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Antagonistic Properties of Human Prolactin Analogs That Show Paradoxical Agonistic Activity in the Nb2 Bioassay*

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Vincent Goffin‡§, Sandrina Kinet¶‖, Fatima Ferrag‡, Nadine Binart‡, Joseph A. Martial¶, and Paul A. Kelly‡

From ‡INSERM unit 344, Endocrinologie Moléculaire, 156 rue de Vaugirard, 75730, Paris Cedex 15, France and the ¶Laboratory of Molecular Biology and Genetic Engineering, Allée du 6 Août, University of Liège, 4000 Sart-Tilman, Belgium

Based on the assumption that the prolactin receptor (PRLR) is activated by PRL-induced sequential dimerization, potential human PRL (hPRL) antagonists were designed that sterically interfere with binding site 2. We previously reported the unexpected agonistic properties of these hPRL analogs in the rat Nb2 bioassay (Goffin, V., Struman, I., Mainfroid, V., Kinet, S., and Martial, J. A. (1994) *J. Biol. Chem.* 269, 32598–32606). In order to investigate whether such paradoxical agonistic behavior might result from characteristic features of the Nb2 assay (*e.g.* species specificity), we transfected in the same cell system the cDNA encoding the PRLR from rat or human species along with reporter genes containing PRL-responsive DNA sequences. We characterized the agonistic, self-antagonistic and/or antagonistic effects of wild type rat PRL, wild type hPRL, and three hPRL analogs, mutated either at binding site 1 or at binding site 2. Our results clearly show that the agonistic/antagonistic properties of PRLs are species-specific. We thus propose different models of receptor activation, depending on the relative affinities of each hormonal binding site, which is directed by species specificity. Finally, this is the first report of hPRL binding site 2 analogs showing antagonistic properties on human and, to a lesser extent, rat receptors.

Prolactin (PRL)[1] and growth hormone (GH) are homologous hormones primarily secreted by the anterior pituitary in all vertebrates (for review, see Ref. 1). They are involved in a wide spectrum of biological activities, mainly related to lactation, reproduction, and immunomodulation for PRL and growth and morphogenesis for GH (for review, see Refs. 2 and 3). These bioactivities are mediated by homologous membrane receptors, the PRL receptor (PRLR), also known as the lactogen receptor, and the GH receptor (GHR), also referred to as the somatogen receptor. Both belong to the cytokine receptor superfamily (for reviews, see Refs. 4–6).

Activation of the GHR and the PRLR is assumed to occur upon ligand-induced dimerization. For the GHR, mutational (7) and structural (8) studies have demonstrated that a single human GH (hGH) molecule binds to two molecules of hGH binding protein (hGHbp) through two regions referred to as binding sites 1 and 2. Binding site 1 is delimited by a pocket encompassed within helix 1, helix 4, and loop 1, whereas binding site 2 involves a cleft delimited by the opposite side of helix 1 and helix 3 as well as few residues of the N terminus. A sequential dimerization model for membrane-bound receptor has been proposed (9). In a first step, one hGH molecule binds to one hGHR molecule through its site 1 to form a 1:1 ligand-receptor complex that remains inactive. In a second step, the hGH involved in this intermediate 1:1 complex binds, through its site 2, to a second hGHR molecule, and an active 1:2 hormone-receptor complex results. For the PRLR, a similar mechanism of receptor activation is anticipated, since we have identified on hPRL two binding sites involving regions of the protein topologically equivalent to those forming the binding sites of hGH (10–14). Moreover, the hGH-hGHbp and hGH-hPRLbp 1:1 complexes were found to have very similar overall structures (8, 15, 16), strengthening analogies between these interactions involving these homologous hormones and receptors.

Based on the critical role of the cleft at binding site 2, which buries two Trp residues of the receptor upon binding (Refs. 8, 17, and 18; for review, see Ref. 15), a GHR antagonist has been designed. By replacing the hydrogen side chain of Gly^{120} (helix 3) in hGH with the much larger side chain of an Arg (so-called G120R hGH analog), the cleft within the helix 1/helix 3 interface is sterically hindered, and this analog, unable to induce receptor dimerization, fails to exhibit any agonistic activity (9). Conversely, it acts at high concentrations as an antagonist of WT hGH by blocking hormone-receptor complexes in the inactive 1:1 stoichiometry (9). The conservation of the helix 3 glycine within the PRL/GH family (19) argues for a conserved functional role of this residue in maintaining the cleft at binding site 2. In agreement, the antagonistic properties of such GH analogs, first assessed on the GHR from humans (9), were then observed on rat and mice GHR (20, 21) as well as on human and rat PRLR (22). In contrast, we recently showed that the G129R hPRL analog (homologous to G120R hGH) failed to antagonize hPRL-induced proliferation of rat Nb2 cells (12). These observations led us to postulate that the mechanism of PRLR dimerization shows some differences depending on the intrinsic properties of PRL *versus* GH (12).

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§ Recipient of a fellowship from the European Communities (Program Biotechnology). To whom correspondence should be addressed: INSERM unit 344, Endocrinologie Moléculaire, 156 rue de Vaugirard, 75730, Paris Cedex 15, France. Tel.: 33-1-40-61-53-10; Fax: 33-1-43-06-04-43.

‖ Recipient of fellowships from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (Belgique) (FRIA).

[1] The abbreviations used are: PRL, prolactin; hPRL, human PRL; rPRL, rat PRL; PRLR, PRL receptor; GH, growth hormone; hGH, human GH; GHR, growth hormone receptor; hGHR, human GHR; WT, wild type; hGHbp, hGH binding protein; PRLbp, PRL binding protein; hPRLbp, hPRL binding protein; PL, placental lactogen; LHRE, lactogenic hormone response element.

