THE BIOLOGICAL, STRUCTURAL AND KINETIC PROPERTIES OF PROLACTIN, PROLACTIN RECEPTOR ANTAGONISTS, GROWTH HORMONE AND THE PROLACTIN RECEPTOR

DISSertation

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By

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ABSTRACT

Human prolactin is a member of the class I cytokine family and is able to bind, activate and produce a biological response when associated with the human prolactin receptor. Prolactin has been shown to bind one prolactin receptor and undergo a conformational change in order to be available to bind a second prolactin receptor. Upon binding the second receptor, an active trimeric complex is formed and is able to produce a biological response. Specific locations on prolactin have been identified as binding sites for the first (site 1) and second (site 2) prolactin receptor and the order of binding to each of these receptors have been shown to be important. A coupling motif consisting of specific amino acids has been identified in human growth hormone upon receptor binding that allows for the binding of a second receptor. A similar motif has yet to be identified in human prolactin. I aimed to identify a similar coupling motif within human prolactin using mutagenesis. A coupling motif was unable to be identified, however, and binding of the second receptor at site 2 was shown to be very weak. A strong correlation was observed that links binding of the first receptor at site 1 with biological activity and was determined to be the event that activates the hormone/receptor complex. This observation was also made with growth hormone binding the prolactin receptor. Receptor binding growth hormone was seen to be the activating event and binding of the second prolactin receptor at site 2 was observed to be very weak. This model for receptor activation
opposes the current model where both receptors need to bind the hormone with high affinity at both sites 1 and 2 in order to create an active trimeric complex.

Prevention of a biological response produced by the prolactin receptor using receptor antagonists has utility as a pharmaceutical for cancers of the breast or prostate. A successful antagonist would be capable of binding a receptor in competition with \textit{in vivo} hormone without inducing a biological response. Several receptor antagonists have been reported and works done by various groups to characterize these receptor antagonists have not been concise and the physical properties of each are unclear. I investigated several antagonists for their structural, biological, and kinetic properties. Several of these antagonists, including; Δ1-14, G129R, Δ41-52 and S179D, were each shown to not be successful antagonists because of instability and/or the induction of a biological response. One antagonist, Δ1-14/G129R, was shown to be structurally sound, did not induce a biological response, and was able to compete with wild-type prolactin to inhibit cell proliferation. This molecule was shown to have all the properties needed to be a successful receptor antagonist.

The prolactin receptor extracellular domain contains regions that have been shown to be important for biological activity. The extracellular domain consists of two subdomains termed S1 and S2. Studies have shown that deletion of certain regions of the extracellular domain either inhibits receptor activation or creates constitutively active receptors \textit{in vivo}. Regions of the receptor are thought to bind to adjacent regions and inhibit receptor activation. I have investigated each of the subdomains for their biological and kinetic properties. Each subdomain was not observed to bind prolactin
suggesting that regions of both the S1 and S2 subdomains are required for hormone binding. The S2 subdomain was shown to not have an affinity for any region of the receptor or induce cell proliferation. The S1 subdomain has a high affinity for the extracellular domain of the prolactin receptor and a low affinity for the S1 subdomain. The S1 subdomain was also observed to have agonist activity in biological assays in the absence of hormone. The indication is that the S1 subdomain can bind regions of the extracellular domain and activate the receptor. Activation of the receptor by binding to these specific regions would suggest the presence of an inhibitory element that prevents activation. Binding to this inhibitory region frees this inhibition allowing the receptor to assume an active conformation and induce a biological response.
To Dr. Charles Brooks, who kept pushing me to daylight.

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CHAPTER 1

INTRODUCTION

1.1 Project Overview

Human prolactin (hPRL) and its ability to bind the human prolactin receptor (hPRL receptor) have been reported previously in regards to both structure and kinetics of the interaction. Further, several candidates have been reported as potential antagonists of the human hPRL receptor with the aim of being used as a pharmaceutical to suppress hPRL \textit{in vivo}. The work done here looks to extend the knowledge of hPRL and its interaction with the hPRL receptor. The ability of hPRL to undergo a conformational change upon binding the first hPRL receptor and its interaction with a second receptor are not well understood. A better understanding of the interaction of hPRL with the second receptor will give insight into the mechanics of receptor antagonists that attempt to block biological activity by preventing second receptor binding. Site 2 binding of human growth hormone (hGH) to the hPRL receptor will also be investigated. Additionally, this work will describe the biological, structural, and kinetic features of several potential antagonists to assess their structural stability and ability to prevent a biological response which will be important factors in their pharmaceutical utility. Finally, the hPRL receptor
will be investigated for the structural, biological and kinetic significance of specific regions on the hPRL receptor.

1.2 Background

Prolactin (PRL) is a protein first discovered in 1928 that was found to be a pituitary factor that stimulated lactation in rabbits, sows and cows after being injected with extracts from the anterior pituitary (1). As a consequence of this, the name “prolactin” was given to this molecule (2). Early attempts to separate hGH from hPRL were unsuccessful and some endocrinologists at the time doubted the existence of hPRL (3). With overlapping biological functions and hGH existing at a 100-fold excess concentration to that of hPRL in human pituitaries, the separation of these two molecules proved to be difficult. There was evidence, however, that hPRL was present. Patients with acromegaly had elevated serum hGH determined by radioimmunoassay, yet they did not exhibit clinical symptoms of elevated hPRL. In contrast, patients with galactorrhea and normal postpartum women showed low serum levels of hGH, yet there was evidence of a milk-producing hormone. In 1969, a patient with galactorrhea had a pituitary tumor removed and the pituitary extract was treated with antiserum to hGH. This antiserum failed to inhibit hPRL activity in extracts and hPRL was shown to indeed exist as a separate molecule (3-5). A bioassay was created shortly after this that was able to identify hPRL in patients who had elevated levels in their blood (6, 7). The amino acid sequence of hPRL was first identified in sheep in 1969 and was shown to be 199 amino acids in length (8). hPRL was first purified by two independent research groups in the
early 1970's (9, 10) and the first amino acid sequence of human hPRL was identified in 1979 (11).

1.3  **Lactogenic Hormones**

1.3.1  **Prolactin, Growth Hormone and Placental Lactogen Cytokine Family**

hPRL, hGH and human placental lactogen (hPL) are members of a superfamily of class I helical protein hormones (12-14). The mature forms of the proteins range in size from 191 to 199 residues in length and have a wide variety of physiological functions in all vertebrates (15,16). hPRL is grouped into this larger family containing hGH and hPL because of their similar structural and biological features (17,18). hPRL, hGH and hPL are members of a class-I cytokine superfamily (13,14) hPRL shares only 21 and 22% sequence homology with hGH and hPL, respectively. hGH and hPL, however, share 85% homology.

1.3.2  **Prolactin**

PRL has over 300 different biological functions including; reproduction, osmoregulation, immune regulation, growth and metabolism (19-21). These hormones are delivered to target tissues through both endocrine and autocrine/paracrine mechanisms (14). PRL targets many tissues including; mammary gland, ovary, prostate, cells of the immune system and many other tissues (22-24). PRL production primarily occurs in the lactotrophic cells of the anterior pituitary and the placenta during pregnancy, however, production has also been demonstrated in human mammary
epithelial cells, skin, spleen, lacrimal glands, circulating lymphocytes, tissues of the
brain, decidua and myometrium (20, 25, 26). With very few exceptions, PRL production
is primarily located in the pituitary in other species (13). In 1991, hypophysectomized
rats showed that in the absence of the pituitary, extra-pituitary cells were not only able to
secrete PRL, but increased synthesis to compensate for the lack of pituitary PRL (27).

hPRL has been linked to cancers of the breast and prostate (28, 29). It has also
been discovered that not only do breast carcinomas express hPRL, but healthy breast
tissue also express both hPRL and its receptor (26, 30). Breast carcinomas express the
hPRL receptor at a much higher level than healthy breast tissue (31). Postmenopausal
women with elevated plasma hPRL were shown to be at an increased risk for breast
cancer (32).

The gene for hPRL is located on chromosome 6 (33). It is more than 15kb in
length and encompasses 5 exons, with exon 1a being differentially expressed in
extrapituitary pituitary cells and 1b expressed in the pituitary (12, 34, 35). Transcription
of hPRL is regulated by two promoters. Pituitary hPRL is regulated by a proximal
promoter approximately 5 kb upstream and approximately 2kb in length (36).
Extrapituitary hPRL is regulated by a second promoter that is approximately 5.8kb
upstream and results in a transcript that is an additional 150bp in length. This additional
150bp lies in the 5’ untranslated region. Depending on the promoter the mRNA products
differs, however, they encode an identical 227 residue human pre-protein containing a
signal sequence created in both pituitary and extrapituitary cells (37, 38). The 28 residue
signal peptide is cleaved in the endoplasmic reticulum prior to secretion resulting in a
mature 199 residue protein approximately 23kDa (38, 39). The mRNA consists of 914 nucleotides with a 681 nucleotide open reading frame encoding the immature 227 residue protein (40).

Posttransitional modifications have been reported, including phosphorylation, glycosylation and proteolitic cleavage (41, 42). Posttranslational modifications are not required for the protein to become biologically active and more often than not posttranslational modifications hinder the biological activity (41, 43). Glycolosylation has been shown to lower biological activity, phosphorylation in certain species creates a receptor antagonist, and proteolytic cleavage can results in a product unable to bind the hPRL receptor. A 16kDa product has been described and it corresponds with the first 148 residues of the mature protein (38, 44, 45). This variant inhibits capillary endothelial cell proliferation (48), prevents angiogenesis (49, 50) and binds weakly to the PRL receptor (51). It is unclear if this variant exists as a product of alternative RNA splicing or proteolityc cleavage. hPRL has also been found to be bound to immunoglobulins both in the glycosylated and un-glycosylated state (52, 53). Phosphorylation of rat PRL at serine 177 has been identified and corresponds to hPRL serine 179 (54). An S179D mutation mimics a phosphorylated serine and it was previously unclear whether or not this mutation activates or inhibits hPRL activity (55, 56).

Secondary structure of hPRL shows a primarily α-helical protein. Circular dichroism (CD) reports hPRL to contain roughly 50% α-helix (57). hPRL folds into a four helix bundle, with the α-helices connecting in an up-up-down-down pattern similar to hGH (18, 58-61). Helix 1 is composed of 30 residues; residues 14-43. Helix 2 is
composed of 26 residues; residues 78-103. Helix 3 is composed of 27 residues; residues 11-137. Helix 4 is composed of 33 residues; residues 161-193. This can be visualized in Figure 1.1 and 1.2 created from the Protein Data Bank (PDB) no. 1RW5 (62) using Visual Molecular Dynamics (VMD) software (64). There are three internal disulfide bonds at C4-C11, C58-C174, and C191-C199 (Figure 1.3) and their reduction greatly reduce their biological activity (48). Structures of hPRL (62, 65-69), hGH (70) and hPL (71) have been reported either free or bound to PRL receptor(s) and an NMR structure of hPRL in the absence of receptor (62).

Residues that support biological activity and binding are located on helix 1 and helix 4 (72-74). Crystal and NMR structures of PRL, GH and placental lactogen (PL) and various analogs of human and other species both bound and free of receptor, have provided insight into the binding of the receptor and subsequent conformational changes that occur (60, 62, 70, 71, 75-79). hPRL binds and activates its receptor in a manner similar to that of hGH and hPL (80). hPRL binds to two hPRL receptors in an obligate-ordered manner as shown by structural, biochemical and biophysical studies (81-83). hPRL binds the hPRL receptor in a sequential manner (72). The location on hPRL where the first receptor binds is termed site 1 and the location on the hormone where the second receptor binds is termed site 2 (Figure 1.4). Eighteen residues of hPRL are thought to be involved in site 1 binding to the hPRL receptor, however, only 9 have been shown to be functionally important (66, 84). Two hPRL receptors binding the hPRL hormone at sites 1 and 2 creates an active heterotrimeric complex (69, 81-83). Site 2 of hPRL is not available to be bound.
Figure 1.1 Structure of hPRL
Structure of hPRL, PDB no. 1RW5 (62). The four \( \alpha \)-helices are represented. Helix 1, residues 14–43 (red); helix 2, residues 78–103 (yellow); helix 3, residues 111–137 (green); and helix 4, residues 161–193 (purple). Images are rotated 180° around the \( z \)-axis (center) and 90° on the \( y \)-axis (right).
Figure 1.2 Structure of hPRL
Structure of hPRL, PDB no. 1RW5 (62). The four α-helices are represented. Helix 1, residues 14–43 (red); helix 2, residues 78–103 (yellow); helix 3, residues 111–137 (green); and helix 4, residues 161–193 (purple). Images are rotated 180° around the z-axis (center) and 90° on the y-axis (right).
Figure 1.3 Disulfide Bonds in hPRL
Structure of hPRL highlighting the disulfide bonds (yellow) formed by C4-C11, C58-C174, and C191-C199 (48). Image is rotated 90° along the y-axis (right).
Figure 1.4 The hPRL Receptor Bound to the hPRL Hormone
Structure of hPRL receptor bound to the hPRL hormone via site 1, PDB no.3N06 (68). Displayed is the S1 (green), proline rich hinge (red), and the S2 subdomain (cyan) of the prolactin receptor complexed to hPRL hormone (red). Images are rotated 180° on the z-axis (right).
by the second receptor until site 1 is occupied by the first receptor (81-83). Both 1:1 (69) and 1:2 (70) stoichiometries of homrone:receptor binding have been demonstrated previously. Formation of 1:2 complexes have also been demonstrated by surface plasmon resonance (SPR) and extremely rapid dissociation from a 1:2 complex to a 1:1 were observed (82, 83). This explains why previous gel filtration experiments were unsuccessful in showing a 1:2 stoichiometry (85).

1.3.3 Growth Hormone

hGH was first purified in 1956 (86) and its effect on growth was discovered shortly after in sexually underdeveloped adolescents in 1958 (87). In 1971, the amino acid sequence of hGH was first reported and the gene was first cloned in 1979 (88, 89). It is primarily known for its anabolic affects that largely promote skeletal growth and body composition (90). The concentration in the body, either high or low, has been linked to changes in body fat, lean muscle mass and bone density (91). hGH is primarily released from the anterior pituitary, and like hPRL, acts in a paracrine/autocrine or an endocrine manner (92-94).

The gene for hGH is located on chromosome 17 and exists in a hGH/hPL gene cluster (95). Two 22kDa variants of hGH are created by alternative splicing. hGH-N (normal) and hGH-V (variant) both consist of 5 exons spread over 2kb, however, they differ by 13 of the 191 residues. hGH-N is expressed in the pituitary while hGH-V is expressed in the placenta. A 217 residue pre-protein is first translated and proteolytic cleavage of the 26 residue N-terminal signaling peptide yields the mature 191 residue
protein (96). In addition, alternative splicing at exon 3 yields the 20kDa hGH-V hormone with a deletion of residues 23 to 46 (97, 98). This variant is capable of binding to the hGH receptor but it lacks the ability to promote biological activity by binding to the hPRL receptor (97, 99).

Porcine growth hormone (GH) was the first of this family of cytokines to have its three-dimensional structure elucidated in 1987 (59). Like hPRL, hGH is primarily an α-helical protein composed of a four helix bundle in an up-up-down-down motif (Figure 1.5 and Figure 1.6). Helix 1 is composed of 30 residues (6–35), helix 2 is composed of 28 residues (72–99), helix 3 is composed of 20 residues (110–129), and helix 4 is composed of 33 residues (150-183) (Figures 1.5 and 1.6) (63, 75, 96). There are two disulfide bonds formed at C53-C165 and C182-C189 (Figure 1.7) (96). The C53-C165 disulfide bond has been shown to be absolutely essential for biological activity (100).

1.4 Prolactin Receptor

The hPRL receptor was first discovered in the 1970s, identified as a membrane-bound protein from rabbit mammary glands (101-105). The cDNA was isolated in 1988 from the rat PRL receptor. The hPRL receptor was first cloned from cDNA libraries of liver and breast cancer cells (106). The PRL receptor has been identified in a large number of mammalian tissue, including: mammary gland, prostate, testes, epithelial cells, skeletal muscle, stomach, intestine, pancreas, kidney, bone, pituitary, brain, etc. (20). It exists in virtually all tissues and there are estimates of between 200 to roughly 30,000 receptors per cell in those which express the receptor (20).
Figure 1.5 Structure of hGH
Structure of hGH, PDB no. 1A22 (78). The four α-helices are represented. Helix 1, residues 6-35 (red); helix 2, residues 72–99 (yellow); helix 3, residues 110–129 (green); and helix 4, residues 150-183 (purple) (64, 81, 104). Images are rotated 180° on the z-axis (center) and 90° on the y-axis (right).
**Figure 1.6 Structure of hGH**

Structure of hGH, PDB no. 1A22 (78). The four α-helices are represented. Helix 1, residues 6-35 (red); helix 2, residues 72–99 (yellow); helix 3, residues 110–129 (green); and helix 4, residues 150-183 (purple) (64, 81, 104). Images are rotated 180° on the z-axis (center) and 90° on the y-axis (right).
Figure 1.7 Disulfide Bonds in hGH
Structure of hGH, PDB no. 1A22 (78), highlighting the disulfide bonds (yellow) formed by C53-C165 and C182-C189 (104).
The hPRL receptor and the hGH receptor are closely related with a single-pass transmembrane, despite their low sequence homology of about 30% (20, 107, 108). The hGH and hPRL receptors share several structural and functional features (108-110). The sequence comparison of these and other receptors led to a new family named class I cytokine receptors (108, 111-113). This family includes receptors for various interleukins, granulocyte-colony stimulating factor, erythropoietin, thrombopoietin, gp130 and leptin (113-117). These receptors contain regions that are highly conserved and the hPRL receptor also binds both hGH and hPL (18).

The hPRL receptor gene is located on chromosome 5 and was thought to consist of 10 exons that exceed 10kb (118, 119). Exons 3 through 10 encode the mature hPRL receptor. Exons 1 and 2 are a part of a 5’-untranslated region and there are 3 different Exon 1s. The transcription of the various Exon 1s depends on the promoter used, however, the mature protein, irrespective of which first exon is used, are identical in all cases (118, 120). The 5’-untranslated region contains several promoters whose usage is tissue specific (118, 121, 122). The hPRL receptor is composed of three major domains; the extracellular domain (ECD), the transmembrane domain and the intracellular domain. The human receptor is found in several forms created by proteolytic cleavage or alternative RNA splicing (123-129). The various isoforms are composed of various lengths of the intracellular domain including two short forms (126, 128) an intermediate form (125) and a long form (124) of the receptor. In 1999, the intermediate form hPRL receptor was first discovered in a breast cancer cell line (125). This form of the receptor is a result of alternative RNA splicing at a consensus site on exon 10 that removes
nucleotides 1009 to 1582. The 573 nucleotide deletion creates a frameshift and introduces a stop codon 13 nucleotides after the splice site resulting in a truncated intracellular domain of the receptor. The intermediate form does not induce cell proliferation, however, it does increase cell survival by inhibiting apoptosis. An additional exon, exon 11, was discovered in 2001 and alternative splicing between exons 10 and 11 creates two short forms of the hPRL receptor (S1_a and S1_b) (126). These short forms will bind hPRL but both have truncated intracellular domains. They act as dominant negative inhibitors for signaling of the long form of the receptor. Variants of other class I cytokine receptors, including the hGH receptor (130) and the erythropoietin receptor (131), have been reported but the variants are not analogous to the isoforms seen with the hPRL receptor.

A soluble form of the receptor has been identified and contains just the ECD of the receptor (132-134). It is unclear whether this form of the receptor is created as a result of proteolytic cleavage or of alternative splicing. It has been identified in the blood and is thought to buffer rapid changes in hPRL concentration and a portion of plasma hPRL is thought to circulate complexed to the soluble form of this receptor (129, 132).

The long form of the hPRL receptor contains 598 residues which includes a 210 residue ECD, a 24 residue transmembrane and a 364 residue intracellular domain. The first 100 residues of the ECD is termed the S1 subdomain and the next 110 residues (residues 101-210) is termed the S2 subdomain (Figure 1.8 and Figure 1.9) (20). Each subdomain contains a seven-stranded antiparallel β-sheet fibronectin type III motif (112). One is composed of three strands, termed A, B and E, and the other is composed of four strands termed C, C’, F and G (60, 70, 110, 117, 135). The two domains are connected by
Figure 1.8 Structure of the hPRL Receptor
Structure of hPRL receptor, PDB no.3N06 (68). Displayed is the S1 subdomain, residues 1-100 (green), the proline rich hinge, residues 101-106 (red), and the S2 subdomain, residues 107-210 (cyan). Images are rotated 180° on the z-axis (right).
Figure 1.9 Structure of the hPRL Receptor
Structure of hPRL receptor, PDB no.3N06 (68). Displayed is the S1 subdomain, residues 1-100 (green), the proline rich hinge, residues 101-106 (red), and the S2 subdomain, residues 107-210 (cyan). Images are rotated 180° on the z-axis (right).
a proline rich hinge (VQPDP) at residues 101-106. The S1 subdomain contains two highly conserved disulfide bonds at C12-C22 and C51-C62 (Figure 1.10). Removal of either of these disulfides removes both the ability to bind ligand (136) and biological activity (137).

A WSXWS motif, located near the membrane interface at residues 191-195, is also highly conserved amongst class I cytokine receptors. Mutations to the WSXWS motif reduces hormone binding affinity by what is thought to be improper folding and reduced transport to the cell surface (110, 136, 138).

The transmembrane spanning domain consists of 24 hydrophobic residues. The intracellular domain consists of two highly conserved regions, Box 1 and Box 2. Box 1 is located proximal to the cell membrane and consists of 8 residues -IFPPVPGP- residue numbers 243-250. Box 2 is less conserved and consists of hydrophobic, negatively charged, followed by positively charged residues (residues 288-298) (20). Both Box 1 and Box 2 have been shown to be critical for signaling (139, 140).

Receptor activation induces downstream signaling of the JAK/STAT pathway (20). JAK1 and JAK2 associate with the hPRL receptor (141-143) with JAK2 being the primary signaling molecule and has been shown to be constitutively associated with the receptor (142, 144, 145). JAK2 activation includes phosphorylation of tyrosines in the intracellular domain, as well as phosphorylation of JAK2, STAT1, STAT3 and STAT5 transcription factors (141-143). STAT5 is the main transcription factor in this pathway and its activation induces STAT5 dimerization and transport to the nucleus where gene
Figure 1.10 Disulfide Bonds and the WSXWS Motif of the hPRL Receptor
Structure of hPRL receptor, PDB no.3N06 (68). Highlighted are the disulfide bonds (yellow) formed by C12-C22 and C51-C62 and the WSXWS motif (orange). Images are rotated 180° on the z-axis (right).
transcription is induced (20, 146). The MAP kinase, PI3 kinase and Protein kinase C pathways have also been associated with the hPRL receptor (146).

