Structural and Thermodynamic Bases for the Design of Pure Prolactin Receptor Antagonists

X-RAY STRUCTURE OF Del1–9-G129R-hPRL

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Competitive antagonists of the human prolactin (hPRL) receptor are a novel class of molecules of potential therapeutic interest in the context of cancer. We recently developed the pure antagonist Del1–9-G129R-hPRL by deleting the nine N-terminal residues of G129R-hPRL, a first generation partial antagonist. We determined the crystallographic structure of Del1–9-G129R-hPRL, which revealed no major change compared with wild type hPRL, indicating that its pure antagonistic properties are intrinsically due to the mutations. To decipher the molecular bases of pure antagonism, we compared the biological, physicochemical, and structural properties of numerous hPRL variants harboring N-terminal or Gly129 mutations, alone or combined. The pure versus partial antagonistic properties of the multiple hPRL variants could not be correlated to differences in their affinities toward the hPRL receptor, especially at site 2 as determined by surface plasmon resonance. On the contrary, residual agonism of the hPRL variants was found to be inversely correlated to their thermodynamic stability, which was altered by all the Gly129 mutations but not by those involving the N terminus. We therefore propose that residual agonism can be abolished either by further disrupting hormone site 2-receptor contacts by N-terminal deletion, as in Del1–9-G129R-hPRL, or by stabilizing hPRL and constraining its intrinsic flexibility, as in G129V-hPRL.

Human (h) prolactin receptor (PRLR) antagonists are a new class of potential drugs developed to target prolactin (PRL)-sensitive pathologies that cannot be treated with current inhibitors of the production of hPRL by the pituitary (1). These include dopamine-resistant prolactinomas (i.e. pituitary tumors of PRL-secreting cells), as well as breast cancer, prostate cancer, and benign prostate hyperplasia, in which evidence for the tumor growth-promoting actions of autocrine PRL has been emerging within the past decade (2, 3). In view of the ubiquitous expression pattern of the PRLR (4), these indications are not necessarily exhaustive and may be extended to other pathologies that remain to be identified (or better characterized) with respect to the involvement of locally produced PRL in their etiology.

Because of their mechanism of action involving competition with endogenous hPRL for receptor binding, competitive PRLR antagonists need to be used in molar excess compared with endogenous hPRL. Therefore, the most promising compounds are anticipated to be those that are devoid of any residual agonistic properties, even at high concentration (5). We recently developed an antagonist exhibiting such unique properties, referred to as Del1–9-G129R-hPRL. In contrast to other antagonists developed to date (for reviews see Refs. 2, 6), Del1–9-G129R-hPRL was shown to be devoid of residual agonism in every cell or animal model in which it has been tested to date (5, 7–11).

As highlighted by its name, Del1–9-G129R-hPRL is a hPRL core protein containing two modifications: deletion of the nine N-terminal residues, and substitution of Gly129 for an Arg (5). The rationale for engineering this variant was based on previous structure-function studies performed in the Goffin laboratory. First, as demonstrated for all members of the PRL/growth hormone (GH)/placental lactogen (PL) family (2), substitution of the conserved helix 3 Gly for an Arg was shown to drastically impair the agonistic properties of hPRL while maintaining its ability to bind to the PRLR (12–14). Second, the sole G129R
mutation appeared to be insufficient to completely abolish the ability to activate the PRLR, as highlighted in sensitive cell bioassays (12, 15) or in transgenic mice overexpressing this variant. Third, mutations of the N terminus, which is interestingly the most divergent region within the PRL/GH/PL family, were shown to slightly modulate the properties of hPRL (16).

Although the combination of the N terminus deletion and the G129R mutation was successful in achieving the goal of generating a pure PRLR antagonist, the mechanism underlying these unique properties remained poorly understood. Although structural studies of the hGH-hGHR complex have clearly identified the helix 3 Gly pocket as a critical characteristic of binding site 2 (17, 18), the roles of the structurally equivalent region (Gly\textsuperscript{129}), and even more of the N terminus, in receptor binding and activation have remained largely speculative for hPRL. Ultimately, this lack of information about the molecular bases of our best antagonist hampers its improvement through knowledge-assisted strategies.

The aim of this work was to elucidate the importance of these two hPRL modifications at the molecular level, using a combination of structural, biophysical, and biological approaches. First, to identify the structural characteristics underlying its pure antagonistic properties, we determined the three-dimensional structure of Del\textsubscript{1–9}-G129R-hPRL by x-ray diffraction. Second, we generated numerous hPRL variants harboring single or combined modifications at the N terminus (deletion/elongation) and the Gly\textsuperscript{129} residue (different substitutions), to either or combined modifications at the N terminus (deletion/elongation) and the Gly\textsuperscript{129} residue (different substitutions), to

### EXPERIMENTAL PROCEDURES

#### Materials

Culture media, fetal calf serum (FCS), genetin (G-418), trypsin, and glutamine were purchased from Invitrogen. Luciferin and cell lysis buffer were from Promega (Madison, WI), and luciferase activity was measured in relative light units using a Lumat LB 9501 (Berthold, Nashua, NH). IODO-GEN was purchased from Sigma, and carrier-free Na\textsuperscript{125}I was obtained from GE Healthcare. Oligonucleotides were from Eurogentec (Liège, Belgium). All immobilization reagents used for surface plasmon resonance experiments were purchased from Biacore (Uppsala, Sweden). Optimization of crystallization conditions was performed in Linbro plates from Hampton. Chemicals were purchased from Sigma, VWR (Fontenay-sous-Bois, France), or Merck.

#### Site-directed Mutagenesis

Expression plasmids encoding WT hPRL, G129R-hPRL, and Del\textsubscript{1–9}-G129R-hPRL were available from previous studies (5, 12). They were used as templates for generating the other mutants of this study, using the QuikChange II mutagenesis kit from Stratagene (La Jolla, CA). Sequences of forward and reverse (complementary) primers are given in Table 1, with mutated codons underlined. The same primers were used for generating Nter-hPRL and Nter-G129R-hPRL plasmids using, respectively, WT hPRL and G129R-hPRL encoding plasmids as templates. For all steps, we strictly followed the recommendations of the manufacturer. After transformation, Escherichia coli BL21(DE3) colonies were analyzed for their DNA content; plasmids were sequenced to verify the presence of the expected mutations.

