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Biological Properties of Human Prolactin Analogs Depend Not Only on Global Hormone Affinity, but Also on the Relative Affinities of Both Receptor Binding Sites*

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Zinc increases the affinity of human growth hormone (hGH) for the human prolactin receptor (hPRLR) due to the coordination of one zinc ion involving Glu-174$_{hGH}$ and His-18$_{hGH}$. In contrast, binding of hPRL to the hPRLR is zinc-independent. We engineered in binding site 1 of hPRL a hGH-like zinc coordination site, by mutating Asp-183$_{hPRL}$ (homologous to Glu-174$_{hGH}$) into Glu (D183E mutation). This mutation was also introduced into G129R hPRL, a binding site 2 mutant (Goffin, V., Kinet, S., Ferrag, F., Binart, N., Martial, J. A., and Kelly, P. A. (1996) *J. Biol. Chem.* 271, 16573–16579). These analogs were characterized using a stable clone expressing both the hPRLR and a PRLR-responsive reporter gene. The D183E mutation *per se* decreases the binding affinity and transcriptional activity of hPRL. However, this loss is partially rescued by the addition of zinc and the effect is much more marked on bioactivity than on binding affinity. These data indicate that the D183E mutation confers zinc sensitivity to hPRL biological properties. Due to an impaired site 2, the agonistic activity of G129R analog is almost nil. Although the double mutant D183E/G129R displays lower affinity (~1 log) compared with G129R hPRL, it unexpectedly recovers partial agonistic activity in the absence of zinc. Moreover, whereas zinc increases the affinity of D183E/G129R, it paradoxically abolishes its agonistic activity. Our results demonstrate that the biological properties of hPRL analogs do not necessarily parallel their overall affinity. Rather, the relative affinities of the individual binding sites 1 and 2 may play an even more important role.

Prolactin (PRL)[1] is a 23,000-dalton polypeptidic hormone mainly secreted by the anterior pituitary in all vertebrates (1, 2). Its biological functions are mediated by a single-pass transmembrane receptor, the PRL receptor (PRLR), also known as lactogen receptor (3), which belongs to the cytokine receptor superfamily (4). We recently listed up to 300 different functions for PRL (5), the best known activities being related to lactation and reproduction as emphasized by the sterility and impaired mammary gland development of female PRLR knockout mice (6). PRL shares several structural and functional similarities with growth hormone (GH), another anterior pituitary hormone (1). Their amino acid sequences show 58% similarity throughout vertebrates (7), and their three-dimensional structures are assumed to be very similar as well (8). The receptors of these hormones are also highly similar with respect to their sequence, three-dimensional structure, and signaling characteristics (5, 9–11).

Both GH and PRL activate their receptors by sequential dimerization, and it is now clearly established that the interaction of one hormone with two receptors involves two different regions of the ligands referred to as binding sites 1 and 2 (12, 13). First, the hormones interact with one molecule of receptor through their binding site 1 to form an intermediate 1:1 complex, which is inactive. Second, the hormone involved in this transient complex interacts via its binding site 2 with a second molecule of receptor to achieve a 1:2 complex that triggers intracellular signaling cascades. This paradigm of receptor activation has been deciphered at the residue/atomic levels by the elucidation of the now classical three-dimensional structure of the complex between human (h) GH and the hGH binding protein (BP), corresponding to the extracellular domain of the hGHR (14). Unfortunately, such data is currently lacking for the hPRL·hPRLR complex.

In addition to binding to the GHR, primate (human and monkey) GHs display the unique ability among growth hormones to also bind to the PRLR. This cross-reaction involves the participation of one zinc ion, since the affinity of hGH for the hPRLBP is increased by 8,000-fold in the presence of $ZnCl_2$ compared with EDTA (15). The crystal structure of the 1:1 complex between hGH and the hPRLBP has shown that the four residues coordinating the zinc ion are His-18$_{hGH}$ and Glu-174$_{hGH}$ of the hormone, both residues belonging to binding site 1, and Asp-187$_{hPRLR}$ and His-188$_{hPRLR}$ of the receptor (16). Zinc mediation is one of the, if not the major, molecular events in the interaction between hGH and the hPRLR. Indeed, the most effective point mutation introduced into hGH (if one excludes those affecting zinc coordination) drops the binding affinity by only 25-fold, compared with the 8,000-fold reduction of affinity of WT hGH in the presence of EDTA (15, 17).

