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UMI®
ELUCIDATION OF A HUMAN GROWTH HORMONE MOTIF CRITICAL FOR
LACTOGENIC ACTIVITY AND THE ROLE OF CONFORMATIONAL CHANGE IN
RECEPTOR ACTIVATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Science in the Graduate
School of The Ohio State University

By
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****

The Ohio State University
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Conformation changes have been observed in enzymes as being integral to their enzymatic activity. My work centers on the notion that protein interactions are also dynamic. This makes the study of the entire molecule, not just the binding epitopes, relevant in the understanding of the overall mechanism of interaction. HGH has served as an excellent model system due to its importance to the pharmaceutical industry and because there is a wealth of crystal structures available.

Although the binding interfaces of hGH and its receptors have been extensively studied, the explicit details of the overall mechanism has not been elucidated. HGH sequentially binds two receptor molecules, yielding an active trimeric complex. HGH binds and activates both the lactogenic and somatotrophic receptors. Comparison of crystal structures of hGH either free in solution or bound to the lactogenic or somatotrophic receptor reveals that hGH binding to the first receptor molecule is associated with structural changes unique to each receptor that influence the spatial arrangement of residues constituting site 2.

Using site-directed mutagenesis, areas undergoing structural modification were scanned in search of the residues that propagated the changes. Proliferation assays using a FDC-P1 cell line transfected with either the lactogenic or somatotropic
receptor identified a contiguous set of hydrophobic residues that forms a motif that communicates between the receptor binding sites. The motif includes Phe44, Tyr160, Tyr164, Leu163, and Leu93. Mutation of these residues to glutamic acid disrupts the hydrophobic interactions and reduces lactogenic activity up to 85 fold without proportionally affecting somatotrophic activity or altering spectroscopic properties. These differential effects indicate that loss of lactogenic activity is not a result of global misfolding.

I propose the loss of lactogenic activity due to these mutations results from the disruption of specific residues in hydrophobic clusters disallowing the binding-induced modification of the second receptor binding site, and thus for lactogenic activity. Fluorescence resonance energy transfer and electron paramagnetic resonance were used to monitor the structure of hGH as it bound to its receptor. The results of these experiments were not conclusive in establishing a physical mechanism for the modification of site 2, but provide enough evidence of a ligand induced conformation change to warrant future investigation into the mechanism using these techniques.
Dedicated to Deb
A true “F”riend
ACKNOWLEDGMENTS

I wish to thank my advisor, Chuck Brooks, for intellectual support and patience in allowing me to make the errors that enabled me to learn to troubleshoot my problems. I would also like to thank him for the encouragement and guidance in my pursuit of not just a degree, but instead, a career in science.

I thank Larry Berliner for the use of his EPR instrument, as well as his time and discussions concerning the evaluation of my EPR data.

I appreciate the support of fellow labmates Uma Sivaprasad and Colleen Almgren. I am especially grateful to Francis Peterson for his meticulous record keeping and organized style, along with his discussions, which made starting my project much easier.

This research was supported by a grant from the National Institutes of Health.
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CHAPTER 1

INTRODUCTION

1.1 Overview of the project

Traditionally, conformation changes have been observed in enzymes as being integral to their enzymatic activity, while other proteins have been described as rigid systems by terms such as “lock and key interactions”. Beginning in the early 1990’s, proteins achieved a new look as their conformational fluidities began to be recognized. As more protein structures were determined, protein “snapshots” were obtained, catching proteins in various conformations.

The ultimate goal of much research lies in the development of new drugs. High throughput screening methods have enabled researchers to scan functional groups to assess their importance in specific protein-protein interactions, such as hormone-ligand interactions. However, it is important to understand how the functional groups interact with each other, both within and outside of the binding domains, to fully utilize high throughput assays.

In the work presented, human growth hormone (hGH) is used as a model to study conformational changes, specifically exosite changes. hGH serves as an excellent model system for several reasons. First, there are many crystal structures available of
hGH alone and in complex with its ligands. Second, it has a large pharmaceutical value, reinforcing the necessity of understanding not only the macroscopic method of action, but also the microscopic details by which activation of its ligands occurs. The following chapters provide data establishing an exosite motif of hGH necessary for its lactogenic proliferative actions.

1.2 History of human growth hormone

People have been recording linear growth patterns in children for more than 200 years, however the first documented study was not performed until 1877 when Count Philibert Guereau de Montbeillard monitored his sons growth (1). The basis for an endocrine system was established in 1889, when Brown-Sequard injected himself with testicular fluid to indicate that there were substances in the fluid that affected his entire body, thus establishing the basis for endocrinology (2). By 1890, Pierre Marie, in reviewing several cases of acromegaly, had established a connection between acromegaly and enlarged pituitaries, although the disease was initially thought to be due to a deficiency (3). In 1900, Hutchinson and Benda independently suggested that the pituitary had a “growth regulating center” and that there may be a link between the pituitary and dwarfism, as well as acromegaly (4) (5). By 1908 it was accepted that an abnormality of the pituitary caused acromegaly, and that the pituitary was connected with growth. Conversely, dwarfism was hotly contended as being a result of pituitary abnormalities because of variations in the pituitary in people with dwarfism. Early hypophysectomies yielded misleading data due to incomplete procedures (6).

Things began to change in 1908 when Nicholas Paulesco developed the first technique for a 100% hypophysectomy, which ultimately proved that the pituitary was...
essential for life (7) (6). Cushing furthered this work through the use of partial hypophysectomies, which showed that it was the anterior lobe of the pituitary that was critical for life, and that you could create dwarfism and sexual infantilism with partial hypophysectomies (8). Initial studies in 1921 by Herbert Evans and Joseph Long found that anterior lobe extracts of beef pituitaries when fed to normal rats could produce giant rats, thus establishing the possibility that a growth-promoting factor could be purified and used to treat dwarfism similar to the use of insulin (9). Thus began the attempts to treat dwarfism with crude purified bovine growth hormone in the 1930's. Unfortunately, conflicting results slowed the work. In 1948, Dingemanse suggested that the possible reason for the mixed results could be the result of inadequate doses, species specificity of GH, or heterogeneity among dwarfism cases (10).

Growth hormone was first purified by Cho Hao Li and Herbert Evans from ox pituitaries, although they only obtained 0.04g per 1 Kg raw material (11). The procedure was improved 50-fold by Wilhelmi, Fishman, and Russell with the ability to crystallize growth hormone (12). The GH produced by these methods were used to show that ox and whale GH caused rats to grow, while monkey or human GH would slow this growth. This data was interpreted as stimulating antibodies that were preventing action, and that primate GH must be structurally different from rat GH. Additionally, ox and pig GH were shown to be inactive in humans, raising the question of whether or not GH was required in humans (13) (14). This question was
answered by Ernst Knobil and Roy Greep in 1959, who discovered that GH was indeed required in humans, and that GH was species specific, with primates only responding to primate GH (15).

Human GH was first purified in 1956 by Li and Papkoff, but the procedure was laborious and unpredictable and the yield low (16). Raben modified the procedure in 1957, such that it included a glacial acetic acid step to remove biological contaminants (17). He initiated the first clinical use of hGH with these preparations in 1958 (18), and from 1959 to 1976, 642 patients were treated with hGH prepared by the Raben method. To prevent a black market in GH, the National Pituitary Agency (NPA) was established in the United States in 1960. The NPA was a collaboration between the National Institute of Arthritis and Metabolic Diseases (NIAMD) and the College of American Pathologists supervising nearly all of the GH treatments in the US from 1960 until 1985 [S. Douglas Frasier, 1997 #44].

In 1985, the US Food and Drug administration began receiving reports of Creutzfeldt-Jakob disease in patients treated with GH in the late 60’s and early 70’s (19). It was established that contamination in the hGH treatments was the culprit. Although there have been no reported cases since column purification was added to the purification procedure in 1977, use of pituitary extracts was soon replaced with recombinant hGH.

HGH was first expressed recombinantly in E. Coli at Genetech, CA in 1979 (20). Since then, the extracellular domains of both the somatotrophic receptor and the lactogenic receptor have also been cloned in complex with hGH (21) (22).
Crystal structures of hGH in various bound states are available, and extensive mutational analysis has been performed. The results of these studies will be discussed in later sections of the introduction.

1.3 Growth hormone physiology

Primate GH’s exhibits a plethora of biological functions via interactions with either the growth hormone (somatotropic) or prolactin (lactogenic) receptors. The majority of these effects involve the promotion of skeletal growth and body composition (23). There are several variants of hGH resulting from 2 genes and alternative splicing. hGH-N (normal) and hGH-V (variant) are both located on chromosome 17 in the GH/placental lactogen gene cluster (24). The proteins encoded for by these genes differ by 13 out of 191 amino acids, and both consist of four introns and five exons with a 500 base pair promoter region. hGH-N is expressed in the pituitary, while hGH-V is expressed in the placenta. Alternative splicing mechanisms of hGH-N at exon three yields a 20 kDa isoform that is missing residues 32-46 (25) (26). The promoter region contains 5 main elements; an enhancer region, a glucocorticoid-responsive element (GRE), a pituitary-specific GH transcription factor site (pit-1), a cAMP-responsive element, and a TATA box (27).

Transcription of GH first produces a prohormone GH consisting of 225 amino acids. This protein is cleaved in the endoplasmic reticulum, removing the signal peptide to yield the 191 amino acid GH. The GH is then translocated to the Golgi apparatus where the GH is packaged into secretory granules where it is stored until release (28). In the secretory granules up to 70% of the GH is stored as dimers or high molecular weight heteropolymers. The formation of the dimers is partially a result of
the presence of a high concentration of zinc, which is present in the secretory granules in approximately equimolar concentrations with the GH (4mM). Zinc, which binds the GH with a $K_a$ of 1μM, serves to stabilize the GH by preventing denaturants from accessing the protein (29) (30). The dimerization also prevents cells proximal to the pituitary from being overstimulated (31).

GH is secreted by somatotrophic cells in the pituitary in pulsitory episodes throughout life (32). The amplitude and frequency of secretion ranges from 50-450 nM and 5-30 seconds respectively, reflects the relative total circulating blood GH levels. The number of somatotrophs, pituitary cells responsible for the production of GH, is also affected by circulating levels of GH by a feedback loop mechanism (33). Regulated GH release is a calcium dependent process, with an increase in cytosolic calcium resulting in secretion of GH (34). The process involves translocation of the secretory vesicles along microtubules to the plasma membrane. At the membrane, the vesicles fuse with the membrane releasing GH. (35). Release is regulated by two hypothalamic peptides. Somatostatin inhibits GH release at the level of the plasma membrane, while growth hormone releasing hormone (GHRH) acts to stimulate GH release by the induction of extracellular calcium entry into the somatotrophs (36). Many factors such as sex, age, sleep, stress, exercise, and nutrition effect GH release by regulating either somatostatin or GHRH (23).

Once released, GH is transported throughout the body in the blood. The lifetime of GH in the bloodstream is increased due to binding to the GH-binding protein (GHBP), a protein structurally homologous to the extracellular portion of the GH receptor (31). Approximately 40-45% of the 22kDa GH in the plasma is bound to
the GHBP. The two proteins interact with a $K_d$ of $10^{-9}$ M. GHBP is derived from the
GH receptor gene, and is produced predominantly in the liver, where the highest
concentration of GH receptors are located [Baumann, 1993 #62].

hGH has the ability to signal through either the lactogenic or somatotropic
receptors. Each GH molecule has two separate receptor binding sites, allowing one
GH molecule to bind with two receptor molecules forming an active trimeric complex.
The interaction occurs in a sequential fashion, with GH binding to the first receptor
molecule at site 1, and then binding to a second, identical receptor at site 2. The
intracellular portions of the trimeric complex binds to JAK 2 or JAK 1, leading to
phosphorylation of the GH receptor and the JAK molecule (37). JAK 2 then
phosphorylates STAT molecules, specifically STAT 5, 1, or 3, which when
phosphorylated are transported to the nucleus where they interact with promoter
regions in the DNA leading to increased transcription of genes such as $c$-$fos$, $c$-$jun$,
and IGF-1 (Fig 1.1)(38) (39).

1.4 Growth hormone structure

GH belongs to a family of polypeptide hormones including prolactin and
placental lactogen (40). The first member of this family to have its three dimensional
structure elucidated was porcine GH in 1987 (41). The fold of the hormone is a left-
handed, anti-parallel four helix bundle with an up-up-down-down connectivity. The
protein has a high degree of $\alpha$-helix (54%), and no $\beta$-sheet, which is consistent with $\beta$-
-sheet and helical content determined previously by circular dichroism (CD)
spectroscopy.
Upon determination of the three dimensional structures for several cytokines including hGH, granulocyte-macrophage colony-stimulating factor (GM-CSF) (42), leukemia inhibitory factor (LIF) (43), and interleukins 2 and 4 (IL-2, and -4) (44) (45) (46), it was discovered they all shared the same four helix fold (47). Additionally, these ligands all interact with receptors in the hematopoietic superfamily. The helical bundle cytokine superfamily has been further divided into two classes. The long chain members, which typically have larger helices and short helical segments in the connector loops forming elongated cylinders, include GH, erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), Leptin, LIF, IL-6, IL-12, oncostatin-M (OSM), PRL, and thrombopoietin (TPO). The second class, the short chain members, are typified by shorter helices, contain two short β-strands, and have an oblate ellipsoid shape. IL-2, -3, -4, -5, -7, -9, -11, -13, and -15 are members of this short chain class (48).

Many physiochemical properties of GH have been investigated. HGH is known to have 4 cysteines, paired in two disulfide bonds, forming a small disulfide loop and a large disulfide loop. Residues 182 and 189 form the small disulfide loop in the far carboxy terminus of the protein. Residues 53 and 165 form the large disulfide loop. Studies with varying concentrations of reducing agents, use of selective carboxymethylation techniques, and subsequent testing of biological activity via the pigeon crop-sac assay and the rat tibia test indicated that the 182/189 disulfide bond was much more labile under reducing conditions, but wasn’t required for full biological activity. The 53/165 disulfide bond was absolutely required for biological activity (49). Further studies on the thermodynamic stability of GH with or without
Figure 1.1: Sequential activation of GH receptors and its correlation to the dose response curve. HGH binds first at site 1. Then, based on hGH concentration, one of two paths are taken. At low concentrations the dimeric complex binds a second receptor at site 2 and recruits JAK, leading to the phosphorylation of the receptor and ultimately activation of STAT (number 2). When a sufficient number of active trimeric complexes are formed to maximally stimulate the cells, a plateau is seen in the dose response curve (number 3). At high GH concentrations (number 4), the receptors are saturated and continue to form unresponsive dimeric complexes.
the disulfides revealed that the short loop disulfide destabilized GH by 3.3 kcal/mol, while removal of both disulfides resulted in a loss of 5.9 kcal/mol (50).

