AN EXAMINATION OF THE KINETIC, STRUCTURAL, AND BIOLOGICAL EFFECTS OF ZINC ON LACTOGENIC CYTOKINE INTERACTION WITH THE HUMAN PROLACTIN RECEPTOR

DISSERTATION

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

By

Jeffrey L. Voorhees, B.S., B.A., M.S.

* * * * *

The Ohio State University
2008

Dissertation Committee:

Dr. Charles L. Brooks, Adviser
Dr. James Cowan
Dr. Ross Dalbey
Dr. Donald H. Dean

Approved by
The Ohio State Biochemistry Graduate Program
ABSTRACT

Human prolactin, human growth hormone, and human placental lactogen are members of the class 1 cytokine family and are individually capable of binding and biologically activating the human prolactin receptor. Structural and biological work have demonstrated that zinc is directly involved in placental lactogen and growth hormone-receptor complex formation but not in the formation of the prolactin-receptor complex. Concentrations of circulating zinc vary significantly throughout the life of an individual and the role and activity of lactogenic hormones vary similarly. Previous examinations of the role of zinc in hormone-receptor complex formation have largely focused on demonstrating cytokine binding and identifying factors that mitigate it. However, little work has been done to assess the importance of zinc concentration in experimental conditions. This work utilizes surface plasmon resonance (SPR), novel mathematical modeling, FRET, and biological assays to illuminate previous work by directly comparing the effects of zinc on the particular kinetic, conformational, and biological changes in the interaction of lactogenic cytokines with the human prolactin receptor.
To Dan Schult and John Schamber, who not only encouraged, but endured my questions.

To Drs. David Fairchild and Clark Butler, who showed me that being challenged can be more fun than challenging others.

To Drs. Robert Gillespie and George Mourad, who opened their laboratories to a someone with nothing to offer but curiosity.

To Dr. Charles Brooks, who never said ‘no.’

To my wife and family, who have in their own ways stood by me throughout this process.
ACKNOWLEDGMENTS

I would like to collectively thank the research community of The Ohio State University who without fail have graciously opened their doors and made freely available the full extent of their resources – both intellectual and technical. This unwritten open-door policy was one of the significant reasons that the Ohio state University attracted me and I have never been disappointed.

I would also like to thank the people of the Department of Veterinary Biosciences for their welcoming and unwaveringly accepting attitude toward an interloper who would hardly knows which end of a mouse to feed, but who was accepted as a contributing member of the academic and social community.

I would like to thank my advisor, Dr. Charles Brooks, who never attempted to recreate himself in me, never discouraged me from trying something, and whose ability to intellectually morph I hope to emulate in my career.

I owe a significant debt of gratitude to my lab mates past and present: Dr. Umamaheswari Sivaprasad, Dr. Coleen Almgren, Laura DePalatis, Michelle Corliss, Wei Liu, TJ Gordon, and Geeta Vittal-Rao. I have learned more from you than you realize. This project would not be where it is today without the work of an unparalleled succession of truly gifted undergraduates: Scott McCardle, Mark Troyer, Jalal Siddiqui, Heather Fortney, Gena Han, and Josephine Bryk.
VITA

July 23, 2977.........................Fort Wayne, Indiana

2001...............................B.S. Biochemistry, Purdue University

2001...............................B.A. Philosophy, Purdue University

2001 – 2002.........................University Fellow, The Ohio State University

2002 – present .....................Graduate Research Associate, The Ohio State University

2003 – 2004.........................Science Teacher, National Science Foundation /Columbus Public Schools

2004...............................M.S. Biochemistry, The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Biochemistry
TABLE OF CONTENTS

Abstract ................................................................................................................................. ii
Dedication .............................................................................................................................. iii
Acknowledgments ................................................................................................................ iv
Vita ........................................................................................................................................ v
List of Tables ....................................................................................................................... x
List of Figures ..................................................................................................................... xi
Forward ................................................................................................................................ xiv

Chapters:

1. Introduction ...................................................................................................................... 1
   1.1 Project Overview ......................................................................................................... 1
   1.2 Background and Discovery of Lactogenic Cytokines .............................................. 2
   1.3 Contemporary Cytokine Research ............................................................................ 3
   1.4 Structural and Physiological Characteristics of Lactogenic Cytokines ............... 4
      1.4.1 Human Prolactin .............................................................................................. 5
      1.4.2 Human Growth Hormone ............................................................................... 12
      1.4.3 Human Placental Lactogen ............................................................................ 14
   1.5 Structural and Physiological Characteristics of the Human Prolactin Receptor .. 18
   1.6 The Role of Zinc in Lactogenic Cytokine Binding to the Human Prolactin Receptor 24

2. Materials and Methods .................................................................................................. 29
   2.1 Vector Design ........................................................................................................... 29
2.1.1 Human Prolactin (hPRL) and Human Growth Hormone (hGH) ..................................................29

2.1.2 Human Placental Lactogen (hPL) ..................................30

2.1.3 The Extracellular Domain of the Human Prolactin Receptor (hPRLr) ........................................31

2.2 Protein Expression and Purification ...........................................32

2.3 Protein Characterization ............................................................33

2.3.1 SDS-Polyacrylamide Gels ....................................................33

2.3.2 UV Absorbance Spectroscopy ..............................................33

2.3.3 Fluorescence Spectroscopy ..................................................39

2.3.4 Circular Dichroism .............................................................39

2.4 Surface Plasmon Resonance .....................................................44

2.4.1 SPR Protein Preparation and Preparation and Chip-Coupling ..............................................49

2.4.2 SPR Binding Experiments ..................................................50

2.4.3 SPR Kinetic Evaluation .......................................................50

2.5 Förster Resonance Energy Transfer ..............................................51

2.5.1 FRET Labeling .................................................................52

2.5.2 FRET: Cytokines with Zinc ..............................................52

2.5.3 FRET: Cytokines with Human Prolactin Receptor and Zinc ..............................................54

2.6 Fluorescence Emission: Cytokines with Zinc ...............................54

2.7 Biological Assays .................................................................54

2.8 Cell Toxicity Study ...............................................................55

3. Surface Plasmon Resonance .......................................................57
3.1 Surface Palsmon Resonance ..................................................57
3.2 Stoichiometry.................................................................63
  3.2.1 Human Prolactin.........................................................64
  3.2.2 Human Growth Hormone..............................................65
  3.2.3 Human Placental Lactogen............................................66
3.3 Kinetics ...........................................................................67
  3.3.1 Binding Models and Analysis.................................67
  3.3.2 Human Prolactin.........................................................69
  3.3.3 Human Growth Hormone............................................72
  3.3.4 Human Placental Lactogen............................................73
4. Förster Resonance Energy Transfer .......................................74
  4.1 FRET ...........................................................................74
  4.2 Cytokine Fluorescence.................................................76
  4.3 FRET: Cytokines............................................................77
  4.4 FRET: Cytokines and Human Prolactin Receptor .............85
  4.5 FRET: Zinc-Knockout Prolactin ....................................91
  4.6 Prolactin Receptor Binding: Site 1 vs. Site 2 ..........91
5. Biological Assessment.........................................................95
  5.1 Cell Assays.................................................................95
  5.2 Effects of Zinc on ED_{50} Values..................................96
  5.3 TPEN Toxicity Study......................................................97
6. Results and Discussion.....................................................100
6.1 Ordered Binding of Lactogenic Cytokines to Human Prolactin Receptor

6.2 Lactogenic Cytokines and Zinc

6.2.1 Kinetics

6.2.2 Conformational Changes

6.2.3 Biological Response

Appendix A

List of References
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Cysteine-mutant hormones</td>
</tr>
<tr>
<td>3.2</td>
<td>Human prolactin receptor binding</td>
</tr>
<tr>
<td>3.3</td>
<td>Rate and equilibrium constants</td>
</tr>
<tr>
<td>4.1</td>
<td>FRET efficiency of labeled cytokines</td>
</tr>
<tr>
<td>4.2</td>
<td>Cytokine FRET response with human prolactin receptor and zinc</td>
</tr>
<tr>
<td>5.1</td>
<td>ED$_{50}$ values determined by biological cell assay</td>
</tr>
<tr>
<td>6.1</td>
<td>Site 1 binding kinetics</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structure of human prolactin</td>
<td>8</td>
</tr>
<tr>
<td>1.2</td>
<td>Structure of human prolactin (α-helices)</td>
<td>9</td>
</tr>
<tr>
<td>1.3</td>
<td>Disulfide bonds in human prolactin</td>
<td>10</td>
</tr>
<tr>
<td>1.4</td>
<td>Significant residues in human prolactin binding</td>
<td>11</td>
</tr>
<tr>
<td>1.5</td>
<td>Structure of human growth hormone</td>
<td>15</td>
</tr>
<tr>
<td>1.6</td>
<td>Structure of human growth hormone (α-helices)</td>
<td>16</td>
</tr>
<tr>
<td>1.7</td>
<td>Disulfide bonds in human growth hormone</td>
<td>17</td>
</tr>
<tr>
<td>1.8</td>
<td>Structure of human placental lactogen</td>
<td>19</td>
</tr>
<tr>
<td>1.9</td>
<td>Structure of human placental lactogen (α-helices)</td>
<td>20</td>
</tr>
<tr>
<td>1.10</td>
<td>Disulfide bonds in human placental lactogen</td>
<td>21</td>
</tr>
<tr>
<td>1.11</td>
<td>The zinc binding pocket human growth hormone</td>
<td>25</td>
</tr>
<tr>
<td>1.12</td>
<td>The zinc binding pocket of human placental lactogen</td>
<td>26</td>
</tr>
<tr>
<td>1.13</td>
<td>Proposed zinc binding pocket of human prolactin</td>
<td>27</td>
</tr>
<tr>
<td>2.1</td>
<td>Reducing SDS-PAGE gels</td>
<td>34</td>
</tr>
<tr>
<td>2.2</td>
<td>Ultraviolet absorbance of human prolactin and mutants</td>
<td>35</td>
</tr>
<tr>
<td>2.3</td>
<td>Ultraviolet absorbance of human growth hormone and mutants</td>
<td>36</td>
</tr>
<tr>
<td>2.4</td>
<td>Ultraviolet absorbance of human placental lactogen and mutants</td>
<td>37</td>
</tr>
<tr>
<td>2.5</td>
<td>Ultraviolet absorbance of the extracellular domain of the</td>
<td></td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.6</td>
<td>FRET: Human prolactin</td>
<td>82</td>
</tr>
<tr>
<td>4.5</td>
<td>FRET: Human growth hormone</td>
<td>83</td>
</tr>
<tr>
<td>4.8</td>
<td>FRET: Human placental lactogen</td>
<td>84</td>
</tr>
<tr>
<td>4.9</td>
<td>FRET: Prolactin with human prolactin receptor</td>
<td>87</td>
</tr>
<tr>
<td>4.10</td>
<td>FRET: Growth hormone with human prolactin receptor</td>
<td>88</td>
</tr>
<tr>
<td>4.11</td>
<td>FRET: Placental lactogen with human prolactin receptor</td>
<td>89</td>
</tr>
<tr>
<td>4.12</td>
<td>FRET: Zinc-knockout prolactin</td>
<td>92</td>
</tr>
<tr>
<td>5.1</td>
<td>Biological assays</td>
<td>98</td>
</tr>
<tr>
<td>5.2</td>
<td>TPEN cell toxicity</td>
<td>99</td>
</tr>
<tr>
<td>6.1</td>
<td>Human prolactin receptor self-dimerization</td>
<td>103</td>
</tr>
<tr>
<td>A.1</td>
<td>Model of two-state binding and dissociation</td>
<td>109</td>
</tr>
</tbody>
</table>
FORWARD

The fundamental understanding of the relationship between cytokine structure and physiological effect is at a crossroads. Until recently it was generally accepted that activation of the prolactin receptor and subsequent cell signaling was brought about by a lactogenic cytokine first binding a membrane-bound receptor, undergoing a conformational change exposing a second receptor binding site, and ultimately binding a second identical prolactin receptor on the cell surface to form an activated trimeric complex. This understanding of binding-induced conformational change in cytokines emerged even as the theory of pre-existing equilibrium distribution took root in the 1990’s. The idea that proteins exist in a distribution of dynamic physical conformations was a progression from the induced fit model which was, itself, an evolution form the pre-World War II-era lock-and-key understanding of enzymatic activity. More current understanding suggests that the relationship between conformational change and the biological activity of proteins exists within the overlapping realms of pre-existing equilibrium and induced fit theory.

This discussion becomes pertinent when we, as scientists, integrate disparate studies to form an understanding of the mechanisms of biochemical interactions and biological response. While this complex model of protein activity lies at the heart of what it means to be a contemporary biochemist, it is more important to bear in mind that
we live and work in the midst of an ever changing, ever evolving field. Today’s dogma may be tomorrow’s quaint notion and, by extension, findings that deviate from the accepted model may ultimately prove indispensable. (Perhaps ironically, the history of science frequently lauds individuals whose findings were initially derided and dismissed.) For this reason, it is necessary to examine the quality and context of science independent of the scientist or the findings.

Without context, isolated discoveries may appear to conflict with one another when, in actuality, they simply describe unique aspects of the same phenomenon. For instance, a crystallographic study may present a static snapshot of the same protein in which an NMR work demonstrates highly variable or unstructured regions. Similarly, kinetic analyses may superficially present different findings by faithfully describing the same event under unique experimental conditions. For this reason it is important to examine findings within their unique contexts and conditions, letting science drive understanding rather than permitting current opinion to unduly weigh upon the significance of discovery.