Several recent studies have shown that hPRL does bind zinc (18–20). Zinc induces hPRL aggregation in secretory granules found in pituitary lactotroph cells, rendering the hormone osmotically inert (18). Otherwise, the concentration of zinc affects

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[1] The abbreviations used are: PRL, prolactin; h, human; GH, growth hormone; PRLR, prolactin receptor; GHR, growth hormone receptor; BP, binding protein; rb, rabbit; FCS, fetal calf serum; WT, wild type; RLU, relative light unit(s); LHRE, lactogenic hormone response element.

neither the affinity of hPRL for its receptor (15) nor its mitogenic activity on rat Nb2 cells (21, 22). Zinc binding by hPRL thus effects its storage and secretion, but not its functional properties. With respect to the four zinc coordinating residues identified in the hGH·hPRLBP complex (16), sequence alignments (12) indicate that the hPRL·hPRLBP complex only differs by the mutation of Glu-174_{hGH} into Asp in hPRL (Asp-183_{hPRL} in hPRL numbering). Despite the fact that Asp is one of the four amino acids able to coordinate zinc ions (the three others are His, Cys, and Glu; see Ref. 23), Asp-183_{hPRL} and His-27_{hPRL} (homologous to His-18_{hGH}) are presumably unable to coordinate a zinc ion in a manner similar to Glu-174_{hGH} and His-18_{hGH} when hGH binds to the hPRLR. Alternatively, if these residues are actually involved in zinc binding, this has no effect on the interaction between hPRL and its receptor.

We have previously generated several hPRL analogs by site-directed mutagenesis (24–27); those carrying a sterically hindering mutation within binding site 2 were shown to act as antagonists due to their reduced ability to induce PRLR dimerization (24). The antagonistic potency of such analogs is relatively weak, however, since their overall affinity only relies on a single interaction, namely that involving the hPRL binding site 1 and one receptor. In comparison, trimeric complexes between wild type hormones (GH, PRL) and their binding proteins involve two additional contacts, one between binding site 2 of the hormone and the second receptor, and one at the receptor interface (14). In order to strengthen the antagonistic properties of PRL/GH mutants, a strategy would be to engineer mutations increasing their affinity at binding site 1. A typical example is the hGH antagonist referred to as B2036, which combines the widely described G120R site 2 mutation (28) with eight point mutations enhancing binding site 1 affinity (see Ref. 29 and references therein). Using the same logic, our aim was to reconstitute in hPRL a putative hGH-like zinc binding site in order to increase the affinity of binding site 1. This was achieved by mutating Asp-183_{hPRL} into Glu-183_{hPRL} in WT hPRL and in the previously reported G129R analog (see "Discussion").

Biological studies of lactogenic hormones are most often performed using the rat Nb2 cell proliferation bioassay (30, 31). However, we have previously shown that the interaction between hPRL (wild type or analogs) and the PRLR is different depending whether the receptor is from rat or human origin, making the rat Nb2 cell assay not suitable for testing antagonistic properties of hPRL analogs (24). In order to test the latter in a homologous system, we developed a new bioassay by generating cells stably expressing the human PRLR cDNA and a PRL-responsive reporter gene.

EXPERIMENTAL PROCEDURES

Materials

Cultures and Reagents—Culture media, fetal calf serum (FCS), Geneticin (G-418), trypsin, and glutamine were purchased from Life Technologies, Inc. Luciferin and cell lysis buffer were from Promega (Madison, WI), and luciferase activity was measured in relative light units (RLU) (Lumat LB 9501; Berthold, Nashua, NH). IODOGEN was purchased from Sigma, and carrier-free $Na^{125}I$ was obtained from Amersham Pharmacia Biotech. Salts were high grade purified chemicals purchased from Sigma or Merck (Darmstadt, Germany). Oligonucleotides were from Eurogentec (Liège, Belgium).