Spectroscopic studies on GH with disulfides removed using several alkylating agents, suggested that the overall structure of the molecule is unchanged, and that biological loss of activity seen in previous studies was due to interference of the alkylating agent rather than the importance of the disulfide bond (51). More recently, raman spectroscopy has been used to detect the number of disulfide bonds present in GH much more sensitively then earlier studies (52).

Spectroscopic studies of GH are aided due to the presence of only one intrinsic tryptophan. Changes in the tryptophan absorption and fluorescence provide information about the solution surrounding the indole ring. The core of GH is primarily hydrophobic with several hydrophobic pockets located throughout the molecule. Residues Ile$^{36}$, Phe$^{44}$, Cys$^{53}$, Phe$^{54}$, and Ile$^{58}$ of mini-helix 1 form a hydrophobic pocket with residues Leu$^{157}$, Tyr$^{160}$, Tyr$^{164}$, Cys$^{165}$, and Phe$^{176}$ of helix 4. Furthermore, residues on the other side of helix 4, Leu$^{162}$ and Leu$^{163}$ form a hydrophobic pocket with residues Leu$^{93}$, Val$^{96}$, and Phe$^{97}$ from the loop between helices 2 and 3 (Fig 1.2) (53). Helix 3 is unique in that there is a distinctive bend at Pro$^{86}$.

1.5 GH receptors

hGH interacts with both the hGH receptor and the hPRL receptor. hGH receptor was first cloned in 1987 by Leung et al. The sequence was not homologous to any other proteins known at the time, suggesting a new class of receptors (54) (55). The hGH binding protein (hGHbp), consisting of the first 238 residues of the full
length hGH receptor, was expressed in *E. Coli* in 1986 (56), and the crystal structure of the hGHbp was determined in 1992 in complex with hGH (29). Cloning of the hPRL receptor followed the work for the GH receptor in 1989 (57), and the sequence homology between the two confirmed a new family of receptors.

Both receptors are members of the hematopoietic superfamily of receptors, which also includes many cytokine receptors. Members of the family consist of three domains, an extracellular domain of 200-250 amino acids, an intracellular domain that is highly variable, and a single pass hydrophobic transmembrane domain. The extracellular domains contain several common features. These include four cysteine residues near the N-terminus and a WSXWS consensus sequence near the C-terminus. The fold of the extracellular domain consists of two immunoglobulin-like domains: that undergo sheet switching as is seen in fibronectin type III domains (53) (58). The two domains are connected by a short four residue hinge region that establishes a relative orientation of the two domains (Fig 1.3).

The stoichiometry of hGH with the hGH receptor was established using several independent physical methods. Size exclusion chromatography, titration calorimetry, and fluorescence quenching all concluded that hGH formed a 1:2 complex with the hGH receptor (29). The stoichiometry of hGH with the hPRL receptor was more difficult to establish, as the site 2 affinity is 10-fold lower than for the hGH receptor. Only recently has dimerization of the hPRL receptor been clearly identified (59).

A sequential mechanism of activation of the receptors by hGH in the formation of the trimeric complex was first suggested when it was observed that addition of excess hGH added to the hGH:(hGHbp)_2 trimeric complex caused
Figure 1.2: View of the hGH structure. The basic structure of hGH showing the four helix bundle. The four helices, as well as mini-helices 1 and 2 and the 2-3 connector loop are labeled. The structural representation was extracted from the crystal structure of hGH bound to two somatotrophic receptors (21).
dissociation into a dimeric hGH:hGHbp complex (29). Mutation of hGH resulting in mutants with minimal binding at either site 1 or site 2 revealed that mutants with a nonfunctional site 2, most notably G120R, could still form a 1:1 complex, whereas a nonfunctional site 1 could not bind at all (29) (60). The second functional observation that the mechanism was sequential came from dose response curves. Treatment of cells with doses of hGH that went beyond saturation of the hGH receptors produced a bell-shaped curve, indicating that hGH serves as a self-antagonist at high doses by predominantly occupying site 1 and depleting the target cells receptor population, thus preventing formation of the active trimeric complex required for a growth promoting signal (60).

The next piece of information regarding the mechanism of activation by hGH came from the crystal structure of hGH:(hGHbp)₂. It was noted that when two hGH receptors dimerize, a substantial portion of their stem regions come into contact. Since site 1 buries approximately 1230 square angstroms, compared to only about 500 square angstroms for site 2, the 500 square angstrom/receptor buried in the stem region upon dimerization may be required to achieve a measurable affinity (21).

Substantial mutagenesis has been performed to further probe specific interactions and binding epitopes for each receptor, which will be discussed in detail in their own corresponding sections.

1.6 HGH interaction with the somatotropic receptor

Since 1992 there have been several three-dimensional structures of hGH solved. The trimeric hGH:(hGHbp)₂ came first (PDB #3HHR) (21), followed in 1994 by the structure of an affinity matured hGH (PDB #1HUW) (61), hGH in the unbound
Figure 1.3 The extracellular domain of the hGH receptor (hGHbp) taken from the crystal structure of hGH bound to 1 hGH receptor molecule (21).
state (PDB #1HGU) (62) was described next in 1995, and finally in 1996 a hGH:hGHbp structure rounded out the three-dimensional database (PDB # 1HWH) (63). These structures were used as starting points for massive mutagenesis projects to uncover both structural and functional binding epitopes. Residues that alter the binding properties of hGH are considered to be a part of the functional epitope, while the structural epitope contains any residue in hGH that is in contact with the receptor as determined by the crystal structures.

The first of such experiments involved homolog-scanning mutagenesis, where regions of hGH 7-30 amino acids in length were replaced with the corresponding segments of placental lactogen, a homologous protein that does not have hGH receptor binding capabilities (64). Replacement of the loop region between residues 54 and 74, the C-terminal segment of helix 4, or the N-terminus of helix 1 reduced binding to the hGHbp. The regions identified in homolog-scanning mutagenesis were further analyzed by alanine-scanning mutagenesis to pinpoint a functional binding epitope, defined as residues that when mutated to alanine showed a greater than 4-fold loss of binding relative to the wild-type hGH. A total of 32 residues, all determined to be part of the structural epitope, were mutated one at a time, with 14 mutants showing a greater than 10-fold decrease in binding (Table 1.1, Fig 1.4) (65). One residue, E174A, actually increased the binding affinity 4-fold (66).

In general, the residues determined to be part of the structural epitope are discontinuous and consist of both hydrophobic and hydrophilic residues, although basic side chains dominate. The functional epitope consists of a “hot-spot” of binding
<table>
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**TABLE 1.1: SITE 1 BINDING EPITOPES***

* data taken from Cunningham et al, 1989
** residues labeled structural are contained in the structural epitope
*** residues in the functional epitope vary in their importance, and are denoted major or minor depending on their significance, such determinations were not delineated for the lactogenic functional epitope and so all participating residues are identified as functional
energy, with 85% of the binding energy coming from only 8 residues, and the remaining 15% from an additional 6 residues. Although the structural epitope contains both hydrophobic and hydrophilic residues, the residues responsible for the binding energy are mostly charged residues (67). The results from a study of the heat and entropy of the binding reaction at site 1 emphasized that although the kinetics and affinity values do not change due to some mutations in the structural epitope, they can produce local structural changes that lead to compensating heat and entropy of binding values (68).

The interface at site 1 has also been shown to have structural plasticity, meaning that mutations in the interface can be compensated for by structural rearrangements of other residues in the structural epitope. It was known from the hGH:(hGHbp)$_2$ crystal structure that Trp$^{104}$ of the hGHbp was critical for the hGH-hGHbp interaction, specifically that Trp inserts into a groove on the surface of the hGH molecule. Using phage display, the five residues (K168, D171, K172, T175, and F176) that pack against Trp$^{104}$ were randomly mutated to search for a hGH mutant that would restore binding to W104A hGHbp, which had a $K_d$ greater than 1000nM. Mutations that increased the size of the residues side chain could fill the groove on hGH, overcoming the W104A mutation (69).

These results were the basis for a series of affinity-matured hGH homologs, and hGH antagonists. Combinations of residues in the site 1 functional epitope have been used to produce a mutant deficient in site 1 binding. The best combination is K172A/F176A hGH, which reduced the $K_d$ almost 700 fold, and shifted the ED 50 value about 1000 fold compared to wt hGH. Site 2 mutations have centered on
**Figure 1.4:** hGH site 1 binding epitope for the hGHbp (21). Correlating with the labeling system used in Table 1.1, residues highlighted in red indicate major contributors of binding energy, while pink residues are minor contributors. Cyan residues represent the remaining structural epitope.
G120R, a mutation designed to fill the pocket in the hGH molecule with the bulky arginine side chain to sterically hinder binding to the second receptor molecule (60).

Through the use of monovalent phage display, a variant of hGH was created that had 15 mutations incorporated resulting in a 400-fold tighter complex with the hGHbp at site 1 (70). These mutations will aid in the development of better antagonists, as stronger binding at site 1 coupled with decreased binding at site 2 should produce the best hGH antagonists. Although site 2 has not been as extensively studied, it was also affinity maturated, identifying 5 residues as a functional epitope; Phe¹, Ile⁴, Arg⁸, Asp¹¹⁶, and Glu¹¹⁹ (Table 1.2, Fig. 1.5). Interestingly, increasing the affinity at both site 1 and site 2 does not translate into an increased biopotency, indicating that the wild-type hGH has sufficient affinity to maximally stimulate the cells (71).

<table>
<thead>
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</thead>
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**TABLE 1.2: hGHbp SITE 2 BINDING EPITOPES**
*data taken from Cunningham et al, 1989*
Figure 1.5: hGH site 2 binding epitope for the hGH receptor (21). Residues in red represent the functional epitope, while cyan residues represent the structural epitope.
1.7 HGH interaction with the lactogenic receptor

The structural and functional binding epitopes of hGH with the hPRLbp were determined using homolog-scanning mutagenesis and alanine scanning mutagenesis as for the hGHbp (Table 1.1, Fig 1.6)(72). The structural epitope overlapped the hGHbp structural epitope, while the functional epitopes overlap but are not identical. One specific example is E174A, which when binding to the hGHbp had a 4-fold increase in binding affinity, but when binding to the hPRLbp had a 350-fold decrease.

Initial studies measuring the binding affinity of the hPRLbp were highly variable. Variation of chelating agents and divalent metal ions revealed a zinc requirement for tight binding to the hPRLbp. Optimal binding occurred at 50 μM ZnCl₂, with the affinity increased 8000-fold compared with binding in the presence of EDTA (73). Size exclusion chromatography data in the presence of increasing concentrations of zinc show that careful consideration must be taken when determining which zinc concentration to use, as dimeric and aggregated hGH form at higher concentrations of zinc. Maintaining a zinc concentration that is equal to or less than the hGH concentration, and is below 50 μM, prevents formation of non-monomeric hGH forms (74). Equilibrium experiments indicated that zinc binds noncooperatively to a single site in the hGH:hPRLbp complex. Mutational analysis implicated His¹⁸, His²¹, and Glu¹⁷⁴ as the zinc binding coordinates in hGH (73). The crystal structure of hGH bound to one molecule of hPRL receptor was elucidated in 1994 (22). Analysis of the structure indicated that the residues originally thought to be responsible for the zinc
Figure 1.6: hGH site1 binding epitope for the hPRLbp (22). Red residues represent a functional epitope, while cyan residues indicate a structural epitope. The yellow residues are His$^{18}$ and Gln$^{174}$, the two residues responsible for binding to zinc.
binding were only partially correct. There is only one zinc molecule bound to the hGH:hPRLbp complex, but it is complexed using His\textsuperscript{18} and Glu\textsuperscript{174} from hGH and Asp\textsuperscript{217} and His\textsuperscript{218} of the hPRLbp.

Antagonists of hGH for the hPRL receptor were designed based on existing antagonists for the hGH receptor. The leading candidate is G120R, although in the hPRL receptor system, G120R shows zinc-dependent agonist activity, and is therefore not as competent as an antagonist as it is in the hGH receptor systems (75).

1.8 Comparison of the hGH-hGH receptor and hGH-hPRL receptor interactions

With four different crystal structures of hGH available; hGH free, hGH:(hGHbp), hGH:hGHbp, and hGH:hPRLbp, comparisons of the structures can be made (Fig 1.7).

Several regions of hGH undergo structural changes upon binding to the various receptors. First, there is the helical formation of mini-helix 1, which although seen upon receptor binding to both the hGH and hPRL receptor types, is subtly different for each. Second, there is a lengthening of helix 2, which reduces the number of unstructured residues of the 2-3 loop. Third, helix 3 rotates approximately 30 degrees relative to helix 1. As site 1 is nestled between helix 1 and 3, this rotation could be extremely important.

Elucidation of the mechanism and reasoning behind such conformational changes lie in hGH residues that are critical for activity in one receptor system but not the other. The first of such residues identified was Phe\textsuperscript{44}, which when mutated to alanine or leucine, decreased hPRL receptor binding and lactogenic action, without affecting the hGH receptor binding or somatotropic bioactivity. The loss of lactogenic activity was inversely related to the bulk and hydrophobicity of the side chain added.
Figure 1.7: Comparison of hGH free in solution (left) (62) with hGH in complex with the hPRLbp (right) (22). Highlighted regions undergo structuring upon receptor binding. Mini-helix 1 is in yellow, helix 2 is in purple, and the 2-3 loop is green.
The crystal structure revealed that Phe\textsuperscript{44} does not contact either receptor when binding at site 1 (21) (62) (76). The work presented in this dissertation investigates the role of several other residues required specifically for hPRL receptor activation.

1.9 *Theories on the propagation of conformational changes*

Conformational changes have been established as a part of the mechanism of action for several proteins. Phosphorylase \textit{a} (77) and hemoglobin (78) are two examples of this that are most similar to the situation in hGH. In both cases the active sites are too far apart to interact directly, and conformational changes may be transmitted across the molecule.

Hemoglobin is the model protein for allosteric interactions and cooperativity of binding. Hemoglobin consists of two pairs of similar chains arranged symmetrically. It has four oxygen binding sites that do not act independently. When oxygenated, the hemoglobin tertiary structure undergoes small changes in the binding pockets, and the two subunits rotate 15 degrees relative to each other. Binding studies demonstrated that deoxyhemoglobin has a lower affinity for oxygen than does oxyhemoglobin, and the affinity increases with each molecule of oxygen that is bound (79).