Plasmids encoding the extracellular domain (ECD) of human or rat (r) PRLR were generated by PCR amplification using plasmids containing the full-length receptor cDNA (19, 20); sequences encoding residues 1–210 of WT PRLR were inserted at Sp\textsubscript{Hi1}-BamHI sites into the pQE-70 expression plasmid containing a His\textsubscript{6} tag at the C-terminal end (Qiagen, Courtaboeuf, France). Subcloning constraints led to the addition of four amino acids just before the His\textsubscript{6} tag (Gly\textsuperscript{211}-Ser\textsuperscript{212}-Arg\textsuperscript{213}-Ser\textsuperscript{214} for hECD, and Arg\textsuperscript{211}-Ser\textsuperscript{212}-Arg\textsuperscript{213}-Ser\textsuperscript{214} for rECD) as described (21).

#### Production and Purification of hPRL Variants and hPRLR-ECD

Recombinant WT hPRL, hPRL variants, and PRLR ECDs were overexpressed in 0.5–1-liter cultures of E. coli BL21(DE3) and purified as described previously (22), with minor modifications. Briefly, when the A\textsubscript{600} of bacterial cultures reached 0.7–
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0.9, overexpression was induced using 2 mM isopropyl-1-thio-
β-ν-galactopyranoside for 4 h (A₆₀₀ = 2–2.5 after 4 h). Cells
were broken using high pressure (French press). Proteins
were overexpressed as insoluble inclusion bodies, which were
solubilized in 8 M urea (5 min at 55 °C and then 2 h at room
temperature) and refolded by continuous dialysis (72 h, 4 °C)
against 100 volumes of 25 mM NH₄HCO₃, pH 8.6. Solubilized
proteins were centrifuged and then loaded onto a HiTrap Q
anion exchange column (GE Healthcare) equilibrated in 25 mM
NH₄HCO₃, pH 8.6. Prolactin (WT and variants) and receptor
ECDs were eluted along a NaCl gradient (0–500 mM), and
the major peak was collected, quantified, and kept frozen until use.
Purity of the various hPRL variant/receptor ECD batches was
>95% as judged from SDS-PAGE analysis.

Crystallization of Del1–9-G129R-hPRL and X-ray Diffraction
Data Collection

Initial crystallization screening was performed in 96-well sit-
ting drop crystallization plates (Greiner Bio-One) using a Cybi-
Disk robot from Cybio. Crystallization screens were set up using
several commercially available high throughput crystalliza-
tion screening kits (Hampton Research). Crystals appeared as
needle clusters after 1 week at 18 °C in several conditions (29,
36, 41) of the MemFac crystallization kit.

The initial crystals were refined using standard techniques,
which lead to isolated needles too thin to be collected. A second
screening was performed by mixing 75% of the optimized crys-
tallization solution with 25% of the crystal screen kits from
Hampton. Larger needles appeared in five new conditions.
After manual optimization in Linbro plates using the hanging
drop method, only one led to diffracting crystals. The corre-
sponding reservoir was composed of 100 mM Tris-HCl, pH 8.5,
675 mM K₂HPO₄, 45 mM (NH₄)₂PO₄, 50 mM LiSO₄, 7.5% glycerol,
8% PEG4000. The drop was formed by mixing 1.5
against 100 volumes of 25 mM NH₄HCO₃, pH 8.6. Solubilized
protein at 10 mg/ml with 1.5

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675 mM K₂HPO₄, 45 mM (NH₄)₂PO₄, 50 mM LiSO₄, 7.5% glycerol,
8% PEG4000. The drop was formed by mixing 1.5 µl of protein
at 10 mg/ml with 1.5 µl of reservoir. The largest final
crystal size was 40 × 40 × 200 µm³. Best diffracting crystals
were flash-frozen in liquid nitrogen without previous soaking.
A complete data set at 2.6 Å resolution was obtained on the
ID29 beam line at ESRF (Grenoble, France).

The diffraction images were reduced, scaled, and merged
with programs MOSFLM and SCALA (23, 24). The intensities
were then converted to the structural factor amplitudes with
TRUNCATE (23, 24). A summary of the crystallographic data
and refinement statistics is given in Table 2. The crystals
belonged to space group I4 with unit cell dimensions of a = b =
122.59 Å, c = 28.68 Å. The Matthews coefficient (2.25) and
solvent content analysis (~42%) indicated the presence of one
antagonist molecule in the asymmetric unit.

| Table 2 |
| --- |
| X-ray diffraction data collection and refinement statistics for Del1–9-G129R-hPRL |
| Parameter | Value |
| Data collection | 14 |
| Space group | 20.0-2.7 (2.77-2.77) |
| Resolution (Å) | 5139 (375) |
| Total no. of reflections | 11/632 |
| Mean B value (Å²) | 31.8 |
| Rmerge (%) | 21.1/30.2 (29.0/35.6) |
| Free Rmerge (%) | 0.919/0.831 |
| Total no. of residues/water | 191/48 |
| Allowance | 9.9 |
| Refinement | 9.0/5.0 |
| Rfactor | 21.1/30.2 (29.0/35.6) |
| Resolution (Å) | 31.8 |
| Rfactor | 21.1/30.2 (29.0/35.6) |
| Resolution (Å) | 31.8 |
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Values in parentheses are for the highest resolution shell.

