STRUCTURAL BASIS AND FUNCTIONAL IMPACT OF LIGAND-INDEPENDENT DIMERIZATION FOR HUMAN PROLACTIN RECEPTOR

DISSERTATION

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ABSTRACT

The human prolactin receptor (hPRLr) is a member of the class 1 cytokine receptor family, which also includes growth hormone receptor (GHr) and erythropoietin receptor (EPOr). The classic mechanism for class 1 cytokine receptor activation describes that ligand-induced receptor dimerization triggers downstream signaling and is supported by biochemical and biophysical evidence. However, the recent discoveries of ligand-independent dimerization of class 1 cytokine receptors in the plasma membrane, including hPRLr, hGHr, and mouse EPOr, have challenged this classic mechanism. Several mechanistic models have been proposed for the activation of GHr and EPOr, including the scissor model, the piston model, and the rotation model. In contrast, it is unknown if hPRLr shares a similar mechanism. The specific amino acid residues that mediate ligand-independent hPRLr dimerization have not been determined, although the transmembrane (TM) domain has been suggested to be important. The role of ligand-independent hPRLr dimers in hPRLr activation is unclear. Furthermore, the presence of ligand-independent hPRLr dimers does not rule out the classic mechanism, because hPRLr may exist in equilibrium between monomers and preformed dimers in the plasma membrane. This dissertation has focused upon the structural basis and the functional impact of ligand-independent dimerization of hPRLr.

We aimed to systematically evaluate in hPRLr the proposed models for class 1 cytokine receptor activation. An extensive series of alanine or glycine insertions were
introduced at the junctions between the TM domain and either the extracellular or intracellular domain to manipulate the relative orientations of different hPRLr domains. The basal and ligand-stimulated activities of these hPRLr insertion variants were examined in transiently transfected 293T cells. Our data demonstrate that altering the spatial relationships of hPRLr domains does not induce constitutive activity or impair ligand-induced activation. Such results do not support the rotation or piston model for hPRLr.

We also identified a population of covalently linked ligand-independent hPRLr dimers that are redox-sensitive and investigated the involvement of intermolecular disulfides in ligand-independent hPRLr dimerization. Twelve cysteines in various domains of hPRLr were replaced with serines, and the dimerization status of these hPRLr mutants was examined under reducing and non-reducing conditions. Iodoacetamide, an alkylation reagent for cysteine, was employed to distinguish in vivo physiological disulfides from ex vivo artifactual disulfides. Our data indicate that multiple cysteines from different domains of hPRLr, including but not limited to C184, C225, and C242, participate in forming intermolecular disulfides in ligand-independent hPRLr dimerization.

We next examined the role of these disulfide-linked hPRLr dimers in hPRLr activation. Abolishing the formation of ligand-independent disulfide-linked hPRLr dimers by removing twelve C-terminal cysteines did not impair ligand-induced activation or affect the speed of activation, indicating that disulfide-linked hPRLr dimers are not required for hPRLr activation. Furthermore, wild-type disulfide-linked hPRLr dimers
were not phosphorylated after ligand stimulation. Only hPRLr dimers that were not covalently linked became phosphorylated. We conclude that disulfide-linked hPRLr dimers are unlikely to participate in hPRLr activation. Collectively, the data in this dissertation suggest the validity of the classic mechanism of ligand-induced dimerization for hPRLr activation and illustrate the uniqueness of hPRLr in this receptor family.
Dedicated to my parents, Changlong Liu and Lanying Wang,
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# TABLE OF CONTENTS

Abstract ......................................................................................................................... ii

Dedication ..................................................................................................................... v

Acknowledgments ......................................................................................................... vi

Vita ................................................................................................................................. viii

List of Tables ................................................................................................................. xiii

List of Figures ............................................................................................................... xiv

Chapters:

1 Introduction ................................................................................................................. 1

1.1 Human Prolactin Receptor .................................................................................. 1

1.1.1 Structural Characteristics of the Long Form hPRLr ....................................... 1

1.1.2 Other Isoforms of hPRLr .............................................................................. 4

1.1.3 Biological Functions of hPRLr ..................................................................... 5

1.1.4 Ligands for hPRLr ....................................................................................... 6

1.1.5 Molecular Mechanism for hPRLr Ligand Binding ....................................... 7

1.1.6 hPRLr Signal Transduction ....................................................................... 9

1.1.7 hPRLr Internalization ............................................................................... 10

ix
3.3.2 hPRLr Mutants Form Ligand-Independent Homodimers Similar to WT hPRLr .......................................................... 48

3.3.3 Rotation of the IC or EC Domain Relative to the TM Helix of hPRLr Does Not Provide Constitutive Activity But Retains hPRL-induced Activation .......... 49

3.3.4 Providing Increased Degrees of Structural Freedom Between the TM and IC Domains Does Not Produce Constitutive hPRLr Activity or Eliminate Activation by hPRL .......................................................... 50

3.3.5 Heterodimerization of WT and Various Mutant hPRLrs Does Not Produce Functionally Active Receptors in the Absence of hPRL ......................... 51

3.4 Discussion ........................................................................................................................................ 52

4 Intermolecular Disulfide Linkages in Ligand-Independent Dimerization of Human Prolactin Receptor ....................................................................................... 67

4.1 Abstract ........................................................................................................................................ 67

4.2 Introduction ................................................................................................................................... 68

4.3 Results .......................................................................................................................................... 69

4.3.1 A Population of hPRLr Exists as Ligand-Free Disulfide-Linked Dimers ....... 69

4.3.2 Multiple Cysteine Residues in Different Domains of hPRLr Are Involved in Ligand-Free Intermolecular Disulfide Linkages ................................. 72

4.4 Discussion ................................................................................................................................... 77
5 Disulfide-Linked Human Prolactin Receptor Dimer in Ligand-Induced Human Prolactin Receptor Activation

5.1 Abstract ........................................................................................................................................ 92

5.2 Introduction .................................................................................................................................. 93

5.3 Results ........................................................................................................................................ 94

5.3.1 Disulfide-Linked hPRLr Dimers Are Not Required for hPRLr Activation .... 94

5.3.2 Disulfide-Linked hPRLr Dimers Are Not Phosphorylated after Ligand Stimulation ................................................................................................................................. 96

5.4 Discussion .................................................................................................................................... 97

6 Conclusions and Future Directions ............................................................................................... 106

References........................................................................................................................................ 112
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Site-directed mutagenesis primers for amino acid insertions</td>
<td>41</td>
</tr>
<tr>
<td>2.2 Site-directed mutagenesis primers for amino acid substitutions</td>
<td>42</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Stucture of hPRLr EC domain</td>
<td>30</td>
</tr>
<tr>
<td>1.2 Structure of hPRL</td>
<td>31</td>
</tr>
<tr>
<td>1.3 Structure of the hPRL/rPRLr 1:2 complex</td>
<td>32</td>
</tr>
<tr>
<td>1.4 Amino acid sequence comparison of hPRLr, hGHr, and hEPOr</td>
<td>33</td>
</tr>
<tr>
<td>2.1 293T cells constitutively express JAK2 but not endogenous hPRLr</td>
<td>43</td>
</tr>
<tr>
<td>3.1 Schematic representation of WT hPRLr and variant insertion mutants</td>
<td>62</td>
</tr>
<tr>
<td>3.2 WT and mutant hPRLr form both monomers and ligand-independent homodimers</td>
<td>63</td>
</tr>
<tr>
<td>3.3 Rotation of hPRLr domains relative to the TM helix does not provide constitutive activity or impair the ligand-induced activity</td>
<td>64</td>
</tr>
<tr>
<td>3.4 Increased degree of freedom in the movement of the IC domain relative to the TM helix fails to yield constitutive activity in hPRLr and minimally affects the ligand-induced activity</td>
<td>65</td>
</tr>
<tr>
<td>3.5 Piston movement of different domains does not result in the constitutive activity of hPRLr</td>
<td>66</td>
</tr>
<tr>
<td>4.1 The structure and function of epitope-tagged hPRLrs was intact</td>
<td>82</td>
</tr>
<tr>
<td>4.2 Epitope-tagged hPRLrs form ligand-independent dimers in transfected 293T cells</td>
<td>83</td>
</tr>
<tr>
<td>4.3 A population of transfected hPRLrs exists as ligand-free disulfide-linked dimers in 293T cells</td>
<td>84</td>
</tr>
</tbody>
</table>
4.4 A population of endogenous hPRLrs exists as ligand-free disulfide-linked dimers in MCF-7 cells ................................................................. 85

4.5 Comparison of cysteine residues in hPRLr, hGHr, and hEPOr .............................. 86

4.6 Multiple cysteine residues in different domains of hPRLr are involved in ligand-free intermolecular disulfide linkages; removal of 12 C-terminal cysteine residues abolishes ligand-free disulfide-mediated hPRLr dimerization .............................................. 88

4.7 C184, C225, and C242 participate in ligand-independent intermolecular disulfide bond formation, but the contribution of C184 and C225 requires the presence of Box 1 in the ICD ........................................................................................................... 90

4.8 Besides C242, other but not all IC cysteines are involved in ligand-independent intermolecular disulfide linkages ............................................................................................................. 91

5.1 Disulfide-linked hPRLr dimers are not required for ligand-induced receptor activation ................................................................................................................................. 102

5.2 Eliminating disulfide-linked hPRLr dimers does not suppress the speed of ligand-induced receptor activation .................................................................................................................................. 103

5.3 hPRL activation does not affect the formation of disulfide-linked hPRLr dimers ... 104

5.4 Disulfide-linked hPRLr dimers are not phosphorylated upon ligand stimulation .... 105
CHAPTER 1

INTRODUCTION

1.1 Human Prolactin Receptor

The human prolactin receptor (hPRLr) was first cloned in 1989 (1) and is a member of the class 1 cytokine receptor family (2). It contains 598 amino acid residues in the mature long form (LF) with an apparent molecular mobility of 100kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It shares several similar structural features with other members of this family such as human growth hormone receptor (hGHR) and human erythropoietin receptor (hEPOr). These structural features include a ligand-binding extracellular (EC) domain, a single-pass transmembrane (TM) domain, and a structurally uncharacterized intracellular (IC) domain.

1.1.1 Structural Characteristics of the Long Form hPRLr

1.1.1.1 Extracellular Domain

The EC domain of the LF hPRLr (Figure 1.1) is comprised of the N-terminal 210 residues and can be divided into two distinct subdomains (S1 and S2) (2, 3). Each subdomain contains approximately 100 residues analogous to the fibronectin type III module and folds into seven anti-parallel β-strands. A four-residue proline-rich linker region joins the S1 and S2 subdomains near residue 105. The S1 subdomain contains two intramolecular disulfide bonds formed between cysteines 12 and 22 and between...
cysteines 51 and 62. These two disulfides are critical for ligand binding and activation (4) and are conserved in the class 1 cytokine receptor family. The S2 subdomain contains another conserved feature, the WSAWS motif (residues 191-195). Although the WSAWS motif is distal to the ligand-binding interface (5), mutations in this motif reduce the binding affinity of receptor to its ligand on cell surface (6), most likely due to perturbed protein folding and trafficking (7). The S2 subdomain also contains the receptor’s Zn²⁺ binding half site (D187 and H188) (5). Zn²⁺ concentrations affect hPRLr behaviors differently for different ligands of hPRLr (8-12). In addition, among the six asparagines in the EC domain of hPRLr, at least two (N35 and N83) are postulated to be glycosylated, based on sequence alignment with rat PRLr (rPRLr), which has been shown to be a glycoprotein (13). Glycosylation of rPRLr is not required for ligand binding or activation but influences receptor trafficking to cell surface (13).

1.1.1.2 Transmembrane Domain

The single-pass TM domain of hPRLr consists of 24 residues flanked by two charged residues (D210 and K235) and is predicted to be an α-helix. The TM sequence is reminiscent of elements of a leucine zipper (14, 15). It has been suggested that the interaction between the TM domains is sufficient for the formation of ligand-independent hPRLr dimers on the plasma membrane because ligand-independent dimerization still occurs when either the EC or IC domains are removed (16). The specific TM residues responsible for this interaction have not been identified.
1.1.1.3 Intracellular Domain

The IC domain of the LF hPRLr is comprised of the C-terminal 364 residues. Like other members in the cytokine receptor family, the IC domain of the LF hPRLr is devoid of intrinsic enzymatic activity (17). Although no structural data are available for this domain, several motifs (Box 1, V Box, Box 2, and X box) have been described in the first 100 residues of the hPRLr IC domain. Box 1 (residues 243-250) is a proline-rich hydrophobic region located proximal to the membrane and is highly conserved in the class 1 cytokine receptor family (3, 18, 19). This motif is required for the constitutive association of hPRLr with Janus kinase 2 (JAK2) (20, 21). Box 2 (residues 288-298) consists of mostly hydrophobic and acidic residues and is much less conserved among the class 1 cytokine receptors than Box 1 (3, 22). The region between Box 1 and Box 2 is described as V Box, and the region immediately C-terminal to Box 2 is called X Box (22). The functions of Box 2, V Box, and X Box remain to be determined. The IC domain of the LF hPRLr contains 10 tyrosine residues and undergoes tyrosine phosphorylation upon ligand activation (23), but the information on the sites of tyrosine phosphorylation in the IC domain is limited. Among the 10 tyrosine residues, only half of them are conserved in prolactin receptors of other mammalian species, and none of them is conserved when compared to hGHR and hEPOr. Nevertheless, based on studies with rPRLr where sporadic efforts have been made to examine tyrosine phosphorylation (24, 25), Y587, the most C-terminal tyrosine residue in the LF hPRLr, has been suggested to be phosphorylated (7).
1.1.2 Other Isoforms of hPRLr

The gene for hPRLr is located on chromosome 5 (5p13-14) and contains 11 exons with a total length of over 200 kb (7). While exons 3-10 yield the long form hPRLr, several naturally occurring isoforms are generated from alternative splicing. The ΔS1 isoform exhibits a deletion of exons 4 and 5 and lacks the S1 subdomain of the EC domain. It binds to hPRL at a lower affinity than the LF, but is able to activate downstream signaling upon ligand binding (26). The ΔS2 isoform is derived from a 303 bp deletion and lacks the S2 subdomain. This isoform has been associated with constitutive activation (27). The intermediate isoform results from a 573 bp deletion in exon 10. The portion C-terminal to X Box in the IC domain is truncated, and 13 residues are added due to frame shift. A different preference in signaling pathways was observed for the intermediate isoform compared to the LF (28). Alternative splicing of exons 10 and 11 generates two short forms of hPRLr (29). The S1a isoform lacks the portion C-terminal X Box and contains 39 additional residues, while the S1b isoform is terminated shortly after Box 1 with three additional residues. Both short forms of hPRLr show dominant negative properties to the LF, with a stronger inhibitory effect from S1b (29).

In addition, the hPRL binding protein (hPRLbp) is a soluble form corresponding to the EC domain of hPRLr and is believed to be a product of proteolytic cleavage of the LF hPRLr (30, 31). It has been shown that hPRLbp inhibits hPRL action by acting as antagonist (30), although others argue that hPRLbp may increase the half-life of circulating hPRL by decreasing the hPRL clearance rate in the blood (32, 33). This cleavage event also creates a TM-ICD isoform of hPRLr. The TM-ICD isoform cannot be
activated by hPRL, but can enhance the signaling of the LF hPRLr when both are present (16). More than one isoform of hPRLr may be expressed in a hPRL target cell, but the temporal pattern of the expression of these hPRLr isoforms at different stages of development or their combinatorial effects in signaling under normal and pathological conditions have not been systematically examined.

1.1.3 Biological Functions of hPRLr

hPRLr is widely distributed in many types of cells and tissues, from mammary gland to circulating lymphocytes (7), and is associated with a great number of biological functions. These biological functions of hPRLr can be divided into six categories (7): 1) water and electrolyte balance, 2) growth and development, 3) endocrinology and metabolism, 4) brain and behavior, 5) reproduction, and 6) immunoregulation and protection. Among these biological functions, hPRLr is classically known for its ability to stimulate mammary gland development and lactation. The terminal differentiation of mammary gland is directly modulated by hPRL and hPRLr (34). The production of major components of milk, including milk proteins, lactose, and lipids, is mediated by hPRLr signaling (35-37).

hPRL and hPRLr have been implicated in cancer development (7), such as breast cancer (38). Both healthy breast tissues and breast carcinoma express hPRL and hPRLr (39, 40), but an elevated level of hPRLr is expressed in breast tumors (41). In addition, hPRL has been shown to stimulate the proliferation of human breast cancer cell lines both in vivo and in vitro (38, 42). The design of hPRL antagonist and the study on inhibiting
hPRLr activity thus have been of interest for developing new therapeutic approaches for breast cancer.

1.1.4 Ligands for hPRLr

The proteins capable of activating hPRLr were identified a number of years ago and include hPRL, hGH, human placental lactogen (hPL) (7). These lactogenic hormones belong to the class 1 cytokine family and exhibit similar structures of four-helix bundle arranged in an up-up-down-down pattern (43). Although hGH and hPL share 85% sequence homology, hPRL has limited sequence homology (~20%) with hGH and hPL (44).

1.1.4.1 Human Prolactin

The gene for hPRL is located on chromosome 6 (6p22.2-21.3) and codes for a 227-residue protein including a 28-residue signal sequence (45, 46). The mature secreted hPRL (Figure 1.2) has 199 residues and a molecular weight of approximately 23 kDa (44). The first nine residues in the N-terminus of hPRL are unique and are not present in hGH or hPL. Removal of these nine residues (Δ1-9), in combination with the G129R mutation (see Section 1.1.5), creates a highly effective antagonist of hPRL (47). Three intramolecular disulfide bonds are formed between cysteines 4 and 11, cysteines 58 and 174, and cysteines 191 and 199. In ovine PRL, disruption of the disulfide bonds is severely detrimental to the structure and function of the hormone (48). H27 is hPRL’s Zn$^{2+}$ binding half site (8), although Zn$^{2+}$ only has modest effect on the affinity of hPRL for hPRLr (12). The global affinity for hPRL/hPRLr binding is determined in the
nanomolar range (49, 50) and is in agreement with the ED$_{50}$ values of hPRL in in vitro biological studies (12, 51).

1.1.4.2 Human Growth Hormone and Human Placental Lactogen

The genes for hGH and hPL are located as a cluster on chromosome 17 (17q22-24) (52). The prehormones for both hGH and hPL have 217 residues including 26-residue signal sequences, and the mature secreted proteins are both 191-residue long with molecular weights of approximately 22 kDa (44). hGH and hPL are highly conserved with a sequence homology of 85% (44, 53). Both hormones contained two intramolecular disulfide bonds formed between cysteines 53 and 165 and cysteines 182 and 199, which mirror two of the three intramolecular disulfide bonds found in hPRL (44, 53, 54). The affinities of hGH and hPL for hPRLr are both highly dependent on Zn$^{2+}$, and the Zn$^{2+}$ binding half sites in hGH and hPL are H18 (homologous to H27 in hPRL) and E174 (not present in hPRL) for both proteins (12).

1.1.5 Molecular Mechanism for hPRLr Ligand Binding

Stoichiometric analysis of the interaction between hPRLr and the lactogenic hormones has shown that one molecule of hPRL binds with two molecules of hPRLr to form a heterotrimeric complex (51). The interactions between hPRL and the two hPRLrs occur at two unique binding surfaces on hPRL, designated site 1 and site 2 (Figure 1.3) (55, 56).

Site 1 binding epitopes involve residues located in helix 1, helix 4, loop 1, and the C-terminus of hPRL (57, 58), while site 2 epitopes have been identified in helix 1 and helix 3 of hPRL (55, 56). Among these identified residues in hPRL, G129 is arguably
best studied (59). This glycine residue is situated in helix 3 of hPRL and is critical for site 2 binding. The small side chain of G129 forms the bottom of a cavity where W72 in hPRLr plugs. Mutation of G129 to a bulky arginine results in a hPRL antagonist. Notably, this glycine residue is conserved in hGH and hPL. The mutation of G120R (homologous to G129R in hPRL) creates antagonists of hGH and hPL for hGHR and hPRLr, respectively (60, 61).