Several models for allosteric binding were developed based on hemoglobin. The Monod-Wyman-Changeux (MWC) concerted mechanism is based on the assumption that a small amount of deoxyhemoglobin exists in the oxyhemoglobin structure, and that once oxygen binds to this form, the oxyhemoglobin structure becomes the predominante structure. This model does not allow for mixed states, and therefore is
only a two-state model. This model provides basic guidelines and simple parameters for establishing experiments and explaining the data, however there are many phenomena that are not explained (80).

A second model for allosteric interactions is the Koshland-Nemethy-Filmer sequential model (81). This model avoids the assumption of symmetry, instead assuming that the transition from one state to another is a sequential process. A series of tertiary changes occur with each binding, creating several mixed states in the transition from the unbound to the bound state. A major portion of this model was based on Koshland’s earlier theory of induced fit, which stated that binding of a substrate to an enzyme could cause conformational changes that align the catalytic groups in a more optimum spatial arrangement (82) (83).

Since the 1960’s, many proteins have been determined to undergo conformational changes based on either crystallographic or solution structures of the protein in the presence and absence of its ligand. Most fit into Koshland’s induced fit model. The most widely accepted examples are the induced fit interaction of antibodies with their antigens (84), sugars binding with ribokinase (85) and glucokinase (86), and in the activation of tyrosine by tyrosyl-tRNA synthetase (87). The above examples have been studied using various spectroscopic methods and site-directed mutagenesis of residues in or near the binding sites, but have not been extended to regions outside of the binding interfaces.

Studies on superoxide dismutase (SOD) (88) and factor VIIa (89) have been conducted that investigate the role of residues outside of the binding epitopes. In the case of SOD a residue 18 angstroms away from the binding site was identified that
increased the efficiency of the enzyme. Studies on factor VIIa discovered a region that was near, but not included in the binding site that could bind to peptides that would inhibit anticoagulation. In both cases the identified residue was located on the surface of the protein. My research on the conformational change in hGH differs from other conformational studies in two ways. First, the regions being studied are not located in or near the binding epitopes. Second, I have discovered not one but a series of communicating residues extending across the entire hGH molecule, linking the binding sites with each other and the regions that undergo a conformational change.
CHAPTER 2
MATERIALS AND METHODS

Materials and methods used in the following chapters will be presented in detail in this chapter. General techniques will not be described, rather can be found in Current Protocols in Molecular Biology (90). Except for rare cases where the proper persons have been credited, all methods described here have been performed by myself.

2.1 The pT7-7 phagemid

The pT7-7 expression plasmid was kindly provided by S. Tabor (Harvard Medical School, Boston, MA). The system includes a T7 RNA polymerase promoter to drive transcription of target genes in E. Coli strains containing a copy of the T7 RNA polymerase gene. Since the DNA manipulation techniques used require the formation of single stranded DNA, Dr. Francis Peterson incorporated an fl origin of replication into the pT7-7 plasmid generating the pT7-7fl(-) phagemid (76, 91). This phagemid is used for all DNA manipulation and protein expression.

2.2 Cloning of the lactogenic and somatotropic receptor extracellular domains.

PCR was used to amplify the coding sequence for the extracellular domains of the lactogenic (hPRLr-ECD) and somatotropic (hGHr-ECD) receptors. 5' primers were designed to exclude the signal peptide, incorporate an unique Nde I site that
simultaneously adds a methionine codon as the first residue, and to silently alter the first eight codons to include bacterially preferred codons. 3' primers introduced a stop codon and a unique Sal I site after the stop codon. The hPRLr-ECD was initially cloned to include the transmembrane domain, but was later altered to remove the transmembrane domain.

5' hPRLr-ECD forward primer
5' CC TGC CTT CTG CAT ATG CAG TTG CCG CCG GGC AAA CCG GAA ATC TTT AAA TGT CG 3'

3' hPRLr-ECD reverse primer (plus transmembrane domain)
5' GGC GGA AAG ATG CAG GTC GAC ATG CTA TAG CCT AAC AAA GCC ACT GCC C 3'

3' hPRLr-ECD reverse primer (minus the transmembrane domain)
5' GCC ACA GAG GTC GAC ACG GTT AAA TCA TTC ATG G 3'

5' hGHr-ECD forward primer
5' GGC AGG ATC AAG TCA TAT GTT TTC CGC CAG CGA AGC CACCGC CGC CAT CCT TAG C 3'

3' hGHr-ECD primer
5' CCA AAG ATA ATA GTC GAC AGC CAT GGT TAG TAG AAA TCT TCT TCA CAT GTA AAT TGG C 3'
The hPRLr-ECD PCR product was amplified from normal human breast tissue cDNA by Dr. Yasuro Sugimoto. The hGHr-ECD PCR product was amplified from a cDNA library derived from a female human liver. The PCR products were agarose gel separated, purified using GENE CLEAN SPIN (Bio101, Carlsbad, CA), and ligated into the TOPO TA cloning vector, version J (Invitrogen, Carlsbad, CA). Colonies selected by ampicillin resistance were screened by PCR using the cloning primers to confirm the presence of the correct size gene insert. Colonies with TA vectors containing the proper insert were expanded to larger cultures and sufficient DNA was prepared for several subcloning experiments. The TA vector was digested with Nde I and Sal I to excise the gene insert. The insert was then uni-directionally ligated into the pT7-7(-) phagemid, which was also digested with Nde I and Sal I. Colonies were screened for ampicillin resistance and by restriction digest, and ultimately confirmed by DNA sequencing (92). The hGH ECD was incorrect, and the proper gene was never obtained.

2.3 Site-directed mutagenesis

2.3.1 The Kunkel method of mutagenesis

_in vitro_ mutagenesis was performed by the Kunkel method (93). This method was chosen over the increasingly common PCR mutagenesis based on two factors: multiple mutations can be made simultaneously, and no sub-cloning is required.

Briefly, RZ1032 E. Coli, a bacterial strain deficient in dUTPase and uracil N-glycosylase, is transformed with the gene of interest. The missing enzymes allows for the incorporation of uracil into the DNA. Addition of R408 helper phage (Promega, Madison, WI) induced the production of single stranded DNA. ssDNA was then purified
from the growth media and used as the mutagenesis template. Mutagenic primers (described in 2.3.2) were phosphorylated, annealed to the uracil containing ssDNA template, and extended by T7 DNA polymerase (New England Biolabs, Beverly, MA) to produce double stranded DNA. The dsDNA products were transformed into DH5α E. Coli where the uracil-containing original strand of DNA is targeted for degradation, leaving only the mutated DNA. Colonies were selected by ampicillin resistance, screened by restriction digest, and confirmed by DNA sequencing.

2.3.2 Mutagenic primer design

Primers were designed to incorporate the desired amino acid change and to include or remove a translationally silent restriction site. Design was aided by the Primer Generator web site, www.med.jhu.edu/medcenter/primer/primer.cgi (94), which located silent restriction sites within the primer. Each primer was between 20 and 40 base pairs in length and included two to three G or C bases at both ends of the primer to aid in the annealing process.

2.4 Protein production

2.4.1 Protein expression

Purified phagemids containing the gene of interest were transformed into BL21(DE3) E. Coli (Novagen, Madison, WI) and grown on ampicillin plates to select for positive transformants. Colonies were grown in 10 ml LB cultures containing 100 μg/ ml ampicillin, overnight (6-8 hours). This overnight culture was used to inoculate a one liter LB culture (with 100 μg/ ml ampicillin). The one liter culture was grown to an OD600 of 0.3. Expression of the recombinant protein was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside, and the culture incubated with shaking for four hours. Cells were
collected by centrifugation and resuspended in 50 ml of 100mM Tris pH 7.5, 25 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Inclusion bodies were harvested following cell lysis via two passages through a French pressure cell at 5000 psi, and subsequent centrifugation of the lysate.

2.4.2 Protein folding

Two different folding procedures were used for the various proteins expressed. Which protocol was used is based on the disulfide and free cysteine content in the protein, which is related to the difficulty of correctly folding the protein, and the amount of protein dimer produced. In general, the more cysteines and disulfides present, the more difficult the protein is to fold. These more difficult proteins use larger dialysis volumes to lower protein concentration, thus reducing dimer formation and reducing conditions to slow disulfide bond formation.

hGH proteins collected in the inclusion bodies were resuspended in 100 ml of 4.5 M urea, 50 mM Tris and then the pH was adjusted to values between 11.0 and 11.5. The inclusion bodies were solubilized by stirring at room temperature for two hours. Solubilized protein solutions were clarified by centrifugation and allowed to oxidize for 1-2 days at 4° C. Oxidized protein was dialyzed at 4° C into 20 mM Tris pH 7.5 (4 X 4L).

Receptor extracellular domains and hGH cysteine mutants were resuspended in 400 ml of 4.5 M urea, 50 mM Tris, 5 mM β-mercaptoethanol, 0.5 mM 2-hydroxyethyl disulfide and the pH adjusted to 11.0-11.5. The solutions were solubilized and clarified as for hGH, although no air oxidation was required. Protein was dialyzed 1 X 4L into
20 mM Tris pH 7.5, 5 mM β-mercaptoethanol, and 0.5 mM 2-hydroxyethyl disulfide, and against 4 X 4L of 20 mM Tris pH 7.5. All dialysis was changed at approximately 12 hour intervals.

2.4.3 Protein purification

Initially proteins were purified using DE52 anion exchange resin (Whatman, Clifton, NJ). hGHs were purified using resin equilibrated in 20 mM Tris pH 9.0, while hPRLr-ECD were purified using resin equilibrated in 20 mM Tris pH 7.5. Protein was loading at 1ml/min in the same buffer as the equilibration buffer. The column was washed for one hour, and then the protein was eluted in a 500 ml, 0-500 mM sodium chloride gradient in 20 mM Tris. Fractions were monitored for the presence of protein by absorbance readings at 280 nm. Eluted protein was dialyzed against 5 mM ammonium bicarbonate (6 X 4L), freeze-dried, and stored at -30°C in a desiccator.

Halfway through my graduate career, the lab obtained an ATKA purification system for our protein purifications. POROS affinity resin (Perseptive Biosystems, Farmington, MA) was equilibrated with either 20 mM Tris pH 9.0 (for GHs) or with 20 mM TEA pH 7.5 (for PRLr-ECD). Proteins were loaded at 20 ml/min, and eluted in a 0-500 mM sodium chloride gradient over 30 min. Outflow was monitored at 280nm, 250nm, and 350nm. The 280 and 250 readings allowed me to monitor for correctly folded protein. Incorrectly folded proteins alter the length and bond angle of the disulfide bonds thus yielding different 250/280 ratios. Since proteins should not absorb light at 350 nm, any readings in this region are instead due to light scattering thus indicating the presence of aggregated protein.
2.5 Physical characterization of proteins

2.5.1 Protein concentration determination

Protein concentrations were determined using BCA reagents (Pierce, Rockford, IL), standardized with bovine serum albumin samples. The reaction uses the bicinchoninic acid/copper sulfate colorimetric principles (95).

2.5.2 Ellman's reaction

Free thiol concentrations were determined using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) (96). A 0.57 mg/ml solution of Ellman's reagent was prepared in 0.1 M degassed sodium phosphate pH 7.4 immediately prior to use. Approximately 5 mg of protein (dry weight) was reconstituted in 0.5 ml degassed sodium phosphate buffer pH 7.5, 10 mM DTT, and then immediately separated from the excess DTT by passage over a 1cm X 26 cm Sephadex G50 column. 175 μl of protein was mixed with 25 μl of Ellman’s reagent in a 96 well plate. OD readings were taken at 415 nm. Free thiol concentrations were calculated using the extinction coefficient 14,500 cm⁻¹M⁻¹.

2.5.3 SDS-polyacrylamide gel analysis

SDS-polyacrylamide gel electrophoresis was used to evaluate the size, purity, and disulfide formation of the recombinant proteins. Size and purity were determined from gels run under reducing conditions. Correct disulfide bond formation was evaluated using gels run under non-reducing conditions, by comparison of the hydrodynamic radius of the recombinant protein with that of a biological isolate (or a recombinant wild-type hGH previously compared with a biological isolate). Proteins with improper disulfide
bond formation would have a slightly altered hydrodynamic radius, and thus would be detected as having a larger or smaller molecular weight. Gels were recorded using an alpha imager and stored as bitmap files.

2.5.4 Absorption spectroscopy

Protein spectra in the near UV region (~210-350) have three regions that contain valuable information. At 350 nm soluble proteins should not absorb light, and thus absorption in this region is due to aggregated protein. At 280 nm, absorption is dominated by three chromophores; Trp, Tyr, and Phe. This peak is directly related to both the total protein concentration in the sample and the hydration of the three chromophores. Absorption at 250 corresponds in part to the presence of disulfide bonds with a 90° dihedral angle. When mutations or incorrect folding causes the disulfide bond angles and/or lengths to change, the absorptivity of the disulfide also changes. Used together, absorption spectra yield information about the disulfide bonds, and the environment and the amount of chromophores present. Protein samples were resuspended at 25 μM in 10 mM Tris pH 8.2, 150 mM NaCl. Spectra were collected from 190-350 nm at room temperature in an Uvicon model 930 spectrophotometer.

2.5.5 Fluorescence spectroscopy

The three chromophoric amino acids, Trp, Tyr, and Phe also emit fluorescence spectra. Trp is the most intense, with a maximum absorption wavelength of 280 nm. The emission spectrum of Trp varies from 307-353 nm depending on how solvent accessible the Trp residues are, with the latter value being fully associated with water. In contrast, the emission spectra for Tyr and Phe, which have maximum absorptions at 274 and 257 nm respectively, only change slightly in response to an altered environment (97). The
fluorescence spectra were used to monitor the packing of the interior of the protein. Since looser packing would allow the sole Trp residue in hGH to be more exposed to water, the emission spectra would be red shifted. To ensure that the emission being observed was from the Trp residue alone, an excitation wavelength of 290 nm was used, which while lowering the Trp signal also excludes Phe and Tyr. Protein samples were resuspended at 1 μM in 10 mM Tris pH 8.2, 150 mM NaCl, and emission collected from 300-400 nm at 20° on a Perkin Elmer model LS 50B fluorimeter.

