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Review

Prolactin Receptor Antagonists

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Most prolactin (PRL) in the circulation is produced by the pituitary. However, a wide variety of traditional target tissues of the hormone have also been shown to produce their own prolactin. The amount produced per cell is low, but may well be sufficient for autocrine/paracrine activity. Although dopamine agonists allow one to study the target tissue effects of pituitary PRL, other agents, such as PRL receptor (PRLR) antagonists, are needed to analyze autocrine/paracrine loops. With PRLR antagonists, it should be possible to dissect out the role of extrapituitary prolactin in both the normal physiology, and the pathophysiology of various tissues. In tissues where the locally produced PRL may promote disease, such antagonists have the potential to be important therapeutics.

This article briefly, but critically, reviews current understanding of PRL–receptor interactions and initial signaling, and describes the development of both growth hormone (GH) and PRL antagonists within that context. In the final section, results with a very potent PRL antagonist further one theme of the article, which is whether the simple receptor dimerization model explains all signal transduction following PRLR binding.

Key Words: Prolactin antagonist; prolactin receptor; prolactin–receptor interaction; signaling.

Introduction

Growth hormone (GH) and prolactin (PRL) receptor (R) antagonists are excellent tools for analysis of the function of GH and PRL in experimental systems. They also have potential as pharmaceuticals in a wide variety of diseases promoted by centrally or locally produced GH and PRL (e.g., *1–4*). This article highlights the development of such antagonists and, at the same time, critically analyzes the current view of PRLR signaling. The article begins with a brief description of PRL–receptor interactions and initial signaling events. This cannot be accomplished without frequent reference to GH–receptor interactions. Next discussed is the development and proposed mode of action of GH antagonists. This is followed by a description of PRL antagonists, developed by analogy to GH antagonists. In the final section, antagonists that mimic the natural antagonist, phosphorylated PRL, are described. The properties of one of these lead to questions concerning some aspects of the current model of PRLR signaling. The purposes of the article are to collect information about the antagonists into one article and to stimulate inquiry that moves us beyond what has been a very useful, but simplistic model of PRL–receptor interactions.

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The Cytokine Family

PRL and GH belong to a family of cytokines that share both ligand and receptor structural similarities. The common structure of the ligands is a four α-helix bundle, which interacts with more than one receptor/receptor subunit through nonsymmetrical sites. The receptors in this family include GH and PRL, erythropoietin, interleukin 2 (β- and γ-subunits), interleukin 3 (α- and β-subunits), interleukin 4 (α-subunit), interleukin 7, interleukin 9, gp130 (a β-subunit for several receptors), thrombopoietin, ciliary neurotrophic factor, leukemia inhibitory factor, granulocyte-stimulatory factor, and granulocyte-macrophage colony-stimulating factor *(5,6)*. The receptors/receptor subunits are single-transmembrane domain proteins, characterized by four positionally conserved cysteines and one tryptophan and an additional characteristic sequence, Trp-Ser-X-Trp-Ser *(5,6)*. The role of this sequence is unknown, apparently not participating (at least for GH) in either receptor–receptor or hormone–receptor interactions *(7)*. Since the receptor/receptor subunits have no intrinsic tyrosine kinase activity, cytokine effect involves ligand binding to two or more receptors/receptor subunits and interaction of these complexes with one or more signal transducing proteins.