Site 1 and site 2 of hPRL bind similar but not identical surfaces of the two hPRLrs (6, 55, 56). The overlapping region between the two binding surfaces on the receptors is located from I132 to F140 in loop 5’ of the S2 subdomain, as suggested in a recent study that utilized structural data of hPRL bound to two EC domains of rPRLr (56). Two tryptophan residues in hPRLr are also implicated in ligand binding (5, 7). W72 interacts with G129 in site 2 of hPRL (59), and W139 sits within the stretch of residues 132-140 (56).

The binding mechanism for hPRL and hPRLr EC domain is an obligate ordered reaction, where site 1 binding not only facilitates but is required for site 2 binding (51). In surface plasmon resonance (SPR) studies, when both sites 1 and 2 of hPRL were available, hPRLr EC domain bound to hPRL at a 2:1 ratio. When only site 1 was available but site 2 was blocked, a 1:1 ratio was observed. When only site 2 was available but site 1 was blocked, hPRLr EC domain could not bind. Similar results have also been reported for hGH and hPL binding to hPRLr EC domain (50) and the binding between hGH and hGHR EC domain (62).
The obligate ordered binding mechanism suggests that site 1 and site 2 on hPRL are functionally coupled and that site 1 binding induces a conformational change in hPRL that organizes site 2 for binding (50, 58). Comparison among the structural data of free hPRL (63), Δ1-9 G129R hPRL mutant bound to one hPRLr EC domain (59), and hPRL bound to two rPRLr EC domains (56) supports this hypothesis. FRET analysis also demonstrated the conformational change of hPRL induced by receptor binding (50). More recently, the residues that are contiguous between and functionally couple site 1 and site 2 in hPRL have been identified by mutagenesis studies (58). Taken together, these data support an allostery mechanism for hPRL binding to hPRLr.

1.1.6 hPRLr Signal Transduction

The IC domain of hPRLr is devoid of intrinsic enzymatic activity (17) and utilizes associated protein kinases to transmit signal upon ligand activation.

1.1.6.1 JAK/STAT Pathway

The JAK2/STAT5 pathway is the principle signaling pathway activated when ligand binds to hPRLr (7, 22), as indirectly evidenced by the very similar phenotypes between PRLr and STAT5 knockout mice (7). JAK2 constitutively associates with hPRLr via the Box 1 motif in the IC domain (20, 21). When ligand binds to hPRLr, JAK2 is rapidly activated through autophosphorylation, and then transphosphorylates tyrosine residues in the hPRLr IC domain (64-66), which serve as SH2 docking sites for signal transducer and activator of transcription 5 (STAT5) (7, 22). After being recruited to the hPRLr-JAK2 complex, STAT5 is also phosphorylated by JAK2 and subsequently dissociates from the complex, forming homodimers that translocate to the nucleus (7, 67),
where they activate the transcription of several genes that contain the γ-IFN-activated sequence (GAS) in their promoter regions (67), such as β-casein (35, 68), interferon regulatory factor-1 (IRF-1) (69, 70), and B-cell lymphoma-2 (Bcl-2) (71). Other regulators in this pathway include the tyrosine phosphatases SH2-containing phosphatase-2 (SHP-2) and suppressors of cytokine signaling (SOCS). SOCS1 and SOCS3 inhibit hPRLr signaling by blocking phosphorylation (22, 72), while SHP-2 enhances hPRLr signaling through dephosphorylation of inhibitory phosphotyrosine residues of JAK2 (22, 73, 74). In addition, JAK1, STAT1, and STAT3 also have been reported to interact with hPRLr (75, 76), although JAK1 is not necessary for hPRL-induced gene expression (77).

1.1.6.2 Other Signaling Pathways

Independently of JAK2 activation, other protein kinases that associate with and are activated by hPRLr include Src and Fyn (78, 79). They lead to the activation of the PI3K/Akt pathway (80) and the PLC-γ/PKC pathway (81, 82). In addition, the Ras/Raf/MEK/MAPK pathway is also involved in hPRLr signaling (83-86). The guanine nucleotide exchange factor Vav, which can be activated by Src/Fyn and PI3K (22), underlies the signaling of the MAPK pathway (22, 87). Finally, PKC-independent intracellular Ca^{2+} increase has been reported for hPRLr (3).

1.1.7 hPRLr Internalization

The magnitude and duration of hPRLr signaling is further regulated by receptor internalization and degradation after ligand stimulation. Early studies using rPRLr indicate that the IC domain contains motifs that are required for receptor recycling (4).
This is later confirmed in bovine PRLr (bPRLr) (88). F290 and several dileucines upstream to F290 are necessary to mediate bPRLr internalization. Curiously, F290 is not present in hPRLr. In hPRLr, the lysosomal pathway involving ubiquitination is responsible for its internalization and degradation (89). This process is enhanced by hPRL-induced JAK2 activity (90). Polyubiquitination of hPRLr is critical for recruiting assembly polypeptide 2 (AP2) to hPRLr, which proceeds through a clathrin-dependent pathway (89). Phosphorylation of S349 in hPRLr is required for the interaction between hPRLr and β-TrCP-containing E3 ubiquitin ligase (90, 91). It has been shown that glycogen synthase kinase 3β (GSK3β) phosphorylates S349 of hPRLr (92).

It is noteworthy that in breast cancer tissues GSK3β is inactivated by HER2 and Ras pathways via inhibitory phosphorylation on S9 of GSK3β (92). The impaired hPRLr phosphorylation on S349 leads to less efficient association between hPRLr and β-TrCP in breast cancer cells and thus decreases hPRLr degradation (93). This may contribute to the increased level of hPRLr in breast tumors. However, other studies have shown that in breast cancer cells hPRLr is internalized by a dynamin-2 mediated proteosomal pathway that does not require ubiquitination (94, 95). Src family kinases, but not JAK2, facilitate this internalization process of hPRLr in breast cancer cells (95).

1.1.8 Constitutively Active hPRLr Variants

Efforts have been made on finding and constructing constitutively active hPRLr to elucidate the mechanism that may regulate the activation of hPRLr. The requirements for various portions of the hPRLr EC domain have been evaluated. Elimination of the S1 subdomain [ΔS1, residues 1-100 (26) or residues 3-103 (27)] did not produce a
constitutively active receptor but did greatly reduce the affinity for hPRL. However, the receptor mutant could still be activated by a higher concentration of hPRL (26). Removal of the S2 subdomain (ΔS2, residues 106-206) produced a constitutively active receptor (27). Similar result was also observed in rabbit PRLr (rbPRLr) S2 (residues 103-203) deletion, but wild type (WT) rbPRLr inhibited the constitutive activity of ΔS2 rbPRLr (96). Constitutive activity of hPRLr was also obtained after deletion of residues 9-187, which also abolished that ability of hPRLr to bind hPRL (97). When the entire EC domain [residues 1-210 (16) or residues 3-206 (27)] was removed, no constitutive activity was observed and the mutant receptor could not be activated by hPRL. Interestingly, the ΔEC domain hPRLr was able to enhance the signaling of WT hPRLr when both were present (16). Taken altogether, these results demonstrate that an alteration in bulk in the hPRLr EC domain invariably impairs ligand binding, which is not surprising. However, these results also indicate that the S1 subdomain may be dispensable for hPRLr activity and that a structural feature situated in the S2 subdomain, specifically between residues 106 and 187 (including the unpaired C184), inhibits ligand-independent constitutive activity. Further, one or more unrecognized structural features located in the proline-rich N-terminus (residues 3-9) and the C-terminus (residues 206-210) are required for constitutive hPRLr activity. It can be hypothesized that the S2 subdomain functions as an inhibitory domain and prevents hPRLr activity in the absence of ligand and that ligand binding to hPRLr releases the inhibition. The release of the inhibition may also be achieved by the removal of the inhibitory elements in the S2 subdomain.
Finally, two hPRLr variants, each with a single point mutation (I76V and I146L), have been identified in some patients with multiple fibroadenomas (MFA), a type of benign breast tumors (98, 99). Both variants confer constitutive activity, but the activity of I76V is weaker than that of I146L (100). In addition, the effect of I146L can be reversed by hPRL antagonist Δ1-9 G129R (98). The mechanism for the constitutive activity from these point mutations remains to be determined.

1.2 Class 1 Cytokine Receptor Family

The class 1 cytokine receptor family was first proposed by J. F. Bazan in 1990 to describe a group of receptors for hematopoietic factors, growth hormones, and interleukins that trigger cell proliferation and differentiation (2). This group of receptors share a striking structural homology in their EC ligand-binding domains, which are approximately 200 residues in size and can be divided into two duplicated subdomains. Each subdomain is approximately 100-residue long and contains seven β-strands that further fold into an antiparallel β-sandwich related to fibronectin type III structures. Other conserved motifs in the EC domains include four N-terminal cysteine residues forming two intramolecular disulfide bonds and the C-terminal WSXWS motif. In contrast, the IC domains show little to no sequence homology except for the proline-rich Box 1 motif (101). None of the class 1 cytokine receptors possesses intrinsic enzymatic activity in the IC domain (101).

Among the members in the class 1 cytokine receptor family, hPRLr, hGHR, and hEPOr exhibit close relationships in their tertiary structures, although the alignment of amino acid sequences for these three receptors shows only modest to minimal sequence
homology of less than 30% (Figure 1.4) (3). Comparison of the structures of hGHR and hEPOr with that of hPRLr and discussion on activation mechanisms for hGHR and hEPOr may lend information about hPRLr activation mechanism.

1.2.1 Human Growth Hormone Receptor

Among the class 1 cytokine receptors, hGHR is the first to be cloned (102) and the first to have the structure of the EC domain bound by its ligand determined (103). It is distributed in almost every cell of the body and mediates a great variety of physiological actions, including postnatal growth, hepatic metabolism, and immune function, among others (104, 105). The gene for hGHR is located on chromosome 5 (5p13) and codes for a 638-residue glycoprotein including an 18-residue signal sequence (106). The hGHR isoforms are different from those reported for hPRLr (107-111). Most notably, the hGHbp isoform is generated by alternative splicing (108), instead of a proteolytic event as for hPRLbp (30, 31).

When compared with other class 1 cytokine receptors, the hGHR EC domain can be distinguished by two unique features. First, an additional pair of cysteine residues (C108 and C122) are present and form an intramolecular disulfide bond, but this disulfide bond is less important than the two conserved ones regarding receptor folding, ligand binding, and signaling (112). Second, the WSXWS motif in hGHR is less than canonical (YGEFS, residues 222-226), but the critical role of this motif in ligand binding is not negated since mutations within this motif cause hGH insensitivity and dwarfism (Laron syndrome) (105). The two tryptophan residues that are important for ligand binding in hPRLr are conserved in hGHR (W104 and W169). W104 of hGHR interacts with G120 of
hGH (analogous to G129 of hPRL), leading to the discovery of two potent hGH antagonists, G120R and G120K (60, 113). Finally, in contrast to the binding between hGH and hPRLr, the binding between hGH and hGHR is not dependent on Zn$^{2+}$ (9, 12).

Similar to hPRLr and hEPOr, the TM domain of hGHR mediates ligand-independent receptor dimerization via leucine zipper-like interactions (104, 114). The single cysteine residue seen in the center of the hPRLr TM domain is not present in the hGHR TM domain.

Although the tyrosine residues in the IC domain and the residues surrounding them are not conserved in hPRLr and hGHR, the JAK2/STAT5 pathway is also central to hGHR signaling and further induces a number of target genes, among which the most prominent and best characterized is the expression of insulin-like growth factor 1 (IGF-1) (105). Besides STAT5, STAT1 and STAT3 are also activated by hGHR (115). In contrast to hPRLr where JAK2 is constitutively associated, the interaction between JAK2 and hGHR via the Box 1 region is enhanced upon ligand activation (116-118). Alternatively, other signaling pathways initiated by ligand binding to hGHR include those activated by Src family kinases such as the MAPK pathway and the PI3K pathway (104, 119, 120). hGHR internalization is independent of ligand induction and utilizes a lysosomal, rather than proteosomal, pathway, where ubiquitination is involved, but not required (121-124).

More than 60 non-functional hGHR mutants have been identified in Laron syndrome patients (105). Almost all of these mutations are in the EC domain (125). However, currently there is no known constitutively active hGHR mutant (105). Finally, increased expression of hGHR has been detected in a number of cancers (126, 127).
Excessive hGHR activation by autocrine hGH signaling is implicated in tumor progression (128-132).

1.2.2 Human Erythropoietin Receptor

The gene for hEPOr is located on chromosome 19p and encodes a 508-residue glycoprotein including a 24-residue signal sequence (133, 134). hEPOr is transiently expressed in erythroid progenitor cells in fetal liver and adult bone marrow during erythropoiesis and is crucial for the survival, proliferation, and differentiation of erythroid progenitor cells (135). The reported isoforms of hEPOr are different from those described for hPRLr (136-138), with the soluble isoform of hEPOr resulted from alternative splicing rather than proteolytic cleavage (136).

The X-ray crystallographic structure of two hEPOr EC domains bound with hEPO (139) revealed that the ligand-binding surface on hEPOr is similar to that found in the hGH/hGHR complex (103). Interestingly, the same surface can also interact with each other and mediate the dimer formation of unliganded hEPOr EC domains in crystal (140). This interaction has not been observed in solution, nor have similar results been observed for hPRLr or hGHR. The S1 subdomain of the hEPOr EC domain contains the two conserved intramolecular disulfide bonds, but their specific locations in hEPOr are not identical to those in hPRLr. The WSAWS motif is conserved in the S2 subdomain. Mutations in this motif impair the efficient folding of hEPOr and its trafficking to the plasma membrane (141). In addition, F93 in S1 and F205 in S2 are important for ligand binding (142). Of these two residues, F93 is analogous to W72 in hPRLr and W104 in hGHR. Finally, the R129C mutation in mouse EPOr (mEPOr) (R130C in human) provides
receptor constitutive activity (143). This mutation results in an aberrant intermolecular disulfide bond that covalently dimerizes the receptor mutant in the absence of ligand (144). Scanning in the flanking region of mEPOr has identified two additional constitutively active mutants, E132C and E133C, which also undergo aberrant intermolecular disulfide bond formation (145). Similar results have not been reported for hPRLr or hGHR.

Ligand-independent dimerization mediated by the TM domain has been demonstrated in mEPOr (146). Notably, the substitution of the TM domain with that of glycophorin A, which possesses a strong dimerizing ability, retained normal ligand-induced activity of mEPOr (146). The single cysteine residue seen in the center of the hPRLr TM domain is not present in either mEPOr or hEPOr.

The IC domain of hEPOr is the shortest among the three related receptors (235 residues in hEPOr vs. 364 residues in hPRLr and 351 residues in hGHR). Two functional regions have been described for the hEPOr IC domain. The membrane proximal region is required for downstream signaling cascades (147, 148) and contains the conserved Box motifs, albeit reduced homology in the Box 2 motif in hEPOr compared to hPRLr and hGHR. JAK2 constitutively binds hEPOr in the membrane proximal region (149). Besides Box 1 (150), W282 in mEPOr (W284 in human) in the V Box motif between Box 1 and Box 2 is also critical for JAK2 binding (148, 151). Although JAK2/STAT5 pathway is the primary pathway activated by hEPOr upon ligand binding (152), STAT5 is not required for erythropoiesis (153) while JAK2 is essential (154, 155). In addition, PI3K is constitutively associated with hEPOr via the sequence immediately C-terminal to Box 2.

17
and is activated after stimulation (156, 157). Other signaling molecules induced by hEPOr include Lyn (158, 159) and the ras/MAPK cascade (152, 160). Among the targeted genes of hEPOr activation are Bcl-xl (161, 162) and c-Myc (163, 164), which promote the survival and growth of erythroid progenitor cells, respectively. In contrast to the membrane proximal region, the distal C-terminal region in the hEPOr IC domain is not required for initiating signal transduction and is defined as a negative regulatory region (147). Several tyrosine residues in this region are phosphorylated after stimulation and serve as docking sites for down-regulators such as SHP-1 and CIS (165-167). Deletion of this region enhances the activity of hEPOr without affecting receptor trafficking or ligand binding (147).

The internalization and degradation of hEPOr involves two successive processes (168, 169). First, ubiquitination of hEPOr by β-TrCP occurs at the cell surface after ligand stimulation, and the hEPOr IC domain is rapidly degraded by proteosomes. S462 is critical for β-TrCP binding (169). Second, the remaining ligand-receptor complex is internalized and degraded by lysosomes. This step does not require proteosome activity (168).

A truncated hEPOr has been identified in patients with familial autosomal dominant benign erythrocytosis (170). The deletion of the C-terminal negative regulatory region increases the sensitivity of hEPOr to hEPO and causes elevated erythrocyte production. The expression of hEPOr has been detected in both hematopoietic and nonhematopoietic cancer tissues but its role in cancer development is controversial (160). Finally, the interaction between mEPOr and gp55, a membrane glycoprotein encoded by
the *env* gene of Friend spleen focus-forming virus (SFFV), results in constitutive activity of mEPOr and causes Friend erythroleukemia (171). This interaction involves the TM domains of both mEPOr and gp55 (172, 173). However, gp55 does not activate hEPOr or other members of class 1 cytokine receptor family (174).

1.2.3 Dimerization of Class 1 Cytokine Receptors

The biological effects of class 1 cytokines exerted through specific cell surface receptors have been identified for decades, but the molecular mechanism by which ligands activate this family of receptors and trigger the downstream cascades of molecular interactions is poorly described. Ligand-bound class 1 cytokine receptor dimers have been associated with receptor activity, suggesting receptor dimerization is critical for ligand-stimulated receptor activation (3, 113, 175, 176).

1.2.3.1 Ligand-Induced Dimerization of Class 1 Cytokine Receptors

Based on the X-ray crystallographic structures of the 1:2 ligand/receptor EC domain complex (5, 55, 56, 103, 139) and the obligate ordered reaction of ligand binding with the receptor EC domain (50, 51, 62), the classic mechanism of ligand-induced receptor dimerization was proposed (113, 176, 177). In this mechanism, ligand binding induces the dimerization of receptor, and the proximity of the induced dimeric receptors triggers downstream signaling.

Several lines of evidence from *in vivo* studies also support this model. First, selected bivalent monoclonal antibodies (mAb) raised against the EC domains of class 1 cytokine receptors are capable of activating receptors or chimeric receptors in cells (113, 178-180). This observation is further extended for hEPOr since EPO mimetic peptides
(EMP), a group of small dimerizing peptides that bind hEPOr but are not homologous to EPO, can act as hEPOr agonists (181). Second, point mutations of three residues in the mEPOr EC domain to cysteine (R129C, E132C, and E133C) and a homologous mutation in hEPOr (R130C) provide receptors with constitutive activity due to aberrant intermolecular disulfide bond formation that covalently dimerizes receptors in the absence of ligand (143-145). Mutations of other residues to cysteine in the same region do not form intermolecular disulfide bond and do not exhibit constitutive activity (145). Third, chimera receptors constructed by replacing the EC domain of mEPOr with that from mouse stem cell factor receptor (mSCFr) or human epidermal growth factor receptor (hEGFr) can be activated by the corresponding ligands known to dimerize their receptors (182). Similar results have been reported for rbPRLr/mEPOr chimera receptor (183) and human granulocyte-macrophage colony-stimulating factor receptor (hGM-CSFr)/rat PRLr (rPRLr) chimera receptor (184). Furthermore, G129R hPRL, G120R hPL, and G120R hGH are impaired in their site 2 binding to hPRLr or hGHr and fail to interact with the second receptor to form an active heterotrimeric complex. These mutated ligands can act as antagonists for receptor-mediated cellular response (59-61, 113). Finally, the dose-response curves of hPRLr, hGHr, and hEPOr to their ligands exhibit a biphasic bell shape (113, 179, 180, 185-187). Low concentrations of ligands are agonistic for cellular activation by receptors, while high concentrations of ligands are antagonistic. The bell-shaped dose-response curve can be explained by ligand-induced receptor dimerization. At very high concentrations of ligands, the majority of the receptor population at the cell
surface are engaged in a heterodimeric complex with ligand, and few free receptors are available to form an active heterotrimeric complex.

Ligand-induced receptor dimer is stabilized by the interactions between the S2 subdomains of the receptor EC domains (55, 56, 62, 103, 139). The residues involved in the S2/S2 interaction have been identified for rPRLr (56) and hGHRr (103) and are not conserved between these two receptor systems. The S2/S2 interface in hEPOr has also been identified (139) and is much smaller than hGHRr. Interestingly, the R130C mutation of hEPOr (R129C in mouse) is located close to the S2/S2 interface. The S2/S2 interaction in the absence of ligand has not been observed for soluble EC domains.