1.5 Research Goals

The focus of this research began with the investigation of hPRL. Site 1 binding of hPRL induces a conformational change that allows site 2 to be available for binding and leads to a subsequent biological response (81-83). Chapter 3 of this document investigates this conformational change trying to discover which residues, if any, may be important for this conformational change. To accomplish this, mutations were made to residues between sites 1 and 2 to determine what affect this has on the ability of hPRL to bind the hPRL receptor at both sites 1 and 2. In doing so, site 2 binding will be characterized and residues important for binding at sites 1 and 2 will also be evaluated.

The characterization of both site 1 and site 2 binding is important in understanding the dynamics that take place between the hPRL receptor and hPRL at sites 1 and 2, as well as the change that hPRL undergoes upon binding at site 1. By investigating these properties, the relationship between hPRL and the hPRL receptor are better understood and are necessary in order to create antagonists that reduce biological activity. Antagonists were investigated in Chapter 4 with our gained knowledge of sites 1 and 2. All known protein hPRL antagonists were investigated to characterize their binding at sites 1 and 2. The goal of receptor antagonism is to modulate biological activity and the extent to which this was achieved was also investigated. The physical
properties of the antagonists were also evaluated and will be useful information in the pharmaceutical production of a successful antagonist.

Regions of the hPRL receptor were investigated to determine which are important for binding the hormone. In Chapter 5 I look at the ECD of the hPRL receptor as well as the S1 and S2 subdomains to determine the role each play in binding to the hormone. In addition to the role each subdomain plays in binding the hormone, I also investigated the interactions between the various receptor subdomains in the absence of hormone. Biological assays were also performed to determine the extent to which the ECD of the hPRL receptor and the S1 and S2 subdomains hinder or promote a biological response in both the absence and presence of hormone.

The hPRL receptor is also able to bind hGH despite the low sequence homology between hPRL and hGH. Site 2 binding of hGH to the hGH receptor has previously been described and the affinity is quite high in comparison to the low affinity site 2 of hPRL for the hPRL receptor (65, 67, 147). It is unclear if the weak site 2 affinity of hPRL to the hPRL receptor is a consequence of the hormone or the receptor. It is known that hGH is able to bind and activate the hPRL receptor, however, the affinity of the hPRL receptor to site 2 of hGH is unknown. In chapter 6 I investigated the interaction between hGH and the hPRL receptor at site 2. This investigation will determine whether the weak affinity of the hPRL receptor to hPRL at site 2 is due to properties of the hormone or the receptor and will shed light on the mechanism by which the receptor is activated.

The sum of these of these chapter will provide mechanisms by which hPRL interacts with the hPRL receptor and the properties of hPRL antagonists. It will compare
the hPRL/hPRL receptor interaction with that of the hPRL receptor to site 2 of hGH.

Finally, studies with hPRL receptor ECD and subdomains will provide new insights into the mechanism by which receptor activity is mediated.
CHAPTER 2

MATERIALS AND METHODS

2.1 Vectors Design

2.1.1 Human Prolactin (hPRL) and Human Growth Hormone (hGH)

The pT7-7 expression plasmid was used to express all proteins being investigated. This vector was originally created by and received from S. Tabor (Harvard Medical School, Boston, MA) (148). This vector contains an ampicillin resistance gene used for selection of positive transformations and a T7 RNA promoter used to express proteins in E. coli that contain a T7 RNA polymerase. Drs. Francis Peterson and Patricia Anderson introduced an f1 origin of replication, creating the pT7-7f(-) phagemid that is able to produce single stranded DNA (149). The methionyl coding sequence for hPRL gene was ligated into the pT7-7 phagemid by Dr. Francis Peterson and the methionyl coding sequence for hGH was introduced into the phagemid by Dr. Karen Duda.

Mutations of single residues in hPRL were performed by the procedure of Kunkel et al. (1991) (150). Phagemids containing mutant hPRL DNAs were sequenced to confirm the presence of the desired mutation and the integrity of the desired sequence. Proteins were expressed, purified, and characterized as subsequently described. Proteins were dried from 5mM ammonium bicarbonate and stored under desiccation at -30°C.
2.1.2 *The Extracellular Domain of the hPRL Receptor*

The methionyl coding sequence for the ECD of the hPRL receptor was ligated into the pT7-7 phagemid by Dr. Umasundari Sivaprasad from a PCR product created from a human cDNA library (Invitrogen, Carlsbad, CA) as previously described (82). The DNA sequences encoding the mature methionyl forms of the receptor consisted of the ECD of the hPRL receptor (ECD-receptor, residues 1 through 210) and the first 14 amino acids of the transmembrane domain. A stop codon was introduced at residue position 211 by mutagenesis to give a product that would express only hPRL ECD-receptor, residues 1-210.

2.2 *Protein Expression and Purification*

Wild-type and mutant hPRL, hGH, various forms of the hPRL receptor and hPRL receptor antagonists were expressed as previously described (82, 149). Phagemids containing the nucleic acid sequence of interest were transformed into BL21(DE3) *E.coli* (Novagen, EMD Millipore, Darmstadt, Germany) and were grown on Lysogeny Broth (LB) agar plates containing ampicillin. A single colony was selected and grown overnight at 37°C with shaking in 10mL of ampicillin-containing LB broth creating a starter culture. Flasks containing 1L of LB Broth with antibiotic were warmed, aerated, and inoculated the following morning with 5mL of the starter culture and were allowed to grow at 37°C with shaking until an OD$_{600nm}$ of 0.400 was reached. Protein expression was induced with the addition of 0.4mM isopropyl-β-D-thiogalactopyranoside for four hours. Cells were collected by centrifugation for 10 minutes at 5000g. Cells were
resuspended in a 45mL solution containing 100mM Tris-base, 1mM PMSF (Sigma-Aldrich, St. Louis, MO), 25mM dithiothreitol (Invitrogen, Carlsbad, CA), pH 7.5 and were lysed using a French pressure cell (SLM-Amico, Urbana, IL) with two passes at 5000psi. Inclusion bodies were collected by centrifugation at 12,500 RPM for 30 minutes and were resuspended in 100mL of 4.5M urea, 50mM Tris-base, pH 11.5 and were stirred slowly for two days with air oxidation at 4°C to allow for complete denaturation of the proteins. Samples were dialyzed at 4°C against 4L of 20mM Tris, pH 7.5 for a minimum of four hours with four additional buffer changes to allow for refolding with removal of urea and neutralization of pH. Proteins were purified by anion-exchange chromatography being run over DEAE Fast Flow Sepharose (GE Healthcare, Chalfont St. Giles, United Kingdom) using an ÄKTA Explorer 100 chromatograph (GE Healthcare, Chalfont St. Giles, United Kingdom) in 20mM Tris, pH 7.5. The desired protein product was removed from the column by eluting with a gradient of NaCl (0-500mM over 40 minutes) and samples were collected when the 280nm:260nm absorbance ratio was greater than 1.5:1. Proteins were applied to a 2.6x100cm Superdex 75 sizing column (Sigma-Aldrich, St. Louis, MO) in 10mM ammonium bicarbonate to separate the proteins based on molecular size. Fractions were collected and analyzed spectrophotometrically and fractions with a 280:260 absorbance ratio approaching 2:1 were pooled, frozen, lyophilized, and stored under desiccation at -30°C.
2.3 Protein Characterization

2.3.1 Protein Concentration by Extinction Coefficient

The concentrations of protein samples were determined using the Edelhoch method which utilizes the extinction coefficient of a protein (151). The extinction coefficient indicates how much light a particular protein should absorb at a specific wavelength. Knowing the amino acid composition of a particular protein, it is possible to calculate the molar extinction coefficient. The extinction coefficient of a protein can be calculated from the molar extinction coefficient of tryptophan, tyrosine and cystine at 280 nm (Table 2.1). The concentration of the sample can then be calculated using the Beer-Lambert Law and the absorbance of the sample at 280 nm.

\[
\varepsilon_{280} \text{ (cm}^{-1}\text{M}^{-1}) = (#\text{Trp})(5,500) + (#\text{Tyr})(1,490) + (#\text{cystine})(125)
\]

\[
c = \frac{A_{280}}{\varepsilon_{280}b}
\]

**Table 2.1 Extinction Coefficients and Equations for Calculating Concentration**

Extinction coefficients of tyrosine, tryptophan and cystine. Equations for calculating the extinction coefficient of a protein, \(\varepsilon\), and Beer-Lambert Law for calculating the concentration of a protein using absorbance (\(A_{280}\), path length (b), and concentration (c).

2.3.2 SDS-Polyacrylamide Gel Electrophoresis

The purity and size of recombinant proteins was evaluated using 20-25\(\mu\)g protein samples and were displayed on 15% SDS-polyacrylamide gels. Samples were run under both reducing and non-reducing conditions. Under reducing conditions, the size and
purity of the recombinant protein would be evaluated. Non-reducing conditions would verify the formation of the correct disulfide bonds formation of various mutants. Incorrect disulfide bond formation would alter the hydrodynamic radius and could be verified by differences in apparent molecular weight when compared to wild-type hPRL. Gel images were captured using an AlphaImager EC (Cell Biosciences, Santa Clara, CA).

2.3.3 UV Absorbance Spectroscopy

UV absorbance spectra of proteins between 200-350nm can determine any significant conformational variances when comparing mutant proteins to that of the wild-type. Aromatic groups have maximum absorbance between 260-280nm (280, 274 and 258 for tryptophan, tyrosine and phenylalanine, respectively), disulfide bonds absorb between 250-270nm, and proteins that have not aggregated and are lacking metal ions do not have significant light scattering or absorption at 350nm. 20µM samples were prepared in 150mM NaCl, 10mM Tris-base, pH 7.4. The UV spectrum was measured from 200nm to 350nm on a Lambda 45 UV/VIS Spectrometer (Perkin Elmer, Wellesley, MA).

2.3.4 Fluorescence Spectroscopy

Aromatic amino acids tryptophan, tyrosine and phenylalanine are able to fluoresce if they are excited in their range of absorption. The max absorption wavelength is 280nm, 274nm and 257nm, respectively. While the range of absorption overlaps between the three residues, only tryptophan has significant absorption at 295nm. The
resulting emission peak from this excitation ranges from 307nm to 350nm and reaches a peak around 340nm. Exposure to water can influence the emission spectra of tryptophan. Changes in protein structure can cause a change in hydration and will result in a change in the emission spectra of tryptophan. In order to assess a change in hydration due to conformation, mutant hormones were compared to wild-type hPRL at 1µM in 150mM NaCl, 10mM Tris-base, pH 7.4. Proteins were excited at 295nm and the emission spectrum was measured between 300nm to 550nm. These measurements were done on an LS 55 Luminescence Spectrometer (Perkin Elmer, Wellesley, MA).

2.3.5 Circular Dichroism

CD can assess secondary structure of proteins pertaining to α-helices, β-sheets and random coils. Each type of secondary structure has a consistently unique CD spectrum. α-helical proteins will display two negative peaks at 208nm and 222nm. β-sheet proteins will show a characteristic negative peak at 218nm and will rise to a positive value at 200nm with a positive peak at 196nm. Random coil proteins will have a positive peak at 212nm and a negative peak at 195nm. Changes in secondary structure of the protein due to mutations will alter the CD spectrum. 20µM samples of wild type and mutant proteins were prepared in 150mM NaCl, 10mM Tris-base, pH 7.4. The spectrum was read between 260nm to 190nm. Samples were analyzed on a Jasco J-815 Circular Dichroism Spectrometer (Jasco, Easton, MD).

CD data was analyzed to determine percent α-helix and β-sheet. Data is entered into the K2D3 algorithm, which fits the spectrum to determine percent α-helix and β-
sheet (Figure 2.1) (152). Based on the fit, a prediction can be made for the percentage of α-helix and β-sheet secondary structure present in the protein.

Figure 2.1 Circular Dichroism Fit to Predict Secondary Structure
An example of the fit of CD data into the K2D3 algorithm. The example shows hGH (red) and the predicted curve fit (green) to determine percent α-helix. Spectrum was converted to Molecular Circular Dichroism to be fit by the K2D3 algorithm.

2.4 Biological Assays

2.4.1 Half Maximal Effective Dose (ED$_{50}$) Agonist Assays

Agonist assays establish dose-response relationships between wild-type and mutant hPRLs that stimulate cell growth. FDC-P1 cells stably expressing the hPRL receptor (82, 124) were obtained from Genentech, Inc. (San Francisco, CA). These cells respond to lactogen by an increased rate of cell division. We measure this biological endpoint by determining relative cell numbers in each well with a vital-dye assay. The cells were maintained in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 10ng/mL recombinant mouse Interleukin-3 (Calbiochem, EMD Biosciences, La Jolla, CA), 10µM
β-mercaptoethanol (Gibco, Invitrogen, Carlsbad, CA) and 220µg/mL G418 sulfate (Hyclone, Logan, UT). Cells were incubated at 37°C in a humidified 5% CO₂ environment. Cells in log phase growth were washed in sterile PBS (Gibco, Invitrogen, Carlsbad, CA) and were serum-starved in lactogen-free media for 24 hours consisting of RPMI 1640 without phenol red (Gibco, Invitrogen, Carlsbad, CA), 10% equine serum (Hyclone, Logan, UT), 10µM β-mercaptoethanol (Gibco, Invitrogen, Carlsbad, CA), and 220 µg/mL G418 sulfate (Hyclone, Logan, UT). Serum-starved cells are resuspended in fresh starvation media without β-mercaptoethanol and aliquoted into 96 well plates with each well containing approximately 15,000 cells. Triplicate wells containing cells were exposed to hormone concentrations over six orders of magnitude from 0.01nM to 10µM in a final volume of 100µL/well. Assays included cells receiving a 0.0nM hPRL dose to measure basal cell growth and serve as an inter-assay control. Assays were performed in groups containing several mutant or antagonist hPRLs; a wild-type hPRL positive control assay was performed with each group. Cells were incubated for 48 hours at 37°C in a humidified 5% CO₂ environment. The growth endpoint of the assay was measured as described in section 2.4.3.

2.4.2 Half Maximal Inhibitory Concentration (IC₅₀) Antagonist Assays

Cells were grown, treated, and staved in the same manner stated above to obtain an ED₅₀ value. Serum-starved cells were resuspended in fresh starvation media without β-mercaptoethanol and aliquoted into 96 well plates with each well containing approximately 15,000 cells. Antagonist or potentially inhibitory proteins in
concentrations over six orders of magnitude ranging from 0.01nM to 10µM were supplemented with 10nM hPRL, which was observed to be the lowest concentration of hormone needed to stimulate cells to near maximal growth. Triplicate wells containing cells were exposed to a final volume of 100µL/well. Assays included cells treated with neither wild-type nor an antagonist or inhibitory protein to measure basal cell growth and serve as an inter-assay control. A wild-type hPRL ED50 assay was performed with each IC50 assay to act as a positive control that demonstrated the responsiveness of cells to lactogen hormone and to determine minimum and maximum cell proliferation. Cells were incubated for 48 hours at 37°C in a humidified 5% CO2 environment. The growth endpoint of the assay was measured as described in section 2.4.3.

2.4.3 Measurement of Assay Endpoint

The endpoint of these assays measured the relative number of cells in each well by their metabolism of a vital dye. Cells were treated with 10µL of a vital dye (AlamarBlue, Invitrogen, Carlsbad, CA) and incubated for 2 to 6 hours to assess hormone-induced proliferation. AlamarBlue is metabolized in the cellular mitochondria and the reduced and oxidized forms have maximum absorbances at 570 and 600nm, respectively. A greater number of cells in a well are reflected by a greater dye reduction. Absorbance was measured in each well at 570 and 600nm to measure reduction of the vital dye and the percent of vital dye reduction is calculated and used to establish dose-response curves. The extinction coefficients and equations are listed in Table 2.2 (153). The dose-response curves were used to calculate ED50 and ID50 values using a four-
parameter fit with SigmaPlot version 12.3. Assays for each hormone were performed three times and the results were averaged. Data was accepted if triplicate wells show similar dye reductions, a complete dose response curve was described, and repeated assays provided similar ED\textsubscript{50}s and IC\textsubscript{50}s.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reduced</th>
<th>Oxidized</th>
</tr>
</thead>
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<tr>
<td>Reduced</td>
<td>570nm</td>
<td>80586cm\textsuperscript{-1}M\textsuperscript{-1}</td>
</tr>
<tr>
<td>Oxidized</td>
<td>600nm</td>
<td>14652cm\textsuperscript{-1}M\textsuperscript{-1}</td>
</tr>
</tbody>
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\[
C_{\text{red}} = \left( \varepsilon_{\text{ox}} \text{ 570nm} * A_{\text{570nm}} \right) - \left( \varepsilon_{\text{ox}} \text{ 600nm} * A_{\text{600nm}} \right)
= \left( \varepsilon_{\text{ox}} \text{ 570nm} * \varepsilon_{\text{red}} \text{ 600nm} \right) - \left( \varepsilon_{\text{ox}} \text{ 570nm} * \varepsilon_{\text{red}} \text{ 600nm} \right)
\]

\[
C_{\text{ox}} = \left( \varepsilon_{\text{red}} \text{ 600nm} * A_{\text{600nm}} \right) - \left( \varepsilon_{\text{red}} \text{ 570nm} * A_{\text{570nm}} \right)
= \left( \varepsilon_{\text{red}} \text{ 600nm} * \varepsilon_{\text{ox}} \text{ 570nm} \right) - \left( \varepsilon_{\text{red}} \text{ 600nm} * \varepsilon_{\text{ox}} \text{ 570nm} \right)
\]

Table 2.2 AlamarBlue Extinction Coefficients and Equations for Cellular Reduction

2.5 Surface Plasmon Resonance

SPR is a tool that allows observation of the adsorption of molecules from an injected analyte to an immobilized molecule over time. SPR is an optical method that measures the result of a change in the refractive index near a thin metal sensor surface resulting from the change in analyte retained. The instrument follows a change in refractive index by its effect on a plasmon wave induced by absorption of a low angle polarized light and the result is a decrease in the intensity of reflected light. Changes in the angle of absorbed and reflected light is a result of the refractive index coupled through the plasmon wave. This metal surface serves as the floor to which the ligand is immobilized. An aqueous running buffer passes over the surface in a microfluidic
channel called a flow cell. As the analyte is injected into the running buffer through the flow channel, any interaction between the ligand and analyte will result in accumulation of analyte and a change in to the refractive index. The change in the refractive index can be observed on the opposite side of the sensor surface. Low angle polarized light is directed at the metal sensor surface and the angle at which light is absorbed is plotted. The refractive index greatly influences the angle at which the polarized light is absorbed. As molecules associate or dissociate from the chip surface the refractive index changes which is observed by a change in the angle of absorbance of the polarized light. This change is measured in resonance units (RU) which is roughly a change of 1 pg/mm² of protein absorbed at the chip surface. The result of the change in RU versus time is plotted (Figure 2.2). Additionally, a change in refractive index can occur because of differences in the running and sample buffer and creates a background response. This response can be recorded and subtracted by using a flow channel with no immobilized ligand. The difference between the signals from this reference cell and test flow cells allows observation of the actual binding between the ligand and analyte.

2.5.1 Protein Preparation and Thiol-Coupling for Surface Plasmon Resonance

Proteins were specifically designed to be immobilized to the chip surface by thiol-coupling. Thiol-coupling is preferred because it allows for the immobilization of the protein to the sensor surface at a specific residue. Thus, all immobilized proteins have an identical coupling orientation. The protein is thiol-coupled to the surface of a Biacore CM5 Sensor Chip (GE Healthcare, Chalfont St. Giles, United Kingdom) and is monitored
Figure 2.2 Surface Plasmon Resonance Sensogram
Example SPR sensogram showing the binding and dissociation of several concentrations of wild-type hPRL to the ECD-receptor. An injection of hormone was made between 75 and 375 sec. Buffer was flowed over the chip surface after the injection until the end of the experiment.
in a Biacore model 3000 (GE Healthcare, Chalfont St. Giles, United Kingdom). Thiol-coupling requires the presence of a free cysteine. Proteins meant for thiol-coupling were designed to have a free cysteine residue in an opportune location on the proteins surface and distant from binding sites. Proteins designed for thiol-coupling were prepared by first being solubilized in 10mM ammonium bicarbonate, pH 7. Proteins were incubated for 5 minutes in a 5µM excess of dithiothreitol (DTT, Invitrogen, Carlsbad, CA) to eliminate inter-molecular thiols. The DTT was subsequently removed by four cycles of ultrafiltration in an 10K Amicon Ultra Centrifugal Filter (Millipore, Billerica, MA). Lost volume was replaced with 20mM sodium acetate, pH 4.5.

The chip surface was prepared for thiol-coupling by injection of 25µL of freshly prepared 50mM N-Hydroxysuccinimide (NHS) and 20mM 1-Ethyl-3-[3-dimethylaminopropyl] carbinomide hydrochloride (EDC), followed by the injection of 40µL of freshly prepared 80mM 2-(2-pyridinylthio) ethanolamine (PDEA) in 100mM boric acid, pH 8.5. These reactions create amine binding sites on the chip surface to add the pyridinyl thiols. Unreacted amine-binding sites were blocked by a 50µL injection of 1M ethanolamine. Step-wise injections of DTT treated protein were then added to be immobilized through disulfide bond to the chip surface until a desired protein density is reached in RU. The aim of the experiments can differ and can require a varying amount of immobilized protein to the chip surface. The protein density of most experiments ranged from 250 to 2000 RU. Unreacted thiol groups on the chip surface were blocked by the addition of 60uL of 50mM cysteine in 20mM sodium acetate, 1M NaCl, pH 4.5. One lane of each chip was not coupled with protein and was used to measure the background.
signal. A reference cell was created this way as a blank by being activated and subsequently blocked by cysteine.

2.5.2 Binding Experiments

Proteins of varying concentrations were prepared in 150mM NaCl, 3mM EDTA, 0.005% Surfactant P20, 10mM HEPES, pH 7.4 (HBS Running Buffer). All experiments included a buffer injection across all lanes. This will be used as a background subtraction for any change in the signal due to the injection itself or non-binding artifacts due to increasing concentrations of protein in the injections. At the conclusion of each study the chip was stripped of non-covalently bound proteins by a brief exposure to a 2.5 M MgCl₂, followed by buffer injections to ensure the clearance of any residual MgCl₂ that could potentially alter subsequent binding experiments. All assays and washes were performed at a 50 μL/min flow rate. We have previously shown that this flow rate does not create diffusion-limited binding resulting from depletion of analyte immediate to the chip surface linked to this density of ligand (82). Between 5 and 7 concentrations of protein are flowed over the chip surface. The concentrations used are dependent on the experiment but most range in concentration from 10nM to 100μM.

