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Functional Impact of Manipulation on the Relative Orientation of Human Prolactin Receptor Domains

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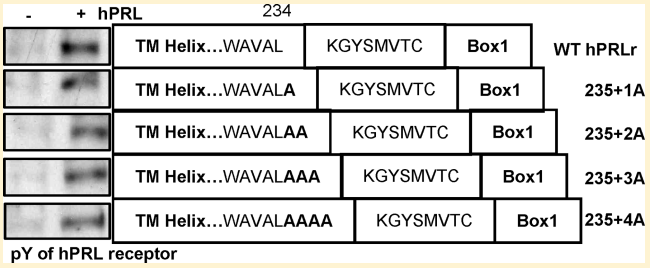
Supporting Information

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 human erythropoietin receptor and human growth hormone receptor and is supported by evidence showing that additions of alanine at the junction of the transmembrane (TM) and intracellular (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 wished to determine if a similar mechanism was at play with human prolactin receptor (hPRLr). Up to four alanines were added between the TM and either the IC or extracellular (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. Wild-type hPRLr or various mutant receptors were expressed in human embryonic kidney 293T cells that express endogenous Janus kinase 2. In the absence of human prolactin (hPRL), none of the alanine or glycine additions increased the level of receptor phosphorylation above that of wild-type hPRLr. In the presence of hPRL, both wild-type 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 wild-type 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.



The human prolactin receptor (hPRLr) is a member of the class 1 cytokine receptor family and shares many similar structural features with other members of this family such as growth hormone receptor (GHr) and erythropoietin receptor (EPOr).[1,2] These structural features include a ligand-binding extracellular (EC) domain that is composed of two β-sheet subdomains (S1 and S2), a single-pass α-helical transmembrane (TM) domain, and a structurally uncharacterized intracellular (IC) domain. Ligand-bound PRLr dimers are associated with receptor activation.[3,4] The JAK2/STAT5 pathway is the principle signaling pathway activated when ligand binds to hPRLr.[2,5,6] The IC domain of hPRLr is devoid of intrinsic enzymatic activity,[7,8] like other members of the cytokine receptor family. The hormonal signal is transmitted within the cell via Janus kinase 2 (JAK2)[9−11] constitutively associated with the highly conserved proline-rich Box 1 motif in the membrane proximal region of the hPRLr IC domain.[12−14] When ligand binds to hPRLr, JAK2 phosphorylates tyrosine residues in the hPRLr IC domain, which serve as docking sites for signal transducer and activator of transcription 5 (STAT5).[15,16] After being recruited to the hPRLr−JAK2 complex, STAT5 is also phosphorylated by JAK2 and then dissociates from the complex, forming homodimers that translocate to the nucleus, where they activate the transcription of several genes involved in proliferation and lactation by binding to specific DNA sequences.[2,5,6]

The proteins capable of activating hPRLr were identified a number of years ago and include hPRL, hGH, human placental lactogen (hPL), and several bivalent monoclonal antibodies,[7,17] 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.[3,4,18] More recently, receptor dimers in the absence of ligand have been found for many class 1 cytokine receptors.[19−24] Some studies indicate that the helical TM domain forms the interface necessary for ligand-independent receptor dimerization,[20,22,24] but the role of these ligand-independent dimers 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

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and/or phosphatases, or are they remnants of the activation process and waiting to be removed by a ubiquitin-mediated mechanism?[25,26] Furthermore, although several recent publications have documented the presence of dimeric hPRLr in the absence of lactogenic hormones,[23,24] monomeric receptors form the majority of receptors as provided by Western analysis.[23] It is unclear if ligand-independent hPRLr dimers are the species activated by ligand binding, if only monomeric receptors are able to be bound and activated by hPRL, or if both mechanisms are available for activation, although it has been argued that the speed of hPRLr activation indicates that preformed dimers are the form to be activated.[27]

Three mechanistic models have been suggested for class 1 cytokine receptor activation. The first model is the scissor model and was proposed on the basis of structural studies of hEPOr.[28] 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 phosphorylation while ligand binding brought the IC domains into sufficient proximity to allow phosphorylation. 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.

The second model is the piston model and was suggested by structural studies of two hGHr EC domains bound by hGH.[29−31] 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 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 hPRLr EC domains supports such a mechanism by showing a similar offset of the C-termini of the hPRLr EC domains.[32] Unfortunately, there are no structure data for dimeric hPRLr in the absence of ligand. Thus, whether the piston action results from hPRL binding or is present in the ligand-independent hPRLr dimerization cannot be determined.

The third and most recent model for receptor activation is the rotation model. 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 hEPOr[33] where selected alanine additions at the TM domain−IC domain junction or within the TM domain impaired the ligand-induced activity of hEPOr in vivo. On the basis of alanine's strong helix-forming character,[34] it was proposed that alanine additions extended the TM helix and induced incremental rotations of approximately 100° in the IC domain relative to the TM helix and that the relative and precise rotations of the two IC domains in the hEPOr dimer were the key step in activation.[33] This model requires an association between the TM domains[20] 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.[33] 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[22,35] 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.[22]

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.

MATERIALS AND METHODS

Construction of the Eukaryotic Expression Vector for Full-Length hPRLr. The full-length hPRLr coding region was amplified from pBluescriptR-hPRLr (Open Biosystems, Huntsville, AL)[36] by polymerase chain reaction (PCR) using the following primers: forward, 5′-GCCAACATGAAGGAAAATGTGGC-3′; and reverse, 5′-GTGAAAGGAGTGTGTAAAACATGCG-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 gene fusion. The YFP gene was subsequently removed between the HpaI and PmeI 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 below). The presence of the desired coding sequence was confirmed by diagnostic restriction enzyme digestion and DNA sequencing. Preparations of the hPRLr plasmid were prepared and stored in water. DNA concentrations were determined by 260 nm absorption.

