Evidence for a Second Receptor Binding Site on Human Prolactin*

Vincent Goffin‡, Ingrid Struman, Véronique Mainfroid, Sandrina Kinet, and Joseph A. Martial§

From the Laboratory of Molecular Biology and Genetic Engineering, Allée du 6 Août, University of Liège, 4000, Sart-Tilman, Belgium

The existence of a second receptor binding site on human prolactin (hPRL) was investigated by site-directed mutagenesis. First, 12 residues of helices 1 and 3 were mutated to alanine. Since none of the resulting mutants exhibit reduced bioactivity in the Nb2 cell proliferation bioassay, the mutated residues do not appear to be functionally necessary. Next, small residues surrounding the helix 1-helix 3 interface were replaced with Arg and/or Trp, the aim being to sterically hinder the second binding site. Several of these mutants exhibit only weak agonistic properties, supporting our hypothesis that the channel between helices 1 and 3 is involved in a second receptor binding site. We then analyzed the antagonistic and self-antagonistic properties of native hPRL and of several hPRLs analogs altered at binding site 1 or 2. Even at high concentrations (~10 μM), no self-inhibition was observed with native hPRL; site 2 hPRL mutants self-antagonized while site 1 mutants did not. From these data, we propose a model of hPRL-PRL receptor interaction which slightly differs from that proposed earlier for the homologous human growth hormone (hGH) (Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., and Goeddel, D. V., and Well, J. A. (1992) Science 256, 1677–1680). Like hGH, hPRL would bind sequentially to two receptor molecules, first through site 1, then through site 2, but we would expect the two sites of hPRL to display, unlike the two binding sites of hGH, about the same binding affinity, thus preventing self-antagonism at high concentrations.

Prolactin (PRL)[1] is a pituitary-secreted hormone. It belongs to a protein family which also includes growth hormone (GH) and placental lactogen (for reviews, see Miller and Eberhardt, 1983; Nicoll et al., 1986). PRL is involved in a wide variety of biological functions, mainly related to reproduction, lactation, osmoregulation, and immunomodulation (reviewed in Clarke and Bern, 1980). The biological activities of PRL are mediated by specific membrane receptors called lactogenic receptors (PRLR; Kelly et al., 1991, 1993). On the basis of several con-served features (a single transmembrane domain, conserved amino acid sequences in the extracellular domain), the PRLR has been linked to the cytokine (or hematopoietic) receptor superfamily (Bazan, 1989; Sprang and Bazan, 1993). Interestingly, the specific GH receptor (GHR, called the somatogenic receptor) belongs to the same receptor superfamily.

Over the past 5 years, there have been several reported mutational studies aimed at elucidating structure-function relationships within the PRL/GH protein family. In 1991, Chen and colleagues expressed a G119R mutant (Gly replaced with Arg) of bovine GH (bGH) in transgenic mice and found it to produce a dwarf phenotype. The reason was unclear, at first, because Gly[119] is on helix 3 (Abdel-Meguid et al., 1987), and the binding site of GH had been unambiguously linked to a region lying on another face of the protein, delimited by portions of helix 1, helix 4, and loop 1 (Cunningham et al., 1989; Cunningham and Wells, 1989). The explanation came from later mutational (Cunningham et al., 1991) and structural (de Vos et al., 1992) studies of human GH (hGH), demonstrating the involvement of a second region, including the helix 3 glycine (Gly[120] in hGH, Gly[119] in bGH) and surrounding amino acids, in the binding of hGH to a second GHR molecule. When the helix 3 Gly is mutated to Arg, this binding site 2 is sterically hindered and the hormone can no longer induce receptor dimerization. The fact that GH mutants carrying the Gly → Arg mutation are biologically inactive and act, moreover, as perfect hGH antagonists led Fuh et al. (1992, 1993) to propose that receptor dimerization is an absolute requirement for signal transduction by the GHR.

Formation of receptor homo- or heterodimers (or oligomers) has also been reported for several other members of the cytokine receptor family (Fukunaga et al., 1990; Watowich et al., 1992; Stahl et al., 1993; for reviews, see Young, 1992; Stahl and Yancopoulos, 1993). Association of membrane proteins is thus anticipated for all cytokine receptors. PRL-induced dimerization of the PRLR has neither been proved nor disproved. However, the observation that bivalent, but not monovalent, monoclonal antibodies raised against PRLR exhibit PRL agonistic properties brought some indirect evidence that activation of the PRLR probably occurs upon dimerization (Elberg et al., 1990). Moreover, Hooper et al. (1993) observed the formation of 1:2 complexes between ovine PRL and the extracellular domain of the rat PRL receptor and anticipated a similar stoichiometry for the membrane-anchored receptor. Studies using the extracellular domain of the receptor remain controversial, however, since other investigators have reported 1:1 complexes in similar experiments (Gertler et al., 1993; Bignon et al., 1994).

Another way to investigate the occurrence of PRL-induced dimerization of the PRLR is to identify on the hormone a region involved in contact with a second PRLR molecule, or in other words, a second receptor binding site. Binding site 2 of hGH consists of a hydrophobic channel bordered by residues of the N-terminal tail, helix 1, and helix 3 (Cunningham et al., 1991; de Vos et al., 1992). Two residues of the GHR extracellular domain, Trp[104] and Trp[169], play a critical role in the interaction with hGH binding site 2 (Bass et al., 1991; de Vos et al., 1992).

[1] The abbreviations used are: PRL, prolactin; hPRL, human prolactin; bPRL, bovine prolactin; hGH, human growth hormone; bGH, bovine growth hormone; PRLR, prolactin receptor; GHR, growth hormone receptor; FCS, fetal calf serum; IPTG, isopropyl β-thiogalactoside; PAGE, polyacrylamide gel electrophoresis.
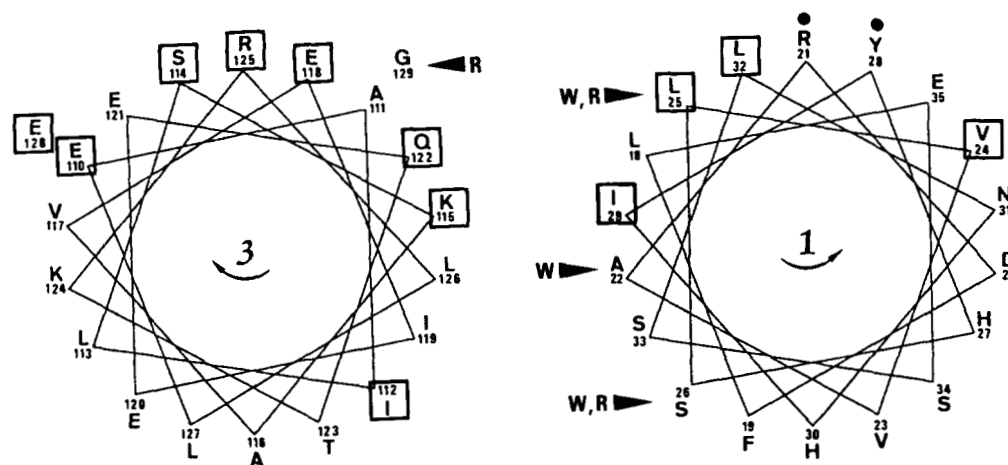
FIG. 1. **Helix wheel representation of helices 1 and 3.** *Curved arrows* below helix numbers symbolize the antiparallel sense of rotation of both α-helices. *Boxed* amino acids correspond to positions which were mutated to alanine. *Arrowheads* show the 4 residues which were mutated to Trp (*W*), Arg (*R*), or both (*W, R*). Arg[21] and Tyr[28], which were previously mutated (Luck *et al.*, 1990, 1991), are labeled with *black points*. Helices 2 and 4 (not shown) are roughly located, respectively, below helices 3 and 1 (Abdel-Meguid *et al.*, 1987).

