cleaves sialic acid, and PNGase F and O-glycosidase hydrolyze $N$-acetylglucosaminylasparagine and $N$-acetylgalactosaminylserine/threonine linkages, respectively [48].

The efficacy of deglycosylation by neuraminidase, PNGase F, and O-glycosidase following two different methods of denaturing hCG was examined (Figure 3-4). Prior to the addition of deglycosidases, $^{125}$I-hCG and $^{125}$I-hCG $\beta$ were denatured by boiling for 10 minutes in 0.1% SDS and 10mM DTT (Figure 3-4, A). As evidenced by the lack of band shifting, neither $^{125}$I-hCG $\alpha$ or $^{125}$I-hCG $\beta$ were deglycosylated. In contrast, when $^{125}$I-hCG and $^{125}$I-hCG $\beta$ were denatured by boiling for 10 minutes in 0.5% SDS and 1% $\beta$-mercaptoethanol, followed by the addition of 1% NP-40 (a non-ionic detergent), bands representing each hormone subunit shifted significantly (Figure 3-4, B). This indicates that carbohydrate moieties were effectively removed.

4. CNBr and Glu C Digestion of Photolabeled hCG $\alpha$ and Photolabeled hCG $\beta$

One problem with the in-tube digestions of photoaffinity labeled hormone is the difficulty in identification of the photolabeled hCG fragment. It is hard to determine with certainty which
subunit the photolabeled hCG fragment was derived from, let alone the amino acid sequence of the fragment. Separate, in-gel digestions of photolabeled hCG α and photolabeled hCG β solved this problem.

hCG was photoaffinity labeled with \(^{125}\text{I-hLHR}^{17-36(C22Bpa)}\), deglycosylated, and electrophoresed. Bands representing photolabeled, deglycosylated hCG α and photolabeled, deglycosylated hCG β were cut from the gel and digested separately with either CNBr or Glu C (Figure 3-5). The molecular weight of the photolabeled fragments in gel lanes 2, 3, 4, and 5 were determined according to the migration of \(^{125}\text{I-hCG}\), \(^{125}\text{I-hCG β}\), and \(^{125}\text{I-hLHR}^{17-36(C22Bpa)}\). The migration coefficient of each of these molecular weight markers was calculated and used to construct a standard graph. This graph was then used to estimate the molecular weight of the photolabeled hCG fragments.

Based on this analysis, the fragment of photolabeled, CNBr-cleaved hCG α in gel lane 2 was assigned a mass range of 1.9 – 5.5 kd (kilodaltons). Complete CNBr digestion of hCG α generates four fragments (Figure 3-6). The first fragment, hCG α\(^{1-29}\), has a mass of 1998 daltons (2112 daltons when iodoacetamide is used as an alkylating agent). This is a close match to the estimated fragment mass of 1.9 kd. If the cleavage site following Met\(^{29}\) were missed by CNBr, the resulting fragment would have a mass of 5413 daltons, close to the 5.5 kd estimate of the hCG α fragment in lane 2. Masses of the third and fourth CNBr-generated fragments, hCG α\(^{48-71}\) and hCG α\(^{72-92}\), are 2726 and 2543 daltons respectively. Neither of these matches the estimated mass of the fragment.

The smaller fragment of photolabeled, CNBr-cleaved hCG β in lane 3 was assigned a mass of 4.8 kd. The other band corresponds to photolabeled, deglycosylated, and alkylated hCG β. There is only one cleavage site for CNBr in the β subunit of hCG (Figure 3-6). The first fragment, hCG β\(^{1-41}\), has a mass of 4672 daltons when alkylated with iodoacetamide. The second fragment, hCG β\(^{42-145}\) has a mass of 11,505 daltons, much larger than the photolabeled fragment in lane 3. These results suggest that \(^{125}\text{I-hLHR}^{17-36(C22Bpa)}\) photoaffinity labeled the N-terminal portion of hCG β.