In addition, a recent publication reported a novel mechanism for ligand-induced hPRLr dimerization involving acetylation in the IC domain (188). Upon ligand stimulation, multiple, but not all, lysine residues along the hPRLr IC domain became acetylated by CREB-binding protein. The neutralization of the positive charge on the lysine residues dampens the electrostatic repulsion and allows two receptors to come into proximity and consequently activation. A similar acetylation-dependent mechanism was also reported for type 1 interferon receptor, a member of class 2 cytokine receptor family (189).

1.2.3.2 Ligand-Independent Dimerization of Class 1 Cytokine Receptors

Ligand-independent receptor dimerization was first reported in the X-ray crystallographic structure of unliganded hEPOr EC domain (140). This dimer formation is mediated by the same key residues involved in ligand binding rather than the S2/S2 interaction. However, a similar interaction cannot be detected in solution between the
hEPOr EC domains. Instead, a later study using antibody-mediated fluorescence copatching demonstrated that the TM domain of mEPOr mediates ligand-independent mEPOr dimerization at the cell surface (146).

Following this discovery, the existence of receptor dimers on the plasma membrane in the absence of ligand has been reported for hGHR (114) and hPRLr (16, 190) with evidence from fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and coimmunoprecipitation (CoIP) experiments. It has been suggested that the interaction between the TM helices is sufficient for ligand-independent receptor dimerization because preformed receptor dimers still occur when the EC domain, the IC domain, or both domains are removed (16, 114). Based on calculations, elements reminiscent of leucine zipper motifs in the TM domain are thought to mediate hPRLr dimerization (14, 15), but the specific TM residues responsible for this interaction have not been identified in hPRLr.

However, other conflicting results were reported regarding TM-mediated ligand-independent receptor dimerization. A chimera receptor containing the EC and TM domains of rbPRLr and the IC domain of mEPOr failed to dimerize with another chimera receptor containing the EC domain of mEPOr and the TM and IC domains of rbPRLr in antibody-mediated fluorescence copatching (146). In rbGHR, the TM domain was not required for ligand-independent dimerization because the substitution of the rbGHR TM domain with that of human low density lipoprotein receptor (hLDLR), a receptor that dimerizes via the IC domain but not the TM domain (191), resulted in no difference in the detection of ligand-independent receptor dimers (192). Instead, the S2/S2 interface in
the EC domain, specifically H150 in this region, is critical for ligand-independent rbGHr dimerization (192). Again, the S2/S2 interaction in the absence of ligand has not been observed for hPRLr. Since the residues involved in the S2/S2 interaction are not conserved between hGHr and rPRLr (56, 103), this discrepancy is not surprising.

1.2.4 Mechanistic Models for Class 1 Cytokine Receptor Activation

The ability of selected bivalent mAb to activate class 1 cytokine receptors supports the dimerization mechanism for receptor activation, but not all bivalent mAb are capable of creating active receptors (179, 193, 194). Furthermore, an antagonist peptide can dimerize the hEPOr EC domains but in a nonpermissive orientation (195). These results indicate that although receptor dimerization is necessary, it is not sufficient for receptor activation. The discovery of ligand-free receptor dimers leads to the proposal of a new mechanism for receptor activation where ligand binding in the EC domain must induce a conformational change that can be transmitted through the TM domain to the IC domain and activates preformed receptor dimers. Three mechanistic models have been suggested for class 1 cytokine receptor activation: the scissor model (196), the piston model (103, 197, 198), and the rotation model (114, 199).

1.2.4.1 The scissor model

The first model is the scissor model and was proposed on the basis of structural studies of hEPOr (140, 196). X-ray studies of hEPOr EC domains showed a pronounced scissor-like reorientation of these domains upon ligand binding. In the absence of ligand, the C-termini of the EC domains were separated by a greater distance than when ligand was present. This observation was interpreted to indicate that preformed receptor dimers
held the IC domains at a sufficient distance to preclude JAK2-mediated transphosphorylation while a scissor-like movement caused by ligand binding brought the IC domains into sufficient proximity to allow transphosphorylation. In addition, these structures demonstrated that under the conditions for crystal formation the EC domains were sufficient for dimer formation. However, this interaction between the EC domains has not been further defined under physiological conditions. Similar data supporting the scissor model have not been presented for either hGHR or hPRLr.

1.2.4.2 The Piston Model

The second model is the piston model and was suggested by structural studies of two hGHR EC domains bound by hGH (103, 197, 198). The C-termini of the hGHR EC domains were offset from each other in relation to an assumed plane of the plasma membrane. This offset suggests that hGH binding may produce a piston-like movement in which the two receptors in the hGH-bound dimer would be afloat in the membrane at different heights. This model assumes that the ligand-induced piston action of the EC domains influences the relative positions of the IC domains and is responsible for the availability of receptor-associated JAK2 for phosphorylation. The most recent structure of hPRL bound to two rPRLr EC domains supports such a mechanism by showing a similar offset of the C-termini of the rPRLr EC domains (56). Unfortunately, there are no structure data for dimeric PRLr in the absence of ligand. Thus, whether the piston action results from hPRL binding or is present in ligand-free hPRLr dimers cannot be determined.
1.2.4.3 The Rotation Model

The third and most recent model for receptor activation is the rotation model (114, 199). This model proposes a ligand binding-induced rotation of the receptors around an axis perpendicular to the assumed plane of the plasma membrane that moves the receptor-associated JAK2 into the positions where it auto- and/or transphosphorylates JAK2, the receptor IC domain tyrosines, and subsequently other proteins that bind the phosphorylated receptors via the SH2 domain. The first work suggesting a rotation movement was conducted with mEPOr (199) where selected alanine additions at the TM/IC domain junction or within the TM domain impaired the ligand-induced activity of mEPOr \textit{in vivo}. On the basis of alanine’s strong helix-forming character (200), it was proposed that alanine additions extended the TM helix and induced incremental rotations of approximately $100^\circ$ in the IC domain relative to the TM helix and that the relative and precise rotations of the two IC domains in the mEPOr dimer were the key step in activation (199). This model requires an association between the TM domains (146) that in the absence of ligand anchors the receptor dimers in an inactive (“off”) conformation. Further, receptor activation requires breaking the “off” conformer held by the TM/TM association and rotation of the IC domains upon ligand binding to the EC domains. The energy required to break the TM/TM association would most likely be harvested from ligand/receptor binding. In addition, it is critical to note that this model also must require a rigid connection between the TM and IC domains (199). In the absence of ligand, this rigid connection locks the IC domains in an “off” conformer defined by the TM/TM association. In the presence of ligand, this rigid connection transmits the conformational
change (e.g., a rotation movement) caused by ligand binding from the EC domain to the IC domain. More recently, this model has been extended to hGHR (114) where specific alanine additions at or near the junction of the TM and IC domains activated hGHR in the absence of hGH. Further, the investigators in this study also demonstrated a rigid structural connection between the TM helix and the Box 1 region (114).

1.3 Project Overview

For more than a decade, the classic mechanism of ligand-induced receptor dimerization (113, 176, 177) had been the paradigm for hPRLr activation. The discovery of ligand-independent hPRLr dimerization (16, 190) has challenged this classic mechanism. Ligand-free hPRLr dimers are inactive in the absence of ligand (16, 190). However, their role in ligand-induced hPRLr activation is unclear: are they dormant and waiting to be activated by ligand, are they the inactive form of the receptor and acting as decoys for inhibitors such as phosphatases, or are they remnants of the activation process and waiting to be removed from the plasma membrane? These questions remain to be addressed. Nevertheless, several mechanistic models based on ligand-independent receptor dimerization have been proposed for the activation of hGHR and mEPOr, including the scissor (196), piston (103, 197, 198), and rotation (114, 199) models. To date, no similar models have been proposed for hPRLr. In addition, the specific amino acid residues that mediate ligand-independent hPRLr dimerization have not been identified, although the TM domain has been suggested to be important (16).

It should also be noted that the presence of ligand-free hPRLr dimers does not rule out the classic mechanism of ligand-induced dimerization, because hPRLr may exist
in dynamic equilibrium between monomers and preformed dimers in the plasma membrane. Indeed, monomeric receptors form the majority of hPRLr in cells as provided by Western analysis (190). It remains to be determined which population of hPRLr is activated by ligand binding or if both mechanisms are at play. It has been argued that the speed of hPRLr activation indicates that the preformed dimers are the species to be activated (38), but whether hPRLr activation is slowed or suppressed when the formation of ligand-free hPRLr dimers is abolished has not been further investigated.

The discovery of ligand-independent hPRLr dimers demonstrates the close relationship of hPRLr to other members of the class 1 cytokine receptor family, but researchers in the scientific community often make overly sweeping assumptions about the similarities among the proteins in this receptor family. In contrast, significant variations in the mechanistic details depict an inconsistent picture for these receptors. The objectives of this study are: 1) to characterize the conformational change of hPRLr during ligand stimulation by systematically evaluating the proposed models for class 1 cytokine receptor activation; 2) to determine the structural role of cysteine residues in the process of ligand-independent hPRLr dimerization; and 3) to investigate the functional role of disulfide-linked hPRLr dimers in ligand-induced hPRLr activation. In this study, we hypothesize that: 1) hPRLr domains adopt a specific relative orientation in the active dimeric conformer upon ligand stimulation; 2) intermolecular disulfide bonds mediate ligand-independent dimerization of hPRLr; and 3) the formation of disulfide-linked hPRLr dimers facilitates ligand-induced hPRLr activation.
Various numbers of alanine or glycine residues were inserted at the junctions of the TM domain with either the EC or IC domain to manipulate the relative orientations of different hPRLr domains. The relationship between the spatial orientation of hPRLr domains and hPRLr activation by ligand binding was examined in 293T cells transiently transfected with the hPRLr insertion mutants. The data demonstrated that altering the physical relationship among the EC, TM, and IC domains does not induce constitutive activity or impair ligand-induced activation. These results do not support the hypothesis of the rotation or piston model for hPRLr (Chapter 3). A population of ligand-free hPRLr dimers was determined to be covalently linked and redox-sensitive, suggesting the involvement of intermolecular disulfide bonds. The physiological relevance of this finding was demonstrated in MCF-7 cells that express endogenous hPRLr. To examine the requirement of cysteine residues for redox-sensitive hPRLr dimer formation, twelve cysteine residues in various domains of hPRLr were replaced by serines. Iodoacetamide (IAM), an alkylation reagent for cysteine, was employed to distinguish in vivo physiological disulfide bonds from ex vivo artifactual disulfide bonds. We determined that multiple cysteine residues from different domains of hPRLr participate in the formation of intermolecular disulfide bonds in ligand-independent hPRLr dimerization (Chapter 4). After the identification of disulfide-linked hPRLr dimers, their functional role in ligand-induced activation was investigated. The hPRLr mutant, where the formation of ligand-free disulfide-linked dimers was abolished, was compared with WT hPRLr regarding the time course of ligand-induced activation. We found that ligand-free disulfide-linked hPRLr dimers were not required for hPRLr activation. Further, in WT
hPRLr, only receptors that were not covalently linked, but not disulfide-linked dimers, were phosphorylated following ligand stimulation. We conclude that disulfide-linked hPRLr dimers are unlikely to participate in hPRLr activation (Chapter 5).

This work illustrates the uniqueness of hPRLr in the class 1 cytokine receptor family and is a critical step toward the understanding of the basic mechanism of this ligand/receptor system. The molecular mechanistic details for hPRLr activation are required to guide the rational design of pharmaceuticals that modulate the biological functions of hPRL and hPRLr in pathological conditions such as breast cancer. Finally, the findings presented here on hPRLr dimerization and activation contribute to our overall understanding of protein structure and function.
Figure 1.1 Structure of hPRLr EC domain

The structure is adapted from the H27A hPRL/hPRLr EC domain 1:1 complex (201). S1 and S2 subdomains are represented in turquoise and green, respectively. Selected structural features are indicated. PDB#: 3N06
Figure 1.2 Structure of hPRL

The four α-helices are represented in blue (helix 1), dark green (helix 2), light green (helix 2), and orange (helix 4) (63). PDB#: 1RW5
Figure 1.3 Structure of the hPRL/rPRLr 1:2 complex

The three binding interfaces are indicated (56). PDB#: 3NPZ
| hPRLr 1 | -------------------------QLPPG--KPEIFCRSPNKEFTCWW | 24 |
| hGhr 1  | FSGSEATAIALSRAPWSLQSVPNGLTNSKEPKFTKCRSPERTEFSCWH | 50 |
| hEPOR 1 | -------------------------APPNLPDPKFESKAALARGPEELLCFTERLEDLVCW | 40 |
| hPRLr 25 | RPTGDDLPTN--------YSLTYHREGETLMHCYPTGPPNSCHFGKQY | 68 |
| hGhr 51  | TDEYHTKIGLPIQLFYTRRNTQWEWKTQKEPCPVDVAGPENVGCFSF | 100 |
| hEPOR 41 | EEAASAGVGPGN-YSFSYOLEDEPWKLRLHQAPTARGAVRFWCSLPTAD | 89 |
| hPRLr 69 | TSMWRTYIMMVNATNMQSSFDELTVYIVQPDPLELAVOEK--QP | 116 |
| hGhr 101 | TSIWIPYCIKLTSN--------GTYDEKCFSEIVQDPDIALNINLNVSL | 146 |
| hEPOR 90 | TSSFVPLELRVTAAS--GAPRYHRVIHINEVLLDAVPGLVARLA----- | 132 |
| hPRLr 117 | EDRKPYLWIKWSPPTLIDKGWFTLLYEIRPKKEAANEIHFAGQPTE | 174 |
| hGhr 147 | TGIHADIQVRWEAPRNADIQKGWMVLEYELQYKEVNETKWKMMPDPLTTS | 196 |
| hEPOR 133 | -DESHVHLRLPPPETMPTS-HIYEVDSAGNAGSVQVSEIQLRMTE | 181 |
| hPRLr 167 | FIKILSHPGQKYLQVR-------CP-DHGYWSAWSPATFIQIP--SDFTMND | 210 |
| hGhr 197 | VPYSLKVDELVEVRVR--------SQQRNSQNYGGEFSEVLVVLTPQMSQFTCEE | 243 |
| hEPOR 182 | CVLSNLGRTRTYTFAVRARMAEPSFGFWSAWSFEPVSLLTP--------S | 276 |
| hPRLr 211 | TT--WVISVAVLSAVICLITTVAWALKG-YSMVTCIFPPVPGPKIKGFDA | 257 |
| hGhr 244 | DFYFPWLLIIIIFGIGLTVMLVFLFSKQRESLIMPPVVPVKIGIDP | 293 |
| hEPOR 224 | DLDPLILTSLLILVVLVVILVLLALLSHRALKQKIWPGISPESEFGL | 273 |
| hPRLr 258 | HLEKGLKEEILSSLALGCQD-FPPTSDYEDDLLVEYLVDSE-DQHLSVH | 305 |
| hGhr 294 | DLLEKGKILEEVTIAIHDSYKPEFHSDDSWEFIEIDEPDEKTEESD | 344 |
| hEPOR 274 | FTHKGNQLWLYQNDGCLWSPCPTFTE------------------------ | 301 |
| hPRLr 306 | SKEHPSQGMKPTYLDP------DTDSGRGSCDSPSSLEKCEE--PQANPSTF | 350 |
| hGhr 345 | TDRLLSSDHEKSHSNLGVKGDGDSGRTCCEPDILETDFANDIHEGTSEV | 394 |
| hEPOR 302 | -DEPPASLELSERCGTWQAVEPGTD | 327 |
| hPRLr 351 | YDEPVEIEKPNETTHTWDPQCISMEMKIPYFHAAG------GSKCSTWPLQQ | 396 |
| hGhr 395 | AQPQRKGEADLCLDQQNKQNNSSYPYDACPATQPSVIQAENKPKQPLPT | 444 |
| hEPOR 328 | DEGPLLEPVGSEAHDQTY------------------------LVLDKWLLPR | 354 |

Continued

**Figure 1.4 Amino acid sequence comparison of hPRLr, hGhr, and hEPOR**

Alignment was performed by the Clustal W multiple sequence alignment program (202) in the UniProt suite (203). The amino acid number does not include the N-terminal signal sequences. WSXWS motifs are highlighted in yellow. TM domains are underlined. Box 1 motifs are highlighted in turquoise.
Figure 1.4 continued

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<td>QEDIYITTELTTAGRPVGEH----------------VPGSEMPVP</td>
</tr>
<tr>
<td>hEPOr</td>
<td>440</td>
<td>455</td>
<td>ISTDYSSGDSQGAQG-------------------------------</td>
</tr>
<tr>
<td>hPRLr</td>
<td>546</td>
<td>595</td>
<td>ACFEESAKEAPPSLEQNQAEEKALANFTATSSKCRQLGLDYLDPACFTH</td>
</tr>
<tr>
<td>hGHRr</td>
<td>576</td>
<td>620</td>
<td>DYSIHIVQSPOGLILNATLPDKFELSSCGVSTDQLNIMP-----</td>
</tr>
<tr>
<td>hEPOr</td>
<td>456</td>
<td>484</td>
<td>---LSDGPYSPYENSLIPAAEPLPPSYVACS-------------------</td>
</tr>
<tr>
<td>hPRLr</td>
<td>596</td>
<td>598</td>
<td>SFG ---</td>
</tr>
<tr>
<td>hGHRr</td>
<td>---</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hEPOr</td>
<td>---</td>
<td></td>
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</tr>
</tbody>
</table>
CHAPTER 2

MATERIALS AND METHODS

2.1 Construction of Eukaryotic Expression Vectors for hPRLr

The full-length hPRLr coding region was amplified from pBluescriptR-hPRLr (Open Biosystems, Huntsville, AL) (204) by polymerase chain reaction (PCR) using the following primers (Integrated DNA Technologies, Coralville, IA): forward, 5’-GCCAAC ATGAAGGAAAATGTGGC-3’; and reverse, 5’-GTGAAAGGAGTGTGTGTAAGGATG CG-3’. These primers yielded the coding region of full-length hPRLr containing the signal sequence but no stop codon. This PCR product was inserted into pcDNA6.2/C-YFP-GW/TOPO (Invitrogen, Carlsbad, CA) by the TOPO reaction, which resulted in a hPRLr-YFP fusion gene. The YFP gene was subsequently removed between the HpaI and Pmel restriction sites. Subsequently, two consecutive stop codons were added in frame immediately after the 3’ end of the full-length hPRLr coding region by site-directed mutagenesis (see Section 2.2). This resulted in the pcDNA6.2-hPRLr eukaryotic expression vector.

To construct the eukaryotic expression vector of hPRLr with V5 tag at the C-terminus, the N-terminal KpnI restriction site and the C-terminal SacII restriction site were incorporated into the full-length hPRLr gene by PCR using pcDNA6.2-hPRLr as template and the following primers (Integrated DNA Technologies): forward, 5’-GGTATGAAAGGAGTGTGTGTAAGGATG CG-3’; and reverse, 5’-GCTAGGATCCATGCGTGTTGTAAGGATG CG-3’.
CCGCAACATGAAGGAAAATGTG-3’; and reverse, 5’-CCGCGGGTGAAAGGAGTGTGTAACAC-3’. These primers yielded the full-length hPRLr gene containing the desired terminal restriction sites and the signal sequence but no stop codon. This PCR product was ligated into pCR2.1-TOPO (Invitrogen) by the TOPO reaction. The DNA fragment containing the full-length hPRLr gene was isolated by KpnI/SacII double digestion and then ligated using T4 DNA ligase (New England BioLabs, Ipswich, MA) into pcDNA3.1/V5-His B (Invitrogen) that had been linearized at the same restriction sites. This resulted in a V5/His double tagged hPRLr gene. A stop codon was added in frame immediately after the 3’ end of the V5 tag by site-directed mutagenesis (see below). This resulted in the pcDNA3.1-hPRLr-V5 eukaryotic expression vector.