2.5.3 Surface Plasmon Resonance Data Analysis

The association rates (k₁), dissociation rates (k₋₁) and residuals were used to calculate the k₁ and k₋₁ constants with BiaEvaluation® 3.0 software (GE Healthcare, Chalfont St. Giles, United Kingdom) (Figure 2.3). Kinetic data was accepted if the
Figure 2.3 Surface Plasmon Resonance Curve Fitting
An example of SPR curve fitting by BiaEvaluation® 3.0 software. Shown is a sensogram of the interaction between the ECD-receptor and hPRL. This study bound ECD-receptor to the chip surface and sequentially pumped 50, 100, 500nM and 1µM hPRL across the chip surface. Binding was observed from 80 to 420 seconds followed by a switch to a buffer solution. The signal was then followed for over 900 seconds to record the dissociation of hPRL from the ECD-receptor. This data represents the binding data after correction of background. An injection of hormone was made between 75 and 425 sec. Buffer was flowed over the chip surface after the injection until the end of the experiment.
residuals were evenly distributed and were less than 10% of the signal. The dissociation constant ($K_d$) was calculated from the rate constants. The kinetic data was double corrected for background (154). This was done by subtracting both reference cell and buffer injection values from each experimental curve.

2.6 **Differential Scanning Calorimetry**

Differential scanning calorimetry (DSC) measures the thermal stability and domain folding integrity of a protein. The differential scanning calorimeter contains two vessels, one for the sample and the other is used as a reference. The sample and reference vessels are maintained at a near identical temperature as the temperature of both vessels rise and fall throughout the experiment. Endothermic or exothermic heat from the phase transitions of the protein in the sample cell cause the temperature of the sample vessel to deviate from the reference. More or less heat is applied to the sample vessel in response to this in order to try to maintain the same temperature as the reference vessels during a controlled increase in both vessels. The amount of energy needed to control the temperature is observed by the calorimeter and the amount of heat absorbed or released during the phase transition is calculated from the differences between the energy applied to the two vessels. As the temperature rises, the peak reached during the phase transition equates to 50% denaturation of the protein ($T_m$) from a folded state to an unfolded state.

Thermal melts of proteins samples were performed on VP-DSC Differential Scanning Calorimeter (GE Healthcare, Chalfont St. Giles, United Kingdom). Samples were prepared in 40mM KH$_2$PO$_4$, pH 7.3, and were heated from 15°C and 110°C over 90
minutes. Buffer was initially used in both vessels and the instrument cycled from heating to cooling repeatedly overnight to determine a background pattern of energy utilization. If a stable background was observed, a roughly 500µL sample ranging from 10µM to 200µM of protein was injected into the sample vessel when the instrument was in a cooling phase between 30°C and 20°C. Once the instrument reached 15°C and was stable for five minutes, the temperature rose to 110°C at 90°C per hour. Data was recorded on VPViewer™ software (GE Healthcare, Chalfont St. Giles, United Kingdom) and was analyzed on Origin® software (GE Healthcare, Chalfont St. Giles, United Kingdom). Select samples were prepared in 25mM HEPES, 150mM NaCl, pH 7.8 or 100mM NaH₂PO₄, 100mM Arginine, pH 7.0 in an attempt to prevent or decrease aggregation.
CHAPTER 3

hPRL SITE 1 BINDING ACTS AS A GATEKEEPING EVENT FOR BIOLOGICAL ACTIVITY

3.1 Introduction

hPRL binding creates hPRL receptor dimers that are aligned so they may initiate biological activities in target cells. hPRL receptors are unique in that they bind three human hormones including hGH, hPL, as well as hPRL (83, 155). These hormones bind the receptor on two distinct binding surfaces (sites 1 and 2). There is low sequence conservation between hPRL, hGH, and hPL, with hGH and hPRL being roughly 28% conserved. Each, however, display an ordered binding of hPRL receptor to sites 1 and 2 (82, 83) suggesting that each of the lactogens undergo a conformational change induced by receptor binding at site 1 resulting in site 2 to become available for receptor binding. Biophysical studies of hPRL, hGH, and hPL show that receptor binding changes FRET signals of these hormones (82, 83) indicating site 1 receptor binding induces a change in the hormone’s conformers.

Binding sites 1 and 2 of hPRL are approximately 20 Å apart. This distance and the ordered binding of receptors (82, 83) require sites 1 and 2 to be functionally coupled.
The $K_d$ of receptor binding to site 1 is approximately 1nM (65, 67, 83). The affinity for site 2 binding, which includes the site 2 hormone/receptor interface and a receptor/receptor interface is 30,000-fold weaker with an approximate $K_d$ of 30µM (65, 67).

It has previously been demonstrated that selected residues constitute a contiguous receptor-coupling motif within hGH when binding hPRL receptors (156). Mutations to residues between but not including sites 1 and 2 did not affect the affinity at site 1, however the affinity at site 2 was weakened. This suggests that mutagenesis of residues in hPRL may be used to identify the coupling motif. Mutations in the coupling motif linking sites 1 and 2 alter the number of available conformers that are able to be visited by the hormone upon site 1 binding the receptor. This would limit the availability of an active site 2 and reduce the biological activity of the mutated hormone. The goal in this work was to identify residues that form an analogous coupling motif in hPRL. In this work I will determine if a coupling motif linking sites 1 and 2 similar to the one previously identified in hGH can be identified.

3.2 Materials and Methods

Measurements of receptor binding at sites 1 or 2 were performed by SPR on CM5 chips monitored in a BiaCore model 3000 (GE Healthcare) in two experiments. First, C-terminal cysteines of the ECD-receptor were covalently linked to CM5 chips with densities of 500, 1000 and 2000 RU.
Affinity measurements of ECD-receptor binding to site 2 were performed for wild-type and selected mutants of hPRL. These studies were performed by the trimeric method first described by Joss et al. for heterotrimeric antigen/antibody complexes (157). The rapid $k_1$ and $k_{-1}$ constants observed for site 2 binding precluded accurate kinetic measurements; these observations were similar to those of Jomain et al. (2007) and Broutin et al. 2010 (65, 67). In addition, mutations that severely reduced site 1 binding were not suitable for trimeric analysis because sufficient amounts of heterodimers could be generated and retained to provide a suitable signal for the binding ECD-receptor at site 2. In these studies, 1500 RU of ECD-receptor were covalently bound to a CM5 chip through C211. This residue was chosen because C211 is located at the C-terminus of the ECD distant from the hPRL binding sites. To build a heterodimeric complex 200 μL of 5μM wild-type or mutant hPRL were flowed over the chip surface producing a sufficient population of heterodimers to discern binding of a second ECD-receptor. This was followed by a short buffer wash during which the extent of dimer formation and the stability of the heterotrimeric complex were assessed for appropriate quantity and stability to bind the second receptor. If sufficient heterodimer was formed and the complex was sufficiently stable, 100μL of the ECD-receptor was flowed over the chip surface at concentrations ranging between 0 and 100μM (Figure 3.1 A). The signals were double corrected for background (154) and used to prepare binding isotherms (Figure 3.1 B) and calculate affinity constants for ECD-receptor binding at site 2 of the heterodimeric complex.
Figure 3.1 SPR of Wild-Type hPRL Site 2 Binding and Langmuir Binding Isotherm

(A) SPR of Site 2 binding of ECD-receptor to a wild-type hPRL/ECD-receptor heterodimer. SPR data was collected for ECD-receptor concentrations of 1, 10, 50, 100, and 500nM as well as 1, 5, 10, 50, and 100µM. Displayed is a representative figure of experiments performed. An injection of hormone was made between 75 and 175 sec followed by a second injection of ECD-receptor between 650 and 850 sec. Buffer was flowed over the chip surface after the injection until the end of the experiment. (B) Langmuir binding isotherms derived from SPR experiments of wild-type hPRL and a representative mutant V99D.
The second SPR trimeric study was performed by coupling hPRL to the chip surface. Two mutants were thiol-coupled to the chip surface, M158C and G129C. M158 is distal from both sites 1 and 2 and thiol-coupling at position 158 should not interfere with receptor binding at either site. G129 is located at site 2 and thiol-coupling at this location should prevent site 2 binding, but site 1 should be fully capable to bind ECD-receptor. Both mutants have been characterized and used in SPR experiments to bind ECD-receptor previously (83) and the mutants used here were created, expressed, and purified by Dr. Jeffrey Voorhees. Roughly 2000 RU of both mutants were coupled to the chip surface in adjacent lanes. Increasing concentrations of ECD-receptor were flowed over the chip surface from 5nM to 100µM to determine the stoichiometry of ECD-receptor:hormone for both M158C and G129C.

3.3 Results

3.3.1 Selection of Mutations, Preparation, and Characterization of hPRLs

In an earlier publication a coupling motif was present in hGH when it bound to the hPRL receptor (156). This motif formed a contiguous collection of largely hydrophobic residues whose mutation diminished the site 2 receptor binding surface upon site 1 binding-induced activation while leaving the site 1 binding unaltered. I decided to explore a similar but enlarged structural space in hPRLs. Twenty one residues, approximately 10% of the total in hPRL, were chosen for mutation, recombinant expression, and functional evaluation (Table 3.1). These residues were largely hydrophobic, frequently located in the interior of hPRL between sites 1 and 2, and
<table>
<thead>
<tr>
<th>hPRL Mutation</th>
<th>Activity Relative to Wild-type*</th>
<th>hGH Coupling Motif for hPRL Receptor Binding**</th>
<th>ΔΔG for Ala Mutations (kCal/mole)***</th>
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</thead>
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<tr>
<td>T45E</td>
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<td>Y169A (-1.85)</td>
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<tr>
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<td>L163</td>
<td></td>
</tr>
<tr>
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<td>Y164</td>
<td>L172A (-0.17)</td>
</tr>
<tr>
<td>H173E</td>
<td>1569 (1)</td>
<td></td>
<td>H173A (-0.34)</td>
</tr>
</tbody>
</table>

**Table 3.1. Mutations Explored and Biological Activities (ED$_{50}$) Relative to hPRL**

* Ratio of ED$_{50}$ for mutant hPRL divided by ED$_{50}$ for Wild-type hPRL, (n) = number of replicates.

** Motif residues identified in hGH by Duda and Brooks (156).

*** ΔΔG = ΔG$_{Mutant}$ − ΔG$_{WT}$, negative ΔΔG indicates a weakened affinity. Significant changes in ΔΔG are > 1.20 kCal/mole, data taken from Rao and Brooks (84).

**** Not determined.
provided exploration of a contiguous molecular space (Figure 3.2). The choice of the replacement amino acid was individualized for each residue (Figure 3.2) and sought to replace hydrophobic residues with charged residues (F, I, A, L, Y, and V were replaced with E or D), charged residues were replaced with oppositely charged or small neutral residues (H, K, and R were replaced with E and A), and one G was replaced with F. These mutations are not conservative and were not meant to be. Mutations were sought that would reduce biological activity and those that did would be further explored in kinetic studies. The aim is to seek mutations that would reduce biological activity but would leave site 1 kinetics unaltered, similar to what was observed with hGH. This would point to a similar binding mechanism and induction of biological activity that would identify a coupling motif connecting sites 1 and 2 of the hormone.

Wild-type and mutated hPRLs were expressed in the BL21 (DE3) strain of *E. coli*, folded, and purified as previously described (149). I was unable to prepare L172E hPRL; thus, it was not determined if this residue should be included in a motif functionally coupling sites 1 and 2 of hPRL. It could be speculated that the leucine to glutamic acid change in this location is associated with an inability to fold correctly. The analogous residue in hGH (L163) has previously been shown to be required for proper folding (158). All hPRLs were characterized for purity and molecular weight in SDS-containing gel electrophoresis under either non-reducing or reducing conditions (Figure 3.3 and Figure 3.4) and showed at least 95% purity and migrated to similar positions as wild-type hPRL.
Figure 3.2 Motif Coupling Sites 1 and 2 of hPRL
Top panel: (A) Backbone structure of hPRL (PDB no. 1RW5) (62) with solid filled residues that were explored by mutagenesis. (B) Backbone structure of hPRL (PDB no. 1RW5) with solid filled residues identified as members of the motif that functionally couples sites 1 and 2. (C) Backbone structure of hGH (PDB# 1BP3) (70) with solid filled residues that constitute the motif that couples sites 1 and 2 when binding the hPRL receptor. Bottom panel: Comparison of the residues forming the motifs coupling sites 1 and 2 of hPRL and hGH when binding the hPRL receptor.
Figure 3.3 SDS-PAGE of Wild-Type and hPRL Mutants
15% acrylamide electrophoretic gels with (top panel) and without (bottom panel) 2-mercaptoethanol for 15-20 µg of hPRLs containing mutations in loop 1. Mutants were observed to be pure in both the reducing and non-reducing gels and migrated to the same location as wild-type hPRL.
Figure 3.4 SDS-PAGE of Wild-Type and hPRL Mutants
15% acrylamide electrophoretic gels with (top panel) and without (bottom panel) 2-mercaptoethanol for 15-20 µg of hPRLs containing mutations in helices 2 and 4. Mutants were observed to be pure in both the reducing and non-reducing gels and migrated to the same location as wild-type hPRL.
3.3.2 *UV and Fluorescence Spectroscopy*

Recombinant proteins were characterized by UV spectroscopy. Each protein showed an absorption peak at 277nm, characteristic of aromatic amino acids (Figure 3.5 and Figure 3.6). All preparations had a 280/260 ratio greater than 1.5 similar to that of pure preparations of hPRL. Absorption at 350nm was absent indicating an absence of light scattering by aggregated proteins. The shape of the absorbance was similar for all hPRLs.

Fluorescence spectra of the mutants, in comparison to that of wild-type hPRL, did not shift greatly from the maximum peak height at 340nm and the overlaid spectrum showed minimal variation (Figure 3.7 and 3.8). These findings suggest the environment surrounding the tryptophan residues were not greatly influenced by a change in hydration indicating the proteins were folded similarly to that of wild-type.

3.3.3 *Circular Dichroism*

CD of mutants when compared to wild-type hPRL showed characteristic curves for α-helical proteins (Figure 3.9 and 3.10). Two negative peaks at 220nm and 208nm and a positive peak approaching 190nm were observed. The CD spectrum of each protein was measured with at least two preparations of each protein. Different batches of protein were also analyzed to ensure a lack of variation between batches. This lack of variation suggests that similar folds were achieved for various batches of protein. The CD spectrum of each protein was converted into molar ellipticity.
Figure 3.5 Absorbance Spectra of Wild-Type and hPRL Mutants
Absorbance spectra for hPRLs containing mutations in loop 1. Proteins (25µM) were in 10mM Tris, pH 8.2, 150mM. Spectra were collected at 25°C. Data in panel insert has been normalized at 277 nm. The spectra for all mutants were similar to wild-type hPRL and absorbance at 350nm was not observed which indicates no aggregation.
Figure 3.6 Absorbance Spectra of Wild-Type and hPRL Mutants
Absorbance spectra of hPRLs containing mutations in helices 2 (top panel) and 4 (bottom panel). Proteins (25µM) were in 10mM Tris, pH 8.2, 150mM. Spectra were collected at 25°C. Data in panel insert has been normalized at 277nm. The spectra for all mutants were similar to wild-type hPRL and absorbance at 350nm was not observed which indicates no aggregation.
Figure 3.7 Fluorescence Spectra of Wild-Type and hPRL Mutants
Fluorescence spectra of hPRLs containing mutations in loop 1 which is the unstructured region that connects helix 1 and 2. Proteins (1µM) were in 10mM Tris, pH 8.2, 150mM. Spectra were collected at 25°C. Data in panel insert has been normalized at 340 nm. Fluorescence spectra of mutant hPRLs did not shift greatly from wild-type hPRL at 340nm.
Figure 3.8 Fluorescence Spectra of Wild-Type and hPRL Mutants

Fluorescence spectra for hPRLs containing mutations in helices 2 (top panel) and 4 (bottom panel). Proteins (1µM) were in 150mM NaCl, 10mM Tris, pH 8.2. Spectra were collected at 25°C. Data in panel insert has been normalized at 340 nm. Fluorescence spectra of mutant hPRLs did not shift greatly from wild-type hPRL at 340 nm.
Figure 3.9 Circular Dichroism of Wild-Type and hPRL Mutants
CD spectra for hPRLs containing mutations in loop 1. Proteins (25μM) were in 10mM Tris, pH 8.2, 150mM NaCl. Spectra were collected at 25°C. Data in panel insert has been normalized at 222nm. Characteristic CD spectrum was observed for mutant and wild-type hPRLs indicating the retention of secondary structure.
Figure 3.10 Circular Dichroism of Wild-Type and hPRL Mutants
CD spectra for hPRLs containing mutations in helix 2 (top panel) and helix 4 (bottom panel). Proteins (25µM) were in 10mM Tris, pH 8.2, 150mM NaCl. Spectra were collected at 25°C. Data in panel insert has been normalized at 222 nm. Characteristic CD spectrum was observed for mutant and wild-type hPRLs indicating the retention of secondary structure.
3.3.4 Agonist Activities of Mutant hPRLs

The biological activities (ED$_{50}$) of twenty mutant hPRLs were determined in the FDC-P1 agonist assay (149) and compared to activity of wild-type hPRL (Table 3.1, Figure 3.11). The biological activities of several mutant hPRLs was reduced by as much as 34-fold when compared to wild-type hPRL ED$_{50}$s of approximately 1nM. Ratios of mutant to wild-type hPRL biological activities greater than 2 were considered significant and identified 9 residues (F50, K53, A54, L95, V99, Y169, L171, and H173) as potential members of a motif coupling sites 1 and 2. The ED$_{50}$ values for H97 was increased but was not consistent, values fell within a >10-fold range, thus this residue could not be included in this group. None of these residues are within site 1 of hPRL (65-68).

Individual mutation of the remaining 11 residues did not influence their ED$_{50}$ suggesting that the chosen amino acid replacements were not unduly disruptive of hPRL’s structure.

3.3.5 Site 1 Kinetics and Affinities of Mutant hPRL

Eight of the nine mutant hPRLs with significant changes in ED$_{50}$s, two mutant hPRLs with unchanged ED$_{50}$s, and wild-type hPRL were selected for determination of their kinetics and affinities for site 1 binding to the ECD-receptor. The k$_1$ and k$_{-1}$ constants were calculated from kinetic binding experiments of several concentrations of the ligand as measured by SPR (Table 3.2) (Figure 3.12). For each protein the dissociation constant was calculated from the rate constants. The k$_1$ constants varied over 30-fold range (3.05 x 10$^4$ to 9.19 x 10$^5$ moles/min) with those for wild-type and non-affected (R48A and Y96E) hPRLs being the most rapid k$_1$ constants. The k$_{-1}$ constants
Figure 3.11 Agonist Assays Comparing Wild-Type and V99D hPRLs
ED$_{50}$ agonist assays performed using FDC-P1 cells that constitutively express the long-form of the hPRL receptor. (A) hPRL and (B) V99D concentrations were between 10nM and 100µM. Above are representative figures of experiments performed. Experiments were repeated three times and values were averaged. Variation in individual data points was small and error bars were not included.
Figure 3.12 Site 1 Binding of Wild-Type and Mutant hPRL to the ECD-Receptor
SPR assays with residuals measuring the kinetics of site 1 binding of (A) hPRL and (B) V99D to ECD-receptor. Concentration of hormone varied between 10nM and 1µM. Figures are representative of experiments performed. The residuals for all SPR experiments performed were evaluated and the fits to determine kinetics were accepted only if the residual value was less than 10% of the response. An injection of hormone was made between 75 and 400 sec. Buffer was flowed over the chip surface after the injection until the end of the experiment.
### Table 3.2 Rate Constants and ED$_{50}$s for Selected hPRL Mutants

* Indicate that that ED$_{50}$s were similar to wild-type hPRL.

**Site 2 K$_d$ not measured due to rapidly dissociating dimeric hPRL/EDC-receptor complex.

ND: not determined

$C_V$ = Standard Deviation / Mean

<table>
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<tr>
<th>Site 1 Mutant</th>
<th>Dissociation Constant (K$_d$) (nM)</th>
<th>$C_V$</th>
<th>Mut/WT Ratio of K$_d$</th>
<th>Mut/WT Ratio of ED$_{50}$</th>
<th>K$_d$ (µM)</th>
<th>Std. Dev.</th>
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varied over a 21-fold range (1.91 x 10^{-2} to 8.84 x 10^{-4} sec^{-1}) with those for wild-type and non-affected hPRLs among the slowest. There was no correlation between the changes in $k_1$ and $k_{-1}$ among the various mutant hPRLs. The dissociation constants were calculated as the ratio of $k_{-1}$ divided by $k_1$. These $K_d$s varied over approximately a 40-fold range (1.08 to 43.8nM). The $K_d$s of wild-type and non-affected hPRLs were the strongest. The remaining hPRLs affinities were weakened by their specific mutations. Mutation of the eight hPRLs with reduced affinities of site 1 were; F50, K53, A54, L95, H97, V99, Y169, and L171. Each of the mutant hPRLs identified by FDC-P1 agonist assays by their increased $ED_{50}$s also experienced a reduced site 1 affinity.

3.3.6 Correlation of Site 1 $K_d$ with $ED_{50}$

The relationship between the $K_d$ of site 1 and biological activity ($ED_{50}$) for wild-type and mutant hPRLs were explored (Figure 3.13). The Pearson product-moment correlation coefficient of these two data sets, ratios of mutant to wild-type values for $ED_{50}$s and $K_d$s, was positive ($R = 0.891$, $n = 10$) displaying a slope approaching unity with an intercept near the origin ($Y = 0.7283 X - 0.7587$).