Interestingly, these 2 Trp residues are ubiquitous within the PRL-GH receptor family (Kelly *et al.*, 1993) but mutated in all other members of the cytokine receptor superfamily (Cosman *et al.*, 1990). Conservation of these Trp residues suggests that, similar to what is observed at binding site 1 (Goffin *et al.*, 1992, 1993), PRL might bind to a second PRLR by a mechanism similar to that described for hGH (de Vos *et al.*, 1992).

To date, there is no three-dimensional structure available for any PRL. To circumvent this lack of data, we recently constructed a model of hPRL,[2] and proposed a location for the putative second binding site. On the basis of helical positions and of their side chain conformations and orientations, we initially selected a dozen residues potentially involved in the interaction with a second PRLR (Fig. 1): four on helix 1 (Val[24], Leu[25], Ile[29], and Leu[32]) and eight on helix 3 (Glu[110], Ile[112], Ser[114], Lys[115], Glu[118], Gln[122], Arg[125] and Glu[128]). In a first step, alanine-scanning site-directed mutagenesis was used to characterize the involvement of these 12 residues in the biological function of hPRL. We monitored the bioactivity of each single mutant in the widely used Nb2 cell proliferation bioassay (Gout *et al.*, 1980; Tanaka *et al.*, 1980). The results obtained with this first set of mutants led us subsequently to design a second set of mutations, using a different mutational strategy.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany), Amersham International (Buckinghamshire, United Kingdom), Life Technologies Inc., and Eurogentec (Seraing, Belgium). Iodogen and bovine γ-globulin were purchased from Sigma and carrier-free Na-^{125}I was obtained from Amersham International. Ampholytes (5–7 pH range) and pI protein markers were from Pharmacia (Uppsala, Sweden). Site-directed mutagenesis was performed by the M13 procedure, using the oligonucleotide-directed *in vitro* mutagenesis systems of either Amersham International or Boehringer Mannheim. Purification of native hPRL and hPRL analogs was performed by gel filtration chromatography using Sephadex G-100 (Pharmacia, Uppsala, Sweden) packed in a 100 × 2.6-cm column (Pharmacia). Apparent molecular mass (MM) of PRL analogs were estimated by gel filtration chromatography using a Superose 12 column (25 ml, Pharmacia) mounted on a fast protein liquid chromatography system (Pharmacia). Culture media and sera were purchased from Life Technologies Inc.

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[2] V. Goffin, J. A. Martial, and N. L. Summers, manuscript submitted for publication.

Methods

Oligonucleotide-directed Mutagenesis

All mutated hPRL cDNAs were constructed by the oligonucleotide-directed mutagenesis method of Sayers *et al.* (1988), using the single-stranded M13 as the vector. We used the oligonucleotide-directed mutagenesis system of Amersham or Boehringer Mannheim and strictly followed the manufacturer's instructions. Clones containing the expected mutation were identified by DNA sequencing, and the mutated cDNAs were digested with *Nde*I (initial ATG) and *Hin*dIII (3'-noncoding region of the hPRL cDNA; Cooke *et al.*, 1981). The isolated cDNA fragments (660 base pairs) were reinserted into the pT7L expression vector (Paris *et al.*, 1990). The sequences of the mutated oligonucleotides are reported below (5' → 3' noncoding strand, mutated codon underlined).

Alanine substitutions are as follows. V24A, 5'-GTG GGA CAG GGC GAC GGC GCG-3'; L25A, 5'-GTA TGT GGA CGC GAC GAC GGC-3'; I29A, 5'-GAG GTT ATG GGC GTA GTG GG-3'; L32A, 5'-TTC TGA GGA GGC GTT ATG GAT-3'; E110A, 5'-TAG GAT AGC CGC CGG GGC TTC-3'; I112A, 5'-C TTT GGA TAG GGC AGC CTC CGG-3'; S114A, 5'-C TAC AGC TTT GGC TAG GAT AGC-3'; K115A, 5'-CTC TAC AGC TGC GGA TAG GAT-3'; E118A, 5'-CTC CTC AAT CGC TAC AGC TTT G-3'; Q122A, 5'-CCG TTT GGT TGC CTC CTC AAT C-3'; R125A, 5'-CTC TAG AAG CGC TTT GGT TTG-3'; E128A, 5'-CTC CAT GCC CGC TAG AAG CCG-3'.

The Trp/Arg substitutions are as follows. A22W, 5'-CAG GAC GAC CCA GCG GTC AAA C-3'; L25W (R), 5'-GTA TGT GGA CCA (T) GAC GAC GGC-3' (degenerate primer); S26W (R), 5'-GAT GTA GTG CCA (T) CAG GAC GAC-3' (degenerate primer); G129R, 5'-CAG CTC CAT CCT CTC TAG AAG-3'.

Expression and Purification of Proteins

Recombinant native hPRL and hPRL analogs were overexpressed in 500-ml cultures of *Escherichia coli* BL21(DE3) and purified as described previously (Paris *et al.*, 1990). Briefly, when the OD_{600} of the bacterial cultures reached 0.9, overexpression was induced with 1 mM IPTG. Maximal overexpression was obtained by a 4-h induction (OD_{600} ~2.5). Human PRL was overexpressed as insoluble inclusion bodies which were solubilized in 8 M urea (5 min/55 °C, then 2 h/room temperature) and refolded by continued dialysis (72 h, 4 °C) against 20 mM NH_4HCO_3, pH 8. Renatured hPRL was concentrated in a DIAFLO ultrafiltration cell with a YM10 membrane (Amicon Co, MA) and purified on a Sephadex G-100 molecular sieve; fractions corresponding to monomeric hPRL were collected and pooled. Purified proteins were lyophilized for at least 24 h and stored at 4 °C.

Quantification of Proteins—Proteins were quantified physically by weighing the lyophilized powder on a precision balance (Electrobalance, Cahn 26) and chemically by the Bradford (1976) method. The disparity between weight and chemical measurements never exceeded 20%.

Electrophoretic Analyses

SDS-PAGE—Protein size and purity were assessed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (2-mer-

captoethanol) according to Laemmli (1970). Electrophoresis was performed for 1 h at 150 V in vertical slab gels (Hoefer Scientific Instruments, CA). The gels (15% polyacrylamide) were stained with Coomassie Blue.

Isoelectrofocusing—The isoelectric point of hPRL analogs was estimated by isoelectrofocusing. Electrophoresis was performed on vertical slab gels under continuous cooling; electrode solutions were 20 mM acetic acid and 20 mM NaOH. The gels contained polyacrylamide (5.5%), glycerol (10%), and ampholytes in the 5–7 pH range (5.5%). Prior to loading the protein samples, the pH gradient was allowed to form during a 15-min prerun at 200 V. One µg of each protein diluted in sample buffer (ampholytes 5.5%, glycerol 10%) was loaded on the gel. The run was performed at 200 V and stopped when the visible band corresponding to methyl red (pI = 3.75) was focused. Gels were fixed in 20% trichloroacetic acid, then in 40% ethanol, 10% acetic acid, 0.25% SDS. They were then washed twice in 40% ethanol, 10% acetic acid, stained with 0.125% Coomassie Blue, and destained in 40% ethanol, 10% acetic acid. Isoelectric points of the hPRL samples were estimated by comparison with the migration of pI marker proteins.