To construct the eukaryotic expression vector of hPRLr with His tag at the C-terminus, the N-terminal KpnI restriction site and the C-terminal AgeI restriction site were incorporated into the full-length hPRLr gene by PCR using pcDNA6.2-hPRLr as template and the following primers (Integrated DNA Technologies): forward, 5’-GGTA CCGCAACATGAAGGAAAATGTG-3’; and reverse, 5’-ACCGGTGTGAAAGGAGTGTGTAACAC-3’. These primers yielded the full-length hPRLr gene containing the desired terminal restriction sites and the signal sequence but no stop codon. This PCR product was ligated into pCR2.1-TOPO (Invitrogen) by the TOPO reaction. The endogenous AgeI restriction site located in the full-length hPRLr gene between the codons for Thr 56 and Gly 57 was substituted with a silent mutation by site-directed mutagenesis (see below) using the following primers: forward, 5’-GTCCAGACTACAT AACAGGTGGCCCCAACCTCCTG-3’; and reverse, 5’-CAGGAGTTGGGGCCACCTG
The DNA fragment containing the full-length hPRLr gene, where the endogenous AgeI restriction site had been removed, was isolated by KpnI/AgeI double digestion and then ligated using T4 DNA ligase (New England BioLabs) into pcDNA3.1/V5-His B (Invitrogen) that had been linearized at the same restriction sites. Linearization of pcDNA3.1/V5-His B using these two restriction sites also removed the V5 tag in the vector. This resulted in the pcDNA3.1-hPRLr-His eukaryotic expression vector.

The presence of the desired coding sequences was confirmed by diagnostic restriction enzyme digestion and DNA sequencing. Plasmids containing WT or tagged hPRLr were prepared and stored in water at -20°C. DNA concentrations were determined by absorption of 260 nm radiation.

2.2 Site-Directed Mutagenesis

Site-directed mutagenesis was performed following the protocol described for the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the pcDNA6.2-hPRLr eukaryotic expression vector whose preparation was characterized above. The designed mutations included up to four alanine or glycine residues inserted at the specified positions in the coding sequence of hPRLr, substitutions of various cysteine residues with serine residues in hPRLr, and introductions of the stop codon at the specified positions to generate truncated hPRLr variants. Forward and complementary reverse primers are listed in Tables 2.1 and 2.2. These alterations in the hPRLr gene were verified by DNA sequencing of the entire coding sequence.
2.3 Cell Culture and Transfection

Human embryonic kidney 293T cells (205), provided by Dr. Michael Lairmore (Department of Veterinary Biosciences, The Ohio State University), were maintained in DMEM (Invitrogen) with 10% FBS (Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine (Invitrogen), and antibiotics and cultured in a 5% CO₂ incubator at 37°C. Endogenous expression of JAK2 but the absence of hPRLr in 293T cells was demonstrated by reverse transcription PCR (RT-PCR) (Figure 2.1). The various hPRLr expression vectors were transfected into 293T cells following the protocol described for the TransIT-LT1 transfection reagent (Mirus, Madison, WI). In the cases of cotransfection experiments, the two plasmids were used at a 1:1 ratio, and the total amount of DNA was equal to that used in the single transfection. Twenty-four hours after transfection, 293T cells were switched to starvation medium [DMEM/F-12 (Invitrogen) with no FBS] and incubated for an additional 24 hours. For the hPRL-activated samples, starved 293T cells were then treated with 100 nM recombinant hPRL (206), a hormone concentration shown to induce rapid hPRLr activation (20, 21, 97, 208), at 37°C for 15 minutes or various periods of time otherwise specified in Chapter 5, immediately prior to solubilization and collection of whole cell extract (WCE).

MCF-7 cells were maintained in MEM (Invitrogen) with 10% FBS (Thermo Fisher Scientific), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen), 10 µg/ml insulin (Sigma, St. Louis, MO), and antibiotics and cultured in a 5% CO₂ incubator at 37°C. Prior to WCE collection, MCF-7 cells were switched to starvation medium [MEM without phenol red (Invitrogen), 1% gelding serum (Gemini Bio-
Products, West Sacramento, CA), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM MEM non-essential amino acids (Invitrogen), 10 µg/ml insulin (Sigma), and antibiotics] and incubated for 24 hours.

2.4 Immunoprecipitation (IP) and Western Blot (WB) analysis

MCF-7 cells or 293T cells were solubilized in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, and 1% NP-40] containing protease inhibitor cocktail Set III (EMD Chemicals, Gibbstown, NJ) and phosphatase inhibitor cocktail Set II (EMD Chemicals). For IAM-treated samples, RIPA buffer also contained freshly prepared 20 mM IAM (Sigma) as a cysteine alkylation reagent, and the samples were kept in the dark. For coimmunoprecipitation (CoIP), 293T cells were solubilized in CoIP buffer [50 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 0.25% NP-40, and 0.25% Triton X-100] containing protease inhibitor cocktail Set III (EMD Chemicals) and phosphatase inhibitor cocktail Set II (EMD Chemicals). Insoluble material was removed by centrifugation at 17,900g and 4°C for 10 minutes. The protein concentration in WCE was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) (208). 250 µg of WCE was incubated overnight at 4°C with 1.5 µg of the appropriate antibody: rabbit polyclonal anti-hPRLr antibody H-300 (Santa Cruz Biotechnology, Santa Cruz, CA) that recognizes the IC domain of hPRLr, mouse monoclonal anti-V5 antibody (Invitrogen), or mouse monoclonal anti-His (C-term) antibody (Invitrogen). The immune complexes were collected on Protein A agarose (Invitrogen) after incubation at 4°C for 1 hour. The immunoprecipitates were resuspended in either reducing [10% 2-mercapto ethanol (2-ME)] or nonreducing SDS-
containing electrophoresis loading buffer and boiled for 10 minutes. Proteins were resolved by electrophoresis on precast 4-15% polyacrylamide gels (Bio-Rad) and transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ) in transfer buffer (25 mM Tris base, 0.192 M glycine, and 20% methanol) at 100 V and 4°C for 90 minutes. Blots were blocked with 5% nonfat dry milk in TBST [10 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween-20] and then probed with the appropriate dilution of the primary antibody: mouse monoclonal anti-hPRLr EC domain antibody 1A2B1 (Invitrogen) at a 1:500 dilution, mouse monoclonal anti-phosphotyrosine antibody 4G10 (Millipore, Billerica, MA) at a 1:1000 dilution, mouse monoclonal anti-V5 antibody (Invitrogen) at a 1:2500 dilution, or mouse monoclonal anti-His (C-term) antibody (Invitrogen) at a 1:2500 dilution. The blots were subsequently treated with HRP-linked sheep anti-mouse antibody (GE Healthcare) at a 1:2000 dilution. Visualization of the HRP-bound hPRLr immune complexes was performed using ECL detection reagents (GE Healthcare), followed by exposure to high-performance chemiluminescence film (GE Healthcare). Data are representative of three independent experiments.
<table>
<thead>
<tr>
<th>mutagenesis site</th>
<th>amino acid(s) inserted</th>
<th>primer (nucleotide insertions underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>between G236 and Y237</td>
<td>+1A</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GCT</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>AGC</strong> GCC CTT CAA AGC CAC-3'</td>
</tr>
<tr>
<td></td>
<td>+2A</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GCT GCC</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>AGC</strong> GCC CTT CAA AGC CAC-3'</td>
</tr>
<tr>
<td></td>
<td>+3A</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GCT GCC GCT</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>AGC</strong> GCC CTT CAA AGC CAC-3'</td>
</tr>
<tr>
<td></td>
<td>+4A</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GCT GCC GCT GCC</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>AGC</strong> GCC CTT CAA AGC CAC-3'</td>
</tr>
<tr>
<td>between L234 and K235</td>
<td>+1A</td>
<td>forward, 5'-C TGG GCA GTG CTT TGG <strong>GCT</strong> TAT AGC TAT AGC ATG-3' reverse, 5'-CAT GCT ATA GCC CTT <strong>AGC</strong> CAA AGC CAC TGC CCA G-3'</td>
</tr>
<tr>
<td></td>
<td>+2A</td>
<td>forward, 5'-C TGG GCA GTG CTT TGG <strong>GCT GCC</strong> TAT AGC TAT AGC ATG-3' reverse, 5'-CAT GCT ATA GCC CTT <strong>AGC</strong> GCC CAA AGC CAC TGC CCA G-3'</td>
</tr>
<tr>
<td></td>
<td>+3A</td>
<td>forward, 5'-C TGG GCA GTG CTT TGG <strong>GCT GCC GCC</strong> TAT AGC TAT AGC ATG-3' reverse, 5'-CAT GCT ATA GCC CTT <strong>AGC</strong> GCC CAA AGC CAC TGC CCA G-3'</td>
</tr>
<tr>
<td></td>
<td>+4A</td>
<td>forward, 5'-C TGG GCA GTG CTT TGG <strong>GCT GCC GCC GCC</strong> TAT AGC TAT AGC ATG-3' reverse, 5'-CAT GCT ATA GCC CTT <strong>AGC</strong> GCC CAA AGC CAC TGC CCA G-3'</td>
</tr>
<tr>
<td>between D210 and T211</td>
<td>+1A</td>
<td>forward, 5'-GAC TTC ACC ATG AAT GAT <strong>GCT</strong> ACA ACC GTG TGG ATC-3' reverse, 5'-GAT CCA CAC GGT TGT <strong>AGC</strong> ATT CAT GGT GAA GTC-3'</td>
</tr>
<tr>
<td></td>
<td>+2A</td>
<td>forward, 5'-GAC TTC ACC ATG AAT GAT <strong>GCT GCC</strong> ACA ACC GTG TGG ATC-3' reverse, 5'-GAT CCA CAC GGT TGT <strong>AGC AGC</strong> ATT CAT GGT GAA GTC-3'</td>
</tr>
<tr>
<td></td>
<td>+3A</td>
<td>forward, 5'-GAC TTC ACC ATG AAT GAT <strong>GCT GCC GCC</strong> ACA ACC GTG TGG ATC-3' reverse, 5'-GAT CCA CAC GGT TGT <strong>AGC GCC AGC</strong> ATT CAT GGT GAA GTC-3'</td>
</tr>
<tr>
<td></td>
<td>+4A</td>
<td>forward, 5'-GAC TTC ACC ATG AAT GAT <strong>GCT GCC GCC GCC</strong> ACA ACC GTG TGG ATC-3' reverse, 5'-GAT CCA CAC GGT TGT <strong>AGC GCC GCC GCC</strong> ATT CAT GGT GAA GTC-3'</td>
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<tr>
<td>between G236 and Y237</td>
<td>+1G</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GGA</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>TCC</strong> GCC CTT CAA AGC CAC-3'</td>
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<td>+2G</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GGA GCC</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>GCC TCC</strong> GCC CTT CAA AGC CAC-3'</td>
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<tr>
<td></td>
<td>+3G</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GGA GCC GGA</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>TCC GCC TCC GCC</strong> CTT CAA AGC CAC-3'</td>
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<tr>
<td></td>
<td>+4G</td>
<td>forward, 5'-GTG GCT TTG AAG GGC <strong>GGA GCC GGA GCC</strong> TAT AGC ATG GTG ACC-3' reverse, 5'-GGT CAC CAT GCT ATA <strong>GCC TCC GCC TCC GCC</strong> CTT CAA AGC CAC-3'</td>
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Table 2.1 Site-directed mutagenesis primers for amino acid insertions
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<th>primers (point mutations underlined)</th>
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<tr>
<td>C184S</td>
<td>forward, 5'-CTT GTC CAG GTT GGC AGC AAA CCA GAC CAT G-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-C ATG GTC TGG TTT GCT GCG AAC CTG GAC AAG-3'</td>
</tr>
<tr>
<td>C225S</td>
<td>forward, 5'-GTC CTT TCT GCT GTG ATC TCT TTG ATT ATT GTC TGG GC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-GC CCA GAC AAT AAT CAA AGA GAT GAC AGC AGA AAG GAC-3'</td>
</tr>
<tr>
<td>C242S</td>
<td>forward, 5'-CAT AGC ATG GTG ACC TCC ATC TTT CCG CCA GTT CCT G-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-AGG AAC TGG CCG AAA GAT GGA GGT CAC CAT GCT ATA G-3'</td>
</tr>
<tr>
<td>C274S</td>
<td>forward, 5'-CTG AGT GCC TTG GGA TCC AGA GAC TTT CCT CCC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-GGG AGG AAA GTC TTG GGA TCC CAA GGC ACT CAG-3'</td>
</tr>
<tr>
<td>C330S</td>
<td>forward, 5'-CA GGC CGG GGG AGC TCT GAC AGC CCT TCC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-GGA AGG GCT GTC AGA GCT CCC CCG GCC TG-3'</td>
</tr>
<tr>
<td>C340S</td>
<td>forward, 5'-CCT TTT TGT TCT GAA AAG AGT GAG GAA CCC CAG GCC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-GGC CTG GGG TTC CTC ACT CTT AGA CAA AAG G-3'</td>
</tr>
<tr>
<td>C372S</td>
<td>forward, 5'-ACC TGG GAC CCC CAG TCC ATA AGC ATG GAA GGC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-GCC TTC CAT GCT TAT GGA CTG GGG GTC CCA GGT G-3'</td>
</tr>
<tr>
<td>C389S</td>
<td>forward, 5'-CAT GCT GGT GGA TCC AAA TCT TCA ACA TGG CCC TTA CC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-GG TAA GGG CCA TGT TGA AGA TTT GGA TCC ACC AGC ATG-3'</td>
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<tr>
<td>C413S</td>
<td>forward, 5'-CAC AAT ATT ACT GAT GTG AGT GAG CTA GCT GTG GCC-3'</td>
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<td>reverse, 5'-GCC CAC AGC CAG CTC ACT CAC ATG AAT ATT GTG-3'</td>
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<tr>
<td>C547S</td>
<td>forward, 5'-CCA CAT GCT AAA AAC GTG GCT AGC TTT GAA GAA TCA GCC AAA G-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-CTT TTC GGC TGA TTC AAA GCT AGC CAC GTT TTT AGC ATG TGG-3'</td>
</tr>
<tr>
<td>C578S</td>
<td>forward, 5'-GCA ACA TCA AGC AAG TCC AGG CTC CAG CGT GGT G-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-ACC CAC CAG CTG GAG GCT GGA CCT GCT TGA TGT TGC-3'</td>
</tr>
<tr>
<td>C592S</td>
<td>forward, 5'-GAT TAC CTG GAT CCC GCA AGT TTT ACA CAC TCC TTT CAC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-GTG AAA GGA GTG TGT AAA ACT TGC GGG ATC CAG GTC AAT-3'</td>
</tr>
<tr>
<td>C242X</td>
<td>forward, 5'-CAT AGC ATG GTG ACC TGA ATC TTT CCG CCA GTT CCT G-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-AGG AAC TGG CCG AAA GAT GGA GGT CAC CAT GCT ATA G-3'</td>
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<tr>
<td>K262X</td>
<td>forward, 5'-GAT GCT CAT CTG TGG GAG TAG GGC AAG TCT GAA GAA C-3'</td>
</tr>
<tr>
<td></td>
<td>reverse, 5'-G TTC TCC AGA CTT GCC CTA CTC CAA CAG ATG AGC ATC-3'</td>
</tr>
</tbody>
</table>

**Table 2.2** Site-directed mutagenesis primers for amino acid substitutions
Figure 2.1 293T cells constitutively express JAK2 but not endogenous hPRLr

Total RNA was extracted from 293T cells. Endogenous expression of JAK2 and hPRLr were examined by RT-PCR using primers complementary to the exon junctions in the mature mRNA. Nucleotide-free water was included as blank control.
CHAPTER 3

FUNCTIONAL IMPACT OF MANIPULATION IN RELATIVE ORIENTATION OF HUMAN PROLACTIN RECEPTOR DOMAINS

3.1 Abstract

Hormone binding creates active receptor dimers for class 1 cytokine receptors; however, the detailed molecular mechanism by which these receptors are activated by their ligands is not well characterized, and it is unknown if these receptors share common mechanisms. A rotation model has been proposed for the activation of mEPOr and hGHr and is supported by evidence showing that additions of alanine at the junction of the TM and IC domains and/or within the TM domain influenced receptor activities. This evidence suggests that alanine additions changed the relative orientations of the IC domains and their subsequent activation. We sought to determine if a similar mechanism was at play with hPRLr. Up to four alanines were added between the TM and either the IC or EC domains to extend the TM helix and to rotate the IC or EC domains. Also, up to four glycines were placed between the TM and IC domains to provide increased flexibility between these two domains. WT hPRLr or various mutant receptors were expressed in human embryonic kidney 293T cells that express endogenous JAK2. In the absence of hPRL, none of the alanine or glycine additions increased the level of receptor phosphorylation above that of WT hPRLr. In the presence of hPRL, both WT hPRLr and each of the mutant receptors were successfully phosphorylated. These data do not support
a rotation mechanism for hPRLr activation or a requirement of a fixed spatial relationship between the TM and IC domains for hPRLr activation. In a second set of experiments, both WT hPRLr and either alanine- or glycine-extended receptors were coexpressed in 293T cells. In the absence of hPRL, there was no detectable phosphorylation of hPRLr. Such data do not support a piston movement between the hPRLr pair in their activation.

3.2 Introduction

The human prolactin receptor is a member of the class 1 cytokine receptor family and shares many similar structural features with other members of this family such as GHr and EPOr (2, 3). These structural features include a ligand-binding EC domain that is composed of two β-sheet subdomains (S1 and S2), a single-pass α-helical TM domain, and a structurally uncharacterized IC domain. Ligand-bound PRLr dimers are associated with receptor activation (3, 175). The JAK2/STAT5 pathway is the principle signaling pathway activated when ligand binds to hPRLr (7, 22).

The proteins capable of activating hPRLr were identified a number of years ago and include hPRL, hGH, hPL, and several bivalent mAb (7, 178), but the mechanism by which lactogenic hormones activate this cascade of molecular interactions through receptor binding is poorly described. Previous studies indicate that ligand-induced receptor dimerization is associated with and necessary for receptor activation of class 1 cytokine receptors such as hPRLr (113, 175-177). More recently, receptor dimers in the absence of ligand have been found for many class 1 cytokine receptors (16, 114, 140, 146, 190). Some studies indicate that the helical TM domain forms the interface necessary for
ligand-independent receptor dimerization (16, 114, 146), but the role of these ligand-independent dimers is unclear.

Three mechanistic models have been suggested for other class I cytokine receptor activation: the scissor model (196), the piston model (103, 197, 198), and the rotation model (114, 199), as described in Chapter 1. However, specific physical models for the activation of hPRLr have not yet been proposed by similar studies. In this study, we used site-directed mutagenesis to create hPRLr mutants with various amino acid additions at the junctions of the TM helix with either the EC or IC domain. The addition of these residues extended the length of the TM helix, rotated either the EC or IC domain relative to the TM helix, or disrupted the fixed spatial relationship between the IC domain and the TM helix. Using these approaches, we investigated the relationship between the spatial orientations of these domains and hPRLr activation by ligand binding. The results presented here demonstrate that altering the physical relationships among the EC, TM, and IC domains by adding either alanines or glycines at or near domain junctions does not affect the formation of hPRLr dimers, does not induce constitutive activity, and does not influence ligand-mediated activation.