3.3.7 Site 2 Affinities of hPRL Mutants

Site 2 affinities were determined in trimeric complex studies using an equilibrium SPR approach for wild-type and five mutant hPRLs (R48A, L95E, Y96E, H97, and V99D) (Table 3.2, Figure 3.1). The mutants that were selected had a $K_d$ below 10nM which was stable enough to allow for the binding of a second ECD-receptor. The
Figure 3.13 Correlation Between ED₅₀s and Site 1 Kᵦs of Mutant hPRLs

ED₅₀s were determined for wild-type and twenty one mutant hPRLs in agonist assays stimulating the growth of FDC-P1 cells expressing hPRL receptors. Typically, two or three replicate experiments were performed and normalized by calculating the ratios from mutant to wild-type ED₅₀s. These normalized values were averaged. Kᵦs for wild-type and nine mutant hPRLs were calculated from the on and off rate constants determined by SPR. Only nine mutants were chosen for kinetic analysis because most of the 21 mutations did not alter ED₅₀ values beyond a 2-fold change from wild-type hPRL. Mutants with ED₅₀ values that deviated greater than 2-fold from wild-type hPRL were selected. Kᵦs were normalized by calculating the ratios of mutant and wild-type hPRLs. The ED₅₀ and Kᵦ ratios for the various mutant hPRLs show a significant correlation (R = 0.891).
remainder of mutant hPRLs could not report site 2 $K_d$s because heterodimers did not provide a sufficiently stable complex to bind a second ECD-receptor. The site 2 $K_d$ for ECD-receptor binding to the wild-type heterodimeric complex was 60.1$\mu$M. This value was similar to those reported by Jomain et al. (2007) and Broutin et. al. 2010 (65, 67) and confirms that site 2 binding is quite weak. The site 2 $K_d$s for the R48A and Y96E hPRLs, mutations that did not influence their ED50s, were 80.9$\mu$M and 38.0$\mu$M. Site 2 $K_d$s for three hPRLs (L95E, H97, and V99D) whose mutations were associated with a weaker site 1 bindings fell within the range of the control $K_d$s and were within approximately 2-fold of each other (38.0-80.9$\mu$M). The approximate 2-fold range of site 2 $K_d$s of various mutant hPRLs is small compared to the 40-fold range of site 1 $K_d$s.

There was no correlation between the normalized site 2 $K_d$s and either the ED50s or the site 1 $K_d$s.

### 3.3.8 Site 2 Binding of Coupled hPRL to the ECD-Receptor

A second investigation of site 2 was performed by coupling hPRL to the chip and flowing ECD-receptor over the chip surface. Two hPRL cysteine mutants (M158C and G129C) were thiol-coupled to the chip surface at specific locations of the hPRL.

The molecular weight of ECD-receptor and hPRL are roughly equivalent, so a 1:1 stoichiometry would be reached if the number of RU of hPRL coupled to the chip surface was equivalent to the RU of ECD-receptor retained on the chip surface were equal. A greater than 1:1 stoichiometry would indicate more than 1 receptor was bound per hPRL coupled to the chip surface and would indicate ECD-receptor binding at site 2.
G129C was observed to bind ECD-receptor nearing a 1:1 stoichiometry with site 2 blocked (Figure 3.14 A). The binding and dissociation of ECD-receptor was similar to the curve shown in Figure 3.12. A 1:1 stoichiometry was approached at the highest concentrations of ECD-receptor with roughly 1800 RU of receptor bound to the chip surface. The blocking of site 2 by coupling the hormone to the chip surface at residue 129 prevents any site 2 binding. All observed change in RU would be the result of site 1 binding.

M158C was observed to bind ECD-receptor with a 1.6:1 stoichiometry of receptor:hormone (Figure 3.14 B). The increase in RU beyond 2000 RU indicates site 2 binding. Roughly 3200 RU of ECD-receptor was bound to the chip at the highest concentrations. The end of the injection of ECD-receptor at roughly 800 seconds shows a rapid decrease in RU and falls almost instantaneously to 2000RU. This would indicate a 1:1 post-injection stoichiometry of receptor:hormone and is the only species remaining on the chip surface. This would indicate that site 2 dissociation is very rapid and site 2 affinity is very weak. The observation here confirms the previous observation of a weak site 2 affinity and a rapid site 2 dissociation.

3.4 Discussion

The residues explored in this report are found in the N-terminal portion of loop 1, the C-terminal portion of helix 2, and the N-terminal portion of helix 4 (Figure 3.2 A). None of these residues is within the structural epitopes of site 1 or 2 (67, 84). Thus, we have explored residues that may represent a motif coupling sites 1 and 2 of hPRL.
Figure 3.14 Surface Plasmon Resonance of ECD-Receptor Binding hPRL

SPR showing of the interaction between hPRL and ECD-receptor. Coupled to the chip is (A) hPRL M158C and (B) G129C. An injection of hormone was made between 75 and 800 sec. Buffer was flowed over the chip surface after the injection until the end of the experiment. ECD-receptor was flowed over the chip surface at concentrations of 1, 10, 50, 100, and 500nM, 1, 5, 10, 50, and 100µM. The M158C mutation is distal from both site 1 and 2 and should allow for binding at both sites. G129C is located in site 2 and should not allow for site 2 binding. Roughly 2000 RU of both M158C and G129C are coupled to the chip surface. A 1:1 receptor/hormone stoichiometry is reached in (A) and a 1.6:1 receptor/hormone stoichiometry is reached in (B) indicating site 2 binding.
Biophysical studies have shown a structural coupling of sites 1 and 2 (82, 83). We have previously identified residues in an analogous region of hGH that when binding the hPRL receptor were interpreted to function as a coupling motif between sites 1 and 2 (156). In that work F44, L93, Y160, L163, and Y164 were identified and closely articulate in that structure (Figure 3.2 C). When the amino acid sequences for hPRL and hGH are aligned, several residues identified in hPRL were also identified in hGH when binding to the hPRL receptor (Figure 3.2 bottom panel). The residues identified in hPRL by increases in ED50s and site 1 Kd can be broken into three groups. Residues F50, K53, and A54 were identified and within the loop connecting helices 1 and 2. F50 aligns with F44 of hGH which is critical for lactogen function (156, 159). The second group in hPRL is located in helix 2 and contains L95 and V99; helix 2 also contained H97. Helix 2 contains no residues of either sites 1 or 2 and is the structural element most distant from these binding surfaces (65, 67). In hGH L93 was identified in a similar area of helix 2 but it does not align with the residues identified in hPRL. These data indicate a functional role for helix 2 in producing lactogenic activity. Finally, residues Y169, L171, and H173 are located in helix 4. In hGH Y160, L163, and Y164 were previously identified and two of these align with the residues identified in hPRL. Spatial comparison of the residues identified in hPRL and hGH show a tightly packed motif in hGH while the residues in hPRL are found with a looser articulation. The loose packing of these residues in hPRL may explain why these mutations do not form a contiguous structural motif able to link the Kd for sites 1 and 2. Thus, although the residues affected by mutation in hPRL and hGH share several sequence similarities, they are functionally nonequivalent. Mutations in this work that
lowered biological activity also reduced the $K_d$s at site 1. We found no correlation between mutation-induced changes in the $K_d$s for sites 1 and 2. Our data does not identify a motif that couples sites 1 and 2. Although residues other than those explored in this work may provide a coupling motif, this report has not identified a contiguous structural motif that explains observed FRET-based data (82, 83) that showed structural coupling of sites 1 and 2. These FRET studies show a physical conformation change in hPRL induced by ECD-receptor binding at site 1. Tsai et al. (2008) (160) propose that allostery may or may not involve a detectable binding-induced conformation change but more importantly that allosteric behavior results from changes in the ensemble of conformers visited by the protein. Such a mechanism would explain observed FRET studies (82, 83) and appear to be plausible for regulating site 2 binding. Our data show a significant correlation ($R = 0.891$) between site 1 $K_d$s and ED$_{50}$s for proteins with mutations between sites 1 and 2. This tight relationship along with weak site 2 binding affinity suggests that hPRL receptor binding to site 1 of hPRL serves as the gatekeeping event for the induction of biological activity. It also suggests that mutations to these residues reduce the ability for the hormone to bind at site 1. This is in contrast with hGH where alterations in the analogous residues appear to leave site 1 unaltered while reducing the affinity at site 2.

The normal range of serum hPRL in adult women is 0.16 to 1.01nM (3.8 to 23.2ng/mL) (Burtis and Ashwood, 2001) (161). This concentration range fits the 1.26nM site 1 $K_d$ reported in this work and describes a functional binding isotherm at site 1 creating a *heterodimeric* complex. This work and that of others (65, 67) reports the site 2 affinity of hPRL to be in the $\mu$M range. This second binding event occurs in the plane of
the plasma membrane through one or both of two proposed mechanisms including a sequential binding of two independent receptor monomers and/or binding to pre-formed hPRL receptor dimers (162, 163). hPRL’s weak site 2 affinity requires some additional interface to create hPRL-induced receptor dimers. This may be provided by the transmembrane domain dimerization interface (162). In such a mechanism the juxtaposition of hPRL’s site 2 is brought about by receptor binding at site 1 and the second receptor could provide an interaction where the dominant conformers of hPRL could position the two receptors into a biologically active position.
CHAPTER 4

PROLACTIN RECEPTOR ANTAGONISTS

4.1 Introduction

hPRL is known for its role in mammary growth, development and lactation (164) and more recently for its mitogenic activity and a role as a viability factor (165). Elevated serum concentrations of hPRL have been associated with a higher risk of breast cancer (166) and the overexpression of hPRL in prostate tissue has been shown to drastically increase the size of the prostate while reducing apoptosis (167). hPRL has even been shown to act as an antagonist to certain chemotherapy drugs such as cisplatin, doxorubicin, and taxol by activating the detoxifying enzyme glutathione-S-transferase which binds to the drugs and removes them from cells (168, 169). There have been attempts to diminish circulating hPRL by repressing its release from the pituitary, however, these attempts were not successful in the treatment of breast cancer, possibly by failure to suppress paracrine/autocrine hPRL secretion in the breast (170). The failure of pituitary hPRL suppression to reduce the growth of breast cancers is now thought to be due to paracrine/autocrine hPRL production. This realization has led to the hPRL receptor as a new target for suppressing the activity of hPRL.
Currently, the target for therapy to prevent signaling through the hPRL receptor is to develop a receptor antagonist. Several groups have proposed modified hPRLs as potential antagonists. Receptor activation happens in an obligate ordered fashion where one receptor binds hPRL at site 1 which induces a conformational change in the hormone to allow a second receptor to bind at site 2 of the hormone (82). Attempts to antagonize receptor activity have largely relied on impeding or reducing the affinity of hormone for the receptor at site 2 and this became the basis of the first strategy implemented in the creation of a receptor antagonist. One such antagonist, G129R, is thought to have weak site 2 affinity to the hPRL by replacement of a glycine at position 129 of the hPRL with a large and charged arginine residue (171, 172). It is thought that the glycine at position 129 is the location of binding/interaction between the second receptor and site 2 on the hormone by the insertion of a receptor tryptophan (Trp 72) into a pocket of the hormone where glycine forms its bottom (65). The large arginine side chain in the G129R antagonist is thought to occupy this pocket and inhibit the insertion of the tryptophan by steric hindrance, thus blocking site 2 binding between the hormone and the second receptor and not allowing a productive orientation of the two receptors (Figure 4.1). Despite the potential steric hindrance, binding at site 2 was shown to decrease only 10-fold, and in cell proliferation assays exhibited weak agonist activity rather than antagonist (82).

Posttranslational modifications of the hormone do occur, and one such modification includes phosphorylation. Rat PRL was observed to be phosphorylated at serine 177, and such a modification antagonizes proliferation (54). A mimic was created
Figure 4.1 Surface Representation of Δ1-9/G129R

Structures of (A) hPRL, PDB no. 1RW5 (69) and (B) hPRL Δ1-9/G129R, PDB no. 2Q98 (65). G129 of hPRL (left) and R129 of Δ1-9/G129R are highlighted in red.
with hPRL by substituting an aspartate for the serine at residue 179 (Figure 4.2) (55). The S179D mutation was shown to work as an antagonist in a manner where the receptor would become activated and STAT5 would become phosphorylated without activating JAK2 (Figure 4.2) (173). These investigators postulated that the hPRL receptor would indeed become activated, but would activate alternative pathways other than those induced by hPRL for cellular proliferation. This became a second class of receptor antagonist. Instead of altering site 2, alternative signaling pathways would be activated without inducing cell proliferation. Work done by an independent group showed that S179D did not work as an antagonist and was even shown to act as an agonist under certain conditions (56). Thus, the original investigators hypotheses regarding this molecule have been controversial and the mechanism by which S179D antagonizes the actions of hPRL remains unclear.

Both a 22kDa (190 residues) and a 20kDa (175 residues) isomer of hGH are present in the pituitary (174). The smaller 20kDa isoform is a product of alternative splicing that is expressed without residues 32 to 46 (98). These residues constitute the C-terminal portion of helix 1 and the unstructured region that connects to helix 2. Unlike the 22kDa hGH, the 20kDa isoform was shown to lack lactogenic activity (99). The sequences of the 20kDa hGH isoform and hPRL were aligned and it was determined that residues 41 to 52 of hPRL aligned with residues 32 to 46 of hGH (175). In light of this, the protein with the deletion of residues 41-52 (Δ41-52) was expressed and showed a decrease in lactogenic activity of 10,000-fold as opposed to only a 200-fold reduction of lactogenic activity in the 20kDa hGH isoform.
Figure 4.2 Structure of Δ41-52 and S179D
Structure of hPRL, PDB no. 1RW5 (62). Highlighted are (A) residues 41 to 52 (cyan) and (B) residue S179. Both figures also highlight helix 1 (red), helix 2 (yellow), helix 3 (green), and helix 4 (purple).
(Figure 4.2). Residues in site 2 of Δ41-52 are unchanged, however, there was no evidence of site 2 binding. Upon receptor binding site 1, a conformational change occurs within the hormone to allow for site 2 binding. It was hypothesized that the deletion of these residues created a protein that could not undergo the conformational change needed to create a site 2 interface capable of receptor binding (175). Additionally, cell assays showed that Δ41-52 also induced cell death by apoptosis. The lack of functional coupling needed to connect the two binding interfaces after binding a receptor at site 1 became a third strategy in the creation of a receptor antagonist.

Further observations comparing hPRL and hGH showed that the N-terminal region of hPRL and hGH were most divergent, specifically the N-terminus of hPRL is nine residues longer and the remaining N-terminal residues are unique when comparing hPRL and hGH (17, 176). The N-terminal loop of hPRL, consisting of the first 14 residues prior to helix 1, was largely thought to be unimportant in terms of binding until a crystal structure of ovine PL bound to the rat PRL receptor showed that the N-terminus was involved in binding at site 2 (77). This was an important finding because the N-terminal domain of ovine PL is very similar to that of hPRL. Additionally, a recent structure of hPRL bound in a heterotrimeric complex with two rat PRL receptors show the interaction between this 14 residue N-terminal domain of hPRL and the second receptor at site 2 (Figure 4.3) (67). In light of this, several N-terminal hPRL deletion mutants were created. The first involved the deletion of the first nine residues to mimic the N-terminal region of hGH, termed hPRL Δ1-9 (Figure 4.4). The second was a
Figure 4.3 Surface Representation of hPRL Bound at Site 2 to the Rat PRL Receptor
Surface representation of hPRL bound to the rat PRL receptor, PDB no. 3EW3 (77). Represented are S1 (white) and S2 (cyan) of the rat PRL receptor and helix 1 (red), helix 3 (green) and residues 1 to 14 (yellow). Image is rotated 90° along the x axis (right).
Figure 4.4 Surface Representation of hPRL and Δ1-9/G129R
(A) Structure of hPRL, PDB no. 1RW5 (62). Highlighted are residues 1 to 14 (yellow) and residue G129 (red). (B) Structure of hPRL Δ1-9/G129R, PDB no. 2Q98 (65). Highlighted is residue G129R (red). Images in (A) and (B) are rotated 90° on the Z axis (top right and bottom right).
deletion of the first 14 residues which deleted the entire N-terminal loop, termed hPRL Δ1-14 (177). The Δ1-9 deletion was shown to slightly increase biological activity through binding at site 1, while the Δ1-14 deletion was shown to slightly decrease biological activity through binding affinity, hypothesized to affect site 2 (177).

Two double mutants were created, Δ1-9/G129R and Δ1-14/G129R, and were shown to have similar dose-response curves (177), and like G129R, had a 10-fold reduced affinity for the hPRL receptor at site 1 (82). Both double mutants, however, displayed no agonism and failed to stimulate target cell proliferation in bioassays (177). The ability of these two double mutants to both bind the receptor while not inducing cell proliferation gives them the potential to be hPRL receptor antagonists.

Small cyclic peptides have also been proposed as receptor antagonist (178). Library screening discovered cyclic peptides that had an affinity for the hPRL receptor. The affinity of the peptide for the hPRL receptor were shown to be weak (K_d of 2-3µM) and these peptides did not inhibit the binding of hPRL to the ECD-receptor.

The studies involving these various candidate hPRL antagonists have largely been done independently of one another and the experiments that have been performed to characterize these molecules have been limited. The goal of this work is to compare all hPRL antagonists under similar conditions so the observations of antagonism, or lack thereof, are comparable. Further, the goal of creating antagonists is to use them as potential drugs in the treatment of cancer and characterization of the structure and stability of these molecules is necessary. Information such as this would be useful in the
mass production of a successful antagonist and may bring to light any pitfalls that may prevent the production of the antagonist at a pharmaceutical level.

4.2 Results

4.2.1 SDS-PAGE

Each antagonist was expressed as previously described and each preparation yielded 20-50mg per liter of fermentation. The purified antagonists were applied to SDS-PAGE to assess purity and molecular weight migration (Figure 4.5). Single bands at the anticipated molecular weight for each antagonist indicates a single species of each protein.

4.2.2 UV and Fluorescence Spectroscopy

Recombinant proteins were characterized by UV spectroscopy. Each protein showed an absorption peak at 277nm, characteristic of aromatic amino acids (Figure 4.5). All preparations had a 280/260 ratio greater than 1.5, similar to that of pure preparations of hPRL. Absorption at 350nm was absent indicating an absence of aggregated proteins detected by light scatter. The shape of the absorbance was similar for all hPRLs (Figure 4.6).

Fluorescence spectra of the antagonists, in comparison to that of wild-type hPRL, did not shift greatly from the maximum peak height at 340nm and the overlaid spectrum showed minimal variation with the exception of Δ41-52 (Figure 4.6). These findings suggest the environment surrounding the tryptophan residues were not greatly influenced
Figure 4.5 SDS-PAGE and UV Absorbance of Wild-Type hPRL and Antagonists
(A) 15% SDS-PAGE with 2-mercaptoethanol and 15-20μg of wild-type hPRL and antagonists. All proteins migrated to anticipated positions on the gel. (B) Absorbance spectra of wild-type hPRL and antagonists. No significant conformational variances were observed when comparing wild-type hPRL to antagonists. 20μM samples were prepared in 150mM NaCl, 10mM Tris-base, pH 7.4. The ultraviolet spectrum was measured from 200nm to 350nm.
Figure 4.6 Fluorescence Spectra of Wild-Type hPRL and Antagonists
Fluorescence spectra of wild-type hPRL and antagonists at 1µM in 40mM KH₂PO₄, pH 7.3. Spectra were collected at 25°C. All antagonists except for Δ41-52 share a similar fluorescence spectra to that of wild-type hPRL indicating a difference in folding.
by a change in hydration indicating the proteins were folded similarly to that of wild-type. Δ41-52 did have a change in fluorescence spectra indicating the environment surrounding the tryptophan residues are different from that seen in wild-type hPRL. This suggests a difference in folding between the two proteins. There are two tryptophan residues in the mature hPRL at positions 91 and 150. Residue 91 is in helix 2 and the loss of residues to create the Δ41-52 deletion between helix 1 and 2 may create strain on helix 2. This may cause a change in hydration of the tryptophan and decrease the fluorescence.

4.2.3 Circular Dichroism

CD of antagonists when compared to wild-type hPRL showed characteristic curves for α-helical proteins. Two negative peaks at 220nm and 208nm and a positive peak approaching 190nm were observed. The CD spectrum of each protein was measured with at least two preparations of each protein. Different batches of protein were also analyzed to ensure a lack of variation between batches. This lack of variation suggests that similar folds were achieved for various batches of protein. The CD spectrum of each protein was converted into molar ellipticity to account for the molecular weight. This is necessary in order to compare mutants that have a deletion of a significant number of residues. Δ1-14, G129R, and Δ1-14/G129R displayed a similar molar ellipticity when compared to wild-type hPRL (Figure 4.7). The Δ1-14, G129R, Δ1-14/G129R and S179D mutations should not cause the loss of helical content if these changes did not influence folding. Δ41-52 and S179D displayed a significantly decreased
Figure 4.7 Circular Dichroism of Wild-Type hPRL and Antagonists
CD spectra of (A) wild-type hPRL and all antagonists and (B) a magnified view of wild-type hPRL and select antagonists. Δ41-52 and S179D displayed a decrease in molar ellipticity which would indicate a decrease in secondary structure. Δ1-14, G129R and Δ1-14/G129R displayed a similar molar ellipticity to that of wild-type hPRL.
molar ellipticity when compared to wild-type hPRL indicating decreased helical content. The loss of secondary structure for the Δ41-52 deletion was expected. This deletion removes residues from the end of helix 1 and into the unstructured region connecting helix 1 to helix 2. The loss of residues that connect these two helices would be thought to create strain and would potentially disrupt the formation of helix 1 and or helix 2 during folding. The loss in secondary structure of S179D was unexpected. A single mutation would not be thought to cause the loss of nearly half the secondary structure in comparison to wild-type hPRL. One explanation for the loss in secondary structure could be that the side-chain of S179 points directly inward towards the other three adjacent helices. This residue is located halfway down helix 4 and is in the very middle of the protein. A mutation from an uncharged serine to a larger charged aspartic acid could destabilize the protein and/or prevent proper folding.

The CD spectrum of each protein was entered into the K2D3 fitting algorithm (152) to estimate percent α-helix. Multiple spectra were taken of each protein and are displayed in Table 4.1. Δ1-14 displayed a reduced percent α-helix when compared to that of wild-type hPRL. The deletion of the first 14 residues appears to have decreased the helical content. The Δ1-14 deletion removes no residues within the helices. The loss in secondary structure may come from helix 1 considering the deletion of residues includes the unstructured region and the first residue of helix 1. This may affect the formation of secondary structure immediately after this deletion.