Circular Dichroism

Far-UV CD spectra as well as temperature melts of all hPRL
variants were recorded on a Jasco 810 spectropolarimeter. Temperature melts were performed in 0.01 M NaH₂PO₄, pH 7.4, using a water-jacketed cuvette of 1-cm light path length, from 37 to 95 °C, and the change in signal followed at 222 nm. Far-UV CD spectra were run at 37 °C from 250 to 190 nm, with a 1-cm path length. However, because of high absorbance from buffer, no signal below 197 to 195 nm could be interpreted. Protein concentrations were set at ~1–2 µM for the tempera-
ture melts and far-UV CD spectra.

Unfolding was assumed to follow a two-state transition. Cir-
cular dichroism thermal scans were fitted to the Gibbs-Helm-
holz equation as described (33), using Gnuplot. Because of the high melting temperatures, which result in the lack of sufficient post-transition regions, it was assumed that the unfolded state
was unchanged by the mutations. The slope of the post-transi-
tion region was therefore extrapolated for each mutant protein
from the well determined post-transition of G129P-hPRL. The
results of the fits are melting temperature (T_m) and change in
enthalpy on unfolding at T_m (∆H(T_m)).
Surface Plasmon Resonance

Immobilization of PRLR ECD—As a first approach, the ECDs (human or rat) were covalently coupled in a random orientation, through their solvent-accessible primary amine groups, to the carboxymethylated dextran matrix of a CM5 sensor chip, using a Biacore 2000 instrument and the amine coupling kit (Biacore), according to manufacturer’s instructions. Briefly, each flow cell, equilibrated at a flow rate of 5 μl/min in phosphate-buffered saline (PBS, pH 7.4, supplemented with 0.005% Tween 20), was activated for 12 min with an NHS-EDC solution (50 mM N-hydroxysuccinimide and 200 mM N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide), followed by an injection of the ECD of interest (100 nM) in 10 mM sodium acetate, pH 4.5. Methylaminopropyl) carbodiimide), followed by an injection of

Reference Surface, after activation by NiCl2 and NHS-EDC, and loaded with 500 μM NH4HCO3, pH 8.6, 150 mM NaCl, 0.005% Tween 20). For each flow cell, equilibrated at a flow rate of 5 μl/min in PBS supplemented with 0.005% Tween 20 and 50 μM EDTA, was sequentially loaded with 500 μM NiCl2 for 4 min and activated with NHS-EDC for 2 min. The His6-tagged ECD (100 nM in PBS/Tween/EDTA) was injected until 500 – 2,000 resonance units were captured by the Ni-NTA moieties. The surface was finally deactivated by a 4-min ethanolamine injection, followed by a 2-min injection of EDTA 0.35 M. One flow cell was used as a reference surface, after activation by NiCl2 and NHS-EDC, and deactivation by ethanolamine and EDTA.

Real Time Binding Assays—All the binding assays were performed at 25 °C at a flow rate of 20 μl/min, in running buffer (25 mM NH4HCO3, pH 8.6, 150 mM NaCl, 0.005% Tween 20). For site 1 characterization, 5–8 different concentrations (ranging from 0.54 to 350 nM) of the hPRL variants were injected for 12 min onto the PRLR surfaces, followed by a 10-min dissociation period. At the end of each cycle, the sensor chip was regenerated by two 1-min injections of 2 M MgCl2 (followed by a 1-min injection of EDTA 0.35 M for Ni-NTA chips only). For site 2 characterization, the PRLR surface was first saturated with each hPRL variant (350 nM). Five concentrations of ECD (ranging from 1.7 to 350 μM) were then injected for 2 min on the ECD-PRL complexes, followed by a 5-min dissociation period. Control experiments showed the absence of detectable ECD-ECD interaction in the absence of hormone (not shown).

Data Analysis—All the association and dissociation profiles were double-referenced using the Scrubber 2.0 software. The site 1 binding curves were globally analyzed with a nonlinear least squares algorithm implemented in the BIAevaluation 4.1 software (Biacore), using single-exponential functions of time (Langmuir monovalent binding model). The site 2 binding curves were subjected to steady-state analysis. Kinetic parameters (k_on and k_off) and equilibrium dissociation constants (K_d) were determined based on at least two experiments.

Cell-based Binding Assays

Binding affinities of hPRL variants were determined using cell homogenates of HEK 293 cells stably expressing the human PRLR (so-called HL-5 clone), following a procedure described previously (14). Briefly, hPRL was iodinated using IODO-GEN, and binding assays were performed overnight at room temperature using 150 – 300 μg of cell homogenate protein in the presence of 20,000 – 30,000 cpm 125I-labeled hPRL and increasing concentrations of unlabeled competitor (WT or mutated hPRL). Results presented are representative of at least three independent experiments performed in duplicate. The relative binding affinities of hPRL variants were calculated with respect to that of WT hPRL based on their IC50.

Cell-based Bioassays

The biological properties of hPRL variants were analyzed using two homologous bioassays that we recently developed for human lactogens (15). The transcriptional assay involved HEK 293 cells stably expressing the hPRLR and the lactogenic hormone-response element-luciferase reporter gene (HL-5 clone); it was used to determine the antagonistic properties by competing a fixed concentration (9 nM) of WT hPRL with increasing amounts of each variant (14, 15, 35). A proliferation assay was also performed to determine the residual agonistic activity of the antagonists, using Ba/F-03 cells stably expressing the human PRLR (referred to as Ba/F-LP cells); this assay has been shown to be much more sensitive than the HL-5 clone to reveal low level agonistic effects (5, 15, 36). These assays were run as follows.

Antagonism Bioassay (HL-5 Cells)—The HL-5 clone was routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 700 μg/ml G-418 (for clonal selection). The assay was performed in 96-well plates using 50,000 cells/100 μl/well in medium containing only 0.5% FCS. Cells were allowed to adhere for 6 h and then 100 μl of (2×) hormones diluted in 0.5% FCS medium were added to each well. After overnight stimulation, cells were lysed (50 μl lysis buffer; Promega) and then luciferase activity contained in 10–20 μl of cell lysate was measured for 10 s. To avoid inter-assay variations, all variants to be compared were systematically tested in the same experiment, and data obtained in one experiment representative of at least three experiments performed in duplicate are shown. In antagonism experiments, a mix of 50 μl of (4×) hormone variants combined with 50 μl of (4×) WT hPRL (to obtain a final concentration of 9 nM) were added. Experimental data were plotted using the GraphPad software.