Structural Analyses

Circular Dichroism—Lyophilized proteins were resuspended in 50 mM NH_4HCO_3, pH 8, at a concentration of 500 µg/ml. Spectra were recorded with a CD6 dichrograph (Instruments SA-JOBIN YVON, Longjumeau, France) linked to a personal computer for data recording and analysis (dichrograph software, Instruments SA-JOBIN YVON, Longjumeau, France). For each protein, five spectra recorded between 195 and 260 nm were averaged. Measurements were performed in 0.1-cm pathlength quartz cell. The helicity was calculated at 222 nm according to Chen *et al.* (1972).

Apparent Molecular Mass—Apparent molecular mass of the six Trp/Arg hPRL mutants were measured by high pressure liquid gel filtration chromatography. 100-µl samples (500 µg/ml) were loaded on a Superose 12 molecular sieve equilibrated in 20 mM Tris-HCl, pH 8, 100 mM NaCl. Elution was performed in the same buffer at a constant flow rate of 0.5 ml/min, and protein elution was monitored at 280 nm. The column was calibrated with several molecular mass markers: dextran blue (void volume), bovine serum albumin dimers (136 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and myoglobin (17.5 kDa).

### Nb2 Cell Culture and in Vitro Bioassay

The bioactivity of the hPRL analogs was estimated by their ability to stimulate the growth of lactogen-dependent Nb2 lymphoma cells (Gout *et al.*, 1980). The procedure used was that of Tanaka *et al.* (1980). Cells were cultured in Fisher's medium containing 10% horse serum and 10% fetal calf serum (FCS). Twenty-four h before the bioassay, cells were synchronized in culture medium containing only 1% FCS. Bioassays were performed in FCS-free Fisher's medium (referred to as the "incubation medium").

Various amounts of hPRL samples, diluted in incubation medium, were added to 2.5 ml of cells ($1–2 \times 10^5$ cells/ml) plated in 6-well Falcon plates. Two to four experiments were performed in duplicate for each mutant. According to the mitogenic activity of the mutants, appropriate hormone concentration ranges were tested. Nb2 cells were counted with a Coulter counter (Coulter Electronics Ltd., Harpenden Hertsforeshire, U.K.) after 3 days. For each hPRL analog, the ED_{50}, *i.e.* the amount of hormone needed to achieve half-maximal cell growth, was calculated. The relative bioactivity of each mutant with respect to native hPRL was estimated as the ratio of the native *versus* mutant ED_{50} values.

Binding Experiments

Binding of hPRL analogs to the lactogenic receptor was studied on Nb2 cell homogenates in order to avoid any uptake or degradation of iodinated hPRL by intact cells. Preparation of cell homogenates and assay conditions have been described in detail (Goffin *et al.*, 1992). Briefly, homogenates from 3×10^6 cells were incubated for 16 h at 25 °C with 30,000–40,000 counts/min ^{125}I-hPRL in the presence of increasing amounts of unlabeled native hPRL or hPRL analogs (the final reaction volume was 0.5 ml). The assay was terminated by addition of 0.5 ml ice-cold buffer (0.025 M Tris-HCl, 0.01 M $MgCl_2$, 0.2% bovine γ-globulin, pH 7.5) followed by centrifugation (5 min, $11,000 \times g$). The supernatants were removed carefully, and the radioactivity of the pellets was counted in a gamma counter (Hybritech 002011B, Belgium).

Each mutant was tested three times in duplicate, except for S26W, for which a significant displacement curve was obtained from a single experiment. Specific binding was calculated as the difference between radioactivity bound in the absence (B_0, maximal binding) and in the
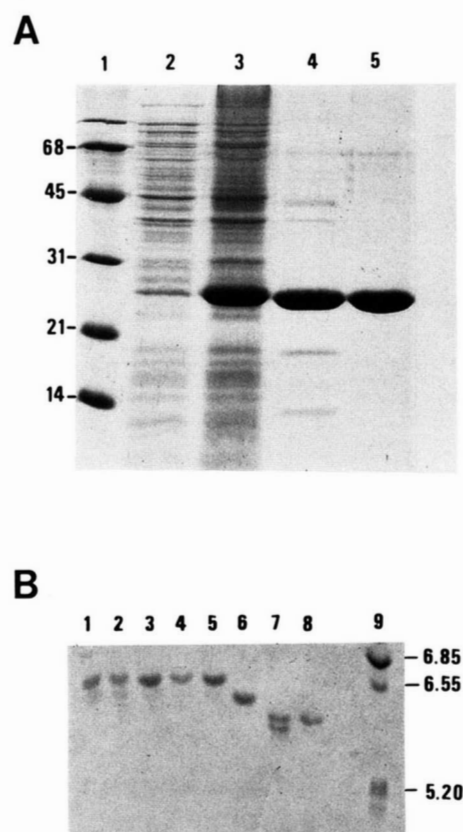
FIG. 2. **Electrophoretic analysis of native hPRL and hPRL analogs.** *A*, SDS-PAGE under reducing conditions (2-ME) of production and successive purification stages of recombinant native hPRL. *Lane 1*, molecular mass markers; *lanes 2 and 3*, 25 µl of BL21(DE3) *E. coli* culture before ($OD_{600} = 0.9$) and after ($OD_{600} = 2.5$) a 4-h induction with 1 mM IPTG, respectively; *lane 4*, 3 µg of insoluble hPRL inclusion bodies; *lane 5*, 3 µg of the monomeric hPRL fraction recovered after Sephadex G-100 gel filtration. *B*, isoelectrofocusing electrophoresis of native hPRL and hPRL analogs. Electrophoresis was performed at 200 V under continuous cooling. Before protein samples were loaded, a pH gradient (5–7 pH range) was allowed to form during a 15-min prerun at the same voltage. One µg of each protein was loaded on the gel. Electrophoresis was stopped when electrofocusing of the methyl red band was achieved (see "Experimental Procedures" for more details). Only PRL analogs with an altered net charge are shown. *Lanes 1–8*, respectively, represent E110A, E118A, E128A, G129R, L25R, native, K115A, and R125A hPRLs. *Lane 9* shows the pI marker proteins.

presence (nonspecific) of 2 µg of unlabeled native hPRL. In the different experiments, nonspecific binding never exceeded 20% of maximal binding. Data are presented as percentages of specific binding. Competition curves were analyzed with the LIGAND PC program (Munson and Rodbard, 1980). The relative binding affinity of each mutant was estimated as the ratio of the native *versus* mutant IC_{50} values.

RESULTS

Production and Purification Yields

Overexpression of recombinant hPRL in *E. coli* was achieved by induction with 1 mM IPTG (4 h) (Fig. 2A, *lanes 2 and 3*). The yield of overexpressed protein was about the same for the 18 hPRL analogs as for native hPRL (±150 mg/liter). In each case, we were able to recover about 100 mg of insoluble inclusion bodies/liter of culture by centrifuging the broken cells (Fig. 2A, *lane 4*). Proteins were solubilized in 8 M urea and refolded during a 72-h dialysis against 20 mM NH_4HCO_3, pH 8. Mutant S26R precipitated extensively during this step. Upon renaturation, recombinant hPRL tends to form covalent (disulfide bonds) and non-covalent aggregates (Paris *et al.*, 1990), and renatured hPRL was routinely purified on a Sephadex G-100 molecular sieve to separate the monomeric hPRL, which eluted

in a single peak (Fig. 2A, *lane 5*), from the aggregated forms, recovered mainly in the void volume of the column (not shown). Usually, monomeric and multimeric peaks were of similar size and around 30 mg of monomeric hPRL was recovered per liter of culture. With the exception of S26R/W mutants, similar amounts of monomer were recovered for all hPRL analogs, attesting a behavior similar to that of native hPRL during renaturation (a similar monomer/aggregates ratio). For the S26R and S26W mutants, however, most of the protein appeared aggregated in the multimeric protein peak. In the monomer peaks, we recovered only 3 mg (S26W) and 0.7 mg (S26R) from the initial 500-ml cultures.