The G129R mutation increased the secondary structure of the protein. The glycine at position 129 is the last residue in helix 3 and this mutation may increase the secondary
structure of this or a neighboring helix. Δ1-14/G129R appears to be the average of percent helix between Δ1-14 and G129R. The loss in helix from the Δ1-14 mutation appears to be offset by the increase in secondary structure of the G129R mutation. Of these three antagonists, the indication would be that the mutations made to G129R and Δ1-14 G129R did not significantly alter the secondary structure but differences were observed. Δ1-14 was shown to have a significant decrease in % α-helix. A t-test of wild-type hPRL showed 95% confidence from 48.38-52.64% which would indicate that the mutations made to Δ1-14 caused a statistically significant loss in secondary structure. Δ41-52 and S179D displayed decreased percent α-helix when compared to that of wild-type hPRL. This would indicate that the mutations made to these proteins had a significant influence on the secondary structure. Δ41-52 lost several residues in the C-terminus of helix 1, but this deletion could not account for the 31.4% reduction in helical content. A single mutations to S179D would not be expected to decrease percent α-helix by 42% indicating serine 179 is an important residue for folding and the resulting secondary structure.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>% α-helix retained vs. wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type hPRL</td>
<td>100%</td>
</tr>
<tr>
<td>Δ1-14</td>
<td>88.09%</td>
</tr>
<tr>
<td>G129R</td>
<td>102.88%</td>
</tr>
<tr>
<td>Δ1-14/G129R</td>
<td>95.92%</td>
</tr>
<tr>
<td>Δ41-52</td>
<td>68.63%</td>
</tr>
<tr>
<td>S179D</td>
<td>57.95%</td>
</tr>
</tbody>
</table>

Table 4.1 Percent α-helix of Wild-Type hPRL and Antagonists
Percent α-helix of wild-type hPRL and antagonists from CD spectra data using a fitting algorithm K2D3 (mean ± SD (replicates)) and percentage of helical content retained vs. that of wt hPRL (% antagonist / % wt hPRL).
4.2.4 Differential Scanning Calorimetry

The thermal stability of each protein was assessed using DSC. The melting temperature ($T_m$) of the wild-type hPRL and each antagonist will give insight into the thermal stability of these proteins. The degree to which there are conformational changes in the structure of the mutant antagonists will be evident in the comparison to the wild-type hPRL. Experimental details are described in the methods section of 2.3.5.

Wild-type hPRL has a melting peak and a smooth transition from a folded state to an unfolded state (Figure 4.8). The DSC data best fit a two-component melting curve for wild-type hPRL as fit by the Origin® software. Each antagonist displayed a transition from a folded to an unfolded state, however, each also displayed a large endothermic spike either during or shortly after the antagonist unfolded (Figure 4.9). The large endothermic spike is presumed to be the cause of aggregation of the proteins following the thermal denaturation. This aggregation was seen in every protein but wild-type hPRL.

In order to fit the plot using the Origin® software, the transition to an unfolded state needs to be near completion in order to calculate an accurate $T_m$. A number of buffer conditions were employed to try to decrease aggregation. Decreasing the protein concentration was seen to be helpful at delaying aggregation to higher temperatures and allows a greater range of temperatures to be recorded and $T_m$s to be calculated. A change from a potassium phosphate buffer to a HEPES buffer was tried but did not decrease aggregation. Addition of arginine has been shown to be effective at reducing aggregation during a thermal melt by DSC (179). A number of different conditions were tried for each antagonist but all resulted in aggregation during or after the transition from folded to
Figure 4.8 Differential Scanning Calorimetry of Wild-Type hPRL
DSC of a 100µM sample of wild-type hPRL in 40mM KH$_2$PO$_4$, pH 7.3. The background has been subtracted using VPViewer™ software and the curve was fit using Origin® software to determine $T_m$. A two-state transition was fit for wild-type hPRL and $T_m$s were observed at 61.79°C and 76.32°C.
Figure 4.9 Differential Scanning Calorimetry Aggregation of Antagonists
DSC of (A) a 50µM sample G129R in 25mM HEPES, 150mM NaCl, pH 7.8 and (B) a 100µM sample of Δ1-14/G129R in 40µM KH₂PO₄, pH 7.3. The background has been subtracted using VPViewer™ software but the curves could not be fit due to aggregation at approximately 80-85°C.
unfolded. Aggregation was able to be delayed for each antagonist and a near complete transition was able to be observed at least once and a T_m could be determined for each. The transition from a folded to an unfolded state was fit in the Origin® software and a T_m for each protein was determined. A delay in aggregation beyond the T_m, and the absence of aggregation allows for the best fit and determination of the T_m and finer details about the transition states are observed. Wild-type hPRL was able to be fit for a two-state transition indicating an intermediate transition from folded to unfolded (Table 4.2).

G129R, using one set of conditions, was also able to be fit for a two-state transition under one set of conditions. The remaining antagonists could only be fit for one T_m. The first T_m (T_m1) for wild-type hPRL was observed to be elongated over a very large temperature range (~35°C to ~85°C) and the peak height of T_m1 was less than T_m2. Because the fit of the antagonists was not precise due to aggregation, it is presumed that the T_m1 of the remaining antagonists could not be determined because aggregation did not allow a smooth melting curve to fit multiple T_m$s.

The T_m for Δ1-14, G129R, and Δ1-14/G129R was each seen to be less but similar to that of wild-type hPRL (Figure 4.10 and Figure 4.11). The curve for Δ1-14/G129R was not able to be fit by the Origin® software, however, a T_m was able to be determined by defining the peak maximum (Figure 4.9). Δ41-52 and S179D were each observed to have a decreased T_m when compared to that of wild-type hPRL indicating decreased thermal stability (Figure 4.11).
Figure 4.10 Differential Scanning Calorimetry of G129R

DSC of (A) a 10µM sample of G129R in 40mM KH$_2$PO$_4$, pH 7.3 with a $T_m$ of 74.65°C. (B) A 50µM sample of G129R in 25mM HEPES, 150mM NaCl, pH 7.8 with a $T_m$ of 73.01 °C. And (C) a 50µM sample G129R in 100mM NaH$_2$PO$_4$, 100mM arginine, pH 7.0 that was fit for a two-state transition with $T_m$s of 65.04 and 74.24. The background has been subtracted using VPViewer™ software and the curve was fit using Origin® software to determine $T_m$s.
Figure 4.11 Differential Scanning Calorimetry of Δ1-14, Δ41-52 and S179D

DSC of (A) a 50μM sample of Δ1-14 in 40μM KH2PO4, pH 7.3 with a Tm of 72.22°C. (B) A 200μM sample Δ41-52 in 40μM KH2PO4, pH 7.3 with a Tm of 65.57°C. And (C) a 50μM sample of S179D in 40μM KH2PO4, pH 7.3 with a Tm of 61.84°C. The background has been subtracted using VPViewer™ software and the curve was fit using Origin® software to determine TmS.
Table 4.2 Melting Temperature of Wild-Type hPRL and Antagonists

$T_m$ determination using DSC of wild-type hPRL and antagonists. Origin® software was used to determine a two-state denaturation ($T_m1$ and $T_m2$) of certain proteins. A one-state fit for the remaining proteins resulted in a single $T_m$.

* Indicates the $T_m$ for this value was determined by defining the peak maximum of the melting curve.

A correlation was observed between the percent $\alpha$-helix and the $T_m$ of wild-type hPRL and the antagonists. The relative loss of secondary structure as determined by percent $\alpha$-helix was observed to diminish proportionately to the loss of $T_m$ for wild-type hPRL and antagonists. The only instance where this didn’t hold true would be the percent $\alpha$-helix of G129R being greater than wild-type hPRL. Wild-type hPRL was observed to have the highest $T_m$ and the second highest percent $\alpha$-helix. It appears G129R is the most conservative of the mutants by these two observations. Δ1-14/G129R has the next highest $T_m$ and percent helix followed by Δ1-14. Δ1-14/G129R appears to be an average between Δ1-14 and G129R in terms of $T_m$ and percent $\alpha$-helix. G129R may serve to stabilize the Δ1-14 mutation indicated by both the increased secondary structure and thermal stability of Δ1-14/G129R. Δ41-52 served as the second lowest $T_m$ and percent $\alpha$-helix followed by

<table>
<thead>
<tr>
<th>Protein</th>
<th>$T_m$ 1</th>
<th>$T_m$ 2</th>
<th>Buffer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt hPRL</td>
<td>61.79</td>
<td>76.32</td>
<td>KH$_2$PO$_4$</td>
<td>100µM</td>
</tr>
<tr>
<td>G129R</td>
<td>x</td>
<td>74.65</td>
<td>KH$_2$PO$_4$</td>
<td>10µM</td>
</tr>
<tr>
<td>G129R</td>
<td>x</td>
<td>73.01</td>
<td>HEPES</td>
<td>50µM</td>
</tr>
<tr>
<td>G129R</td>
<td>65.04</td>
<td>74.24</td>
<td>Arginine</td>
<td>50µM</td>
</tr>
<tr>
<td>Δ1-14</td>
<td>x</td>
<td>72.22</td>
<td>KH$_2$PO$_4$</td>
<td>50µM</td>
</tr>
<tr>
<td>Δ1-14/G129R</td>
<td>x</td>
<td>~73.5*</td>
<td>KH$_2$PO$_4$</td>
<td>100µM</td>
</tr>
<tr>
<td>Δ41-52</td>
<td>x</td>
<td>65.57</td>
<td>KH$_2$PO$_4$</td>
<td>200µM</td>
</tr>
<tr>
<td>S179D</td>
<td>x</td>
<td>61.84</td>
<td>KH$_2$PO$_4$</td>
<td>50µM</td>
</tr>
</tbody>
</table>
S179D. The mutations to both Δ41-52 and S179D have had an impact on the secondary structure and thermal stability when compared to that of wild-type hPRL.

4.2.5 Biological Assays

4.2.5.1 Agonist Assays of Wild-Type hPRL and Antagonists to Determine ED_{50} Values

*In vitro* cell assays were performed using lactogen-responsive eukaryotic cells to determine the biological activity of wild-type hPRL and antagonists. The ability of the hormones and antagonists to stimulate cell growth in agonist assays is reflected in the ED_{50} value and is a reasonable measure of the agonist activity. It is important to measure the ED_{50} of the value of the antagonists to determine the extent to which they promote proliferation. Agonist activity to promote cell growth would be seen as a negative for an antagonist. Assays ranged in concentration from 0.01nM to 10µM for both wild-type hPRL and antagonists. A representative dose-response curve for wild-type hPRL is shown in Figure 4.12. The ED_{50} values were determined using a four parameter sigmoidal fit using SigmaPlot 12.3. A plateau in the percent reduction of the vital dye is needed in order to fit a sigmoidal curve. All fits were only acceptable if the R^2 value exceeded 0.9. Assays were performed three times and the results were averaged (Table 4.3). The percent reduction of the vital dye was small and the plateau of the signal is not a product of substrate depletion. The standard deviation of the replicates in the agonist and antagonist assays were small and the addition of standard error bars was not necessary.
Figure 4.12 Agonist Assay of Wild-Type hPRL
Agonist assay assessing the hormone-induced response of (A) wild-type hPRL. Concentrations of hormone ranged from 0.01nM to 10µM. Decrease in average percent reduced was observed beyond 100nM. (B) Four parameter sigmoidal fit of the dose-response curve to determine the ED$_{50}$. Ascending portion of the curve (0.01nM to 100nM) was used to provide the best fit to determine an ED$_{50}$ value. Assays were performed in triplicate and the results averaged. The standard deviation of these replicates was small and the addition of standard error bars was not necessary.
Table 4.3 ED$_{50}$ Values Determined by Agonist Cell Assays

Agonist assays to determine ED$_{50}$ values with standard deviations for wild-type hPRL and antagonists. Values were determined by a four parameter sigmoidal fit using SigmaPlot 12.3 software.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>ED$_{50}$ (nM)</th>
<th>Antagonist/WT Ratio of ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type hPRL</td>
<td>0.83 ± 0.17</td>
<td>1</td>
</tr>
<tr>
<td>Δ1-14</td>
<td>2.98 ± 1.04</td>
<td>3.59</td>
</tr>
<tr>
<td>G129R</td>
<td>42.06 ± 11.79</td>
<td>50.67</td>
</tr>
<tr>
<td>Δ1-14/G129R</td>
<td>N/A*</td>
<td>N/A</td>
</tr>
<tr>
<td>Δ41-52</td>
<td>N/A**</td>
<td>N/A</td>
</tr>
<tr>
<td>S179D</td>
<td>603.6 ± 67.88***</td>
<td>727.23</td>
</tr>
</tbody>
</table>

* No agonist activity was observed for Δ1-14/G129R

** Agonist assay for Δ41-52 could not be fit and an ED$_{50}$ could not be determined

*** Data for S179D is from the mean of two agonist assays

The ascending portion of each of the curves were over a 3.5 to 4 orders of magnitude hormone concentration and were similar for each hPRL that displayed agonist activity. Maximum response for wild-type hPRL is observed at 10nM and will be useful later when performing antagonist assays to determine IC$_{50}$ values. The percent reduction begins at a minimum of 0.04 and reaches a maximum of 0.19, a change of 0.15. The dose-response curve begins to fall above 100nM. This phenomenon has been documented previously in hGH bioassays using FDC-P1 cells (156). Because of this fall in percent reduction of the vital dye above 100nM, the dose-response curve was fit using the data from .01nM to 100nM. This provided the best fit to determine the ED$_{50}$ value. The significance of the decrease in cell proliferation with increasing concentrations above 100nM is unknown.

Maximum percent reduction for Δ1-14 is observed at 13nM and the percent reduction begins at a minimum of 0.04 and reaches a maximum of 0.13, a change of 0.09.
The curve begins to fall at 100nM which is similar to what was seen in the agonist assay of wild-type hPRL (Figure 4.13). The response at 100nM promoted the same intensity of cell proliferation as wild-type hPRL, but then fell almost to baseline level. This fall in stimulus beyond 100nM was greater than that of wild-type hPRL and the mechanism of this difference is unclear.

Maximal percent reduction for G129R is observed at 300nM and the percent reduction begins at a minimum of 0.06 and reaches a maximum of 0.14, a change of 0.08. G129R maintains maximal proliferation at the highest doses used in the assay (10µM). Unlike wild-type hPRL or Δ1-14 hPRLs, the growth response did not fall at high concentrations (Figure 4.14 A). Δ1-14 and G129R were observed to elicit about half of the change in percent reduction compared to wild-type hPRL of 0.09 and 0.08 respectively as opposed to a change of 0.15 for wild-type hPRL. Decreased change in percent reduction might be explained by a corruption at site 2 where fewer active trimeric complexes could be maintained (156, 158). This is reasonable because both structural changes are proximal to site 2.

Δ1-14/G129R showed little to no response in any of the agonist assays performed. The reduction begins at a minimum of 0.050 and reaches a maximum of 0.065, a change of 0.015. The baseline of the assays here were similar to the baseline of that seen with wild-type hPRL (Figure 4.14B). The response rose slightly as the concentration increased, however, the rise in the response is negligible when compared to that of wild-type hPRL and the remaining antagonists (Figure 4.15A). When the dose-response curve of Δ1-14/G129R is overlaid against that of the wild-type and the remaining antagonists, it
Figure 4.13 Agonist Assay of Δ1-14
Agonist assay assessing the hormone-induced response of (A) Δ1-14. Concentrations of hormone ranged from 0.01nM to 10µM. Decrease in average percent reduced was observed beyond 100nM. (B) Four parameter sigmoidal fit of the dose-response curve to determine the ED$_{50}$. Ascending portion of the curve (0.01nM to 100nM) was used to provide the best fit to determine an ED$_{50}$ value. Assays were performed in triplicate and the results averaged. The standard deviation of these replicates was small and the addition of standard error bars was not necessary.
Figure 4.14 Agonist Assay of G129R and Δ1-14/G129R

(A) Agonist assay and four parameter sigmoidal fit of G129R. (B) Agonist assay assessing the hormone-induced response of Δ1-14/G129R. Assays were performed in triplicate and the results averaged. The standard deviation of these replicates was small and the addition of standard error bars was not necessary.
Figure 4.15 Agonist Assays of Wild-Type hPRL and Antagonists
Overlay of all of the agonist assays of (A) wild-type hPRL, Δ1-14, G129R and Δ1-14/G129R. G129R and Δ1-14 show agonist activity while Δ1-14/G129R shows essentially no biological activity. (B) Overlay of the agonist assay for wild-type hPRL, Δ41-52 and S179D. Both show right-shifted cures with near wild-type hPRL cell proliferation at the highest concentrations.
is evident that there is essentially no biological activity. This data could not be fit by the four parameter method used in the other assays and an ED50 could not be determined.

The agonist assay for Δ41-52 displayed a right shift in the curve in comparison to wild-type hPRL and this would indicate reduced agonist activity (Figure 4.16A). The percent reduction of the vital dye nearing the highest concentration of the agonist assay (0.20) neared the same percent reduction of the vital dye seen in the wild-type hPRL agonist assay (.020) indicating cells were induced to proliferate to the same degree as wild-type hPRL. The change in percent reduction of both Δ41-52 and wild-type hPRL were also the same (0.15). Since the response to the hormone continues to rise at the highest concentration, a maximal dose could not be observed and an ED50 value could not be calculated for this antagonist. A higher concentration of hormone may be able to elicit a maximal cellular response in order to fit a sigmoidal curve for this antagonist, however, the limited solubility of this protein would not allow higher concentrations. The ascension of the percent reduction was over 2 orders of magnitude which is less than that observed with the wild-type hPRL of 3.5 to 4 orders of magnitude. If the ascension in cell proliferation continued in a manner similar to the agonist assay of wild-type hPRL, a higher maximum cell proliferation may have been reached before the plateau was met in the assay. The cells in the assay would be induced to proliferate at a higher rate than that of wild-type hPRL and would be an indication of more total cells in the wells in comparison to wild-type hPRL or the remaining antagonists and a mechanism by which this would occur is unclear.
Figure 4.16 Agonist Assay of Δ41-52 and S179D
Agonist assay assessing the hormone-induced response of (A) Δ41-52 and (B) the agonist assay and four parameter sigmoidal fit of S179D. Both assays show right-shifted curves with cell proliferation at the highest concentrations to be near that of wild-type hPRL.
The agonist assay for S179D displayed a right shift in the curve in comparison to wild-type hPRL indicating reduced agonist activity (Figure 4.16 B). The maximum cell proliferation at the highest concentration of S179D was similar to wild-type hPRL and would indicate cells were stimulated to proliferate to the same degree as wild-type hPRL. Unlike Δ41-52, a plateau was reached at the highest concentrations allowing a sigmoidal fit of the curve and the ED$_{50}$ value could be calculated. Higher concentrations of this hormone would be desired to confirm the plateau of the hormone response but higher concentrations of antagonist could not be achieved due to solubility.

The R$_2$ value of the four parameter fit of the curves to determine an ED$_{50}$ value exceeded 0.95. A wild-type hPRL agonist assay was run in conjunction with each set of agonist assays and was used as a control to ensure the consistency of the agonist assays. Wild-type hPRL consistently displayed a characteristic ED$_{50}$ value as seen in previous agonist assays. The ED$_{50}$ for both wild-type hPRL and Δ1-14 were calculated using the ascending part of the curve until the maximum response was reached in order to calculate an accurate ED$_{50}$. Δ1-14 displayed a near wild-type ED$_{50}$ with only a 3.5-fold reduction in biological activity (Table 4.3). The ED$_{50}$ for Δ-14 was similar to that wild-type hPRL and the indication would be that most agonist activity is retained with the Δ1-14 deletion.

G129R showed a near 50-fold reduction in agonist activity indicating this mutation reduces the biological activity of the hormone. This antagonist still retains agonist activity at low concentration. The Δ1-14/G129R was not observed to have any agonist activity. An ED$_{50}$ for Δ41-52 was not able to be determined because the lack of the maximal response at the highest concentrations needed to properly fit a sigmoidal
curve. S179D was seen to have a near 725-fold reduction in agonist activity. Ascending arms of the Δ41-52 and S179D curves were right-shifted to a similar extent suggesting that the loss in agonist activity for these two hormones was similar.

There appear to be two groups into which these antagonists fall into. One consists of Δ1-14, G129R and Δ1-14/G129R which reduce maximum cell proliferation as seen in Figure 4.15 A. The second group consists of Δ41-52 and S179D which create a right-shift in the agonist curve while still able to stimulate maximal cell proliferation at the highest concentrations (Figure 4.15 B). This indicates that there are two separate mechanisms present for reducing agonist action between these two groups. Agonist activity to induce proliferation was observed with all of the antagonists with the exception of Δ1-14/G129R.

4.2.5.2 Antagonist Assays of hPRL Antagonists to Determine IC₅₀ Values

Antagonist assays were performed to determine the degree to which the antagonists would block wild-type hPRL-induced proliferation. Successful antagonists reduce proliferation of cells able to be activated by hPRL. Antagonists will have to compete with circulating hPRL in order to prevent proliferation in vivo. Each assay contained 10nM wild-type hPRL and the antagonist of varying concentrations. Wild-type hPRL and antagonist were mixed prior to the introduction with the cells in the assay.

An hPRL agonist assay was performed to serve as a positive control and to determine a baseline for the assay and the highest stimulation of cell proliferation at maximum biological activity (Figure 4.17). The percent reduction begins at a minimum
Figure 4.17 Wild-Type hPRL Agonist Assay and Δ1-14 Antagonist Assay
(A) Agonist assay of wild-type hPRL displayed a characteristic curve and ED$_{50}$ value. (B) antagonist assay of Δ1-14 reduced cell proliferation to the baseline. An agonist assay was performed on the same day as antagonist assays and under similar conditions to compare agonist and antagonist activities. Both the minimum and maximum percent reduction observed in the hPRL agonist assay is displayed in all antagonist assays.
of 0.08 and reaches a maximum of 0.24, a change of 0.16 and the ED_{50} of the assay was 0.52nM. The change in the percent reduced and the ED_{50} value is both similar to previous agonist assays and are an indication of the viability of the cells in the assay.

Inhibition of cell proliferation was seen with three of the antagonists (Δ1-14, G129R and Δ1-14/G129R) (Table 4.4). Δ1-14 brought average percent reduction down from 0.27 to a baseline level of 0.08, a change of 0.19 (Figure 4.17). G129R lowered the percent dye reduced from 0.25 to 0.14, a change of 0.11 and this is a 68% reduction in agonist activity (Figure 4.18). Δ1-14/G129R brought average percent reduction down from 0.27 to a baseline level of 0.08, a change of 0.19 (Figure 4.18).

Δ41-52 and S179D did not antagonize the activities of hPRL in the antagonist cell assay (Figure 4.19). Δ41-52 actually showed agonist activity above 700nM. S179D did not show agonist or antagonist activity in these competition assays.