It is in this light that I present my thesis. This body examines the relationship between cytokine conformational changes involved in lactogenic receptor binding and the influence of zinc on conformational changes and biological activity. I have worked to perform these experiments under the most reflective biological conditions possible and I have worked to examine those biological conditions. This study uses current biochemical, biophysical, and biological methods to form a kinetic, stoichiometric, structural, and biological characterization of the hormone binding event that activate the
human prolactin receptor. It is my hope that as the findings presented here contribute to the body of science, that they will also contribute to the interpretation of science.
CHAPTER 1

INTRODUCTION

1.1 Project Overview

Despite being closely related members of a cytokine superfamily, sharing a great deal of sequence identity and profound structural similarity, prolactin, growth hormone, and placental lactogen perform very different biological functions in humans and show very different biological responses with varying availability of zinc. Previous studies have reported on structural and kinetic aspects of the interaction between these lactogenic hormones and the prolactin receptor. However, beyond recognizing the biological and chemical necessity of zinc for activity of growth hormone and placental lactogen, little work has been done to examine the kinetic, structural, or biological effects exerted by zinc on this family of hormones. The work described here uses surface plasmon resonance, Förster resonance energy transfer, and biological assays to compare the binding kinetics, structural rearrangement upon receptor binding, and biological activity of lactogenic hormones in the context of varying zinc availability. Further, this work lends an additional voice to the growing body of technical approaches used in characterizing the interaction between lactogenic cytokines and the lactogenic receptor.
1.2 Background and Discovery of the Lactogenic Cytokines

A previously unreported hormone with “lactogenic” properties was discovered in sheep and cows by Stricker and Grueter in 1928. By injecting castrated virgin rabbits with pituitary extract they were able to induce mammary development and milk production (1, 2). The endocrinology community was skeptical that a unique hormone was responsible for the reported lactogenic qualities of this hormone, preferring instead to maintain that growth hormone served dual functions as lactogen and mitogen. Homogenates of human pituitaries invariably demonstrated both growth hormone and prolactin-like effects, but when analyzed contemporary purification techniques yielded only growth hormone. Purification of prolactin was difficult primarily due to what is now realized to be, compared to prolactin, an approximately 100-fold excess of growth hormone in anterior pituitary tissues. Though it was capable of evoking a biological response, proportionally speaking prolactin was little more than a minor contaminant during what effectively functioned as a growth hormone purification process. During the 1950’s Raben successfully isolated human growth hormone and demonstrated its ability to treat dwarfism (3, 4). Further evidence against the existence of a unique prolactin hormone in humans was the fact that assays of human growth hormone elicited both mitogenic and lactogenic responses while growth hormone form other species did not (5, 6).

However, anecdotal evidence soon emerged supporting the hypothesis of separate prolactin and growth hormones. Patients with acromegaly showed increased blood serum levels of growth hormone without demonstrating any symptoms of increased prolactin levels and, inversely, patients with galactorrhea demonstrated no clinical signs of
increased growth hormone levels while yet clearly demonstrating the effects of a milk-producing hormone (6). The breakthrough came in 1969 when a patient presented with galactorrhea after having earlier had a pituitary tumor removed. The patient showed continued prolactin activity even after presence of growth hormone had been removed (6-8). Not long after this a bioassay was developed capable of measuring prolactin concentrations in circulating blood and confirming that growth hormone and prolactin were, in fact, distinct hormones (9, 10). The amino acid sequence of mature human prolactin was first determined in 1977 by Edman degradation of trypsin-cleaved fragments (11) shortly before the discovery of extrapituitary prolactin production in decidualized endometrial cells (12). The complete amino acid sequence including the 28 residue signaling peptide was determined from cDNA in 1981 (13).

In 1962 Josimovich described a growth hormone-like substance in placenta which was later recognized as placental lactogen because of its ability to induce milk production in rabbit mammary cells (14, 15), however clinical trials showed it to be an ineffective growth promoting agent (6). Significantly less work has been done to examine human placental lactogen than human prolactin and human growth hormone.

1.3 Contemporary Cytokine Research

The seminal studies that began the process of characterizing the interaction between lactogenic cytokines and their receptors were conducted by Cunningham and Wells almost twenty years ago. By way of a painstakingly articulate series of studies primarily using mutagenesis, radio-labeled competitive binding assays, and biological assays, Cunningham, Wells, and their collaborators were able to identify key residues
involved in hormone-receptor interaction and zinc binding, compare the binding and activity of the different hormones, identify sequential receptor binding, comment explicitly on the role of zinc in this interaction, and report some of the earliest measurements of affinity between the lactogenic hormones and growth hormone and prolactin receptors (5, 16-22). In the process they effectively laid the groundwork for studies that continue to this day by members of the prolactin/growth hormone research community.

The advent of surface plasmon resonance technology (SPR) in the early 1990’s and the continued reporting of crystal structures has enabled more refined experiments for characterizing hormone-receptor interaction. Crystal and solution structures of human growth hormone (hGH), human prolactin (hPRL), and human placental lactogen (hPL) as well as analogs from other species both alone and in various complexes with receptor have provided detailed insights to the particular interactions that bind hormone to receptor as well as shed light on conformational changes that occur upon receptor binding (23-31). The first significant examination of the hormone-receptor binding to use SPR was conducted by Gertler, et. al. in 1996 (32). More recently, SPR is quickly becoming the method de jour for refined kinetic analysis of small molecules and has been heavily employed in prolactin/growth hormone research. The versatility of SPR allows questions of molecular interaction to be approached from many directions (33-36).

1.4 Structural and Physiological Characteristics of Lactogenic Cytokines

Human prolactin, growth hormone, and placental lactogen are structurally homologous members of a superfamily of class-I helical cytokines that has expanded to
include prolactin-like proteins, prolactin related proteins, proliferins, and proliferin-like proteins (37, 38). These recent additions are the product prolactin gene duplications in non-primates and have only recently been recognized (38). The mature species of human prolactin, growth hormone, and placental lactogen are 191-199 amino acids in length and range in mass from approximately 22kDa (growth hormone and placental lactogen) to approximately 23kDa (prolactin). Crystal and solution structures reveal that the proteins are each comprised of four anti-parallel $\alpha$-helices in an up-up-down-down motif. While human growth hormone and human placental lactogen share 85% amino acid sequence identity, human prolactin shares only 25% sequence identity with other two hormones. It is believed that these similar proteins are derived from a common ancestral gene, with prolactin diverging 400 million years ago from the gene that ultimately yielded growth hormone and placental lactogen. In non-primates placental lactogen appears to have derived from prolactin, whereas primate placental lactogen is a product of growth hormone lineage (39). The biological affects of these hormones are expansive and biological response can be propagated by delivery in circulating blood or locally by paracrine or autocrine action (38). The biological affects of these hormones will be elaborated upon individually in the following sections.

1.4.1 Human Prolactin

Prolactin is expressed in all vertebrates and more than 300 distinct biological activities have been ascribed to it including roles in reproduction, osmoregulation, immune regulation, growth, and metabolism (40-42). Because of the broad range of both actions and tissues affected by prolactin, alternate names such as “versatilin” and
“omnipotin” have been suggested (43). Due to the broad range of work that has been done on prolactin, this discussion will be kept in the context of human prolactin whenever possible. Prolactin production is primarily associated with lactotrophic cells of the anterior pituitary, though research has demonstrated prolactin production in human decidua, myometrium, skin, mammary epithelial cells, circulating lymphocytes, lacrimal glands, spleen, and tissues of the brain (41, 44, 45) whereas prolactin production is limited to the pituitary in non-human species with few exceptions (37). Perhaps most pertinent to disease has been the realization that not only do breast carcinoma cells express prolactin, but healthy breast epithelial cells express both prolactin and its receptor (46, 47). Further, breast carcinomas express higher levels of prolactin receptor than surrounding healthy tissues (48). This implication of prolactin in human breast cancer has placed great emphasis on the development of prolactin antagonists and new manners of inhibiting activation of the prolactin receptor.

The human prolactin gene is located as single copy on chromosome 6, specifically located in the interval 6p22.2-p21.3 (49, 50). The gene is more than 15kb in length and encompasses five exons (some sources recognize exon 1a and 1b separately) that are differentially expressed in pituitary and extrapituitary cells (44, 51). Pituitary prolactin is regulated by a proximal promoter approximately 2kb in length and transcription begins at exon 1b. In extrapituitary tissues transcription is controlled by a promoter located approximately 5800bp upstream and transcription begins with exon 1a, resulting a transcript that is an additional 150bp in length. This additional 150bp lies in the 5’ untranslated region and DNA transcripts from both pituitary and extrapituitary cells code for a protein that is 227 amino acids in length. A 28 residue signal peptide is cleaved in
the endoplasmic reticulum and the mature 199 amino acid (approximately 23kDa) human prolactin is then secreted (52, 53).

A naturally occurring 16kDa fragment of the mature human protein has been described (54, 55). This isoform is believed to be the product of proteolytic cleavage and corresponds the first 145 N-terminal amino acids of the mature protein and has been detected in serum, pituitary, and amniotic fluid (56-58). This 16kDa fragment is mitogenic in vitro (Nb2 lymphoma cells, rat mammary cells) and lactogenic in vivo (rat mammary tissue) and inhibits angiogenesis in both in vitro and in vivo studies (58-60).

Human prolactin exists as a bundle of four α-helicies arranged in a familiar up-up-down-down motif that describes this family of cytokines (figure 1.1, 1.2) (30). Based on the model of the solution structure generated using Visual Molecular Dynamics software (62), helix 1 is comprised of residues 15-43, helix 2 is formed by residues 77-103, helix 3 is made of amino acids 111-136, and helix four is composed of residues 161-189. The six cysteines are each partnered to form three internal disulfide bonds (C4-C11, C58-C174, and C191-C199) (figure 1.3) and studies using ovine prolactin show that selective reduction of the disulfide bonds significantly and adversely affects the biological activity of the hormone (63). Alanine scanning mutagenesis and amino acid substitution have recognized regions of importance, especially regions on helices 1 and 4. Residues demonstrated to affect receptor binding or biological activity detrimentally when replaced are mapped in figure 1.4 (64-66). Specific mutations used in this work are addressed in later chapters.
Figure 1.1 Structure of human prolactin
A structural representation of human prolactin. The four characteristic α-helices are represented in blue (helix 1), red (helix 2), yellow (helix 3), and green (helix 4) (58, 59). Images are rotated 90° on the z-axis (center) and 90° on the x-axis.
Figure 1.2 Structure of human prolactin (α-helices)
A structural representation of human prolactin. The four characteristic α-helices are represented as tubes in blue (helix 1), red (helix 2), yellow (helix 3), and green (helix 4) (58, 59). Images are rotated 90°.
Figure 1.3 Disulfide bonds in human prolactin
A structural representation of human prolactin highlighting the three internal disulfide bonds formed, respectively, by C4-C11, C58-C174, and C191-C199 (58, 59). Images are rotated 90°.
Figure 1.4 Significant residues in human prolactin binding
A structural representation of human prolactin. The four characteristic α-helices are represented in blue (helix 1), red (helix 2), yellow (helix 3), and green (helix 4) and points of amino acid replacement resulting in reduced activity are shown in orange (58, 59, 61-63). Images are rotated 90°.
1.4.2 Human Growth Hormone

Growth hormone (somatotropin) is most widely recognized for its anabolic affects via its ability to stimulate long bone, muscle, and cartilage development postnatally, however an increasing body of information documents the role of growth hormone in immune function and sexual development and reproduction (67, 51, 68, 69). While growth hormone is primarily produced by anterior pituitary somatotrophs, expression has also been shown in human mammary tumors, testis (mRNA), and immune system (peripheral lymphocytes, thymus, and spleen) suggesting that, like prolactin, growth hormone can act in a paracrine/autocrine as well as endocrine fashion (67, 69, 70). An age-related decrease in growth hormone levels results in increases in body fat, decreases in lean body mass, loss of vitality, and bone loss (71) while exposure to excess levels of growth hormone puts individuals at risk of cardiovascular, respiratory, and metabolic disease. Additionally, excess growth hormone is implicated in breast, prostate, and colorectal cancer (72) via processes that may rely heavily on its ability to bind the prolactin receptor.

In humans, growth hormone is part of a family of five genes which includes the genes for placental lactogen and are clustered within a 47kb stretch on chromosome 17 (specifically, 17q22-q24). Included in the gene cluster are, in transcriptional order, “normal” growth hormone (GH-N), placental lactogen pseudogene (PL-1), placental lactogen A (PL-4), growth hormone variant (GH-V), and placental lactogen B (PL-3). Nomeclatures and numbering systems for placental lactogen and its genes vary and the term “chorionic somatomammotropin” is used synonymously. Placental lactogen A and B express identical proteins, though the “A” form is expressed preferentially in a ratio of...
5:1 (73, 74). The members of this gene family share 95% sequence identity and are transcribed in the same direction. Transcriptional control of this entire group of genes is governed by a single control region located 23kb upstream from the growth hormone gene (38). Growth hormone is the only member of this gene family found in all vertebrates with other family members being found only in primates. While this may seem to jar with the fact that many non-primates produce placental lactogen, it is reconciled by recognizing that primate placental lactogens are a product of growth hormone gene duplications and non-primate placental lactogens are derived from prolactin. Expression of growth hormone occurs in the pituitary while expression of GH-V and the placental lactogens take place in the placenta (37, 39, 67, 75, 76). The gene for growth hormone is made of five exons that are spread over approximately 2kb. The expressed form of the protein is 217 amino acids in length with the N-terminal 26 serving as a signaling sequence that is cleaved in the endoplasmic reticulum to yield a mature protein of 191 amino acids (77).

A variant 20kDa form of growth hormone exists, resulting from a partial splicing out of exon 3 with intron 2 during mRNA synthesis. The result is a growth hormone missing residues 32-46 (78). Limited biological examinations show that 20kDa growth hormone retains some of the capabilities of the full length form (most notably growth promotion in rats and pigeon crop sac stimulation in vitro) and is capable of binding growth hormone receptor, but is incapable of biologically activating the prolactin receptor (78, 79). Despite comprising 5-15% of pituitary growth hormone, the biological significance is unclear (80, 81).
Like all members of this cytokine family, human growth hormone has four easily identifiable α-helices (figure 1.5 and 1.6). Based on crystallographic data these helices span amino acids 6-35 (helix 1), 72-99 (helix 2), 110-129 (helix 3), and 150-183 (helix 4) (25, 62, 77), which is structurally analogous to human prolactin and placental lactogen (figure 1.5 and 1.6). Additionally, two internal disulfide bonds are formed by C53-C165 and C182-C189 (77) which mirror the disulfides formed in placental lactogen and two of the three disulfide bonds formed in prolactin (figure 1.7).

1.4.3 Human Placental Lactogen

The primary role of human placental lactogen (also called chorionic somatomammotropin) is in regulating metabolic functions during pregnancy that benefit the fetus by suppressing regular insulin response in the mother, thereby ensuring steady glucose supply, increasing insulin response during hyperglycemia, and increasing fat utilization (15, 80). Placental lactogen is placentally expressed and production increases gradually during pregnancy and peaking during the final four weeks of pregnancy before abruptly falling to pre-pregnancy levels within 24 hours of parturition (11, 15, 82). At its peak, placental lactogen is produced in quantities of grams per day and circulating placental lactogen level show a positive correlation with birth weight (83, 84).