3.3 Results

3.3.1 Rationale for the Design of hPRLr Mutants

In mEPOr and hGHr systems, incremental additions of alanine residues at or near the junction between the TM and IC domains have been employed to determine the mechanisms for receptor activation (114, 199). These alanine additions are believed to both lengthen the TM helices and rotate the IC domains relative to each other in receptor
dimers because of the rigid linkage between the TM and IC domains (114, 199). Each additional alanine residue will lengthen the TM helix by approximately 1.5 Å and rotate the IC domain by approximately 100°. Constitutive ligand-independent activity of hGHR has been detected only with certain numbers of alanines inserted (114). Therefore, the orientations of the IC domains in these rotated receptors are believed to mimic the “on” receptor conformer. On the basis of this evidence, a rotation model for receptor activation has been proposed (104, 114). Because of the structural and functional similarities between hPRLr and hGHR (3), the possibility of a rotation mechanism for hPRLr activation was investigated in this study. Up to four alanine residues were inserted at the cytoplasmic end of the TM helix (Figure 3.1), specifically between leucine 234 and lysine 235 (235+1~4A), to rotate the IC domain relative to the TM helix. This set of alanine insertions is similar to what was introduced into hGHR, where up to four alanines were introduced between the last hydrophobic residue of the hGHR TM helix (F269) and the first hydrophilic residue of the hGHR IC domain (S270) (114). Another set of alanine insertions was placed above the JAK2-binding Box 1 region in the IC domain (Figure 3.1), between glycine 236 and tyrosine 237 (237+1~4A). Adding alanines at this position (six residues upstream from Box 1) is slightly different from adding them at the position tested in hGHR or mEPOr (five residues upstream from Box 1) (114, 146). hPRLr shares little sequence homology with hGHR or mEPOr in this region. Alanine insertions adjacent to G236 allow our results to be comparable with those for glycine insertions described below and also will not interrupt the small conserved hydrophobic motif immediately preceding Box 1. In addition, a third set of alanine insertions was introduced at the
extracellular end of the TM helix (Figure 3.1), between aspartic acid 210 and threonine 211 (211+1-4A), to rotate the EC domain relative to the TM helix because the interaction between the TM helices has been reported to hold the two receptors as a ligand-independent dimer (16). Alanine insertions at similar positions have not been examined in either hGHR or mEPOR. Lastly, up to four glycine residues were inserted above the IC domain (Figure 3.1), between glycine 236 and tyrosine 237 (237+1-4G). These glycine insertions were placed adjacent to the wild-type G236 and have a strong tendency to form an extended flexible hinge that increases the degree of structural freedom of the IC domain relative to the TM helix, therefore releasing the specific orientation of the two IC domains in a ligand-independent hPRLR dimer. Glycine repeats have been used in mEPOR as flexible linkers between the TM and IC domains to probe the interaction within the dimer pair (196). We tested each of these receptors for constitutive activity and for hPRL-induced tyrosine phosphorylation of hPRLR, a proximal end point of receptor activation.

3.3.2 hPRLR Mutants Form Ligand-Independent Homodimers Similar to WT hPRLR

The different hPRLR mutants were transiently expressed in 293T cells. IP and WB results showed that these mutants were expressed at a level similar to that of WT hPRLR (Figure 3.2, top panels). Further, the formation of ligand-independent dimers in these hPRLR mutants, as well as the relative ratio of monomer to dimer, was similar to that in WT hPRLR, as indicated in nonreducing gels (Figure 3.2, bottom panels). These results confirmed that the insertions of alanine or glycine residues at various positions do not affect the expression or dimerization of hPRLR.
3.3.3 Rotation of the IC or EC Domain Relative to the TM Helix of hPRLr Does Not Provide Constitutive Activity But Retains hPRL-induced Activation

To determine whether a rotation mechanism is at play for the activation of hPRLr, between one and four alanines were introduced following leucine 234. These mutants or WT hPRLr were transiently expressed in 293T cells, which also express endogenous JAK2 kinase. hPRLr expression and phosphorylation were analyzed by IP and WB following serum starvation for 24 hours and treatment for 15 minutes with either 100 nM hPRL or control medium. Both WT and 235+1~4A hPRLrs were expressed in 293T cells at similar levels (Figure 3.3 A, top panel). In the absence of hPRL, none of the hPRLr alanine insertion mutants exhibited higher tyrosine phosphorylation levels than the background observed with WT hPRLr (Figure 3.3 A, bottom panel). Thus, rotation of the IC domain relative to the TM helix did not induce constitutive hPRLr activity. Moreover, each of these mutants was activated by hPRL treatment, as evidenced by enhanced hPRLr tyrosine phosphorylation, comparable to that observed for hPRL-activated WT hPRLr (Figure 3.3 A, bottom panel). The functional potential of hPRLr was not impaired by the IC domain rotation mediated by alanine addition.

Because it is commonly accepted that it is JAK2 that phosphorylates tyrosine residues in the IC domain of hPRLr, we investigated whether rotation of the Box 1 region where JAK2 binds is involved in receptor activation. A second set of alanine residues was inserted after glycine 236 above Box 1. In contrast to a similar experiment conducted in hGHr (114), no constitutive activity was observed in any of these hPRLr mutants in the absence of ligand (Figure 3.3 B). In addition, these mutants retained the capability of
being activated by hPRL, although that capability was weakened by 2~4 folds (Figure 3.3 B). Therefore, rotating the JAK2-binding Box 1 region appeared to have a minimal effect, if any, on the function of hPRLr and failed to constitutively activate hPRLr.

In a third set of experiments, we examined whether rotating the EC domain would mimic the “on” conformer of hPRLr bound by ligand and render tyrosine phosphorylation without ligand and whether the rotated EC domains would be capable of inducing the IC domains to the “on” conformer in the presence of hPRL. In these experiments, up to four alanine residues were placed between the EC and TM domains, after aspartic acid 210, to rotate the EC domain (rather than the IC domain) relative to the TM helix because the interaction between the TM helices has been reported to hold the two hPRLrs in ligand-independent dimerization (16). Again, these EC domain-rotating mutants failed to yield constitutively active hPRLr in the absence of hPRL (Figure 3.3 C). Surprisingly, these mutant hPRLrs were successfully activated by hPRL treatment regardless of the 100° incremental alteration in the orientation of the ligand-binding EC domain (Figure 3.3 C), although the level of tyrosine phosphorylation of the 211+4A mutant in the presence of hPRL was decreased compared to that in WT hPRLr. Taken together, these data do not support rotational action as a mechanism required for hPRLr activation upon ligand binding.

**3.3.4 Providing Increased Degrees of Structural Freedom Between the TM and IC Domains Does Not Produce Constitutive hPRLr Activity or Eliminate Activation by hPRL**

Ligand-independent hPRLr dimers have been shown to be inactive (16, 190). This observation suggests that hPRLr dimers are held in an “off” conformer in the absence of
ligand. A rigid linkage between the TM and IC domains is required to lock the IC domain in the “off” conformer in the absence of hPRL and to transmit the conformational change induced by ligand binding from the EC domain to the IC domain. We sought to determine whether an increased degree of spatial freedom of the IC domain relative to the TM helix would unlock the receptors and result in an “on” conformer without the addition of ligand. Up to four glycine residues were added between the TM and IC domains, after glycine 236. These glycine additions have a strong tendency to form increasingly flexible hinges, so that a fixed relationship between the TM helix and the IC domain was disrupted. However, no tyrosine phosphorylation was detected in these glycine-insertion mutants in the absence of hPRL (Figure 3.4). Thus, an enhanced ability of the IC domain to move relative to the TM helix did not result in an increased level of formation of the “on” conformer. More interestingly, the treatment of hPRL successfully activated these four hPRLr mutants to a level above the background observed with the nontreated receptors, although the intensity of tyrosine phosphorylation decreased with increasing numbers of glycine residues inserted (Figure 3.4). These data indicate that the flexible glycine hinge does not buffer or counteract the conformational change caused by the binding of hPRL and that a rigid connection between the TM and IC domains is not required for hPRLr activation.

3.3.5 Heterodimerization of WT and Various Mutant hPRLrs Does Not Produce Functionally Active Receptors in the Absence of hPRL

The predicted TM helix of hPRLr is slightly larger than the required number of residues for crossing the hydrophobic core of the plasma membrane and thus is
compatible with a movement of several angstroms perpendicular to the plane of the membrane. Therefore, the piston model for receptor activation was also explored in this study. WT hPRLr was cotransfected with various mutant hPRLrs described above at a 1:1 ratio in 293T cells expressing endogenous JAK2. The added residues elongate the mutant hPRLrs and alter the relative vertical positions of the domains with respect to WT hPRLr. This resembles a piston movement when the mutant hPRLr heterodimerizes with WT hPRLr, assuming the insertion mutations do not disturb heterodimer formation (see Section 3.4). In addition to hPRLr heterodimer formation, there were also homodimers of WT hPRLr as well as those of two mutant hPRLrs. As shown in Figures 3.3 and 3.4, the homodimers were not active in the absence of hPRL and will not obscure tyrosine phosphorylation resulting from heterodimeric hPRLrs. None of the cotransfection experiments in the absence of hPRL provided receptors with tyrosine phosphorylation comparable to that observed with the ligand-activated WT hPRLr, or with tyrosine phosphorylation above the background level observed with the nonactivated WT hPRLr (Figure 3.5). The piston movement that shifts the vertical alignment of different domains in hPRLr is not capable of producing constitutive activity and thus does not mimic the “on” conformer.

3.4 Discussion

On the basis of structural and functional studies, three models for class 1 cytokine receptors have been proposed to reveal how ligand binding to the EC domain induces intracellular activation: a scissor model (196), a piston model (103, 197, 198), and a rotation model(114, 199), as described in Chapter 1. It is generally assumed that these
mechanisms must physically move the receptor-associated JAK2 to the positions where auto- and/or transphosphorylations of JAK2, the receptors, and subsequently other proteins bound to the phosphorylated receptors via the SH2 domain occur. It should be noted that these models are not mutually exclusive and that several components of these mechanisms may be present in any system.

When compared to that of GHr and EPOr, the molecular mechanism by which hPRL activates hPRLr is not described well. Investigators often assume that these structurally similar receptors will function through similar mechanisms. Indeed, the proteins involved in the downstream activation of these receptors have been identified and are largely similar (3, 7, 209). However, other data do not provide a consistent picture of the details for EPOr, GHr, and PRLr activation. For example, in mEPOr, addition of selected numbers of alanines at the TM/IC domain junction impaired EPO-induced receptor tyrosine phosphorylation and cell proliferation but had little effect on JAK2 tyrosine phosphorylation (146). No constitutive activity for mEPOr was produced by alanine additions (146). In contrast, a similar study conducted with hGHr (114) yielded constitutive tyrosine phosphorylation of receptor-associated JAK2 and cell proliferation in the absence of hGH, but the activity of hGHr in the presence of hGH was not evaluated. The data presented here in our study of hPRLr, however, are significantly different from those for both mEPOr and hGHr.

We aimed to evaluate the proposed activation models, and particularly the rotation model, in hPRLr. We have used site-directed mutagenesis to manipulate the structural relationships among the EC, TM, and IC domains of hPRLr. We examined the
basal and hPRL-stimulated activities of these receptors *in vivo* to determine the effects of our various structural interventions, including helical extensions at the EC/TM and TM/IC domain junctions and immediately preceding Box 1 by various alanine additions. This approach mimics the rotation model because alanine additions rotate either the EC or IC domains in approximately 100° increments (114, 146, 200). In hPRLr activated by hPRL binding, the additions of alanines also rotate the domains and may be capable of inactivating the heterotrimeric complex. We have also attempted to disrupt any rigid relationship between the TM and IC domains by the addition of increasing numbers of glycines. If a rigid relationship between the domains is required to lock the ligand-free “off” conformer and to transmit a ligand binding-induced conformational change, then we reason that the addition of glycine hinges would increase the degree of freedom of movement between the domains and disrupt both the TM-defined “off” conformer of the IC domain and the “on” conformer induced by hPRL binding. Furthermore, if the ligand-free receptor dimers were being held in an “off” conformation, then the increased degree of freedom of movement might allow activation in the absence of ligand. In addition, alanine and glycine additions lengthen the connection between the domains. When only one species of extended hPRLr is expressed in a cell, all receptors in this cell are extended equally, but if both WT and extended receptors are simultaneously expressed in a cell, a heterodimeric subpopulation of receptors will mimic the piston model, assuming the insertion mutations do not disturb heterodimer formation (see below).

In our study, we found no support for the rotation model in hPRLr as proposed and demonstrated for both the mEPOr and hGHr systems (104, 114, 146), where mimicry
of the IC domain rotation by incremental alanine insertions affected the activities of these receptors. None of the alanine additions placed at the junction between the TM and IC domains (235+1~4A) provided an increase in the basal level of hPRLr tyrosine phosphorylation above that of WT hPRLr. We have shown that these additions of residues did not affect the formation of ligand-independent receptor dimers and the ratio of ligand-independent dimers to monomers was similar to that in WT hPRLr (Figure 3.2). Each of these series of alanine additions between the TM domain and the JAK2 binding site should rotate the IC domain relative to the TM helix, creating dimeric hPRLr pairs with a variety of IC domain orientations. None of these unique dimeric hPRLr pairs demonstrated constitutive activity. This suggests that the incremental rotation of the IC domains of hPRLr pairs fails to provide an active orientation. In addition, each of the hPRLr mutants with added alanines was able to be stimulated by a brief exposure (15 min) to an hPRL concentration of 100 nM (20, 21, 97, 208) (Figure 3.3 A). Equivalent hPRL-stimulated receptor activity for both WT and alanine-inserted receptors indicated that the IC domain rotations failed to create receptor conformers that were unable to be activated by hPRL binding. This failure of the various alanine-mediated IC domain rotations to influence dramatically hPRL-induced activity demonstrates that these alanine additions do not disrupt the elements of receptor structure critical for hPRL-induced function and suggests that a rotational conformer of the hPRLr pair is not relevant to their activation. Similar results were observed when alanine additions were placed closer to the proline-rich JAK2 binding site (237+1~4A). Thus, the failures of alanine additions to either
induce constitutive activity or reduce hPRL-induced activity do not support a molecular mechanism whereby hPRL binding rotates the IC domains to an active conformer.

When incremental alanine additions were added immediately succeeding the TM domain (235+1~4A), the intensities of hPRL-induced receptor phosphorylation were similar to that in WT hPRLr, but when similar increment alanine additions were placed closer to Box 1 (237+1~4A), the phosphorylation responses of receptors to hPRL inducement were weakened, although still higher than the background observed with non-stimulated samples. This difference may be due to the interference near the JAK2 binding site at Box 1.

We also explored if the structural relationship between the EC and TM domains would influence the basal or hPRL-induced activity of hPRLr. Alanine additions at the EC/TM domain junction rotate the EC domain relative to the TM helix, assuming that these domains are rigidly connected and that the TM domains anchor the receptor dimer in the absence of ligand. On the basis of several structures of lactogens bound to pairs of rPRLr EC domains (5, 55, 56), lactogen binding holds the two EC domains in similar mirrored orientations. In addition, investigators have suggested that the EC domains enhance the formation of ligand-independent hPRLr dimers (16). However, in this study, alanine additions at the EC/TM domain junction did not disrupt the formation of hPRLr dimers in the absence of hPRL (Figure 3.2), nor does the EC domain rotation in hPRLr dimers display a conformation with constitutive activity. Finally, each of these alanine additions does not eliminate hPRL-stimulated JAK2-mediated receptor tyrosine phosphorylation (Figure 3.3 C). These results clearly show that disruption of the
relationship between the EC and TM domains does not influence the ability of hPRL to stimulate the target receptors. Thus, a specific structural relationship of the EC domains induced by hPRL binding in WT hPRLr does not appear to be necessary for receptor activation. Again, these results do not support the rotation model for the activation of hPRLr.

Each inserted alanine residue induces an incremental rotation of approximately 100°. Introducing four additional alanine residues will complete a full turn of the helix exploring all available orientations with a minimal extension of the TM helix, but this method of exploration is limited and granular because it fails to visit all possible orientations. If a precise rotation is required for hPRLr activation and is not mimicked by 100° stepwise alanine insertions, then our method may not visit this orientation, but if this were the case, then manipulating the orientations of hPRLr domains by inserting alanine residues would also abolish any ligand-induced activation of hPRLr. This was not observed in our experiments.

In contrast to alanine additions, which promote the formation of stable helices that rotate the adjoining domains relative to one another, glycine additions at the junction between the TM and IC domains of hPRLr have a strong predisposition to increase the degree of structural freedom between these domains. If a rigid spatial relationship between the TM and IC domains is necessary to maintain the “off” conformation of the two IC domains in a ligand-free hPRLr dimer and to transmit the ligand binding-induced conformational change from the EC domain to the IC domain, as the rotation model would require, then the disruption of this relationship by glycine additions should
influence the activities of these receptors in the absence or presence of hPRL stimulation. Our results show that insertion of one to four glycines between G236 and Y237 (237+1~4G) did not create receptor constitutive activity in the absence of hPRL or drastically impair the ability of hPRLr to be activated by hPRL, although the activation level in the presence of hPRL decreased with increasing numbers of inserted glycines. Thus, the disruption of the relationship between the TM and IC domains by increasing their relative degree of freedom does not unlock the IC domains from an “off” conformer or significantly affect hPRL-stimulated receptor activity. These data suggest that a rigid connection between the TM and IC domains that would hold the two IC domains of a receptor dimer in fixed spatial relationships is not required for either the inhibition or activation of hPRLr, further implying that a rotation mechanism is not involved in hPRLr activation.

Our additions of alanines or glycines also increased the distance between the EC or IC domains and the plasma membrane. Therefore, these receptors were used to mimic the elements of the piston model of receptor activation. The range of piston displacements has been reported to vary between ~2 Å for bacterial chemoreceptors (198, 210) and ~8 Å for hGhr (114). Because each additional alanine residue will lengthen the TM helix by approximately 1.5 Å, the range of the piston movement can be mimicked by inserting up to four alanine residues. We cotransfected both the WT and various forms of the alanine- or glycine-extended hPRLrs into 293T cells to allow the formation of a subpopulation of receptor heterodimers in which WT and extended hPRLrs would associate. We observed no increase in basal activity in any of these receptor heterodimers.
Thus, these data do not support a model in which a piston movement of the hPRLr dimer produces active receptors. The premise to this conclusion is that the insertion mutations do not affect the ability of the hPRLr mutants to heterodimerize with WT hPRLr. Although this premise remains to be further investigated, given the report that the TM domain mediates the ligand-independent dimerization of hPRLr (16) and the fact that the alanine or glycine insertions, which do not disturb the core of the TM helix, do not affect the homodimerization of hPRLr (Figure 3.2), the likelihood that the dimerization between the mutant and WT hPRLr is abolished by these mutations is arguably low. However, it should also be noted that the piston model cannot be completely excluded because the elongation displacements resulted from alanine insertions are also accompanied by the rotation movement. It is possible that hPRLr activation employs a one-dimensional piston sliding that does not involve any rotation, which is determined for bacterial chemoreceptors (198, 210). Signaling by such a mechanism would be difficult to mimic using insertions or deletions.

In this work, we have explored the structural relationships among the EC, TM, and IC domains of hPRLr. We have taken the previously used technique of alanine additions at domain interfaces and extended these studies with glycine mutations and the evaluation of the ability of hPRL to activate mutant receptors. Our data do not support the rotation or piston models of hPRLr activation. GHr, EPOr, and PRLr share several common properties, including their activation induced by ligand binding, the formation of active trimeric ligand/receptor complexes, and the presence of ligand-free inactive receptor dimers. In addition, these receptor systems utilize many similar intracellular

59
signaling pathways. Although GHr, EPOr, and PRLr have similar global structures (2, 3), many of the details are significantly different. For example, comparison of the amino acid sequences of either the IC or TM domains among hGHr, hEPOr, and hPRLr shows only modest to minimal sequence homology (3, 7). Thus, it should not be surprising that these three receptors use different mechanisms.

Although the rotation and piston models are not supported by our data, we have not tested the scissor model and therefore cannot exclude the possibility of hPRLr adopting a scissor model or a combination of elements from the scissor model and other proposed models. Determining if a scissor action is involved in hPRLr activation requires additional experiments.