Isoelectric Point

The major isoform of purified recombinant hPRL exhibits a pI of 6.2 (Paris *et al.*, 1990). Introduction or removal of charged residues was assumed to modify the net charge of native hPRL, and indeed, removal of a negative charge or addition of a positive charge (E110A, E118A, E128A, L25R, and G129R mutants) enhanced the pI by almost 0.3 units. Removal of a positive charge (K115A and R125A mutants) had an opposite effect (Fig. 2B). These observations correlate well with theoretical pI calculations predicting values of 6.59 for native hPRL and 6.77 and 6.42, respectively, for the two groups of mutants. In some cases, a second isoform was detected. The presence of more than one isoform has been reported for native hPRL (Paris *et al.*, 1990).

Structural Characterization of the Trp/Arg hPRL Mutants

Small-to-large side chain mutations can generate steric hindrance and lead to protein misfolding (for a review, see Eigenbrot and Kossiakoff, 1992). If this occurs, the observed modifications of biological properties can be erroneously attributed to the mutated residue when in fact a global alteration of protein structure is responsible. Trp/Arg mutants were therefore first structurally characterized.

Circular Dichroism—Prolactins are all α-proteins; circular dichroism is thus appropriate for estimating their overall secondary structure content (Goffin *et al.*, 1992, 1993). Five Trp/Arg mutants were analyzed; the supply of S26R hPRL was insufficient for CD analysis. The spectra are reported in Fig. 3 and the helical contents in Table IA.

The analyzed mutants exhibited spectra typical of all α-proteins, with two minima, at 208 and 222 nm, and a maximum around 195 nm. Spectra obtained with native, A22W, L25R, L25W, and G129R hPRL were almost superimposable; the curve obtained for the G129R mutant is presented in Fig. 3 as an illustration. The helical content was calculated as described previously (Chen *et al.*, 1972). For the above mentioned mutants, helicity lies in the 50–55% range, in keeping with previous analyses of native hPRL (Goffin *et al.*, 1992, 1993).

The spectrum of the S26W mutant is slightly different in that the minimum at 222 nm is less pronounced than the minimum at 208 nm (Fig. 3). Consequently, the calculated helicity is a few percent lower (45%).

Apparent Molecular Mass—The apparent molecular mass of a protein is related to its global folding (shape, compactness). Retention time on a molecular sieve was used to estimate the apparent molecular mass of the six Trp/Arg hPRL mutants. Results are reported in Table IB. None of the six mutants analyzed differed significantly in apparent molecular mass from native hPRL.

Biological Analysis of the hPRL Mutants

To estimate the bioactivity of the hPRL mutants, we measured their ability to stimulate proliferation of rat lymphoma Nb2 cells whose growth is lactogen-dependent. As described
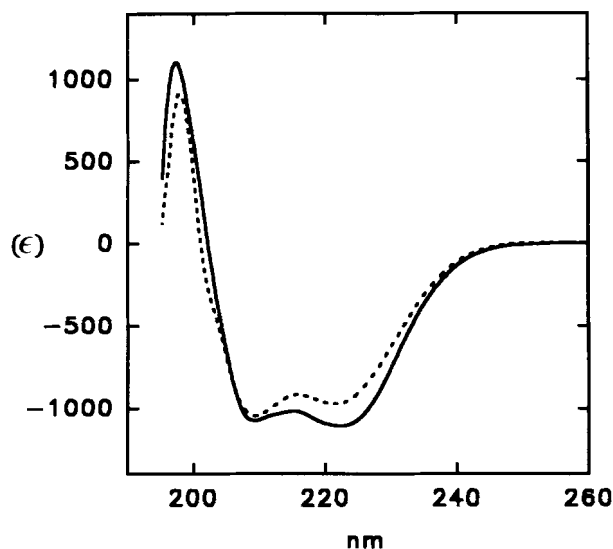

Fig. 3. **Circular dichroism analysis of Trp/Arg hPRL analogs.** Lyophilized protein samples were resuspended at 0.5 mg/ml in 50 mM NH₄HCO₃, pH 8. Spectra were measured in a 0.1-cm pathlength quartz cell in the shortwave UV range (195–260 nm); *ordinate axis* is expressed in M^{-1} cm^{-1}. All spectra exhibit the profile typical of all α-proteins, with two minima (208, 222 nm) and one maximum (195 nm). Spectra of G129R (*continuous line*) and S26W (*broken line*) analogs are shown. Spectra obtained with native, A22W, L25R, L25W, and G129R hPRLs were almost indistinguishable. S26W exhibited a slightly different curve with a less pronounced minimum at 222 nm. Helicity contents of the different proteins are reported in Table IA.

TABLE I
Structural analysis of Trp/Arg hPRL analogs

Part A, the helical content was measured by circular dichroism (see Fig. 3) and calculated at 222 nm according to Chen *et al.* (1972). Mutant S26R could not be tested because of low amounts available. Part B, the apparent molecular mass (MM) of the mutants was estimated by gel filtration on a Superose 12 column mounted on a fast protein liquid chromatography apparatus. The column was eluted with 20 mM Tris-HCl, pH 8, 100 mM NaCl at a flow rate of 0.5 ml/min. The column was calibrated with different MM markers: dextran blue (void volume), dimers (136 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and myoglobin (17.5 kDa). 100 μl (0.5 mg/ml) of each hPRL sample were loaded on the column. Retention times and the corresponding calculated apparent MM are presented.

	Native hPRL	Trp/Arg mutants					
		A22W	L25R	L25W	S26R	S26W	G129R
A. Helicity (%)	55.8	51.6	52.2	50.9		45.0	52.5
B. Retention time (s)	1564	1562	1567	1562	1570	1568	1570
Apparent MM (kDa)	23.5	23.6	23.3	23.6	23.0	23.2	23.0

previously (Goffin *et al.*, 1992), recombinant native hPRL stimulates Nb2 cells as effectively as pituitary-derived hPRL, with half-maximal growth around 100–200 pg hPRL/ml (ED₅₀). Results obtained with the 12 alanine-substitution mutants are summarized in Fig. 4. We defined the "mitogenic potency" of each mutant as the ratio of the ED₅₀ of native hPRL to the ED₅₀ of the mutant hormone. None of the analogs was significantly less potent than native hPRL (mutant I29A was the least mitogenic, with a potency of 67%). Actually, several mutations of residues belonging to helix 3 (Glu[110], Ile[112], Lys[115], Glu[128]) slightly but reproducibly increased the mitogenic potency.