Digestion of photolabeled hCG α and photolabeled hCG β with Glu C neither confirms or refutes these findings. Two faint bands can be seen in lane 4. The upper band corresponds to photolabeled, deglycosylated, and alkylated hCG α. The lower band is similar in mass to \(^{125}\text{I-}\)
hLHR\textsuperscript{17-36(C22Bpa)}, suggesting that the Glu C generated fragment of hCG \(\alpha\) labeled by \(125\text{I}-\)hLHR\textsuperscript{17-36(C22Bpa)} is very small or that there was some problem with the digestion. A similar pattern can be seen in lane 5. There are two faint bands: the upper band corresponds to photolabeled, deglycosylated, and alkylated hCG \(\beta\), and the lower band is similar in mass to \(125\text{I}-\)hLHR\textsuperscript{17-36(C22Bpa)}. The bands in lanes 4 and 5 are very faint, illustrating again that Glu C does not efficiently digest photolabeled hCG.

D. Conclusions

CNBr was able to cleave the hCG/ hLHR\textsuperscript{17-36(C22Bpa)} complex more efficiently than Glu C. In-gel digestions of photolabeled hCG \(\alpha\) and photolabeled hCG \(\beta\) with CNBr indicate that the N-terminal regions of both hCG \(\alpha\) and hCG \(\beta\) were photoaffinity labeled by hLHR\textsuperscript{17-36(C22Bpa)}. Computer modeling of hCG shows that the N-terminal regions of each subunit are located on the convex side of the heterodimer (Figure 3-7). These results support the idea that the N-terminal portion of the receptor wraps around the back of hCG, contacting the convex face of the hormone dimer [38].

The faintness of the Glu C generated bands in Figure 3-5 makes interpretation of the digestion data difficult. Use of a different chemical cleavage reagent or enzyme in conjunction with CNBr could potentially help in further narrowing down the labeling site in hCG \(\alpha\) and hCG \(\beta\). Difficulty lies in finding a cleaving agent that will not digest \(125\text{I}-\)hLHR\textsuperscript{17-36(C22Bpa)}, resulting in loss of the \(125\text{I}\) radiolabel. For example, trypsin could not be used in these experiments because it cleaves on the carboxyl side of arginine and lysine residues. \(125\text{I}-\)hLHR\textsuperscript{17-36(C22Bpa)} contains two Arg residues, Arg\textsuperscript{21} and Arg\textsuperscript{31} (Figure 2-3). While cleavage on the carboxyl side of Arg\textsuperscript{21} would be acceptable, due to formation of a covalent bond by the photoprobe at the Cys\textsuperscript{22} position, cleavage on the carboxyl side of Arg\textsuperscript{31} would effectively remove the radioiodinated Tyr residue from the photolabeled hCG/peptide complex. Chymotrypsin, which preferentially cleaves on the carboxyl side of aromatic residues, could be a candidate.

Future studies should also include using tandem mass spectrometry to identify the actual residues which are covalently modified by hLHR\textsuperscript{17-36(C22Bpa)}. Several, largely unsuccessful attempts at this were made. The fragments of interest, the pieces of hCG \(\alpha\) and hCG \(\beta\) photolabeled by hLHR\textsuperscript{17-36(C22Bpa)}, must be isolated in a quantity great enough to allow for such
analysis. Use of high-performance liquid chromatography (HPLC) during sample preparation would facilitate generation of a pure sample. Edman degradation sequencing could also potentially be used to identify the residues which are covalently modified by hLHR\textsuperscript{17-36(C22Bpa)}. However, this method typically requires more sample than mass spectrometry.