Our data suggest that the overall structure of hPRLr appears to possess a strong plasticity that can accommodate the presumed significant structural alterations caused by the additions of alanines or glycines without losing its function. Since the discovery of ligand-independent dimerization in hPRLr (16, 190), the classic model in which the proximity of the ligand-induced dimeric receptors triggers activation (177) is largely deemed invalid because these ligand-free preformed hPRLr dimers have been shown to be inactive (16, 190). To support such argument, ligand binding to the EC domains of preformed hPRLr dimer must bring about a conformational change that is transmitted to the IC domains and subsequently activate the IC domains. However, the results presented here have demonstrated that our variety of spatial manipulations that attempted to mimic this presumed activating conformational change had a minimal impact, if any, on hPRLr activation and that a rigid connection between the TM and IC domains, which would
have been critical to transmit the aforementioned ligand-induced conformational change from the EC domain to the IC domain, was not required for hPRLr activation. These data, especially the results from the glycine insertion mutations, prompted us to re-evaluate the significance of ligand-independent hPRLr dimers. Do they exist in dynamic equilibrium with hPRLr monomers among the total hPRLr population? What is their role in the process of activation? Are they remnants of previous activation processes? These questions remain to be investigated.
Figure 3.1 Schematic representation of WT hPRLr and variant insertion mutants

The TM helix sequence and JAK2 binding Box 1 sequence are shown in bold. The amino acid number does not include the N-terminal signal sequence of 24 amino acids. Up to four alanine residues were inserted at various positions, between L234 and K235 (235+1~4A), between G236 and Y237 (237+1~4A), and between D210 and T211 (211+1~4A), to rotate the IC domain, the Box 1 region, and the EC domain, respectively. Up to four glycine residues were inserted between G236 and Y237 (237+1~4G) to disrupt the fixed relationship between the TM helix and the IC domain.
293T cells were transiently transfected with WT hPRLr or various insertion mutants (see below) and incubated in the absence of hPRL. hPRLr monomers and dimers were detected by WB on reducing gels (top) and nonreducing gels (bottom) using anti-hPRLr EC domain antibody 1A2B1 after IP with anti-hPRLr IC domain antibody H-300. Mock transfections were included as negative controls (lane 1 in panels A and B). (A) WT hPRLr and hPRLr mutants with up to four alanine residues inserted between D210 and T211 (211+1~4A, lanes 3~6) or between L234 and K235 (235+1~4A, lanes 7~10). (B) WT hPRLr and hPRLr mutants with up to four alanine (237+1~4A, lanes 3~6) or glycine (237+1~4G, lanes 7~10) residues inserted between G236 and Y237.
Figure 3.3 Rotation of hPRLr domains relative to the TM helix does not provide constitutive activity or impair the ligand-induced activity

293T cells were transiently transfected with WT hPRLr or various alanine insertion mutants (see below) and not treated or treated with hPRL (100 nM) for 15 min. Tyrosine phosphorylation in hPRLr was analyzed by WB using phosphotyrosine antibody 4G10 after IP with anti-hPRLr IC domain antibody H-300. Mock transfections were included as negative controls (lanes 1 and 2 in panels A ~ C). Top panels show blotting with anti-hPRLr EC domain antibody 1A2B1 for hPRLr total expression. Bottom panels show blotting with anti-phosphotyrosine antibody 4G10 for hPRLr activation. (A) Up to four alanine residues were introduced between L234 and K235. Rotation of the IC domain relative to the TM helix did not result in constitutive activity of hPRLr or impair hPRL-induced activity. (B) Up to four alanine residues were introduced between G236 and Y237. Rotation of the JAK2-binding Box 1 region had a minimal effect on basal or hPRL-induced hPRLr activity. (C) Up to four alanine residues were introduced between D210 and T211. The basal hPRLr activity was not affected, and the ability of hPRLr to be activated by hPRL persisted regardless of rotation of the ligand-binding EC domain relative to the TM helix. Data are representative of three independent experiments.
Figure 3.4 Increased degree of freedom in the movement of the IC domain relative to the TM helix fails to yield constitutive activity in hPRLr and minimally affects the ligand-induced activity.

293T cells were transiently transfected with WT hPRLr or 237+1–4G mutants and not treated or treated with hPRL (100 nM) for 15 min. Tyrosine phosphorylation in hPRLr was analyzed by WB using phosphotyrosine antibody 4G10 after IP with anti-hPRLr ICD antibody H-300. Mock transfection was included as a negative control (lanes 1 and 2). The top panel shows blotting with anti-hPRLr ECD antibody 1A2B1 for hPRLr total expression. The bottom panel shows blotting with anti-phosphotyrosine antibody 4G10 for hPRLr activation. Data are representative of three independent experiments.
293T cells were cotransfected with WT hPRLr and various insertion mutants at a 1:1 ratio and incubated in the absence of hPRL. Tyrosine phosphorylation in hPRLr was analyzed by WB using phosphotyrosine antibody 4G10 after IP with anti-hPRLr IC domain antibody H-300. Mock transfections were included as negative controls (lanes 1 and 2 in panels A and B). Single transfections with WT hPRLr were included as positive controls (lanes 3 and 4 in panels A and B). - , treated without hPRL; +, treated with hPRL (100 nM). Top panels show blotting with anti-hPRLr EC domain antibody 1A2B1 for hPRLr total expression. Bottom panels show blotting with anti-phosphotyrosine antibody 4G10 for hPRLr activation. (A) WT hPRLr was cotransfected with 211+1~4A (lanes 5~8) or 235+1~4A (lanes 9~12). (B) WT hPRLr was cotransfected with 237+1~4A (lanes 5~8) or 237+1~4G (lanes 9~12). Data are representative of three independent experiments.
CHAPTER 4

INTERMOLECULAR DISULFIDE LINKAGES IN LIGAND-INDEPENDENT DIMERIZATION OF HUMAN PROLACTIN RECEPTOR

4.1 Abstract

Receptor dimers in the absence of ligand have been demonstrated for hPRLr, but the specific amino acid residues that mediate ligand-independent hPRLr dimerization have not been determined, although the TM domain has been suggested to be important. A population of the ligand-free hPRLr dimers was determined to be redox-sensitive by WB under reducing and nonreducing conditions. In this study, we demonstrated the physiological relevance of this finding in MCF-7 cells that express endogenous hPRLr and investigated the role of intermolecular disulfide bonds in ligand-independent hPRLr dimerization using transfected 293T cells. Twelve cysteine residues in various domains of hPRLr were mutated to serine residues to determine if they were required for redox-sensitive hPRLr dimer formation. Four N-terminal EC cysteine residues form intramolecular disulfide bonds critical for receptor activity and thus were not altered in this study. IAM, an alkylation reagent for the thiol group of cysteine, was employed to prevent ex vivo formation of disulfide bonds during solubilization of the cells and immunoprecipitation of hPRLr. Removal of all 12 C-terminal cysteine residues abolished the formation of ligand-free redox-sensitive hPRLr dimers, confirming the involvement of intermolecular disulfide bonds in this process. Our data indicate that multiple cysteine
residues, including but not limited to C184, C225, and C242, participate in the formation of intermolecular disulfide linkages in ligand-free hPRLr dimers, although the contribution from C184 and C225 requires the presence of the Box 1 region of the IC domain.

4.2 Introduction

The mature long form of hPRLr contains 598 amino acid residues and has an apparent molecular weight of 100 kDa. Seven additional isoforms of hPRLr have been described. The ΔS1 isoform is generated from alternative mRNA splicing and lacks the S1 subdomain of the EC domain (26). A constitutively active ΔS2 isoform has also been reported (27). The intermediate form (28) and two short forms (S1a and S1b) (29) are also generated from alternative mRNA splicing and have various lengths of the IC domain shorter than that in the LF. Finally, the hPRL binding protein (hPRLbp) is the EC fragment of hPRLr and is believed to result from proteolytic cleavage of LF hPRLr (30, 31). This cleavage event also creates a TM-ICD isoform of hPRLr (16).

Biochemical and biophysical studies have suggested that receptor dimerization is critical for ligand-induced hPRLr activation (3, 51, 175, 177). One molecule of hPRL binds with two molecules of hPRLr to form a heterotrimmeric complex (56, 175). In the past few years, hPRLr dimers in the absence of ligand have been reported to exist on the plasma membrane (16, 190). Ligand-independent dimerization of hPRLr has been associated with redox sensitivity (190), but the nature of the critical bonds involved in this redox-sensitive process has not been characterized. Dimerization by disulfide bond formation has been demonstrated for other members in the class 1 cytokine receptor
family including murine erythropoietin receptor (mEPOr) (211), human growth hormone receptor (hGHr) (212), and rabbit growth hormone receptor (rbGHr) (213), as well as tyrosine kinase growth factor receptors including colony-stimulating factor-1 receptor (CSF-1R) (214) and platelet-derived growth factor receptor (PDGFR) (215). In hPRLr, the S1 subdomain of the EC domain contains two intramolecular disulfide bonds. These two disulfides are critical for ligand activation (4, 216) and are conserved in the class 1 cytokine receptor family. Twelve additional cysteines are distributed in the three domains of hPRLr; these cysteines are unique for hPRLr and their role in redox-sensitive hPRLr dimers has not been described.

In this study, we examined the structural role of cysteines in ligand-independent hPRLr dimerization in transfected 293T cells and identified a population of ligand-free disulfide-linked hPRLr dimers. IAM, a cysteine alkylation reagent, was employed in our biochemical studies to distinguish between the in vivo and ex vivo formations of disulfide bonds. We determined that multiple cysteine residues from different domains of hPRLr, including but not limited to cysteines 184, 225, and 242, participated in ligand-independent disulfide-mediated hPRLr dimerization. The physiological relevance of intermolecular disulfide linkages in ligand-independent hPRLr dimerization was also demonstrated in MCF-7 cells that express endogenous hPRLr.

4.3 Results

4.3.1 A Population of hPRLr Exists as Ligand-Free Disulfide-Linked Dimers

To confirm ligand-independent dimerization of hPRLr, we prepared DNA constructs of LF hPRLr tagged with poly-histidine or V5 epitope at the C-terminus for
CoIP. We first assessed the expression level and the functional integrity of the epitope-tagged hPRLrs. These constructs or WT hPRLr were transiently transfected in 293T cells. IP and WB results showed that hPRLr-His and hPRLr-V5 were expressed at levels similar to that of WT hPRLr (Figure 4.1, top panel). Upon hPRL treatment, both hPRLr-His and hPRLr-V5 exhibited induced tyrosine phosphorylation comparable to that observed for WT hPRLr (Figure 4.1, bottom panel). These results demonstrated that the structure and the function of epitope-tagged hPRLrs were intact.

To examine ligand-independent dimerization of hPRLr, 293T cells were cotransfected with hPRLr-His and hPRLr-V5 at a 1:1 ratio and were deprived of hormones in serum-free medium for 24 hours. hPRLr-V5 was coimmunoprecipitated with hPRLr-His by anti-His antibody and was detected by anti-V5 antibody in WB (Figure 4.2, Lane 4). No signal was detected in 293T cells singly transfected with hPRLr-His, hPRLr-V5, or WT hPRLr (Figure 4.2, Lanes 1–3), indicating the specificity of the antibodies used in IP and WB. The overall expression of the various constructs was demonstrated by IP and WB against V5 epitope (Figure 4.2, Lanes 5–8) and by IP and WB against hPRLr (Figure 4.1, top panel). These results confirmed ligand-independent dimerization of hPRLr.

Previous study of hPRLr in the absence of ligand has observed a high molecular weight form of hPRLr corresponding to hPRLr homodimers under nonreducing conditions (190), suggesting that disulfide linkage is involved in ligand-independent hPRLr dimerization. Because the ambient environment after cell lysis is relatively oxidative compared to that in vivo (217), this observation cannot exclude the possibility
that unpaired cysteine residues are brought together during sample preparation and disulfide-linked hPRLr dimers are formed in an *ex vivo* manner. The present study utilized IAM, a reagent that alkylates the thiol group of free cysteine and prevents the formation of artifactual intermolecular disulfide bonds but does not cleave existing disulfide bond (211, 218, 219).

293T cells were transiently transfected with WT hPRLr, incubated in the absence of hPRL, and lysed with RIPA buffer in the presence or absence of 20 mM IAM. After IP with H-300, an anti-hPRLr IC domain antibody, and separation in SDS-containing polyacrylamide gels under nonreducing or reducing conditions, hPRLr monomers and dimers were identified by WB using 1A2B1 antibody (anti-hPRLr EC domain). In the absence of 2-ME, the IAM-treated IP revealed a heterogeneous population of hPRLr with an approximate molecular weight of 200 kDa, as well as an approximate 100 kDa hPRLr population (Figure 4.3, bottom panel, Lane 4). The 200 kDa population was abrogated upon reduction (top panel). A separate band observed at 65 kDa on reducing gel and at 60 kDa on nonreducing gel is a non-specific band because it also appeared in mock transfected 293T cells and we have demonstrated by RT-PCR that 293T cells do not express endogenous hPRLr (Figure 2.1). These results confirm the involvement of cystine in ligand-independent dimerization of hPRLr and support *in vivo* intermolecular disulfide bond formation. Notably, the WT hPRLr transfectant without IAM treatment displayed a stronger and more heterogeneous signal at the high molecular weight position (bottom panel, Lane 3), consistent with the hypothesis of additional *ex vivo* intermolecular disulfide bond formation in the absence of IAM treatment. Thus, besides
the *in vivo* intermolecular disulfide cystines, hPRLr also contains unpaired free cysteine residues capable of artifactual intermolecular disulfide formation. These *ex vivo* disulfide linkages were eliminated in subsequent experiments by including IAM during WCE preparation, allowing us to discern only hPRLr dimers formed *in vivo*.

To examine the physiological relevance of ligand-independent disulfide-mediated hPRLr dimerization, we also performed similar studies with MCF-7 human breast cancer cells that express low level endogenous hPRLr (220). MCF-7 cells were incubated in the absence of lactogenic hormones for 24 hours and were lysed with or without IAM before hPRLr dimerization was analyzed under reducing or nonreducing conditions. Disulfide-linked hPRLr dimers were again detected under nonreducing condition for MCF-7 cells in the presence of IAM (Figure 4.4). Such results further confirm the *in vivo* formation of ligand-free disulfide-linked hPRLr dimers in the physiological context of endogenous hPRLr with low level expression.

4.3.2 Multiple Cysteine Residues in Different Domains of hPRLr Are Involved in Ligand-Free Intermolecular Disulfide Linkages

We sought to determine which cysteine residues are responsible for ligand-independent disulfide-mediated dimerization of hPRLr. Full-length hPRLr contains 16 cysteine residues (Figure 4.5). To dissect the roles of these 16 cysteine residues, we have categorized them into three groups. First, cysteines 12 and 22 and cysteines 51 and 62 are located near the N-terminus of the EC domain and form two conserved intramolecular disulfide bonds. These intramolecular disulfide bonds are critical for ligand binding and biological activity of hPRLr (4, 216). Therefore, these four residues are very unlikely to
participate in intermolecular disulfide linkage and were not altered in this study. Second, cysteine 184 is unpaired in S2 subdomain close to the TM domain, and cysteine 225 is within the TM domain (residues 210-234). Finally, the IC domain contains 10 uncharacterized cysteines (residues 242, 274, 330, 340, 372, 389, 413, 547, 578, and 592). The unpaired EC cysteine is also observed in hEPOr and hGHr but in unique positions. Cysteines homologous to the TM C225 of hPRLr are not found in either of these receptors. IC domain cysteines are not conserved in these three receptors. To evaluate the participation of these twelve cysteines in the formation of redox-sensitive hPRLr dimers, we have mutated the twelve cysteines to serines in different combinations and compared these hPRLr mutants for their abilities to form ligand-free disulfide-linked dimers.

Plasmids expressing WT hPRLr or various hPRLr mutants (C184/225S, C242-592S, and C184-592S) were transiently transfected in 293T cells. Expression and dimerization status of hPRLr were analyzed by IP and WB following serum starvation and treatment with or without IAM. Both WT and mutant hPRLrs were expressed in 293T cells at similar levels as assessed by WB under reducing condition (Figure 4.6, top panels). Mutation of all 12 cysteines to serines (C184-592S) eliminated the appearance of any hPRLr above 180 kDa under nonreducing condition, regardless of the IAM treatment (bottom panels, Lanes 7, 8, 15, and 16), thus abolishing ligand-free disulfide-linked hPRLr dimers. This again confirms the in vivo formation of intermolecular disulfide bonds in ligand-independent hPRLr dimerization. The intensity of the 100 kDa hPRLr WB signals was not affected by the mutation of these cysteines to serines, indicating that the avidities of anti-hPRLr antibodies used in IP and WB were not disturbed by the
cysteine mutations. A separate band below 180 kDa was observed for the C184-592S mutant and in various intensities for other hPRLr variants. For the reasons described in Section 4.4 Discussion, we conclude that this band represents a misfolded hPRLr species involving the formation of intermolecular disulfide bonds by the four N-terminal cysteine residues that are normally utilized in the formation of two intramolecular disulfide bonds. Surprisingly, when the two cysteines within or close to the TM domain (C184/C225S) or those ten cysteines in the IC domain (C242-592S) were mutated to serines, under nonreducing condition, disulfide-linked dimeric hPRLrs were detected for both mutants in the presence of IAM (bottom panels, Lanes 6 and 14, respectively). Thus, neither of these two groups of cysteines was solely responsible for ligand-free disulfide-mediated dimerization of hPRLrs. In addition, when compared between the presence and absence of IAM, the C184/225S hPRLr mutant displayed a change of banding pattern at the high molecular weight position (bottom panel, Lanes 5 and 6), similar to that for WT hPRLr (bottom panel, Lanes 3 and 4), albeit less prominent. This result suggests that among the remaining 10 IC cysteines one or more are free cysteines and can form ex vivo disulfide bonds. In contrast, the heterogeneity of the disulfide-linked dimers in the C242-592S mutant was not affected by the lack of IAM treatment (bottom panel, Lanes 13 and 14). The lack of free cysteines capable of ex vivo intermolecular disulfide formation in C242-595S indicates that both remaining cysteines (C184 and C225), are involved in the formation of in vivo intermolecular disulfide linkages for ligand-independent hPRLr dimerization.
A previous study from Dufau and colleagues (190) demonstrated that the S1b short form of hPRLr possessed the capacity of forming ligand-free redox-sensitive dimers. The S1b hPRLr is a 264-residue isoform produced by alternative mRNA splicing and terminates shortly after the Box 1 region in the IC domain. S1b consists of the first 261 residues of the full-length hPRLr and three additional amino acids. Cysteine 242 is located between the TM domain and Box 1. To examine the implication of cysteine 242, we have generated a truncated hPRLr in which lysine 262 is mutated to a stop codon (K262X). This truncated hPRLr is very similar to S1b and was tested for ligand-independent disulfide-mediated dimerization. Consistent with the S1b hPRLr study, an approximate 40 kDa monomeric K262X hPRLr and an approximate 80 kDa dimeric K262X hPRLr were detected under nonreducing conditions with IAM treatment (Figure 4.7, bottom panel, Lane 5). Additional cysteine-to-serine mutations for the remaining three cysteine residues (C184, C225, and C242) in the K262X truncated hPRLr showed similar behaviors to the full-length hPRLr C/S mutants: removal of all three remaining cysteines (C184-242S K262X) abolished ligand-independent disulfide-linked dimerization (bottom panel, Lane 8), while neither the mutation of the two cysteines within or close to the TM domain (C184/225S K262X) nor the mutation of the one in the IC domain (C242S K262X) eliminated ligand-free disulfide-linked hPRLr dimers (bottom panel, Lanes 6 and 7, respectively). These dimers of the K262X truncated hPRLr disappeared under reducing condition (Figure 4.7, top panel, Lanes 5-8). These data indicate that cysteines 184, 225, and 242 all participate in the ligand-independent intermolecular disulfide bond formation that creates dimers of the truncated hPRLr.
To further examine the role of cysteines 184 and 225, a more severely truncated hPRLr was generated, where a stop codon was introduced at residue 242 (C242X) immediately before Box 1. The C242X hPRLr lacks all 10 IC cysteines as well as Box 1. Interestingly, despite the presence of cysteines 184 and 225 in this mutant, C242X failed to exhibit ligand-independent disulfide-mediated dimerization under nonreducing conditions when treated with IAM (Figure 4.7, bottom panel, Lane 3). A comparison between the C242X mutant and the C242S K262X mutant attributes this result to the difference between the absence and the presence of Box 1. Therefore, the contribution of cysteines 184 and 225 to ligand-independent intermolecular disulfide linkages requires the presence of the Box 1 region in the IC domain.

We next investigated the role of cysteine 242 in the context of the full-length hPRLr. When cysteine 242 is mutated to serine (C242S), even if both cysteines 184 and 225 are also mutated to serines (C184-242S), disulfide-linked hPRLr dimers were still detected under nonreducing condition when treated with IAM (Figure 4.8, bottom panel, Lanes 6 and 8), indicating the involvement of other remaining IC cysteine residues in forming in vivo intermolecular disulfide linkages. Similarly to WT hPRLr (bottom panel, Lanes 3 and 4), the lack of IAM treatment increased the intensity and the heterogeneity of the dimeric hPRLr signal for C242S (bottom panel, Lane 5) and C184-242S (bottom panel, Lane 7), indicating additional artifactual ex vivo disulfide-mediated dimerization due to free cysteines in the remaining IC cysteines. Again, removal of all 12 cysteine residues (C184-592S) eliminated the appearance of any disulfide-linked hPRLr dimer above 180 kDa under nonreducing conditions, regardless of the IAM treatment (bottom
panels, Lanes 9 and 10). The presence of 2-ME abolished disulfide-mediated dimerization in these hPRLr mutants (Figure 4.8, top panel). Taken together, these data indicate that besides cysteines 184, 225, and 242, other but not all IC cysteines are involved in \textit{in vivo} ligand-independent intermolecular disulfide formation, and suggest that multiple and complex disulfide linkages are at play for hPRLr dimerization.