Human placental lactogen is a member of the human growth hormone gene family and is discussed in section 1.4.2. The genes for placental lactogen A and B are the product of a gene duplication event and encode the same peptide sequence. They differ in their coding regions by only eight nucleotides, none of which result in coding alteration and the only differences between genes being in the 5’ and 3’ untranslated
Figure 1.5 Structure of human growth hormone
A structural representation of human growth hormone. The four characteristic α-helices are represented in blue (helix 1), red (helix 2), yellow (helix 3), and green (helix 4) (11, 59). Images are rotated 90° on the z-axis (center) and 90° on the x-axis.
Figure 1.6 Structure of human growth hormone (α-helicies)
A structural representation of human growth hormone. The four characteristic α-helicies are represented as tubes in blue (helix 1), red (helix 2), yellow (helix 3), and green (helix 4) (11, 59). Images are rotated 90°.
Figure 1.7 Disulfide bonds in human growth hormone
A structural representation of human growth hormone highlighting the two internal disulfide bonds formed, respectively, by C53-C165 and C182-C189 (11, 59, 85). Images are rotated 90°.
regions. Despite this amino acid sequence identity, the gene for placental lactogen A is expressed with approximately five times the frequency of placental lactogen B (73). The placental lactogen gene itself spans 1.65kb located on chromosome 17 (17q21-qter) and includes five exons and four exons. Like growth hormone, the expressed protein includes a 26 amino acid signaling peptide that is enzymatically cleaved yielding a mature protein of 191 amino acids (85, 86).

Since human placental lactogen and human growth hormone share 85% amino acid sequence identity, it is not surprising that their structures would be remarkably similar. Like the other proteins described here, placental lactogen has four characteristic \( \alpha \)-helices (figure 1.8 and 1.9). Models based on a crystal structure recognize these \( \alpha \)-helices as spanning residues 6-35 (helix 1), 72-93 (helix 2), 111-129 (helix 3), and 154-184 (helix 4) which coincides well with the structure of human growth hormone and matches well with published predictions (28, 62). Additionally, placental lactogen shares internal disulfide bonds C53-C165 and C182-C189 with human growth hormone (figure 1.10).

1.5 Structural and Physiological Characteristics of the Human Prolactin Receptor

Prolactin receptors and prolactin binding sites have been recognized in a large number of mammalian tissues (mammary gland, epithelial cells, endometrium, placenta, testes, prostate, skeletal muscle, liver, pancreas, stomach, esophagus, intestine, heart, lung, kidney, bone tissue skin, pituitary, brain, lymphoid tissue, etc.) (41). This seemingly ubiquitous expression lends to the far-reaching biological affects of lactogenic hormones. Currently, two theories exist as to the mechanism of prolactin receptor-
Figure 1.8 Human placental lactogen
A structural representation of human placental lactogen. The four characteristic α-helicies are represented in blue (helix 1), red (helix 2), yellow (helix 3), and green (helix 4) (14, 59). Images are rotated 90° on the z-axis (center) and 90° on the x-axis.
Figure 1.9 Human placental lactogen (α-helices)
A structural representation of human placental lactogen. The four characteristic α-helices are represented as tubes in blue (helix 1), red (helix 2), yellow (helix 3), and green (helix 4) (14, 59). Images are rotated 90°.
Figure 1.10 Disulfide bonds in human placental lactogen
A structural representation of placental lactogen highlighting the two internal disulfide bonds formed, respectively, by C53-C165 and C182-C189 (14, 59). Images are rotated 90°.
induced cellular response. The classical understanding is that response is initiated by a lactogenic cytokine binding to the extracellular domain of a membrane-bound receptor. Upon (or as a result of) binding to the receptor, a conformational change in the lactogenic cytokine exposes an additional prolactin receptor binding site which permits the formation of a heterotrimeric (hormone-receptor-receptor) complex external to the cell surface. More recent evidence suggests that additional pre-formed dimers of prolactin receptor may exist on the cell surface, to which lactogenic cytokines may bind and shift into an active conformation. The formation of this complex or, more specifically, the dimerization of the prolactin receptors in an active conformation upon complex formation, brings together the intracellular regions of the receptors in a manner that is not as of yet completely understood. The result, however, is the initiation of Jak2 phosphorylation of specific tyrosine residues in the cytoplasmic domain of the receptor leading to Stat5 phosphorylation. Phosphorylated Stat5 then dimerizes and moves across the nuclear membrane where it acts as promoter of DNA transcription, effectively upregulating cellular activity (41, 87, 98).

Prolactin receptors are members of the class 1 cytokine receptor superfamily that includes the closely related growth hormone receptor, gp130, and receptors for erythropoietin, leptin, and many interleukins (41). Prolactin receptors contain a multi-subunit extracellular ligand binding domain of 210 amino acids, a single membrane spanning region of approximately 24 hydrophobic residues, and in intracellular domains of variable length. The N-terminal extracellular region of the receptor can be divided into two distinct subdomains, domain 1 and domain 2, each comprised of approximately 100 residues and each folding into seven anti-parallel β-sheets joined by a hinge of...
approximately four residues centered at residue 100. Characteristic of the class 1 superfamily, the extracellular region of prolactin receptor contains two pairs of disulfide-linked cysteines in domain 1 (formed C12-C22 and C51-C62) and a tryptophan-serine WSXWS motif near the C-terminal membrane interface. While these conserved structural features do not interact directly with bound ligand, studies that alter these regions in receptors for growth hormone, IL-2, and erythropoietin receptors showed greatly reduce ligand binding and ligand-induced cellular response. This is most likely due to the critical structural role played by the disulfide bonds in the extracellular domain and the similarly vital role of the WSXWS domain in protein folding and trafficking (41, 89, 90).

The gene for human prolactin receptor is located near the gene for growth hormone receptor on chromosome 5 (5p13-p14) and includes 11 exons spanning more than 200kb. While the extracellular and membrane spanning domains of the expressed protein do not vary, alternate splicing results in various isoforms of the intracellular domain. Exons 3-10 encode for the 622 residue full length form of the receptor from which a 24 residue signal peptide is cleaved. A deletion of 573bp from exon 10 results in the intermediate form (325residues) and short forms of 376 and 288 amino acids (S1a and S1b, respectively) result from alternate splicing of exons 10 and 11. Additionally, a soluble form corresponding to the extracellular domain of the receptor has been reported (prolactin binding protein), though it is not clear if this is a product of proteolytic cleavage or another product of alternate mRNA splicing (41, 87, 88, 91-94). It is speculated that the soluble prolactin binding protein may extend the half-life of
circulating prolactin \textit{in vivo} by binding it and decreasing the rate of clearance from the blood (45).


\textbf{1.6 The Role of Zinc in Lactogenic Cytokine Binding to the Human Prolactin Receptor}

By 1991 research groups lead by Cunningham and Wells had discovered that human growth hormone and placental lactogen require zinc to bind the human prolactin receptor and that zinc has no significant effect on human prolactin binding. Using little more than mutagenesis, $^{125}$I competitive binding assays, and an early crystal structure of free porcine growth hormone they were able to publish dissociation constants for the receptor, determine the relative binding contribution of individual amino acids, and identify the individual amino acids involved in zinc binding in both hormone and prolactin receptor (16, 17, 19, 95). It would be four years before a crystal structure demonstrated the postulated binding site in growth hormone and 16 years before the analogous residues were confirmed in placental lactogen (24, 28). As a direct result of their work it is now recognized that the affinities of human growth hormone and placental lactogen for the prolactin receptor are highly zinc dependant whereas the affinity of human prolactin for the receptor is much less affected by zinc.

Crystalographic and mutagenic studies show a zinc binding pocket formed at the interface of growth hormone and placental lactogen and the receptor. A tetravalent zinc ion is coordinated at the first receptor binding site by D187 and H188 of the prolactin receptor and H18 and E174 of growth hormone and placental lactogen with H21 serving in a structural capacity to coordinate the two hormone residues (5, 19, 20, 21, 24, 28) (figure 1.11 and 1.12). Amino acid sequence alignment of growth hormone, placental
Figure 1.11 The zinc binding pocket human growth hormone
A structural representation of the proposed zinc binding pocket (residues H18, H21, and E174) of human growth hormone (11, 59). Images are rotated 90°.
Figure 1.12 The zinc binding pocket of human placental lactogen
A structural representation of the proposed zinc binding pocket (residues H18, H21, and E174) of human placental lactogen (14, 59). Images are rotated 90°.
Figure 1.13 Proposed zinc binding pocket of human prolactin
A structural representation of the proposed zinc binding pocket (residues H27, H30, and D183) of human prolactin structure (58, 59). Images are rotated 90°.
lactogen, and prolactin show the analogous residues in prolactin to be H27, H30, and D183 (figure 1.13). It is noteworthy that in growth hormone and placental lactogen – the two hormones for which zinc is required for prolactin receptor binding – the substituent groups of the residues forming the zinc binding pocket are no more than 4.5Å and 3Å apart, respectively, where as in prolactin D183 is approximately 8Å and 13Å from H27 and H30, respectively. The implications of this will be discussed in more detail in Chapter 4.

The role of zinc in growth hormone and placental lactogen binding is important not only physiologically but also experimentally. Total zinc concentrations in serum have been estimated at 5-23uM, though at equilibrium as much as 95% is bound by circulating protein resulting in a concentration of free zinc in the range of 1-2uM (19, 96, 97). Biological cell assays often come closest to approximating in vivo zinc conditions, if inadvertently, by including FBS or equine serum that contains endogenous zinc while purely in vitro examinations of the interaction between lactogenic cytokines and the prolactin receptor have typically ignored the zinc requirement or used a single 50uM concentration based on the early work of Cunningham and Wells. This work examines the interaction of the lactogenic cytokines with the human prolactin receptor in the context of various concentraitons of zinc.
CHAPTER 2

MATERIALS AND METHODS

2.1 Vector Design

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

The pT7-7 expression plasmid used in this research was originally received from S. Tabor (Harvard Medical School, Boston, MA) (99). This plasmid contains a gene for ampicillin resistance and a T7 RNA polymerase promoter which makes it suitable for protein expression in \textit{E. coli} expressing T7 RNA polymerase. Dr. Francis Peterson incorporated an f1 origin of replication, resulting in the pT7-7f(-) phagemid that is suitable for the production of single stranded DNA. The hPRL gene was cloned into the pT7-7 phagemid by Dr. Peterson and hGH was cloned into pT7-7 by Dr. Karen Duda. Pituitary mRNAs for hPRL and hGH were reverse transcribed and used as PCR templates that provided for the replacement of the signal sequence with an N-terminal methionine immediately upstream from the first residue of the mature protein. Additionally, NdeI and HindIII restriction sites were incorporated N-terminal and C-terminal to the sequence coding for the protein. The PCR product was incorporated into the pCR6.1 vector, amplified, digested with NdeI and HindIII, gel purified, and ligated into similarly digested and purified pT7-7 phagemend. Kunkel method site-directed mutagenesis was
used by Dr. Umasundari Sivaprasad to produce hPRL M158C and hGH N152C (34, 100). Briefly, the Kunkle method utilizes dut- ung- (i.e. lacking dUTPase and uracil N-glycosylase enzymes) RZ1032 E. coli and R408 helper phage (Promega, Madison, WI) to express single stranded DNA. DNA primers (Integrated DNA Technologies, Coralville, IA) containing the desired point mutations are then phosphorylated with polynucleotide kinase (New England Biolabs, Ipswich, MA) and T7 DNA polymerase (New England Biolabs, Ipswich, MA) and deoxynucleotide triphosphates are used to synthesize mutant-containing complimentary DNA before being ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA) and transformed into DH5α E. coli (Invitrogen, Carlsbad, CA) which removes the uricilated template DNA and replaces it with complimentary native DNA, resulting in double stranded mutant DNA.

2.1.2 Human Placental Lactogen (hPL)

hPL was cloned into pET-28b(+) (Novagen, EMD Chemicals, Darmstadt, Germany) by Geeta Vittal-Rao. The pET-28b(+) vector is encoded to expresses six consecutive histidine residues at the N-terminus of a protein cloned into the polyclonal site. This was overcome by digesting the vector at an NcoI restriction site (which lies between the ribosomal binding site and the His-tag) and at a HindIII restriction site in the polycloning region. The linearized vector was then extracted by gel purification. The hPL gene was PCR amplified using mutagenic primers incorporating N-terminal NcoI and C-terminal HindIII restriction sites. The gene was then double digested and gel purified. The linearized vector and purified hPL gene product were ligated together and transformed into XL-10 Gold cells (Stratagene, Cedar Creek, TX), grown on selective
kanamycin media, and cultured for DNA extraction. hPL N152C was created using QuikChange® Site-Directed Mutagenesis (Stratagene, Cedar Creek, TX). This method utilizes complimentary DNA primers, each encoding for the desired mutation, to PCR amplify the entire vector in which the gene of interest is contained. The PCR product is then digested with DpnI to cleave methylated (parental) DNA and transformed into E. coli for DNA amplification.

This work used wild-type (i.e. as found in vivo) forms of hPRL, hGH, and hPL as well as three mutant forms of each hormone. Mutant forms were designed to corrupt receptor Site 1 binding, corrupt receptor Site 2 binding, or elicit no effect on receptor binding and thereby serve as a control (see Table 3.1). All DNA sequences were confirmed by Sanger dideoxy sequencing (incorporating fluorescent dye labeled dideoxynucleotides that are read mechanically) at The Ohio State Plant-Microbe Genomics Facility.