2.5.6 Circular dichroism spectroscopy

The characteristic patterns associated with the major secondary structures, α-helix and β-sheet, result from the chromophoric properties of the amides in the protein backbone. α-helix structures have three peaks; two negative peaks at 222 nm and 208 nm, and one positive peak at 190 nm. When the peak values are used in conjunction with the sample concentration and protein molecular weights, circular dichroism (CD) can be used to calculate the percentage of α-helix present. Losses of helical structure greater than 5% indicate that the overall protein structure has been perturbed, and any corresponding loss in biological activity may be due to the perturbation of the secondary structure. Differential CD can be used to determine changes in structure of one molecule upon binding to another, by subtracting one spectra from another. Protein samples were resuspended at 25 μM in 10 mM Tris pH 8.2, 150 mM NaCl, and spectra collected from 200-260 nm at 20° on an Aviv model 202.

2.5.7 Computer modeling

VMD software (98) was used to graphically represent hGH free in solution [Chantalat, 1995 #22], the hGH:hGH receptor complex (63), the hGH:hGHR:hGHR
complex [de Vos, 1992 #24] and the hGH:1hPRL receptor complex [Somers, 1994 #25] crystal structures. These representations were then used to measure distances between residues.

2.6 Electroparamagnetic resonance spectroscopy (EPR)

I have used EPR as a tool to monitor conformational changes in hGH. Briefly, EPR detects the energy absorbed in the process of reversing the spin of an unpaired electron by the application of an oscillating magnet. The length of time it takes for the unpaired electron to relax, directly relates to the range of motion that it has on a microenvironment scale. Since proteins do not have any native unpaired electrons, they are undetectable by the EPR instrumentation. I have used this to my advantage by attaching spin labels specifically to the free thiol group of cyteine residues that I have engineered into the protein at various locations.

2.6.1 Site-directed spin labeling

MTSL ((1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate) (Reanal, Hungary, Budapest) can be covalently attached to a protein via reduced thiols. One advantage of MTSL over other thiol spin labels is that it is does not participate in side reactions with other functional groups on the protein. The four endogenous cysteines in hGH are all paired in disulfide bonds, leaving no reactive thiols available for labeling. I prepared hGHs with one cysteine mutation engineered in as a site for spin labeling. However, the small disulfide loop connecting C182 to C189 is fairly labile, and reduces under very mild conditions causing specificity problems during the labeling procedure, and so C182 and C189 were mutated to serine to eliminate the problem.
Protein was evaluated by BCA for protein concentration, and by the Ellman's reaction to measure the free thiol content. The two measurements were then combined to determine the free thiol to total protein ratio. Samples with thiol/protein ratios between 0.5 and 1.0 were then incubated with 25 $\mu$l of an 8 mg/ml MTSL solution for a minimum of four hours, with less accessible residues requiring overnight incubation. To remove excess label, a size exclusion (Sephadex G50) column was run, which removed excess label and dimeric species, and exchanged the buffer to ammonium bicarbonate, allowing the protein to be freeze-dried.

2.6.2 Mass Spectroscopy

After labeling, proteins were resuspended in 10 mM ammonium bicarbonate and concentrations determined by BCA assay. 100 $\mu$g of each protein were dried in a speed vac and then resuspended in 500 $\mu$l deionized water to remove volatile salts. This wash step was repeated three times. Samples were sent to the Ohio State University CCIC mass spectroscopy unit and analyzed by electrospray TOF (Time of Flight) mass spectroscopy. Results yielded information about the percent protein labeled, and detected the presence of dimers not removed in the purification step.

2.6.3 Spectra collection

Labeled proteins were resuspended in 10 mM Tris pH 7.5, 150 mM NaCl. Protein concentrations ranged from 20 $\mu$M to 80 $\mu$M, depending on the percentage of protein labeled and the experiment being performed. Spectra were recorded on a Bruker instrument. Integrations and other data manipulations were performed using EPR Ware (99).
2.7 Fluorescence Resonance energy transfer (FRET)

FRET experiments are used to monitor the distance between two chromophores located in the same sample by quantifying the amount of energy transferred from a donor chromophore to an acceptor chromophore. The donor chromophore is excited at one wavelength, and then the emission spectra is monitored over a range covering the emission wavelengths of both the donor and acceptor chromophores. The closer the two chromophores are, the more efficiently energy can be transferred between them. The method is most useful in determining distances between 5 and 100 angstroms.

Proteins were labeled with CPM (7-diethylamino-3-(4’maleimidylphenyl)-4-methylcoumarin) using the same procedure as for MTSL (see site-directed spin labeling, section 2.6.1). Labeled proteins were resuspended in 10 mM Tris pH 7.5, 150 mM NaCl. Proteins were diluted to 500 nM. Experiments were conducted using tryptophan as the donor chromophore and CPM as the acceptor chromophore. Samples were excited at 285 nm, and the emission spectra monitored from 300-500 nm, with the expected tryptophan emission at 337 nm and CPM at 468 nm. Spectra were obtained from the protein in the labeled and unlabeled form in the presence of equimolar amounts of hPRLbp to compare relative levels of tryptophan emission.

2.8 FDC-P1 lactogenic and somatotrophic proliferation assays

FDC-P1 cells containing either the human prolactin receptor or the human somatotrophic receptor were gifts from Genentech Incorporated (South San Francisco, CA, USA). Cells were maintained in RPMI-1640 containing 10% FBS, 440 µg/ml G418 or 5 µg/ml gentamycin sulfate, and 1 nM wild type hGH (100). Log phase cells were collected and washed with non-supplemented RPMI 1640. 24 hours prior to the assay
cells were washed and suspended in media devoid of hGH and phenol red, but supplemented with 10% gelding horse serum. Hormone stocks were diluted with phenol red-free media to the desired concentrations and added to 96 well plates in triplicate sets. Each well contained 15,000 FDC-P1 cells in a total volume of 100 μl. Plates were gently agitated and then incubated at 37ºC in a 5% CO₂/95% air atmosphere for 48 hours. Hormone induced proliferation of the cells was assessed by a vital dye method upon addition of 10 μl of Alamar Blue (Accumed International, West Lake, OH, USA) per well, followed by a 4 hour incubation. The oxidation-reduction of Alamar Blue was evaluated at 570 and 600nm using a UVmax plate reader (Molecular Dynamics, CA). These values were used to calculate the percent reduction of the dye, which is highly correlated with the number of cells (r²>0.99). The values obtained from dose-response studies were used to calculate ED 50s for the agonist phases by a four parameter fit method (92).

2.9 Binding assays

2.9.1 \( ^{125}\text{I} \)hGH receptor binding assays

Dr. Brooks generously iodinated hGH using Iodogen and carrier free \( ^{125}\text{I} \) iodine. Reaction buffer contained Fisher’s media supplemented with 0.5% bovine albumin, 25 mM HEPES, 5 mM MgCl₂, 1 mM ZnSO₄, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin at pH 7.4. Binding reactions contained the membranes of 2 x 10⁶ FDC-P1 hPRLr cells, 1.0-2.0 ng of \( ^{125}\text{I} \)hGH, and recombinant hormone doses ranging from 0-10 μg/ml in a total of 500 μl of reaction buffer. Reactions were incubated for approximately 20 hours at room temperature. Membranes were collected by
centrifugation, washed with 25 mM Tris, 10 mM MgCl₂ pH 7.5, and receptor associated [¹²⁵I] hGH counted. Non-specific binding was determined by the addition of 10 μg of hGH. Relative affinities were calculated by the Scatchard method.
CHAPTER 3
IDENTIFICATION OF A MOTIF REQUIRED FOR LACTOGENIC ACTIVITY

3.1 Introduction

Members of the hematopoietic cytokine family initiate signaling via homo- or heterodimerization of their receptors (48). Many of the members achieve this dimerization in a sequential fashion. GH is an excellent model to study sequential binding as there is extensive structural and functional data available. Information on the detailed molecular mechanism of the sequential interaction of hGH with its receptors will aid in the understanding of the entire family. Additionally, there is a need for hGH antagonists and super-agonists for use in the treatment of several disease states such as dwarfism and acromegaly. As hGH is able to signal through both the hGH receptor and the hPRL receptor, it is also necessary to obtain agonists and antagonists that are specific for only one receptor type to prevent unwanted side effects.

Receptor dimerization has been shown to occur in an ordered, sequential fashion (29) (101). X-ray crystallography structures are available for unbound hGH (Protein Data Base number 1HGU), hGH bound to one extracellular domain of the human lactogenic receptor (22), and hGH bound to either one (PDB number 1A22 and 1HWH) (63) (61), or two (PDB number 3HHR and 1HWG) (63) (21) extracellular domains of the somatotrophic receptor. The receptor binding sites are structurally
distinct with the first receptor molecule binding at site 1, and the second receptor molecule binding at site 2. The structural and functional binding epitopes of the hGH:hGHr complex at site 1 (67), and site 2 (29) have been identified. Competitive binding experiments with hGH and either the lactogenic or somatotrophic receptor describe extensively overlapping, but non-identical sites on the surface of hGH (72). The current model for the mechanism of this sequential interaction is based on two independent binding sites, distinguished by their difference in binding affinities (71) (102).

Comparison of the multiple crystal structures of hGH reveals that hGH undergoes structural changes associated with receptor binding that are unique to binding either the lactogenic or somatotrophic receptors (Fig 1.7) (103). These changes induce helical structuring in residues 38-49 and 90-99 (mini-helix 1 and the 2-3 loop, respectively) resulting in formation of mini-helix 1 and a lengthening of helix 2, respectively. These large structural changes are associated with a 15° movement of helix 3 relative to the helical bundle. The articulation of helices 1 and 3 provide an ordered groove that constitutes site 2 for either the lactogenic or somatotrophic receptor. Specific comparison of the structures of hGH bound to one extracellular domain of the somatotrophic receptor and hGH bound to two extracellular domains of the somatotrophic receptor suggest that these changes occur upon binding to the first receptor molecule. Previous mutational analysis has shown that Phe44, located in the mini-helix region, is required for lactogenic activity, but not for somatotrophic activity (76). While affecting biological activity, Phe 44 is not located in the functional epitope for either site 1 or site 2.
Based on the crystallographic evidence, as well as the mutational data provided, I hypothesized that only the first receptor binding site is present on hGH as it is found unbound in solution. Further, a conformational change induced by receptor binding at site 1, transduces across the hGH molecule through a series of hydrophobic interactions, resulting in the structuring of a functional site 2. To locate the hydrophobic residues that articulate the conformation change, I began by alanine-scanning the N-terminus of helix 4, as it is proximal to mini-helix 1 and Phe \(^{44}\). Residues showing significant loss of lactogenic receptor-induced cell proliferation were further mutated to glutamic acid. Since the proposed theory is based on hydrophobic interactions, the negatively charged glutamic acid should result in a larger loss of activity. In addition to scanning helix 4, hydrophobic residues in the 2-3 loop that are close enough to interact with the side of helix 4 opposite from mini-helix 1 were also mutated to glutamic acid.

3.2 Results

3.2.1 Alanine-scanning of Helix 4

hGH residues 157 through 164 were mutated to alanine. Purified proteins were characterized by SDS-gel electrophoresis, fluorescence, UV/VIS, and circular dichroism spectroscopy (Dr. Francis Peterson, unpublished results). Bioassays were performed at concentrations extending to 1000 nM in both the FDC-P1 hPRL receptor and FDC-P1 hGH receptor cell lines. Anything less than a 2-fold increase in the ED 50 was due to biological variation and considered insignificant. Four mutants, L157A, Y160A, L163A, and Y164A, had small but significant loss of activity in the hPRL
Figure 3.1: FDC-P1 hPRL receptor Bioassay. Hormone doses were prepared and administered to FDC-P1 cells transfected with the hPRL receptor as described in materials and methods. Data are representative of at least two separate experiments, with data for each experiment collected in triplicate for each dose. The maximum responses were normalized to the wild-type hGH maximal response.
Figure 3.2: FDC-P1 hGH receptor Bioassay. Hormone doses were prepared and administered to FDC-P1 cells transfected with the hGH receptor as described in materials and methods. Data are representative of at least two separate experiments, with data for each experiment collected in triplicate for each dose. The maximum responses were normalized to the wild-type hGH maximal response.
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<th><strong>FDC-P1 hPR L receptor</strong></th>
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<td><strong>ED 50</strong></td>
<td><strong>Relative loss of activity</strong></td>
<td><strong>ED 50</strong></td>
</tr>
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Table 3.1: ED 50 values for alanine scanning of helix 4
* Data not determined

receptor assay (Figure 3.1, Table 3.1). All four mutants retained full biological activity in the hGH receptor assays (Figure 3.2, Table 3.1).

3.2.2 Glutamic acid scanning of helix 4 and 2-3 loop residues

Residues identified by alanine scanning to be involved in lactogenic activity, including Phe\textsuperscript{44}, were mutated to glutamic acid. Additionally, Val\textsuperscript{90}, Leu\textsuperscript{93}, Val\textsuperscript{96}, Phe\textsuperscript{97}, and Tyr\textsuperscript{103}, all residues in the 2-3 loop, were mutated to glutamic acid. Wild type and mutant proteins were expressed and purified, yielding an average of 40
mg/liter of fermentation. All mutants, with the exception of L163E, co-migrated with wild-type protein on SDS polyacrylamide gel electrophoresis under both reducing and non-reducing conditions. Reducing conditions showed the proteins to be greater than 95% pure, while the non-reducing conditions suggested that all proteins were folded correctly with only small traces of dimerized protein. L163E did not elute from the anion exchange column in one sharp peak, but instead eluted very broadly. Additionally, the SDS polyacrylamide non-reducing gel of L163E contained many bands, corresponding to variously folded and dimeric proteins.

UV absorbance spectra were collected, normalized to 277 nm, and compared to wild-type spectra (Fig. 3.3). All mutants had similar curves, although five proteins varied significantly in their 250nm/280nm ratio. These five proteins, L93E, R94F, L157E, L163E, and Y164E showed increased 250/280 ratios of 42, 36,36,34,and 35%, respectively. With the exception of L163E, all fluorescent spectra had similar emission spectra when normalized at 338nm (Fig. 3.4). L163E had a reduction in quantum yield of 29%, as well as a side peak near 318nm when compared to wild-type hGH. Only two other proteins had any significant variation in the spectra. V90E was red shifted 4nm, and R94F had an increased quantum yield of 18%. The circular dichroism spectra (Fig. 3.5) all described a typical helical protein shape. Mutant proteins had molar ellipticity values at 222 nm within 10% of wild-type values except for V90E and L163E, which had 117.7% and 74.7% of wild-type values respectively.