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Within the past 2 years, however, conflicting data have been reported that question the ubiquitous antagonistic properties of GH analogs mutated at binding site 2. First, a recent report demonstrates that the G120R hGH analog actually acts as an agonist on rat Nb2 cells in the absence of zinc ions (23). Second, Mode *et al.* (24) failed to observe any GHR antagonism of the hGH G120R analog when infused in rats but moreover found that this variant displayed agonistic activity via the PRLR. Third, although the I4A hGH analog is poorly able to dimerize the hGHbp *in vitro* (7), it acts as a perfect GH agonist in transgenic mice (25, 26). Accordingly, a hGH variant lacking the first seven N-terminal residues displayed full agonistic activity in the rat Nb2 assay (27). Taken together, these data suggest that the antagonistic properties of PRL/GH analogs mutated at binding site 2 might not only depend on the properties of the hormones themselves, as usually expected, but also on some features (*e.g.* species specificity, sensitivity) of the bioassays used for their functional characterization.

Studies on lactogens are most often performed using the rat Nb2 cell proliferation bioassay (10–12, 14, 27–31). In order to investigate if the use of this bioassay might be misleading for assessing antagonistic properties of lactogen analogs, such as G129R hPRL (12), we have analyzed several PRLs, wild type (WT) or mutated, using an *in vitro* bioassay in which the transcriptional activation of PRL-responsive reporter genes is mediated by exogenous PRLR from rat or human species, which allows their comparison in a strictly identical cell system. Our data clearly show that (i) the rat PRLR displays a higher affinity than human PRLR for binding site 2 of PRLs, (ii) consequently, the agonistic and antagonistic properties of a given analog are driven by species specific features, and (iii) the hPRL analog G129R displays antagonistic properties on PRLR from both species, in contradiction to what we previously observed using the Nb2 proliferation bioassay (12).

EXPERIMENTAL PROCEDURES

Materials

Cultures and Bioassays—Culture media and sera were purchased from Life Technologies, Inc. and dexamethasone from Sigma. Luciferase activity was measured in relative light units (Lumat LB 9501, Bethold, Nashua, NH).

Hormones—For stimulation, we used recombinant WT and mutated hPRL produced in *Escherichia coli*. Site-directed mutagenesis, production, and purification procedures have been extensively described in previous publications (10–12, 14, 32). In this study, we used recombinant WT hPRL, one binding site 1 analog (K181E, Lys^{181} replaced with Glu) (14), and two binding site 2 analogs (A22W, Ala^{22} replaced with Trp; G129R, Gly^{129} replaced with Arg) (12). Pituitary-purified rat PRL (rPRL) (NIDDK-rPRL-B-7; 25 IU/mg) was kindly provided by the Pituitary Hormones and Antisera Center of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

Plasmids—For experiments performed on rat PRLR, we used the cDNA encoding the intermediate (Nb2) form of the receptor (33). For human PRLR cDNA, we used a construct previously reported (34) in which the nucleotide sequence encoding the human PRLR signal peptide has been replaced by the sequence encoding the signal peptide from rat PRLR, which was found to give rise to higher receptor expression at the membrane. Both rat and human PRLR cDNAs were inserted into plasmids (pR/CMV and pC/DNA3, respectively; InVitrogen) under the control of a cytomegalovirus promoter. Reporter genes carry the sequence encoding the luciferase gene, which is under the control of either the β-casein gene promoter (34, 35) or a six-repeat sequence of the LHRE DNA element, followed by the minimal thymidine kinase promoter (36). The sequence of a single LHRE copy is 5′- CTGCAGTGTGGACTTCTTGGAATTAAGGGACTTTTGCTGCAG-3′, with the Stat5 consensus binding sequence underlined (6, 37). The plasmid encoding β-galactosidase (pCH110 vector) was purchased from Pharmacia Biotech, Inc.

Methods

Quantification of Proteins—Proteins were quantified physically by weighing the lyophilized powder on a precision balance (Electrobalance, Cahn 26) and chemically by the Bradford method (38). The disparity between weight and chemical measurements never exceeded 20%.

Transient Transfection of 293 Fibroblasts and Luciferase Assay—We used the human embryonic kidney fibroblast 293 cell line for transient transfection, which has been shown to highly express cDNAs controlled by the cytomegalovirus promoter (35). 293 cells were routinely cultured at 37 °C and 5% CO_2 in DMEM/F12 medium containing 10% fetal calf serum, 2 mM glutamine, and antibiotics and passaged every 3–4 days. Before transfection, cells were plated in 6-well plates at a density of 500,000 cells/well (~50% confluency) in 2 ml of a rich medium (2 parts DMEM/F12 and 1 part DMEM containing 4.5 g/liter glucose, 10% fetal calf serum, 2 mM glutamine, antibiotics). Transfections were performed using the calcium phosphate precipitate procedure as described earlier (35). In each well, we co-transfected 100 ng of plasmid containing the PRLR cDNA (rat or human), 100 ng of reporter plasmid (β-casein or LHRE promoter) and 500 ng of pCH110 (β-galactosidase). Cells were incubated overnight at 3% CO_2 and returned to at 5% CO_2 the next day. Twenty-four hours after transfection, cells were shifted to serum-free DMEM/F12 containing the hormone to be analyzed. When using the β-casein reporter gene, the assay was performed in the presence of 250 nM dexamethasone. After 20–24 h of stimulation, cells were lysed, and luciferase and β-galactosidase activities were measured.