4.4 Discussion

The evidences for ligand-independent dimerization of hPRLr on the plasma membrane were reported several years ago in two independent studies (16, 190) and were confirmed in this study by CoIP of epitope-tagged hPRLr (Figure 4.2). However, the structural details about ligand-free hPRLr dimers, including the specific covalent and/or non-covalent bonds responsible for dimerization, have not been well described. In this study, we investigated a redox-sensitive population of ligand-free hPRLr dimers, and the results presented here reveal the involvement of intermolecular disulfide linkages formed \textit{in vivo} in ligand-independent dimerization of hPRLr. A critical factor one has to consider when biochemically evaluating the physiological significance of disulfide bonds is the oxidative \textit{in vitro} environment compared to the \textit{in vivo} environment (217). After the cells are lysed and the membranes are solubilized, it is possible that proteins with unpaired cysteines are locally concentrated and brought together to form \textit{ex vivo} disulfide bonds in the conducive ambient oxidative environment during the process of IP. IAM alkylates the thiol group in unpaired cysteine without disrupting any preexisting disulfide cystine (211, 218, 219). Therefore, including IAM in the lysis buffer prevents disulfide-mediated \textit{ex vivo} hPRLr dimerization. Another advantage of this approach is that the comparison
between the IAM-treated and the nontreated samples allows us to distinguish between physiological and artifactual disulfide bonds and provides us with information regarding the existence of unpaired free cysteines in hPRLr.

In the presence of 20 mM IAM, a population of hPRLrs are covalently associated to form approximately 200 kDa homodimers that are detected by WB in nonreducing SDS-PAGE but eliminated by reduction (Figure 4.3). Such results strongly suggest a role of cystine in creating ligand-free redox-sensitive hPRLr dimers via intermolecular disulfide bonds in vivo. Although studies with transfected 293T cells raise the concern that such observation could be due to the overexpression of exogenous hPRLr, our data from MCF-7 cells expressing a low level of endogenous hPRLr (Figure 4.4) address this concern and further support the physiological relevance of in vivo ligand-independent disulfide-mediated hPRLr dimerization. The low amount of hPRLr in MCF-7 cells could also account for the lack of the effect of IAM, because during sample preparation, the concentration of endogenous hPRLr from MCF-7 cells may not be high enough to facilitate ex vivo artifactual disulfide bond formation even in the absence of IAM.

Among the total 16 cysteine residues in the long form of hPRLr, the 12 C-terminal cysteine residues are possible candidates for intermolecular disulfide bond formation and were evaluated in our study. The four N-terminal cysteines form two pairs of intramolecular disulfide bonds (C12-C22 and C51-C62) in the S1 subdomain that are critical for receptor activity (4, 216) and were not mutated in this study. By examining different combinations of mutations in the 12 C-terminal cysteines and by comparing the results obtained from IAM-treated and nontreated receptors, we determine that multiple
cysteine residues from different domains of hPRLr are involved in the *in vivo* formation of intermolecular disulfide bonds that mediate ligand-independent redox-sensitive hPRLr dimerization. These participating cysteines include, but are not limited to, C184 in the EC domain, C225 in the TM domain, and C242 in the IC domain (Figures 4.6, 4.7, and 4.8). Ligand-free redox-sensitive hPRLr dimers can be eliminated by mutating all 12 C-terminal cysteines to serines. These results are in accord with a previous study from Dufau and colleagues on the S1b hPRLr short form (190) where C184, C225, or C242 was mutated individually. A C184/C225 double mutant of S1b was also examined, but a triple mutant of S1b was not tested. None of the mutants tested in that study abolished ligand-independent dimerization of S1b hPRLr, as examined by BRET analysis (190). It should also be noted that BRET analysis does not specifically probe disulfide-mediated dimerization, as nonreducing SDS-PAGE would. Noncovalent dimerization may also contribute to a positive BRET signal and therefore mask the contribution from covalently linked receptor dimers.

C184 resides in the S2 subdomain of the EC domain and is close to both the receptor’s Zn2+ binding half site (D187 and H188) (5) and the WSAWS motif (residues 191-195) that is conserved in the class 1 cytokine receptor family. C225 is located within the TM domain (residues 211 through 234). The interaction between the TM domains has been shown to be sufficient for ligand-independent hPRLr dimerization because the hPRLr mutant lacking the entire EC domain can dimerize with the hPRLr mutant lacking the entire IC domain (16). Participation of the IC domain cysteines, including C242, in forming intermolecular disulfide bonds is rather surprising, because the relative reducing
environment in the cytosol usually renders intracellular disulfide bonds unstable (217). However, several membrane and juxtamembrane proteins have been shown to modulate intracellular disulfide bond formation to regulate signaling and transportation (221-224). C242 is immediately above the proline-rich Box 1 motif (residues 243-251) where JAK2 constitutively associates with hPRLr (20, 21). Our results also indicate that Box 1 is essential for the participation of C184 and C225 in forming intermolecular disulfide bonds (Figure 4.7) and suggests that the association of JAK2 with hPRLr is involved in ligand-independent disulfide-mediated hPRLr dimerization. It has been reported that JAK2 binding increased hGHR maturation and stability on the plasma membrane (124, 225) and is required for hEPOr biogenesis (226).

A separate band with a molecular weight below 180 kDa was observed in hPRLr mutants that contain mutations at C184 and C225 (C184/225S, C184-592S, and C184-242S), and was also detected with less intensity in other hPRLr variants including the WT. The less-than-180 kDa molecular weight of this hPRLr population is larger than that of a correctly folded hPRLr monomer (100kDa) and smaller than that of the corresponding hPRLr dimer (200kDa). Therefore, this hPRLr population is most likely a misfolded species. This species was redox sensitive because it was abolished by 2-ME in reducing gels. This species was observed in the C184-592S mutant where the 12 C-terminal cysteine residues were removed, indicating that the four N-terminal cysteine residues are involved in the formation of the misfolded hPRLr. The treatment of IAM did not eliminate this species, suggesting that it is not an ex vivo artifact from sample preparation but results from a cellular process. The signal from the misfolded hPRLr was
relatively weak in the hPRLr variants where C184 and C225 were intact, including the WT, and was enhanced by the mutations of C184 and C225, suggesting a role of these two residues in this abnormality. As shown in Chapter 5, hPRLr with C184 and C225 mutations exhibited reduced tyrosine phosphorylation upon hPRL stimulation. This observation can be attributed to the increased level of misfolded hPRLr from the mutations of C184 and C225. The involvement of the four N-terminal cysteine residues in the mutation-induced misfolding that correlates with reduced receptor phosphorylation is consistent with previous report that the proper formation of intramolecular disulfide bonds from these four N-terminal cysteine residues is critical for ligand activation (4). A similar hypothesis of cysteine-mediated receptor misfolding has been proposed for mEPOr (211).

Intermolecular disulfide linkages have been implicated in the dimerization of other members in the class 1 cytokine receptor family such as hGHR (212), rbGHR (213) and mEPOr (211). C241 in the EC domain of rbGHR (analogous to C240 in hGHR) was solely responsible for disulfide-mediated receptor dimerization (213), while in the EPOr system the specific cysteine residues involved in intermolecular disulfide linkages were not identified. Our data demonstrate that in hPRLr multiple cysteine residues from different domains mediate disulfide-linked hPRLr dimerization. Such results point out the difference among hPRLr and other class 1 cytokine receptors in the structural basis for receptor dimerization.
293T cells were transiently transfected with WT hPRLr, C-terminal V5-tagged hPRLr (hPRLr-V5), or C-terminal poly-histadine-tagged hPRLr (hPRLr-His) and not treated or treated with hPRL (100 nM) for 15 min. Tyrosine phosphorylation in hPRLr was analyzed by WB using phosphotyrosine antibody 4G10 after IP with anti-hPRLr ICD antibody H-300. Mock transfection was included as a negative control (lanes 1 and 2). The top panel shows blotting with anti-hPRLr ECD antibody 1A2B1 for hPRLr total expression. The bottom panel shows blotting with anti-phosphotyrosine antibody 4G10 for hPRLr activation.
Figure 4.2 Epitope-tagged hPRLrs form ligand-independent dimers in transfected 293T cells

293T cells were singly transfected with WT hPRLr, hPRLr-His, or hPRLr-V5, or cotransfected with hPRLr-His and hPRLr-V5, and incubated in the absence of hPRL. After IP with anti-His antibody (Lanes 1–4) or anti-V5 antibody (Lanes 5–8), hPRLr dimerization was analyzed by WB using anti-V5 antibody.
Figure 4.3 A population of transfected hPRLrs exists as ligand-free disulfide-linked dimers in 293T cells

293T cells were transiently transfected with WT hPRLr, incubated in the absence of hPRL, and lysed with or without 20 mM IAM. After IP with anti-hPRLr IC domain antibody H-300, hPRLr monomers and dimers were detected by WB on reducing (top) or nonreducing gels (bottom) using anti-hPRLr EC domain antibody 1A2B1. Mock transfections were included as negative controls (lanes 1 and 2). Molecular weight standards are presented to the right of the panels. hPRLr dimers are indicated by red box. Data are representative of three independent experiments.
Figure 4.4 A population of endogenous hPRLrs exists as ligand-free disulfide-linked dimers in MCF-7 cells

MCF-7 cells or nontransfected 293T cells were incubated in the absence of hPRL and lysed with or without 20 mM IAM. After IP with anti-hPRLr IC domain antibody H-300, hPRLr monomers and dimers were detected by WB on reducing (top) or nonreducing gels (bottom) using anti-hPRLr EC domain antibody 1A2B1. Molecular weight standards are presented to the right of the panels. hPRLr dimers are indicated by red box. Data are representative of three independent experiments.
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<td>QLPG--KPEIFKRSNKETFTCWW</td>
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<td>FSSEAALTSAINPWSLQSNPGKTKNSKEFKTGERPERTFSSCWH</td>
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Continued

**Figure 4.5 Comparison of cysteine residues in hPRLr, hGHr, and hEPOr**

Alignment was performed by the Clustal W multiple sequence alignment program (202) in the UniProt suite (203). The amino acid number does not include the N-terminal signal sequences. TM domains are underlined. Cysteine residues are highlighted in red.
Figure 4.5 continued

hPRLr 397  P-SQHNPRSSYHNITDEVELAVGPAGAPATLNEAGKDALKSSQTIKSRE 445
hGHr 445  EGAESTHQAAHIQLSNPSSLSNIDFYAQVSDITPAGSVLSPGQKKNKAGM 494
hEPOr 355  N-----PPSEDLPGGSVDIVAMEGSEASSSSALASKPSP------ 393

hPRLr 446  EGKATQREVESFHSETDQTPWLLPQEKTPFGSAKPLDYVEIHKVNKD 495
hGHr 495  SQCDMHPEMVSLQENFLMDNAYFEDAKKEIPVAPHIKVESHIQPSL 543
hEPOr 394  EGASAASFETYILDPSQLLRPWTLPELP----PTPPHLKYLYLVVSDSG 439

hPRLr 496  ALSLLPKQRENSGKPKPGTPENNKEYAKVSGVMDNNILVLVPDPHAKNV 545
hGHr 544  QEDIYITTESLTTAAGRPGTGEH------------------VPGSEMPVP 575
hEPOr 440  ISTDYSSGDSQGAQGG---------------------------------------- 455

hPRLr 546  AFEESAKEAPPSEQNQAEXKALANFTATSSKRLQLGLDYLDPARFTH 595
hGHr 576  DYSIHVTQPSQGLILNATALPLPDKEFLSSGVSTDQNLKIMP----- 620
hEPOr 456  --LSDGPYSPYENSLIPAEOPLPPSYVALS--------------------------------- 484

hPRLr 596  SFG  598
hGHr  ---  598
hEPOr  ---  598
Figure 4.6 Multiple cysteine residues in different domains of hPRLr are involved in ligand-free intermolecular disulfide linkages; removal of 12 C-terminal cysteine residues abolishes ligand-free disulfide-mediated hPRLr dimerization

293T cells were transiently transfected with WT hPRLr or various cysteine-to-serine mutants, incubated in the absence of hPRL, and lysed with or without 20 mM IAM. After IP with anti-hPRLr IC domain antibody H-300, hPRLr monomers and dimers were detected by WB on reducing (top) or nonreducing gels (bottom) using anti-hPRLr EC domain antibody 1A2B1. Mock transfections were included as negative controls (lanes 1, 2, 9, and 10). Molecular weight standards are presented to the right of the panels. hPRLr dimers are indicated by red boxes. Lanes 4a, 6a, 12a, and 14a are double-time exposure of lanes 4, 6, 12, and 14, respectively, and are provided to better visualize hPRLr dimers. Data are representative of three independent experiments.
Figure 4.6
Figure 4.7 C184, C225, and C242 participate in ligand-independent intermolecular disulfide bond formation, but the contribution of C184 and C225 requires the presence of Box 1 in the ICD.

293T cells were transiently transfected with WT hPRLr or various truncated forms with cysteine-to-serine mutations, incubated in the absence of hPRL, and lysed in buffer containing 20 mM IAM. hPRLr monomers and dimers were detected by WB on reducing (top) or nonreducing gels (bottom) using anti-hPRLr EC domain antibody 1A2B1. Mock transfections were included as negative controls (lane 1). Molecular weight standards are presented to the right of the panels. hPRLr dimers are indicated by red boxes. Data are representative of three independent experiments.
Figure 4.8 Besides C242, other but not all IC cysteines are involved in ligand-independent intermolecular disulfide linkages

293T cells were transiently transfected with WT hPRLr or various cysteine-to-serine mutants, incubated in the absence of hPRL, and lysed with or without 20 mM IAM. After IP with anti-hPRLr IC domain antibody H-300, hPRLr monomers and dimers were detected by WB on reducing (top) or nonreducing gels (bottom) using anti-hPRLr EC domain antibody 1A2B1. Mock transfections were included as negative controls (lanes 1 and 2). Molecular weight standards are presented to the right of the panels. hPRLr dimers are indicated by red boxes. Data are representative of three independent experiments.
CHAPTER 5

DISULFIDE-LINKED HUMAN PROLACTIN RECEPTOR DIMER IN LIGAND-INDUCED HUMAN PROLACTIN RECEPTOR ACTIVATION

5.1 Abstract

The discovery of ligand-independent dimerization of hPRLr has challenged the classic model for hPRLr activation whereby receptor proximity caused by ligand-induced receptor dimerization triggers downstream activation. However, the role of ligand-independent hPRLr dimers in ligand-induced receptor activation is unclear. We have previously identified a population of covalently linked ligand-free hPRLr dimers that involve intermolecular disulfide bonds. Similar intermolecular disulfide linkages have also been reported for other class 1 cytokine receptors including GHr and EPOr, but significant variations in mechanistic details for disulfide-mediated dimerization depict an inconsistent picture for these receptors. In this study, we investigated the functional role of disulfide-linked hPRLr dimers in hPRLr activation. Ligand stimulation did not affect the formation of disulfide-linked hPRLr dimers. Abolishing the formation of ligand-independent disulfide-linked hPRLr dimers by removing 12 C-terminal cysteines did not impair ligand-induced activation or suppress the speed of activation, indicating that disulfide-linked hPRLr dimers are not required for ligand-induced receptor activation. Furthermore, we demonstrated that WT disulfide-linked hPRLr dimers were not phosphorylated after ligand stimulation. Only hPRLrs that were not covalently linked
became phosphorylated. We conclude that disulfide-linked hPRLr dimers are unlikely to participate in hPRLr activation.

5.2 Introduction

The human prolactin receptor is a member of the class 1 cytokine receptor family, which also includes GHr and EPOr, among others (2). This receptor is devoid of intrinsic kinase activity (17) but associates constitutively with JAK2 via the structural elements in the IC domain including the Box 1 motif (20, 21). Upon ligand binding to hPRLr, JAK2 phosphorylates tyrosine residues in the hPRLr IC domain, which serve as docking sites for STAT5 (7, 22). After being recruited to the hPRLr/JAK2 complex, STAT5 is also phosphorylated by JAK2 and then dissociates from the complex, forming homodimer that translocates to the nucleus, where they activate the transcription of several genes involved in proliferation and lactation by binding to specific DNA sequences (7, 22, 67). Alternatively, other signaling pathways initiated by ligand binding to hPRLr include those activated by Fyn/Src such as the MAPK pathway and the PI3K pathway (22, 78, 80, 85).

X-ray crystallographic structures of the 1:2 ligand/receptor EC domain complex (5, 55, 56) and the obligate ordered reaction of ligand binding with the hPRLr EC domain (50, 51) support the classic mechanism of ligand-dependent receptor dimerization (177). In this mechanism, ligand binding induces the dimerization of hPRLr and the proximity of the induced dimeric receptors triggers downstream signaling. Several lines of evidence from in vivo studies also support this model (61, 178, 183, 184, 186). However, in recent years, hPRLr dimers in the absence of ligand have been reported (16, 190). Ligand-free
hPRLr dimers are inactive in the absence of hormone (16, 190) and may be capable of activation after hPRLr binding. It is currently unclear if lactogenic hormones activate hPRLr by binding through the classic mechanism, or by binding to preformed hPRLr dimers, or perhaps by both mechanisms because hPRLr may exist in dynamic equilibrium between monomers and preformed dimers in the plasma membrane. It has been argued that the speed of hPRLr activation indicates that the preformed dimers are the species to be activated (38), but whether hPRLr activation is slowed or suppressed when the formation of ligand-free hPRLr dimers is abolished has not been further investigated.

In Chapter 4, we have identified a population of ligand-free disulfide-linked hPRLr dimers that involve multiple cysteines from different domains of hPRLr. In this chapter, we examined the functional role of these disulfide-linked hPRLr dimers in receptor activation. The hPRLr mutant C184-592S, where the formation of ligand-free disulfide-linked dimers was abolished, was compared with WT hPRLr regarding the time course of ligand-induced receptor activation. We found that ligand-free disulfide-linked hPRLr dimers were not required for hPRLr activation. Further, in WT hPRLr, only receptors that were not covalently linked became phosphorylated following ligand stimulation, while disulfide-linked dimers were not phosphorylated. These results reveal that disulfide-linked hPRLr dimers are unlikely to participate in hPRLr activation.

5.3 Results

5.3.1 Disulfide-Linked hPRLr Dimers Are Not Required for hPRLr Activation

We first examined if the elimination of disulfide-mediated hPRLr dimers would influence ligand-induced receptor activation. WT or cysteine mutants of hPRLr were
transiently expressed in 293T cells that express endogenous JAK2 kinase. Following a 24 hour serum starvation and a 15 minute treatment with either 100 nM hPRL or control medium, hPRLr phosphorylation was analyzed by IP and WB. Mutations of the two cysteine residues within or close to the TM domain (C184/225S) or the ten cysteine residues in the IC domain (C242-592S) did not eliminate the capability of being activated by hPRL as evidenced by ligand-mediated hPRLr tyrosine phosphorylation on a reducing gel (Figure 5.1, bottom panel, Lanes 6 and 8). Serine mutations of cysteines 184 and 225 in hPRLr only slightly decreased the ligand-induced tyrosine phosphorylation when compared to WT hPRLr, while serine mutations of the ten IC cysteines displayed a phosphorylation level similar to WT hPRLr. More importantly, the hPRLr mutant C184-592S (all 12 C-terminal cysteines were mutated and the formation of ligand-free disulfide-linked dimers was abolished; see Chapter 4) was successfully activated by hPRL treatment (bottom panel, Lane 10) to an intensity above that observed for control medium-treated receptors, suggesting that disulfide-linked hPRLr dimers were not required for ligand-induced receptor activation. Comparison of tyrosine phosphorylation between C184-592S and C242-592S hPRLr mutants confirms a reduced tyrosine phosphorylation resulting from the serine mutation of cysteines 184 and 225.