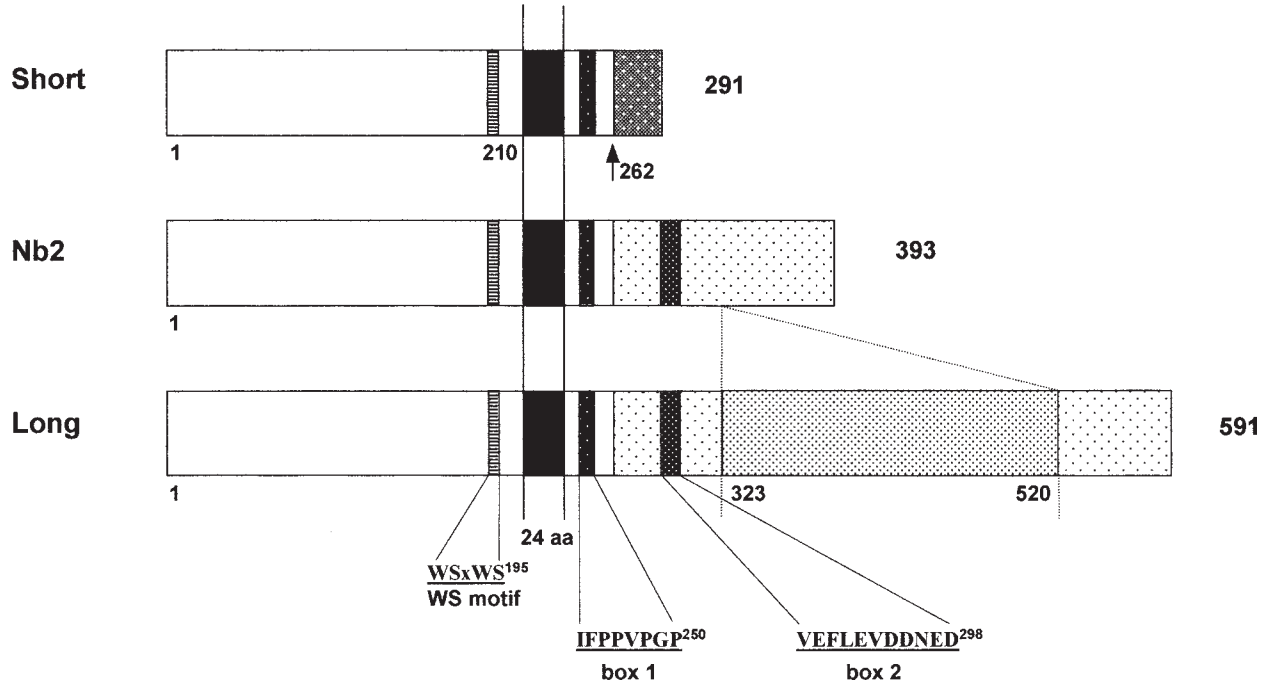

Fig. 1. Schematic structure of the rat PRLRs. Identified are the short, Nb2 (intermediate), and long forms of the rat PRLR. The extracellular (ligand binding) domain is to the left, the transmembrane domain (24 amino acids [aa] in length) is in black, and the cytoplasmic domain is to the right. Numbers without arrows or dotted lines designate the first and last aa of the extracellular domain and the last aa of the mature receptor. Identical regions are represented by the same shading, with the exception of T^{324} in the Nb2 form, which is M^{522} in the long form. The arrow at 262 represents the site of alternative splicing to produce a 30 aa unique cytoplasmic region in the short receptor after the first 27 common cytoplasmic aas. The dotted lines between the Nb2 and long form indicate the 198 aa deletion, which occurs to produce the Nb2 PRLR, beginning with aa #323 and ending at aa #520 with respect to the long form of PRLR. Below the long form PRLR, the WS motif, box 1, and box 2 are designated with the last aa numbered. Box 1 and box 2 were first observed and named by Murakami et al. *(83)* and further delineated for the PRLR and the cytokine receptor superfamily members by O'Neal and Yu-Lee (*20*; proline-rich motif, box 1). The box 2 sequence here is adapted from He et al. *(84)*.

Current View of PRL–Receptor Interactions and Signaling

The PRLR

Three versions of the PRLR have been described in rat tissues: a short form of 291 amino acids *(8)*, a long form of 591 amino acids *(9)* and a 393 amino acid form, found so far only in Nb2 T lymphoma cells, designated the intermediate form *(10)*. Each has an identical extracellular domain and differs in the cytoplasmic region as a result of alternate splicing of a single gene product *(11)* (Fig. 1).

In all normal rat tissues expressing the PRLR, both long and short forms are present and, with the exception of the liver, the long form predominates or is equally expressed with the short *(12)*. A long and short form of the receptor have been described in several species, including the human *(13–16)*. However, the distribution of long and short forms may differ somewhat in different species *(13–16)*.

Signaling

Homodimerization of the long and intermediate forms of the PRLR is thought to result from PRL binding *(17)* and to result in activation of a constitutively associated tyrosine kinase, Jak 2 *(17)* (Fig. 2). This activation of Jak 2 is followed by tyrosine phosphorylation of the PRLR (tyrosine phosphorylation of the short receptor does not occur) *(18)*, although it is currently unclear whether Jak 2 is directly responsible. Tyrosine phosphorylation of the receptor is a step thought to be required for recruitment of the transcription factor, Stat 5, which is then activated by Jak 2 phosphorylation (Fig. 2). There are conflicting results, however, concerning the importance of receptor phosphorylation in the recruitment of Stat 5. These are well summarized by Goupille et al. *(19)* whose own study concludes that there are three regions of the cytoplasmic portion of the receptor involved in PRL signaling to milk protein genes. The first of these is box 1, which contains the most crucial domain for interaction with Jak 2. This domain is present in all three forms of the receptor *(20,21)* (Fig. 1). A second domain in the box 2 region appears necessary for Stat 5 activation. This is present in only the long and intermediate forms. A third domain, toward the C-terminus of the receptor, contains a tyrosine phosphorylation site that appears to be involved in amplification of the signal *(19)*. Tyrosine phosphorylation of this site, however, is not an absolute requirement for Stat 5 activation *(19)*.

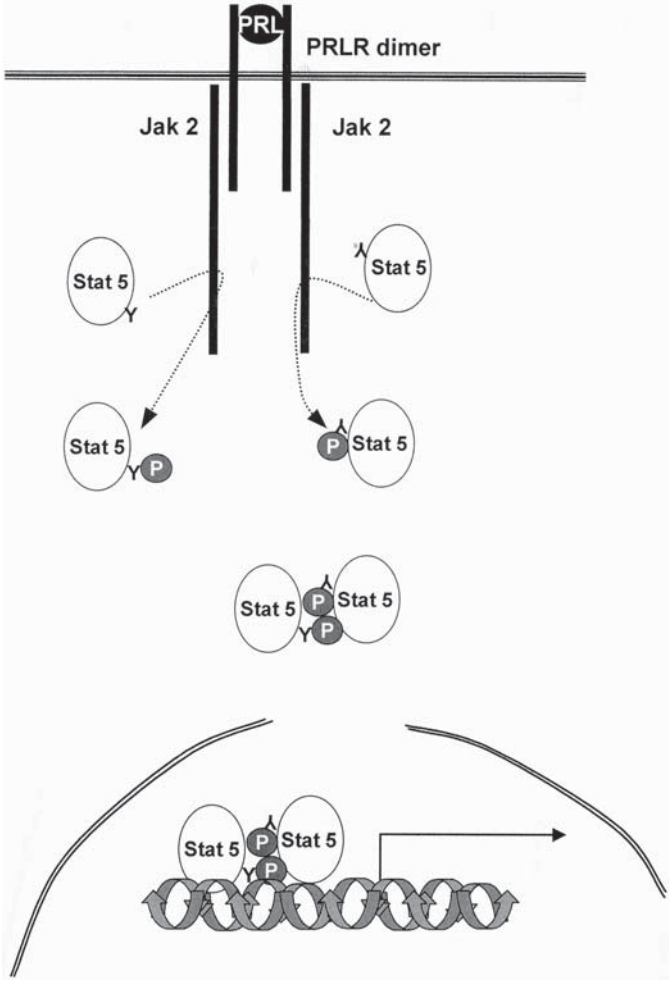

Fig. 2. This shows the current view of PRL–receptor interactions and signaling through the Jak 2-Stat 5 pathway. PRLRs are dimerized by PRL. This results in Jak 2 transphosphorylation events, which activate the C-terminal kinase domains. Phosphorylation of the receptor, probably by Jak 2, increases the efficiency of Stat 5 recruitment to the receptor–Jak 2 complex, ultimately resulting in Stat 5 phosphorylation. Phosphorylated Stat 5 molecules dimerize and are transported into the nucleus, where they bind to specific regions of DNA and regulate gene transcription.