The Trp/Arg substitutions were found to affect the biological properties of hPRL much more strongly. This was demonstrated by measuring, as above, the mitogenic effect of each analog on Nb2 cells (Fig. 5B) and by estimating its binding affinity for the Nb2 receptor (Fig. 5A).
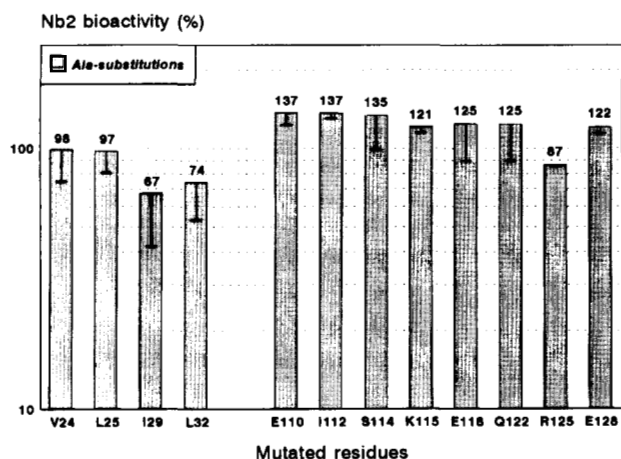
FIG. 4. **Mitogenic activity toward Nb2 cells of the 12 alanine substitution hPRL analogs.** Nb2 cells were cultured as explained under "Experimental Procedures." Bioassays were performed in 2.5 ml of incubation medium containing $1-2 \times 10^5$ cells/ml. Fifty to 100 µl of serial protein dilutions were added to each well. After 3 days, cells were counted in a Coulter counter. The relative mitogenic potency of each mutant was estimated as the amount of native *versus* mutant hPRL required to produce half-maximal proliferation of Nb2 cells (ED_{50}). Each mutant was tested two to four times in duplicate; average values expressed as percentages are indicated. None of the alanine mutants exhibited a significantly decreased mitogenic potency.

Binding affinities were estimated from the ability of each mutant hormone to displace ^{125}I-native hPRL from the Nb2 lactogenic receptor. Typical displacement curves are shown in Fig. 5*A*. As described previously (Goffin *et al.*, 1992), the concentration of unlabeled hPRL producing half-maximal displacement of ^{125}I-hPRL (IC_{50}) is around 2 ng/ml (~ 100 pM). Since all the competition curves are almost parallel in the linear part of the sigmoid curve, comparing IC_{50} values is a good way to estimate the relative affinity of each mutant for the lactogenic receptor. Averaged over three different experiments, the IC_{50} ratio (native value *versus* mutant value) was $16.5 \pm 3\%$ for L25W and $17.9 \pm 0.14\%$ for L25R, while the other analogs exhibited much lower affinity: $0.33 \pm 0.11\%$ (A22W), $0.75 \pm 0.08\%$ (S26R), and $1.07 \pm 0.03\%$ (G129R). Tested in a single experiment because of the limited supply, the measured IC_{50} of mutant S26W was 4853 ng/ml, so its affinity for the receptor is about 0.03% of the affinity of native hPRL.

The mitogenic activity of the Trp/Arg analogs was also strongly altered. Mutations L25R and L25W led to a 2-fold reduction. Averaged over three independent experiments, the mitogenic potencies of the four remaining mutants were 2 to 3 orders of magnitude lower than for native hPRL: $0.217 \pm 0.11\%$ (A22W), $1.7 \pm 1\%$ (S26R), $0.058 \pm 0.04\%$ (S26W), and $0.47 \pm 0.2\%$ (G129R).

To investigate the antagonistic properties of the Trp/Arg mutant hormones, we measured their ability to inhibit native hPRL-stimulated Nb2 cell growth. These competition experiments were made difficult by the intrinsic growth-stimulating effect of each mutant. At concentrations of native hPRL producing maximal cell growth (about 1 ng/ml), no inhibition of cell proliferation by the mutant proteins was observed. At low concentration of native hPRL, however, a slight inhibition was detected (Fig. 6*A*). In the experiment shown, $45 \pm 2\%$ ($n = 4$) of maximal cell growth was achieved at 0.05 ng/ml of native hPRL. Under these experimental conditions, A22W and S26W analogs present at concentrations ranging from 0.1 to 5 ng/ml caused cell proliferation to decrease by about 10%. Inhibition was never more acute. At analog concentrations exceeding 10 ng/ml, mutant-induced cell proliferation occurred. The slightly higher intrinsic activity of the G129R mutant (see above) can be linked
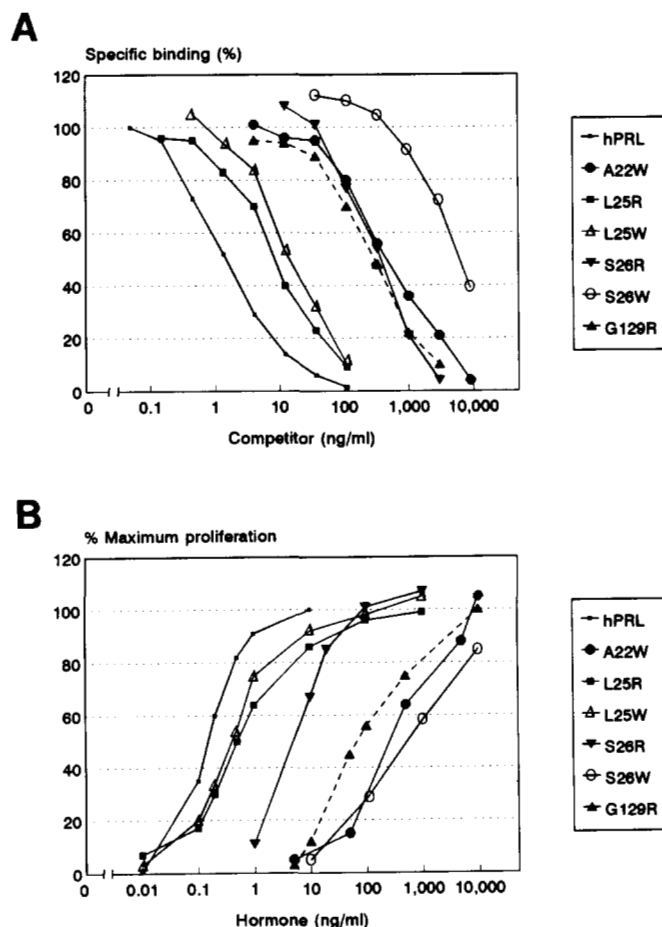

FIG. 5. **Biological analysis of the Trp/Arg hPRL analogs.** *A*, competition curves for the displacement of ^{125}I-labeled native hPRL by unlabeled native hormone and Trp/Arg hPRL analogs. In this experiment, Nb2 cell homogenates prepared from 3×10^6 cells were incubated with 32,000 counts/min of ^{125}I-labeled native hPRL (tracer) and serial dilutions of unlabeled native hPRL or hPRL analogs (competitor). Specific binding is the difference between the radioactivity bound in the absence (B_0) and in the presence (nonspecific) of 2 µg of unlabeled hPRL. In this experiment, nonspecific binding was 17% of B_0. All curves presented in this figure are taken from the same experiment and presented as percentages of specific binding. Each point is the average of duplicate measurements; maximal disparity between duplicate values is 12% of specific binding. The *dotted curve* corresponds to the G129R analog. *B*, Nb2 cell proliferation in the presence of Trp/Arg hPRL analogs. Bioassay conditions are described under "Experimental Procedures." Each mutant was tested in at least three different experiments. Each point is the average of duplicate measurements. Disparity between duplicate values did not exceed 15%. Arg/Trp substitutions of Leu[25] decreased the mitogenic potency by 2-fold. Point mutations at Ala[22], Ser[26], and Gly[129] produced drastically decreased mitogenic potency by 2 to 3 orders of magnitude (see "Results" for accurate values).

with the fact that it causes no real inhibition and that it induces more rapid cell growth (Fig. 6*A*). As expected, mutants L25R and L25W did not compete at all with native hPRL (data not shown); mutant S26R was not tested due to the limited supply.