Bioassays were performed at concentrations extending to 10,000 nM, a concentration at which the antagonistic portion of the curve could be seen. Protein aggregation at these concentrations was not observed based on light scattering data at
350 nm, which was performed at twice the highest concentration used for the bioassays. Only F44E, L93E, R94F, and L163F showed modest decreases in hGH receptor activity (Fig 3.6, Table 3.2). Anything less than a 2-fold loss was due to biological variation and considered insignificant. In the FDC-P1 hPRL receptor assay (Fig. 3.7, Table 3.2), F44E and Y164E had the greatest loss of activity relative to wild type at 83.8 and 86.5 fold, respectively. Other proteins with significant loss in activity included L93E and Y160E at 4.7 and 9.0 fold, respectively. In both proliferation assays L163E, while promoting some proliferation, did not yield curves that could be analyzed for ED 50 values. Since Tyr	extsuperscript{164} is part of the structural binding epitope, binding studies were performed to ensure that loss of biological activity wasn’t a result of a loss of binding at site 1. Wild-type and Tyr	extsuperscript{164} hGH’s were placed in competitive I	extsuperscript{125} binding assay and the relative K	extsubscript{d} values were determined to be 0.047 and 0.070 nM, respectively.

3.2.3 Leu	extsuperscript{163} mutants

Since Leu	extsuperscript{163} hGH shows a highly altered structure by spectroscopic studies and was inactive in the FDC-P1 hGH receptor bioassay as well as the FDC-P1 hPRL receptor bioassay, it was determined that the residue was critical to the global structure of the protein. To resolve whether or not Leu	extsuperscript{163} is a member of my proposed motif, more conservative amino acid substitutions that varied both the size and hydrophobicity were prepared. Specifically, the residue was further mutated to alanine and phenylalanine. Both of these mutant proteins had full activity in the hGH receptor assay. In the hPRLr assay they showed a 3.5 and a 9.0 fold loss with alanine or phenylalanine substitution respectively (Table 3.3).
<table>
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<td>ED 50</td>
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</tr>
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<td>F44E</td>
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Table 3.2: ED 50 values of glutamic acid scanning

*** Values could not be calculated
Figure 3.3: UV absorbance spectra of hGHs. Protein concentrations were 20 μM in 10 mM Tris, pH 8.2, containing 150 mM NaCl as determined by BCA assay. Spectra were collected at room temperature on a Uvicon model 930 spectrophotometer. The large panel contains the unmodified spectra of proteins that had reduced lactogenic ED 50 values. The inset contains spectra for all proteins normalized to the wild-type hGH 277 nm value. Inset scaling is identical to the large panel.
Figure 3.4: Fluorescence spectra of hGHs. Protein concentrations were 1 μM in 10 mM Tris, pH 8.2, containing 150 mM NaCl as determined by BCA assay. Emission spectra were collected at 25° C on a Perkin-Elmer model LS-50B with a 290 nm excitation. The large panel contains the unmodified spectra of proteins that had reduced lactogenic ED 50 values. The inset contains spectra for all proteins normalized to the wild-type hGH value at 338nm (peak maximum). Inset scaling is identical to the large panel.
Figure 3.5: Circular dichroism spectra of hGHs. Protein concentrations were 25 μM in 10 mM Tris, pH 8.2, containing 150 mM NaCl as determined by BCA assay. Spectra were collected at 25°C on an Aviv model 202.
Figure 3.6 FDC-P1 hGH receptor bioassay. Hormone doses were prepared and administered as described in the bioassay section of chapter 2. Data are representative of at least two separate experiments, with data for each experiment collected in triplicate at each dose. The maximum responses were normalized to the wild-type hGH maximal response.
Figure 3.7 FDC-P1 hPRL receptor bioassay: Hormone doses were prepared and administered as described in the bioassay section of chapter 2. Data are representative of at least two separate experiments, with data for each experiment collected in triplicate at each dose. The maximum responses were normalized to the wild-type hGH maximal response. The large panel contains the dose response curves for mutants that had reduced lactogenic ED 50 values. The inset contains dose response curves for all proteins tested.
<table>
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<th>FDC-P1 hPRL receptor</th>
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Table 3.3: ED 50 values for the Leucine 163 mutant hGH’s

*** Data could not be calculated

3.3 Discussion

The residues identified to be biologically relevant by mutagenesis experiments are rearranged to form a more tightly packed structure upon binding to the lactogenic receptor (Figure 3.8). Specific changes include a structuring of mini-helix 1, a structuring of the 2-3 loop resulting in the lengthening of helix 2, and a rotation of helix 3 with respect to helix 1. The mechanism of the proposed conformational change begins with Phe$^{44}$. The lactogenic receptor binding at site 1 constrains Phe$^{44}$ and induces its interaction with Tyr$^{160}$ and Tyr$^{164}$. Leu$^{163}$ shares helix 4 as a backbone with Tyr$^{160}$ and Tyr$^{164}$, but extends approximately 180° away from this hydrophobic cluster into the interior of the protein. Lactogenic receptor binding promotes interaction of Leu$^{163}$ with the hydrophobic residue Leu$^{93}$ located in the 2-3 loop. This association drives the restructuring of the 2-3 loop by extension of helix 2. This structuring also causes Tyr$^{103}$, a site 2 determinant, to rotate towards site 2 creating a
functional site 2. Disruption of this mechanism is much more efficient closer to site 1, rather than site 2, as is seen in the ED 50 values. F44E and Y164E, required early in the structuring, have relative losses of activity of 83.8 and 86.5 respectively. L93E at the end of the mechanism only has a relative loss in activity of 4.7 fold.

The molecular graphics package VMD (98) was used to view the arrangement of residues in these regions in the lactogenic receptor bound and free form of hGH (Figure 3.8). All of the residues are found in a contiguous hydrophobic array lying between the two receptor binding sites. The residues form two hydrophobic clusters that are on either side of helix 4. The two clusters may interact, as each cluster contains residues on helix 4. Phe$^{44}$, Leu$^{157}$, Tyr$^{160}$, and Tyr$^{164}$ are located in hydrophobic cluster one, although Leu$^{157}$ is not a member of the functional lactogenic motif.

Hydrophobic cluster two contains Leu$^{163}$, Val$^{90}$, Leu$^{93}$, Val$^{96}$, and Phe$^{97}$. Leu$^{163}$ and Leu$^{93}$ were the only two residues in this cluster whose mutation reduced lactogenic activity. Using the crystal structure of hGH bound to one molecule of the lactogenic receptor, distances between residues were measured, and a correlation between distance and loss of lactogenic activity was discovered. Phe$^{44}$ to Tyr$^{164}$ and Phe$^{44}$ to Tyr$^{160}$ were the closest at 3.287 and 3.282 angstroms respectively. Leu$^{163}$ to Leu$^{93}$ was next closest at 4.285 angstroms, while other residues in the clusters had distances greater than 7 angstroms. Distances were measured from the two closest atoms in each residue pair using the Rasmole software. Distances above 7 angstroms may be too great for single glutamic acid residues to disrupt hydrophobic packing. L163E was the only mutant whose properties varied significantly in all three types of
Figure 3.8: Hydrophobic packing of hGH in the unbound form (left) (62) and the 1:1 hGH/hPRL receptor complex (right) (22). hGH is pictured in both panels as a ribbon structure in dark blue to provide a point of reference. The light blue represents the Van der Waals space occupied by the entire molecule, to indicate areas of high and low atom densities. The motif residues are represented as Van der Waals space fill in solid red to highlight the alteration of packing in the interior of hGH due to lactogenic receptor binding at site 1. Distance measurements between residues in the motif decrease by 5-11 angstroms when comparing hGH free and hGH bound to the lactogenic receptor.
spectroscopy, electrophoresis, and biological activity indicating that the mutation had altered the global folding of the protein. Accordingly, the somatotrophic assay also revealed a severe decrease in activity. The ED 50 values could not be calculated for L163E for either the lactogenic or somatotrophic assays. Interestingly, the somatotrophic assay curve showed several peaks and did manage to achieve 66% of the maximum response. The corruption of hydrophobic packing by replacing Leu^{163} with glutamic acid demonstrates the importance of packing in the interior of the protein. Loss of packing by hydrophilic residues results in a protein that may assume multiple conformations, resulting in a protein solution that contains multiple transient folding states.

The data for Leu^{163} shows that mutation to a glutamic acid created large disturbances in the structure of hGH and emphasizes the centrality of this hydrophobic residue for protein stability. Unfortunately, due to the global consequences of this mutation, its membership in the hydrophobic motif that propagates a conformation change could not be either supported or dismissed based on this mutation alone. To further investigate this critical residue, the additional mutations L163A and L163F were prepared. Both of these mutations alter the packing based on steric properties of each residue, without removing the hydrophobicity. L163A had a modest 3.5 fold loss in lactogenic activity. This may be the result of a shorter side chain that offers less surface for hydrophobic packing and may no longer be close enough to Leu^{93} to exert as strong of an effect. L163F had a lactogenic loss of activity of 9.0 fold and a somatotrophic loss of activity of 3.3 fold. The increased bulk in going from a leucine to a phenylalanine most likely prevents tight packing in the second hydrophobic
cluster, which appears to be more important in the lactogenic receptor interaction than in the somatotrophic receptor interaction. These two mutations provide evidence for including Leu\textsuperscript{163} in the lactogenic motif, as well as to support the argument for its centrality in maintaining the overall structure of hGH by hydrophobic packing.

Many of the mutants studied in this paper are located in the interior of the hGH protein, making it important to establish that the mutants were folded correctly. This was verified in several ways. Full biological activity in the somatotrophic assay indicated that the structure had not been globally changed, and that any local change only effected lactogenic activity. The various spectroscopic methods yielded more detailed information on the fold of the mutants. Circular dichroism revealed that the secondary structure was intact. The fluorescent spectra monitors the environment of the single tryptophan located in the hydrophobic core of the protein. The data indicated that the hydrophobic interior of the protein had not been disturbed. The 250/280 ratios in the UV spectra are important for evaluation of disulfide bond formation, as disulfide bonds absorb at 250, while free cysteines do not. The amount of absorption of a disulfide bond at 250 is dependent on bond angle and tension. The 250/280 ratio is thus a measure of disulfide bond characteristics normalized to total protein concentration (91). The spectra indicate that although several of the mutants have disulfide bonds that are strained, all of the bonds are intact. Combined, this data indicates that loss in lactogenic activity is not due to a gross disturbance in the structure, rather a disruption in the mechanism by which hGH signals through the lactogenic receptor.
The hPRL receptor bioassay dose response curves seen for these mutations are unlike typical bell-shaped dose-response curves. Several other papers have described the effects on biological and binding assays for hGH mutated within site 1 or site 2 (102) (104). Hormones that activate via receptor dimerization are known to have bell-shaped dose-response curves. Mutations within site 1 that alter binding affinities either right shift or left shift both the agonist and the antagonist phases of the curve. Mutations in site 2 are indicated by modulations in the maximal biological response.

The curves seen here for the exosite mutations show a shift in the agonist phase without a concurrent and proportional shift in the antagonist phase. The maximal biological activity is hard to discern in some cases, as the antagonist phase is reached before the agonist phase is finished. Since the antagonist phase represents a population of receptors that are predominantly in a 1:1 complex with hGH due to saturation, the ID 50 is representative of site 1 binding affinity. Therefore a shift in the ED50 value without a shift in the ID50 indicates that loss of activity is not due to binding at site 1, which is in agreement with the binding data generated. This interpretation of the bioassay data has been previously published by Duda and Brooks (105). These curve shapes also support the notion of an induced-fit model, where site 1 is always available for binding, but where site 2 requires binding at site 1 to induce the conformation change required to create site 2.

The proposed model of a contiguous hydrophobic motif that propagates a conformational change that activates site 2 has not previously been considered. To conclude, we have found a unique motif that is required for the lactogenic activity of
hGH. Additionally, the concept of this type of motif may also be relevant in other ligand-receptor interactions among the proteins in the hGH superfamily.
CHAPTER 4
THE ROLE OF ZINC IN hGH LACTOGENIC ACTIVITY

4.1 Introduction

Human growth hormone (hGH) transmits its actions through either the somatotrophic or lactogenic receptors. Dimerization of the receptors is required for activation (29). The dimerization occurs in a sequential fashion, with hGH binding to the first receptor molecule at site 1, and then binding a second receptor molecule at site 2 (60). The hGH binding sites for the hGH and hPRL receptors overlap, but are not identical (72). One difference in the hGH-hPRL receptor interaction from that of the hGH-hGH receptor is that the lactogenic interaction requires one zinc molecule per complex for tight binding at site 1 (22) (73).

Zinc deficiency has been observed to play an important role in many endocrine functions, as it can affect various aspects of activity ranging from secretion, excretion, transport, and tissue binding (106). Binding studies of hGH with the hPRL receptor report a 8000-fold increase in site 1 binding in the presence of zinc as compared to binding in the absence of zinc (73). However, these studies may be exaggerated, as the affinities determined in the absence of zinc were also devoid of other divalent ions as well. Crystallographic data has identified His\textsuperscript{18} and Glu\textsuperscript{174} of hGH, and Asp\textsuperscript{217} and
His$^{218}$ of the hPRL receptor as the residues that chelate zinc (22). Additionally, hGH His$^{21}$ may play a role in the interaction by pre-orienting Glu$^{174}$ to enhance chelation through a hydrogen bond.

Comparison of the multiple crystal structures of hGH reveals that hGH undergoes structural changes associated with receptor binding that are unique to binding either the lactogenic or somatotrophic receptors (Fig 1.6) (103). These changes structure residues 38-49 and 90-99 into ordered helical structures (mini-helix 1 and the 2-3 loop, respectively) resulting in a lengthening of helix 2. These large structural changes are associated with a 15° movement of helix 3 relative to the helical bundle. The articulation of helices 1 and 3 provide an ordered groove that constitutes site 2 for either the lactogenic or somatotrophic receptor. Specific comparison of the structures of hGH bound to one extracellular domain of the somatotrophic receptor and hGH bound to two extracellular domains of the somatotrophic receptor suggest that these changes occur upon binding to the first receptor molecule.

The effect of zinc on the biological activity of hGH mutants has been variable (75), and therefore unable to provide a clear mechanism by which zinc influences lactogenic activity. From the crystallographic evidence, it is not clear if zinc plays a role in the conformational changes described. Circular dichroism (CD) can be used to monitor the structuring of helices. Since one of the major structural changes in hGH involves additional helical content due to the lengthening of helix 2, CD measurements can be used to detect helical content changes in hGH as a result of the addition of zinc.
I have previously identified five residues, Phe^{44}, Leu^{93}, Tyr^{160}, Leu^{163}, and Tyr^{164}, that form an articulating motif critical to lactogenic activity, but that are not required for somatotrophic activity (chapter 3). The current chapter probes the effect of zinc on the effect of this motif, and the overall role of zinc in lactogenic activity. It was my theory that zinc played a role in the structuring of the hGH molecule, and that zinc could prevent the loss of biological activity due to these mutations.