Hormones—In this study, we used exclusively recombinant proteins: WT hPRL, the binding site 2 analog G129R (Gly-129 replaced with Arg) (26), the zinc binding analog D183E (Asp-183 replaced with Glu), and the double mutant D183E/G129R, combining both site 1 and site 2 mutations. The pT7L expression vector has been described previously (32). Recombinant WT and mutated hPRL were produced in *Escherichia coli* and purified as described previously (24–26, 32). Recombinant hGH was a generous gift from Ares-Serono (Geneva, Switzerland).

Plasmids—The human PRLR cDNA inserted into the pc/DNA3 eukaryotic vector (Invitrogen, Carlsbad, CA) has been used and described previously (24, 33). The PRLR-responsive reporter gene carries the sequence encoding the luciferase gene under the control of a 6-repeat sequence of the lactogenic hormone response element (LHRE) followed by the minimal thymidine kinase promoter (24). LHRE is the DNA binding element of the signal transducer and activator of transcription Stat5 (34), one of the signaling proteins activated by dimerized PRLR (5). Plasmids were purified using the cesium chloride gradient procedure and quantified by absorbance at 260 nm.

Methods

Site-directed Mutagenesis—Construction of the D183E/G129R mutated hPRL cDNA was performed by the oligonucleotide-directed mutagenesis method using the Chameleon mutagenesis kit from Stratagene (La Jolla, CA), strictly following the manufacturer's instructions. The D183E mutation was introduced into the cDNA encoding the G129R hPRL analog (26) using the following mutated oligonucleotide: GAG ATA ATT CTC GAT TTT ATG (5′–3′ non-coding strand, mutated codon underlined). The D183E single mutant was obtained by substituting the *EcoRI*-Bgl*II* fragment (encoding amino acids 40–145) of WT hPRL cDNA for the corresponding fragment of the D183E/G129R cDNA. Clones containing the expected mutation were identified by DNA sequencing.

Circular Dichroism—Circular dichroism was performed as described previously (25) using a CD6 dichrograph (Instruments SA-Jobin Yvon, Longjumeau, France), and the helicity was calculated at 222 nm (35).

Apparent Molecular Mass—Apparent molecular mass of hPRL proteins were measured by high pressure liquid gel filtration chromatography. One hundred-μl samples (500 μg/ml) were loaded on a Superose 12 molecular sieve column 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).

Transfection and Stable Expression in 293 Fibroblasts—We used the human embryonic kidney fibroblast 293 cell line to generate a clonal cell line stably expressing the hPRLR, since this cell line has been shown to highly express cDNAs controlled by the cytomegalovirus promoter (24, 36). Cells were routinely cultured as described previously (36). They were transfected with both plasmids encoding the hPRLR and the LHRE-luciferase reporter genes (2 μg each) using the calcium-phosphate precipitate procedure (24, 36). Twenty-four hours after transfection, cells were shifted to growth medium containing 500 μg/ml active G-418. From this step, G-418 was systematically added to all culture media. After 15–20 days, single G-418-resistant colonies were localized by microscope, picked out individually by local trypsinization, and amplified in 24-well plates before being characterized for their ability to respond to hPRL, as monitored by the induction of luciferase activity. Clones were referred to as HL (H for human PRLR, L for LHRE reporter plasmid) and numbered starting at HL1.

Luciferase Assay—After trypsinization, cells were counted and aliquoted in 96-well plates at a density of 50,000 cells/100 μl/well. Plating medium contained 0.5% FCS to allow cell adhesion. Eighteen hours (overnight) after plating, 100 μl of 2-fold concentrated hormones diluted in FCS-free medium were added to each well. For assays involving ions (see below), solutions containing 4-fold concentrated hormone (50 μl) and 4-fold concentrated ion salts (50 μl) were added separately to avoid any interference of the ion concentration on hormone dilutions (*e.g.* protein aggregation, precipitation, etc). In addition, due to possible effects of the different salts on cell metabolism, a standard dose-response curve using WT hPRL (including non stimulated conditions) was performed separately for each ion tested. Typically, we assayed the different hormones (WT or analogs) at concentrations ranging from ~10 ng/ml to ~250 μg/ml (~10 μM) in duplicate. After 18–24 h of stimulation, culture medium was aspirated and cells were lysed for at least 10 min in 50 μl of lysis buffer. Luciferase activity for each experimental condition was counted in 10–20 μl of cell lysates for 10 s. The difference between duplicates never exceeded 15% of RLU values.