Besides studying the antagonistic properties of the Trp/Arg mutants, observable only at low protein concentrations (<10 ng/ml), we also investigated their self-antagonistic effects. Self-antagonism is indicated by the occurrence of bell-shaped curves at extremely high protein concentrations (Fuh *et al.*, 1992, 1993). Protein concentrations up to 250 µg/ml (~ 10 µM) were used; solubility problems prevented our testing higher concentrations. The results are presented in Fig. 6*B*. We repeatedly observed no self-inhibition with native hPRL. Mutants R125A and L25R likewise failed to self-antagonize. Mutants G129R (Fig. 6*B*) and A22W (not shown), on the other hand, exhibited

A

B
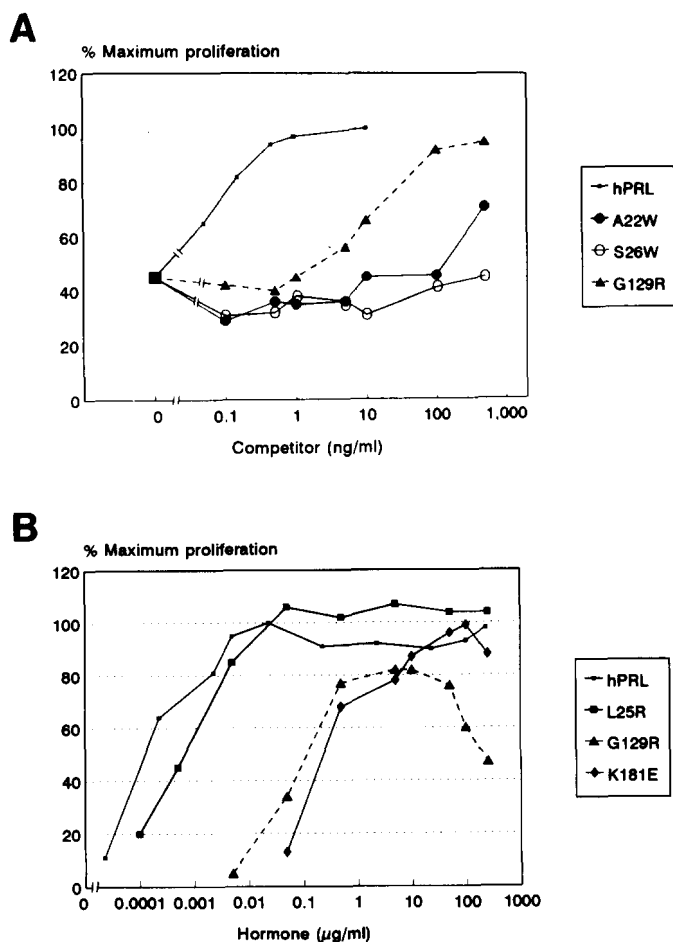
FIG. 6. **Antagonistic and self-antagonistic effects of native hPRL and hPRL analogs in the Nb2 cell proliferation bioassay.** *A,* competition between native hPRL and Arg/Trp analogs. Three Arg/Trp analogs (A22W, S26W, and G129R) were tested for their ability to antagonize native hPRL in the Nb2 cell proliferation bioassay. Proliferation in presence of native hPRL is shown as control. The curves presented are averages of duplicate measurements; maximum disparity between duplicate values was 4% of the maximum effect. In this experiment, 0.05 ng/ml of native hPRL produced $45 \pm 2\%$ ($n = 4$) maximum cell proliferation. In the presence of low concentrations of competitor (0.1–5 ng/ml), cell proliferation decreased to 31–38% with S26W and to 29–40% with A22W; it remained virtually unchanged with G129R. At higher concentrations, the intrinsic relative activity of each mutant (G129R > A22W > S26W, see Fig. 5*B*) additively increased cell division. *B,* self-antagonistic effect at very high concentrations. The occurrence of bell-shaped curves in the Nb2 cell proliferation bioassay at very high hormone concentrations (about 250 µg/ml) was indicative of self-antagonism. Native hPRL and both site 1 and site 2 analogs were tested. The curves presented are averages of duplicate measurements; maximum disparity between duplicate values was 15% of the maximum effect. With native hPRL, no self-inhibition was observed, even at 250 µg/ml. The L25R and R125A mutants (not shown), whose site 2 is altered weakly or not at all, did not self-antagonize either. Mutants G219R whose site 2 is markedly weakened, exhibited self-inhibition in the 50–250 µg/ml range. Analog A22W behaved similarly (not shown). Mutants K181A (not shown) and K181E, whose site 2 is intact but whose site 1 is weakened,[3] failed to exhibit a bell-shaped curve.

a bell-shaped curve with detectable self-inhibition in the 50–250 µg/ml range. By extrapolating the self-inhibition curves in several independent experiments, we were able to determine IC_{50} values for self-inhibition ("self-IC_{50}"). The values obtained were: 252, 256, and 570 µg/ml for G129R, and 680 and 630 µg/ml for A22W. Finally, we tested K181A and K181E hPRL, two mutants whose binding site 1 is dramatically weakened.[3] Neither exhibited self-inhibition at the concentrations tested.

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[3] V. Goffin and G. Guillaume, unpublished results.

DISCUSSION

Alanine Substitutions—To date, no NMR or x-ray structure is available for any PRL. To provide an atomic structure on which to base our mutational studies, we recently constructed a theoretical three-dimensional model of hPRL[2] derived from the x-ray coordinates of porcine GH, the first elucidated PRL/GH protein structure (Abdel Meguid *et al.,* 1987). This model enabled us to select 12 residues assumed to surround the putative binding site 2 of hPRL. Residues Arg[21] and Tyr[28], also presumed to be involved in this site, have already been studied by others (Luck *et al.,* 1990, 1991) and were not reconsidered. The alanine-scanning approach, previously used successfully to identify certain residues involved in the biological properties of hGH (Cunningham *et al.,* 1989, 1991), hPRL (Goffin *et al.,* 1992), or hGHR (Bass *et al.,* 1991), was applied to the dozen residues selected on the basis of our model (see Fig. 1). Unexpectedly (and excepting I29A and L32A mutations whose effects were very slight), none of the alanine substitutions diminished the mitogenic effect of the hPRL analogs on the Nb2 lymphoma cell line (Fig. 4). This means that none of the 12 residues initially selected on the basis of the hPRL model is essential to the hormone's bioactivity. As shown on Fig. 1, our study includes nearly the entire exposed, mutually facing sides of helices 1 and 3, covering some two (helix 1, amino acids 24–32) or five (helix 3, amino acids 110–128) helix turns. Therefore, systematic misprediction of residues presumably involved in the second binding site seems very unlikely. Moreover, the proposed location for hPRL-binding site 2 is in good agreement with the observed decrease of bioactivity of bPRL when Arg[21] or Tyr[28] are mutated or Arg[125] deleted (Luck *et al.,* 1990, 1991). Studies performed on the binding site 1 of hGH have revealed that polypeptide regions can remain virtually insensitive to alanine substitution (Cunningham and Wells, 1989; Cunningham *et al.,* 1989) although their involvement at the hormone-receptor interface is demonstrated by structural (de Vos *et al.,* 1992) or energetic (Cunningham and Wells, 1993) analysis. Thus, while interactions predicted by alanine substitution do occur, some residue contacts can be missed by this mutational approach. This could account for the lack of effect in the present study.