2.1.3 The Extracellular Domain of the Human Prolactin Receptor (hPRLr)

The extracellular domain of the human prolactin receptor was cloned from a human cDNA library (Invitrogen, Carlsbad, CA) into pT7-7 phagemid by Dr. Umasundari Sivaprasad as described previously (3). This clone expressed the mature methionyl extracellular domain of the prolactin receptor (amino acids 1-210) as well as 14 amino acids of the membrane-spinning region. Kunkel mutagenesis was used to introduce a stop codon at native residue 211 resulting in a phagemid expressing the 210 N-terminal residues of the human prolactin receptor corresponding to only the extracellular region.
2.2 Protein Expression and Purification

Wild-type and mutant hPRL, hGH, hPL and hPRLr 1-210 were expressed as described previously (34, 101). Briefly, vectors containing the respective genes of interest were transformed into BL21(DE3) E. coli (Novagen, EMD Chemicals, Darmstadt, Germany) and grown on selective media. 1L flasks of pre-warmed and aerated Lysogeny Broth (LB) (102) containing appropriate antibiotic were inoculated with individual colonies and allowed to grow with shaking at 37° until an OD$_{600nm}$ between 0.300 and 0.400 was achieved. Protein expression was induced by the addition of isopropyl-β-d-thiogalactopyranoside (0.4mM final concentration for wild-type and mutant hPRL, hGH, and hPRLr and 1.0mM final concentration for wild-type and mutant hPL) and cultures were allowed to grow for an additional 8-12 hours. Cells were collected by centrifugation at 5000g for 10 minutes, resuspended in a buffered solution containing PMSF (Sigma-Aldrich, St. Louis, MO), and disrupted by two passes through a French pressure cell (SLM-Aminco, Urbana, IL) at 5000psi. Inclusion bodies were collected by centrifugation before being resuspension in 100mL of a solution of 4.5M urea, 50mM Tris, pH 11-11.5 and the inclusion bodies dissolved first by mechanical agitation followed by two days of gentle stirring and air oxidation to completely denature the proteins. Protein samples were then dialyzed extensively against four changes of four liters of 20mM Tris, pH 7.5 to allow refolding of the proteins before purification by anion-exchange chromatography using DEAE Fast Flow Sepharose (GE Healthcare, Chalfont St. Giles, United Kingdom) with an AKTA Explorer 100 chromatograph (GE Healthcare, Chalfont St. Giles, United Kingdom) in 20mM Tris, pH 7.5. Proteins were
eluted from the column with an increasing gradient of NaCl (0-600mM over 30 minutes) and collected in the region displaying a 280nm:260nm absorbance ratio approaching 2:1. Samples were then run over a 2.6x100cm Superdex 75 (Sigma-Aldrich, St. Louis, MO) column in 10mM ammonium bicarbonate to separate molecular species by size. Collected fractions were analyzed spectrophotometrically and fractions displaying a 280nm:260nm ratio of approximately 2:1 were pooled, frozen, and lyophilized.

2.3 Protein Characterization

2.3.1 SDS-Polyacrylamide Gels

20ug protein samples were displayed on a 15% SDS-polyacrylamide gels to demonstrate purity and relative size of expressed proteins (figure 2.1).

2.3.2 UV Absorbance Spectroscopy

Disulfide bonds absorb at 250-270nm and aromatics groups have maximum absorbances in the range of 260-280nm (258, 274, and 280nm for phenylalanine, tyrosine, and tryptophan, respectively) and proteins free of metal ions do not absorb significantly at 350nm (103, 104). Assessing the absorbance spectra of wild-type and mutant proteins from 200-350nm permits the determination of any significant conformational variance. 20uM samples of each protein were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and the ultraviolet absorbance spectra were measured for each protein from 200nm to 350nm (figures 2.2-2.5).
Figure 2.1 Reducing SDS-PAGE gels
20µg samples of recombinantly expressed protein were resolved on reducing 15% SDS-polyacrylamide gels.
Figure 2.2 Ultraviolet absorbance of human prolactin and mutants
20μM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and UV absorbance recorded from 220-350nm. Data were normalized to 278nm.
Figure 2.3 Ultraviolet absorbance of human growth hormone and mutants

20μM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and UV absorbance recorded from 220-350nm. Data were normalized to 278nm.
Figure 2.4 Ultraviolet absorbance of human placental lactogen and mutants
20μM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and UV absorbance recorded from 220-350nm. Data were normalized to 278nm.
Figure 2.5  Ultraviolet absorbance of the extracellular domain of the human prolactin receptor
A 20uM protein sample was prepared in 150mM NaCl, 10mM Tris, pH 7.4 and UV absorbance recorded from 220-350nm.
2.3.3 *Fluorescence Spectroscopy*

Aromatic amino acids (phenylalanine, tyrosine, and tryptophan) are capable of fluorescing if excited in their ranges of absorbance. While the absorbance spectra of these residues overlap significantly, only tryptophan has significant absorbance at 295nm and the resultant emission peaks near 340nm. Changes in the electron environment, particularly as a result of exposure to water, can influence the emission spectra of tryptophan. In order to assess changes in the electron environment of tryptophan in each hormone mutant, 1uM samples of each wild-type and mutant protein were prepared in 150mM NaCl, 10mM Tris, pH 7.4. The proteins were illuminated at 295nm and the fluorescent emission spectra were measured from 300-550nm using a Varian Cary Eclipse Fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA) (figure 2.6-2.9)

2.3.4 *Circular Dichroism*

Circular dichroism can permit assessment of protein secondary structure as it pertains to α-helices, β-sheets, and random coil structures, each of which provides a unique and characteristic CD spectrum. α-helical proteins will typically demonstrate negative peaks at 208nm and 222nm corresponding to π→π* and n→π* electron shifts, respectively and β-helical proteins will typically give readings in the positive range immediately above 200nm (positive peak at 196nm resulting from the n→π* transition) and a negative peak at 218nm due to the π→π* transition. Random coil proteins will
**Figure 2.6 Fluorescence spectrum of human prolactin and mutants**

1μM samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and illuminated at 295nm and the emission spectra recorded from 300-550nm
Figure 2.7 Fluorescence spectrum of human growth hormone and mutants
1uM samples were prepared in 150mM NaCl, 10mM Tris, pH 74 and illuminated at 295nm and the emission spectra recorded from 300-550nm
Figure 2.8  Fluorescence spectrum of human placental lactogen and mutants
1μM samples were prepared in 150mM NaCl, 10mM Tris, pH 74 and illuminated at 295nm and the emission spectra recorded from 300-550nm
Figure 2.9 Fluorescence spectrum of the extracellular domain of the human prolactin receptor
A 1uM sample was prepared in 150mM NaCl, 10mM Tris, pH 74 and illuminated at 295nm and the emission spectra recorded from 300-550nm
demonstrate a negative peak at 195nm (n→π*) and a positive peak at 212nm (π→π*).

20mM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and analyzed using an AVIV Circular Dichroism Spectrophotometer Model 202 (AVIV Biomedical, Inc., Lakewook, NJ) and CD spectra were recorded from 200-260nm (figure 2.10-2.13).

2.4 Surface Plasmon Resonance

Surface plasmon resonance (SPR) is the common name for a technique that allows the real-time measurement of the binding and dissociation of selected molecules. In SPR experiments, polarized light in the infrared spectrum is directed at a thin metal surface. Some of the energy from this light interacts with delocalized electrons in the metal (the plasmon) and reduces the proportion of light reflected. The angle at which the light is reflected is heavily affected by the refractive index of the opposite side of the metal film. It is on the side opposite the light that chemically active species are tethered in a flow cell. When this system is operated in an aqueous medium, secondary molecules can be washed over the chemically active surface. As molecules bind or dissociate from the chip, the refractive index changes, and the angle of reflection similarly changes. By measuring the change in angle of reflection over time, the binding or dissociation of biologically active molecules can be measured in a real-time fashion and kinetic information can be determined. The change in mass on the chip surface is measures in response units (“RU”), where on RU corresponds to a change of approximately 1pg/mm².
Figure 2.10  Circular dichroism analysis of wild-type and mutant human prolactin
20uM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and analyzed from 200-260nm at 20-25°. Results were normalized to 223nm.
Figure 2.11 Circular dichroism analysis of wild-type and mutant human growth hormone
20uM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and analyzed from 200-260nm at 20-25°. Results were normalized to 223nm.
Figure 2.12  Circular dichroism analysis of wild-type and mutant human placental lactogen
20uM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and analyzed from 200-260nm at 20-25°.
Results were normalized to 223nm.
Figure 2.13  Circular dichroism analysis of the extracellular domain of the human prolactin receptor
20uM protein samples were prepared in 150mM NaCl, 10mM Tris, pH 7.4 and analyzed from 200-260nm at 20-25°.
2.4.1 SPR Protein Preparation and Chip-Coupling

Proteins were designed and expressed to be thiol-coupled to Biacore CM5 Sensor Chips (GE Healthcare, Chalfont St. Giles, United Kingdom). This coupling method permits the covalent attachment of a protein to the chip surface at a specific residue. Thiol-coupling requires a free cysteiny1 sulfhydryl, which in these experiments was provided by the introduction of unbound cysteines in specific locations on the surface of the hormones. Proteins were prepared for thiol-coupling by solubilization in 10mM ammonium bicarbonate, pH 7 before a five minute room-temperature incubation in 5uM excess of dithioldithreitol (DTT, Sigma-Aldrich, St. Louis, MO). The DTT was removed by three centrifugations in YM-10 Centricon Centrifugal Filter Devices (Millipore, Billerica, MA), each time replacing lost volume with 20mM sodium acetate, pH 4.5.

The carboxylated dextran surface of the chip was prepared for thiol-coupling by injecting 25uL of freshly combined 50mM N-Hydroxysuccinimide (NHS) and 20mM 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), followed by 40uL of freshly prepared 80mM 2-(2-pyridinylthio) ethanolamine (PDEA) in 100mM boric acid, pH 8.5. Unreacted amine-binding sites were blocked by injecting 50uL of 1M ethanolamine, pH 8.5. Sequential injections of prepared hormone were then made, binding protein in a step-wise fashion until the chip surface of contained approximately 200-800RU of bound protein. A blank lane was chemically activated and unreacted thiol-binding groups on all lanes were blocked by injecting 30uL of 50mM cysteine, 20mM sodium acetate, 1M NaCl, pH 4.5. All preparative injections were made at 5uL/minute using a Biacore 3000 instrument (150mM NaCl, 3mM EDTA, 0.005% Surfactant P20, 10mM HEPES, pH 7.4).
2.4.2 SPR Binding Experiments

Various concentrations of hPRLr (10nM-100uM) were prepared in each of two separate buffers: 150mM NaCl, 3mM EDTA, .005% Surfactant P20, 10mM HEPES, pH 7.4 (HBS-EP, GE Healthcare, Chalfont St. Giles, United Kingdom) or 150mM NaCl, 15uM ZnSO4, 10mM HEPES, pH 7.4 (JBS). The chips were primed with buffer appropriate to the experimental conditions and 300uL injections of various concentrations of prepared hPRLr were injected over the thiol-coupled hormones at 50uL/minute before being allowed to dissociate for one hour and the resulting sensograms recorded. Chips were regenerated between runs with a 25uL injection of 4.5M MgCl2, 150mM NaCl, 3mM EDTA, 0.005% Surfactant P20, 10mM HEPES, pH 7.4. Experiments were performed two to five times and the results averaged.

2.4.3 SPR Kinetic Evaluation

Kinetic binding models for hPRL M158C, hGH N152C, and hPL N152C presume a 1:2 stoiciometric model where one surface-bound hormone is capable of simultaneously binding two receptors. While previous work has determined that the heterotrimeric complex forms as a result of an ordered binding event (hormone binds receptor at Site 1 before binding hormone at Site 2), an ordered model of dissociation has not been established. In order to determine meaningful kinetic interpretations for a two-state binding model, it was presumed that receptor dissociation occurs in the opposite order of heterotrimeric formation (figure 2.14). It should be noted that a more complete mathematical models have been developed for data interpretation (Appendix 1), but modeling software capable of implementing it has yet to be developed.
Figure 2.14 Proposed model of ordered receptor binding and dissociation

Curve fitting and data analysis was performed using Scrubber 2.0 (David Myszka, Center for Biomolecular Interaction Analysis, University of Utah) and BIAevaluation 3.0. (GE Healthcare, Chalfont St. Giles, United Kingdom) (figure 2.15). Residuals were generally less than 2% of binding and calculated association and dissociation rate constants were used to determine equilibrium constants.

2.5 Förster Resonance Energy Transfer

Förster resonance energy transfer (FRET) permits the measurement of energy transferred from an excited fluorophore to a nearby acceptor. In these experiments, tryptophan illuminated at 295nm serves as the fluorophore and the reporter moiety is a 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) tag (Invitrogen, Carlsbad, CA). Excited tryptophan has a maximum emission at approximately 350nm, which coincides with the absorbance region of CPM (105). The CPM then maximally re-emits absorbed energy in the range of 460-480nm. The efficiency of this energy transfer is highly distance dependent ($1/r^6$) and effective over a short distance (<70Å) (106). In this way, intramolecular conformational changes can be observed by recording changes in FRET emission.
Figure 2.15  SPR curve fitting
An example of curve-fitting by non-linear regression.
2.5.1 FRET Labeling

Cysteine-mutant hormones (hPRL M158C, hGH N152C, and hPL N152C) were prepared for FRET labeling by solubilization in 10mM ammonium bicarbonate, pH 7 before a five minute room temperature incubation in 5μM excess of dithiolthreitol (DTT, Sigma-Aldrich, St. Louis, MO). The DTT was removed by three centrifugations in YM-10 Centricon Centrifugal Filter Devices (Millipore, Billerica, MA), each time replacing lost volume with 150mM NaCl, 10mM Tris, pH 7.4 (FRET buffer). 10mM CPM was prepared in DMSO and then added in a 10:1 molar ratio to the reduced hormone. Samples were stored away from light at 4° for 8-12 hours before being filtered through a Sephadex G75 column to separate unbound CPM. Fractions containing labeled hormone were pooled and dialyzed away from light against FRET buffer for 8-12 hours.

2.5.2 FRET: Cytokines with Zinc

250nM CPM-labeled cysteine-mutant hormone (hPRL M158C, hGH N152C, and hPL N152C) and 750nM corresponding wild-type hormone (1μM total protein) were combined in FRET buffer with varying concentrations (0-25μM) of either ZnSO₄ or Na₂SO₄ and allowed to come to equilibrium in darkness for 1 hour at room temperature. Samples were illuminated at 295nm using a Varian Cary Eclipse Fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA) and the emission spectra recorded from 300 to 550nm.
2.5.3 FRET: Cytokines with Human Prolactin Receptor and Zinc

Samples were prepared and experiments performed as described above. One-to-one ratio: 250nM CPM-labeled mutant hormone (hPRL M158C, hGH N152C, and hPL N152C) was combined with 750nM corresponding wild-type hormone and 1uM hPRLr (1uM hormone, 1uM hPRLr total). One-to-five ratio: 250nM CPM-labeled mutant hormone (hPRL M158C, hGH N152C, and hPL N152C) was combined with 1.25uM hPRLr (.25uM hormone, 1.25uM hPRLr total).

2.6 Fluorescence Emission: Cytokines with Zinc

1uM wild-type hPRL, hGH, and hPL prepared in FRET buffer with varying concentrations (0-40uM) of either ZnSO₄ or Na₂SO₄ and allowed to come to equilibrium in darkness for 1 hour at room temperature. Samples were illuminated at 295nm using a Varian Cary Eclipse Fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA) and the emission spectrums recorded from 300 to 550nm.