Agonism Bioassay (Ba/F3-LP cells)—Ba/F3-LP cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 500 – 1000 μg/ml G-418, and 10 ng/ml WT hPRL instead of interleukin-3 (nontransfected Ba/F3 cells are dependent on that cytokine for survival/growth). For the assay, cells were starved for 6 h in 1–2% FCS RPMI medium (with additives) and then distributed in 96-well plates at a density of 50,000 cells/100 μl/well in the same medium (excluding hormones). One hundred μl of [2x] hormones were added to
each well. Cell proliferation was monitored after 2 days of hormonal stimulation using 10–15 μl of cell proliferation reagent WST-1 (Roche Applied Science). Experiments were performed at least three times in triplicate. Experimental data were plotted using the GraphPad software.

RESULTS

Determination of the Del1–9-G129R-hPRL Three-dimensional Structure

The totality of the crystallized sequence has been positioned in the electron density. The refined structure is composed of 1567 protein atoms and 48 water molecules. The secondary structure corresponds to the classical long chain cytokine fold consisting of four main α-helices (Leu15 to His46, Gln77 to Arg103, Glu110 to Val137, and Glu161 to Ile194) arranged in an up-up-down-down topology with two long overhand loops termed 1 and 3 (Fig. 1A). A short 3₁₀ helix is present from His⁵⁹ to Ser⁶² (helix 1’), and two additional small α-helices are located from Lys⁶⁹ to Gln⁷⁴ (helix 1”) and from Gly¹⁵² to Gln¹⁵⁷ (helix 3’). Helix 3’ was not observed in the NMR structure of WT hPRL, and helix 1 is four residues longer in the crystal structure of the antagonist, possibly reflecting the more dynamic state of the soluble protein.

There is one disulfide bond involving residues 58 and 174, which links loop 1 to helix 4. The disulfide bond between Cys¹⁹¹ and Cys¹⁹⁹ described in both the crystallographic structure of hPRL (PDB code 1Z7C; Ref. 37) and in the NMR structure of WT hPRL (PDB code 1RW5; Ref. 21) is not present in the antagonist. Instead, the C-terminal Cys is in close contact with Ser¹¹ in a symmetry-related molecule (Fig. 1E). The natural residue at position 11 in WT hPRL is a Cys, which is involved in a disulfide bond with Cys⁴. In the antagonist, as the N-terminal nine first
residues have been omitted, Cys11 has been mutated to Ser (to avoid a free Cys; Ref. 5), and residues 10–14 are thus free to be oriented differently. This loop packs on the C-terminal part of a symmetry related molecule. Accordingly, these structural features move in a coordinated manner in the channel formed by the I4 packing (Fig. 1E).

The structure of the antagonist, colored according to temperature factors, is shown in Fig. 1A. The core of the structure composed of the four classical α-helices is quite stable, with a mean B factor calculated on C-x of 28.6 Å², to be compared with the mean B factor of the whole structure of 33.8 Å² (Table 2). The most dynamic parts are composed of the N and C termini of the protein, of loop 2 (located between helix 2 and helix 3), and of the long loop-helix-loop-helix located between helices 1 and 2. In particular, the first part of the latter (loop 1) and helix 1” are very dynamic, with B values up to 57 Å². This region of the hormone is poorly defined in most of PRL/GH/PL structures found in the PDB (also when determined by NMR), and it has been hypothesized to adopt a defined structure only when the hormone is receptor-bound (17). In our case, this is also partly the case because of the location of this loop in the crystal packing, as loop 1 and helix 1” point inside the I4 space group channel (Fig. 1E). Fig. 1D represents the B factor and the accessibility (multiplied by minus one for clarity) in the crystal as a function of residue number. The two curves evolve in parallel, with the sole exception of the N and C termini of the protein. This is because of the reasons listed above, i.e. that those two regions are surrounded by solvent and are in close contact; therefore their respective movements are correlated.

Comparison of Del1–9-G129R-hPRL and WT hPRL Structures

Two structures of PRL exist in the PDB, 1N9D (38) and 1RW5 (21) (Fig. 1B). Both are unbound, natural PRL of human origin, produced in bacteria and resolved by NMR. Those two structures are significantly different with a root mean square deviation (r.m.s.d.) value of 3.8 Å, as noted previously (21). Our structure is the first PRL-core structure resolved by crystallography. It was fitted to both NMR structures, with r.m.s.d. values on C-x of 5.2 Å and 1.53 Å, respectively. When limited to the four-helix bundle core, the r.m.s.d. fit improved to 4.08 and 0.90 Å, respectively. As we (21) and Hodsdon and co-workers (39) recently agreed that 1RW5 is more accurate than 1N9D, this strongly suggests high structural similarity between the antagonist and the WT hormone (Fig. 1C). We plotted the r.m.s.d. between our structure and 1RW5 as a function of residue number (Fig. 1D). Fig. 1D also shows temperature factors and solvent accessibility of Del1–9-G129R-hPRL in the crystal packing. The three curves are synchronized, indicating that the only noticeable differences between the WT hPRL NMR structure and the antagonist crystallographic one are localized in accessible dynamic regions. This further argues that the mutations performed in
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binding site 2 do not generate major structural rearrangement (Fig. 1C).