Site-Directed Mutagenesis. Single or multiple amino acids were added to the coding sequence of hPRLr using to the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the

Table 1. Primers for Site-Directed Mutagenesis

mutagenesis site	amino acid(s) inserted	primer (nucleotide insertions underlined)
between G236 and Y237	+1A	forward, 5′-GTG GCT TTG AAG GGC GCT TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA AGC GCC CTT CAA AGC CAC-3′
	+2A	forward, 5′-GTG GCT TTG AAG GGC GCT GCC TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA GGC AGC GCC CTT CAA AGC CAC-3′
	+3A	forward, 5′-GTG GCT TTG AAG GGC GCT GCC GCT TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA AGC GGC AGC GCC CTT CAA AGC CAC-3′
	+4A	forward, 5′-GTG GCT TTG AAG GGC GCT GCC GCT GCC TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA GGC AGC GGC AGC GCC CTT CAA AGC CAC-3′
between L234 and K235	+1A	forward, 5′-C TGG GCA GTG GCT TTG GCT AAG GGC TAT AGC ATG-3′
		reverse, 5′-CAT GCT ATA GCC CTT AGC CAA AGC CAC TGC CCA G-3′
	+2A	forward, 5′-C TGG GCA GTG GCT TTG GCT GCC AAG GGC TAT AGC ATG-3′
		reverse, 5′-CAT GCT ATA GCC CTT GGC AGC CAA AGC CAC TGC CCA G-3′
	+3A	forward, 5′-C TGG GCA GTG GCT TTG GCT GCC GCT AAG GGC TAT AGC ATG-3′
		reverse, 5′-CAT GCT ATA GCC CTT AGC GGC AGC CAA AGC CAC TGC CCA G-3′
	+4A	forward, 5′-C TGG GCA GTG GCT TTG GCT GCC GCT GCC AAG GGC TAT AGC ATG-3′
		reverse, 5′-CAT GCT ATA GCC CTT GGC AGC GGC AGC CAA AGC CAC TGC CCA G-3′
between D210 and T211	+1A	forward, 5′-GAC TTC ACC ATG AAT GAT GCT ACA ACC GTG TGG ATC-3′
		reverse, 5′-GAT CCA CAC GGT TGT AGC ATC ATT CAT GGT GAA GTC-3′
	+2A	forward, 5′-GAC TTC ACC ATG AAT GAT GCT GCC ACA ACC GTG TGG ATC-3′
		reverse, 5′-GAT CCA CAC GGT TGT GGC AGC ATC ATT CAT GGT GAA GTC-3′
	+3A	forward, 5′-GAC TTC ACC ATG AAT GAT GCT GCC GCT ACA ACC GTG TGG ATC-3′
		reverse, 5′-GAT CCA CAC GGT TGT AGC GGC AGC ATC ATT CAT GGT GAA GTC-3′
	+4A	forward, 5′-GAC TTC ACC ATG AAT GAT GCT GCC GCT GCC ACA ACC GTG TGG ATC-3′
		reverse, 5′-GAT CCA CAC GGT TGT GGC AGC GGC AGC ATC ATT CAT GGT GAA GTC-3′
between G236 and Y237	+1G	forward, 5′-GTG GCT TTG AAG GGC GGA TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA TCC GCC CTT CAA AGC CAC-3′
	+2G	forward, 5′-GTG GCT TTG AAG GGC GGA GGC TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA GCC TCC GCC CTT CAA AGC CAC-3′
	+3G	forward, 5′-GTG GCT TTG AAG GGC GGA GGC GGA TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA TCC GCC TCC GCC CTT CAA AGC CAC-3′
	+4G	forward, 5′-GTG GCT TTG AAG GGC GGA GGC GGA GGC TAT AGC ATG GTG ACC-3′
		reverse, 5′-GGT CAC CAT GCT ATA GCC TCC GCC TCC GCC CTT CAA AGC CAC-3′

pcDNA6.2-hPRLr eukaryotic expression vector whose preparation was characterized above. Forward and complementary reverse primers are listed in Table 1. The expected mutations were verified by DNA sequencing.

Cell Culture and Transfection. Human embryonic kidney 293T cells[37] were obtained from M. D. Lairmore's laboratory (Department of Veterinary Biosciences, The Ohio State University) and maintained in DMEM (Invitrogen) with 10% FBS (Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine (Invitrogen), and antibiotics and cultured in a 5% CO_2 incubator at 37 °C. We demonstrated by reverse transcription PCR (RT-PCR) that 293T cells constitutively express JAK2 but not endogenous hPRLr (see the Supporting Information). Variant hPRLr expression constructs were transfected into 293T cells following the protocol described for 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 DMEM/F-12 (Invitrogen) starvation medium with no FBS and incubated for an additional 24 h. For the hPRL-activated samples, starved cells were then treated with 100 nM hPRL (expressed and purified in our lab),[38] a hormone concentration previously shown to induce rapid hPRLr activation,[13,14,39,40] at 37 °C for 15 min immediately prior to solubilization and whole cell extract collection.

Immunoprecipitation (IP) and Western Blot (WB) Analysis. Transfected hPRL-treated and nontreated 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). Insoluble material was removed by centrifugation at 17900*g* and 4 °C for 10 min. The protein concentration in the whole cell extract was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA);[41] 250 μg of whole cell extract was incubated with 1.5 μg of rabbit polyclonal anti-hPRLr antibody H-300 (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes the IC domain of hPRLr, at 4 °C overnight. The immune complexes were collected on Protein A agarose (Invitrogen) after incubation at 4 °C for 1 h. The immunoprecipitates were resuspended in either reducing or nonreducing sodium dodecyl sulfate-containing electrophoresis loading buffer and boiled for 10 min. Proteins were resolved by electrophoresis on precast 4 to 15% polyacrylamide gels (Bio-Rad) and transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ) at

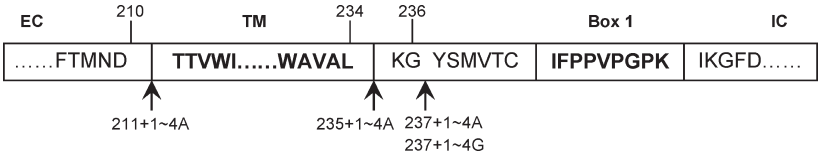

Figure 1. Schematic representation of wild-type hPRLr and variant insertion mutants. The transmembrane (TM) helix sequence and JAK2 binding Box 1 sequence are shown in bold. The amino acid number does not include the N-terminal signal sequence of 24 amino acids. Up to four alanine residues were inserted 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.