2.7 Biological Assays

FDC-P1 cells stably expressing the human prolactin receptor were provided by Genentech, Inc. (San Francisco, CA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FBS (Hyclone, Logan UT), 10ng/mL IL-3 (BD, Franklin Lakes, NJ), and 220µg/µL G418 sulfate (Hyclone, Logan, UT). Cytokine dose-response curves were obtained as previously described (34). Cells were washed in sterile PBS (Invitrogen, Carlsbad, CA) and placed in starvation consisting of RPMI 1640 without phenol red (Invitrogen, Carlsbad, CA) with 10% gelding serum (Hyclone, Logan UT), β-
mercaptopoethanol (Invitrogen, Carlsbad, CA), 220ug/uL G418 sulfate (Hyclone, Logan, UT) and maintained for 24 hours. Serum-starved cells were diluted in starvation media lacking β-mercaptopoethanol and aliquoted in 96-well plates (~15,000 cells/well) and exposed to hormone concentrations ranging from 0nM to 10uM and supplemented with final concentrations of 15uM ZnSO4, 2uM N,N,N’,N’-tetrakis(2-pyridylmethyl)ethylenediamine) (TPEN, Sigma Aldrich, St. Louis, MO), or no additional supplementation. Cells were incubated for 30 hours at 37° in a 5% CO2 environment. Cells were then treated with 10uL of vital dye (Alamar Blue, Accumed International, West Lake, OH) and incubated for 3.5 hours. Alamar Blue is metabolized and reduced in the cellular mitochondria and the reduced and oxidized forms have maximum absorbances at 570 and 600nm, respectively. As such, Alamar Blue can be effectively used to gauge relative cell viability. The absorbance spectrums of the reduced and oxidized dye overlap one another, so it is necessary to take this into account mathematically when calculating the percent of dye oxidized and reduced. Plates were spectrophotometrically evaluated at 570 and 600nm to measure mitochondrial reduction of the vital dye and establish dose-response curves (figure 2.16) (107). ED50 values were determined using a four parameter fit with Sigma Plot software. Assays were performed three times and the results averaged.

2.8 Cell Toxicity Study

Cells maintained in RPMI 1640 with 10% FBS, 10ng/mL IL-3, and 220ug/uL G418 sulfate were given a supplementation of 2uM TPEN or no supplementation. Cells were stained with Tripan Blue (Invitrogen, Carlsbad, CA) at 0, 24, 36, and 48 hours and
hemocytometeric cell counts recorded total cell count as well as number living versus dead.

Alamar Blue extinction coefficients

<table>
<thead>
<tr>
<th></th>
<th>Red</th>
<th>Ox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced 570nm</td>
<td>570nm</td>
<td>570nm</td>
</tr>
<tr>
<td>Oxidized 570nm</td>
<td>600nm</td>
<td>600nm</td>
</tr>
<tr>
<td>Reduced 600nm</td>
<td>570nm</td>
<td>600nm</td>
</tr>
<tr>
<td>Oxidized 600nm</td>
<td>80586cm⁻¹M⁻¹</td>
<td>117216cm⁻¹M⁻¹</td>
</tr>
</tbody>
</table>

\[
C_{\text{red}} = \frac{(\varepsilon_{\text{red}570nm} \cdot A_{570nm}) - (\varepsilon_{\text{ox}570nm} \cdot A_{600nm})}{(\varepsilon_{\text{red}570nm} \cdot \varepsilon_{\text{ox}600nm}) - (\varepsilon_{\text{ox}570nm} \cdot \varepsilon_{\text{red}600nm})}
\]

\[
C_{\text{ox}} = \frac{(\varepsilon_{\text{red}570nm} \cdot A_{600nm}) - (\varepsilon_{\text{red}600nm} \cdot A_{570nm})}{(\varepsilon_{\text{red}570nm} \cdot \varepsilon_{\text{ox}600nm}) - (\varepsilon_{\text{ox}570nm} \cdot \varepsilon_{\text{red}600nm})}
\]

Alamar Blue reduction (%) = \frac{C_{\text{red}}}{(C_{\text{ox}} \text{ of negative control cells})}

**Figure 2.16  Extinction coefficients and equations for calculating cellular reduction**
3.1 Surface Plasmon Resonance

Surface plasmons are electromagnetic polarities that spread across the interface of two materials with dielectric constants of opposite sign. Typically this is achieved at the interface of a metal (having a negative dielectric constant) and either air or water (both having positive dielectric constants) (108-111). The metal component is most commonly a thin layer of gold or silver though copper, titanium, and chromium can also been used (108). The collective oscillation of electrons (resonance) is brought about in the film by exposure to $p$-polarized light in the visible or near-infrared spectrum. Resonance propagates along a water-metal interface (for systems using gold or silver) in the range of 50-500nm from the point of incidence while penetrating perpendicularly 150-450nm into the water and 20-200nm into the metal (108, 112). This perpendicular penetration of the metal which decays evanescently with depth is central to the utilization of surface plasmon resonance in biophysical applications.

The conditions under which resonance is achieved is a function of wavelength and the incident angle of projected light (figure 3.1). As the incident light strikes the surface of the metal film, resonance causes absorbance at particular wavelengths while light of other wavelengths is reflected. Changes in the electromagnetic, dielectric, or physical
Figure 3.1 Surface plasmon reflectivity plot
Regions of resonance, shown by dark lines, are functions of wavelength and angle of incidence. *Left inset:* Reflectivity surrounding the critical angle. *Right inset:* Reflectivity versus wavelength. (108)

properties of the metal measurably affect its refractive index and angle of reflection. A Ketchman configuration (used by most commercial SPR instruments, figure 3.2) places a prism at the optical surface (108, 111). Reflected light is directed through the prism to a spectrophotometer where changes in the angle of reflection and distribution of light are recorded (113). These changes in the angle of reflection correspond to changes in the chemical properties of the film. The binding or dissociation of small molecules during standard biochemical experiments increase or decrease the mass of the sensor chip which consequently alters the angle at which the incident light is reflected.
Figure 3.2 Ketchman configuration
A prism placed at the surface of the metal film distributes reflected light. (114)

Many commercial platforms for surface plasmon resonance (SPR) are available. Experiments described here were performed using a Biacore 3000 instrument (GE Healthcare, Chalfont St. Giles, United Kingdom). Biacore makes available gold sensor chips for SPR that are prepared with carboxylated or carboxylated dextran surfaces, ready made for covalently binding biological molecules by various chemical coupling methods (amine, thiol, aldehyde, maleamide). Experiments described hereafter used Biacore Research Grade CM5 Sensor chips (GE Healthcare, Chalfont St. Giles, United Kingdom) which are composed of a 50nm thick gold film coated with dextran fibers approximately 100nm in length (114). These dextran fibers make available multiple carboxyl groups which permit the thiol coupling chemistry that binds the lactogenic hormones to the chip surface.

Lactogenic hormones (prolactin, growth hormone, and placental lactogen) underwent site-directed mutagenesis that replaced a native amino acid with a cysteine
residue at one of three specific locations on the surface of the hormones. Previous work has demonstrated the ability of mutations of hPRL K181 and hPRL G129 to affect binding and biological activity, with the role of each being to specifically impede receptor binding at Site 1 or Site 2, respectively, and that mutations at hPRL M158 do not inhibit receptor binding or biological activity (34, 64, 66). Further, work on hGH G120 and hPL G120 (analogous to hPRL G129) and hGH K172 and hPL K172 (both analogous to hPRL K181) elicit effects that are similar to those seen in prolactin when mutated (28, 115-121). This information was used and extended to create an analogous series of cysteine mutants designed to disrupt Site 1 receptor binding, disrupt Site 2 receptor binding, or to elicit no effect on Site 1 or Site 2 binding (Table 3.1).

The hPRL mutants implemented here have been used in previous work and behave as predicted (34). The design of the prolactin, growth hormone, and placental lactogen cysteine mutants was based on crystallography, sequence alignment and structural homology (28, 66, 115, 122, 123). Structural studies have demonstrated that the six native cysteines in prolactin and four native cysteines in growth hormone and placental lactogen all form internal disulfide bonds (24, 28, 30). In this way the introduction of an additional surface-exposed cysteine yields a single free sulfhydryl that will permit thiol coupling of the hormones to the surface of a Biacore chip at a known position. In these experiments 300-500RU of mutant hormones were coupled to three of four available lanes on a Biacore chip. The remaining lane was chemically activated and blocked with a saturating injection of cysteine to serve as a reference channel. The extracellular domain of the prolactin receptor was allowed to interact with the hormones
Table 3.1 Cysteine-mutant hormones

Cysteine mutant hormones were created to selectively corrupt receptor binding Site 1, Site 2, or elicit no effect on receptor binding by being flowed over the chip in concentrations ranging from 10nM to 100uM and the relative responses were recorded.

Biacore measures the change in resonance angle in terms of “response units” (RU), an esoteric term in which a change of 1RU conveys a change in the resonance angle of .0001° and correlates to a change in mass on the surface of the chip of 1pg/mm² (114). Knowing the molecular masses of ligand and analyte permits calculation of the approximate mass of total ligand bound to the chip as well as the total amount of analyte bound at saturation, permitting stoichiometric comparison. Saturation can be calculated by plotting equilibrium responses vs. analyte concentration (figure 3.3). However, it is not possible in all experiments to reach a point of equilibrium between the on rate ($k_{on}$) and the off rate ($k_{off}$) during the binding phase. In these cases, evaluation software can be used to calculate $k_{on}$ and $k_{off}$ for a given binding and dissociation curve using nonlinear
Figure 3.3 Calculating saturation
(A) a graph showing injections of increasing concentration of analyte (114) (B) a graph of response vs. analyte concentration (114) (C) an example of curve-fitting by non-linear regression

The relative amount of hormone bound to the chip (in terms of RU) was followed during chip preparation and recorded. Because of the difference in mass between the hormones and the prolactin receptor, an adjustment is necessary to correlate the change in RU upon binding to the proportion of prolactin receptor bound per molecule of hormone. Because the extracellular domain of the human prolactin receptor has 7-11% greater mass than the hormones, the response elicited by one molecule of receptor binding is greater
than that of one molecule of hormone binding to the chip surface. The relative number of receptors bound to each surface-bound molecule of ligand at saturation was calculated according to the following equation:

\[
\text{Molecules of hPRLr bound/hormone} = \frac{A \times B}{C \times D}
\]

- \(A\) = response at saturation (RU)
- \(B\) = molecular weight of surface-bound hormone
- \(C\) = molecular weight of hPRLr
- \(D\) = amount of hormone bound to the chip (in RU)

This mathematical correction addresses discrepancies in response brought about by species of differing mass interacting with the chip surface and allows for the determination of stoichiometric relationships.

### 3.2 Stoichiometry

Because the native forms of the hormones used in this study ideally bind hPRLr in a 1:2 heterotrimeric complex, it would be presumed that a hormone on the surface of the chip bound in such a way as to expose two active binding sites would be able to bind approximately twice the amount of receptor as hormones coupled with only one available binding site. Previous work has shown that prolactin binds the receptor in a sequential fashion (with prolactin receptor binding to Site 1 before Site 2) and that blocking Site 1 can almost entirely abrogate binding of the receptor (34). If this motif is maintained across this family of hormones, the sequential binding of human growth hormone and human placentla lactogen should be demonstrated by hormone controls (with two active binding sites) binding approximately twice the amount of prolactin receptor that is bound.
by the Site 2 knockouts (as they leave Site 1 available for binding) and Site 1 knockouts binding little or no prolactin receptor (table 3.1). It is to be noted that, while less than optimal, the particular experimental method used for these experiments subjected the surface of the chip to numerous fluid priming events. Subsequent experience has taught that this has a detrimental affect on the responsiveness of a chip, most likely due to the extremely high flow rates (>1mL/minute) that are encountered during chip priming. As a result, the amount of hormone bound to the chip during binding experiments is reduced from the amount initially bound during chip preparation. Up to 100uM human prolactin receptor was flowed over the chip to saturate each hormone in buffers containing EDTA or 15uM ZnSO₄ and the relative response of each binding and dissociation event was recorded.

3.2.1 Human Prolactin

The responses of the control hormones were used as a baseline of response for each respective mutant hormone set and this baseline response was labeled as 100% of maximum binding (table 3.2). Prolactin mutants behaved as described previously in buffer containing EDTA (34) as well as buffer containing 15uM ZnSO₄. hPRL G129C was able to bind approximately half the amount of receptor (52-56% of maximum) that was bound by hPRL M158C. Being that Site 2 is disrupted in hPRL G129C, any receptor binding can be attributed to Site 1 of the hormone. hPRL K181C binding was negligible both in quantity of response (1% of maximum) and quality. While technically demonstrating a “maximum” binding, examination of the sensogram shows no typical qualities of binding and/or dissociation (i.e. an accumulation of RU during the binding
Table 3.2  Human prolactin receptor binding  
Human prolactin receptor bound per molecule of hormone on the chip surface as measured by surface plasmon resonance.

<table>
<thead>
<tr>
<th></th>
<th>hPRLr/hormone EDTA</th>
<th>15uM Zinc</th>
<th>Binding (% of control) EDTA</th>
<th>15uM Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRL M158C</td>
<td>0.88</td>
<td>0.70</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>hPRL G129C</td>
<td>0.46</td>
<td>0.39</td>
<td>52%</td>
<td>56%</td>
</tr>
<tr>
<td>hPRL K181C</td>
<td>0.01</td>
<td>0.01</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>hGH N152C</td>
<td>0.75</td>
<td>0.69</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>hGH G120C</td>
<td>0.08</td>
<td>0.34</td>
<td>11%</td>
<td>49%</td>
</tr>
<tr>
<td>hGH K172C</td>
<td>0.01</td>
<td>0.04</td>
<td>1%</td>
<td>6%</td>
</tr>
<tr>
<td>hPL N152C</td>
<td>0.12</td>
<td>0.98</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>hPL G120C</td>
<td>0.12</td>
<td>0.56</td>
<td>100%</td>
<td>57%</td>
</tr>
<tr>
<td>hPL K172C</td>
<td>0.02</td>
<td>0.02</td>
<td>17%</td>
<td>2%</td>
</tr>
</tbody>
</table>

phase, a gradual decrease in RU during the dissociation phase, or dose/response relationship). For this reason the trivial “binding maximum” that was detected for this protein can likely be dismissed as an experimental artifact. This lack of observed response for hPRL K181C is explained by the ordered binding mechanism of prolactin that requires Site 1 binding before binding can occur at Site 2. Site 1 binding is disrupted in hPRL K181C, which effectively prevents any receptor from binding to the hormone.