When the agonist assay and the antagonist assays are overlaid, it is clear that Δ1-14, G129R and Δ1-14/G129R are inhibitors of wild-type hPRL for the hPRL receptor in the assay (Figure 4.20). At the highest concentrations of antagonist, cell proliferation was antagonized to the greatest degree by Δ1-14/G129R followed by Δ1-14 and G129R being the weakest antagonist action of the three. The overlay of the agonist and antagonist assays in Figure 4.20 shows an almost equal level of cell proliferation at the highest concentrations of antagonist. This is an indication that the biological activity of the cells is primarily dictated by the antagonist in the assay. Of note is the absence of biological activity in the antagonist assay of Δ1-14/G129R to a baseline level where there
Figure 4.18 Antagonist Assays of G129R and Δ1-14/G129R
Antagonist assays of (A) G129R and (B) Δ1-14/G129R. Assays were supplemented with 10nM hPRL. Both the minimum and maximum cell proliferation observed in the agonist assay of hPRL from Figure 4.14 are displayed in the antagonist assays above. G129R reduced cell proliferation 68% while Δ1-14/G129R reduced proliferation to the baseline.
Figure 4.19 Antagonist Assays of Δ41-52 and S179D
Antagonist assays of (A) Δ41-52 and (B) S179D. Assays were supplemented with 10nM hPRL. Both the minimum and maximum percent dye reduction observed in the agonist assay of hPRL from Figure 4.14 is displayed in antagonist assays above. Δ41-52 showed agonist activity at the highest concentrations and S179D displayed no response.
Figure 4.20 Antagonist Inhibitory Assays
Antagonist assays of (A) all antagonists including Δ1-14, G129R, Δ1-14/G129R, Δ41-52 and S179D and (B) Δ1-14, G129R and Δ1-14/G129R. All assays were supplemented with 10nM hPRL. Both the minimum and maximum percent dye reduction observed in the agonist assay of hPRL from Figure 4.14 is displayed in antagonist assays above. Δ1-14 and Δ1-14/G129R reduced cell proliferation to the baseline. G129R reduced cell proliferation by 68% and S179D did not agonize or antagonize proliferation. Δ41-52 was observed to stimulate cell growth in the antagonist assay.
Table 4.4 IC$_{50}$ Values Determined by Antagonist Assays

Mean IC$_{50}$ values and standard deviations of antagonists supplemented with 10nM hPRL. Values were determined by a four parameter sigmoidal fit using SigmaPlot 12.3 software. Each of the antagonist assays were repeated three times and the results were averaged.

* IC$_{50}$ values could not be calculated because no antagonism was observed.

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ1-14</td>
<td>354.4 ± 85.84</td>
</tr>
<tr>
<td>G129R</td>
<td>101.85 ± 20.82</td>
</tr>
<tr>
<td>Δ1-14/G129R</td>
<td>247.91 ± 33.78</td>
</tr>
<tr>
<td>Δ41-52</td>
<td>N/A*</td>
</tr>
<tr>
<td>S179D</td>
<td>N/A*</td>
</tr>
</tbody>
</table>

is essentially no cell proliferation occurring.

Both Δ41-52 and S179D were shown to be full agonists in the agonist assays and showed no antagonist activity in the antagonist assay. Rather, at high doses there remain agonist activities and the proliferation is additive to the already wild-type hPRL stimulated cells. Note that no high-dose fall in cell proliferation was observed as seen in wild-type agonist studies (Figure 4.12 A). These responses in the antagonist assays were shown to mimic this rise in proliferation when the assays are overlaid (Figure 4.21).

4.2.6 Kinetic Evaluation of Antagonists Binding the hPRL Receptor

4.2.6.1 Site 1 Kinetic Evaluation

The ECD of the hPRL receptor underwent site-directed mutagenesis to add a cysteine residue to the C-terminal. The receptor has been used previously and behaved as predicted (84). The introduction of an exposed cysteine yields a free sulfhydryl that will be used to thiol-couple the ECD-receptor to the surface of a Bicacore CM5 chip. In these experiments between 700 and 2000 RU of ECD-receptor was bound to the chip surface.
Figure 4.21 Overlaid Assays of \( \Delta 1-14 \), G129R and \( \Delta 1-14/G129R \)

Overlaid plots of agonist and antagonist assays of (A) \( \Delta 1-14 \) taken from Figures 4.13 A and 4.17 B, (B) G129R taken from figures 4.14 A and 4.18 A and (C) \( \Delta 1-14/G129R \) taken from Figures 4.14 B, and 4.18 B. Biological activity of the cells in the three antagonist assays show cell proliferation is dictated by the antagonist at the highest concentrations in the assay.
on three of the available four lanes of the Biacore chip. The fourth lane served as a reference channel that was chemically activated and blocked with a saturating concentration of cysteine. Increasing concentrations of antagonists were flowed over the chip surface and allowed to interact with the ECD-receptor ranging in concentration from 10nM to 10µM as well as a blank injection containing only buffer (Figure 4.22). The response from the reference lane was subtracted from the corresponding lanes. Additionally, an injection containing only buffer would be flowed over each lane and subtracted as suggested by Myszka and colleagues (154). Data was recorded, used to calculate $k_1$, $k_{-1}$ and $K_d$ for site 1, and is listed in Table 4.5.

Wild-type hPRL displayed a characteristic $K_d$ of 1.47nM. Δ1-14, G129R and Δ1-14/G129R each displayed a moderately reduced site 1 affinity, each being around 10nM. Δ41-52 showed a roughly 16-fold reduction in site 1 affinity and S179D showed a greatly diminished $K_d$ nearing almost a 100-fold reduction compared to wild-type hPRL. The majority of the loss in affinity can be seen in the on $k_1$. All antagonists showed at least a four-fold reduction in the $k_1$. The $k_{-1}$ for all was at most a two-fold reduction. It is interesting to note that the $k_{-1}$ for Δ41-52 is slower than that of wild-type hPRL and is the only antagonist to display this characteristic.

4.2.6.2 Site 2 Kinetic Evaluation

CM-5 sensor chip with thiol-linked ECD-receptor were prepared as described in section 4.2.6.1. Δ1-14, G129R and Δ1-14/G129R were evaluated for their site 2 kinetics and hPRL was also evaluated and used as a positive control. The dissociation of Δ41-52
Figure 4.22 Overlaid Agonist and Antagonist Assays of Δ41-52 and S179D
Overlaid plots of agonist and antagonist assays of (A) Δ41-52 taken from Figures 4.16 A and 4.19 A which shows agonist activity in both assays. (B) S179D taken from figures 4.16 B and 4.19 B which shows no agonist or antagonist activity.
<table>
<thead>
<tr>
<th>Antagonist</th>
<th>On Rate Constant (k₁)</th>
<th>Mut/WT Ratio of k₁</th>
<th>Cᵥ</th>
<th>Off Rate Constant (k₋₁)</th>
<th>Mut/WT Ratio of k₋₁</th>
<th>Cᵥ</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>6.60 x 10⁵</td>
<td>1.0</td>
<td>0.34</td>
<td>8.69 x 10⁻⁴</td>
<td>1.0</td>
<td>0.04</td>
</tr>
<tr>
<td>Δ1-14</td>
<td>1.25 x 10⁵</td>
<td>0.19</td>
<td>0.03</td>
<td>1.07 x 10⁻³</td>
<td>1.23</td>
<td>0.03</td>
</tr>
<tr>
<td>G129R</td>
<td>1.05 x 10⁵</td>
<td>0.16</td>
<td>0.09</td>
<td>1.04 x 10⁻³</td>
<td>1.20</td>
<td>0.01</td>
</tr>
<tr>
<td>Δ1-14/G129R</td>
<td>1.87 x 10⁵</td>
<td>0.28</td>
<td>0.12</td>
<td>1.79 x 10⁻³</td>
<td>2.06</td>
<td>0.09</td>
</tr>
<tr>
<td>Δ41-52</td>
<td>1.25 x 10⁴</td>
<td>0.02</td>
<td>0.23</td>
<td>2.96 x 10⁻⁴</td>
<td>0.34</td>
<td>0.21</td>
</tr>
<tr>
<td>S179D</td>
<td>1.26 x 10⁴</td>
<td>0.02</td>
<td>0.08</td>
<td>1.85 x 10⁻³</td>
<td>2.13</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Dissociation Constant (K_d)</th>
<th>Antagonist/WT Ratio of K_d</th>
<th>Cᵥ</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>1.47 x 10⁻⁹</td>
<td>1.47</td>
<td>1.00</td>
</tr>
<tr>
<td>Δ1-14</td>
<td>8.54 x 10⁻⁹</td>
<td>8.54</td>
<td>5.81</td>
</tr>
<tr>
<td>G129R</td>
<td>9.99 x 10⁻⁹</td>
<td>9.99</td>
<td>6.80</td>
</tr>
<tr>
<td>Δ1-14/G129R</td>
<td>9.66 x 10⁻⁹</td>
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<td>6.57</td>
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<tr>
<td>Δ41-52</td>
<td>2.39 x 10⁻⁸</td>
<td>23.9</td>
<td>16.26</td>
</tr>
<tr>
<td>S179D</td>
<td>1.46 x 10⁻⁷</td>
<td>146</td>
<td>99.31</td>
</tr>
</tbody>
</table>

**Table 4.5 Antagonist Site 1 Rate and Equilibrium Constants**

Site 1 rate and equilibrium constants of the antagonist binding to the ECD-receptor. The ECD-receptor was coupled to the chip and antagonists were flowed over the chip surface. Studies were repeated three times and the coefficient of variation of k₁, k₋₁ and K_d are listed (Cᵥ).

Cᵥ = Standard Deviation / Mean
and S179D from the chip surface was rapid and left too few hormone/receptor dimers to
determine their site 2 kinetics. Δ1-14, G129R and Δ1-14/G129R were flowed over the
chip surface at a concentration of 10μM. This injection produced a saturation of
heterodimeric complexes. These heterodimers were used to measure site 2 binding of a
second ECD-receptor. Injections of a second ECD-receptor followed at concentrations
ranging from 500nM to 100μM. The data was recorded and can be seen in Figure 4.23.
The heterodimeric complex was dissociated with a brief injection of 2.5M MgCl₂ and
was rebuilt for each concentration of ECD-receptor.

The antagonists and hPRL bound in sufficient quantity and were retained on the
chip surface for a period sufficient for site 2 binding studies. Site 2 binding of hPRL to
the ECD-receptor was observed and the binding isotherm is shown in Figure 4.24. The \( K_d \)
at site 2 for hPRL to the ECD-receptor was shown to be 21.57μM. There were no site 2
interactions observed between the ECD-receptor and site 2 of any of the antagonists. No
increase in RU was observed for any concentration of ECD-receptor. Therefore, deletion
of residues 1-14, changing residue 129 to arginine or both mutations eliminated
interaction of ECD-receptor at site 2.

4.3 Discussion

Several putative hPRL antagonists have been created by various groups to try and
successfully suppress receptor signaling. There have been a diversity of experimental
strategies utilized to demonstrate antagonism of receptor function. These studies typically
state that their antagonist modulate receptor binding and site 2 and thus
Figure 4.23 Antagonist Interaction with the hPRL Receptor at Site 2
Representative figure of the site 2 interaction between antagonist (Δ1-14) and the ECD-receptor. Initial injection of Δ1-14 at 75 sec flowed over the chip surface between 75 and 250 sec. This was followed by a second injection of ECD-receptor between 425 and 575 sec. No interaction was seen between antagonist and ECD-receptor at site 2 for the concentrations of ECD-receptor used.
will fail to create an active heterotrimer. In this chapter I have investigated the structural, biological, and kinetic properties of each antagonist for the first time. I believe a comparison of all antagonists under similar conditions will give a clearer picture of their relative characteristics and functions. These studies are the first direct comparison of these hormones’ capacity to antagonize wild-type hPRL. Below I will describe the characteristics, structural, and kinetic properties of each, their agonist activities, and the success or failure to antagonize wild-type hPRL.

4.3.1 Wild-Type hPRL

Wild-type hPRL was consistently and predictably the most well behaved protein of all those studied in this chapter. The secondary structure showed roughly 50% α-helix, which is consistent with what has been previously reported by CD studies (57). Wild-type
hPRL displayed the highest thermal stability of 76.32°C when determined by DSC. A second T_m of 61.79°C was also observed. This indicates the presence of a two-stage denaturation and suggests that wild-type hPRL folds through a two-step process. The significance of this is unclear. Previous thermal melts of hPRL as monitored by a change in absorbance at 280nm upon heating showed a T_m of 65°C (175). The T_m observed in my studies was significantly greater. The method by which I obtained these values was more precise and was a direct measure of heat radiated rather than an interpretation of melting based on absorbance. The T_m of 65°C observed by DePalatis and the T_m of 61.79°C observed here by DSC may represent an observation of the same phenomena. The change in the A_{280} and resulting T_m may be a result of the hydration of tryptophan from a change in the tertiary structure. The second and higher T_m observed here by DSC may be a result of the loss of secondary structure at higher temperatures. The two T_m's would indicate a two-step fold that would include dehydration and the formation of the helices.

The ED_{50} of hPRL was 0.83nM and is also consistent with previous studies showing an ED_{50} of 0.81nM (180). Site 1 kinetics in this chapter was consistent with the site 1 kinetics seen in Chapter 3 (Table 4.6).

<table>
<thead>
<tr>
<th>Wild-type hPRL</th>
<th>On Rate Constant (k_1)</th>
<th>Off Rate Constant (k_{-1})</th>
<th>Dissociation Constant (K_d) (nM)</th>
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</thead>
<tbody>
<tr>
<td>Chapter 3</td>
<td>7.02 x 10^5</td>
<td>8.41 x 10^{-4}</td>
<td>1.26 x 10^{-9}</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>6.60 x 10^5</td>
<td>8.69 x 10^{-4}</td>
<td>1.47 x 10^{-9}</td>
</tr>
</tbody>
</table>

Table 4.6 Wild-Type Site 1 Kinetics
Site 1 kinetics of hPRL to the ECD-receptor using SPR. The kinetics of site 1 binding gathered in independent experiments displays the consistency and reliability of the data obtained.
The data recorded in this chapter is consistent with data observed for hPRL previously by other investigators or has been repeated from previous chapters within this document. There is confidence in the observations made of the antagonists because the reliability of the data observed with wild-type hPRL. The observation of hPRL in the agonist assays showing a decrease in percent reduction at higher concentrations has not been previously reported, however. This observation has been made previously with agonist assays of hGH using the same FDC-P1 cell line and is consistent with the shape of the curve that was observed here (156). The significance of this observation and the mechanism to create a decrease in cell proliferation with increasing concentration of hormone is unclear. A hypothesis of the mechanism may be that as the concentration of hPRL increases, only 1:1 hormone:receptor complexes form and insufficient receptor remains to form a trimeric complex. This observation does not change the confidence in the data that has been obtained and is another facet of this system that would require further investigation to determine the mechanism that creates this observed phenomenon.

4.3.3  \textit{S179D}

Of all antagonists, S179D has the lowest correspondence to wild-type hPRL in terms of structure, kinetics and biological activity. The secondary structure by percent $\alpha$-helix of S179D was almost half of what was observed with wild-type hPRL. This mutation was a single residue which caused great destabilization in the protein. The mutation is at the midpoint of helix 4 and projects the residue’s side-chain inward towards the other 3 helices. The location of this mutation and the change from an
uncharged serine to a larger charged aspartic acid residue may have caused destabilization in this and adjacent regions on the other 3 helices. Partial secondary structure may form but the fold may only progress to a molten-globule form and fails to collapse into the folded state. The thermal stability of this antagonist is decreased in comparison to wild-type hPRL and is consistent with the decrease in secondary structure.

The agonist activity of S179D is also greatly decreased in comparison to wild-type with a right-shifted dose-response. S179D showed little agonist activity below 100nM, but was able to stimulate cell proliferation to a near maximal level described by wild-type at the concentrations nearing 1µM. The shift in the dose-response with a maximum response similar to that of wild-type hPRL at the highest concentrations would indicate decreased affinity at site 1 and would also suggested a functional site 2. The agonist assay showed no reduction of cell proliferation with increasing concentration of S179D in the presence of 10nM wild-type hPRL. S179D is a poor antagonist and this is consistent with the observations made previously to show S179D to not work by competitive inhibition but by a noncompetitive means (56, 173).

The K\(_d\) for S179D at site 1 was decreased 146-fold in comparison to wild-type. This is consistent with the agonist assays which indicated a decreased site 1 affinity. This is also consistent with a previous study that mutated S179 to alanine (84). The site 1 K\(_d\) observed with S179A to the ECD-receptor at site 1 was 7.69µM which was very similar to the K\(_d\) of 14.6µM observed here with S179D. The greatly weakened affinity for site 1 did not allow for studies at site 2. The dissociation of the protein from the receptor at site 1 was rapid and an inadequate amount of heterodimer was present to evaluate site 2.
binding with a second injection of receptor. Such behavior would not allow antagonism by extensive site 1 occupancy.

The mutation to create this antagonist has greatly decreased its’ structure, thermal stability, biological activity and affinity at site 1 for the receptor. Even a modest mutation of S179 to alanine showed a greatly reduced site 1 affinity and indicates the functional importance of this residue. This protein stimulates cell proliferation in agonist assays and does not inhibit cell proliferation in antagonist assays. Walker and colleagues belief as to how this protein inhibits cell proliferation is to activate alternate pathways from those activated by wild-type hPRL (54). Alternate cell types may be able to show reduced proliferation with increasing amounts of S179D, however, such observations were not made here with this cell type. Based on these data I concluded that S179D did not successfully antagonize hPRL’s activity. A mechanism to explain the activity seen here and elsewhere may be that phosphorylation of S179 serves to modulate the function of the hormone. Phosphorylation of a residue that is very sensitive to change may act as a switch to change the structure and thus reduce the biological activity and inactivate the hormone.

4.3.4 Δ41-52

The secondary structure of Δ41-52 hPRL as determined by CD was decreased when compared to that of wild-type hPRL. Loss of secondary structure could be explained by the deletion of residues that occurred at the end of helix 1 and proceeds into the unstructured region that connects helix 1 to helix 2. To speculate, the loss of helical
content would be expected for helix 1 and potentially helix 2 if the shortening of the unstructured region creates strain and prevents the start of the $\alpha$-helix in the first few residues of helix 2. The thermal stability was also reduced in comparison to wild-type hPRL. The reduced secondary structure and thermal stability would be expected with a deletion of a significant number of internal residues.

The dose-response of $\Delta41-52$ hPRL was right-shifted and was observed to increase to a maximal response similar to wild-type hPRL at the highest concentrations. The shift in the dose-response indicates decreased affinity at site 1 and the near wild-type hPRL maximal response indicates an unaltered site 2. This would not be consistent with the previous investigation of $\Delta41-52$ hPRL which was thought to act by binding at site 1 and not allowing for the conformational change to create an active site 2 (175). Site 1 affinity appears to be reduced while site 2 appears to be unaltered. The antagonist assay shows that not only does $\Delta41-52$ hPRL not inhibit cell proliferation, but it stimulates cell proliferation at the higher concentrations in the assay. In the presence of 10nM wild-type hPRL, the maximal proliferative response is almost double that seen of wild-type hPRL alone.

Interpretation of biological data regarding receptor affinity is confirmed by kinetic binding studies. The $K_d$ for site 1 was weaker by almost 24-fold in comparison to wild-type hPRL. Overall, this is consistent with the biological assays which indicated a decreased site 1 affinity. The affinity of $\Delta41-52$ hPRL for the receptor was weak and sufficient heterodimer could not be created on the chip surface to accurately measure site 2 affinity.
These studies have shown that Δ41-52 hPRL has failed to act as an antagonist for the hPRL receptor and retains agonist activity. Δ41-52 hPRL is thought to antagonize proliferation by binding at site 1 and not allowing a conformational change that allows for an active site 2 (171). Here, Δ41-52 does not bind site 1 with great affinity compared to wild-type hPRL and site 2 appears to be functional. Δ41-52 would not be considered to be a successful antagonist based on these studies.

4.3.5 Δ1-14

Δ1-14 hPRL appears to have similar but reduced secondary structure when compared to wild-type hPRL as determined by CD and was considered to be statistically significant. A decrease in helical content was observed and is surprising considering the first 14 residues of the protein are unstructured. Perhaps the loss in helical content would come from helix 1 because this helix begins at residue 14 and removal of the N-terminus may destabilize the start of helix 1. Adjacent helices may also be affected by the loss of these first 14 residues, however, the Δ1-14/G129R mutant retains an increased helical content in comparison and is missing the same 14 N-terminal residues. The thermal stability of Δ1-14 hPRL was also near wild-type hPRL indicating the thermal stability is not affected by this deletion.

Agonist assays showed that Δ1-14 hPRL was most similar to wild-type hPRL in its ability to stimulate cell proliferation of all the antagonists. The ED_{50} of Δ1-14 was reduced 3.5-fold in comparison to wild-type hPRL. The near wild-type hPRL ED_{50} would indicate that the mutation made did not affect site 1, but the maximal cell proliferation
induced by Δ1-14 was only half that observed of wild-type hPRL, indicating the mutation affected site 2. Additionally, the response at 100nM promoted the highest intensity of cell proliferation, which was also seen with wild-type hPRL, but began to fall approaching baseline activities at the highest concentrations which was also similar to wild-type hPRL. An explanation of this fall may be that as the concentration of hormone rises, the formation of dimeric receptor/hormone complexes dominates the cell surface. Because of a strong site 1 affinity, this leaves very few free receptors available to bind the heterodimers and create a heterotrimeric complexes capable of stimulating cell proliferation because of the weakened site 2 affinity. This argument has been previously presented (177). The Δ1-14 hPRL-induced fall of cell proliferation to near baseline activities supports this hypothesis. Not only would the number of free receptors available to bind at site 2 diminish as the concentration of hormone rises and consumes unbound receptor, but weakened site 2 affinity would decrease formation of heterotrimeric complexes required to stimulate cell proliferation. The antagonist assay shows that Δ1-14 inhibits biological activity and the reduction in cell proliferation approaches basal activity only at the highest concentrations.

Site 1 kinetics show a six-fold weaker $K_d$ when compared to wild-type. Site 2 affinity could not be determined because there was no binding observed with the ECD-receptor concentrations used in SPR. The Site 2 $K_d$ for wild-type hPRL was measured and was similar to values found in Chapter 3, so I can conclude that the profound loss in site 2 binding was real and not a technical problem.
The deletion to create this antagonist did not greatly alter the structure and Δ1-14 was able to bind site 1 in the kinetic studies with a relatively high affinity when compared to wild-type hPRL. The agonist assays showed a near wild-type hPRL ED$_{50}$, but reduced maximal cell proliferation, thus this hormone acts as a partial agonist. How this biological activity can be observed with what appears to be a complete loss of site 2 affinity by kinetic studies is unclear. Perhaps site 2 binding is not required but site 2 docking is necessary. The reduction in cell proliferation at concentrations higher than 100nM indicates site 2 is affected by this deletion. This is in agreement with previous studies that the Δ1-14 mutation affects site 2 (177). This deletion appears to contain some of the elements needed to create a receptor antagonist but the induction of cell proliferation at low concentrations indicates that this would not make for a clinically successful antagonist.