100 V and 4 °C for 90 min. 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 appropriate dilutions of primary antibodies: mouse monoclonal anti-hPRLr EC domain antibody 1A2B1 (Invitrogen) at a 1:500 dilution or mouse monoclonal anti-phosphotyrosine antibody 4G10 (Millipore, Billerica, MA) at a 1:1000 dilution. The immune-complexed 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.

RESULTS

Rationale for the Design of hPRLr Mutants. In hGHr and hEPOr 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.[22,33] 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.[22,33] 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.[22] 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.[22,35] Because of the structural and functional similarities between hPRLr and hGHr,[1,2] 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 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).[22] Another set of alanine insertions was placed above the JAK2-binding Box 1 region in the IC domain (Figure 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 hEPOr (five residues upstream from Box 1).[22,33] hPRLr shares little sequence homology with hGHr or hEPOr 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 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.[24] Alanine insertions at similar positions have not been examined in either hGHr or hEPOr. Lastly, up to four glycine residues were inserted above the IC domain (Figure 1), between glycine 236 and tyrosine 237 (237+1−4G). These glycine insertions were placed adjacent to the wild-type G236 and function as 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. We tested each of these receptors for constitutive activity and for hPRL-induced tyrosine phosphorylation of hPRLr, a proximal end point of receptor activation.

hPRLr Mutants Form Ligand-Independent Homodimers Similar to Wild-Type hPRLr. The different hPRLr mutants described above were transiently expressed in 293T cells. IP and WB results showed that these mutants were expressed at a level similar to that of wild-type hPRLr (Figure 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 wild-type hPRLr, as indicated in nonreducing gels (Figure 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.

Rotation of the IC or EC Domain Relative to the TM Helix of the hPRL Receptor Does Not Provide Constitutive Activity But Retains Prolactin-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 wild-type 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 h and treatment for 15 min with either 100 nM hPRL or control medium. Both wild-type and 235+1−4A hPRLrs were expressed in 293T cells at similar levels (Figure 3A, top panel). In the absence of hPRL, none of the hPRLr alanine insertion mutants exhibited significantly higher tyrosine phosphorylation levels than the background observed with wild-type hPRLr (Figure 3A, 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 wild-type hPRLr (Figure 3A, bottom panel). The functional potential of hPRLr was not impaired by the IC domain rotation mediated by alanine addition.