Receptor Dimerization

The current model holds that PRL in solution binds first to one receptor and then recruits a second from the surrounding area of membrane. This is believed, by analogy to other cytokine signaling, to bring two Jak 2 molecules in close proximity to one another, causing transphosphorylation and therefore activation *(22)*. This model is based in part on analogy to GH–receptor interactions and in part on experimental data. Both GH *(23)* and, recently, placental lactogen (PL) *(24)* have been crystallized with the extracellular domains (ECD) of the growth hormone receptor (GHR) and PRLR, respectively. These crystals demonstrated the presence of one ligand–two receptor complexes. No one has yet crystallized PRL with the PRLR. However, a number of studies have demonstrated PRL-like activity of bivalent, but not monovalent, anti-PRLR antibodies *(25)* and a 1:2 stoichiometry between PRL and the ECD of the PRLR in solution has been reported *(26,27)* when hormone and receptor complexes from heterologous species are used. Such heterologous complexes have a slower dissociation rate for the second receptor than homologous ligand–receptor complexes *(28)* and, therefore, the 1:2 stoichiometry is more stable and more easily detected.

In addition, under circumstances where bivalent anti-PRLR antibodies have been shown to both activate Jak 2 and induce Nb2 cell proliferation, monovalent anti-PRLR Fab fragments have been shown to be inactive. Their activity was 40% restored by crosslinking with bivalent anti-Fab antibodies *(17)*.

Consistent with the binding of one PRL to two receptors are biphasic dose–response curves, which have long been a hallmark of PRL endocrinology (e.g., *29*). Biphasic dose–response curves are correlated to one ligand–two receptor complexes, being the active combination as illustrated in Fig. 3. Thus, when the maximal number of two receptor–one ligand complexes are formed, a maximal response is obtained. At higher ligand concentrations, more one receptor–one ligand complexes are formed, reducing the cell response. The defining assumptions of this model are that receptor dimers are necessary for signal transduction and that the ligand–first receptor affinity is a little higher than second receptor affinity for already bound ligand. This latter explains the conversion of 1:2 complexes to 1:1 complexes with increasing ligand concentration.

Using experimental systems in which receptors are overexpressed, biphasic dose–response curves have proven difficult to demonstrate. This is in large part because ligand:receptor ratios of 1:2 must be exceeded before self-antagonism is evident. If receptors are highly overexpressed, the measured cell-response parameter may be limiting and maximal through many orders of magnitude increases in ligand before self-antagonism is evident. Using a relatively low number of Nb2 cells/mL, Rui et al. were able to demonstrate self-antagonism with their most mitogenic antireceptor antibody, T6, at about 1000-fold the dose that produced a mitogenic response *(17)*. Using even fewer cells per milliliter, such that the number of receptors, and not cell proliferation, was limiting, we have recently demonstrated self-antagonism within a 10-fold range of mitogenic response *(30)*.

Direct evidence of the existence of one PRL–two receptor complexes in cells was first obtained in crosslinking studies by Rui et al. *(31)*, although the identity of the appropriate gel band was not confirmed. Recently, Sakal et al. have produced evidence that ovine PL makes a one ligand–two receptor complex with the rat PRL receptors on Nb2 cells *(32)*. What is additionally of interest in the study is the reported presence of receptor dimers in the absence of ligand *(32)*.

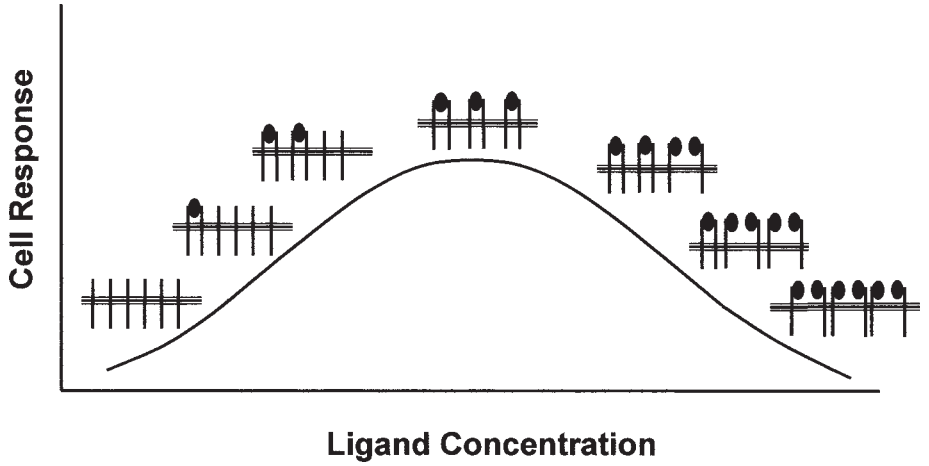

Fig. 3. Diagrammatic representation of agonism and self-antagonism in a system requiring receptor dimerization for biological activity.

Is Dimerization Enough?