Some helix 3 mutants displayed weak, but reproducible, increased bioactivity (Fig. 4). Replacement of exposed residues within α-helices by alanine tends to stabilize a protein (for a review, see Fontana, 1991). In the present case, increased stability might explain the slightly enhanced activity. Alternatively, Ala substitution of large, hydrophilic residues (Glu[110], Lys[115], Glu[128]) might favor the interaction with the large, hydrophobic Trp residues of the receptor. These hypotheses and others deserve further analysis.

Tryptophan/Arginine Substitutions—Alanine scanning having proved unsuccessful for our purpose, we turned to the opposite strategy, *i.e.* mutating some residues to much larger ones (Trp or Arg). The aim was to fill the helix 1-helix 3 cavity assumed to form binding site 2 of hPRL. Four amino acids were selected for their proximity to the helix-helix interface and for the size of their side chains: Ala[22], Leu[25], Ser[26], and Gly[129] (Fig. 1).

Of the four selected residues, Ser[26] is the most buried (Fig. 1). Extensive aggregation and precipitation of S26W/R analogs upon renaturation step probably reflects an effect of the mutations on protein folding. Expectedly, introducing a charge (Arg) near the hydrophobic core was more detrimental to folding than the steric effect alone (Trp). Although we detected no modification of the apparent molecular mass for these mutants (Table I), their structure is likely to be affected locally around the Ser[26] mutations. None of the four remaining Trp/Arg mu-

tants (A22W, L25R, L25W, G129R) appeared to be misfolded, since the renaturation yield, the CD analysis, and the apparent molecular mass were practically unchanged (Table I). Any effects on bioactivity observed with these mutants can thus be attributed to effects of the mutations on the local environment rather than to a major alteration of protein structure.

Mutations A22W and G129R reduced both the binding affinity and the Nb2 bioactivity by 2 to 3 orders of magnitude. This result strengthens our starting hypothesis concerning the critical functional role of the hydrophobic channel between helices 1 and 3 (Fig. 1). Mutations at position 26 caused similar (S26R) or even worse (S26W) alterations of biological properties. As mentioned above, these data must be considered with caution since structural effects might be involved. Although S26R appears to be more strongly affected structurally (see above), S26W hPRL is 30-fold less active (binding and bioactivity; Fig. 5); this suggests that the effect of the latter mutation is due, at least in part, to the steric hindrance produced by the Trp substitution. Mutating Leu[25] reduced bioactivity and binding by only 2- and 5-fold, respectively. As Leu[25] borders on the central channel and is oriented toward the solvent,[2] replacement of this residue by either Trp or Arg is more liable to interfere with receptor docking than to fill the central channel and thereby block binding site 2. Moreover, leucine is a medium-sized residue, and the steric effect of the mutations, especially to Arg, should be considerably less marked than when smaller residues are replaced (Ala, Ser, Gly).

The positions of the residues selected for the Trp/Arg mutational study are incompatible with an involvement in binding site 1, delimited by the opposite face of helix 1, helix 4, and loop 1 (Goffin *et al.*, 1992, 1993). Our results thus strengthen the hypothesis that the region delimited by the mutually facing sides of helices 1 and 3 has an important functional role. They thereby provide some experimental evidence as to the existence and location of a second binding site on hPRL. Residues directly involved in contacts with the receptor are probably not limited to those which were mutated, since the steric hindrance resulting from small-to-large side chain substitutions can prevent other surrounding amino acids from interacting with the receptor as they do in the native hormone. However, since alanine scanning failed to point out any binding residue, exhaustive identification of the amino acids involved in the interaction with the receptor awaits determination of the three-dimensional structure of the PRL·PRLR complex.

Model of PRL-PRLR Interaction—From the behavior of the G120R analogue, a full hGH antagonist, Fuh and colleagues (1992, 1993) proposed a sequential dimerization model: hGH would first bind to one receptor through binding site 1 to form an intermediate, inactive 1:1 complex. Then the receptor-bound hGH would interact with a second receptor through binding site 2 to produce the active 1:2 complex (Fig. 7A). Occurrence of sequential dimerization can be indirectly visualized in a dose-dependent cell growth bioassay (Fuh *et al.*, 1992, 1993). At low hGH concentrations (<1 nM), 1:2 complexes are progressively formed and the growth-promoting effect is observed. At high hGH concentrations (>100 nM), excess hormone progressively disrupts the 1:2 complexes in favor of inactive 1:1 complexes, and cell growth is progressively inhibited. Such proliferation curves, referred to as "bell-shaped" curves (Fuh *et al.*, 1992), thus constitute experimental evidence of GH self-antagonism linked with sequential dimerization of its receptor. Given the similarity between hGH and hPRL, we expected hPRL to behave like hGH in the hGH-GHR interaction and thus also to exhibit a bell-shaped curve in the Nb2 cell proliferation bioassay. Testing concentrations up to 250 μg/ml (about 10 μM), we failed to detect any significant self-inhibition at high concen-

A. Model of the hGH/hGHR interaction
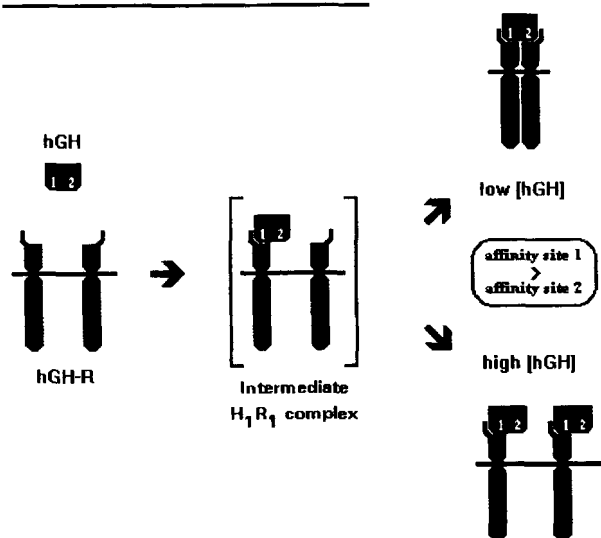
B. Model of the hPRL/PRLR interaction
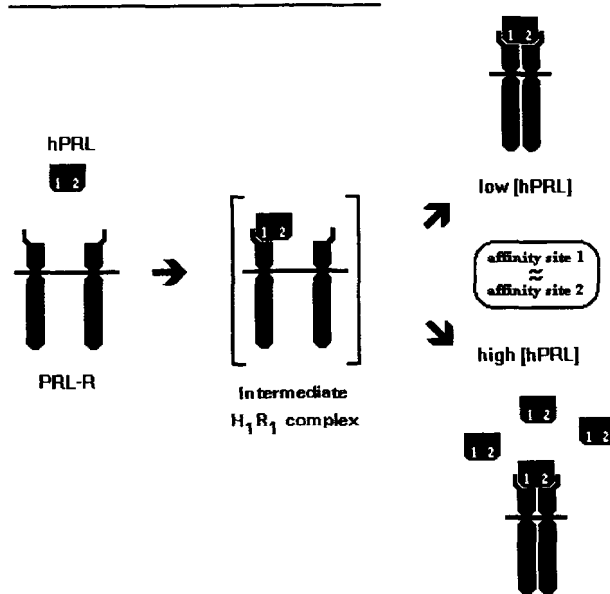
FIG. 7. **Models of hormone-receptor interaction: comparison of hPRL and hGH.** The *upper panel* represents the model recently proposed for the interaction of hGH with the somatogenic receptor (Fuh *et al.*, 1992); the interaction between hGH and the lactogenic receptor (Nb2) is assumed to be similar (Fuh *et al.*, 1993). The *lower panel* represents the model we propose for the interaction of hPRL with the lactogenic receptor (Nb2). In both cases, an inactive intermediate hormone $(H)_1$-receptor$(R)_1$ complex is formed first, always via receptor binding to site 1 of the hormones. At low hormone concentration, an active $H_1 \cdot R_2$ complex is formed. The behavior of hPRL and hGH differs at high hormone concentration. For hGH (*upper panel*), since the affinity of binding site 2 is much lower than that of binding site 1, excess of hormone binds preferentially through its binding site 1, and active $H_1 \cdot R_2$ complexes are progressively disrupted in favor of inactive $H_1 \cdot R_1$ complexes; this model accounts for the self-antagonism of hGH in both lactogenic and somatogenic bioassays (Fuh *et al.*, 1992, 1993). In the case of hPRL (*lower panel*), we propose that the affinity of both binding sites is similar. Therefore, excess of hormone cannot disrupt the previously formed $H_1 \cdot R_2$ but remains free in solution. This model accounts for the absence of self-inhibition at high native PRL concentrations (Fig. 6B). The A22W and G129R mutants, whose binding site 2 is markedly weakened, behave like hGH and self-antagonize at high concentrations.