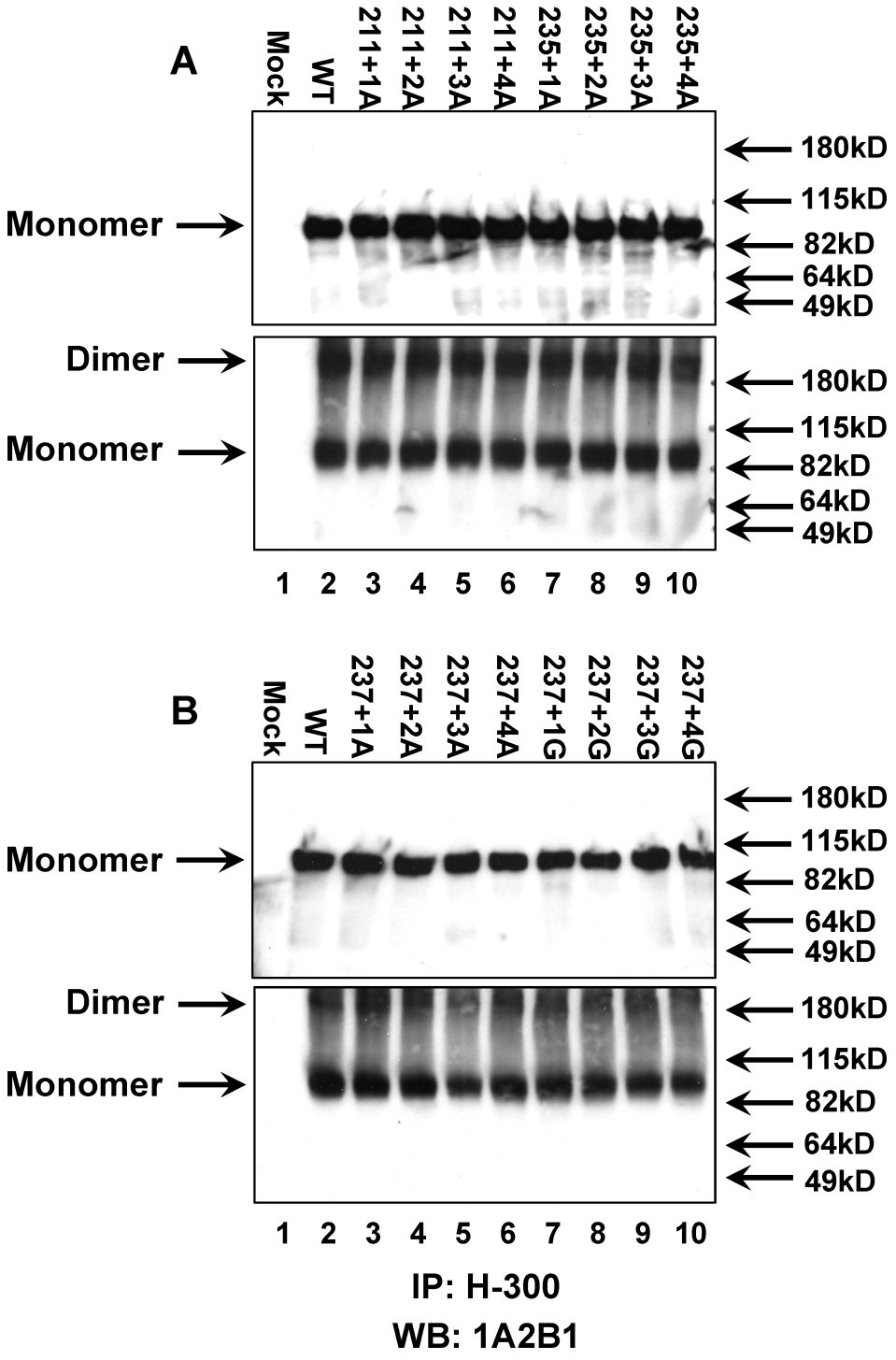

Figure 2. Wild-type and mutant hPRLr form both monomers and ligand-independent homodimers. 293T cells were transiently transfected with wild-type hPRLr (WT) or various insertion mutants (see below) and incubated in the absence of hPRL. hPRLr monomers and dimers were detected by Western blot on reducing 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.

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,[22] no constitutive activity was observed in any of these hPRLr mutants in the absence of ligand (Figure 3B). In addition, these mutants retained the capability of being activated by hPRL, although that capability was weakened by 2−4-fold (Figure 3B). Therefore,

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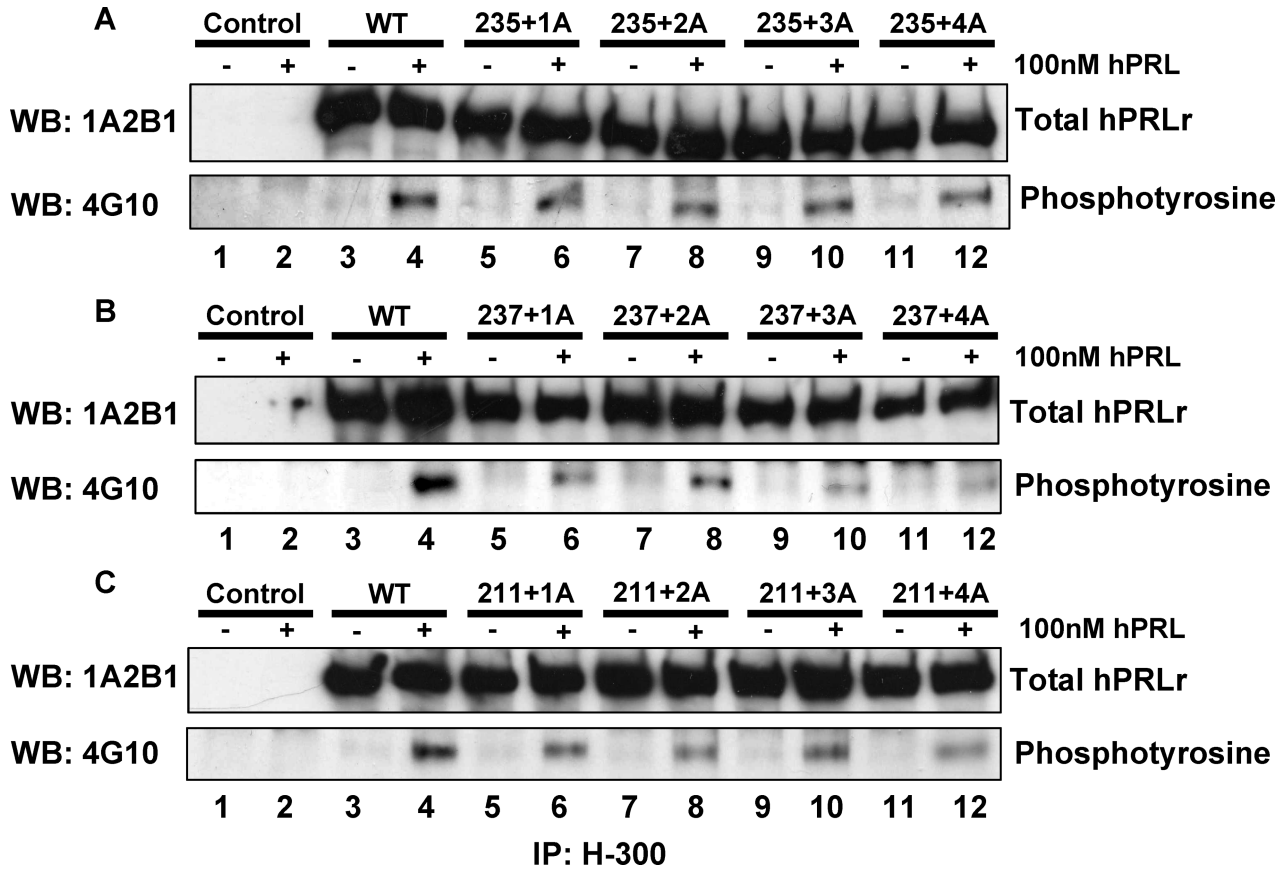

Figure 3. Rotation of hPRLr domains relative to the TM helix does not provide constitutive activity or impair the ligand-induced activity. 293T cells were transiently transfected with wild-type hPRLr (WT) or various alanine insertion mutants (see below) and not treated or treated with hPRL (100 nM) for 15 min. Tyrosine phosphorylation in hPRLr was analyzed by Western blot 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.

rotating the JAK2-binding Box 1 region appeared to have a minimal effect, if any at all, 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.[24] Again, these EC domain-rotating mutants failed to yield constitutively active hPRLr in the absence of hPRL (Figure 3C). Surprisingly, these mutant hPRLrs were successfully activated by hPRL treatment regardless of the presumably significant alteration in the orientation of the ligand-binding EC domain (Figure 3C), although the level of tyrosine phosphorylation of the 211+4A mutant in the presence of hPRL was slightly decreased compared to that in wild-type hPRLr. Taken together, these data do not support rotational action as a mechanism required for hPRLr activation upon ligand binding.