The preceding section has illustrated that there is significant evidence in favor of one ligand–two receptor complexes being the active species. It may not be true, however, that ligand initiates dimerization, nor that dimerization alone is sufficient to activate signal transduction. Instead, ligand–receptor interactions may induce conformational changes in the two receptor molecules that are transmitted through the intramembranous region to the intracellular domains. Given the flexibility of the receptor close to the membrane, the most effective transmembrane transmission might be a twist of the transmembrane amino acids, thereby bringing two associated Jak 2 molecules in the intracellular region into the most favorable position for transphosphorylation. The Nb2 form of the receptor has been reported to have a higher affinity for PRL than the long receptor *(33)*. Since the extracellular domains of the long and Nb2 forms are identical, this suggests an effect of the intracellular domain, or an intracellular domain-associated protein, on conformation of the extracellular domain. Further, mutants lacking 55 of the 57 intracellular amino acids showed a four- to fivefold increase in affinity for PRL *(34)*, and the PRL binding protein (soluble ECD of the receptor) found in rabbit milk has a 10-fold greater affinity *(35)*. If, as these results demonstrate, the intracellular domain can affect the conformation of the extracellular domain, it seems reasonable to suggest that hormone binding to the extracellular domain can affect the conformation of the intracellular domains and, hence, signal transduction.

Until now, the discussion has considered the PRL–receptor complex a stable one, being undone only by additional ligand. There is evidence, however, for rapid on–off reactions between the complex and second receptor *(32)*. Thus, it is possible that multiple second receptors with their associated Jak 2 can interact with the first receptor–PRL complex, thereby amplifying the signal. Since Shiu et al. *(36)* demonstrated that 30% receptor occupancy (in 1983 they assumed a 1:1 complex) produced a maximal growth response in Nb2 cells, this would, after dimerization, leave one spare receptor per two involved in the active complex. This spare receptor could potentially exchange places with the second receptor.

Jak 2

All three forms of the receptor have been shown to associate with Jak 2, and PRL binding to all forms results in Jak 2 phosphorylation *(18)*. All three forms have been shown to promote cell proliferation *(37,38)*, although the short form of the receptor probably uses a somewhat different signaling route *(18,19,38)*. Only the long and intermediate forms can activate Stat 5-mediated β-casein gene transcription *(39)*.

Different regions of the Jak 2 molecule can activate different signaling pathways, which result in the activation of different genes *(40)*. Thus, Stat 5 signaling is only one consequence of Jak 2 activation.

Role of the Short Receptor

When the short form of the receptor is cotransfected with the long form and a β-casein reporter construct into a recipient mammalian cell, the short form of the receptor inhibits PRL activation of the β-casein gene promoter *(41)*. Thus, it has been suggested that the short form of the receptor, by heterodimerization to the long form, contributes to the decline in signaling to β-casein in the second half of lactation *(41)*. This purely downregulating role of the short form, however, seems at odds with previous reports of a two-fold induction of the short form between day 20 of pregnancy and day 7 of lactation in rats *(12)*. Induction of the short form during early, rather than late, lactation may imply an entirely different role for short-form homo- rather than heterodimers and a mechanism within the normal, polarized mammary epithelial cell for regulating receptor homo- and heterodimerization.

In the corpus luteum, Duan et al. *(42)* recently identified a molecule that associates with the short form of the PRLR. Although prolactin receptor-associated protein (PRAP) itself is specific to the corpus luteum, there may be similar short receptor-associated proteins in other tissues, each of which may play a role in short receptor signaling. The presence or absence of particular PRAPs may account for the ability of the short receptor to mediate proliferation when transfected into some, but not other cell types *(37,38)*.

With a second protein implicated in the functioning of the short receptor, it is important to re-emphasize that most analyses of short receptor function have been performed in transfection systems where expression of this second protein may or may not occur. It remains possible, therefore, that more signaling through the short form occurs in natural tissues. To date, whole animal studies that address this issue have revealed that PRL treatment leads to both Jak 2 and PRLR phosphorylation in mammary gland, where the long receptor exceeds the short by 10:1, but not in the liver where the ratio is reversed *(43)*. Whether this is evidence of lack of signaling or just alternate signaling pathways for the short form remains to be determined.

GH Antagonists

Prior to the crystallization of GH with the extracellular domain of the GHR, mutagenesis studies had defined two sites on the GH molecule that were important for binding to receptors *(44)*. To find amino acids involved in binding to the first receptor, the results of mutagenesis on binding were analyzed by monoclonal antibody (MAb) precipitation of one ligand–one receptor complexes *(44,45)*. To recognize those involved in binding the ligand–receptor complex to a second receptor, a fluorescence quenching approach was used. In this instance, the fluorescent tag was placed near the C-terminus of the receptor ECD and production of a one GH–two receptor ECD complex reduced fluorescence *(45)*. The crystal data, however, allowed full appreciation of these results and suggested further mutagenic analyses of the amino acids, which became buried at each contact site. What became clear from these continued studies was that dimerization of the receptors by GH was a sequential process, i.e., that GH reacts first through site 1 and then through site 2. The rationale for this statement is that mutations at site 2 do not affect the ability of GH to form 1:1 GH–receptor complexes, whereas mutations at site 1 do *(46)*. Further studies established that binding of the second receptor involved not only site 2 regions on the GH, but also a region of the first receptor *(23)*.

Because the receptor uses essentially the same region to bind to either site 1 or 2 on GH and, knowing that sites 1 and 2 are not symmetrical, one can appreciate why the two receptor molecules in the complex are nonsymmetrical. They are close in the GH–GHR ECD complex, but to superimpose one GHR with the second in the complex, it is necessary to rotate by 159°, not 180°, and to translate by 8 Å *(23)*. Thus, simplistic diagrams, such as Fig. 2, showing bilaterally symmetrical receptors have their uses, but they omit a potentially important part of signal transduction and that is the conformational change in the receptor(s) on ligand binding. Recent work on the crystal structure of PL-PRLR ECD dimers shows a greater angle in one receptor in the complex. For the first receptor, there are approx 108° between the N- and C-terminus. For the second receptor, there are 114°. There is also a twist of the C-terminal domain of the ECD with respect to the N-terminus (Elkins et al., personal communication). Thus, there is evidence of conformational changes in the PRLR that could be transmitted to the cytoplasmic domain.