Typically, we assayed the different hormones (WT or analogs) at concentrations ranging from 10 ng/ml to 250 μg/ml (~10 μM), so that each single experiment (performed in duplicate) required around 1.5–2 mg of hormone. Therefore, depending on the availability of the different hPRL analogs, experiments were performed from two to five times and, whenever possible, concentrations up to 500 μg/ml were also tested. For pituitary-purified rPRL and K181E hPRL analog, concentrations higher than 100 μg/ml could not be tested due to limited supply. Finally, for investigation of the antagonistic properties of hPRL analogs, a fixed concentration of WT hPRL producing maximal effect (2–10 μg/ml) was added to each well and competed with increasing amounts of each analog. Due to limited supply, data for extreme A22W concentrations (500 μg/ml) are from a single experiment performed in duplicate.

Presentation of Data—Arbitrary luciferase units were normalized for β-galactosidase activity in each experiment. Fold induction is calculated as the ratio between the normalized light units of stimulated *versus* unstimulated (no hormone added) cells. Luciferase fold induction levels obtained varied depending on hormone and receptor species. In order to accurately compare the biological activities of the different hormones mediated by either receptors, all results have been expressed as a percentage of maximal fold induction obtained with WT PRL. Bioactivity of hPRL analogs is given with respect to maximal activity of WT hPRL on each PRLR. Self-IC_{50} (self-antagonsim) was defined as the concentration at which the hormone activity is decreased by 50% of maximal activity at high concentration (right part of bell-shaped curves). The IC_{50} (antagonism) was calculated as the amount of hPRL analog producing 50% inhibition of WT hPRL maximal activity

RESULTS

Bell-shaped Curves and Receptor Species Specificity—The luciferase fold induction using the β-casein promoter was routinely in the range of 3–5, whereas it was much higher using the LHRE construct for which we obtained maximal activation of 32.6 ± 10 (n = 6) for the rat PRLR, and 68 ± 14 (n = 5) for the human PRLR upon hPRL stimulation. The LHRE construct was thus preferentially used for its higher response, whereas the β-casein construct was used as a control “physiological” promoter. Using rPRL, we obtained a maximal activation of the LHRE construct of 26.5 ± 1.1 (n = 3) for the rat PRLR and 18.8 ± 3.7 (n = 3) for the human receptor. Luciferase activity obtained in dose-response experiments has been normalized to these maximal fold induction values and is expressed as a percentage of these values. Results obtained for hPRL analogs on either receptor are given with respect to WT hPRL maximal activity.

As shown in Fig. 1*A*, agonistic activity is almost identical for both human and rat PRLR, with a maximal effect achieved in the range of 1–10 μg/ml hormone. It is noteworthy that maximal effect requires 1000-fold more hormone than in the Nb2 cell proliferation assay (12), which probably reflects the high number of PRLRs expressed in 293 cells as well as the requirement of nearly total receptor recruitment for maximal lucifer-

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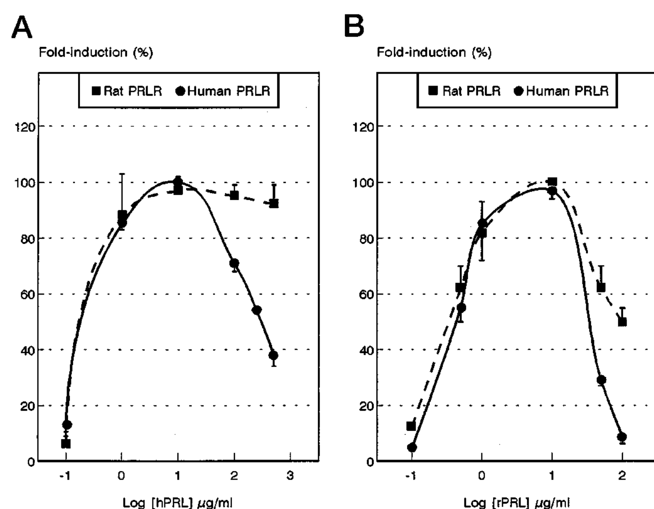

FIG. 1. **Agonistic and self-antagonistic properties of wild type human PRL (*A*) or rat PRL (*B*) on PRLR from rat (■, *broken line*) and human (●, *continuous line*).** 293 fibroblasts were co-transfected with the plasmid carrying the cDNA encoding the PRLR from either rat or human, the plasmid carrying the luciferase coding sequence under the control of a six-repeat sequence of the LHRE element (37), and the pCH110 vector encoding β-galactosidase (see "Experimental Procedures" for details). Twenty-four hours after transfection, cells were shifted to a serum-free medium containing appropriate hormone concentrations (given in log units) ranging from 0.1 to 100 μg/ml (rPRL) or 500 μg/ml (hPRL). After 20–24 h of stimulation, the luciferase activity was measured and normalized to the β-galactosidase activity. Dose-response curves are expressed in percentage of the maximal activity, observed at 10 μg/ml PRL for all interactions. Using hPRL (*panel A*), 100% corresponds to a luciferase induction of 68-fold on human PRLR and of 32.6-fold on rat PRLR. Using rPRL (*panel B*), 100% corresponds to 26.5-fold induction for rat PRLR and 18.8-fold induction for human PRLR. Whatever the hormone used, self-antagonism was more pronounced for human than for rat PRLR.

ase induction (see "Discussion"). At high hPRL concentrations, self-antagonism was observed only with human PRLR, with a self-IC_{50} of 380 μg/ml averaged from two independent experiments (Table I). Similar curves were obtained using the β-casein promoter (not shown), with a self-IC_{50} slightly lower (Table I). No bell-shaped curve was observed using rat PRLR, for which >90% activity was maintained, even at 500 μg/ml. In order to control the effect of species specificity with respect to the ligand, we also analyzed the dose-response of rat PRL in the same assay (Fig. 1*B*). Stimulation occurs in the same concentration range as with hPRL (maximal induction at 1–10 μg/ml), whatever the species of the receptor used. In contrast to hPRL, however, self-antagonism of rPRL is observed for both receptors, although the self-IC_{50} occurs at a 3-fold lower concentration for the human PRLR (32 μg/ml) compared with the rat receptor (94 μg/ml) (Table I). From the sequential dimerization model of GH and PRL receptors (9, 22), self-antagonism (*i.e.* bell-shaped curve) reflects the progressive disruption of active 1:2 hormone-receptor complexes in favor of inactive 1:1 complexes (hormone bound through its site 1) due to the higher affinity of binding site 1 compared with binding site 2. Our data show a direct correlation between the profile of bell-shaped curves and the receptor species. Whatever the species origin of the hormone, self-antagonism is more pronounced for human than rat PRLR, suggesting that the affinity of rat PRLR for PRL binding site 2 is higher than that of human PRLR.