To investigate the effect of zinc ions on luciferase activity, we first used a zinc-free medium (Life Technologies, Inc.). The residual concentration of zinc in this medium as estimated by atomic absorption spectroscopy was ~0.2 μM, which is ~10-fold lower than that of normal medium (~2 μM). To ensure cell adhesion, functional tests required being performed in the presence of 0.25% FCS, the zinc content of which was measured as ~0.2 μM. As shown under "Results," the effect of zinc

on biological properties of zinc-sensitive analogs required the addition of at least 5 μM zinc salts and, in agreement, we never saw any significant difference using either "zinc-free" (~0.4 μM zinc measured, including 0.25% FCS) or normal (~2.2 μM zinc) medium. Therefore, normal medium was used for all functional studies and was referred to as 0 μM zinc, indicating no addition of exogenous ions. The indications 5, 10, 25, and 50 μM (experimentally estimated at 54 μM zinc) correspond to the concentrations of $ZnSO_4$ added to normal medium.

The addition of EDTA to cell cultures to remove any traces of divalent ions appeared detrimental to cell attachment and metabolism, and the effect was more apparent as EDTA concentration increased. EDTA was thus only used for some experiments to reverse the effect of added zinc (see below), at a concentration of 0.1 mM that had minimal side effects on cell response (meaning absolute RLU values).

Presentation of Luciferase Data—-Fold induction is calculated as the ratio between the RLU of stimulated *versus* non-stimulated (no hormone added) cells. In order to accurately compare the biological activities of the different hormones in the various conditions tested (see "Results"), data obtained within each experiment were expressed as a percentage of the maximal -fold induction obtained with WT hPRL in the absence of ion added, and these percentages were averaged to calculate EC_{50} and IC_{50} values. One experiment representative of at least three experiments is presented in the figures. For assays of agonists, EC_{50} values correspond to the concentration of hormone required to reach 50% of the maximal activation of the reporter gene achieved by WT hPRL. The bioactivity of hPRL analogs is given as the ratio of its EC_{50} with respect to that of WT hPRL. The IC_{50} of self-inhibition (self-IC_{50}) was calculated as the hormone concentration leading to 50% decrease of maximal cell response. For assays of antagonists, the IC_{50} values were calculated as the amount of hPRL analog producing 50% inhibition of WT hPRL maximal activity.

Binding Studies—Binding experiments of hPRL analogs to the hPRLR were performed on cell homogenates, which eliminates any detrimental effect of chemicals (ions, EDTA) on intact living cells. 293 cells stably expressing the hPRLR (clone HL5) were amplified, then starved in serum-free medium for 18–24 h. Cells were scrapped, centrifuged, and resuspended in the same medium at ~40×10^6 cells/ml. Cell homogenates were prepared by three freeze/thaw cycles, followed by homogenization using a Dounce grinder as described previously (25). The protein content was quantified by the Bradford method, and cell homogenate aliquots were frozen (−80 °C). Human PRL was iodinated using IODOGEN as reported previously (25). Its specific activity was in the range of 40–50 μCi/μg.

For binding assays, 150–300 μg of cell homogenate proteins, corresponding to 1–2 $\times 10^6$ cells, were incubated overnight at room temperature with 20,000–40,000 cpm of ^{125}I-hPRL in the presence of increasing amounts of non-labeled WT hPRL or hPRL analogs in medium containing 0.1% bovine serum albumin (the final reaction volume was 0.5 ml). Ion content is specified for each experiment. The assay was terminated by addition of 0.5 ml of ice-cold buffer (25 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.4) followed by centrifugation (15 min, 15000 $\times$ *g*). The supernatants were aspirated carefully, and the radioactivity of the pellets was counted in a γ counter. To assess the role of divalent ions on binding affinities, we systematically added 1 mM EDTA to the condition referred to as "0 μM" zinc. The condition referred to as 25 μM zinc (or other ions) corresponds to the final concentration of divalent ion salts added into the medium ($ZnCl_2$, $MgCl_2$, $MnCl_2$, $NiSO_4$, $CaCl_2$, $CuSO_4$).