Sequential dimerization in the GH–receptor interaction suggested to investigators that GH mutants, which prevented the binding of a second receptor, could be GH antagonists. One mutant in particular (G120R), first described by Chen et al. *(47)* and independently described by Fuh et al. *(48)*, has proven to be very potent, and a modified form with additional site 1 mutations to increase affinity is currently in clinical trials for use in acromegaly (Sensus Corporation, Austin, TX, 1997). Attempts to demonstrate the formation of 1:2 GH–receptor complexes with wild-type GH and 1:1 GH–receptor complexes with the G120R mutant in intact cells were, however, confounded by an inability to show anything but 1:1 complexes with either form of the hormone *(49)*. Crystallization studies, however, produced 1:1 complexes between G120R and the ECD of the receptor *(50)*. Interestingly, there is evidence that treatment of cells with the G120R mutant can result in the phosphorylation of a molecule with a molecular weight consistent with that of Jak 2 *(49)*, but not in the phosphorylation of a molecule consistent with the molecular weight of Stat 5. Thus, G120R may signal, but the signaling does not result in Stat 5 activation.

Development of PRL Antagonists Based on GH Antagonists

Human GH can bind to the PRLR, and this binding is increased 8000-fold for human GH–human PRLR interactions by the addition of 50 μ*M* Zn^{2+}. By contrast, neither the human GH–human GHR nor the human PRL–human PRLR interactions require Zn^{2+} *(51)*. If signaling through the PRLR involves receptor dimerization and the G120R mutant of GH prevents dimerization, then as Fuh et al. predicted and found, the G120R mutant of human GH should serve as a PRLR antagonist in the presence of Zn^{2+} *(52)*. Proliferation of Nb2 cells in response to 1 n*M* human PRL was inhibited 50% by 30 n*M* G120R GH in the presence of 10 μ*M* added $ZnSO_4$. This mutant was also effective at inhibiting the growth of breast cancer cells *(2)*. These early studies of PRLR antagonists led to PRL-based versions that did not require Zn^{2+} addition.

The human PRL residue equivalent to G120 in GH is G129. Mutation of the site by Goffin et al. produced a PRL with only weak agonist and weak antagonist activity in the Nb2 bioassay *(53)*. Based on this and other results presented, these authors suggested at the time that the binding of the second receptor has a higher affinity in the PRL vs GH system. Later studies, however, suggest that heterologous hormone–receptor interactions (i.e., hormone and receptor from different species) can vary significantly from homologous ones. Thus, when the G129R mutant of human PRL was tested using human receptors, it showed no agonist activity and proved to be a much more effective antagonist than was appreciated using the Nb2 assay *(54)*. The same was true for a second site 2 mutant, A22W, although it was not as effective an antagonist as G129R. By direct comparison with rat receptors transfected under equivalent circumstances, it appears that rat receptors bind more tightly to site 2 regions on human PRL than rat PRL *(54)*.

An interesting study by Gertler et al. *(28)* has investigated the interspecies phenomenon using a BIAcore system. By analyzing the interactions between the ECD of either the rabbit, bovine, or rat receptor and ovine, rabbit, or rat PRL, or human GH, they concluded that species-homologous systems had a much faster dissociation rate for the second receptor. Species-heterologous systems were more stable in a two receptor–one ligand complex.

One important lesson to be learned from these and other analyses, elegantly reviewed by Goffin et al. *(55)*, is that binding, and even activation, measured with heterologous hormone preparations may not reflect binding and activation with homologous hormone.

Other mutations in human GH have also led to antagonists of GH and PRL activity. A deletion mutant in which the N-terminal 13 amino acids of GH are missing blocks the lactogenic activities and Nb2 cell proliferative activities of ovine PRL and human GH *(56)*. Fifty percent inhibition of Nb2 cell proliferation in response to 2 ng/mL ovine PRL was achieved at 10 μg/mL. Although this antagonist requires high doses to be effective, its mechanism of action is likely very similar to the G120R GH mutants, since the N-terminal region of GH participates in site 2 binding (at least of GH to the GHR ECD). Thus, this deletion mutant should retain most of its ability to bind to the first receptor, but lose a large part of the site 2 binding domain.

Other Potential Antagonists

In 1989, Davis and Linzer described a mutant rodent PL II, which binds to, but does not activate the PRLR *(57)*. This molecule therefore has the potential to be an antagonist, although this was not directly investigated. Likewise, other work from our laboratory has described a covalent IgG–PRL complex, which binds to, but does not activate the PRL receptor *(58)*. In this instance, it is probable that the large IgG molecule interferes with PRL's ability to interact with more than one PRLR.

Development of PRL Antagonists Based on the Natural Antagonism of the Phosphorylated Hormone

The main approach taken by the authors' laboratory has been somewhat different from that described above. Rather than trying to produce antagonists by preventing the binding of a second receptor, we worked instead through a series of previous observations that demonstrated that naturally phosphorylated rat PRL very effectively antagonized the growth-promoting effects of unmodified PRL *(59,60)*. This had been demonstrated in the Nb2 bioassay system *(59)* and in the antagonism of GH_3 cell growth, 70% of which is dependent on an autocrine PRL growth loop *(60)*. PRL phosphorylation occurs in secretory granules just prior to exocytosis *(61,62)* and the ratio of phosphorylated to nonphosphorylated PRL varies reproducibly with the estrous cycle *(63)*, pseudopregnancy, pregnancy *(64)*, and lactation (unpublished results). A rise in estrogen, for example, decreases the proportion of phosphorylated PRL, and hence, estrogen's effects are to increase both the growth-promoting activity and amount of PRL produced *(62,63)*.