Self-antagonism of hPRL Analogs—The A22W hPRL analog is a binding site 2 variant that shows weakened agonistic properties in the Nb2 proliferation assay and self-antagonizes at high concentration (12). In the LHRE-luciferase assay, using the rat PRLR, this analog shows a maximal activity of 77% compared with WT hPRL. Furthermore, this analog displays a bell-shaped curve (self-antagonism) at high concentrations (Fig. 2*A*), in agreement with an alteration of affinity at binding site 2. The interactions of A22W with rat PRLR (Fig. 2*A*) and WT hPRL with human PRLR (Fig. 1*A*) display similar curves, confirming that the affinity of the PRLR for binding site 2 of hPRL varies depending on the receptor species. Accordingly, the A22W analog is a very weak agonist for the human PRLR (maximal activity < 10% that of WT hPRL) (Fig. 2*A*).

G129R hPRL is another binding site 2 analog. From data obtained in the Nb2 proliferation assay, binding site 2 of this variant is even more hindered than in the A22W analog, since self-antagonism is observed at lower concentrations (12). In agreement, the maximal agonistic activity of this analog is weaker than that of A22W, 5.9% on the rat PRLR and <1% on the human PRLR (Fig. 2*B*). As expected, G129R displays a bell-shaped curve on both species of PRLR.

In order to confirm that self-antagonism of PRLs at high concentration actually results from low affinity at binding site 2, we tested a site 1 analog (K181E) in the LHRE-luciferase assay. From the Nb2 assay, this analog exhibits agonistic properties in the same range as both site 2 analogs (12, 14). In the luciferase assay, however, dose-response curves of K181E are displaced toward the right by ~1.5 log units compared with WT hPRL for each species of PRLR (Fig. 2*C*), and only submaximal activity (85–90%) could be obtained in the range of concentrations tested (up to 100 μg/ml). Thus, we failed to observe any self-antagonism for this site 1 analog.

These data clearly indicate that point mutations reducing the affinity of either binding site 1 or binding site 2 by the same range, as deduced form the Nb2 assay (12, 14), result in analogs behaving differently in the assay used in the present study. Compared with WT hPRL, the dose-response curve obtained with site 2 analogs is not shifted with respect to the range of hormone concentration, but varies in the amplitude of the response; the more the affinity at binding site 2 is weakened, the more are maximal effect reduced and self-antagonism pronounced. In contrast, the bioactivity curve of the site 1 analog is displaced toward high concentrations compared with WT hPRL but maintains the ability to achieve near-maximal activity; consequently, no self-antagonism can be observed in working concentration ranges.

Antagonism of Site 2 hPRL Analogs—Hormone antagonism is observed for analogs blocked at binding site 2 while maintaining the ability to bind to the receptor through site 1. We then tested the ability of the three hPRL analogs to antagonize WT hPRL in the LHRE-luciferase assay. Expectedly, an inverse correlation between agonistic and antagonistic properties was observed (Table I). For example, A22W hPRL (Fig. 3*A*), which is nearly a full agonist for the rat PRLR, was almost unable to antagonize WT hPRL, while some antagonism was observed at very high concentrations (IC_{50} = 110 μg/ml) for the human PRLR, in agreement with a lower agonistic activity for that receptor (Table I). The G129R hPRL analog, which only produces minimal luciferase activity for both receptor species, is able to antagonize the maximal effect of WT hPRL with an IC_{50} of 22 μg/ml and 53 μg/ml in cells expressing the human or rat PRLR, respectively (Fig. 3*B*). Control experiments using the β-casein promoter and the human PRLR gave similar values (Table I). As a general rule, antagonism appeared more pronounced on human than on rat receptors, in agreement with the lower affinity of the former for binding site 2 of PRLs. Finally, site 1 analog K181E failed to exhibit any significant antagonism at the concentrations tested (Fig. 3*C*), confirming that antagonistic properties correlate with site 2 alteration.

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TABLE I
Agonistic, self-antagonistic, and antagonistic effects of WT rPRL, hPRL, and hPRL analogs on activation of the β-casein promoter or the LHRE sequence mediated by the rat and human PRL receptors

Two WT hormones (rPRL and hPRL) and three hPRL analogs, mutated either at binding site 2 (A22W, G129R) or at binding site 1 (K181E), have been analyzed for their ability to activate the PRL receptor from rats or humans. Reporter genes are composed of the luciferase coding sequence under the control of either the β-casein promoter or a six-repeat sequence of the LHRE element (see "Experimental Procedures" for details). Results obtained using either reporter genes are similar.