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.[23,24] 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 function as 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 4). Thus, an enhanced ability of the

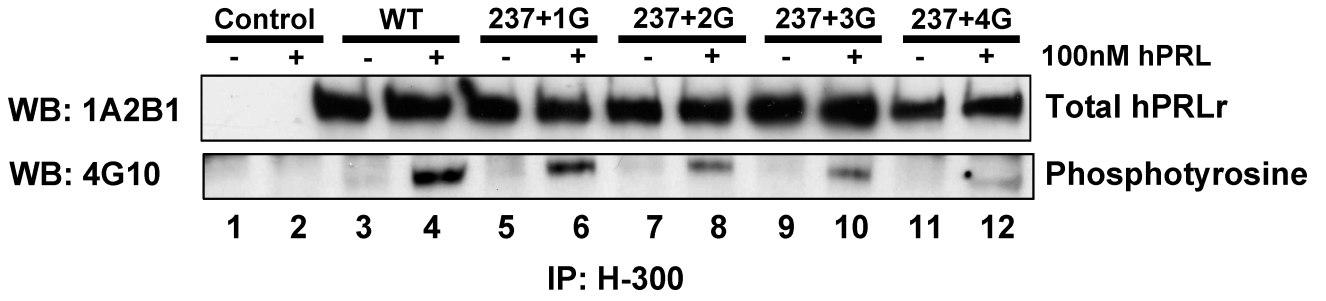

Figure 4. Increased degree of freedom in the movement of the IC domain relative to the TM helix that fails to yield constitutive activity in hPRLr and minimally affects the ligand-induced activity. 293T cells were transiently transfected with wild-type hPRLr (WT) or 237+1−4G mutants and not treated or treated with hPRL (100 nM) for 15 min. Tyrosine phosphorylation in hPRLr was analyzed by Western blot 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.

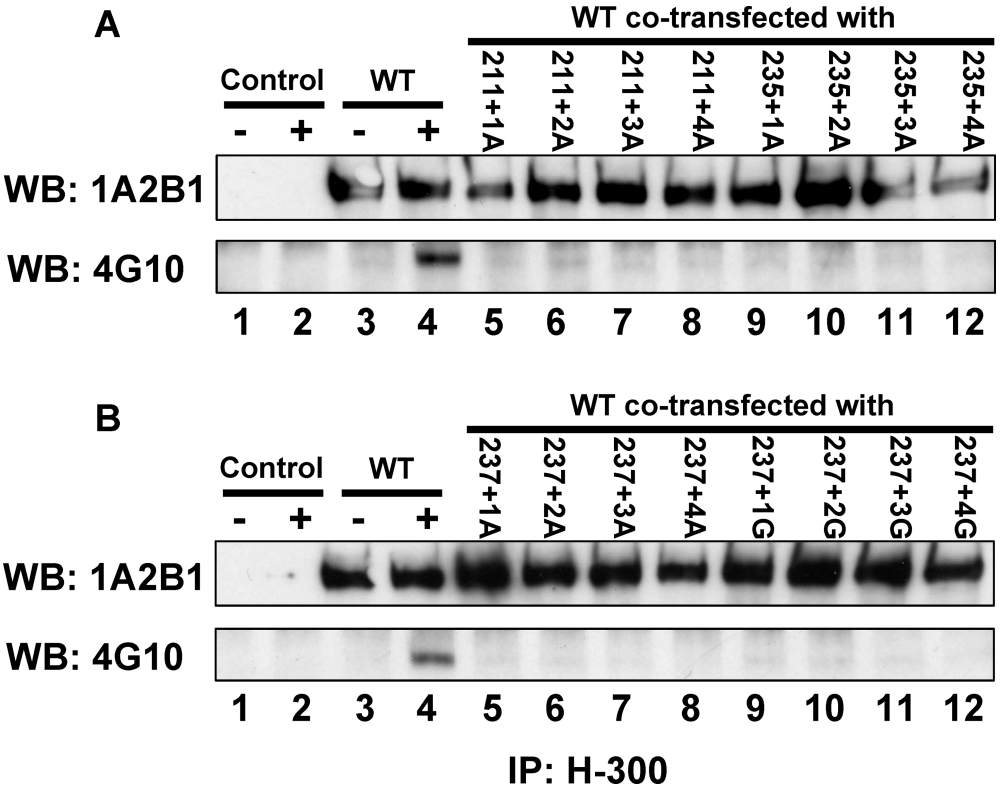

Figure 5. Piston movement of different domains does not result in the constitutive activity of hPRLr. 293T cells were cotransfected with wild-type hPRLr (WT) and various insertion mutants at a 1:1 ratio and incubated in the absence of hPRL. Tyrosine phosphorylation in hPRLr was analyzed by Western blot 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 wild-type hPRLr (WT) 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).

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 significant level above the background observed with the nontreated receptors, although the intensity of tyrosine phosphorylation slightly decreased with increasing numbers of glycine residues inserted (Figure 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.

Heterodimerization of Wild-Type 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. Wild-type 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 wild-type hPRLr. This resembles a piston movement when the mutant hPRLr heterodimerizes with wild-type hPRLr, assuming the insertion mutations do not disturb heterodimer formation (see Discussion). In addition to hPRLr heterodimer formation, there were also homodimers of wild-type hPRLr as well as those of two mutant hPRLrs. As shown in Figures 3 and 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 wild-type hPRLr, or with tyrosine phosphorylation significantly above the background level observed with the nonactivated wild-type hPRLr (Figure 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.