Others subsequently directly identified phosphorylated bovine *(65)*, ovine, chicken, and turkey *(66)* PRL, and confirmed the presence of the PRL kinase in PRL secretory granules *(67)*, and an increase in Nb2 biological activity of bovine pituitary PRL *(68)* or human milk PRL *(69)* after phosphatase treatment. Charge isomers, unrelated to glycosylation and consistent with phosphorylation, have been described for all species thus far examined *(70–73)*. Because of the limited availability of nontumorous, viable human tissue, direct demonstration of intracellular human PRL phosphorylation has not occurred. We have shown, however, that dephosphorylation of human PRL, derived from cadaverous tissue, does result in an increased biological activity *(30)* and, hence, indirectly, that human pituitary PRL is also phosphorylated.

Other previous work from our laboratory had demonstrated that rat PRL was primarily phosphorylated on serine 177 *(74)*. This is an absolutely conserved residue in all PRLs *(75)*, is a serine or threonine in the bovine PL family, is absent from all GHs, human PL, and rodent PLI, and is again a threonine in the rodent PLII family (reviewed in ref. *55*). The equivalent residue in the human hormone is serine 179 *(75)*.

Use of the phosphorylated hormone itself as an antagonist has several disadvantages, the most important of which is the potential conversion of the antagonist to an agonist by phosphatase activity during the course of an experiment. Although we have demonstrated that phosphorylated PRL is sufficiently resistant to phosphatase activity, to have biological activity in the circulation *(59,60)*, stagnant in vitro assays, particularly of tissues with a high phosphatase output, such as the prostate, could well be complicated by conversion. Also, slow-release forms designed for thera-

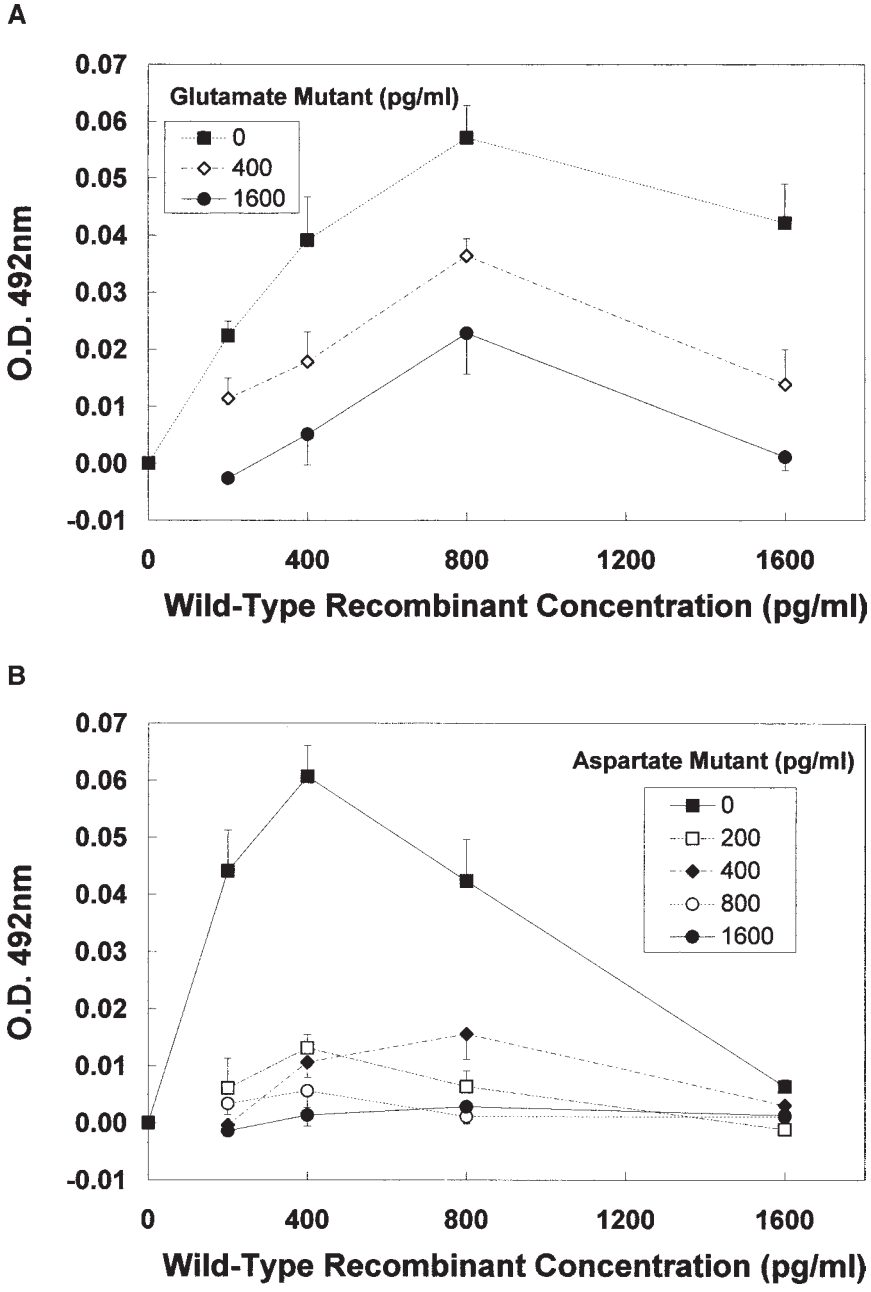

Fig. 4. Titration of glutamate and aspartate PRL mutants against wild-type recombinant hormone. Stationary phase Nb2 cells were plated at 1000/well and incubated in wild-type PRL with or without the addition of glutamate or aspartate mutant PRLs at the concentrations indicated for 3 d. OD_{492} is a measure of viable cell number using the MTS assay *(37)*. Data are presented as the mean ± SE. Figure reproduced with permission from The Endocrine Society. Original figure appeared in Chen et al. *(30)*.