Hormones	Properties	Rat PRLR		Human PRLR	
		β-casein	LHRE	β-casein	LHRE
hPRL (WT)	Agonism[a]	100% (3.1-fold)	100% (32.6-fold)	100% (3.5 fold)	100% (68-fold)
	Self-antagonism[b] (self-IC_{50})	No (n = 3)	No (n = 6)	182 μg/ml (n = 5)	380 μg/ml (n = 5)
rPRL (WT)	Agonism	—[c]	100% (26.5-fold)	—	100% (18.8-fold)
	Self-antagonism (self-IC_{50})	—	94 μg/ml (n = 3)	—	32 μg/ml (n = 3)
A22W hPRL (site 2)	Agonism (max)	—	77% (n = 2)	<1% (n = 2)	8.6% (n = 2)
	Antagonism (IC_{50})	—	>500 μg/ml (n = 1)	89 μg/ml (n = 3)	110 μg/ml (n = 2)
G129R hPRL (site 2)	Agonism (max)	—	5.9% (n = 2)	<1% (n = 2)	<1% (n = 2)
	Antagonism[d] (IC_{50})	—	53 μg/ml (n = 2)	14.5 μg/ml (n = 3)	22 μg/ml (n = 2)
K181E hPRL (site 1)	Agonism (max)	—	89% (n = 2)	—	85% (n = 2)
	Antagonism (IC_{50})	—	No	—	No

[a] Agonism, maximal fold induction (indicated in parentheses) of both reporter genes by WT PRLs mediated by human or rat PRL receptor is assigned to 100%. Agonistic effects of hPRL analogs are expressed in percentage of maximal induction with respect to WT hPRL.

[b] Self-antagonism, the ability of WT hormones to self-antagonize at high concentrations is given by the self-IC_{50}, *i.e.* the hormone concentration at which maximal activity is decreased by 50%.

[c] —, not tested.

[d] Antagonism, the ability of hPRL analogs to antagonize WT hPRL is given by the IC_{50}, which represents the concentration at which 50% inhibition is achieved. For binding site 2 analogs (A22W and G129R), an inverse proportional correlation is observed between the agonistic and antagonistic effects. In this bioassay, G129R is a more potent antagonist than A22W. On the contrary, site 1 analog K181E cannot antagonize WT hPRL, whatever the receptor used.

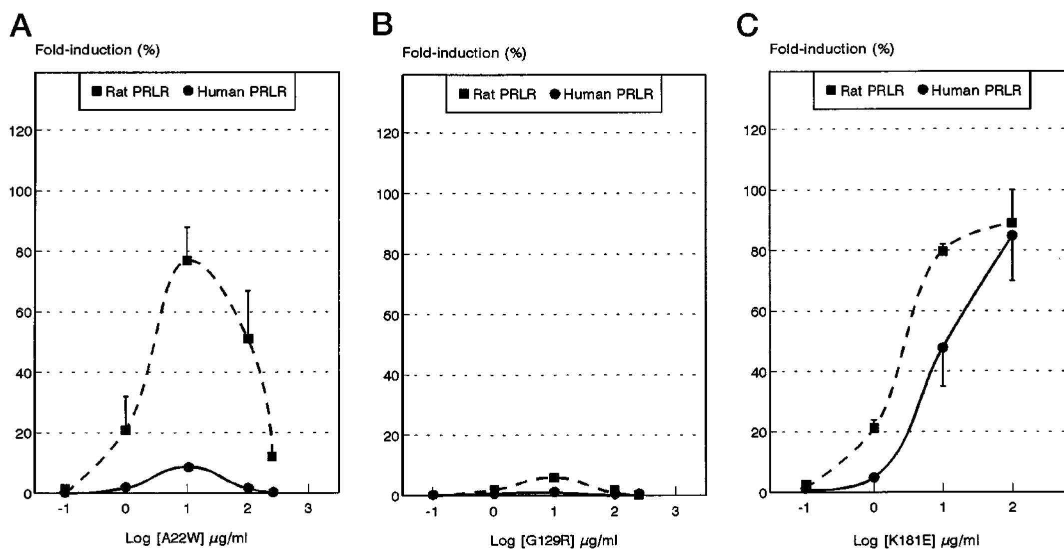

FIG. 2. **Agonistic and self-antagonistic properties of hPRL analogs mutated at binding site 2, A22W (*A*) and G129R (*B*), or at binding site 1, K181E hPRL (*C*), on PRLR from rat (■, *broken line*) and human (●, *continuous line*).** The experiment presented is identical to that described in the legend to Fig. 1 (for more detail, see "Experimental Procedures"). Stimulation of luciferase activity by the hPRL analogs is given as percentage of maximal induction by WT hPRL on each receptor (see legend to Fig. 1). A22W hPRL (*panel A*) shows nearly maximal agonistic activity on the rat PRLR and self-antagonizes at high concentrations. In contrast, only minimal induction of the LHRE-luciferase gene is observed with human PRLR. G129R hPRL (*panel B*) shows weak agonistic activity on rat PRLR and almost none on human. For both site 2 analogs, maximal activity is achieved at 10 μg/ml on both receptors. In contrast, dose-response curves obtained with K181E hPRL (*panel C*) achieved nearly maximal activity with both PRLR, but at higher concentrations compared with WT hPRL or site 2 hPRL analogs.