Greater understanding of hCG/LH-CG receptor contact sites is the crucial first step in the design of hCG-based fertility-enhancing pharmaceuticals and contraceptives. Innovative drugs based on the glycoprotein hormones and their receptors would have a much greater specificity than the steroid hormone based contraceptives currently on the market. These drugs are known to impact gene expression in most body cells and can cause serious side effects. Glycoprotein hormone and glycoprotein hormone receptor based pharmaceuticals would target primarily the gonads, greatly reducing such side effects [27].
Figure 3-1: Enzymatic digestion of photoaffinity labeled hCG by Endoproteinase Glu C. Radioiodinated Bpa-substituted peptides representing the N-terminal region of the hLHR were incubated with hCG and exposed to UV irradiation for 1.5 minutes as described in "Materials and Methods". Samples were digested with 6% Glu C (by weight) for 18 hours at 25°C as indicated. Following electrophoresis, the gel was dried and exposed to a PhosphorImager screen (Molecular Dynamics) overnight. The imaging screen was scanned on a model 860 Storm System Optical Scanner (Molecular Dynamics). A photolabeled, Glu C generated fragment of hCG is indicated with an arrow.
Figure 3-2: Enzymatic digestion of photoaffinity labeled deglycosylated hCG by Endoproteinase Glu C. Radioiodinated Bpa-substituted peptides representing the N-terminal region of the hLHR were incubated with deglycosylated hCG and exposed to UV irradiation for 2 minutes as described in "Materials and Methods". Samples were digested with 6% Glu C (by weight) for 18 hours at 25°C as indicated. Following electrophoresis, the gel was processed as described in the legend to Figure 3-1. Photolabeled, Glu C generated fragments of deglycosylated hCG are indicated with arrows.
Figure 3-3: Chemical Cleavage of Photoaffinity Labeled hCG by CNBr.
A) $^{125}$I-hCG, $^{125}$I-hCG $\beta$, and hCG photolabeled with $^{125}$I-hLHR$^{17-36}$(C22Bpa) were digested with CNBr in either 70% formic acid or 70% TFA as indicated. Gel was processed as described in the legend to Figure 3-1. B) Contrast was adjusted in image of lanes 8-13. Photolabeled, CNBr generated fragments are indicated with arrows.
### A

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-dg hCG</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{125}$I-hCG</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{125}$I-hCG β</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNGase F</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-glycosidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-dg hCG</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{125}$I-hCG</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{125}$I-hCG β</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNGase F</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-glycosidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 3-4: Enzymatic deglycosylation of $^{125}$I-hCG and $^{125}$I-hCG $\beta$.** A) Prior to the addition of deglycosidases, $^{125}$I-hCG and $^{125}$I-hCG $\beta$ were denatured by boiling for 10 minutes in 0.1% SDS and 10mM DTT as described in “Materials and Methods”. Samples were cooled to room temperature and neuraminidase, PNGase F, and O-glycosidase were added as indicated. Following electrophoresis, gels were processed as described in the legend to Figure 3-1. B) Prior to the addition of deglycosidases, $^{125}$I-hCG and $^{125}$I-hCG $\beta$ were denatured by boiling for 10 minutes in 0.5% SDS and 1% β-mercaptoethanol as described in “Materials and Methods”. 1% NP-40 was then added to each tube. After allowing samples to cool completely, neuraminidase, PNGase F, and O-glycosidase were added as indicated.
Figure 3-5: CNBr and Glu C digestion of photolabeled hCG α and photolabeled hCG β. hCG was photoaffinity labeled with $^{125}$I-hLHR<sub>17-36(C22Bpa)</sub> then deglycosylated with neuraminidase, O-glycosidase, and PNGase F. Bands representing photolabeled, deglycosylated hCG α and photolabeled, deglycosylated hCG β were excised from the gel and digested separately with either CNBr or Glu C as described in "Materials and Methods". Digestion products were electrophoresed, and the gel was processed as described in the legend to Figure 3-1.
Cleavage of hCG by CNBr

hCG α subunit
APDVQ DCPEC TLQEN PFFSQ PGAPI LQCMG CCFSR AYPTP LRSKK TMLVQ
KNVTS ESTCC VAKSY NRVTN MGGFK VENHT ACHCS TCYYH KS

hCG β subunit
SKEPL RPRCR PINAT LAVEK EGCPV CITVN TTICA GYCPT MTRVL QGVLP
ALPQV VCNYR DVRFE SIRLP GCPRG VNPVV SYAVA LSCQC ALCRR STTDC
GGPKRD HPLTC DDPRF QDSSS SKAPP PSLS PSLP GPSDT PILPQ