DISCUSSION

The molecular mechanisms by which members of the class 1 cytokine receptor family, including hEPOr, hGHr, and hPRLr, are activated by their ligands have been investigated for a number of years. The ability of hormones and selected bivalent antibodies to create active receptors supports the concept that heterotrimeric complexes are necessary and sufficient for the activation of hEPOr, hGHr, and hPRLr.[7,17,42−44] Physical and structural studies typically using the EC domains of these receptors also support this idea,[18,29,45−49] but not all bivalent antibodies are capable of creating active receptors,[17,43,50] indicating that although the dimerization of the receptors is necessary, it is not sufficient for activation. More recently, the presence of hEPOr, hGHr, and hPRLr dimers in the absence of ligands has been documented.[19−24] These hormone-free receptor dimers are not active.[20,22−24] These data suggest that ligand binding is not required for receptor dimerization but ligand binding guides receptor dimers to conformations associated with activity.[20,22,35] It has been argued that on the basis of the speed of JAK2-mediated hPRLr phosphorylation, preformed hPRLr dimers are required to meet this timeline of ligand activation,[27] but whether hPRLr activation is slowed or abolished when the preformation of hPRLr dimers is suppressed has not been further investigated. Thus, there are few data to support the concept that the actions of hPRL are mediated through preformed hPRLr homodimers.

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, a piston model, and a rotation model. 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. The scissor model was proposed on the basis of several structures of the hEPOr EC domain in which ligand binding produced a scissoring action between the two receptors.[28] The piston model was proposed for hGHr on the basis of the crystallographic heterotrimeric structure of hGHr EC domains bound by hGH where ligand binding created receptors that are off set along the *Z*-axis to the presumed *X*−*Y* plane of the plasma membrane.[29−31] The rotation model was suggested for both hEPOr and hGHr activation. Addition of specific numbers of alanines at the TM domain−IC domain junction of hEPOr or hGHr affected the activities of these receptors.[22,33] It must 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 hGHr and hEPOr, 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.[2,4,42] However, other data do not provide a consistent picture of the details for hEPOr, hGHr, and hPRLr activation. For example, in hEPOr, addition of selected numbers of alanines at the TM domain−IC domain junction impaired EPO-induced receptor tyrosine phosphorylation and cell proliferation but had little effect on JAK2 tyrosine phosphorylation.[33] No constitutive activity for hEPOr was produced by alanine additions.[33] In contrast, a similar study conducted with hGHr[22] 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 hEPOr and hGHr.

We wished 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 domain−TM domain and TM domain−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.[22,33,34] 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 wild-type 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 hEPOr and

hGHr systems,[22,33,35] 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 wild-type 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 wild-type hPRLr (Figure 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[13,14,39,40] (Figure 3A). Equivalent hPRL-stimulated receptor activity for both wild-type 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 preceding the TM domain (235+1−4A), the intensities of hPRL-induced receptor phosphorylation were similar to that in wild-type 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 significant. 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 domain−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 hPRLr EC domains,[32,51,52] 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.[24] However, in this study, alanine additions at the EC domain−TM domain junction did not disrupt the formation of hPRLr dimers in the absence of hPRL (Figure 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 3C). 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 wild-type 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 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 have been reported to vary between ∼2 Å for bacterial chemoreceptors[31−33] and ∼8 Å for hGHr.[22] 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 wild type 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 wild-type 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 wild-type hPRLr. Although this premise remains to be further investigated, given the report that the TM domain mediates the ligand-independent dimerization of hPRLr[24] 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 2), the likelihood that the dimerization between the mutant and wild-type 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.[31,53] 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. hGHr, hEPOr, and hPRLr 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 signaling pathways. Although hGHr, hEPOr, and hPRLr have similar global structures,[1,2] many of the details are significantly different. For example, comparison of the amino acid sequences of either the EC or TM domains among hGHr, hEPOr, and hPRLr shows only modest to minimal sequence homology.[2,4] 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,[23,24] it has been argued that preformed hPRLr dimers are the species activated by ligand binding because of the speed of JAK2-mediated receptor phosphorylation.[27] The classic model in which the proximity of the ligand-induced dimeric receptors triggers activation[4,18] is deemed invalid because these ligand-free preformed hPRLr dimers have been shown to be inactive.[23,24] On the basis of these arguments, 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 at all, 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.

Several studies appear to provide clues about a mechanism that may regulate the activation of hPRLr. They have evaluated the requirements for various portions of the EC domain of hPRLr. Elimination of the first 100 residues of the EC domain (ΔS1) did not produce a constitutively active receptor but did greatly reduce the affinity for hPRL.[54] Removal of residues 11−185 in the EC domain produced a constitutively active receptor but abolished the ability of hPRLr to bind hPRL.[39] Constitutive activity was also obtained after deletion of the S2 subdomain of rabbit PRLr,[55] but when the entire EC domain (residues 1−210) was removed, no constitutive activity was observed and the mutant receptor could not be activated by hPRL.[24] Interestingly, this EC domain-removed hPRLr mutant was reported to enhance the signaling of wild-type hPRLr when present.[24] These results indicate that a structural feature situated between residues 100 and 186 inhibits ligand-independent constitutive activity and that an unrecognized structural feature located between residues 186 and 210 is required for constitutive hPRLr activity. An entire EC domain appears to be necessary for ligand-induced activation of hPRLr. Ligand-independent hPRLr dimerization was not eliminated by the deletion of the entire EC or IC domains.[24] This suggests that the TM domain is the primary focus of ligand-independent hPRLr dimerization.[24] These authors also observed that the EC or IC domains influenced the ligand-independent TM-driven receptor dimerization.[24] Taken together, the portions of hPRLr required for ligand-independent dimerization do not correspond to the structures involved in hPRL binding or those required for constitutive hPRLr activity. Our results indicate that the rigid structural connections among the three domains of hPRLr are not necessary for either hPRLr dimerization or activation.

■ ASSOCIATED CONTENT

Supporting Information. Gel showing 293T cells constitutively express JAK2 but not endogenous hPRLr. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Bazan, J. F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. U.S.A. 87*, 6934–6938.

(2) Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P. A. (1998) Prolactin (PRL) and its receptor: Actions, signal transduction

pathways and phenotypes observed in PRL receptor knockout mice. *Endocr. Rev. 19*, 225–268.

(3) Hooper, K. P., Padmanabhan, R., and Ebner, K. E. (1993) Expression of the extracellular domain of the rat liver prolactin receptor and its interaction with ovine prolactin. *J. Biol. Chem. 268*, 22347–22352.