DISCUSSION

Species Specificity—Bell-shaped curves in dose-response biological assays are typical of hormone-receptor interactions sharing two typical features: first, receptor activation occurs through ligand-induced sequential dimerization; second, the affinity of the first site of interaction is higher than that of the second site. When these two conditions are met, formation of inactive 1:1 complexes are favored at high hormone concentration, and self-antagonism occurs. According to the model first proposed for the hGH-hGHR interaction (9), bell-shaped curves have been reported by several authors analyzing GH-dependent bioactivities, including the Nb2 proliferation assay (9, 20, 22, 23). Although PRLR activation is assumed to follow this sequential dimerization model (12, 22, 39), we almost always failed to observe any bell-shaped curve when stimulating rat Nb2 cell proliferation with WT hPRL, even at concentrations as high as ~20 μM (500 μg/ml) (12, 40). Furthermore, G129R hPRL, an analog anticipated to exhibit no agonistic activity, was able to produce nearly maximal Nb2 cell growth and failed to antagonize WT hPRL (12), in contrast to its homolog G120R

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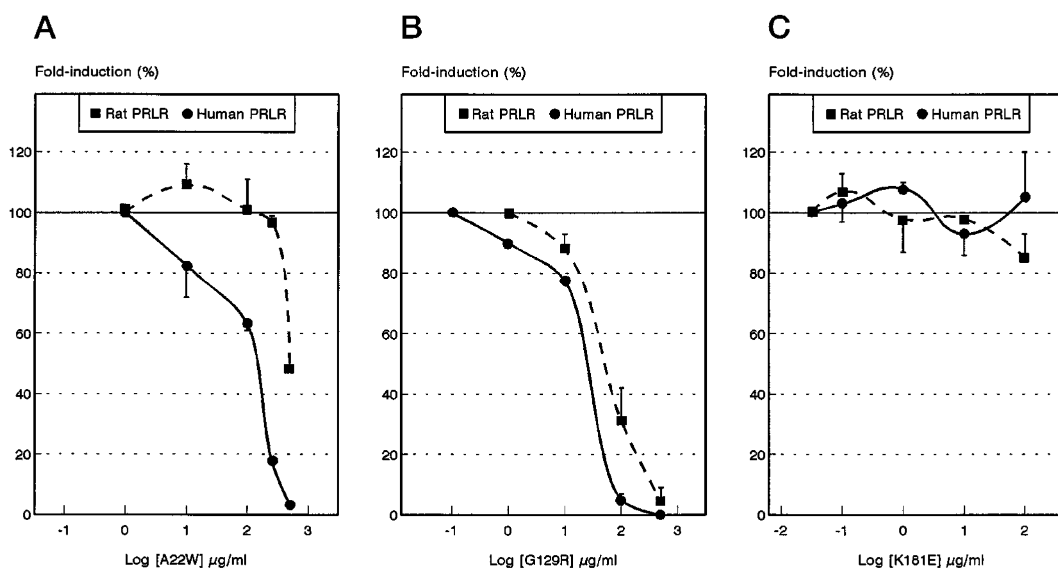

FIG. 3. **Antagonistic properties of hPRL analogs mutated at binding site 2, A22W (*A*) and G129R (*B*), or at binding site 1, K181E hPRL (*C*), on PRLR from rat (■, *broken line*) and human (●, *continuous line*).** In these experiments, 2–10 μg/ml hPRL (producing ~100% effect; *continuous line*) were competed with increasing amounts of either analog. Antagonism of site 2 analogs (*panels A* and *B*) was obtained at a lower concentration for human than for rat PRLR (see Table I). The site 1 analog K181E (*panel C*) failed to antagonize either receptor at the concentrations tested.

hGH, which has been shown to antagonize both hPRL and hGH in the Nb2 bioassay (22). From these observations, we thus proposed a model of PRLR activation in which the affinity of both binding sites of hPRL would be roughly identical, so that formation of 1:1 complexes would not be favored at high concentrations (12). In agreement, G129R hPRL, in which affinity at binding site 2 is reduced, follows the GH model and self-antagonizes at high concentration (12).

In contradiction with the initial publication (22), the G120R hGH analog was recently reported to be a potent agonist in the rat Nb2 cell proliferation assay in the absence of zinc ions (23). Interestingly, the curve obtained with this analog is reminiscent of that obtained with the G129R hPRL analog in the same assay (12), with an EC_{50} in the nanomolar range and a self-IC_{50} in the micromolar range. This report, as well as other conflicting data concerning the agonistic/antagonistic properties of the site 2 hGH analogs (see Introduction), led us to hypothesize that the differences between the models of receptor activation proposed for hGH (9, 22) and hPRL (12), at first sight assumed to differ by the relative affinity of both binding sites, could not only depend on intrinsic properties of the hormones *per se* (GH *versus* PRL), but also on some specific features of the bioassays used, such as sensitivity or species specificity of receptors (*e.g.* human *versus* rat). The present report confirms this assumption. Using the self-antagonism phenomenon as a reflection of the relative affinity of both binding sites, our results strongly suggest that the affinity of the rat PRLR for the second site of WT PRLs (either rat or human) is higher than that of the human PRLR (Fig. 1, Table I). The profiles of dose-response curves using site 2 hPRL analogs confirm this hypothesis, with a maximal agonistic effect in all cases lower for human PRLR compared with rat PRLR (Table I), indicating that a given mutation within site 2 differently alters the biological activity of the hormone depending on the species origin of the receptor used.

The curves obtained in this study are in absolute agreement with the computerized model of receptor dimerization proposed by Ilondo *et al.* (20). In that model, reducing the affinity at binding site 2 decreases the overall fraction of receptor dimers but does not shift the peak of dimer formation toward high hormone concentrations. This is observed for both site 2 hPRL analogs (Fig. 2 and Table I), with a level of maximal activity proportional to the affinity of site 2 (A22W rat PRLR > A22W human PRLR > G129R rat PRLR > G129R human PRLR). The model of Ilondo and colleagues (20) predicts, in contrast, that mutations affecting binding site 1 will shift the curve of dimer formation to the right (higher concentrations) without affecting the maximal dimer concentration (*i.e.* maximal activity achieved). The behavior of the binding site 1 hPRL analog K181E is in agreement with this model, although we could not test concentrations producing maximal activity (>100 μg/ml) due to lack of sufficient protein. The high affinity of rat PRLR for binding site 2 of PRLs (and probably GHs also) presumably accounts for several poorly understood, or even contradictory, observations. For example, ovine PRL or hGH were reported to form 1:2 complexes with the rat PRLbp (41, 42), while only 1:1 complexes were observed between ovine PRL and/or hGH with PRLbp from rabbit (43, 44), human (16), or bovine (45) species. Other authors also observed that injection of G120R hGH to rats led to unexpected agonistic effects on the PRLR-mediated effect (24), thus corroborating the results obtained in the rat Nb2 assay (23). Human PRL receptors seem particularly sensitive to alterations to the second hormonal binding site, in agreement with the lower affinity of the interaction involving this site (compared with rat PRLR). Replacement of the helix 3 glycine in hGH, human PL, or hPRL is detrimental to human PRLR-mediated effects thus far analyzed (Refs. 22 and 39; present study). The molecular basis of the higher affinity of the rat PRLR (and perhaps of other species also) for the second binding sites of PRL/GH hormones is currently unknown. Analysis of primary sequences correlated with the three-dimensional structure of the human PRLbp (16) might help direct further mutational studies.