4.2 Results

4.2.1 Protein Characterization

Wild-type, F44E, L93E, Y160E, L163F, and Y164E hGH proteins were characterized in chapter 3. The site 2 mutant G120R was characterized by fluorescence and absorbance spectroscopy (Figures 4.1 and 4.2 respectively). The fluorescence spectrum shape is identical to that of the wild-type hGH. The absorbance spectrum shows a decrease in the 250/280 ratio. SDS-PAGE analysis in the presence of 1% 2-mercaptoethanol indicated that the protein was greater than 95% pure, while samples without 2-mercaptoethanol indicated that the disulfide bonds were correctly formed (Figure 4.3).

4.2.2 FDC-P1 lactogenic proliferation assays

Wild-type, F44E, L93E, Y160E, L163F, and Y164E hGH’s were used to stimulate the growth of FDC-P1 cells stably transfected with the hPRL receptor. Extended dose-response curves were performed to obtain both the agonist and antagonist phases. Assays were conducted using either assay medium with endogenous zinc (estimated to be approximately 3 μM), or with medium supplemented with 25 μM zinc (Figure 4.4). Three of the mutants, L93E, Y160E, and
Figure 4.1 Fluorescence spectra of G120R. Protein concentrations were 1 μM in 10 mM Tris, pH 8.2, containing 150 mM NaCl as determined by BCA assay. Emission spectra were collected at 25° C on a Perkin-Elmer model LS-50B with a 290 nm excitation.
Figure 4.2 Absorbance spectra of G120R. Protein concentrations were 20 μM in 10 mM Tris, pH 8.2, containing 150 mM NaCl as determined by BCA assay. Spectra were collected at room temperature on an Uvicon model 930 spectrophotometer.
Figure 4.3 SDS-PAGE 15% SDS-PAGE of wt hGH (lane 1 and 4) and G120R (lanes 2 and 5). Samples were prepared in the presence (lanes 1 and 2) or absence (lanes 4 and 5) of 1% 2-mercaptoethanol. Lane 3 contains reduced molecular weight markers (size in kDa recorded on the right margin).
<table>
<thead>
<tr>
<th></th>
<th>Endogenous Zinc</th>
<th>25 μM Zinc supplemented</th>
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</thead>
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<tr>
<td></td>
<td>ED 50 (nM)</td>
<td>Relative loss of activity</td>
</tr>
<tr>
<td>wt hGH</td>
<td>3.25 ± 0.30</td>
<td>1.0</td>
</tr>
<tr>
<td>F44E</td>
<td>907 ± 110</td>
<td>2.79</td>
</tr>
<tr>
<td>L93E</td>
<td>13.7 ± 1.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Y160E</td>
<td>20.4 ± 1.8</td>
<td>6.3</td>
</tr>
<tr>
<td>L163F</td>
<td>9.60 ± 0.73</td>
<td>3.0</td>
</tr>
<tr>
<td>Y164E</td>
<td>300 ± 33</td>
<td>92.2</td>
</tr>
</tbody>
</table>

Table 4.1: Lactogenic ED 50 values for the GH's containing mutations included in the motif, in the presence and absence of supplemental zinc

*Percent activity recovered was determined by comparing the relative loss of activity with or without supplemental zinc. In the cases where relative loss of activity with supplemental zinc was within the 2-3 fold range, percent activity recovered was 100%.

L163F, were restored to full lactogenic activity by the addition of zinc. F44E and Y164E recovered 88.2 and 87.3% activity respectively (Table 4.1). The ID 50 of each protein shifts approximately 10-fold with the exception of F44E and Y164E, which do not show a shift at all. The maximum response for each protein was unaffected, although the zero-dose response was increased 10-15% by the addition of zinc regardless of the mutant. The site 2 mutant G120R was also used to stimulate cell
Figure 4.4: FDC-P1 lactogenic bioassay: FDC-P1 cells transfected with the hPRL receptor stimulated with hGH’s supplemented with 25 μM zinc (bottom panel) or with endogenous zinc levels (upper panel).
Figure 4.5 FDC-P1 lactogenic Bioassay: Effect of zinc on the lactogenic activity of the site 2 mutant G120R. FDC-P1 cells transfected with the hPRL receptor stimulated with hGH's supplemented with 25 μM zinc or with endogenous zinc levels.
growth with or without supplemental zinc (Figure 4.5). The addition of 25 μM zinc failed to produce a left-shift in the ED50 or the ID50, but did decrease the maximum response from 22% of the wild-type response to 9% of the wild-type response.

4.2.3 Spectroscopy

Circular dichroism spectra of wild-type hGH did not change upon addition of 25 μM zinc (Figure 4.6). Similarly, the fluorescence spectrum was also unchanged by the addition of zinc (Figures 4.7).

4.3 Discussion

G120R is a hGH mutant that has been traditionally used to study the hGH system with a highly diminished site 2 binding. Fuh et al (100) previously used the FDC-P1 hPRL receptor assay to describe the effects of G120R. Their work, which used 10 μM zinc, indicated that G120R had less than 5% of wt hGH activity. Dattani et al (75) used an alternative cell line, Nb2 cells, to perform the same studies. The Nb2 cells express the rat PRL receptor at a higher number of receptors per cell than the FDC-P1 hPRL receptor cell line. Dattani and colleagues performed their experiments both without additional zinc and in the presence of 25 μM zinc. They observed maximal stimulation with a narrower bell-shaped dose-response curve in response to G120R without zinc. They also showed that the agonist activity of G120R could be titrated out with increasing zinc concentrations. I have shown in the FDC-P1 hPRL receptor system that G120R does have agonist activity, and that this agonist activity is reduced in the presence of zinc.

Interestingly, addition of zinc to the G120R mutant does not left shift the dose-response curve, instead it decreases the maximal stimulation. If zinc is only required
Figure 4.6: Effect of zinc on circular dichroism. Protein concentrations were 25 μM in 10 mM Tris, pH 8.2, containing 150 mM NaCl as determined by BCA assay. Spectra were collected at 25 degrees on a 201 Aviv CD instrument. Spectra were measured either in the presence of 1mM EDTA or 25 μM Zn.
Figure 4.7: Effect of zinc on Fluorescence. Wt hGH concentration was 1 μM in 10 mM Tris, pH 8.2, containing 150 mM NaCl as determined by BCA assay. Emission spectra were collected at 20° C on a Perkin-Elmer model LS-50B with a 290 nm excitation. Fluorescence spectra was measured either in the presence of 40 μM EDTA or 25 μM zinc.
for increased affinity of hGH for the hPRL receptor, than this observation is in direct
opposition with the standard dose-response curve interpretation that a site 2 mutation
would only affect the maximal stimulation (102). This indicates that either G120R
effects more than just binding at site 2, or that zinc effects more than just the affinity
at site 1.

The motif mutants studied here are all located distal to both receptor binding
sites. Since site 1 binding is not altered by these mutations, addition of zinc should
shift the agonist and antagonist phases of the dose-response curves equally. That is
not what is observed. Three of the mutants, L93E, Y160E, and L163F, all undergo the
expected 10-fold left shift in the antagonist phase predicted by an increase in site 1
affinity. The remaining two mutations, F44E and Y164E, do not undergo this shift in
the antagonist phase indicating that site 1 affinity is not increasing due to increased
zinc concentrations. However, F44E and Y164E do regain 87-88% of their lactogenic
activity relative to wild-type hGH even without the concurrent increase in site 1
affinity.

The recovery of lactogenic activity in the motif mutations in response to
additional zinc could be the result of several factors. First, the increase in binding
affinity at site 1 increases the proportion of 1:1 complexes available to bind a second
receptor molecule. Second, the zinc may induce the conformation changes observed
in the crystal structures (22) (62). The spectroscopy studies performed in this paper do
not agree with the second option. CD and fluorescence spectra of wt hGH are not
altered by the addition of zinc, indicating that neither the interior packing nor the
helical content of the hGH is changing. While the increase in affinity at site 1 may
play a role in overcoming the effect of the motif mutations, it is not the only factor as indicated by F44E and Y164E, which recovered a majority of the loss of activity without an increase in site 1 affinity.

A third explanation for the role of zinc in lactogenic activity is that it decreases the number of conformations that the molecule can populate by linking residues 18 and 174, thus increasing the proportion of molecules that are in conformations available for binding at site 2. This theory incorporates the data for G120R as well as for the motif mutants. When the relative positioning of G120R is compared in the hGH unbound form to that of the hGH: hPRL receptor complex (figure 4.8), it can be seen that due to the twisting of helix 3 in the hGH: hPRL receptor complex the side chain of residue 120 faces into the groove formed between helices 1 and 3. In the hGH unbound structure the side chain of residue 120 does not point directly into the groove. If additional zinc leads to a population of hGH molecules with a bias towards a functional site 2, then the G120R mutation is in the correct position to sterically block binding at site 2. In the absence of excess zinc, there are more opportunities for a hGH molecule to bind a second receptor before the G120R side chain is positioned to sterically block binding. In the case of the motif mutations, the addition of zinc and the subsequent conformational limitations, allows the hGH molecule to overcome the packing effects produced by mutations within the motif.

In summary, the mutants described here are all zinc sensitive. Using observations from the available crystal structures, as well as detailed analysis of the dose-response curves, a plausible theory for the mechanism of action of zinc in the lactogenic activity of hGH has been proposed.
Figure 4.8: Gly^{120} in hGH free and hGH:hPRL receptor complex structures In the unbound form (right) hGH is more loosely packed, allowing residue 120 to extend away from the groove formed by helices 1 and 3. In contrast, when bound to the hPRL receptor (left) (22) hGH is more tightly packed, pulling residue 120 into the groove.
CHAPTER 5

SALT BRIDGE SUBSTITUTION OF HYDROPHOBIC CLUSTERS

5.1 Introduction

Salt bridge interactions have been studied by replacing the charged residues of interest with non-charged residues. Salt bridges may have more free energy to contribute to an interaction than a hydrophobic interaction, and the presence of salt bridges in proteins generally produces more stable proteins (107).

The engineering of a salt bridge must take several factors into account. First, the charged residues must come into close enough proximity to be able to interact. Two oppositely charged residues should be 3 angstroms apart to form an optimum salt bridge, but the distance can be much larger, as the electrostatic force deteriorates as 1/d (108). If the residues are too close, other factors such as steric hindrance can play a role. The second factor is the degree of freedom of rotation that the charged residue side chain has. Depending on where in the protein the charged residues are substituted too much freedom of rotation can allow extra water molecules to interact, changing the solvation of the region and thus affecting the packing of salt bridge and surrounding areas (109).
The previous chapters have dealt with a series of hGH residues which function together as a hydrophobic motif. This packing of the side chains of the residues in this motif is critical for lactogenic activity, but is not necessary for somatotrophic activity. Using the available crystal structures of hGH free in solution and hGH bound to one molecule of the hPRLbp, the distance between residues 44 and 160, and between 44 and 164 were calculated to be 3.9 and 4.2, respectively. These distances indicate that substitution of these residue pairs with oppositely charged side chains could support the formation of a salt-bridge. It was my theory that if the replacement of hydrophobic residues with glutamic acid disrupted a conformational packing required for lactogenic activity, then replacing the hydrophobic pockets with salt bridges should restore activity, and possibly even create a more potent hGH. As the most critical residues are located in the first hydrophobic cluster, the salt bridges were engineered within this cluster. Various combinations were constructed using either lysine or arginine at position 44 and glutamic acid at either position 160, 164, or both.

5.2 Results
5.2.1 Protein characterization

The proteins were analyzed by SDS-PAGE and were determined to be approximately 80% pure. Non-reducing gel analysis indicated that the disulfide bonds were properly formed. The proteins were characterized using absorbance (Fig. 5.1), fluorescence (Fig. 5.2), and circular dichroism (Fig. 5.3) spectroscopy. The absorbance and fluorescence spectra did not significantly differ from the wild-type protein. The CD spectra were identical to the wild-type hGH with the exception of minor distortions near 208nm.
Figure 5.1 Absorbance spectra: Absorbance spectra of hGH mutants with incorporated salt bridge mutations. The upper panel contains spectra for the series of mutants utilizing arginine at position 44. The lower panel contains the series with lysine at position 44. Proteins were prepared at 20 μM in 10 mM Tris, 150 mM NaCl pH 7.5. Spectra were collected at room temperature on an Uvicon spectrophotometer.
Figure 5.2 Fluorescence Spectra: Spectra of hGH mutants with incorporated salt bridge mutations. The upper panel contains spectra for the series of mutants utilizing arginine at position 44. The lower panel contains the series with lysine at position 44. All curves were normalized at 340nm. Proteins were prepared at 1 μM in 10 mM Tris, 150 mM NaCl pH 7.5. Spectra were collected at 20° C.
Figure 5.3 Circular Dichroism: Spectra of hGH mutants with incorporated salt bridge mutations. The upper panel contains spectra for the series of mutants utilizing arginine at position 44. The lower panel contains the series with lysine at position 44. All curves were normalized to the wild-type protein at 222 nm. Proteins were prepared at 25 μM in 10 mM Tris, 150 mM NaCl pH 7.5. Spectra were collected at 25°C.
5.2.2 Biological activity of the salt-bridge mutants

The single mutations of F44R, Y160E, and Y164E caused an 80, 6, and 92-fold decrease in the ED 50 values, respectively. When combined as double mutants, both F44R/Y160E and F44R/Y164E had significantly lower losses of activity, with a 50 and a 33-fold decrease in the ED 50 values, respectively. The triple mutant, F44R/Y160E/Y164E showed additive affects with a 178-fold decrease in ED 50 (Table 5.1, Figure 5.4).

The second series of salt bridge substitutions incorporated lysine at position 44 instead of arginine. The single mutation F44K had a 42-fold decrease in ED 50. The double mutants all displayed a greater-than additive effect. The ED 50 values of F44K/Y160E and F44K/Y164E were decreased by 75 and 336-fold, respectively (Table 5.1, Figure 5.5).