A detailed comparison of the two structures focused on the Gly129 pocket is presented on Fig. 2. This highlights the cup formed by Gly129 and the large residues surrounding it in WT hPRL and the absence of a cavity because of the Arg mutation in the antagonist. It also shows the N terminus on the edge of the Gly pocket in WT hPRL, and its absence in the antagonist mutant. Residues involved in the formation of the site 2 binding pocket are part of both helix 1 and of helix 3 (Fig. 2C). There are correlated movements of Leu18, Leu132, and Glu228 which position at van der Waals distances of Arg129. Leu25 is not affected by the presence of the mutated residue. The rearrangements of Arg125, Arg21, and Gln136 are not because of the presence of Arg129, but because of two symmetry-related molecules. Sym-Glu161 forms a hydrogen bond with Gln136 and with the carbonyl of the N-terminal methionine (initiation codon). As can be seen in the WT structure (Fig. 1B), the N terminus is undefined because of the lack of distance information. In the antagonist structure, the presence of the symmetry molecule forces the N terminus to reorient toward the Arg129 cup. Despite that, the backbone of residues 11–14 is superimposed on the corresponding region of the NMR structure (Fig. 1C). In conclusion we can argue that the replacement of Gly129 by an Arg does not affect the global structure of hPRL and generates only small displacements of residues surrounding locally the arginine.

Rationale for the Design of hPRL Variants

To investigate the individual contribution of the two modifications in Del1–9-G129R-hPRL (N terminus and Gly129) to the ability of hPRL to activate the hPRLR, we generated several mutants in addition to those already available. All mutants analyzed in this study are shown in Fig. 3. Two N-terminal variants were generated to mimic those of the two other members of the hormone family. Deletion of the nine first residues (Del1–9-hPRL) mimics the N terminus of hGH as described previously (16). As for the elongated Nter-hPRL variant, it contains the sequence AQHPPY at its N terminus instead of the natural LPI sequence of hPRL; in ovine PL, this AQHPPY motif was shown to contact the PRLR and to contribute to a large extent of the energy of site 2 interaction (40).

With respect to the Gly129 mutations, seven new substitution variants were generated and compared with the original G129R variant (12): Pro, Asp, Asn, Val, Leu, Tyr, and Phe. These residues were chosen to explore the influence of different parameters such as volume (Pro < Asp < Asn < Val < Leu < Tyr < Phe < Arg), charge (negative (Asp), positive (Arg), or none (Pro, Asn, Val, Leu, Tyr, Phe)), and polarity (Asp, Asn, Tyr). N-terminal variants and Gly129 mutations were combined in Del1–9-G129R-hPRL and in Nter-G129R-hPRL variants.

Circular Dichroism

None of the mutations induced any major structural alteration, as far-UV circular dichroism analysis revealed similar spectral properties for all variants compared with WT hPRL, which is shown on Fig. 4A. All, except for G129N, had a double trough ratio of $\theta_{222}/\theta_{208}$ larger than one and identical to WT hPRL, with an average of 1.05 ± 0.01 as expected (12, 16). G129N, however,
showed an increased contribution to the ellipticity at 222 nm, indicating either a more pronounced coiled-coil structure or other subtle structural changes involving aromatic residues (Fig. 4). This was not investigated further.

The heat denaturation of all hPRL variants was then followed by measuring the change in helical ellipticity at 222 nm with temperature. As shown in Table 3, N-terminal mutations (truncation or elongation) did not induce significant changes of $T_m$, whether these modifications were introduced in the WT or the G129R contexts. In contrast, the Gly$^{129}$ substitutions led to a decrease in $T_m$ in two cases with 2 K for G129R-hPRL and 10 K for G129P-hPRL (Fig. 4B), the latter as expected for a proline substitution at a mid-helical position. All other Gly$^{129}$ substitutions led to a large and significant increase in $T_m$ for the G129N-hPRL (Fig. 4B), G129D-hPRL, and G129F-hPRL (not shown) mutants could be determined by extrapolating post-transition curves, whereas this was not possible for variants G129L-hPRL (Fig. 4B), G129V-hPRL, and G129Y-hPRL (not shown), indicating that their $T_m$ values were even higher than 368 K. For these last three variants, three assumptions were made to estimate their $T_m$ values as follows: 1) the unfolded state was unchanged by the mutation; 2) the percentage of signal change at 222 nm was expected to be the same as the average measured for other variants (32 ± 2%); and 3) the slope of the post-transition region was unchanged compared with WT. Errors on these estimates were given as 4 K. All data are summarized in Table 3.

**Table 3**

| Thermodynamic parameters for heat denaturation of hPRL variants |
|---------------------------------------------------------------|
| **Hormones** | $\Delta H_{Tm}$ | $T_m$  |
|----------------|-------------|--------|
| **N-terminal variants** | | |
| HPRL | $-261 \pm 4$ | $354.3 \pm 0.1$ |
| Del1–9-hPRL | $-261 \pm 6$ | $355.6 \pm 0.1$ |
| Nter-hPRL | $-202 \pm 3$ | $355.8 \pm 0.1$ |
| G129R-hPRL | $-234 \pm 3$ | $352.1 \pm 0.1$ |
| Del1–9-G129R-hPRL | $-254 \pm 4$ | $353.9 \pm 0.1$ |
| Nter-G129R-hPRL | $-206 \pm 3$ | $352.2 \pm 0.1$ |
| **Gly$^{129}$ variants** | | |
| G129R-hPRL | $-234 \pm 3$ | $352.1 \pm 0.1$ |
| G129D-hPRL | $-243 \pm 4$ | $357.4 \pm 0.1$ |
| G129F-hPRL | NC$^a$ | $367 \pm 3$ |
| G129L-hPRL | NC | $370 \pm 4^b$ |
| G129V-hPRL | NC | $363 \pm 2$ |
| G129Y-hPRL | $-210 \pm 2$ | $344 \pm 1$ |
| G129R-hPRL | NC | $374 \pm 4^b$ |
| G129Y-hPRL | NC | $370 \pm 4^b$ |

แค่ NC indicates not calculable.

$^b$ Extrapolated from experimental data (see text).