(4) Goffin, V., and Kelly, P. A. (1997) The prolactin/growth hormone receptor family: Structure/function relationships. *Journal of Mammary Gland Biology and Neoplasia 2*, 7–17.

(5) Clevenger, C. V., and Kline, J. B. (2001) Prolactin receptor signal transduction. *Lupus 10*, 706–718.

(6) Chilton, B. S., and Hewetson, A. (2005) Prolactin and growth hormone signaling. *Curr. Top. Dev. Biol. 68*, 1–23.

(7) Kelly, P. A., Djiane, J., Postel-Vinary, M. C., and Edery, M. (1991) The prolactin/growth hormone receptor family. *Endocr. Rev. 12*, 235–251.

(8) Nagano, M., and Kelly, P. A. (1992) Absence of a putative ATP/GTP binding site in the rat prolactin receptor. *Biochem. Biophys. Res. Commun. 183*, 610–618.

(9) Cambell, G. S., Argetsinger, L. S., Ihle, J. N., Kelly, P. A., Rillema, J. A., and Carter-Su, C. (1994) Activation of JAK2 tyrosine kinase by prolactin receptor in Nb2 cells and mouse mammary gland explants. *Proc. Natl. Acad. Sci. U.S.A. 91*, 5232–5236.

(10) Rui, H., Kirken, R. A., and Farrar, W. L. (1994) Activation of receptor-associated tyrosine kinase JAK2 by prolactin. *J. Biol. Chem. 269*, 5364–5368.

(11) Lebrun, J. J., Ali, S., Sofer, L., Ullrich, A., and Kelly, P. A. (1994) Prolactin-induced proliferation in Nb2 cells involves tyrosine phosphorylation of the prolactin receptor and its associated tyrosine kinase. *J. Biol. Chem. 269*, 14021–14026.

(12) Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) Identification of ten amino acid proline-rich SH3 binding site. *Science 259*, 1157–1161.

(13) Lebrun, J. J., Ali, S., Ullrich, A., and Kelly, P. A. (1995) Proline-rich sequence-mediated JAK2 association to the prolactin receptor is required but not sufficient for signal transduction. *J. Biol. Chem. 270*, 10664–10670.

(14) Pezet, A., Buteau, H., Kelly, P. A., and Edery, M. (1997) The last proline of Box 1 is essential for association with JAK2 and functional activation of the prolactin receptor. *Mol. Cell. Endocrinol. 129*, 199–208.

(15) Wakao, H., Gouilleux, F., and Groner, B. (1994) Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J. 13*, 2182–2191.

(16) Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994) Prolactin induces phosphorylation of Tyr694 of STAT5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J. 13*, 4361–4369.

(17) Rui, H., Lebrun, J. J., Kirken, R. A., Kelly, P. A., and Farrar, W. L. (1994) JAK2 activation and cell proliferation induced by antibody-mediated prolactin receptor dimerization. *Endocrinology 135*, 1299–1306.

(18) Gertler, A., Grosclaude, J., Strasburger, C. J., Nir, S., and Djiane, J. (1996) Real-time kinetic measurements of the interactions between lactogenic hormones and prolactin-receptor extracellular domains from several species support the model of hormone-induced transient receptor dimerization. *J. Biol. Chem. 271*, 24482–24491.

(19) Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1999) Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science 283*, 987–990.

(20) Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H.-S., Henis, Y. I., and Lodish, H. F. (2001) Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *Proc. Natl. Acad. Sci. U.S.A. 98*, 4379–4384.

(21) Gent, J., van Kerkhof, P., Roza, M., Bu, G., and Strous, G. J. (2002) Ligand-independent growth hormone receptor dimerization occurs in the endoplasmic reticulum and is required for ubiquitin system-dependent endocytosis. *Proc. Natl. Acad. Sci. U.S.A. 99*, 9858–9863.

(22) Brown, R. J., Adams, J. J., Pelekanos, R. A., Wan, Y., McKinstry, W. J., Palethorpe, K., Seeber, R. M., Monks, T. A., Eidne, K. A., Parker, M. W., and Waters, M. J. (2005) Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nat. Struct. Mol. Biol. 12*, 814–821.

(23) Qazi, A. M., Tsai-Morris, C-H, and Dufau, M. L. (2006) Ligand-independent homo- and heterodimerization of human prolactin receptor variants: Inhibitory action of the short forms by heterodimerization. *Mol. Endocrinol. 20*, 1912–1923.

(24) Gadd, S. L., and Clevenger, C. V. (2006) Ligand-independent dimerization of the human prolactin receptor isoforms: Functional implications. *Mol. Endocrinol. 20*, 2734–2746.

(25) Li, Y., Kumar, K. G., Tang, W., Spiegelman, V. S., and Fuchs, S. Y. (2004) Negative regulation of prolactin receptor stability and signaling mediated by SCF(β-TrCP) E3 ubiquitin ligase. *Mol. Cell. Biol. 24*, 4038–4048.

(26) Varghese, B., Barriere, H., Carbone, C. J., Banerjee, A., Swaminathan, G., Plotnikov, A., Xu, P., Peng, J., Goffin, V., Lukacs, G. L., and Fuchs, S. Y. (2008) Polyubiquitination of prolactin receptor stimulates its internalization, postinternalization sorting, and degradation via the lysosomal pathway. *Mol. Cell. Biol. 28*, 5275–5287.

(27) Clevenger, C. V., Gadd, S. L., and Zheng, J. (2009) New mechanisms for PRLr action in breast cancer. *Trends Endocrinol. Metab. 20*, 223–229.

(28) Remy, I., Wilson, I. A., and Michnick, S. W. (1999) Erythropoietin receptor activation by a ligand-induced conformation change. *Science 283*, 990–993.

(29) De Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. *Science 255*, 306–312.

(30) Kossiakoff, A. A., Somers, W., Ultsch, M., Andow, K., Muller, Y. A., and De Vos, A. M. (1994) Comparison of the intermediate complexes of human growth hormone bound to the human growth hormone and prolactin receptors. *Protein Sci. 3*, 1697–1704.