It should be noted that stoichiometric results of prolactin binding to its receptor were similar independent of the availability of zinc in solution. This re-establishes the findings of Cunningham and Wells and other by showing that zinc has little bearing on prolactin binding to its receptor (19, 61).
3.2.2 Human Growth Hormone

The stoichiometric responses of human growth hormone in the presence of 15uM zinc were similar overall to those of prolactin, though the responses differed in the absence of zinc. (The kinetic implications of differing responses to zinc of hGH and hPL mutants will be commented upon more fully in the section 3.3.) The proportional response of the human growth hormone Site 2 knockout (hGH G120C) was enhanced by the presence of zinc (table 3.1 and 3.2). The availability of zinc permitted the Site 2 knockout, which retains one available receptor binding site, to bind approximately half as much receptor as the control (hGH M158C) with two active binding sites (Table 3.1 and 3.2). Further, the corruption of Site 1 (hGH K172C) showed very little receptor binding despite the availability of one binding site (table 3.1 and 3.2). This echoes the behavior of prolactin and implies that receptor binding is an ordered process.

3.2.3 Human Placental Lactogen

The stoichiometric binding response of placental lactogen in 15uM zinc was very similar to those encountered for prolactin and growth hormone. The Site 2 knockout (hPL G120C) with one available receptor binding site bound approximately half as much receptor per molecule as the hormone possessing two available receptor binding sites (hPL N152C), and when Site 1 was corrupted (hPL K172C) very little receptor was bound despite possessing an active receptor binding site. This further points to the sequential binding motif of lactogenic cytokines whereby a hormone must first bind the prolactin receptor at Site 1 before it can bind receptor at Site 2 (table 3.1 and 3.2).
On the surface, examination of the numerical responses of receptor binding to placental lactogen in the presence or absence of zinc may appear incongruent with those described above. (A Site 2 knockout placental lactogen binding as much receptor as the control and a Site 1 knockout with substantial binding would not be anticipated.) However, maximum receptor binding to placental lactogen in the absence of zinc was so low that mild variations in recorded responses could cause artificially high stoichiometric comparisons, hence normal comparison of binding proportions is not representative of true placental lactogen-receptor binding.

3.3 Kinetics

3.3.1 Binding Models and Analysis

Surface plasmon resonance experiments were performed as described above. Concentrations of prolactin receptor were allowed to bind to the surface-bound hormones for 300 seconds and then allowed to dissociate. Curves were fitted to binding models using BIAevaluation 4.1 software (GE Healthcare, Chalfont St. Giles, United Kingdom). The curves for binding of receptor to Site 1 and Site 2 knockouts (Table 3.1) were fit using a simple Langmuir 1:1 binding model. Non-linear regression of these plots permits determination of on and off rates ($k_1$ and $k_{-1}$) and dissociation constants ($K_D$) of binding. Curves for the binding of receptor to control hormones with two active binding sites were fit to a model proposing sequential binding of the receptor (Site 1 before Site 2), as suggested by the stoichiometric analysis, and ordered dissociation where receptor dissociates from Site 2 before Site 1 (figure 2.12). This allowed for calculation of on rates, off rates, and dissociation constants for the interaction at Site 1 as well as global
dissociation constants describing the overall binding reaction. It should be noted that this model makes explicit presumptions about the order of receptor dissociation from the complex that go beyond the scope of this work. Additionally, as will be discussed, the kinetic role Site 1 seems to be the significant determinant in trimeric complex formation. For both of these reasons kinetic calculations determined for Site 2 were judged to be potentially mathematically and mechanistically unrepresentative of the true reaction and were omitted from this work. A complete mathematical model for two-state binding and dissociation has been devised (Appendix 1), however, the limited flexibility of SPR analysis software does not permit it to be utilized at this time.

The differential requirement for zinc of human prolactin and the more closely related growth hormone and placental lactogen has been widely reported (16, 17, 19, 24, 28, 61, 95, 115, 119). While zinc shows little affect on the binding activity of prolactin, it is required for growth hormone and placental lactogen to bind the human prolactin receptor. The zinc binding pocket (discussed in Chaper 1) formed by H18, H21, and E174 of growth hormone and placental lactogen coordinate and hold a zinc ion at the face of the first prolactin receptor binding site and mutations corrupting the zinc binding site of hGH and hPL have shown marked decreases in affinity for prolactin receptor (16, 17, 19, 28, 95). A tetravalent zinc ion is coordinated at the first receptor binding site by D187 and H188 of the prolactin receptor and H18 and E174 of growth hormone and placental lactogen with H21 serving in a structural capacity to coordinate the two binding residues of the hormone (5, 19, 20, 21, 24, 28). For these reasons it was expected that the availability of zinc would influence the kinetics of growth hormone and placental
lactogen binding human prolactin receptor more than it would influence the binding of prolactin.

It should be noted that reports often vary widely in the determination of particular kinetic values of a biological reaction. For instance, reported values for the global dissociation constant describing the binding of prolactin to its receptor vary over a range of two orders of magnitude. However, it seems that very rarely is the same experiment reported twice and just as rarely does experimental design carry over perfectly from one group to another. Some groups have determined $K_D$'s using $^{125}$I competitive binding assays while other used surface plasmon resonance. Some used surface bound receptor in their SPR experiments while other couple the hormone. Some use the extracellular domain of the prolactin receptor, some use truncated versions, and some contain portions of the membrane spanning region. Experimental conditions further vary in pH, buffer, analyte concentrations, etc., to the point that while we may not be comparing apples and oranges, we may be comparing Fuji with Granny Smiths. For this reason it is important the results of these experiments are examined beyond the particular numbers reported and against the backdrop of the experimental context.

3.3.2 Human Prolactin

Sources describing the binding of prolactin to its receptor report global dissociation constants that range from 3-110nM (19, 28, 34, 61). A surface-bound prolactin molecule possessing two active binding sites was allowed to bind free prolactin receptor extracellular domain (hPRL M158C) (table 3.1). Here a global $K_D$ of 79nM is observed for binding experiments conducted with no zinc (EDTA) which coincides
nicely with previously published work (table 3.3). Binding experiments conducted in 15uM zinc resulted in a KD of 3uM. While this 40-fold decrease in measured affinity of prolactin for its receptor is novel, this is the first study of this kind to measure receptor binding to a surface-bound receptor in the presence of zinc. The greatest kinetic contributor to this decreased affinity in the presence of zinc is the decreased on-rate (k_{on}) of the receptor. One explanation for the observed change in receptor binding is that the receptor dimerizes at high concentrations in the presence of zinc. The only previous study to use surface-bound hormone injected concentrations of prolactin receptor of less than 1uM (34). In the zinc-coordinated binding of the receptor to growth hormone and placental lactogen, two of the four residues that ultimately hold the free zinc ion are in the receptor. It may be that at high concentrations free receptor will coordinate and bind around a zinc ion – a phenomenon that does not occur at low concentrations of receptor or in the absence of zinc. On the surface this may be seen as an experimental artifact, but if true this may be of physiological significance when one recalls the prolactin binding protein. Little is known about the physiological role or expression of the prolactin binding protein. All that is known is that it corresponds to circulating extracellular domain of the prolactin receptor.

The kinetic assessments of Site 1 determined using a prolactin Site 2 knockout (hPRL G129C) were very similar to those corresponding to Site 1 that were derived from a two-state binding model, varying less than two-fold (32nM and 62nM without zinc, respectively, and 2.6uM and 1.2uM with 15um zinc, respectively) (Tables 3.1 and 3.3). The implications of this are two-fold: This supports the validity of these measurements within the context of this experiment by achieving very similar result by
<table>
<thead>
<tr>
<th>Site 1</th>
<th>[Zn2+]</th>
<th>k_{s1} (1/M*s)</th>
<th>k_{d1} (1/s)</th>
<th>K_{D_{Site1}} (M)</th>
<th>K_{D_{global}} (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRL M158C</td>
<td>0uM</td>
<td>1.92E+04</td>
<td>1.19E-03</td>
<td>6.20E-08</td>
<td>7.85E-08</td>
</tr>
<tr>
<td>hPRL M158C</td>
<td>15uM</td>
<td>2.35E+03</td>
<td>6.07E-03</td>
<td>2.58E-06</td>
<td>3.01E-06</td>
</tr>
<tr>
<td>hGH N152C</td>
<td>0uM</td>
<td>3.47E+03</td>
<td>2.00E-02</td>
<td>5.76E-06</td>
<td>6.79E-06</td>
</tr>
<tr>
<td>hGH N152C</td>
<td>15uM</td>
<td>2.73E+03</td>
<td>1.06E-04</td>
<td>3.89E-08</td>
<td>2.49E-07</td>
</tr>
<tr>
<td>hPL N152C</td>
<td>0uM</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hPL N152C</td>
<td>15uM</td>
<td>6.28E+03</td>
<td>9.36E-06</td>
<td>1.49E-09</td>
<td>2.22E-09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site 2</th>
<th>[Zn2+]</th>
<th>k_{s2} (1/M*s)</th>
<th>k_{d2} (1/s)</th>
<th>K_{D} (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRL G129C</td>
<td>0uM</td>
<td>3.42E+04</td>
<td>1.10E-03</td>
<td>3.23E-08</td>
</tr>
<tr>
<td>hPRL G129C</td>
<td>15uM</td>
<td>6.38E+03</td>
<td>1.01E-02</td>
<td>1.15E-06</td>
</tr>
<tr>
<td>hGH G120C</td>
<td>0uM</td>
<td>2.39E+03</td>
<td>1.29E-02</td>
<td>4.49E-06</td>
</tr>
<tr>
<td>hGH G120C</td>
<td>15uM</td>
<td>2.51E+02</td>
<td>2.91E-04</td>
<td>1.06E-06</td>
</tr>
<tr>
<td>hPL G120C</td>
<td>0uM</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hPL G120C</td>
<td>15uM</td>
<td>2.46E+03</td>
<td>6.30E-05</td>
<td>2.62E-08</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site 2</th>
<th>[Zn2+]</th>
<th>k_{s2} (1/M*s)</th>
<th>k_{d2} (1/s)</th>
<th>K_{D} (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRL K181C</td>
<td>0uM</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hPRL K181C</td>
<td>15uM</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hGH K172C</td>
<td>0uM</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hGH K172C</td>
<td>15uM</td>
<td>1.69E+02</td>
<td>2.64E-03</td>
<td>1.70E-05</td>
</tr>
<tr>
<td>hPL K172C</td>
<td>0uM</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hPL K172C</td>
<td>15uM</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Table 3.3 Rate and equilibrium constants**
Top: Receptor binding to hormones with two available binding sites. Middle: Receptor binding to hormones with only Site 1 active. Bottom: Receptor binding to hormones with only Site 2 active. N/A indicates that receptor binding was too low to measure kinetics.
two independent means. It also suggests that the formation of the heterotrimeric complex is heavily determined by the energetics and kinetics of receptor binding at Site 1 of the hormone. If binding at Site 2 were substantially tighter than Site 1, the global dissociation constant would be significantly higher than that of Site 1 alone. Given that the global dissociation constants differ so slightly from those of Site 1, it can reasonably be believed that Site 1 makes the more significant contribution to trimeric complex formation.

Prolactin with a corrupted Site 1 but an available Site 2 showed no measurable binding. This is consistent with previous work (34).

3.3.3 Human Growth Hormone

Previous analyses of human growth hormone binding to the prolactin receptor have provided affinities that range from 33pM to 5.6nM in the presence of 50uM zinc and 270-349nM in the absence of zinc (5, 28). In these experiments a global dissociation constant of 6.79uM is observed in buffer with EDTA and a global dissociation constant of 249nM is shown in the presence of 15uM zinc (table 3.3). The most significant observation in this is that the affinity of human grown hormone for the prolactin receptor is increased almost 30-fold in the presence of zinc. While zinc slightly reduces the rate of receptor binding, it decreases the rate of dissociation significantly. This is expected given the zinc binding pocket formed by the interface of the prolactin receptor and the first receptor binding site of growth hormone.

By examining binding at Site 1 of growth hormone (hGH G120C) it is apparent that zinc greatly decreases the rate of receptor dissociation (table 3.1 and 3.3), as would
be expected. Unexpectedly, a very small amount of receptor binding was seen using hGH K172C in the presence of zinc. This may be evidence that corrupting this residue does not completely prevent binding and may allow a small amount of residual “leaky” binding.

3.3.4 Human Placental Lactogen

Human prolactin receptor extracellular domain binding to human placental lactogen was unable to be demonstrated in buffer containing EDTA. Upon substitution with 15uM Zn₂SO₄ substantial binding was recorded. Placental lactogen demonstrated the tightest binding of the three hormones examined (global Kd of 2nM) and, like the other hormones examined, it appears that Site 1 is the significant contributor to the overall tightness of binding that is observed, owing in particular to the very slow rate of receptor dissociation. No receptor binds to the hPL Site 1 knockout (hPL K172C) is observed regardless of the availability of zinc (Table 3.1 and 3.3).
CHAPTER 4

FÖRSTER RESONANCE ENERGY TRANSFER

4.1 FRET

Förster resonance energy transfer is the transfer of energy between two chromophores without radiation, heat conversion, or kinetic contact. First reported by Theodor Förster in 1948 (125), dipole-dipole coupling allows the transference of resonance energy from an excited donor species to an acceptor species when respective emission and absorption spectra overlap and when the participating species are sufficiently near one another. Typically this requires the donor and acceptor to be 1-8nm from one another (106, 126). Beyond this range the transfer of energy is extremely inefficient and below this range energy is usually transferred by other means (reabsorption, complex formation, or collisional quenching)(127). The ability of donor-acceptor pairs to transfer resonance energy relies on the Förster distance of the particular pair. The Förster distance is defined as the distance of separation at which an average of 50% of the energy from an excited donor can be non-radiatively transferred to an acceptor, the rest being lost by other processes (128). The efficiency of transfer is highly distance-dependant, resulting in sensitivity to changes in distance of angstroms (129).
Figure 4.1 Distance-dependence of FRET Efficiency
The efficiency of resonance energy transfer (E) where $R_0$ equals the Förster distance of the donor-acceptor pair and $R$ equals the distance between the two.