5.3 Discussion

The single mutations F44R, F44K, Y160E, and Y164E all disrupt lactogenic activity as was expected from previous studies (chapter 3). Double mutations incorporating salt bridge pairs restored partial activity when the substitution at position 44 was an arginine, but did not restore activity when 44 was converted to a lysine. This was not surprising, as lysine has more degrees of freedom, and thus is better suited to bind water molecules. Binding to water creates a situation in which F44K has a higher desolvation energy associated with it. This higher energy makes it more energetically disfavorable for the lysine side chain to become buried, thus making formation of a salt bridge more difficult (107). Arginine, because of its more rigid
<table>
<thead>
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<th>ED 50 (nM)</th>
<th>Standard deviation</th>
<th>Relative loss of activity</th>
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<tr>
<td>wt hGH</td>
<td>3.25</td>
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<td>1.0</td>
</tr>
<tr>
<td>F44R</td>
<td>260</td>
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<tr>
<td>F44R/Y160E</td>
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<td>50</td>
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<tr>
<td>F44R/Y164E</td>
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<td>19</td>
<td>33</td>
</tr>
<tr>
<td>F44R/Y160E/Y164E</td>
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<tr>
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<tr>
<td>F44K/Y164E</td>
<td>1092</td>
<td>310</td>
<td>336</td>
</tr>
</tbody>
</table>

Table 5.1: Lactogenic ED 50 values for GH's incorporating novel salt bridges.

Side chain, is not correctly oriented to interact with water and therefore is more common in buried salt bridges (109).

The optimal distance for a salt bridge interaction is 3-4 angstroms (110). For a salt bridge substitution to stabilize an interaction enough to produce a more active hGH, the salt bridge would need to be formed in the absence of hPRL receptor binding at site 1. Comparison of the relative positions of residues 44, 160, and 164 in the crystal structures of hGH unbound and hGH bound to the hPRLbp, reveal that without receptor binding, Phe$^{44}$, which is 11.5 and 17.0 angstroms from 164 and 160,
Figure 5.4 FDC-P1 lactogenic activity of arginine salt bridge mutants: Salt bridge mutants incorporating an arginine at position 44. The curves are representative of 2 separate experiments, each dose was performed in triplicate wells.
Figure 5.5 FDC-P1 lactogenic activity of lysine salt bridge mutants: Salt bridge mutants incorporating an lysine at position 44. The curves are representative of 2 separate experiments, each dose was performed in triplicate wells.
respectively, is not close enough to create a salt bridge. The salt bridge cannot be
formed until the receptor binding interaction induces the residue at position 44 to fold
into the protein within 4.2 and 3.9 angstroms of residues 160 and 164, respectively
(Figure 5.6). Distance were measured using the closest two atoms in each residue.

The most effective salt bridge addition is F44R/Y164E. Individually each
mutation introduces an 80 and a 92-fold decrease in the ED 50 value, respectively.
Together, the loss is decreased by 33-fold, which is lower than either mutation
individually. If there was no salt bridge formation to provide free energy when
binding the receptor then the effects should have been additive with approximately a
170 fold reduction in activity. The 33-fold reduction indicates that a salt bridge likely
forms, although the energetics are not as favorable as the original hydrophobic
packing. F44R/Y160E had a 50-fold decrease in ED 50, which while still greater than
F44R/Y164E, is also less than the sum of the individual substitutions, which would
predict a combined loss of 86-fold. The triple mutant, F44R/Y160E/Y164E had a
decrease in ED 50 of 178-fold, which is almost exactly the additive loss of 179
predicted by the individual ED 50s. This additive loss of activity indicates that the
residues are no longer interacting. This is most likely due to the proximity of the
negatively charged glutamic acid residues at 160 and 164, which would cause 160 and
164 to repulse each other, and which most likely do not allow for the insertion of the
basic arginine group between them. The increased distance between residues 44 and
160 or 164 in the unbound structure further supports the theory that the lysine
Figure 5.6: Spatial arrangement of residues 44, 160, and 164 in hGH unbound (left) (62) and in hGH;hPRLbp complex (right) (22).
substitution is interacting with water molecules. Although the lysine mutants do not form a salt bridge that restores activity, the mutations do act cooperatively as is indicated by the ED 50 values. If two mutations act independently of each other, the resulting loss of activity should be roughly the sum of the loss of activity of each mutation separately. In the case of F44K/Y164E, one would expect a decrease in the ED 50 to be equal to the sum of F44K and Y164E mutations. F44K and Y164E have 41.6 and 92.2 fold decreases in ED 50 values, while the combined mutant has a 336 fold decrease in ED 50 which is three times higher than 133.8, the summation of the two individual substitutions.

This data clearly indicates that the articulation of these residues are critical for lactogenic activity. Previous work revealed that disruption of the hydrophobic packing, leads to a loss of lactogenic activity. While the mutants incorporating novel salt bridges did not create a more active lactogenic hGH, the studies provide further proof that it is the packing of specific residues in the motif that are critical for activity. This packing can be switched from hydrophobic interactions to salt-bridge interactions with some success indicating that although hydrophobic interactions are the preferred method of packing, any interactions that allow the motif residues to pack more tightly together upon binding to the hPRLbp will produce hGH’s with lactogenic activity. An hGH with increased lactogenic activity may be more easily obtained by increasing the hydrophobicity of the residues in the motif, or possibly the residues near the motif.
6.1 Introduction

Human growth hormone assumes several conformations in various crystal structures. Although other groups have commented on these structural changes (22) (66), no one has identified or monitored these changes in solution on a microscopic level. There are several biophysical techniques available to probe conformational changes. Electron paramagnetic resonance (EPR) and fluorescence resonance energy transfer (FRET) are two of the most common.

The basis for EPR is that the resonance of unpaired electrons allows a molecule containing an unpaired electron to absorb radiation in the form of microwaves. The unpaired electron has a paramagnetic center that can align itself in one of two orientations, either parallel or antiparallel with the magnetic field being applied. The interaction of the magnetic moment of the electron with these magnetic fields causes a splitting in the spectra that is referred to as the hyperfine structure.

The hyperfine structure changes as the freedom of rotation changes. The change in rotational freedom can be directly correlated to a change in conformation,
either as an increase in rotational constraint due to additional tertiary structure formation, or a decrease in rotational constraint due to a loss of tertiary structure. The EPR spectra are recorded as the derivative of the absorbance spectra. The information from the spectra is obtained by several measurements. The first measurement is the distances between peaks. A second indication of the conformation of the protein is obtained from the ratios of the heights and widths of each peak in the spectra relative to the other peaks.

A nitroxide spin label randomly tumbling in solution will produce spectra with three sharp peaks equally spaced and equal in height and width. Lowering the temperature, increasing the viscosity, or attaching the label to a larger molecule all decrease the mobility of the label. This causes the spectra to broaden, increasing the distances between the peaks, as well as decreasing the height and increasing the width of the outermost peaks. These spectral observations can be attributed to the orientation of the free electron in solution.

Since the interaction between the free electron and the magnetic field depends on the relative orientation of the electron spin vector and the magnetic field vector, the maximum frequency occurs when the spin and field vectors are parallel. This orientation effect directly relates to the rate of tumbling of the electron, and thus information about the motion of the molecule in solution. In general, the faster an electron can tumble, the narrower the peak will be. This phenomenon is a result of the Heisenberg Uncertainty Principle. As the position of the particle becomes more certain, as is seen when the electron is tumbling slowly, the energy becomes less certain, resulting in a broader peak. Likewise, the position of the electron is less
established when the electron is tumbling rapidly, and therefore the energy is more certain, resulting in a sharper peak (for EPR references see (97) (111) (112).

The largest advantage of EPR is that most proteins do not contain an unpaired electron. This enables a protein that has been derivatized with a spin label to be monitored without any additional signals making the spectra more complicated. Spin labels are nitrooxide radicals that are relatively stable. The most common spin labels employ a reactive functional group, such as iodoacetamide or maleimide, which allows the label to be attached to specific side chain groups, in this case to thiol groups. Protein engineering has furthered the use of EPR, as cysteine residues can be mutated into or out of a protein with ease, insuring a specific and unique location of the spin label (113).

FRET is a long-range, radiative process exploring the transfer of energy between two chromophores to obtain distance measurements between two specific locations in the protein. The technique is used to measure distances between 10 and 100 angstroms. Basic fluorescence is the process where a chromophore absorbs photons at one wavelength, exciting the molecule, and then emits the energy at a longer wavelength. If two chromophores are in close enough proximity to one another, then some of the energy can be transferred between the two chromophores. The distance between the two chromophores can then be measured using the following equation:

\[ D = R_o ((1-\alpha)/ \alpha)^{1/6} \]
D is the distance between the two chromophores, \( R_o \) is a constant related to the donor-acceptor pair, and \( e \) is the efficiency of energy transfer. \( e \) can be easily calculated by obtaining the emission of the donor molecule, both in the presence and absence of the acceptor. The equation is:

\[
e = 1 - \left( \frac{F_{DA}}{F_D} \right)
\]

Where \( F_{DA} \) is the fluorescence intensity of the donor in the presence of the acceptor, and \( F_D \) is the fluorescence intensity of the donor in the absence of the acceptor. The calculations require that there be only one donor and one acceptor chromophore in the system. As with EPR, FRET has become a much more powerful tool now that cysteines can be site specifically mutated into the proteins of interest (for FRET references see (97) (114).

Each of the techniques mentioned has specific advantages and disadvantages. The downfall of EPR is the high protein concentrations required to obtain signals strong enough to optimize the signal to noise ratio. However, the ability to simultaneously have wt receptor and the labeled hGH in solution while only having to interpret the signal from the spin labeled hGH makes it a very valuable tool, if sufficient protein concentrations can be obtained. One advantage of FRET over EPR is its ability to achieve sufficient signals at nanomolar concentrations. One pitfall of the technique is that most proteins contain tryptophan, an intrinsic chromophore that can interfere with the results.

Based on the static information gained from the crystal structures, it was my theory that certain residues in hGH, mostly those located in mini-helix 1 and in the 2-3
loop region, would undergo large structural changes, while other residues would remain unchanged. To test this theory, several experiments were conducted. Residues located throughout the entire hGH molecule, focusing on regions that undergo change or that are located in site 2, were changed one at a time to cysteines. This allowed me to label and probe the specific microenvironments at each position.

The first set of experiments incorporated the thiol-specific spin label MTSL at the cysteine position. Once labeled the freedom of rotation of the label was monitored as hPRLbp, zinc, or both were titrated. The residues chosen for labeling sites were based on several properties. It was assumed that the positions that would yield the largest results would be those that underwent the largest change in respect to their freedom of rotation. Those residues in the 2-3 loop that became part of helix 2 and the residues that form mini-helix 1, in theory, should obtain a much more rigid environment, decreasing the speed of the spin labels' rotation. I also chose residues within site 2, to monitor for any change in environment. The third set of residues were chosen as controls, and included residues that did not show any change in the crystal structures, and would therefore not be expected to yield a change in the microenvironment or EPR spectra.

The second set of experiments labeled the hGH mutants with the extrinsic chromophore CPM. The CPM labeled GH's were then used in FRET experiments, monitoring the energy exchange, and thus the distance, between the only native tryptophan at position 86, and the CPM upon addition of either hPRLbp or zinc. These experiments focused on the mutation at position 92, since it had the largest change in distance from the tryptophan residue compared to the other cysteine mutants.
6.2 Results

6.2.1 Characterization of the hPRLbp

The hPRLbp was cloned and expressed as described in materials and methods. Sequence analysis confirmed the DNA was identical to the published sequence of the hPRLbp. SDS-PAGE electrophoresis indicated that the purified protein product was approximately 95% pure (Figure 6.1). Fluorescence, absorbance, and circular dichroism spectroscopies were performed to characterize the protein so that future batches of protein could be compared. Fluorescence yielded a spectra with a maximum at 343 (Figure 6.2). Absorbance spectra had a peak max at 279 with a side peak indicative of tryptophan absorbance at 291 (Figure 6.3). Circular dichroism spectra was typical of a protein consisting mostly of β-sheet. The spectra also contained multiple peaks in the 200-205 region (Figure 6.4).

The biological activity, and thus the binding capabilities, of the hPRLbp was confirmed with a competitive bioassay. FDC-P1 cells stably transfected with the hPRL receptor were treated with a mixture of 20 nM wt hGH and increasing concentrations of the hPRLbp (Figure 6.5). The ID 50 of the hPRLbp was estimated to be approximately 300 nM.

6.2.2 Characterization of hGH's

Labeling experiments on hGH's with an additional fifth cysteine residue genetically engineered at various positions throughout hGH yielded hGH’s labeled at multiple positions. Mass spectroscopy and Ellman's reactions (data not shown)
Figure 6.1 SDS-PAGE of the hPRLbp. Lane 1 and 2 contain two different batches of hPRLbp. Lane 3 contains molecular weight markers, with the corresponding size labeled to the right. Samples were prepared in the presence of 1% 2-mercaptoethanol.
Figure 6.2 Fluorescence Spectroscopy: hPRLbp samples were prepared at 1μM in 10mM Tris pH 7.6, 150mM NaCl. Spectra were collected at 20° C with an excitation wavelength of 290 nm.
Figure 6.3 Absorbance Spectroscopy: hPRLbp samples were prepared at 20 μM in 10mM Tris pH 7.6, 150mM NaCl. Spectra were collected at 25°C.
Figure 6.4 Circular dichroism: hPRLbp samples were prepared at 25 µM in 10 mM Tris pH 7.6, 150 mM NaCl. Spectra were collected at 25° C
revealed that the multiple labeling sites were a result of the reduction of the 182/189 disulfide bond. To eliminate these multiple labeling sites, a C182S/C189S hGH mutant was prepared. The fluorescent and CD spectra of this 182/189 mutant were not altered from that of wild-type hGH (Figures 6.6 and 6.7). The lactogenic activity was decreased by 7-fold as determined in the FDC-P1 hPRL receptor bioassay (Figure 6.8). Cysteine residues were mutated into the hGH C182S/C189S at various positions throughout the hGH molecule (Table 6.1).

6.2.3 Labeling of hGH's with MTS for EPR

The labeling reaction requires the presence of a reduced thiol. Since the cysteines are oxidized during the folding and purification procedures, all proteins must be reduced prior to labeling. Proteins were resuspended in phosphate buffer pH 7.5 containing 10 mM DTT. Excess DTT was immediately removed by passage over a sephadex G50 column. Ellman's and BCA reactions were run as described in materials and methods. The data from the Ellman's and BCA reactions were used to calculate the percentage of protein with a free thiol (Table 6.2). C182S/C189S hGH had only minimal amounts of free thiol, while the remaining mutant samples had between 10 and 44% of their thiols in the reduced form. During the labeling procedure (see methods for complete details) L117C precipitated out of solution.