4.3.6  G129R

G129R hPRL has similar secondary structural when compared to that of wild-type hPRL. The thermal stability is also near wild-type and the single mutation did not significantly alter the structure of the protein as observed by DSC. G129R displayed nearly a 50-fold reduction in maximal biological activity and is considered a partial agonist. Based on the agonist curve, G129R would not be considered to affect site 1 binding. The maximal response is half that of wild-type hPRL indicating that site 2 is also affected. The reduction of maximal cell proliferation would be expected because the
mutation is thought to hinder site 2 binding. Unlike Δ1-14 hPRL, cell proliferation does not fall as the concentration increases.

Antagonist assays showed G129R to have the lowest IC\textsubscript{50} values of the three proteins with antagonist actions. In these assays, reduction in cell proliferation is only reduced to 68% of the maximal response and inhibition does not reach basal levels even at the highest concentrations.

Site 1 kinetics show a seven-fold weaker K\textsubscript{d} in comparison to wild-type hPRL. Site 1 affinity is reduced compared to that of wild-type hPRL and the site 2 affinity could not be measured because of an inability to reach a plateau needed to calculate a binding isotherm.

The structure of G129R was not different than wild-type hPRL when evaluated by spectroscopy. The agonist assay of G129R hPRL showed a reduced ED\textsubscript{50}, but not significantly less than wild-type hPRL. The antagonist assay showed that G129R did indeed reduce biological activity, however, the reduction in activity was not complete and proliferation was still observed even with the highest concentration of G129R. This protein showed a mixture of agonist and antagonist activities. This is in agreement with previous studies that have also concluded that this is not a potent antagonist and should be considered a weak agonist (82).
4.3.7  Δ1-14/G129R

The structural properties of Δ1-14 /G129 were very similar to wild-type hPRL. CD spectrum of Δ1-14/G129R was also similar to that of wild-type hPRL indicating the secondary structure is not affected by this mutation. The thermal stability was also retained and was observed to be similar to wild-type hPRL.

The most significant finding for Δ1-14/G129R was an inability to induce cell proliferation. The biological response increased slightly with an increasing concentration of antagonist, however, this rise is insignificant in comparison to the biological response seen with the wild-type hPRL. Thus Δ1-14/G129R hPRL is not an agonist. The inhibitory assay showed the reduction in biological activity to a baseline level and a near complete inhibition of biological activity was observed. Thus, Δ1-14/G129R hPRL is a complete antagonist. These properties indicate this protein is the most promising of this group of modified hPRLs to sever as a drug candidate. Our finding agree with those of Kelly and colleagues (177).

Δ1-14/G129R showed a 6.5-fold weaker $K_d$ and was not significantly lower than wild-type hPRL. Site 2 affinity could not be determined because of an inability of increasing Δ1-14/G129R to create a binding isotherm.

The structure of Δ1-14/G129R was not significantly altered by the mutations. There was a complete lack a biological activity observed in the biological assay. The inhibitory assay showed a near complete inhibition of biological activity. Site 1 affinity was near that of wild-type and site 2 affinity could not be determined at the highest
protein concentrations that could be obtained. Δ1-14/G129R has all of the characteristics needed to be a successful antagonist.

4.3.8 Overview of Antagonists and Conclusions

All of the antagonists, with the exception of Δ1-14/G129R, have agonist activity, although several show partial antagonist activity. Δ1-14/G129R shows promise and the remaining antagonists were shown here to be missing one or more of the benchmarks needed to be considered a successful antagonist.

All antagonists, including Δ1-14/G129R, aggregated beyond their T_m which was not seen with the wild-type hormone. The significance of this in terms of pharmaceutical production could potentially be significant. Attempts made to reduce the aggregation were unsuccessful. Perhaps other methods could successfully prevent aggregation. The rise in temperature beyond 80°C, however, would not be anticipated during mass production and the likelihood of aggregation being a hindrance to production would appear to be slim.
CHAPTER 5

STRUCTURAL, BIOLOGICAL AND KINETIC PROPERTIES OF THE EXTRACELLULAR DOMAIN OF THE PROLACTIN RECEPTOR

5.1 Introduction

The hPRL receptor consists of an ECD, a transmembrane domain and an intracellular domain. The ECD binds hPRL, hGH or hPL and induces a signaling cascade. Two subdomains are present in the ECD and most often are termed S1 and S2. The ECD is 210 residues long and the S1 domain consists of the first 100 residues and the S2 consists of the remaining 110 residues (101-210). The two domains are linked by a proline rich region that is thought to act as an unstructured hinge to provide flexibility.

Several features of the ECD have been shown to be important for biological activity located in both the S1 and S2 domains. In the S1 domain, there are two disulfide bonds at C-12-C-22 and C-51-C-62. Removal of either of these disulfide bonds results in the loss of biological activity (136, 137). In the S2 subdomain, there exists a Zn$^{2+}$ half site and the WSXWS motif commonly observed in class I receptors. The Zn$^{2+}$ half site is located at D187 and H188 and the other half site in hPRL at position H27 (70, 181). The presence of Zn$^{2+}$ reduces the affinity of hPRL for the ECD-receptor at site 1 but was also shown to strengthen the global affinity by the formation of a heterotrimeric complex.
(180). Mutagenesis of H27 in hPRL to an alanine residue was shown to produce an insignificant change in affinity for site 1 (84). The ability to secrete hPRL from the pituitary is reduced with the H27A mutation (182). The exact role the half site plays in receptor binding and biological activity remains to be determined. The present data suggest that the Zn$^{2+}$ half site plays a larger role in secretion of the hormone from the anterior pituitary than is does on biological activity. The WSXWS motif is located at residues 191 to 195. This motif is conserved in the hGH receptor (residues 221-225) and the human erythropoietin receptor (212-216) (183). As stated earlier, mutations to this motif reduces the receptors hormone binding affinity by improper folding and reduced transport to the cell surface (110, 136, 138).

The structure of hPRL bound to two rat PRL receptors to create a heterotrimeric complex has identified the binding surfaces involved in this complex (67, 69). These surfaces include site 1 and site 2 of hPRL and an interaction between the two opposing S2 subdomains of the receptor is also observed (S2/S2). These three binding surfaces between the hormone and two receptors create the heterotrimeric complex. Similar but not identical residues of the receptors form the binding surfaces at sites 1 and 2. The S2/S2 binding surfaces between the two receptors share some similar residues but are not identical (Figure 5.1). Upon binding hPRL, the S2 domain has been shown to change confirmation and is thought to play a role in receptor activation (184). Upon binding hPRL, the S2 domain becomes more structured and creates a mature dimerization interface.
Figure 5.1 Heterotrimeric Complex of hPRL to Two Rat PRL Receptors
Structure of hPRL complexed to two rat PRL receptors, PDB no.3EW3 (67). (A) Cartoon representation and (B) surface representation of the heterotrimeric complex. Circed are site 1, site 2, and the S2/S2 binding interfaces. Images are rotated 180° on the z-axis (right).
Deletion of the S1 subdomain (Δ1-100) greatly reduces the affinity of the ECD-receptor for hPRL (127). Deletion of residues 4-102 abolishes the affinity for the hormone (123). This appears to be reasonable because structural data that show that much of the contact surface between hPRL and receptor reside in the S1 subdomain (figure 5.1) (68). Very high hormone concentrations, however, can activate the receptor indicating that regions outside of the S1 subdomain are sufficient but not optimal for receptor activation. Deletion of the S2 subdomain (Δ106-206) results in a constitutively active hPRL-receptor (123). This observation indicates the S2 subdomain is not required for receptor activity. Deletion of both S1 and S2 by Δ1-210 mutation results in a receptor that is incapable of being activated and obviously unable to bind hPRL. These receptors missing the entire ECD can still form receptor dimers (123, 162). The deletion of residues 10-186 results in a constitutively active receptor (185). This receptor is missing residues that bind site 1, site 2 and the S2/S2 receptor binding region, suggesting that none of these regions are necessary for receptor activation. Based on this deletion data one can hypothesize that regions of the ECD play an inhibitory role that is released by hPRL binding or by removing inhibitory structures. Such interactions remain to be identified.

In this chapter I will investigate structural, biological, and kinetic properties of the receptor subdomains and the ECD-receptor. These subdomains will be investigated in vitro for their potential to bind other subdomains, the ECD, or hPRL and to induce or inhibit biological activity.
5.2 Results

5.2.1 Vector Design

The extracellular domain (ECD-receptor) of the hPRL receptor was expressed and purified as previously described. The vector for the S1 subdomain was created using site directed mutagenesis to add a stop codon at position 101 of the ECD-receptor. The S2 vector was created by site directed mutagenesis of the ECD-receptor vector to add a methionine at position 98 of the mature protein. This was followed by mutagenesis by the use of overlapping primers upstream and downstream of the S1 subdomain to delete the codons for the first 98 residues. Mutations were also performed to create C-terminal cysteine mutants to be used as ligands in SPR. The proper coding sequence for each vector was confirmed by DNA sequencing.

5.2.2 SDS-PAGE

Each protein was expressed and purified as previously described and each preparation yielded 20-50mg per liter. The purified receptor subdomains were applied to an SDS-PAGE gel (Figure 5.2). Single bands are shown to migrate to locations on the gel at anticipated molecular weights for each protein and this indicates a positive preparation and purity of the sample.
Figure 5.2 SDS-PAGE of ECD-Receptor and Receptor Subdomains
15% SDS-PAGE with 2-mercaptoethanol for 15-20 µg of ECD-receptor, the S1 subdomain and the S2 subdomain in addition to cysteine mutants of each. All proteins migrated to anticipated positions on the gel and show single bands in each lane.
5.2.3 *Circular Dichroism*

CD was performed on the S1 and S2 subdomains, as well as the ECD of the hPRL receptor. Additionally, CD was performed on proteins where a cysteine was added to the C-terminus. Each protein showed characteristics of a β-sheet protein with a negative peak at 218nm and a positive peak at 200nm (Figure 5.3). The CD spectrum of each protein was converted into molar ellipticity to take into account the molecular weight. This is necessary in order to compare mutants that have a deletion of a significant number of residues. The S1 and S2 subdomains each showed only half the molar ellipticity as that seen with the ECD of the hPRL receptor. The CD spectrum of each protein was entered into the K2D3 algorithm to estimate percent β-sheet (Table 5.1) (152). All of the domains showed roughly the same percentage of β-sheet structure between 36-40%. This is consistent with the crystal structure of the ECD-receptor (41.9% β-sheet) showing β-sheet structure in 42 residues in the S1 (42% β-sheet) and 46 residues in the S2 (41.8% β-sheet) and 88 residues in the ECD-receptor (41.9% β-sheet) (68). Anticipated %β-sheet

<table>
<thead>
<tr>
<th>Protein</th>
<th>% β-sheet</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECD-receptor</td>
<td>40.00% ± 1.02</td>
</tr>
<tr>
<td>ECD-receptor-C</td>
<td>40.74% ± 0.47</td>
</tr>
<tr>
<td>S1</td>
<td>37.51% ± 0.77</td>
</tr>
<tr>
<td>S1C</td>
<td>36.63% ± 0.99</td>
</tr>
<tr>
<td>S2</td>
<td>38.15% ± 0.24</td>
</tr>
<tr>
<td>S2C</td>
<td>37.61% ± 1.76</td>
</tr>
</tbody>
</table>

Table 5.1 Percent β-Sheet of hPRL ECD-Receptor and Subdomains

Percent β-sheet of the ECD-receptor, the S1 subdomain, the S2 subdomain and the cysteine mutant of each from CD spectra data using a fitting algorithm K2D3. Above are the mean values of two sets of CD data.
Figure 5.3 Circular Dichroism of ECD-Receptor and Subdomains
CD spectra of (A) S1, S2 and ECD-receptor and (B) C-terminal cysteine mutants of the ECD-receptor and receptor subdomains. Above is a representative figure of experiments performed.
are listed in the table below. The indication would be that the secondary structure is retained in each subdomain and that each half of the ECD contributes approximately equally to the overall β-sheet content of the ECD. Addition of cysteine did not alter the secondary structure of the receptor. When the CD spectra of the S1 and S2 subdomains are added, they somewhat resemble the curve of the ECD-receptor.

5.2.4 *Differential Scanning Calorimetry*

The $T_m$ of each receptor subdomain was assessed using DSC. The $T_m$ of each subdomain will give insight into the thermal stability of these proteins. The ECD was observed to have a $T_m$ of 44.59°C (Figure 5.4). This is in agreement with a previous thermal melt of the ECD with a $T_m$ of 45°C (175). The S1 and S2 subdomains showed no observable $T_m$. This indicates that each subdomain released very little heat during the melt that it was not able to be detected by the instrument. Aggregation is present for all three proteins. The S1 and S2 subdomains begin to aggregate at approximately 90°C while the ECD began to aggregate at around 70°C. Experiments were repeated twice for the S1, S2 and ECD-receptor.

5.2.5 *Biological Assays*

5.2.5.1 *ED$_{50}$ of ECD-Receptor and the S1 and S2 Subdomains*

*In vitro* FDC-P1 cell assays were performed as previously described to determine if exposure to either ECD-receptor or the S1 or S2 subdomains would create a biological response. Assays ranged in protein concentration from 0.01nM to 10µM for the S1 and
Figure 5.4 Differential Scanning Calorimetry of the hPRL ECD-Receptor and Subdomains

DSC of (A) ECD-receptor. The background has been subtracted using VPViewer™ software and the curve was fit using Origin® software to determine a $T_m$ of 44.59°C. No peak was observed in both (B) S1 and (C) S2. All proteins were at a concentration of 100µM and were solubilized in 40mM KH$_2$PO$_4$, pH 7.3.
S2 subdomains and the ECD-receptor. Dose-response curves for the receptor subdomains can be seen in Figure 5.5. Each of the agonist assays were performed in the absence of hormone (Figure 5.5 B, C and D). Any change in cellular proliferation would be a product of the treatment of the ECD-receptor, or the S1 or S2 subdomain. The dose-response curve of wild-type hPRL is also included as a control to ensure the viability of the cells as well as provide a baseline of unstimulated cells. Wild-type hPRL was shown to have a characteristic ED$_{50}$ of 0.82nM indicating the cells were viable and responsive to hormone treatment (Table 5.2).

The cell bioassays were shown to be nonresponsive to both the ECD - receptor and the S2 subdomain (Figure 5.5 A and D). Cell proliferation stayed near baseline for six orders of ligand concentration. The agonist assay of the S1 subdomain showed an increase in cell proliferation. The ED$_{50}$ of the S1 subdomain was 624.17nM. The ability of the S1 subdomain to stimulate cell proliferation in these cells that express the hPRL receptor was unexpected.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt hPRL</td>
<td>0.82nM ± 0.16</td>
</tr>
<tr>
<td>ECD-receptor</td>
<td>N/A*</td>
</tr>
<tr>
<td>S1</td>
<td>624.17nM ± 12.23</td>
</tr>
<tr>
<td>S2</td>
<td>N/A*</td>
</tr>
</tbody>
</table>

Table 5.2 Agonist Assays of the ECD-Receptor and Subdomains
Agonist assays to determine ED$_{50}$ values with standard deviation for wild-type hPRL and the ECD-receptor and the S1 and S2 subdomains. The wild-type ED$_{50}$ was used as a control to ensure the viability of the cells in the assay and established a baseline value. Values were determined by a four parameter sigmoidal fit using SigmaPlot 12.3 software.
Figure 5.5 Agonist Assays of hPRL, ECD-Receptor and Receptor Subdomains

Biological assay assessing the hormone-induced response of (A) wild-type hPRL, (B) ECD-receptor, (C) the S1 subdomain, and (D) the S2 subdomain. Concentrations of hormone and receptor ranged from 0.01nM to 10µM. A four parameter sigmoidal fit of the dose-response curves to determine the ED$_{50}$ was applied to A and C. Agonist activity was observed with both (A) hPRL and (C) the S1 subdomain. No agonist activity was observed with (B) ECD-receptor or (D) the S2 subdomain.
5.2.5.2 IC_{50} of ECD-Receptor and the S1 and the S2 Subdomains

Inhibitory assays were performed to determine the degree to which the ECD-receptor and subdomains would block wild-type hPRL induced proliferation. Inhibitory assays were performed in the same manner as antagonist assays in Chapter 4. Each assay contained 10nM hPRL and increasing concentrations of ECD-receptor, S1 or S2 subdomains. The wild-type hPRL and receptor proteins were mixed prior to introduction to the assay. The concentration of hPRL was based on the ED_{50} data previously mentioned.

Inhibition of cell proliferation was observed only with the ECD-receptor (Figure 5.6 and Table 5.3). The S1 and S2 subdomains were not observed to have any inhibition of hPRL in the assay (Figure 5.6). The S1 subdomain showed agonist activity beyond the already hPRL-stimulated cells when receptor protein was greater than 700nM. The plots of the biological and inhibitory assays can been seen together in Figure 5.7. Inhibition of cell proliferation is seen with ECD-receptor. The reduction of cell proliferation in the inhibitory assay nears the unresponsive baseline level of the biological assay but total inhibition was not reached. Both the agonist and inhibitory

<table>
<thead>
<tr>
<th></th>
<th>IC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECD-receptor</td>
<td>37.76nM ± 7.07</td>
</tr>
<tr>
<td>S1</td>
<td>N/A</td>
</tr>
<tr>
<td>S2</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Table 5.3 IC_{50} Values of the ECD-Receptor and Subdomains**

Inhibitory assays to determine IC_{50} values with standard deviations for the ECD-receptor and receptor subdomains. The inhibitory assays were supplemented with 10nM hPRL. Values were determined by a four parameter sigmoidal fit using SigmaPlot 12.3 software. Inhibition of cell proliferation was only observed with the ECD-receptor.
Figure 5.6 Agonist and Inhibitory Assays of the ECD-Receptor and Subdomains

(A) Agonist assays of hPRL taken on the same day as the inhibitory assays to serve as a control and as a baseline for 0nM hPRL and the upper limit at 10nM hPRL used in the inhibitory assays. Inhibitory assays of (B) ECD-receptor, and (C) S1 and (D) S2. All assays were supplemented with 10nM hPRL. Included in each inhibitory assay are the baseline percent reduction of 0nM hPRL and the maximum reduction observed at 10nM hPRL from the agonist assay. Above are representative assays. Experiments were repeated three times and the results were averaged. The only inhibition of cell proliferation was observed with the ECD-receptor.
Figure 5.7 Agonist and Inhibitory Assays of ECD-Receptor and Subdomains
Both the agonist and inhibitory assays of (A) ECD-receptor showing inhibition, (B) the S1 subdomain which is showing agonism, and (C) the S2 subdomain showing neither agonist nor antagonist activity. Included in each are the baseline for 0nM hPRL and the percent reduction for 10nM hPRL from Figure 5.6.
assays of S1 subdomain display agonist activity. The biological and inhibitory assays were not affected by the presence of the S2 subdomain.

5.2.6 Kinetic Evaluation of Receptor Subdomains

5.2.6.1 Site 1 Kinetics of hPRL to the Receptor Subdomains

Each of the receptor subdomains underwent site directed mutagenesis to introduce a C-terminal cysteine that will be used to couple to the surface of a Bicore CM5 chip. In these experiments roughly 900 RU of the S1 and S2 subdomains were coupled to two independent lanes on the chip and 1400 RU of ECD-receptor was bound to the third lane of a CM5 chip. The fourth lane served as a reference channel that was chemically activated and blocked with a saturating concentration of cysteine. Additional RU of ECD-receptor was bound to the chip surface to compensate for the relative molecular weights of the proteins and achieve similar ligand densities.

hPRL ranging in concentrations from 100nM to 10µM was flowed over the chip surface and the responses were recorded. The interaction of the ECD-receptor with hPRL at site 1 has been described earlier in this document and the $K_d$ at site 1 was observed to be 1.02nM. hPRL showed no affinity to either the S1 or the S2 subdomains (Figure 5.8).

5.2.6.2 Kinetics of Various Subdomain Interactions

ECD-receptor and the S1 and S2 subdomains were coupled to the chip surface as described above and increasing concentrations of ECD-receptor, the S1 subdomain, and the S2 subdomain were flowed over the chip surface. The interactions that were
Figure 5.8 Surface Plasmon Resonance of hPRL and Receptor Subdomains
Injections of human hPRL between 75 and 250 sec over (A) the S1 subdomain and (B) the S2 subdomain. Both show no interaction between hPRL and the receptor subdomains. Above is a representative figure. Experiments were repeated three times.
observed are presented in Table 5.4. The coupled S1 subdomain bound both ECD-receptor and the S1 subdomain (Figure 5.9). The affinity of coupled S1 subdomain to the ECD-receptor was relatively strong ($K_d$ of 69.2nM) (Table 5.5). The affinity of coupled S1 subdomain to the S1 subdomain was relatively weak ($K_d$ of 2.35µM) (Table 5.5). To be noted is only 10% of the coupled S1 subdomain binds ECD-receptor at the highest concentrations. Only 3% of the coupled S1 binds the S1 subdomain. This is a small percentage in comparison to most SPR experiments and further experiments would confirm this interaction.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>ECD-receptor</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt hPRL</td>
<td>1.02nM</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ECD-receptor</td>
<td>N/A</td>
<td>69.2nM</td>
<td>N/A</td>
</tr>
<tr>
<td>S1</td>
<td>N/A</td>
<td>2.35µM</td>
<td>N/A</td>
</tr>
<tr>
<td>S2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Table 5.4 Intra-Subdomain Binding Interactions**

Intrasubdomain affinities of the hPRL receptor. The ECD-receptor binds hPRL with a characteristic affinity. Interaction between the S1 subdomain to both ECD-receptor and the S1 subdomain are observed. All other injections yielded no affinity and are labeled N/A.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>On Rate Constant $k_1$</th>
<th>Off Rate Constant $k_1$</th>
<th>Dissociation Constant $K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECD-receptor</td>
<td>4.32 x 10^3</td>
<td>2.99 x 10^4</td>
<td>6.92 x 10^{-8}</td>
</tr>
<tr>
<td>S1</td>
<td>1.37 x 10^3</td>
<td>3.22 x 10^{-3}</td>
<td>2.35 x 10^{-6}</td>
</tr>
</tbody>
</table>

**Table 5.5 Kinetics of Receptor Subdomains**

Kinetics of coupled S1 subdomain to either the ECD-receptor or the S1 subdomain.
Figure 5.9 Surface Plasmon Resonance of S1/ECD-Receptor and S1/S1
SPR showing the interaction of the coupled S1 subdomain with injections of (A) the ECD-receptor and (B) the S1 subdomain between 450 and 575 sec at concentrations ranging from 1µM to 100µM. Above is a representative figure. Experiments were repeated twice.
5.2.6.3 Site 2 Kinetic Evaluation of Receptor Subdomains

The conditions of the sensor chip and the proteins coupled to the surface were identical to that of the site 1 kinetics described above. The only receptor subdomain able to bind was the ECD-receptor/hPRL heterodimer. Experiments to determine site 2 affinity involved a site 1 association of coupled ECD-receptor bound to hPRL and the S1 and S2 subdomains were flowed over the chip surface to determine affinity to site 2 of hPRL followed. A 200µL injection of 10µM hPRL was flowed over the ECD-receptor surface. A 100 µL injection of either the S1 subdomain or the S2 subdomain followed at concentrations ranging from 1µM to 100µM. The data was recorded and are presented in Figure 5.10. hPRL was observed to bind in sufficient quantity and was retained on the surface long enough to allow for a second injection of the receptor subdomains. There was no interaction observed between the S1 or S2 subdomains at site 2 of hPRL in the heterodimeric complex.