**FIGURE 5. Binding of hPRL variants to full-length transmembrane hPRL**. Displacement of $^{125}$I-hPRL by unlabeled ligands was performed using HL-5 cell lysates. N-terminal hPRL variants (agonists) and G129R-containing variants (antagonists) are shown in A and B, respectively. Symbols: ■ (dotted line), hPRL; ▲, Nter-hPRL; ●, Del1–9-hPRL; ○, Del1–9–G129R-hPRL; □, G129R-hPRL; △, Nter-G129R-hPRL.
using a 1:1 Langmuir model, the $\chi^2$ values obtained upon global fitting were repeatedly 4–5 times lower for the oriented coupling strategy. We therefore resorted to this immobilization method for all subsequent SPR site 1 and site 2 binding assays.

The $K_a$ value of the interaction between site 1 of hPRL and...
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FIGURE 7. Antagonism and residual agonism displayed by hPRL variants. Representative experiments (performed in triplicate) of antagonism assays against 9 nM hPRL using HL-5 cells (A and B) and agonism assays using Ba/F-LP cells (C and D) are shown for G129R-containing mutants (A and C) and the other Gly129 variants (B and D). Data are expressed as percentage of the maximal activity of hPRL in each panel. Values of IC50 (antagonism) and maximal agonistic potency of all variants are reported in Table 5. Symbols: hPRL; □, Del1–9-G129R-hPRL; □ (dotted line), G129R-hPRL; △, Nter-G129R-hPRL; ▽, G129P-hPRL; □, G129D-hPRL; ×, G129F-hPRL; ●, G129V-hPRL; +, G129L-hPRL; *, G129N-hPRL; ], G129Y-hPRL.

TABLE 5

Anergic and antagonistic properties of hPRL variants

| Hormones          | Antagonism HL-5 cells | Agonism Ba/F-LP cells |
|-------------------|-----------------------|-----------------------|
|                   | IC50 (nM)             | % hPRL maximum activity |
| **N-terminal variants** |                       |                       |
| HPRL              | 100 ± 7.0             |                       |
| Del1–9-hPRL       | 100 ± 9.4             |                       |
| Nter-hPRL         | 100 ± 10.6            |                       |
| G129R-hPRL        | 200 ± 26              | 45.5 ± 12.8           |
| Del1–9-G129R-hPRL | 120 ± 43              | 8.3 ± 3.6             |
| Nter-G129R-hPRL   | 250 ± 33              | 80.0 ± 16.7           |
| **Gly129 variants** |                       |                       |
| G129D-hPRL        | 200 ± 26              | 45.5 ± 12.8           |
| G129Q-hPRL        | 340 ± 50              | 23.6 ± 4.1            |
| G129F-hPRL        | 110 ± 41              | 19.1 ± 6.2            |
| G129H-hPRL        | 160 ± 34              | 16.8 ± 7.2            |
| G129N-hPRL        | 130 ± 19              | 26.4 ± 11.8           |
| G129P-hPRL        | 340 ± 90              | 90.4 ± 7.4            |
| G129V-hPRL        | 110 ± 12              | 14.2 ± 5.0            |
| G129Y-hPRL        | 190 ± 70              | 24.3 ± 10.8           |

*For more information, see Ref. 16.

hPRLR-ECD was ~6 nM (Fig. 6A), with an association rate (k_on) close to $10^3$ M$^{-1}$s$^{-1}$ and a dissociation rate (k_off) of about $5 \times 10^{-4}$ s$^{-1}$ (Table 4). We also determined the $K_a$ value for the interaction between hPRLR-ECD and site 2 of hPRL, which was ~30 μM, i.e. 10,000 times higher than that for site 1 (Fig. 6C and Table 4). As the interaction is very transient (the half-life of the 2:1 complex is just about 1 s), the association and dissociation rates could not be determined, and only steady-state analysis of the curves could be performed.

N-terminal elongation (Nter-hPRL) or deletion (Del1–9-hPRL) did not significantly affect k_on and k_off values of site 1, whereas the affinity for site 2 was unaffected by N-terminal elongation and only slightly reduced (3-fold) by N-terminal truncation (Table 4). In contrast, Gly129 mutations all induced a slight but significant increase (1.5–2.5-fold) in k_off of site 1, with no significant change in k_on. Most strikingly, all the substitutions at position 129 totally abolished site 2 binding, as exemplified by the G129P and G129R mutants for which no signal could be detected for hECD concentrations as high as 350 μM, therefore giving a minimal threshold of 5 mM for the $K_a$ of site 2 for these mutants (Fig. 6D).

Cell-based Bioassays

Antagonism—The antagonistic properties of all hPRL variants were investigated using our routine HL-5 luciferase assay (5, 14, 15, 35). All data presented in Fig. 7, A and B, were obtained by competing the same concentration of WT hPRL (9 nM). In agreement with their full agonistic properties (see below), Nter-hPRL and Del1–9-hPRL failed to exert any antagonism (Table 5). In contrast, all mutants harboring a mutation of Gly129 exhibited antagonism in this assay. With respect to G129R-containing mutants, Del1–9-G129R-hPRL was slightly, but repeatedly, a more potent antagonist than G129R-hPRL (IC50 120 ± 43 nM versus 200 ± 26 nM), confirming our previous observations (5). Elongation of the N terminus (Nter-G129R-hPRL) did not modify G129R-hPRL properties (IC50 250 ± 33 nM) (Fig. 7A). Other substitutions at position Gly129 gave rise to parallel curves of antagonism (Fig. 7B), with similar IC50 values that varied by less than 3-fold between the most (G129V or G129F) and the least (G129P or G129D) potent antagonists. All data are summarized in Table 5.

Residual Agonism—Del1–9-hPRL was shown previously to be a full agonist in the HL-5 assay (16), and the same observations were made for Nter-hPRL in this study (not shown). We showed previously that this reporter gene assay is not very sen-
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Correlations between Bioactivity and Thermodynamic Parameters

We systematically investigated whether the residual agonistic properties of the different PRL variants could be correlated to their physicochemical features, such as their thermodynamic stability, their site 1 binding parameters, and the volume and helix propensity of the residue in position 129. The only significant relation we evidenced was the inverse logarithmic correlation between the residual agonism and the global stability (Tm) of the Gly129 variants (Fig. 8).