(31) Ottemann, K. M., Xiao, W., Shin, Y. K., and Koshland, D. E. (1999) A piston model for transmembrane signaling of the aspartate receptor. *Science 285*, 1751–1754.

(32) van Agthoven, J., Zhang, C., Tallet, E., Raynal, B., Hoos, S., Baron, B., England, P., Goffin, V., and Broutin, I. (2010) Structural characterization of the stem-stem dimerization interface between prolactin receptor chains complexed with the natural hormone. *J. Mol. Biol. 404*, 112–126.

(33) Constantinescu, S. N., Huang, L.-J., Nam, H., and Lodish, H. F. (2001) The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif. *Mol. Cell 7*, 377–385.

(34) Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T. M., and Baldwin, R. L. (1990) Relative helix-forming tendencies of nonpolar amino acids. *Nature 344*, 268–270.

(35) Waters, M. J., Hoang, H. N., Fairlie, D. P., Pelekanos, R. A., and Brown, R. J. (2006) New insights into growth hormone action. *J. Mol. Endocrinol. 36*, 1–7.

(36) Lennon, G. G., Auffray, C., Polymeropoulos, M., and Soares, M. B. (1996) The I.M.A.G.E. consortium: An integrated molecular analysis of genomes and their expression. *Genomics 33*, 151–152.

(37) Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol. 36*, 59–74.

(38) Peterson, F. C., Anderson, P. J., Berliner, L. J., and Brooks, C. L. (1999) Expression, folding, and characterization of small proteins with increasing disulfide complexity by a pT7-7-derived phagemid. *Protein Expression Purif. 15*, 16–23.

(39) Lee, R. C., Walters, J. A., Reyland, M. E., and Anderson, S. M. (1999) Constitutive activation of the prolactin receptor results in the induction of growth factor-independent proliferation and constitutive activation of signaling molecules. *J. Biol. Chem. 274*, 10024–10034.

(40) Neilson, L. M., Zhu, J., Xie, J., Malabarba, M. G., Sakamoto, K., Wagner, K., Kirken, R. A., and Rui, H. (2007) Coactivation of Jak1

positively modulates prolactin-Jak2 signaling in breast cancer: Recruitment of ERK and Stat3 and enhancement of Akt and Stat5a/b pathways. *Mol. Endocrinol. 21*, 2218–2232.

(41) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.

(42) Watowich, S. S., Wu, H., Socolovsky, M., Klingmuller, U., Constantinescu, S. N., and Lodish, H. F. (1996) Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annu. Rev. Cell Dev. Biol. 12*, 91–128.

(43) Elliott, S., Lorenzini, T., Yanagihara, D., Chang, D., and Elliott, G. (1996) Activation of the erythropoietin (EPO) receptor by bivalent anti-EPO receptor antibodies. *J. Biol. Chem. 271*, 24691–24697.

(44) Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V., and Wells, J. A. (1992) Rational design of potent antagonists to the human growth hormone receptor. *Science 256*, 1677–1680.

(45) Yoshimura, A., Longmore, G., and Lodish, H. F. (1990) Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. *Nature 348*, 647–649.

(46) Watowich, S. S., Yoshimura, A., Longmore, G. D., Hilton, D. J., Yoshimura, Y., and Lodish, H. F. (1992) Homodimerization and constitutive activation of the erythropoietin receptor. *Proc. Natl. Acad. Sci. U.S.A. 89*, 2140–2144.

(47) Watowich, S. S., Hilton, D. J., and Lodish, H. F. (1994) Activation and inhibition of erythropoietin receptor function: Role of receptor dimerization. *Mol. Cell. Biol. 14*, 3535–3549.

(48) Cunningham, B. C., Ultsch, M., De Vos, A. M., Mulkerrin, M. G., Clauser, K. R., and Wells, J. A. (1991) Dimerization of the Extracellular Domain of the Human Growth Hormone Receptor by a Single Hormone Molecule. *Science 254*, 821–825.

(49) Sivaprasad, U., Canfield, J. M., and Brooks, C. L. (2004) Mechanism for ordered receptor binding by human prolactin. *Biochemistry 43*, 13755–13765.

(50) Rowlinson, S. W., Behncken, S. N., Rowland, J. E., Clarkson, R. W., Strasburger, C. J., Wu, Z., Baumbach, W., and Waters, M. J. (1998) Activation of chimeric and full-length growth hormone receptors by growth hormone receptor monoclonal antibodies. A specific conformational change may be required for full-length receptor signaling. *J. Biol. Chem. 273*, 5307–5314.

(51) Somers, W., Ultsch, M., De Vos, A. M., and Kossiakoff, A. A. (1994) The X-ray structure of a growth hormone-prolactin receptor complex. *Nature 372*, 478–481.

(52) Broutin, I., Jomain, J. B., Tallet, E., van Agthoven, J., Raynal, B., Hoos, S., Kragelund, B. B., Kelly, P. A., Ducruix, A., England, P., and Goffin, V. (2010) Crystal structure of an affinity-matured prolactin complexed to its dimerized receptor reveals the topology of hormone binding site 2. *J. Biol. Chem. 285*, 8422–8433.

(53) Falke, J. J., and Hazelbauer, G. L. (2001) Transmembrane signaling in bacterial chemoreceptors. *Trends Biochem. Sci. 26*, 257–265.

(54) Kline, J. B., Rycyzyn, M. A., and Clevenger, C. V. (2002) Characterization of a novel and functional human prolactin receptor isoform (deltaS1PRLr) containing only one extracellular fibronectin-like domain. *Mol. Endocrinol. 16*, 2310–2322.

(55) Gourdou, I., Gabou, L., Paly, J., Kermabon, A. Y., Belair, L., and Djiane, J. (1996) Development of a constitutively active mutant form of the prolactin receptor, a member of the cytokine receptor family. *Mol. Endocrinol. 10*, 45–56.