tration. We thus tried to elucidate the disparity between the behaviors of hPRL and hGH.

Self-antagonism of hGH results not only from the existence of two binding sites but also from differences in their properties: site 1 displays a large surface and a high affinity, site 2 a smaller surface and lower affinity (Cunningham *et al.*, 1991; de Vos *et al.*, 1992). At high hGH concentrations, consequently, competition for receptor binding between site 1 (on the free hormone) and site 2 (on the intermediate hormone-receptor complex) is in favor of the former (Fuh *et al.*, 1992). When excess hGH is added to preformed 1:2 complexes, moreover, the equilibrium is displaced toward formation of 1:1 complexes. This indicates that 1:2 complex formation is reversible (Cunningham *et al.*, 1991). In sequential binding of hGH, the molarity at which self-inhibition occurs (formation of 1:1 complexes from 1:2 complexes) seems to correlate with the relative affinities of the two binding sites. As an illustration, the EC_{50} for cell proliferation and the IC_{50} for self-antagonism of native hGH are separated by 10^5 log units; this range drops to 3×10^3 log units in a triple mutant (H21A/R64K/E174A) exhibiting a 30-fold increased affinity at binding site 1 (Fuh *et al.*, 1992). Conversely, a mutant whose affinity at binding site 1 is decreased by 560-fold (K172A/F176A) exhibited no detectable self-inhibition at the concentration tested (Fuh *et al.*, 1992). In other words, the higher the difference in affinity between both binding sites, the smaller the concentration range separating agonistic from self-antagonistic effects.

Linking these observations on hGH mutants with the absence of any detectable self-antagonism of native hPRL, we propose a model of hPRL-PRLR interaction which differs from the hGH model in that binding sites 1 and 2 would have similar, if not identical, receptor binding affinities (Fig. 7B). In such a model, the 1:2 complexes should be much more stable than the 1:1 complexes because they result from two high affinity binding events. Excess hPRL should therefore neither lead preferentially to the formation of 1:1 complexes through binding site 1 nor reverse preformed 1:2 complexes in favor of less stable 1:1 complexes.

Since the difference between the hGH and hPRL models is based on the relative affinities of their two binding sites, we challenged our theory by analyzing several available hPRL mutants at very high concentration (Fig. 6B). As expected, mutants whose site 2 was unaffected (R125A) or weakly affected (L25R) behaved like native hPRL and did not self-antagonize. Conversely, mutant hPRLs with a markedly weakened binding site 2 (G129R and A22W) exhibited self-inhibition as does hGH (Fig. 7A). It was also important to see whether sequential binding occurs preferentially through one of the binding sites. We therefore tested mutants K181A and K181E whose binding site 1 is weakened by 2 and 3 orders of magnitude, respectively.[3] Although this reduction in affinity is similar to that of binding site 2 in A22W and G129R, none of these site 1 mutants exhibited self-inhibition at the concentration tested. This strongly suggests that sequential binding does occur first via site 1. When available, other site 1 analogs should be used to confirm these observations.

Our model can also account for the results of the competition experiments performed with the Trp/Arg mutants. When native hPRL is present at a concentration producing maximum cell growth (we tested the 1–10 ng/ml range, data not shown), almost all the receptors (R) are occupied in $H_1 \cdot R_2$ complexes with the wild-type hormone (H). Added at low concentrations, mutants with a weakened site 2 cannot displace these stabilized complexes and are ineffective. Added in excess, however, such mutants exhibit intrinsic mitogenic activity which adds to that of the native hPRL. When the concentration of native

hPRL is lower (0.05 ng/ml; Fig. 6A), many receptors remain unoccupied by the hormone. The mutants should bind to the free receptors and mainly form $H_1 \cdot R_1$ complexes since the weakened affinity of binding site 2 markedly reduces the occurrence of $H_1 \cdot R_2$ complexes. The receptors thus remain blocked in an almost inactive state. That no more than 10% inhibition of cell proliferation could be achieved in competition experiments even with a 100-fold excess (5 ng/ml) of mutant might reflect the analogs' inability to displace preformed 1:2 complexes between the receptor and native hPRL. At higher concentrations, the analogs exhibit their intrinsic mitogenic activity; their effects and those of native hPRL are additive. Full understanding of the competition studies will require, however, further analysis.

Finally, our model of the hPRL-PRLR interaction can account for the failure of the alanine-scanning study. Due to the high binding affinity of the hPRL-binding site 2, alanine substitutions alone, conversely to mutations which markedly alter the affinity of binding site 2 (A22W, G129R), might be insufficient to reduce cell proliferation. In hGH, the single G120R mutation completely blocks binding site 2, while in hPRL, G129R, and A22W mutations give rise to weak agonists. This also might reflect the higher affinity of binding site 2 in hPRL.

Conclusion—Our study demonstrates that the region delimited by the interface between helices 1 and 3 is crucial to hPRL bioactivity. Alanine scanning was inappropriate for identifying some functionally important residues. The Trp/Arg mutant data suggest, indeed, that at least some of the 12 residues selected for alanine substitution could be involved in contacts with the receptor. The model we propose for the PRL-PRLR interaction differs from the earlier hGH-hGHR model in that a similar affinity is predicted for both binding sites. Our data highlight the steric importance of small residues such as Ala^{22} or Gly^{129} in maintaining the geometry of binding site 2. We would expect PRLs from other species, possessing more cumbersome residues at these and/or other positions (in rodent PRL, for example, position 22 is occupied by a valine residue), to behave differently at high concentration.

Our results do not tally with some of the studies performed with the soluble extracellular domain of the PRLR, predicting an $H_1:R_1$ stoichiometry (Gertler *et al.*, 1993; Bignon *et al.*, 1994). As stated by the authors, however, the (in)ability of the extracellular domain to form 1:2 complexes does not necessarily reflect the biological activity of the hormones tested (Bignon *et al.*, 1994). Dimerization of membrane-anchored receptors might be markedly facilitated by additional interactions involving the transmembrane and/or cytoplasmic regions of the receptor. This would obviously weaken interactions between the two molecules in the presence of the extracellular domain alone. This work calls for further study: the antagonistic properties of the available analogs should be better characterized, new mutants should be designed, and, as usually formulated, the three-dimensional structure of receptor-bound hPRL should be determined.

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