DISCUSSION

Since the discovery that transgenic mice expressing the G119R mutant of bovine GH exhibited a dwarf phenotype (43), replacement of the helix 3 Gly by an Arg (or Lys) has been considered as the most direct and efficient way to design receptor antagonists in the PRL/GH family. Soon after this pioneering study, the same strategy was used by Wells and co-workers (18) for generating competitive antagonists of hGH (G120R), by our group (13) for hPRL (G129R), and by Gertler and co-workers (44) for bovine PL (G133K). The mechanism involved for GHR antagonists was elucidated from the crystal structure of the hGH-hGHR2 complex (17). The small side chain of the helix 3 Gly maintains a cleft (so-called “cup”) at the helix 1/helix 3 interface (site 2), into which Trp104 of the receptor docks upon binding; consequently, when a larger side chain is substituted for the natural Gly, docking of the Trp is impaired, leading to a nonfunctional interaction with the second GHR of the GH-GHR2 complex. The same mechanism is assumed to be involved in the PRL-PRLR interaction. This is supported by the role of the homologous Trp (residue 72) at site 2 in the oPL-rPRLRc complex (40), and by the steric hindrance resulting from the Gly → Arg substitution in Del1–9-G129R-hPRL (compare Fig. 2, A and B).

Although all PRL/GH/PL variants mutated at the helix 3 Gly were shown to exhibit antagonistic properties against the PRLR in various in vitro assays, several reports also provided evidence that they retained the ability to activate this receptor to some extent. We have demonstrated that the detection of residual activity often requires the use of sensitive bioassays, i.e. cell systems in which a measurable biological response is observed at low hormone concentrations (2, 15, 45). As an example, G120K-hGH and G129R-hPRL exhibit no activity in the low sensitivity HL-5 assay (14, 46) or in signaling studies (immuno-precipitates) (35), although they are able to stimulate the proliferation of Ba/F-LP cells and of rat Nb2 cells (the two most sensitive bioassays for lactogens) to mid- and sub-maximal level, respectively (12, 46, 47). It would be misleading to neglect the residual activity of these mutants otherwise referred to as antagonists. First, our ongoing studies indicate that transgenic mice overexpressing G129R-hPRL exhibit phenotypes that are reminiscent of those reported for PRL-transgenic mice,7 supporting the idea that residual agonism predominates over antagonism in vivo. Second, based on their competitive mechanism of action, PRLR antagonists must be used in molar excess compared with endogenous PRL, i.e. at concentrations at which G129R-hPRL exhibits residual agonism. Obviously, such a situation...
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Alterations of the hPRL N terminus appear to have a significant impact on biological activity only when site 2 is impaired through a mutation at position 129. Indeed, in the WT hPRL context, the nine-residue N-terminal deletion only led to a slight decrease of site 2 affinity (3-fold as measured by SPR; see Table 4). As for the N-terminal AQHPPY motif insertion, it had no detectable effect in vitro, as measured by SPR (Table 4), although it only slightly, but reproducibly, enhanced global affinity measured using cell lysates (Fig. 5). These limited effects could be explained by the fact that, in the WT hPRL, binding of the second receptor to the hormone site 2 relies principally on the anchoring of Trp\textsuperscript{72} into the Gly cup (Fig. 2A), independently of the N terminus. In contrast, when hPRL site 2 is impaired by the bulky Arg\textsuperscript{129} side chain (Fig. 2B), the N terminus might still be able to provide enough residual interaction energy to allow a limited second receptor recruitment or to induce a limited reorientation of the second receptor within a pre-formed hPRL dimer (49, 50) leading to a residual agonistic activation. The efficacy of this process could be further enhanced when additional residues leading to more interaction energy are added with the AQHPPY motif insertion, resulting in a clear iterative effect on agonistic activity between Del1–9-G129R-hPRL, G129R-hPRL, and Nter-G129R hPRL (Fig. 5 and 7C). Unfortunately, we could not correlate the difference in agonistic activity of G129R-hPRL and Del1–9-G129R-hPRL to differences in their affinities at site 2, as the latter were beyond the threshold of detectable interactions for both mutants (Fig. 6D).

The main feature of PRL site 2 is clearly the Gly\textsuperscript{129} pocket, as highlighted by the fact that G129R mutation induced at least a 150-fold decrease in site 2 binding affinity in vitro (SPR assays) and reduced the agonistic activity of hPRL by more than 98% as deduced from the luciferase assay (HL-5) (14). This major role prompted us to investigate this region in more detail as, surprisingly, residues other than Arg have never been tested as substitutes for Gly\textsuperscript{129}, with the aim of generating pure PRLR antagonists. Indeed, based on the guess that a large and charged side chain residue should be the most effective in disrupting receptor binding, this prototype mutation was always assumed by us and others to be the best within the Gly pocket. To challenge this hypothesis, we performed various substitutions covering a range of physicochemical properties of natural amino acids (see “Results”). All Gly\textsuperscript{129} mutants exhibited similar antagonism in the HL-5 assay (IC\textsubscript{50} varying by less than 3-fold; see Fig. 7B), confirming that any residue other than Gly at position 129 transforms hPRL into an antagonist. However, we were surprised to observe that all substitutions (except G129P) led to lower residual agonism than G129R (Fig. 7D). In other words, Arg is actually one of the less efficient substitutions of Gly\textsuperscript{129} when attempting to generate a pure antagonist. Val, Leu, and Phe were in this respect the substitutes leading to the lowest residual agonism.