$E = \frac{R_0^6}{R_0^6 + R^6}$

Experiments described here utilize native tryptophan residues in the hormones as the donor species. hPRL contains two tryptophans (residues 91 and 150) while hGH and hPL contain one tryptophan (residue 86 in both proteins). A fluorescent tag, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) (Invitrogen, Carlsbad, CA), was covalently coupled to an engineered surface-exposed cysteine (hPRL M158C, hGH N152C, and hPL N152) that is spatially removed from both prolactin receptor binding sites of the hormones (Table 3.1). Crystal and NMR structures suggest that this should result in a CPM tag being placed 13-20Å from the native tryptophans in the hormones and approximately 31Å from the nearest tryptophan in bound human prolactin receptor (24, 28, 30, 130). The Förster distance ($R_0$) for this donor-acceptor pairing is 31Å (127), indicating that the donor and acceptors are sufficiently near one another to generate a measurable FRET signal and that resonance energy transfer from the hormone tryptophans will be the predominant species contributing to resonance energy transfer.

4.2 Cytokine Fluorescence

1μM samples of wild-type human prolactin, growth hormone, placental lactogen and prolactin receptor were individually prepared in 150mM NaCl, 10mM Tris, pH 7.4
Table 4.1 FRET efficiency of labeled cytokines
The efficiency of resonance energy transfer of the tryptophan-CPM donor-acceptor pair within the labeled cytokines, based on crystal and solution structures and a Förster distance of 31Å

with varying concentrations of ZnSO₄ or Na₂SO₄ and allowed to reach equilibrium by incubating in darkness at room temperature for one hour. Samples were illuminated at 295nm and emission spectrums recorded from 300-450nm. Na₂SO₄ was chosen as a control to rule out the possibility that sulfate ions structurally affect the proteins in samples containing ZnSO₄. 295nm was chosen to illuminate samples because while tryptophan retains significant absorbance at wavelengths as high as 295nm, phenylalanine and tyrosine do not absorb substantially at 295nm (131). The emission spectrum of tryptophan has a maximum at 348nm (132) and changes in the emission spectrums would indicate changes in the solvent exposure of at least one tryptophan (133).

Experimental results show no significant change in the emission spectra for control samples using Na₂SO₄. Similarly, there was no significant change in the emission spectra for human growth hormone, placental lactogen, or prolactin receptor with

FRET efficiency for CPM-labeled hormones

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Efficiency (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRL W150</td>
<td>99.3%</td>
</tr>
<tr>
<td>hPRL W91</td>
<td>98.1%</td>
</tr>
<tr>
<td>hGH W86</td>
<td>95.7%</td>
</tr>
<tr>
<td>hPL W86</td>
<td>97.4%</td>
</tr>
<tr>
<td>hPRLr W72</td>
<td>50.0%</td>
</tr>
</tbody>
</table>
increasing concentrations of ZnSO₄. However, human prolactin showed a significant increase in emission near 340nm with increasing concentrations of ZnSO₄. This increased emission can be attributed to a structural rearrangement moving a tryptophan to a less polar (i.e. less surface exposed) environment (figures 4.2-4.5).

4.3 FRET: Cytokines

1uM samples of CPM-labeled hormones (250nM CPM-labeled hPRL M158C, hGH N152C, or hPL N152C combined with 750nM corresponding wild-type hormone) were prepared with varying concentrations of ZnSO₄ or Na₂SO₄ as described above. Samples were illuminated at 295nm and the emission spectra recorded from 300-550nm. The CPM tag absorbs maximally at 384nm, overlapping with the emission spectrum of tryptophan, and emits maximally at 469nm (127, 133, 134). In this way, energy absorbed by tryptophans in the hormone is able to be transferred to the fluorescent tag and measured as emission near 469nm. increases or decreases in the 469nm emission can be interpreted as decreases or increases in distance between the excited tryptophans and the CPM tag, respectively.

The control Na₂SO₄ samples showed no significant variation and human growth hormone and placental lactogen showed no response to increasing concentrations of ZnSO₄. However, human prolactin showed a significant and regular increase in 469nm emission with increasing concentrations of ZnSO₄ (figure 4.6-4.8). This indicates that upon exposure to increasing concentrations of zinc, the fluorescent tag at residue 158 is brought closer to at least one of the tryptophan residues in the protein.
Figure 4.2 Human prolactin fluorescence
1uM samples of human prolactin were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of Na$_2$SO$_4$ (top) or ZnSO$_4$ (bottom) and illuminated at 295nm and emission spectrums were recorded from 300-450nm.
Figure 4.3  Human growth hormone fluoresce
1uM samples of human growth hormone were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of Na$_2$SO$_4$ (top) or ZnSO$_4$ (bottom) and illuminated at 295nm and emission spectrums were recorded from 300-450nm.
Figure 4.4 Human placental lactogen fluorescence
1uM samples of human placental lactogen were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of Na₂SO₄ (top) or ZnSO₄ (bottom) and illuminated at 295nm and emission spectrums were recorded from 300-450nm.
Figure 4.5 Human prolactin receptor fluorescence
1uM samples of human prolactin receptor were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of Na$_2$SO$_4$ (top) or ZnSO$_4$ (bottom) and illuminated at 295nm and emission spectrums were recorded from 300-450nm.
**Figure 4.6 FRET: Human prolactin**

1uM samples of CPM-labeled human prolactin were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of Na$_2$SO$_4$ (left) or ZnSO$_4$ (right) and illuminated at 295nm and emission spectrums were recorded from 300-550nm.
Figure 4.7  FRET: Human growth hormone
1uM samples of CPM-labeled human growth hormone were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of Na$_2$SO$_4$ (left) or ZnSO$_4$ (right) and illuminated at 295nm and emission spectrums were recorded from 300-550nm.
Figure 4.8 FRET: Human placental lactogen
1uM samples of CPM-labeled human placental lactogen were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of Na$_2$SO$_4$ (left) or ZnSO$_4$ (right) and illuminated at 295nm and emission spectrums were recorded from 300-550nm.
4.4 FRET: Cytokines and Human Prolactin Receptor

In order to examine structural changes upon hormones binding to human prolactin receptor, samples combining CPM-labeled hormone and prolactin receptor were prepared in 1:1 and 1:5 molar ratios. A one-to-one ratio of hormone to prolactin receptor was prepared by combining 250nM CPM-labeled mutant hormone (hPRL M158C, hGH N152C, or hPL N152C) with 750nM corresponding wild-type hormone and 1uM prolactin receptor (1uM total hormone, 1uM prolactin receptor). A one-to-five ratio of hormone to prolactin receptor was prepared by combining 250nM CPM-labeled mutant hormone (hPRL M158C, hGH N152C, or hPL N152C) with 1.25uM prolactin receptor (.25uM hormone, 1.25uM prolactin receptor). Samples were prepared with varying concentrations of ZnSO₄ or Na₂SO₄ and allowed to reach equilibrium in darkness at room temperature as described above.

The ability of samples to achieve equilibrium is essential for these experiments. In solution the stoichiometric distribution of interacting species may differ from their relative molar ratios. Previously published reports indicate that the hormones used here should have global dissociations constants for human prolactin receptor that are approximately 100nM or less (28, 34). By combining hormones with receptor concentrations ten times greater than the Kᵤ, it is reasonable to believe that in samples combining 1uM hormone with 1uM receptor the predominant species should have a 1:1 stoichiometry in solution at equilibrium. Similarly, the samples with 1:5 molar ratios (.25uM hormone and 1.25uM prolactin receptor) should heavily favor the formation of a complex with a 1:2 stoichiometric ratio of hormone to receptor.
In samples combining 1uM hormone with 1uM prolactin receptor (corresponding to a 1:1 stoichiometry in solution), prolactin and growth hormone both showed significant increases in resonant energy transfer as demonstrated by the increased emission in the area near 469nm while sodium sulfate control samples showed no variation (figure 4.9 and 4.10). This indicates a systematic decrease in distance between the CPM-tag at hPRL M158 and at least one tryptophan (W91 and/or W150) and a systematic decrease in distance between the CPM-tag at hGH N152C and W86 with increasing concentrations of zinc. These increases in emission approach a maximum in 25uM zinc. Placental lactogen, however, demonstrated no zinc sensitivity in this experiment (figure 4.11).

Samples with 1:5 molar ratios of hormone to receptor (corresponding to a 1:2 stoichiometry in solution) each demonstrated increased FRET signal with increasing concentrations of zinc, a pattern which differed from that of the 1:1 stoichiometric ratio samples. The 1:5 prolactin samples demonstrated a zinc-induced increase in FRET response that was smaller than that for the 1:1 prolactin samples, the 1:5 growth hormone samples provided an increase in FRET signal that was similar to that produced by the 1:1 growth hormone samples, and the 1:5 placental lactogen samples showed an increase that was greater than that for the 1:1 placental lactogen samples. These results are summarized in Table 4.2.

The implications of these finding vary for each hormone. Human prolactin shows a structural rearrangement upon exposure to zinc whereas growth hormone and placental lactogen do not. The zinc binding pocket of growth hormone and placental lactogen is comprised of H18, H21, and E174 and residues H27, H30, and D183 in prolactin. It is
Figure 4.9 FRET: Prolactin with human prolactin receptor

Samples of CPM-labeled human prolactin were prepared in 1:1 (left) and 1:5 (right) molar ratios with human prolactin receptor in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of ZnSO₄ and illuminated at 295nm and emission spectrums were recorded from 300-550nm. 1:1 molar ratio samples contain 1uM prolactin and 1uM prolactin receptor, 1:5 molar ratio samples contain .25uM prolactin and 1.25uM prolactin receptor. Control samples prepared with Na₂SO₄ showed no change in response (data not shown).
Figure 4.10  FRET: Growth hormone with human prolactin receptor
Samples of CPM-labeled human growth hormone were prepared in 1:1 (left) and 1:5 (right) molar ratios with human prolactin receptor in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of ZnSO4 and illuminated at 295nm and emission spectrums were recorded from 300-550nm.  1:1 molar ratio samples contain 1uM prolactin and 1uM prolactin receptor, 1:5 molar ratio samples contain .25uM growth hormone and 1.25uM prolactin receptor.  Control samples prepared with Na2SO4 showed no change in response (data not shown).
Figure 4.11  FRET: Placental lactogen with human prolactin receptor

Samples of CPM-labeled human placental lactogen were prepared in 1:1 (left) and 1:5 (right) molar ratios with human prolactin receptor in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of ZnSO₄ and illuminated at 295nm and emission spectrums were recorded from 300-550nm.  1:1 molar ratio samples contain 1uM prolactin and 1uM prolactin receptor, 1:5 molar ratio samples contain .25uM placental lactogen and 1.25uM prolactin receptor.  Control samples prepared with Na₂SO₄ showed no change in response (data not shown).
Table 4.2 Cytokine FRET response with human prolactin receptor and zinc
Maximum FRET responses of cytokines combined with ZnSO4 and various molar ratios of prolactin receptor (1:0, 1:1, and 1:5). *The change in signal was within the range of variation for the instrument.

<table>
<thead>
<tr>
<th></th>
<th>1:0</th>
<th>1:1</th>
<th>1:5</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRL:hPRLr</td>
<td>80</td>
<td>115</td>
<td>50</td>
</tr>
<tr>
<td>hGH:hPRLr</td>
<td>&lt;10*</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>hPL:hPRLr</td>
<td>&lt;10*</td>
<td>&lt;10*</td>
<td>35</td>
</tr>
</tbody>
</table>

noteworthy that in growth hormone the residues that comprise the binding pocket are no more than 4.25Å from one another and separated by no more than 3Å in placental lactogen. However, in prolactin a distance of 8Å and 13Å separate H27 and H30 from D183, respectively. The bringing together of these three amino acids to form an active zinc binding pocket would require significant structural changes and cause substantial rearrangement of human prolactin. Inversely, the relative proximity of the residues forming the zinc binding pockets of growth hormone and placental lactogen may explain the lack of structural rearrangement upon binding zinc in these hormones. It is difficult to speculate which of the two tryptophans in hPRL plays a larger role in the demonstrated FRET signaling. The similarity in structure between the hormones places the analogous hPRL W91, hGH W86, and hPL W86 in structurally similar positions on helix 2 of the respective proteins. hPRL has an additional tryptophan (W150) that the other hormones lack. In the zinc-free state these two prolactin tryptophans are nearly equidistant from the CPM tag bound at residue 158 (13.5Å from W150 and 16Å from W91) and each transfer resonance energy with greater than 98% efficiency. If human growth hormone and placental lactogen are used as models, zinc binding would suggest little structural change,
particularly with regard to the distance between CPM and W86 (the analog of hPRL W91), which would suggest that W150 in hPRL experiences the more significant changes and leads to the majority of FRET signaling. However, if hPRL must undergo more significant reorientation than hGH and hPL in order to bind zinc, W91 (perhaps in addition to W150) may contribute to the reaction.

4.5 FRET: Zinc-Knockout Prolactin

Prolactin alone demonstrated a zinc-dependant FRET response in the absence of prolactin receptor. In order to explore the possibility that the observed effects were not due to the binding of zinc in the proposed prolactin binding site, two of the three residues that comprise the proposed site in prolactin were corrupted via alanine substitution. The resulting human prolactin H27A, H30A, M158C mutant was CPM-labeled and FRET experiments were conducted as described above (Section 4.3 and 4.4, respectively). In FRET experiments using only 1uM zinc-knockout prolactin no response to increasing concentrations of zinc was shown. However, experiments conducted with 1:1 molar ratios of zinc-knockout prolactin and prolactin receptor showed a similar zinc-dependant FRET response to those observed with “zinc-responsive” prolactin (Figures 4.6, 4.9, and 4.12).

4.6 Prolactin Receptor Binding: Site 1 vs. Site 2

As demonstrated by SPR experiments in this work and documented elsewhere, the receptor binding of human growth hormone and placental lactogen are greatly influenced
Figure 4.12  FRET: Zinc-knockout prolactin
(left) 1uM samples of CPM-labeled human prolactin H27A, H30A, M158C were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of ZnSO₄ and illuminated at 295nm and the emission spectrums recorded from 300-550nm.
(right) Samples of 1uM CPM-labeled human prolactin H27A, H30A, M158C and 1uM human prolactin receptor (1:1 molar ratio) were prepared in 150mM NaCl, 10mM Tris, pH 7.4 with various concentrations of ZnSO₄ and illuminated at 295nm and emission spectrums were recorded from 300-550nm.
by zinc whereas prolactin is not (19, 28). It has been suggested that a fixed set of residues make up the individual binding sites on the hormones whereas the residues of the receptor that are involved in hormone binding are more variable (and perhaps ligand-specific) and that this variability may afford prolactin receptor its biological promiscuity (28). This suggests that the unstructured zinc binding site may be present even in receptor-bound prolactin and, taken further, that zinc binding may induce a conformational change despite prolactin already having bound a receptor. In these experiments prolactin showed a diminished response to zinc when both receptor binding sites are occupied (1:5 ratio) compared to when only Site 1 was bound to the receptor (1:1 ratio), indicating that prolactin receptor binding to Site 2 of prolactin restricts zinc-induced restructuring of the ligand. The stories are different for growth hormone and placental lactogen. Human growth hormone shows no change in zinc-induced FRET signaling upon Site 2 binding and placental lactogen demonstrates an increased response upon Site 2 receptor binding. Taken together, this indicates a lack in uniformity in structural affects of Site 2 prolactin receptor binding.