The labeled hGH's were characterized by reducing and non-reducing SDS-PAGE electrophoresis (Figure 6.9). Labeled proteins were 95% pure with varying amounts of dimerized protein. Time of flight electrospray mass spectroscopy was used to elucidate the percent of protein labeled, as well as to determine relative
<table>
<thead>
<tr>
<th>mutation</th>
<th>residue position</th>
<th>mutation</th>
<th>residue position</th>
</tr>
</thead>
<tbody>
<tr>
<td>F10C</td>
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<td>L101C</td>
<td>2-3 loop</td>
</tr>
<tr>
<td>L23C</td>
<td>Helix 1</td>
<td>Y103C</td>
<td>2-3 loop</td>
</tr>
<tr>
<td>Y35C</td>
<td>Helix 1</td>
<td>N109C</td>
<td>2-3 loop</td>
</tr>
<tr>
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<td>1-2 loop</td>
<td>Y111C</td>
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<td>K115C</td>
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</tr>
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<td>Mini-helix 1</td>
<td>S132C</td>
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<td>F146C</td>
<td>3-4 loop</td>
</tr>
<tr>
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<td>Helix 2</td>
<td>A155C</td>
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<tr>
<td>F97C</td>
<td>2-3 loop</td>
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Table 6.1: Spin label sites

*residue position is based on the crystal structure of hGH free in solution
Figure 6.5 Competitive Bioassay: Dilutions containing 20 nM wt hGH and increasing concentrations of hPRLbp were prepared and allowed to reach equilibrium before being used to dose FDC-P1 hPRL receptor cells. 100% is defined by the cells stimulated with wt hGH in the absence of hPRLbp. 0% is defined by the cells in the absence of both hGH and hPRLbp.
Figure 6.6 Fluorescence spectroscopy: wt and C182S/C189S hGH samples were prepared at 1 μM in 10mM Tris pH 7.6, 150mM NaCl. Spectra were collected at 20° C, using an excitation wavelength of 290 nm.
Figure 6.7 Circular dichroism: wt and C182S/C189S hGH samples were prepared at 25 μM in 10 mM Tris pH 7.6, 150mM NaCl. Spectra were collected at 20° C.
Figure 6.8 FDC-P1 lactogenic activity: FDC-P1 hPRL receptor cells were treated with either wt or C182S/C198S hGH at various concentrations. Cell number was measured by a vital dye method. Each dose was performed in triplicate wells.
Figure 6.9 SDS-PAGE electrophoresis: The SDS-PAGE gel in the upper panel was loaded with samples in the absence of 2-mercaptoethanol. Starting at the left the lanes contain; molecular weight markers, wt hGH, C182S/C189S, F44C, L45C, E88C, F92C, L101C, Y103C, Y111C, L117C, F146C, and L156C. The lower panel contains the same loading order starting at the right, with samples prepared in the presence of 1% 2-mercaptoethanol.
Table 6.2: Thiol/protein ratios of C182S/C189S GH's containing additional nonnative cysteines.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Active Thiol (μM)</th>
<th>Protein (μM)</th>
<th>Percent thiol available</th>
</tr>
</thead>
<tbody>
<tr>
<td>C182S/C189S</td>
<td>0.57</td>
<td>29.6</td>
<td>1.6</td>
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<tr>
<td>F44C</td>
<td>8.06</td>
<td>31.7</td>
<td>22.2</td>
</tr>
<tr>
<td>L45C</td>
<td>12.8</td>
<td>38.7</td>
<td>29.0</td>
</tr>
<tr>
<td>E88C</td>
<td>26.0</td>
<td>65.8</td>
<td>34.6</td>
</tr>
<tr>
<td>F92C</td>
<td>24.7</td>
<td>72.9</td>
<td>29.7</td>
</tr>
<tr>
<td>L101C</td>
<td>10.9</td>
<td>36.7</td>
<td>25.9</td>
</tr>
<tr>
<td>Y103C</td>
<td>33.1</td>
<td>78.2</td>
<td>37.0</td>
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<td>Y111C</td>
<td>7.85</td>
<td>39.7</td>
<td>17.3</td>
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<td>L117C</td>
<td>5.65</td>
<td>46.2</td>
<td>10.7</td>
</tr>
<tr>
<td>F146C</td>
<td>37.88</td>
<td>75.5</td>
<td>43.9</td>
</tr>
<tr>
<td>L156C</td>
<td>10.67</td>
<td>61.9</td>
<td>15.1</td>
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</table>
Figure 6.10 FDC-P1 lactogenic activity of spin labeled mutants: FDC-P1 hPRL receptor cells were treated with either wild-type or mutant hGH's at various concentrations. Cell number was measured by a vital dye method. Each dose was performed in triplicate wells.
<table>
<thead>
<tr>
<th></th>
<th>Percent Labeled</th>
<th>ED 50*</th>
<th>Relative loss of activity**</th>
<th>Maximum response</th>
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<td>wt hGH</td>
<td>0</td>
<td>2.66</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>182/189</td>
<td>0</td>
<td>20.1</td>
<td>7.6</td>
<td>55</td>
</tr>
<tr>
<td>F44C</td>
<td>51</td>
<td>394</td>
<td>148</td>
<td>104</td>
</tr>
<tr>
<td>L45C</td>
<td>72</td>
<td>150</td>
<td>56</td>
<td>66</td>
</tr>
<tr>
<td>E88C</td>
<td>67</td>
<td>37.0</td>
<td>14</td>
<td>55</td>
</tr>
<tr>
<td>F92C</td>
<td>50</td>
<td>50.3</td>
<td>19</td>
<td>65</td>
</tr>
<tr>
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<td>6.3</td>
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<td>Y103C</td>
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<td>11</td>
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<td>Y111C</td>
<td>38</td>
<td>48.0</td>
<td>18</td>
<td>50</td>
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<td>L117C</td>
<td>24</td>
<td>64.6</td>
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<td>F146C</td>
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<td>L156C</td>
<td>20</td>
<td>38.6</td>
<td>14</td>
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</table>

Table 6.3: ED 50 values and the percent protein labeled for MTSL labeled GH’s.

*ED 50 in the FDC-P1 hPRL receptor bioassay

**relative to wt hGH
percentages of dimer present in each sample. Only three proteins had measurable dimer concentrations. F44C, L45C, and E88C had 11, 8, and 7% dimer respectively. The percentage of protein labeled varied from mutant to mutant, and ranged from 20-100% labeled (Table 6.3). Molecular masses determined by mass spectroscopy were within 2 daltons of the calculated molecular mass. There was no evidence of proteins labeled more than once, indicating that all label was incorporated in the correct position. FDC-P1 hPRL receptor bioassays were run to establish the effect of the label on the biological activity of each protein. The ED 50's and maximum responses are listed in Table 6.3. Three of the labeled proteins, F44C, L45C, and L117C, affected binding at site 1 as determined by a shift in the antagonist phase of the dose-response curve (Figure 6.10).

6.2.4 EPR spectroscopy

Labeled proteins had spectra that varied with the position of the label (Figure 6.11). To obtain spectra with an acceptable signal to noise ratio, a minimum concentration of 30 μM was required. Addition of zinc to the proteins did not alter the spectra. The hPRLbp could not be added to the labeled proteins at equimolar concentrations due to solubility restrictions. Therefore, the effects of receptor binding on hormone concentration could not be effectively determined with the available equipment.

6.3 FRET

A cysteine residue was engineered into the C182S/C189S mutant at position 92 to create the triple mutant F92C/C182S/C189S. This mutant was labeled with the
Figure 6.11: 

**EPR spectra.** EPR spectra of C182S/C189S/L92C-MTSL (upper spectra), and C182S/C189S/Y103C-MTSL (lower spectra). Samples were resuspended at 30 μM in 10 mM Tris pH 7.5, 150 mM NaCl, 30% Ficoll. While the hyperfine splitting distances are equal, the peak heights and the peak height ratios differ.
Figure 6.12 FRET: F92C/C182S/C189S hGH was labeled with CPM as described in chapter 2. The labeled protein was diluted to 500nM in 10 mM Tris pH 7.6, 150 mM NaCl. The sample was excited at 290 nm, and the emission spectra followed in the presence or absence of 1 μM zinc.
Figure 6.13 FRET: F92C/C182S/C189S hGH was labeled with CPM as described in chapter 2. The labeled protein was diluted to 500nM in 10 mM Tris pH 7.6, 150 mM NaCl. The sample was excited at 290 nm, and the emission spectra followed in the presence or absence of 500 nM hPRLbp. The spectra of hPRLbp was subtracted from the spectra of the sample containing both hGH and hPRLbp.
fluorescent label, CPM (7-diethylamino-3-(4’maleimidyIphenyl)-4-methylcoumarin), which attaches specifically to thiols. Samples were diluted to 500 nM, and then either 1 μM zinc or 500 nM hPRLbp was added. The system was excited at 290nm, and the emission was scanned from 300-550nm. The intrinsic tryptophan in hGH at position 86 serves as the donor chromophore, while the CPM attached at position 92 serves as the acceptor chromophore. No difference was seen in the emission spectra with the addition of zinc (figure 6.12). There was an increase in the CPM emission (485nm) concurrent with a decrease in the tryptophan emission upon the addition of hPRLbp in equimolar amounts (figure 6.13). No distances were calculated from this experiment as it could not be established that the intrinsic tryptophans in the hPRLbp were not contributing to the energy transfer.

6.4 Discussion

Initial EPR experiments indicated that wild-type hGH could not be used as a base for the cysteine mutants due to difficulties initiated during thiol reduction. It was observed from the Ellman’s results (not shown) that the DTT was also reducing one or both of the native disulfide bonds. Work published in the 60’s by Bewely and coworkers revealed that the 182-189 disulfide bond is very labile, and that the concentration required to reduce a fifth cysteine is also sufficient to reduce this disulfide bond (51). Reduction of the native disulfide bonds allows aggregation and derivatization at additional sites in the molecule.

Characterization of C182S/C189S hGH revealed that although the structure was intact, there was a 7-fold loss of lactogenic activity. Inspection of the crystal
structures revealed that the 9 residues beyond 182 are normally tethered close to the hGH molecule by the disulfide bond. I suspect that removal of this disulfide bond allowed the 9 residue segment to have more freedom of movement, possibly causing steric interference at the receptor binding sites. Attempts were made to eliminate this effect with the production of a tailless mutant comprising C182S/des 186-191 hGH. Surprisingly, the tailless mutant was almost completely devoid of lactogenic activity (data not shown). Interestingly, when used in a corresponding assay with the hGH receptor, tailless had full activity. Similar results were seen with a tailless mutant in porcine GH (115). This implies that the last few residues in hGH are functional determinants in the interaction with the hPRLbp, but not the hGH receptor. Further investigation of this portion of the molecule may reveal additional information about the mechanism of interaction.

During the labeling procedure L117C precipitated out of solution and required centrifugation before purification on the sephadex G50 column. This was most likely due to the fact that at position 117 the cysteine residue extends into solution, making it extremely solvent accessible. The enhanced accessibility of the reduced cysteine made conditions optimal for the cysteine at position 117 to interact with the 117 cysteine on neighboring hGH molecules, thus resulting in dimerization. While this resulted in a large loss of protein, gel electrophoresis and mass spectroscopy results indicate that the final purified protein was in the proper monomeric form. Some labeling sites adversely affected the biological activity. This was expected, as some of the labeling sites are located in site 2, or are located within the motif proposed in chapter 3 to be required for the proper formation of site 2. The mutants that had
increased ED 50 values that could be correlated solely to a decrease in the maximum response, were considered acceptable. The reasoning being that a decrease in the maximum response is an indication of disrupted site 2 activity, and the changes that I hoped to monitor occur without interaction at site 2. The three mutants, F44C, L45C, and L117C caused a shift in the antagonist phase of the curve, indicating that site 1 may be perturbed. This could prevent binding at site 1, and thus alter the experimental results and so these mutants were not used.

The EPR spectra obtained from the labeled proteins appeared very promising. The base spectra of each mutation varied as would be expected since the microenvironment at each position would be slightly different. When hPRLbp was added to the system, cloudy precipitate could be seen. Various buffers and pH’s were tested with the same result. The use of a loop-gap resonator may have solved the problem as it intensifies the signal, allowing proteins to be run at much lower concentrations. Unfortunately, I did not have access to a loop-gap resonator. Future studies would require access to a loop-gap resonator to alleviate the concentration difficulties. Additionally, incorporation of a G120R mutation in the system would prevent binding at site 2, ensuring that changes in the spectrum were a result of site 1 binding.

The hPRLbp competitive bioassay results indicated an ID 50 of approximately 300 nM, which is much higher than would be expected. Since there is no biologically purified hPRLbp to compare the spectroscopy to, there is no definitive proof that my protein was folded correctly. While the assay confirms binding, the higher than expected ID 50 is consistent with incorrectly folded proteins. The high ID 50 could
also be a result of protein precipitation. As precipitation was seen in solution during EPR experiments at concentrations used in the bioassay, I cannot eliminate the possibility that the hPRLbp was precipitating out or aggregating during the competitive bioassay. While the immediate effect is due to loss of protein, again, the aggregation would indicate either an improper fold or a contaminating factor that promoted aggregation. The CD spectra further supported the theory that the protein may not be completely pure, as there were multiple peaks in the 200-205 region that were not expected from a β-sheet protein.

The FRET results are very promising preliminary results. The entire hGH molecule is only about 40 angstroms at its widest. The majority of FRET experiments use much larger molecules, measuring distances of 30-80 angstroms between the acceptor and donor chromophores. Using proteins already available from the EPR work allowed preliminary experiments that indicate that it is possible to measure distances from 5-10 angstroms. Calculations of distances however were not performed, as the results may have been skewed by energy transferred from tryptophan residues in the hPRLbp which when the hGH binds the hPRLbp would come into close enough range with the CPM label on the hGH to transfer energy. This problem is normally eliminated by the removal of native tryptophans. In the case of the hPRLbp, the tryptophans could not be removed as they have been determined by mutagenesis to be required for activity. Nevertheless, the results are promising because they are proof that the hPRLbp is interacting with the hGH molecules.
Future experiments with FRET could eliminate the tryptophan interference by using two extrinsic labels that do not excite in the 270-300 nm range. Suggested combinations include using CPM as the donor chromophore, and either fluorescein, eosin, or BODIPY derivatives as the acceptor chromophore. These experiments would require the production of hGH mutants with two cysteine residues placed at strategic locations.

This chapter has provided the basis for additional work in studying the proposed conformational change in hGH. Additionally, it provides the first evidence for conformational changes in hGH in solution. Similar techniques may also be applied to studies of the hPRLbp, as it has been suggested that conformational changes occur in the receptor as well.
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