To understand why the Arg substitution was not optimal and whether contacts could persist between the site 2 of G129R-hPRL and the PRLR, we superimposed our three-dimensional structure of Del1–9-G129R-hPRL (which harbors the G129R mutation) on the only currently available structure providing information on the site 2-PRLR interaction, namely the oPL-PRLR\textsubscript{2} ternary complex (PDB code 1F6F) (40). This model (Fig. 9) suggested that, contrary to what was expected, the long side chain of Arg\textsuperscript{129} does not protrude straightforward from the center of the cup but clashes with the aromatic plane of Trp\textsuperscript{72} in the oPL-PRLR\textsubscript{2} complex, site 2 binding involves, in addition to Trp\textsuperscript{72} itself, several bonds between charged amino acids on both sides of the cup; Arg\textsuperscript{125} is hydrogen-bonded to Asp\textsuperscript{96} and Thr\textsuperscript{98} in the oPL-PRLR\textsubscript{2}, and Lys\textsuperscript{124} is linked to Glu\textsuperscript{18}. The homologous residues in hPRL are Arg\textsuperscript{21} and Gln\textsuperscript{122} in hPRL and Gly\textsuperscript{122} in hPRL, respectively. Two additional charged residues (Asp\textsuperscript{17} and Arg\textsuperscript{125}) could form additional electrostatic interactions. To release the prohibited van der Waals contact between Trp\textsuperscript{72} and Arg\textsuperscript{129} in hPRL, we suggest that the
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Trp side chain could rotate upon G129R-hPRL site 2 binding, thereby disrupting the hydrogen bonds at one side of the cup (Gln122 and Arg125), while potentially preserving those at the other side (Asp17 and Arg21). As a result of the high flexibility of the Arg side chain, either the positive charge of Arg129, which is colored in red. Hydrogen bonds are symbolized by dotted lines. The arrows illustrate the hypothesized movements of Trp72 necessary to remove the clash between this residue and Arg129 (see the text), relocating Trp72 side chain in the free space (7.4 Å) between Asp17 (antagonist) and Ser40 (PRLR).

Figure 9. Superimposition of binding sites 2 of Del1–9-G129R-hPRL and PRLR-bound oPL (PDB code 1F6F). The C-α atoms of helices 1 and 3 of Del1–9–G129R-hPRL were superimposed on the equivalent atoms of the helices 1 and 3 of oPL, using PyMOL. The secondary structure elements of oPL-PRLR are colored in white, residues belonging to oPL are colored in orange, and those belonging to PRLR are colored according to the atom type (N in blue, O in red, and white for others). PRL antagonist is colored in green at the exception of Arg129, which is colored in red. Hydrogen bonds are symbolized by dotted lines. The arrows illustrate the hypothesized movements of Trp72 necessary to remove the clash between this residue and Arg129 (see the text), relocating Trp72 side chain in the free space (7.4 Å) between Asp17 (antagonist) and Ser40 (PRLR).

In conclusion, we report here the first PRL core structure solved by crystallography. This structure of the pure antagonist Del1–9-G129R-hPRL revealed no major structural change compared with WT hPRL. Evidence obtained from our structural, physicochemical, and biological results from this protein and from a diverse set of mutant proteins allow us to propose the following. (i) The glycine pocket is the major component of hPRL site 2. (ii) The N terminus of hPRL is an intrinsic component of site 2, whose importance only becomes clearly apparent when the glycine pocket is impaired because of Gly129 substitution. (iii) Structural flexibility of hPRL is required for fully functional site 2 binding. In line with these conclusions, we show that complete abolition of residual agonism can be achieved by two very different strategies, either by totally disrupting hormone site 2-receptor contacts, as in Del1–9-G129R-hPRL, or by stabilizing the hormone and constraining its intrinsic flexibility, as in G129V-hPRL.

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clearly appears to be non-energy-optimized. The vicinity of Gly129 appears to be highly flexible, as shown by the increased amide hydrogen exchange rates of adjacent residues (39). Furthermore, normal mode analysis of WT hPRL and Del1–9-G129R-hPRL structures, as well as temperature factors (Fig. 1A), predicted the presence of a bending region close to Gly129. The amplitudes of these movements, as well as the center of mass, could very well be significantly constrained and shifted, respectively, upon mutation. In good agreement, Keeler et al. (38) observed from NMR relaxation data, high dynamics (nuclear Overhauser effect ≈0.5) at position Glu128 in hPRL. Finally, when superimposing helices 3 from all the structures of GH (PDB codes 1HUW and 1HGU) and GH-GHR complexes (PDB codes 1A22, 1HWH, 1HWG, and 3HHR) available in the PDB, a clear difference in the bending of the helix appeared between unbonded and complexed structures (not shown). One can suggest that this movement is restrained/prohibited depending on the Gly129 mutants and, conversely, that the helix is already properly bent in G129P-hPRL, which could explain the higher residual agonism of this mutant despite the intrinsically hindering effect of introducing a Pro within the cup.

We suggest from these observations and from the stability changes we determined that the intrinsic dynamics of hPRL is a prerequisite for its fully functional interaction with PRLR; global stabilization of hPRL by substitution of Gly129 by any residue except Pro might either lock site 2 in an unproductive state leaving site 1 unaffected, or interfere with the coupling of site 1 and site 2 suggested by Brooks and co-workers (51). For several other signaling proteins such as GH (52), insulin (53), keratinocyte growth factor (54), and fibroblast growth factor (55), similar positive or negative correlations between the stability of specific regions and their functional properties have been underlined, showing the prominence of global or local structural flexibility in the fine-tuning of their activity.

In conclusion, we report here the first PRL core structure solved by crystallography. This structure of the pure antagonist Del1–9-G129R-hPRL revealed no major structural change compared with WT hPRL. Evidence obtained from our structural, physicochemical, and biological results from this protein and from a diverse set of mutant proteins allow us to propose the following. (i) The glycine pocket is the major component of hPRL site 2. (ii) The N terminus of hPRL is an intrinsic component of site 2, whose importance only becomes clearly apparent when the glycine pocket is impaired because of Gly129 substitution. (iii) Structural flexibility of hPRL is required for fully functional site 2 binding. In line with these conclusions, we show that complete abolition of residual agonism can be achieved by two very different strategies, either by totally disrupting hormone site 2-receptor contacts, as in Del1–9-G129R-hPRL, or by stabilizing the hormone and constraining its intrinsic flexibility, as in G129V-hPRL.