Cleavage of hCG by Endoproteinase Glu C

hCG α subunit
APDVQ DCPEC TLQEN PFFSQ PGAPI LQCMG CCFSR AYPTP LRSKK TMLVQ
KNVTS ESTCC VAKSY NRVTN MGGFK VENHT ACHCS TCYYH KS

hCG β subunit
SKEPL RPRCR PINAT LAVEK EGCPV CITVN TTICA GYCPT MTRVL QGVLP
ALPQV VCNYR DVRFE SIRLP GCPRG VNPVV SYAVA LSCQC ALCRR STTDC
GGPKRD HPLTC DDPRF QDSSS SKAPP PSLS PSLP GPSDT PILPQ

Figure 3-6: CNBr and Endoproteinase Glu C cleavage sites in hCG α and hCG β. The chemical cleavage reagent CNBr cleaves on the carboxyl side of methionine residues. The enzyme Endoproteinase Glu C cleaves on the carboxyl side of glutamate residues. N-linked glycosylation sites are shown in red. O-linked glycosylation sites are shown in blue.
Figure 3-7: hCG fragments photoaffinity labeled by $^{125}$I-hLHR$^{17-36}$(C$_{22}$Bpa). Computer modeling of hCG $\alpha$ (A), hCG $\beta$ (B), and the hCG heterodimer (C) showing the CNBr generated fragments photoaffinity labeled by $^{125}$I-hLHR$^{17-36}$(C$_{22}$Bpa). The photolabeled portion of hCG $\alpha$ is shown in red, and the photolabeled fragment of hCG $\beta$ is shown in blue.
LIST OF REFERENCES


CURRICULUM VITAE

Name: Rebecca Louise McCaffrey
Date of Birth: December 18, 1973
Place of Birth: Calgary, Alberta, Canada

EDUCATION

B.S. 1998-1999 University of Wyoming, Laramie, WY
Major: Molecular Biology
B.A. 1991-1996 University of Wyoming, Laramie, WY
Major: Anthropology

RESEARCH EXPERIENCE

1999-present Graduate Student
University of Kentucky, Lexington, KY
Molecular and Cellular Biology Department
Principle Investigator: Dr. Tae H. Ji
1999 Student Investigator
University of Wyoming, Laramie, WY
Molecular Biology Department
Principle Investigator: Dr. Mark Stayton
1998-1999 Lithics Analyst
University of Wyoming, Laramie, WY
Archaeology Department
Principle Investigators: Dr. Mary Lou Larson, Dr. Marcel Kornfeld
1998 Student Investigator
University of Wyoming, Laramie, WY
Biology Department
Principle Investigator: Dr. Steve Miller
SCHOLASTIC HONORS

05/2001  3rd Place Poster Award, Symposium in Reproductive Sciences
        University of Kentucky, Lexington, KY
06/2000-06/2002  National Institutes of Health Fellowship in Reproductive
                 Endocrinology
                 University of Kentucky, Lexington, KY
05/1999  Top Graduating Senior in Molecular Biology:  Award for
          Outstanding Achievement and Academic Excellence
          University of Wyoming, Laramie, WY
President’s List  University of Wyoming, Laramie, WY
                 1995 (Spring), 1995 (Fall), 1998 (Fall)
Dean’s List  University of Wyoming, Laramie, WY
             1994 (Fall), 1996 (Spring), 1999 (Spring)
1991-1995  Superior Student Scholarship