Antagonism of Binding Site 2 hPRL Analogs—Weak, or even no, agonistic effect of PRL/GH site 2 analogs usually correlates with antagonistic properties (9, 22, 39). As summarized in Table I, both site 2 hPRL analogs are able to antagonize the action of WT hPRL, with a potency inversely proportional to their agonistic effect. To the best of our knowledge, this is the first report of binding site 2 hPRL analogs antagonizing a PRLR-mediated biological effect. Although only weak antagonism of A22W was observed on rat PRLR, G129R clearly ex-

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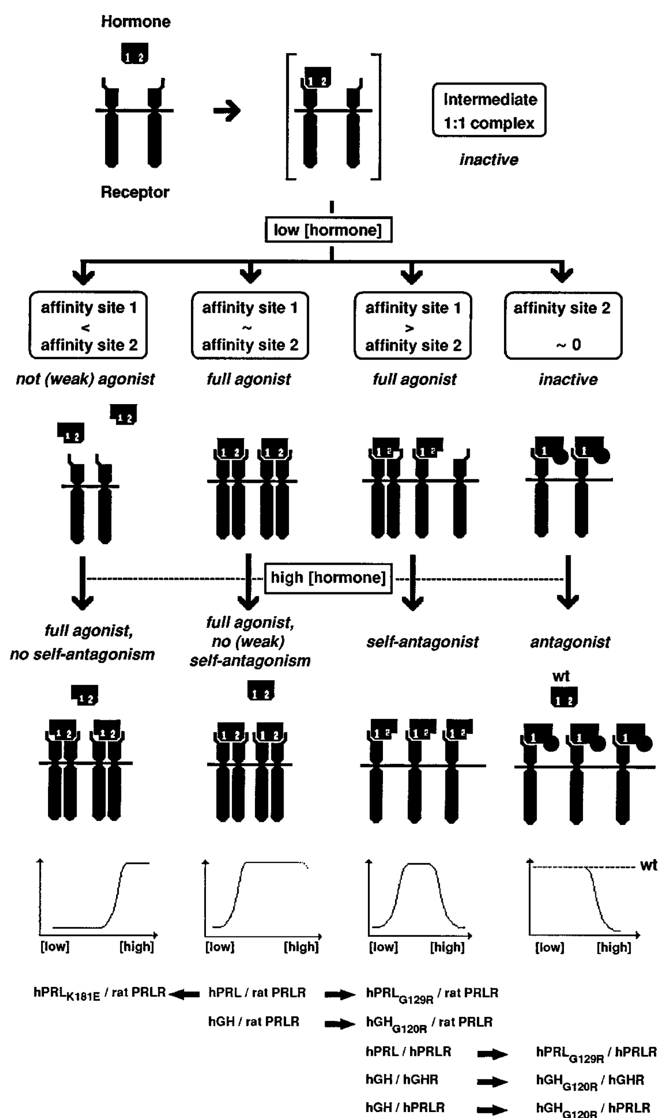

FIG. 4. **Models of hormone-receptor interaction: effect of the relative affinities of both binding sites.** The model of receptor dimerization that we proposed earlier (12) has been completed, and four cases are presented that differ by the relative affinities of both binding sites. The model is assumed to be applied to interactions involving proteins of the PRL/GH/PL family and the PRLR and the GHR, assuming that receptor activation always requires dimerization, with interaction involving binding site 1 being required before site 2 can bind to the receptor. In the *lower part* of the figure, a typical curve is illustrated in which the *x* axis corresponds to the hormone concentration and the *y* axis to any receptor-mediated bioactivity. For each curve, examples of particular hormone-receptor interactions are given. In a first step, the hormone (PRL, GH, or PL) binds to a receptor (PRLR or GHR) through its binding site 1 to form an inactive 1:1 complex (9, 12, 22). Then four situations can be observed. 1) The affinity of binding site 1 is weak; few hormone-receptor complexes are formed at low hormone concentrations and no, or only weak, agonistic effects occur (*e.g.* site 1 analog K181E hPRL on human or rat PRLR; Fig. 2*C*). At high concentrations, the amount of ligand is sufficient to allow formation of 1:2 complexes required for full agonistic activity. 2) The affinity of both binding sites is roughly identical; full agonism is observed at low concentration due to sufficient dimer formation. At high concentration, no (or only weak) self-antagonism is observed, since high stability of 1:2 complexes does not favor the formation of 1:1 complexes (*e.g.* hPRL in the Nb2 assay or in the luciferase assay using rat PRLR). 3) The affinity of binding site 1 is higher than the affinity of site 2; full agonistic activity is observed at relatively low concentration. The more the biological system contains spare receptors, the lower will be the hormone concentration producing maximal effect. At high concentrations, the higher affinity of binding site 1 favors the disruption of 1:2 complexes for 1:1 complexes, and self-antagonism occurs. In such a case, the higher the difference in the affinity between both sites, the lower the maximal effect achieved (see *dotted curve*; Ref. 20). As an example, the curve obtained with hGH G120R analog in the Nb2 proliferation assay follows the *continuous line* in the absence of zinc and the *dotted line* in presence of zinc (which strongly increases the difference of affinity between both sites; Ref. 23). 4) The affinity of binding site 2 is nearly nil; the hormone cannot dimerize the receptor and fails to display any agonistic effect. Due to their ability to bind through their site 1, such analogs behave as antagonists of WT hormones when added in sufficient amounts (which depends on the affinity of the first binding site and on the proportion of spare receptors of the biological system). For example, G129R hPRL can antagonize the rat PRLR in the LHRE-luciferase assay (Fig. 3*B*), but not in the Nb2 cell proliferation assay (12), since maximal activity in the latter system occurs at very low dimer formation.