peutic applications could also potentially be affected by phosphatase activity. To overcome the disadvantages of using the phosphorylated hormone, we attempted to produce a molecular mimic of phosphorylated human PRL by mutating serine 179 to glutamate and aspartate. Molecular mimicry of phosphorylation has been successfully applied to a variety of enzymes turned constitutively on or off by phosphorylation *(76–78)*. In one instance, the structural changes observed after phosphorylation have also been shown to be accurately reproduced by an aspartate mutant *(79)*. As described in detail in a recent publication *(30)*, mutation of serine 179 to either glutamate or aspartate produced an antagonist to unmodified PRL. The glutamate and aspartate mutants have somewhat different properties and efficacies, however, which lead us to expand on points raised earlier in the article. Both mutants have greater efficacies than others described in the literature.

Let us first consider the efficacy of the glutamate mutant. By reference to Fig. 4, one can see that at 400 pg/mL wild-type and 400 pg/mL glutamate mutant, the cell response to

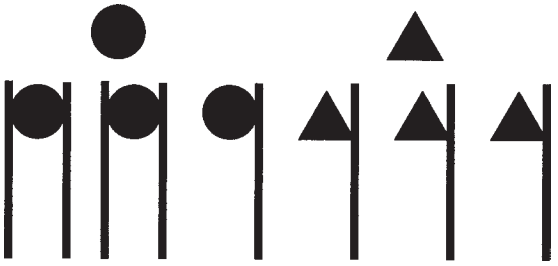

Fig. 5. Model consistent with the efficacy of the glutamate mutant. ○ = wild-type PRL, △ = glutamate mutant. Only the wild-type PRL molecules are able to dimerize the receptor.

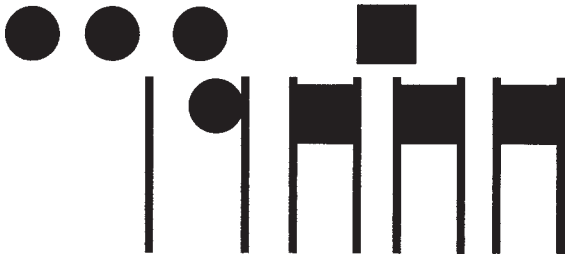

Fig. 6. Model consistent with the efficacy of the aspartate mutant. ○ = wild-type PRL, □ = aspartate mutant. The efficacy suggests that the aspartate mutant has a higher affinity for the receptor and that it occupies two receptors.

wild-type was reduced by half. If we make the assumption that efficacy is a function only of ligand–receptor interaction and we model this as shown in Fig. 5, this result fits very nicely with the concepts that the wild-type and glutamate mutant have very similar affinities for the first receptor, but that the glutamate mutant cannot bind a second receptor. Thus, equal numbers of Os (wild-type) and Δs (glutamate mutant) bind to the first receptor, but only Os can dimerize, thereby reducing the potential of a four-dimer response down to a two-dimer response. This result is easy to accept, because it fits with previous notions of PRLR antagonism.

If we now consider the efficacy of the aspartate mutant, however, something much more complicated must be occurring. At 200 pg/mL wild-type and 200 pg/mL aspartate mutant, there is a more than 75% drop in response from wild-type alone. If for simplicity we call this a 75% drop and use another model (Fig. 6), one can see that the model suggests: that the aspartate mutant (□) has a higher affinity for the PRLR (more □s than ○s are bound), and that two receptor molecules must be occupied by each aspartate mutant in order for it to so effectively block wild-type activity.

Thus, this result, based only on concepts of binding, suggests that the aspartate mutant can dimerize the receptor without producing a growth-promoting signal. It also suggests that the aspartate mutant–second receptor bond is stronger than the wild-type–first receptor bond; otherwise the wild-type, not engaged by a receptor, could displace the mutant.

Consistent with this interpretation are unpublished recent results from our laboratory that show receptor saturation with the aspartate mutant at half the concentration of the wild-type. In other words, consistent with the idea that when wild-type PRL was undoing dimers to increase binding, a parallel action was not occurring with the aspartate mutant. If we think of this in the usual way, it means that the aspartate mutant must have very similar site 1 and site 2 affinities, and that both exceed wild-type site 1 affinity.

Given the site of phosphorylation/mutation to aspartate, what can be predicted about conformational changes in the PRL that could contribute to both increased site 1 and site 2 affinity? Serine 179 is in a region of the molecule that forms part of site 1 (helix 4), but is on the opposite side of the helix from amino acids lysine 187, arginine 177, lysine 181, histidine 173, arginine 176, and histidine 180, which together with tyrosines 169 and 185 have all been suggested to be directly involved in receptor binding (reviewed in *55*, *80*) (Fig. 7). This suggestion is, however, based on mutagenesis studies, and mutagenesis studies can only address the importance of a residue in its natural location. They cannot determine actual interacting residues. That said, however, it seems to make sense to have the hydrophilic residues exposed in solution, although, for GH, it has been demonstrated that it is hydrophobic residues that make facial contact with the receptor and that the charged residues are important stabilizers of the interaction *(46)*. With lysine 187 on the outside, serine 179 would lie in the hydrophobic core of the PRL molecule. If it remained in the interior on phosphorylation, then by facing and distorting helix 3 in the model of Goffin et al. *(55)*, it could affect site 2 binding. It seems more likely to us, however, that phosphorylation of serine 179 induces a twist in helix 4 allowing greater hydrophobic interaction with the receptor, which is stabilized by the phosphoserine/aspartate residue. This twist is likely to result in compensatory rearrangements elsewhere in the molecule and, hence, changes in site 2 binding also. We hypothesize that these changes in conformation result in a molecule that binds tightly to both site 1 and site 2, but does not activate the dimerized receptor to produce a growth response. The possibility exists, however, that it may activate an alternate signaling pathway. Clearly these are testable hypotheses, which are only put forth at this early stage of development to stimulate discussion beyond the simplistic dimerization scheme.