5.3 Discussion

Regions on both the S1 and S2 subdomains have been shown to contain important regions necessary for biological activity. The S1 subdomain holds the majority of the surface that interacts with hPRL at both sites 1 and 2. The S2 subdomain contains the Zn$^{2+}$ half site and the WSXWS motif. Deletion of the S1 subdomain results in a nearly inactive receptor and deletion of the S2 subdomain results in a receptor that is constitutively active. These deletions result in biological activities that are directly opposite of one another. Each subdomain and the ECD-receptor were investigated here.
Figure 5.10 Site 2 Affinity of Receptor Subdomains
SPR showing ECD-receptor bound to the chip surface with injections of 10µM hPRL between 75 and 300 sec followed by injections of (A) the S1 subdomain and (B) the S2 subdomain between 450 and 550 sec. Both show no affinity of either subdomain for site 2 of hPRL. Above is a representative figure of the experiments performed. Experiments were repeated three times.
Each subdomain was expressed and yielded between 20 and 40 mg of lyophilized protein. The SDS-PAGE gel of the subdomains showed the preparations to be pure and at the anticipated molecular weight. The secondary structure showed the ECD-receptor, and the S1 and S2 subdomains to each to have roughly the same percentage of β-sheet. This would indicate that each subdomain contains roughly an equal amount of β-sheet and each subdomain was able to fold in order to obtain the proper secondary structure. The thermal melt of the ECD-receptor was shown to have a $T_m$ of 44.59°C, which was consistent with previous $T_m$ s. This evidence, in addition secondary structure and previous data showing the ability of ECD-receptor to bind hPRL at a high affinity are indications that the receptor is properly folded. The S1 and S2 subdomains did not show a $T_m$. The liberated heat may not have been sufficient to provide for an observable signal. The presence of secondary structure seen by CD would indicate that the subdomains are folded. The procedure for preparing and analyzing proteins by both CD and DSC were quite similar, so the loss of structure due to handling seems improbable. The heat liberated may not have been recognizable above background and the melting may have occurred over a large range considering the ECD-receptor melting curve extended for more than 30°C.

The biological assays for the ECD-receptor were predictable and the results were expected. The biological assay showed no increase in proliferation in the absence of hormone and showed a decrease in cell proliferation with increasing concentrations of ECD-receptor. ECD-receptor has previously been observed to not have an affinity for the hPRL receptor and also has not been observed to stimulate cell proliferation.
Additionally, an affinity for hPRL has been observed and a large concentration of ECD-receptor in the inhibitory assay would diminish the concentration of free hPRL to stimulate cell proliferation. The agonist assay of the S2 subdomain showed no activation of cell proliferation and the inhibitory assay showed no inhibition of cell proliferation. This would indicate that the S2 subdomain has little affinity for the hPRL receptor on the surface of the cells in a manner that would activate the receptor and does not have a mechanism to active cell signaling alone. If inhibitory regions on the receptor exist, the S2 subdomain is also not able to bind to those to allow for activation of the receptor in the absence of hormone. Inhibition was also not observed in the presence of the S2 subdomain which would indicate no affinity for hPRL to reduce the concentration of free hPRL.

The assays of the S1 subdomain provided unexpected results. The S1 subdomain was able to induce cell proliferation in the absence of hormone. The mechanism by which this occurs is unclear. The concentration of the receptor subdomain is relatively high in comparison to the concentration of hormone needed to induce similar cell proliferation. I hypothesize the mechanism by which proliferation could occur consists of the S1 subdomain binding to cell surface receptors in a way that alters the surface receptor into an activated position and allows receptor signaling. It is interesting that the S1 subdomain can perform this function but neither the ECD-receptor nor the S2 subdomains have similar activity. Portions of the hPRL-receptor may be present to act in an inhibitory manner. Binding of a hormone to the receptor prevents the inhibition and activation of the receptor and signaling proceeds. This would be in agreement with observations of the
deletion mutants ΔS2 and Δ10-186 which are both constitutively active (185). The S1 subdomain in these biological assays may be able to bind regions on the cell surface receptors that are causing the inhibition and an active receptor is a result and proliferation proceeds.

Kinetic evaluation of the S1 and S2 subdomains shows that both have no affinity for hPRL at either site 1 or site 2. From structural data, most of the contact between hPRL at both sites 1 and 2 and its receptor is in the S1 subdomain (67). Expectations were that the S1 subdomain would have some affinity for hPRL since ΔS1 mutants result in a receptor that loses almost all affinity for hPRL (127). Deletion of residues 4-102 creates a receptor completely incapable of binding receptor and deletion of residues 106-206 results in the constitutively active receptor indicating the S2 subdomain has little affinity for hPRL (123). Residues that consist of the hinge region that links the S1 and S2 subdomains and provides flexibility at residues 100-106 may be important for hPRL binding. These residues may not bind the hormone directly, but this hinge region may serve to structure the domain or create flexibility and allow for a conformation able to bind the hormone and create an active receptor complex.

The inability of the S2 subdomain to bind site 1 of hPRL is not surprising. Most of the contact between the receptor and hormone appears in the S1 domain of the receptor (67). It is interesting to note that the site 2 kinetic evaluation did not reveal any affinity for the hormone/receptor dimer. The second receptor binding at site 2 of the hormone has a binding surface on both the hormone and the receptor. Still, there was no affinity seen
for site 2 and the S2/S2 interfaces. This is in agreement with data observed in Chapter 3 that shows a µM site 2 affinity.

Most portions of the receptor had no affinity for other subdomains of the receptor in the absence of hormone. The only two interactions observed between the S1 subdomain and both the ECD-receptor and the S1 subdomain. The affinity for ECD-receptor was quite strong with a dissociation constant of 69.2nM, while the affinity for S1 was weaker at 2.35µM. This affinity explains the agonist assay data that showed the S1 subdomain stimulates cell proliferation in the agonist assay. The S1 subdomain may be able to bind regions on the receptor that inhibit activation and upon binding and relieve this inhibition. The low affinity of S1/S1 interaction could be explained in that the region beyond the S1 subdomain is important for binding in terms of either affinity or to structure the receptor in a way that allows for binding. Structuring of the receptor upon binding has been observed previously where the S2 subdomain changes confirmation upon binding of hPRL (184). It is interesting to note that there was no affinity seen between the coupled ECD-receptor and the S1 subdomain. It would be thought that similar affinities would be obtained no matter which receptor proteins were bound to the chip surface. One explanation for our failure to observe a binding might be when one receptor subdomain is coupled to the chip surface, a structural motif is obtained that allows the binding in one direction but not the other. Another explanation could be that concentration plays a role in the ability of receptor subdomains to bind. The concentration of receptor bound to the chip surface is infinitely smaller than the µM concentration that is pumped across the surface. Both of these technical explanations are
in contrast with the agonist assay of the S1 subdomain being able to bind the receptor and stimulate proliferation. Receptors on the cell surface are mimicked by ECD-receptor on the chip surface and it would be assumed that S1 subdomain would be able to bind ECD-receptor if the S1 subdomain is also able to stimulate cell proliferation by binding to cell surface receptors. That is not the case of what was seen here and further investigation is needed to explain these differences.
CHAPTER 6

A COMPARISON OF hGH AND hPRL BINDING TO THE hPRL RECEPTOR

6.1 Introduction

The affinity at site 2 of hPRL for the hPRL receptor was reported in Chapter 3 to be weak with a \( K_d \) of 60.1 \( \mu \)M. This is in contrast with site 2 binding of hGH to the hGH receptor which has previously been described and the affinity is high with a \( K_d \) of 3.8nM (71). The hPRL receptor is able to bind and become activated by both hPRL and hGH despite the low sequence homology between the two hormones. It is unknown if the weak site 2 affinity of hPRL for the hPRL receptor is similar when hGH binds the hPRL receptor or if hGH binds site 2 of the hPRL receptor with a high affinity. Here, I will investigate the affinity of hGH for the hPRL receptor at site 2.

6.2 Results

6.2.1 Circular Dichroism

The CD spectra of hGH showed a characteristic curve for an \( \alpha \)-helical protein and was similar to that of hPRL. Two negative peaks at 220nm and 208nm and a positive peak approaching 190nm were observed (Figure 6.1). The spectrum of each protein was converted into molar ellipticity to take into account the molecular weight.
The spectrum of each protein was entered into the fitting algorithm K2D3 to estimate percent α-helix and is displayed in Table 6.1. hGH displayed a percent α-helix similar to that of wild-type hPRL.

![Circular Dichroism of hPRL and hGH](image)

**Figure 6.1 Circular Dichroism of hPRL and hGH**
Both hPRL and hGH show a characteristic CD spectra of α-helical proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% α-helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRL</td>
<td>52.34% ± 0.96</td>
</tr>
<tr>
<td>hGH</td>
<td>49.68% ± 0.54</td>
</tr>
</tbody>
</table>

**Table 6.1 Percent α-Helix of hPRL and hGH**
Percent α-helix with standard deviations of hPRL and hGH from CD data using the fitting algorithm K2D3.

6.2.2 *Agonist Assay of hPRL and hGH*

*In vitro* cell assays were performed as previously described to determine the biological activity of hPRL and hGH. Assays ranged in concentration from 10pM to 10µM for hPRL and from to 1pM to 1µM for hGH. The agonist assay of hPRL was consistent with previous biological assays and is an indication of the viability of the cells.
in the assay (Figure 6.2). hGH showed a similar dose-response curve to hPRL. Additionally, a decrease in cell proliferation in the hGH agonist assay when compared to that of wild-type hPRL would indicate a lower site 2 affinity. The ED₅₀ values of both hormones were calculated from the dose-response curves and are listed in Table 6.2.

![Figure 6.2 Agonist Assay of hPRL and hGH](image)

**Figure 6.2 Agonist Assay of hPRL and hGH**

Agonist assay assessing the hormone-induced response of hPRL and hGH and sigmoidal fit of the dose-response curves to determine ED₅₀ values. A representative figure is displayed above. Experiments were repeated three times and the results were averaged.

<table>
<thead>
<tr>
<th></th>
<th>ED₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt hPRL</td>
<td>0.82nM ± 0.14</td>
</tr>
<tr>
<td>wt hGH</td>
<td>1.98nM ± 0.37</td>
</tr>
</tbody>
</table>

**Table 6.2 ED₅₀ Values of hPRL and hGH**

ED₅₀ values with standard deviations for hPRL and hGH. Values were determined by a four parameter sigmoidal fit using SigmaPlot 12.3 software.
6.2.3  Kinetic Evaluation of ECD-Receptor and Subdomains

6.2.3.1  Site 1 Kinetics of hGH and hPRL to the hPRL Receptor

The hPRL ECD-receptor was thiol-coupled to the surface of a Bicacore CM5 sensor chip as is described in section 2.5.1. In these experiments between 500 and 2000 RU s of ECD-receptor were bound to the chip surface on three of the four available lanes of the sensor chip. hGH and hPRL were flowed over the chip surface and allowed to interact with the ECD-receptor ranging in concentration from 10nM to 1µM (Figure 6.3). The presence of Zn$^{2+}$ has previously been shown to be necessary for hGH to have sufficient affinity for the ECD-receptor (83). In these experiments, EDTA was omitted and 15µM Zn$^{2+}$ was supplemented to the running buffer. hGH displayed a site 1 K$_d$ of 4.3nM (Table 6.3). hPRL had a reduced site 1 K$_d$ of 328nM in the presence of Zn$^{2+}$.

<table>
<thead>
<tr>
<th></th>
<th>On Rate Constant ($k_1$)</th>
<th>Off Rate Constant ($k_{-1}$)</th>
<th>Dissociation Constant (K$_d$, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGH</td>
<td>$3.50 \times 10^4$</td>
<td>$1.43 \times 10^{-4}$</td>
<td>$4.30 \times 10^{-9}$</td>
</tr>
<tr>
<td>hPRL</td>
<td>$4.02 \times 10^3$</td>
<td>$1.32 \times 10^{-3}$</td>
<td>$3.28 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Table 6.3 Site 1 Kinetics of hGH and hPRL to the ECD-Receptor

Site 1 affinity of hGH and hPRL to the ECD-receptor. Zn$^{2+}$ reduces the site 1 K$_d$ of hPRL nearly 300-fold compared to an EDTA containing Zn$^{2+}$ free buffer shown in previous chapters.

6.2.3.2  Site 2 Kinetic Evaluation of hGH to the hPRL Receptor

The conditions of the sensor chip and the proteins coupled to the surface were identical to that of the site 1 kinetics described above. A 150µL injection of 10µM hGH was flowed over the surface and is a concentration which was determined to be sufficient to achieve saturated binding between ECD-receptor and hGH. A second 100 µL injection
Figure 6.3 hGH Binding to the ECD-Receptor
SPR showing (A) high affinity binding of hGH to the hPRL ECD-receptor at site 1. Shown is an injection of hGH between 75 and 425 sec followed by buffer. (B) An injection of hGH occurred between 75 and 250 sec followed by a second injection of ECD-receptor between 500 and 600 sec. No affinity of hGH for the ECD-receptor at site 2 (bottom).
of ECD-receptor followed at concentrations ranging from 1µM to 100µM. The data was recorded and can be seen in Figure 6.3. hGH was observed to bind in sufficient quantity and was retained on the surface long enough to allow for a second injection of the receptor subdomains. The dissociation of hPRL from the chip surface in the presence of Zn$^{2+}$ was rapid and left too few hormone/receptor dimers to determine the site 2 kinetics. There was no interaction observed between hGH and the ECD-receptor at site 2 within the tested ECD-receptor concentrations.

6.3 Discussion

hPRL and hGH have been shown to bind and activate the hPRL receptor both here and previously. hGH activates the hPRL receptor in a manner similar to hPRL in an ordered fashion (83). Upon binding the first receptor, a conformational change occurs within the hormone to allow for the binding of a second receptor and creates an active receptor complex (80). The affinity at site 2 for hGH to the hGH ECD-receptor has been demonstrated and is quite strong (71) while the affinity for hPRL to the hPRL ECD-receptor at site 2 is weak as described here and previously (65, 67). The stark difference in site 2 affinity of the two hormones to their respective receptors can be a product of the hormone and/or the receptor.

hGH was shown to be viable with secondary structure and ED$_{50}$ values similar to that of hPRL. The ED$_{50}$ value of 1.98nM observed here was similar to that of previous biological assays using the same cell type of 3.25nM (156). The site 1 Kd of hGH to the hPRL receptor by SPR was observed to be 4.3nM. The similarity in ED$_{50}$ and site 1 Kd of
hGH to the hPRL ECD-receptor is similar to the site 1 ED$_{50}$ and K$_d$ correlation of hPRL to the ECD-receptor observed in Chapter 3.

The investigation revealed no site 2 binding between hGH and the hPRL ECD-receptor with the concentrations of ECD-receptor used. This interaction is more akin to that of hPRL to the hPRL receptor than of hGH for the hGH receptor. The mechanism by which the hPRL receptor becomes activated is very different than that of the hGH receptor. The requirement of an even modest site 2 affinity appears to not be necessary for the hPRL receptor to become activated. Because the hGH receptor has a strong site 2 affinity for hGH, it would indicate the necessity for a high affinity site 2 to activate the hGH receptor. hPRL receptor would appear to only require one high affinity binding surface at site 1 to activate the receptor. The similarity in ED$_{50}$ and site 1 K$_d$ of hGH to the hPRL ECD-receptor indicates a similar mechanism of activation to that of hPRL to the hPRL receptor. This observation is similar to those seen in Chapter 3 and would support the binding at site 1 of hPRL as the gatekeeping event for hPRL receptor activation and support the notion that site 2 interaction does not provide sufficient affinity to create a heterotrimeric complex that is required for biological activity. These data suggest that an alternate binding interface must be present between the two hPRL receptors to create the necessary complex.
CHAPTER 7

DISCUSSION

7.1 *Receptor Activation Driven by Site 1 Binding*

Ordered binding has been shown to be necessary for the hPRL receptor to become activated (82). Binding at site 1 is required to create an available site 2 able to bind a second receptor and become activated (80). Previously, a conformational change has been observed in both hGH and hPRL upon binding at site 1 (82, 83). Further, mutations made to residues between but not including those at sites 1 and 2 of hGH have reduced site 2 affinity (82).

Similar mutations were made to hPRL consisting of residues between sites 1 and 2 to determine if these mutations altered site 2 affinity. Mutations made between sites 1 and 2 were not shown to alter site 2 affinity. However, site 1 affinity was affected by various mutations and the affinity changes were highly correlated to the ED$_{50}$s in agonist assays. The $K_d$s and the ED$_{50}$s were reduced proportionately with each mutation. The stimulation of cells in the biological assays would require an active receptor that includes both sites 1 and 2 while the kinetic evaluation is only a measure of site 1. The equal reduction in both $K_d$ and ED$_{50}$ would indicate that site 1 serves as the gatekeeping event needed to create an active receptor able to induce proliferation. Site 1 would appear to be
rate limiting in terms of receptor activation. Site 2 affinities were not affected by the mutations and the affinity at site 2 was also shown to be very weak. This raises the possibility that site 2 needs to be open, but a significant affinity at site 2 is not necessary. Such a model for hPRL/receptor binding and activation opposes the current model where both sites 1 and 2 bind receptors with high affinity.

7.2 Receptor Antagonists

The goal of receptor antagonism is to create a molecule capable of binding a receptor in competition with in vivo hormone without inducing biological activity. Several antagonists have been created over the years but have yet to be compared. In additional, several physical features of each antagonist have yet to be characterized. These physical features are often relevant for production of such proteins.

Here, receptor antagonists were studied together and structural, biological, and kinetic features of each were reported. Δ41-52 hPRL and S179D hPRL were not structured well and were shown to be agonistic in nature rather than inhibit cell proliferation. Additionally, neither protein bound the hPRL receptor with high affinity at site 1. Based on these results, both Δ41-52 hPRL and S179D hPRL would make for poor receptor antagonists.

Δ1-14 hPRL and G129R hPRL showed structural properties similar to that observed for wild-type hPRL. Both proteins displayed a strong affinity for the hPRL ECD-receptor at site 1. In agonist assays, both appeared to have agonist activities
stimulating cell proliferation at low to modest concentrations. Because of this, both proteins would not be considered as viable receptor antagonists.

\Delta 1-14/G129R hPRL displayed structural characteristics similar to that of wild-type hPRL and had a strong affinity for receptor at site 1. Agonist assays revealed that this protein did not stimulate cell proliferation and in antagonist assays showed complete inhibition of cell proliferation. \Delta 1-14/G129R hPRL showed all the characteristics required for a viable receptor antagonist. It is interesting that the \Delta 1-14 deletion or the G129R mutation of hPRL each were poor antagonists that sustained considerable agonist action. When combined, all agonist activities were lost and gained antagonist properties that were not observed in either \Delta 1-14 or G129R hPRLs. Thus, of all the proposed antagonists, \Delta 1-14/G129R hPRL appears to be the only successful antagonist. But the synergism by which these two structural changes combine to make a potent antagonist are unknown.

7.3 Prolactin Receptor Subdomains

The hPRL ECD-receptor consists of two subdomains named S1 and S2. Each subdomain is known to contain regions necessary to bind hPRL and activate the complete receptor. Studies have shown that deletion of certain regions of the receptor either inhibits activation or creates constitutively active receptors \textit{in vivo}. The regions of the receptor that are thought to inhibit activation were postulated to have an affinity for other regions of the receptor to create this inhibition. Further, other regions of the receptor could potentially induce activation when in contact with an adjacent receptor.
Both the S1 and the S2 subdomains showed no affinity for hPRL in the kinetic evaluation at either site 1 or site 2. The S2 subdomain had no effect in either the agonist or antagonist assays and was shown to not bind any portions of the receptor in the kinetic evaluation. The S1 subdomain, however, was shown to stimulate cell proliferation in agonist assays in the absence of hormone. The S1 subdomain when bound to the surface of a sensor chip also showed a relatively strong affinity for the ECD of the hPRL receptor as well as a weak affinity for free S1 subdomain. The activation of cells in the agonist assays could be explained with the observation that the S1 subdomain can bind regions of the hPRL receptor and would explain why cell proliferation occurs with S1 subdomain application in the absence of hormone. The mechanism by which the S1 subdomain activates the cells is unknown but it appears from the kinetic analysis that regions of the S2 subdomain are required in order to obtain high affinity binding between the S1 subdomain and an adjacent receptor. It is also of note that the S1 and S2 subdomains do not bind hPRL alone. Only the full ECD-receptor has been shown to be able to bind hormone. This also demonstrates that regions of the S2 subdomain are required to be present along with the S1 subdomain to allow for hPRL binding.

7.4  
*hGH and the hPRL Receptor*

Until recently the hPRL and hGH systems have been compared and were thought to function through similar mechanisms. This conclusion can be reached when one considers the types of cells each can activate, the 2:1 receptor:hormone complex they form, and the ability of hGH to activate the hPRL receptor. More differences are
surfacing as these systems are studied and one difference is the mechanism by which the receptor becomes activated when bound to hormone. hGH has a high affinity site 2 for the hGH receptor while hPRL has a weak site 2 affinity. The weak site 2 affinity of the hPRL receptor is also present with hGH. No site 2 affinity was observed between the hPRL ECD-receptor and hGH indicating another difference in the ways hGH and hPRL receptors become activated. The mechanism by which the hPRL receptor becomes activated does not involve a high affinity site 2, rather site 1 binding appears to be the gatekeeping event that activates this hormone/receptor complex. The similarity of $K_d$ at site 1 and the $ED_{50}$ of hGH for the hPRL receptor also suggests the notion that site 1 binding functions as the gatekeeping event. Even though there is essentially no site 2 affinity, the cell assay shows an almost equal affinity to what was observed for site 1 in the kinetic study between hGH and the hPRL receptor. This is another piece of evidence that binding at site 1 is the critical step for activation of the receptor and for biological activity. Site 1 binding also creates a functional site 2. Although these site 2 surface interfaces articulate, they do not appear to contribute a significant amount of free energy to the formation of the active hormone/receptor heterotrimeric complex.
LIST OF REFERENCES


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