hibits antagonistic properties on PRLR from both species. These observations are thus in contradiction to those we previously reported using the Nb2 assay (12), in which both A22W and G129R hPRL produced nearly maximal cell growth and failed to display any antagonism of WT hPRL. Although the present study suggests that the weak agonistic effect of site 2 analogs can be magnified in rat bioassays due to the high affinity of the rat receptor compared with the human, this does not explain the discrepancy between data obtained from Nb2 and LHRE-luciferase assays, since the same PRLR isoform (so-called Nb2 or intermediate; Ref. 33) is involved. Another explanation, however, can be proposed. It has been shown that maximal Nb2 cell proliferation is achieved at very low receptor occupancy, indicating that the majority of binding sites in these cells are spare receptors (46). This strongly suggests that a low number of dimerized receptors is sufficient to produce maximal cell growth, in agreement with the extremely high sensitivity of Nb2 cells able to respond to hormone concentrations in the picomolar range. This is likely to explain why G129R hPRL can produce near maximal activity in that assay (>80%; Ref. 12) while, in contrast, it can only achieve 5.9% of maximal activity in the LHRE-luciferase assay. Taken together, these data thus question the appropriateness of the Nb2 cell proliferation assay for assessing agonistic/antagonistic properties of PRL/GH analogs mutated at binding site 2, especially if human hormones are considered. Although this proliferation assay appears reliable and consistent with other bioassays as far as binding site 1 is considered (10, 11, 14, 27, 30, 31), experiments investigating features linked to binding site 2 should probably be interpreted with caution.

Models of Receptor Activation—Based on Nb2 cell proliferation assay, we have proposed a model of receptor activation for hPRL differing from that described for hGH (9, 22), based on the similar affinities of both binding sites (12). In view of the results presented in this study, we can now provide a completed picture of this model of receptor dimerization depending on the properties of the different analogs, receptor type, and species (Fig. 4). A good correlation can be made between the relative affinity of each binding site and the shape of the curves obtained in hormone dose-response bioassays (see above). As a general rule, the greater the difference in affinity between each site, the smaller the concentration range separating agonistic and self-antagonistic effects (12). For example, IC_{50} and self-IC_{50} of hGH on Nb2 cultures are separated by almost 7–8 log units, while only 3–4 log units separate both sides of the bell-shaped curve with G120R hGH in the absence of zinc (23). The four models of hormone-receptor interactions that we propose are each typical of a given ratio between the affinities of both binding sites (Fig. 4). When the affinity of site 1 is weak (*e.g.* hPRL K181E on rat or human PRLR), the hormone acts as a partial agonist, and maximal effect can be achieved only at high hormone concentrations. In such cases, no self-antagonism is observed, since the low affinity of site 1 does not favor the formation of 1:1 complexes. When the affinity of both sites

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is of the same order (*e.g.* hPRL on rat PRLR), an agonistic effect is observed at low hormone concentration. At high concentration, no self-antagonism is observed, probably due to the higher stability of the 1:2 complexes compared with the 1:1 complexes (12). When the affinity of site 2 is lower than that of site 1 (*e.g.* hPRL G129R on rat PRLR), the hormone also behaves as a (partial) agonist at low concentration, with a maximal effect proportional to the affinity at site 2. In such a case, however, the maximal effect achieved by a given hormone can be modulated with respect to the fraction of spare receptors in the bioassay, such as Nb2 cells, which only require very low dimer formation to proliferate. Finally, if the affinity of site 2 is almost nil (hPRL G129R on human PRLR), no agonistic activity is observed, but the analog exhibits antagonistic properties. Once again, receptor spareness will influence the antagonistic potency of a given analog (G129R on Nb2 PRLR in cell proliferation *versus* luciferase assay). Obviously, if the affinity at site 1 is nil, the analog is neither agonist nor antagonist (not represented in Fig. 4).

If these models are reliable, they could account for the paradoxical activity of the G120R hGH analog in the Nb2 proliferation assay depending on zinc concentrations (22, 23). In the presence of zinc, the affinity of hGH binding site 1 for the PRLR is markedly increased (47). Since the affinity of G120R binding site 2 is weak due to the Gly to Arg substitution, the 1:1 hormone-receptor stoichiometry is widely favored due to the huge difference in affinity between both sites, and only minimal cell proliferation is observed (22, 23). However, in the absence of zinc, the affinity of binding site 1 is greatly reduced, and the 1:1 hormone-receptor stoichiometry is less favored. Consequently, even though the formation of 1:2 complexes presumably remains limited, it is sufficient to generate cell proliferation in the very sensitive Nb2 bioassay (23), as observed for G129R hPRL also (12). Although this interpretation appears satisfactory, full understanding of the proliferation curves obtained in the Nb2 assay will require further investigation to accurately integrate the effects of spare receptors in our models.

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