Before leaving these preliminary studies with the aspartate mutant, there is another intriguing aspect of the binding experiment that deserves mention. The binding was conducted at 4°C using precooled cells and solutions. Unless receptors are mobile in the plane of the membrane

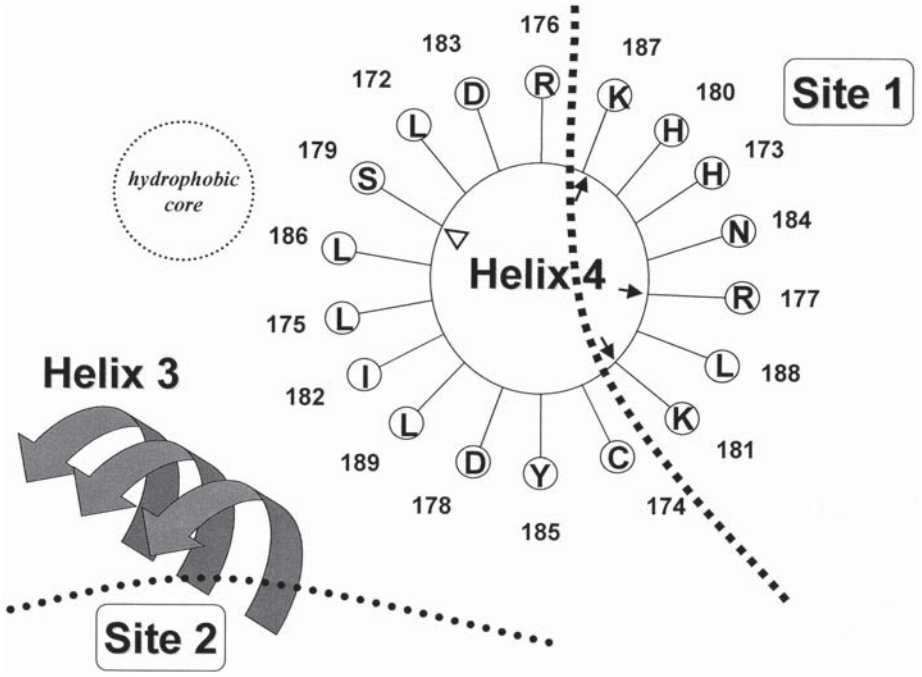

Fig. 7. Schematic representation of the region of PRL helix 4 including serine 179, which becomes phosphorylated or was mutated to glutamate or aspartate (arrowhead). The residues that appear most crucial for maintaining binding to receptor 1 (site 1) are indicated by arrows. Hydrophobic core refers to the hydrophobic interior of PRL. Phosphorylation of serine 179 could potentially distort helix 3 of PRL, thereby affecting interactions with the second receptor (site 2). Alternatively, phosphorylation of serine 179 may result in a twist in helix 4 and likely compensatory changes in the conformation of the site 2 region.

at 4°C or artifactitiously dimerize during cooling, the results, showing receptor saturation with the aspartate mutant at half the concentration of wild type, also therefore imply the pre-existence of dimers. This is a result in accord with those of Sakal et al. discussed earlier *(32)*. There are therefore indications from two laboratories that PRLR dimers may exist prior to ligand binding. Is there any indication in the GH system of such a phenomenon? Thus far, discussions in the GH literature have considered it unlikely that GHRs exist as dimers in the absence of ligand because of the relatively small interaction surface (500 Å) observed in the receptor ECDs *(46)*. This logic, however, makes the large assumption that only extracellular interactions could stabilize dimer formation. Potential intracellular proteins include cytoskeletal elements as well as nonsignal-transducing conformations of molecules such as Jak 2. Also, interesting work from Frank's lab (*81*, *82* and recent personal communication), although suggesting the necessity of GH for dimer formation, nevertheless shows great stability of the dimer in the absence of ligand.

Intriguing suggestions of dimerization in the absence of ligand therefore exist and raise the possibility that dimerization alone is not sufficient for signal transduction. As mentioned earlier, extracellular ligand–receptor interactions may instead produce key intracellular conformational changes.

Summary

In this article, we have tried to critically analyze evidence that supports a simple dimerization phenomenon as being sufficient for GHR and PRLR signaling. In light of data from several labs, we suggest that receptor dimers may exist in the absence of ligand. This in no way contradicts the concept of sequential binding of the ligand to the two receptors, or the proposed mechanism of action of the GH G120R and PRL G129R antagonists, since they may not bind the second receptor, may bind it differently from the unaltered hormone, or may even undo the dimer. Without correct interaction with the second receptor, the presence of ligand may not be appropriately transmitted through a conformational change to the cytoplasm. If a conformational change is important in signaling, the duration of binding (and therefore the species of ligand vs receptor) may be important, as may the differences between binding of human GH, PL, and PRL to the PRLR. These differences in signaling may be subtle and observed only by time-course studies or by analysis of the activation of multiple signaling pathways.

Although the mutation of site 2 regions on GH and PRL has produced effective antagonists, molecules, such as the human PRL aspartate 179 mutant, which appear to very effectively bind two receptors without producing a growth signal, have the potential to be even more effective antagonists.

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