Site 2 binding shows diminished 340nm emission for prolactin and growth hormone but not placental lactogen. Being that this decrease does not correspond to alterations in FRET signaling at 469nm, this specifically implies that the binding of hPRLr at Site 2 changes the environment of tryptophan in prolactin and growth hormone by moving them to or creating a more polar environment. Given the similar responses in prolactin and growth hormone, this implies that hRPL W91 (and not W150) is the affected tryptophan. More broadly, given that there was no change in 340nm emission
for placental lactogen, this finding further indicates that hPRLr does not bind uniformly to Site 2 of the three hormones.
CHAPTER 5

BIOLOGICAL ASSESSMENT

5.1 Cell Assays

In order to assess the biological role of zinc in lactogenic cytokine binding, in vitro cell assays were performed using cytokine-responsive eukaryotic cells in the presence of varying concentrations of zinc. FDC-P1 cells are a murine hematopoietic stem cell line that require the presence of IL-3 in order to be sustained in culture (135-137). The cells used in this study were transfected with and stably express the human prolactin receptor and are responsive to stimulation by lactogenic cytokines (34). The cell assays exposed serum-starved cells to hormone concentrations of 0nM-10uM and measured the relative biological response by measuring the reduction of a non-toxic indicator dye that is reduced in the mitochondria. Dose-dependant response to increasing concentrations of the lactogenic cytokines permitted the determination of ED_{50} values (i.e. the concentration at which one half maximum cellular response is achieved). Cell assays were performed as described previously (34), in media supplemented with 15uM ZnSO_{4}, or supplemented with 2uM N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). TPEN is a heavy metal-specific chelator with a particularly high affinity for Zn^{2+} (10^{15.6} M^{-1}) as well as Fe^{2+} (10^{14.6} M^{-1}) and Mn^{2+} (10^{10.3} M^{-1}) (138). The standard FDC-P1 bioassay containing 10% gelding serum is estimated to have approximately 1uM
endogenous zinc. A 2uM supplementation of TPEN should result in a cell assay essentially devoid of zinc, and bioassays supplemented with 15uM ZnSO₄ would then possess 16uM total zinc. Each cell assay (standard, 2uM TPEN supplement, and 15uM ZnSO₄ supplement) was performed using each of the three hormones described in this work (human prolactin, growth hormone, and placental lactogen). Assays were performed three times and the results averaged (table 5.1). Dose-response curves were used to determine ED₅₀ values by using a four sigmoidal parameter fit (139). Assays were performed three times and the results averaged. Representative dose-response curves for cellular response to prolactin are shown (figure 5.1).

<table>
<thead>
<tr>
<th></th>
<th>hPRL</th>
<th>hGH</th>
<th>hPL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED₅₀ (nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16uM Zn²⁺</td>
<td>0.811</td>
<td>0.059</td>
<td>13.179</td>
</tr>
<tr>
<td>1uM Zn²⁺</td>
<td>0.865</td>
<td>0.327</td>
<td>26.678</td>
</tr>
<tr>
<td>0uM Zn²⁺</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Table 5.1  ED₅₀ values determined by biological cell assay

5.2 Effects of Zinc on ED₅₀ Values

Elevated concentrations of zinc decreased the ED₅₀ values in cellular assays of human growth hormone and placental lactogen. This shift in dose-response indicates that when more zinc is present in the media, maximal cellular response is achieved at lower
concentrations of growth hormone and placental lactogen. Because the interaction between human growth hormone and placental lactogen and the extracellular domain of the human prolactin receptor is so readily influenced by the availability of zinc, it is plausible that an increased availability of zinc facilitates increased binding and activation. The binding of prolactin to the extracellular domain of its receptor has shown to be largely unaffected by zinc and, accordingly, biological response was largely unaffected by increased concentrations of zinc.

However, chelation of zinc in assay media universally nullified any cellular response to cytokines (figure 5.1). No dose-response was observed in assays performed in TPEN-supplemented media. This was surprising in that prolactin can be shown to bind to the extracellular domain of its receptor in the complete absence of zinc.

5.3 TPEN Toxicity Study

It was suggested that TPEN may be toxic to the cells and thereby preventing any measureable cytokine-induced response. A toxicity study was undertaken in which FDC-P1 cells in maintenance media (containing FBS and IL-3) were supplemented with 2uM TPEN or nuclease free water. Aliquots were periodically collected and stained to determine total cell counts as well as the number of living and dead cells. The results showed no significant difference in cellular proliferation or viability between control samples and samples supplemented with TPEN (figure 5.2). This indicates that TPEN alone is not toxic to the cells and that zinc may play a roll in cytokine induction that goes beyond binding to the extracellular domain of the prolactin receptor.
Figure 5.1 Biological assays
Biological assays assessing the cytokine-induced response were performed in the presence of increasing concentrations of human prolactin in media supplemented with 2uM TPEN (left), no supplementation (center), or 15uM ZnSO₄ (right).
Figure 5.2 TPEN cell toxicity
FDC-P1 cells were grown in maintenance media (control) or media supplemented with 2uM TPEN. Total cell counts were conducted at 0, 24, 36, and 48 hours (left axis) and the proportion of living cells was recorded as a percentage (right axis).
CHAPTER 6

RESULTS AND DISCUSSION

6.1 Ordered Binding of Lactogenic Cytokines to Human Prolactin Receptor

In optimal binding conditions, the stoichiometric binding data provided by surface plasmon resonance shows that lactogenic cytokines containing a corrupted Site 2 are capable of binding approximately half (49-57%) the amount of human prolactin receptor per molecule that the corresponding cytokines with two active binding sites are able to bind (Tables 3.1 & 3.3). Further, cytokines with a corrupted Site 1 are able to minimally bind the prolactin receptor (1-6% of max) despite possessing an intact Site 2.

If prolactin receptor bound the two sites on the hormone independently then Site 1 knockouts should bind amounts of receptor similar to Site 2 knockouts. This was not observed. However, if receptor binding occurred in an ordered fashion where receptor binds Site 1 before binding Site 2 it would be expected that a Site 2 knockout (with an active Site 1) would bind half the receptor of the control hormone with two available binding sites, and a Site 1 knockout would not bind any receptor. This hypothesis fits very well with experimental evidence.

It should be noted that Site 2 knockouts sometimes demonstrated greater than 50% of maximum binding and that in no case did a Site 1 knockout show zero receptor binding at saturating conditions. It may be that, while very effective at corrupting a
particular receptor binding site, the mutations used did not completely prevent receptor binding at the target site. The result may be “leaky” binding at the severely impaired binding site and would explain a Site 2 knockout showing greater than 50% maximum binding and a Site 1 knockout showing minimal (but greater than zero) receptor binding.

Taken together, the evidence suggests that all three lactogenic cytokines (human prolactin, growth hormone, and placental lactogen) bind the human prolactin receptor in an ordered fashion where the receptor binds Site 1 of the hormone before binding to Site 2. While this has been previously reported for human prolactin, these are novel observations for human growth hormone and human placental lactogen (34).

6.2 Lactogenic Cytokines and Zinc

6.2.1 Kinetics

Previous reports have noted the zinc-independence of prolactin binding to the prolactin receptor and the zinc-dependance of growth hormone and placental lactogen in the same process. While the work done here demonstrates that zinc has a significant effect on growth hormone and placental lactogen binding to the prolactin receptor, the experiments indicates that zinc also influences the binding of prolactin to its receptor by decreasing the rate of association and decreasing the rate of dissociation.

It is noteworthy that prolactin and growth hormone (the two hormones that showed measurable binding in both the presence and absence of zinc) in zinc-containing buffer elicited decreased receptor on-rates of between five and 10-fold at Site 1. After the completion of these experiments we have learned through similar surface plasmon
resonance experiments that the human prolactin receptor does, in fact, weakly bind itself in buffer containing zinc (figure 6.1). Our observations and those of others show that receptor does not self-dimerize in buffer containing EDTA (36). When growth hormone and placental lactogen bind receptor in the presence of zinc, the tetravalent zinc is directly bound to two residues in the hormone and two residues in the receptor. It may be the case that the extracellular domain of the prolactin receptor can coordinate and self-dimerize around a zinc ion. If this is the case it is likely that for the experiments using prolactin receptor in zinc-containing buffer described in Chapter 3 a portion of that receptor existed in a dimerized state, reducing the actual concentration of unbound receptor in the experiment and ultimately reducing the measured rate of receptor binding. The extracellular domain of the prolactin receptor corresponds to the circulating prolactin binding protein which is itself poorly understood. Little research has been done and, consequently, it would be difficult to speculate on the biological implications of zinc-induced dimerization.

If the rate of receptor association in zinc-containing buffer equal or greater than that in zinc-free buffer, the dissociation constants for experiments done in zinc would fall by almost an order of magnitude (table 6.1). This does not address the likelihood that a hormone capable of coordinating and binding zinc may bind receptor at an even greater rate in the presence of zinc than in the absence and that the zinc-present on-rate may be even greater than perceived. As can be seen particularly well in Site 1 binding, the dissociation rate of receptor in zinc is significantly slower than in buffer containing EDTA and it stands to reason that the rate of association would be positively affected.
Figure 6.1 Human prolactin receptor self-dimerization
The extracellular domain of the human prolactin receptor was coupled to the surface of an SPR chip and extracellular domain of human prolactin receptor in buffer was allowed to flow over it and bind.
Table 6.1 Site 1 binding kinetics
Dissociation constants in zinc-containing buffer decrease significantly when calculations are based upon zinc-free association rates (italics).

Despite this, the presence of zinc still resulted in an increased affinity of human growth hormone for the prolactin receptor, reflecting results shown previously. In kinetic experiments using human placental lactogen, receptor binding was too low to measure kinetics in the absence of zinc and placental lactogen demonstrated the tightest binding of the three hormones in the presence of zinc, also reflecting the results of previous reports.

6.2.2 Conformational Changes
In light of reports dismissing the importance of zinc in receptor binding, experiments here show that prolactin undergoes a distinct conformational change upon exposure to zinc and that these conformational changes do not occur when residues of the proposed prolactin zinc-binding site are replaced with alanine. However, conformational changes associated with zinc-binding in the presence of the prolactin receptor remain
after the zinc-binding site is altered. This suggests that either the zinc-associated conformational changes in prolactin in the presence of receptor are not related to the proposed binding site or, more likely, that the hormone-receptor complex is still able to bind zinc in a diminished capacity.

To that end, human prolactin showed a diminished change in conformation upon exposure to zinc when bound to two receptors versus one. This may indicate a greater rigidity of the trimeric complex or that the conformation of the trimeric complex places the prolactin in a more energetically relaxed conformation (thus binding zinc does not elicits as great a change).

In human growth hormone the conformational changes associated with zinc binding were similar whether binding one or two receptors. This indicates that the hormone does not undergo substantial conformational rearrangements upon binding receptor at Site 2. The opposite effect was seen in human placental lactogen, where zinc-dependant conformational changes were only seen when two receptors were bound. As shown here and elsewhere, placental lactogen does not bind receptor in the absence of zinc. The introduction of zinc elicited no conformational change upon Site 1 binding. However, a zinc-dependant conformational change was shown upon binding two receptors, indicating a distinct conformational change associated with Site 2 binding in placental lactogen.

6.2.3 Biological Response

Increased concentrations of zinc in assay media left-shifted the dose-response curves of human growth hormone and placental lactogen indicating that when more zinc
is available less growth hormone or placental lactogen are required to elicit maximum cellular response. Increased zinc availability had little effect on the dose-response curve of prolactin. These results further indicate the positive correlation between zinc concentrations and the binding of growth hormone and placental lactogen to the human prolactin receptor. Additionally, cells were unable to respond to cytokines including prolactin in media lacking zinc. This may indicate that the role of zinc in cytokine-induced cell response goes beyond its role in cytokine-receptor binding.
The receptor binding model outlined in Section 2.4.3 makes explicit presumptions about the order of receptor binding and dissociation, namely, that prolactin receptor obligately binds Site 1 of the hormone before Site 2 and that receptor dissociation necessarily occurs in the reverse order. While this work shows that binding does occur in a manner consistent with this model, no information is available regarding the order of receptor dissociation from the hormone. Despite this, mathematical analysis requires a function for dissociation in a non-covalent binding event. Given the kinetic contribution of Site 1 to overall trimolecular binding, it is likely that Site 2 binds the receptor more weakly than Site 1 and, on average, dissociates from Site 2 before receptor bound at Site 1. Additionally, early surface plasmon resonance work assessing the Site 1 and Site 2 dissociation constants using class 1 cytokines of various species concluded that Site 2 binding is 5-500 fold weaker than Site 1 binding (32). For these reasons a synthetic binding model (figure 2.14) in which Site 2 dissociates before Site 1 was used for trimolecular binding analysis.

Despite software limitations, a complete binding model taking into account all possible binding and dissociation events can be constructed and the corresponding integral relating mass upon complex formation to said binding model derived (figure A.1). This model leaves open all binding possibilities – even those that can be ruled out.
experimentally. For instance, as is shown in this work, receptor does not bind to Site 2 of
the hormone before Site 1 is bound (that is, k₃, as defined in figure A.1 does not occur).
However, it leaves open the possibility of binding and dissociation that are currently
unable to be addressed. Software capable of integrating this complete integral for two-
state binding would be of use beyond the scope of this research and, as such, has been
shown in its completeness.
Figure A.1 Model of two-state binding and dissociation

Above: Complete binding model defining all possible binding and dissociation events in heterotrimeric complex formation of hormone (H) and receptor (R)

Below: Integral for calculation of hormone-coupled SPR response defining all possible binding and dissociation events and respective masses of species involved, based upon molecular weights of 23kD for a representative cytokine (human prolactin) and 24.5kD for the extracellular domain of the human prolactin receptor.
LIST OF REFERENCES


134. Invitrogen website. www.probes.com