Since JAK2 is rapidly activated within 30 seconds of ligand stimulation to hPRLr (65, 66, 75), we sought to further test whether preformed disulfide-linked hPRLr dimers facilitate the speed of ligand-induced receptor activation. The activation process of the C184-592S hPRLr mutant was compared to that of WT hPRLr over an extended time course. 293T cells were transiently transfected with WT hPRLr or C184-592S, deprived
of hormones for 24 hours, and then stimulated with 100 nM hPRL for various periods of time: 15 seconds, 30 seconds, 1 minute, 2 minutes, 5 minutes, 10 minutes, 15 minutes, 30 minutes, or 1 hour. hPRLr activation by tyrosine phosphorylation was analyzed by IP and WB. Indeed, WT hPRLr was rapidly phosphorylated in 15 seconds of hormone treatment and the phosphorylation signal lasted for at least 1 hour (Figure 5.2, A). However, the C184-592S hPRLr mutant, where preformed disulfide-linked hPRLr dimers were eliminated, exhibited similar, if not identical, phosphorylation kinetics to that observed for WT hPRLr (Figure 5.2, B). Such results indicate that abolishing disulfide-mediated hPRLr dimerization by removing all 12 C-terminal cysteine residues does not suppress the speed of hPRLr activation. Again, disulfide-linked hPRLr dimers are not required for ligand-induced receptor activation.

**5.3.2 Disulfide-Linked hPRLr Dimers Are Not Phosphorylated after Ligand Stimulation**

Since the elimination of disulfide-linked hPRLr dimers did not eliminate the phosphorylation of hPRLr or affect the speed of activation, we next examined whether the presence of disulfide-linked hPRLr dimers contributes to receptor activation. First, hPRL treatment in 293T cells transfected with WT hPRLr did not change the ratio of monomeric to dimeric receptors when evaluated under nonreducing conditions in the presence of IAM (Figure 5.3, right panel, Lanes 7 and 8), indicating that disulfide-mediated hPRLr dimerization is a constitutive process independent of ligand stimulation.

We then addressed the question of which population of WT hPRLr is activated by ligand binding. WT hPRLr-transfected 293T cells were treated with hPRL, solubilized in lysis buffer containing IAM, and analyzed under reducing or nonreducing conditions.
Under nonreducing conditions, a hPRL-induced increase in tyrosine phosphorylation was only observed for monomeric WT hPRLrs, but not for dimeric WT hPRLrs (Figure 5.4, right panel, Lane 8), when compared to non-stimulated WT hPRLr-transfected cells (right panel, Lane 7). Therefore, disulfide-linked hPRLr dimers are not phosphorylated upon ligand stimulation; only hPRLr species that are not covalently linked become activated by hPRL. It should be cautioned that because the amount of disulfide-linked hPRLr dimers observed in the presence of IAM under nonreducing conditions was low, it is possible that our methods of IP and WB are not sensitive enough to detect a phosphorylation signal from these dimers. Taken together, disulfide-linked hPRLr dimers are unlikely to participate in ligand-induced receptor activation by JAK2.

5.4 Discussion

Disulfide-linked receptor dimers have been reported for members of the class 1 cytokine receptor family such as hGHR (212), rbGHR (213), and mEPOr (211). However, the details for the structural and functional roles of disulfide-mediated dimerization do not provide a consistent picture for these receptors. For example, in the GHR system, disulfide-linked GHR dimers were only detected after ligand binding (212, 213). C241 in the EC domain of rbGHR was solely responsible for the ligand-induced disulfide-mediated receptor dimerization (213). These ligand-induced disulfide-linked GHR dimers are tyrosine phosphorylated during the process of ligand activation (212), but are not required for ligand activation since the removal of C241 in rbGHR did not eliminate tyrosine phosphorylation in the receptor or JAK2 (213). Neither did the inhibition of tyrosine kinase block the formation of ligand-induced intermolecular disulfide linkage.
(212). In other words, ligand-induced disulfide-mediated receptor dimerization and ligand-induced receptor activation appear to be two processes independent of each other in the GHr system. In contrast, disulfide-mediated dimerization in mEPOr has been shown to be constitutive and independent of ligand stimulation (211). These ligand-independent disulfide-linked mEPOr dimers became tyrosine phosphorylated upon ligand stimulation (211). Specific cysteine residues responsible for the ligand-independent disulfide-mediated dimerization of mEPOr were not identified. Therefore, it cannot be determined yet whether the elimination of disulfide-linked mEPOr dimers would allow or prohibit ligand-induced receptor activation.

In this study, our data for hPRLr are different from those for either GHr or EPOr. We have demonstrated that in hPRLr, disulfide-mediated dimerization is a constitutive process. hPRL treatment does not increase or decrease the population of disulfide-linked hPRLr dimers (Figure 5.3). Disulfide-linked hPRLr dimers do not become tyrosine phosphorylated after hPRL stimulation (Figure 5.4). This is consistent with the X-ray crystal structures of ligand-bound hPRLr EC domain, where ligand binding stabilizes the hPRLr EC domain dimers in such an orientation that the distance between the two EC C184 residues is unfavorable for intermolecular disulfide bond formation (55, 56, 175), although the structural studies with the hPRLr EC domain cannot resolve the cysteine residues in the TM and IC domains of hPRLr. We also determine that disulfide-linked hPRLr dimers are not required for ligand-induced activation, because eliminating disulfide-linked hPRLr dimers by removing the 12 C-terminal cysteines did not abolish the capability of hPRLr to be tyrosine phosphorylated upon hPRL treatment (Figure 5.1)
or suppress the speed of ligand-induced phosphorylation (Figure 5.2). Taken together, these results indicate that ligand-independent disulfide-linked hPRLr dimers do not appear to participate in ligand-induced hPRLr activation and that hPRLr has a different mechanism of activation than those for GHR and EPOr.

The inconsistency among the three related members of the class 1 cytokine receptor family regarding the structural and functional roles of disulfide-mediated receptor dimers should not be surprising because comparison of the patterns of the cysteine residues among hPRLr, hGHR, and hEPOr shows minimal homology (Figure 4.5 in Chapter 4). Although the two pairs of intramolecular disulfide bonds in the S1 subdomain of the hPRLr EC domain are conserved in hGHR and hEPOr, hGHR contains an additional intramolecular disulfide bond in its EC domain, while the specific locations of the intramolecular disulfide bonds in hEPOr are not identical to hPRLr. A single unpaired cysteine residue in the EC domain is found in hPRLr, hGHR, and hEPOr, but at different positions. C184 in the hPRLr EC domain is 26 residues from the TM domain, and is close to both the receptor’s half of the Zn$^{2+}$ binding site (D187 and H188) in the ligand/receptor complex (5) and the WSAWS motif (residues 191-195). C240 in hGHR is only four residues from the TM domain, analogous to C241 in rbGHR, therefore is believed to be solely responsible for the formation of hGH-induced disulfide-linked hGHR dimers (213, 227). C182 in hEPOr is 45 residues from the TM domain. The hPRLr TM domain contains a unique cysteine residue (C225) that is not present in either hGHR or hEPOr. The sequence homology of the IC cysteines in these three receptors shows an even greater degree of variation in terms of both the numbers and the positions of
cysteines. This is highlighted by the unique presence of C242 in hPRLr, located between the TM domain and the Box 1 motif. Neither hGhr nor hEPOr contains an analogous cysteine in this region. In addition, the PRLr IC cysteines in human are poorly conserved in other mammalian species.

An expanded examination on disulfide-mediated dimerization in other membrane proteins may provide clues to the function of ligand-independent disulfide-linked hPRLr dimers. Ligand-induced dimerization by the formation of intermolecular disulfide bonds has been reported for tyrosine kinase receptors such as CSF-1R and PDGFR (214, 215). In CSF-1R, it is proposed that ligand-induced disulfide-mediated dimerization serves as a portal to receptor down regulation and leads to receptor dephosphorylation and internalization (214). In hPRLr, are the ligand-independent disulfide-linked dimers locked in an inactive form and waiting to be degraded? Or are they remnants of previous activation processes [hGH-induced disulfide-linked hGhr dimers have been shown to be long lived (212)]? Will an extended period of time of hormone exposure increase the formation of disulfide-linked hPRLr dimers? These questions remain to be addressed. Disulfide-mediated dimerization has also been observed in a leukemogenic membrane glycoprotein (gp55) encoded by Friend spleen focus-forming virus (SFFV) (228, 229). It is suggested that intermolecular disulfide formation controls the folding and processing of gp55 to its mature form and the transportation of gp55 to the plasma membrane (228, 229). gp55 forms diverse and heterogeneous intramolecular and intermolecular disulfide linkages, but only one unique disulfide-linked dimer is able to be processed and transported to the cell surface (228, 229). Notably, gp55 is able to associate with mEPOr
via the TM domain and activate cell proliferation without the stimulation from growth factors (171-173, 230). hPRLr is a glycoprotein (13), and our results demonstrate extensive and heterogeneous intermolecular disulfide formation involving multiple cysteine residues in hPRLr; thus it may be of interest to examine in hPRLr the relationship between disulfide formation and cell surface transportation, especially after our observation that the mutations of C184 and C225 correlate with the formation of a misfolded species of hPRLr, as discussed in Chapter 4. Whether disulfide-mediated dimerization of hPRLr is involved in the down-regulation or the processing of hPRLr remains speculative and further studies are needed to investigate these possibilities.

It is highly probable that hPRLr exists in several species in the plasma membrane: disulfide-linked dimers, noncovalently linked dimers, and monomers, because Western analysis in this study has revealed that disulfide-linked receptor dimers only constitute a fraction of the total receptors (Figures 4.3 and 4.4 in Chapter 4, and Figure 5.3). Furthermore, these species of hPRLr may be in dynamic equilibrium. Although our results indicate that disulfide-linked hPRLr dimers are unlikely to participate in ligand-induced receptor activation, the existence of noncovalently linked hPRLr dimers and their role in the activation process have yet to be determined.

In summary, these data have revealed that disulfide-linked hPRLr dimers are unlikely to participate in ligand-induced receptor activation. The results from this chapter, combined with the results from Chapter 3, suggest the validity of the classic mechanism where hPRLr is activated via ligand-induced dimerization, and illustrate the uniqueness of hPRLr in the class 1 cytokine receptor family.
Figure 5.1 Disulfide-linked hPRLr dimers are not required for ligand-induced receptor activation

293T cells were transiently transfected with WT hPRLr or various cysteine-to-serine mutants and not treated or treated with hPRL (100nM) for 15 minutes. After IP with anti-hPRLr IC domain antibody H300, tyrosine phosphorylation in hPRLr was analyzed by WB on reducing gels using anti-phosphotyrosine antibody 4G10. Mock transfections were included as negative controls (lanes 1 and 2). The top panel shows blotting with anti-hPRLr EC domain antibody 1A2B1 for hPRLr total expression. The bottom panel shows blotting with anti-phosphotyrosine antibody 4G10 for hPRLr activation. Data are representative of three independent experiments.
Figure 5.2 Eliminating disulfide-linked hPRLr dimers does not suppress the speed of ligand-induced receptor activation

293T cells were transiently transfected with WT hPRLr (A) or C184-592S mutant hPRLr (B) and stimulated with hPRL (100nM) for various periods of time as indicated. After IP with anti-hPRLr IC domain antibody H-300, tyrosine phosphorylation in hPRLr was analyzed by WB on reducing gels using anti-phosphotyrosine antibody 4G10. Transfected but nonstimulated 293T cells were included as negative controls (lane 1 in A and B). The top panels show blotting with anti-hPRLr EC domain antibody 1A2B1 for hPRLr total expression. The bottom panels show blotting with anti-phosphotyrosine antibody 4G10 for hPRLr activation. Data are representative of three independent experiments.
Figure 5.3 hPRL activation does not affect the formation of disulfide-linked hPRLr dimers

293T cells were transiently transfected with WT hPRLr, not treated or treated with hPRL (100nM) for 15 minutes, and lysed in buffer containing 20 mM IAM. After IP with anti-hPRLr IC domain antibody H-300, hPRLr monomers and dimers were detected by WB on reducing (left) or nonreducing gels (right) using anti-hPRLr EC domain antibody 1A2B1. Mock transfections were included as negative controls (lanes 1, 2, 5, and 6). Molecular weight standards are presented to the right of the panels. hPRLr dimers are indicated by red boxes. Data are representative of three independent experiments.
Figure 5.4 Disulfide-linked hPRLr dimers are not phosphorylated upon ligand stimulation

293T cells were transiently transfected with WT hPRLr, not treated or treated with hPRL (100nM) for 15 minutes, and lysed in buffer containing 20 mM IAM. After IP with anti-hPRLr IC domain antibody H-300, tyrosine phosphorylation in hPRLr was analyzed by WB on reducing (left) or nonreducing gels (right) using anti-phosphotyrosine antibody 4G10. Mock transfections were included as negative controls (lanes 1, 2, 5, and 6). Molecular weight standards are presented to the right of the panels. Tyrosine phosphorylation in hPRLr is indicated by asterisks (*). Total expression of hPRLr is shown in Figure 5.3. Data are representative of three independent experiments.
CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The discovery of ligand-independent dimerization of hPRLr in 2006 (16, 190) added this protein to the list of the class 1 cytokine receptors that form ligand-independent dimers such as GHr (114) and EPOr (140, 146), and was thought to challenge the classic mechanism of ligand-induced dimerization for hPRLr activation. However, little progress following this finding has been made in the scientific community of hPRLr research during the past few years to establish a new activation mechanism that could replace the classic one, making it intriguing and worthwhile to ruminate over the functional role of ligand-independent hPRLr dimers. In this dissertation, we have systematically evaluated in hPRLr the activation models proposed for other class 1 cytokine receptors that also form ligand-independent dimers, and have further investigated the significance of covalently linked ligand-independent hPRLr dimers in ligand-induced hPRLr activation. We found that 1) a specific relative orientation between the TM and IC domains is not required for hPRLr activation; the rotation or piston model is not supported for hPRLr activation by our data; 2) a population of hPRLr exists as disulfide-linked ligand-free dimers that involve multiple cysteine residues from different domains to form intermolecular disulfide bonds; 3) disulfide-linked hPRLr dimers are not
affected by ligand stimulation in their formation and do not participate in ligand-induced receptor activation.

These results collectively suggest the validity of the classic ligand-induced dimerization mechanism for hPRLr activation. If hPRLr is activated by its ligand through the preformed receptor dimer, then ligand binding in the EC domains of the preformed hPRLr dimer must cause a specific conformational change that is transmitted through the TM domain to the IC domain and subsequently activates the molecular cascade associated with the IC domain. However, our systematic spatial manipulations by alanine insertions at the domain junctions that generate an exhaustive variety of relative orientations of hPRLr domains cannot mimic this presumed conformational change to produce any constitutive activity. Furthermore, in the presence of ligand binding, our glycine insertions between the TM and IC domains would act as increasingly flexible hinges to dissipate any specific conformational change to be transmitted into the IC domain and therefore disengage the IC domain from the EC and TM domain. Our data demonstrate that such glycine hinges cannot disrupt the ability of hPRLr to be activated by its ligand, indicating that a rigid connection between the TM and IC domains, which would have been required in the aforementioned ligand-induced conformational change mechanism, is not necessary for hPRLr activation. Finally, our data on the tyrosine phosphorylation status of monomeric and dimeric hPRLr species provide direct evidence that hPRLr activation does not require or involve preformed disulfide-linked receptor dimers. Ligand binding only activates the species of hPRLrs that are not covalently linked.
Interestingly, the fact that disulfide-linked hPRLr dimers are not active and cannot be activated suggests that although the proximity of the two hPRLrs in a dimer is critical for activation, it is not sufficient. It is therefore highly probable that ligand binding will also induce a permissive orientation between the two hPRLrs in the ligand-stabilized receptor dimer to allow activation. This is supported by our results where increasing the numbers of inserted glycine residues is associated with decreasing hPRLr activities as assessed by tyrosine phosphorylation.

However, this ligand-induced permissive orientation must be relatively forgiving to accommodate the observation that structural alterations by alanine or glycine insertions did not abolish the ligand-induced activity of hPRLr. This may also shed light on the unsolved structure of the hPRLr IC domain. The hPRLr IC domain may exist largely as an intrinsically disordered polypeptide. The results from Chapters 4 and 5 also support this idea. The seemingly extensive formation of intermolecular disulfide bonds among the 10 IC cysteines indicates a high level of solvent exposure to these cysteine residues. The observation that the mutations of all 10 IC cysteines to much more hydrophilic serines did not affect the activation of hPRLr by hPRL further supports the idea that in the native state these IC cysteines are not folded inside a hydrophobic core but are rather extended in the aqueous cytosol. This is also consistent with previous unpublished data from our lab that analysis of the hPRLr IC domain using hydropathy and charge suggests that this domain may contain substantial unstructured regions. The overall structure of the hPRLr IC domain appears to possess a degree of plasticity, and the lack of plasticity in disulfide-
linked hPRLr dimers may cause them to be locked in the inactive state. In this regard, the entire IC domain of mEPOr has also been suggested to act as a long flexible linker (196).

Several questions are raised by the studies in this dissertation. Although disulfide-mediated hPRLr dimers do not participate in ligand-induced receptor activation, they may be involved in the processing and the degradation of hPRLr, as suggested for other receptors and membrane proteins. Biotinylation of surface-expressed hPRLr will reveal initial clues on cellular trafficking of disulfide-linked hPRLr dimers, and centrifugation fractionation will provide finer details of the cellular location of these dimers. Meanwhile, analysis using isotope-labeled hPRL/hPRLr will yield useful information regarding ligand binding and internalization of disulfide-linked hPRLr dimers. Among the 12 C-terminal cysteine residues, C184 and C225 are of particular interest because the serine mutations of these two residues are associated with the enhancement of a misfolded species of hPRLr and the decrease of ligand-induced tyrosine phosphorylation in hPRLr. Comparison between the structure of unliganded hPRLr EC domain, which has yet to be determined, and that of ligand-bound hPRLr EC domain may provide insight on the physical and chemical environments of C184, and separate mutations of C184 and C225 may help dissect the roles between these two residues. In addition, the contribution of C184 and C225 to the formation of disulfide-linked dimers of truncated hPRLr requires the presence of the Box 1 motif in the IC domain. Box 1 is also essential for the constitutive association of JAK2 with hPRLr, suggesting a role of JAK2 in the process of disulfide-linked hPRLr dimer formation. Thus, it would be interesting to examine
disulfide-mediated hPRLr dimerization in a JAK2-deficient cell line or using a full-length hPRLr mutant with a corrupted Box 1 motif.

Besides disulfide-linked receptor dimers, whether another population of hPRLr exists as noncovalently linked preformed dimers awaits investigation. Epitope-tagged hPRLr constructs described in this dissertation can serve as an experiment platform to address this question. CoIP and WB under nonreducing conditions would theoretically differentiate between disulfide-linked and noncovalently linked dimers but should be designed with care because under nonreducing conditions the high molecular weight of antibody used in such experiments may present a challenge in discerning the result signal from the noise background. Alternatively, anti-epitope antibody directly conjugated to resin, nickel resin for His-tag pull-down, or other similar affinity precipitation products can be used to overcome this issue. If such experiments indeed identify a population of noncovalently linked preformed hPRLr dimers, the next logical step is to perform mutagenesis, such as alanine scanning, in the TM domain in order to determine the specific amino acid residues critical for this noncovalent interaction, because the TM domain has been suggested to be responsible for ligand-independent hPRLr dimerization. Finally, by combining the mutations of these critical TM residues and the mutations of the 12 C-terminal cysteine residues, the functional roles of covalently and noncovalently linked hPRLr dimers as well as hPRLr monomers can be fully dissected.

The studies in this dissertation demonstrate the differences of hPRLr from other class 1 cytokine receptors such as GHR and EPOr in their molecular mechanisms. These differences include the model of activation, the involvement of cysteine residues in dimer
formation, the ligand dependency of disulfide-mediated receptor dimerization, and the functional significance of disulfide-linked receptor dimers. Combined with other mechanistic differences reported for this receptor family such as the receptor isoforms, the key interfaces in ligand binding, the dependency on Zn$^{2+}$ in ligand binding, and the phosphorylation sites upon activation, they illustrate the uniqueness of hPRLr in the class 1 cytokine receptor family. The significant contrasts among these related receptors suggest that refined regulation mechanisms have developed during the evolution of this receptor family and remind us as scientists to study these receptors with an unbiased opinion.
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