Each experimental condition (hPRL analogs, divalent ions) was tested at least three times in duplicate. Specific binding was calculated as the difference between radioactivity bound in the absence (B_0, maximal binding) and in the presence (nonspecific) of 2–10 μg/ml unlabeled WT hPRL. Data are presented as percentages of specific binding. Competition curves were analyzed with the LIGAND PC program (37). The relative binding affinity of each mutant was calculated as the ratio of its IC_{50} *versus* the IC_{50} of WT hPRL.

RESULTS

Characterization of Stable Clones and Establishment of the 96-well Bioassay—Twenty G-418-resistant clones were isolated and grown independently, then analyzed based upon the maximal -fold induction of luciferase activity in the presence of various hPRL concentrations. This value ranged from 1-fold (no induction) up to 20-fold, and we selected the HL5 clone based on that data. As previously observed using transiently transfected cells (24), the maximal activation of luciferase activity was obtained at ~1 μg/ml of hPRL. From Scatchard analysis, this HL5 clone contains 65,000 ± 13,000 hPRL receptors/cell.

TABLE I
Structural characterization of hPRL analogs

	Helical content	Apparent molecular mass
	%	*kDa*
hPRL	56.8	24.4
D183E	59.9	23.6
D183E/G129R	57.8	23.3

Depending on the experimental conditions (ions, EDTA, analogs), the time of stimulation and the number of culture passages, the levels of luciferase -fold induction were found to vary from 10- to 30-fold. Although this induction was significantly lower than the 68-fold induction obtained by transient transfection of the same plasmids (24), it is sufficient for reliable comparison of dose-response curves obtained with various analogs. The low amount of FCS added in the starvation medium (0.25% FCS) to allow cell adhesion was not found to rise the background of luciferase activity in non-stimulated cells. This is in agreement with the observation that the content of lactogens in 0.25% FCS as estimated from the Nb2 assay (31) is around 0.1–0.2 ng/ml (data not shown), whereas the dose-response curve of luciferase activity in HL5 clone starts rising at >1 ng/ml hPRL. This new bioassay presents three major advantages. First, it circumvents the problem of species specificity (24) when testing human lactogens since it involves the human PRLR. Second, the use of a clone stably expressing both the receptor and the reporter genes makes each measurement within one experiment directly comparable without the need for normalization of RLU values by a second enzymatic activity (such as β-galactosidase) as required when performing transient transfection. Third, this bioassay, as it is set up in 96-well plate, is less time-, cell-, plasmid-, and hormone-consuming than transient transfection experiments (24).

Characterization of hPRL Analogs—Prior to any functional studies, the global folding of each hPRL analog was evaluated by circular dichroism analysis, which measures their helical content, and by the retention time of the proteins on a molecular sieve, which reflects their apparent molecular mass. Data relative to wild type hPRL (25) and G129R (26) have been published previously, and results obtained with the zinc-binding hPRL analogs are reported in Table I. None of the hPRL analogs used in this study differs from WT hPRL in its helical content or compactness, suggesting that any alteration of the biological properties can be specifically linked to the point mutation rather than to an alteration of the global protein folding.

Effect of Zinc on Biological Properties of WT hPRL and hGH—As shown in Fig. 1*A*, the dose-response curve obtained with hPRL in normal medium (no zinc added) shows a maximal activation at ~0.5–1 μg/ml ligand, with an average EC_{50} of 50 ± 13 ng/ml. Self-IC_{50} is achieved at concentrations higher than 250 μg/ml, in agreement with our previous observations (24). The bioactivity of hPRL is not affected by the addition of 25 μM zinc (EC_{50} of 44 ± 10 ng/ml; Fig. 1*A*) or of 0.1 mM EDTA (see Fig. 4*D* below). The affinity of WT hPRL (Fig. 1*B*) calculated by Scatchard analysis indicated a K_d of (3.4 ± 1.3) $\times 10^{-10}$ M in the presence of 1 mM EDTA and (3.5 ± 1.8) $\times 10^{-10}$ M in the presence of zinc (25 μM), thus confirming the zinc independence of hPRL binding.

In order to test for the reliability of our experimental conditions with respect to the addition of zinc in culture media, we tested the biological properties of hGH, which is known to be affected by the addition of zinc (21, 22, 38). In the presence of 25 μM zinc (Fig. 1*C*), hGH activity was slightly less than that of hPRL, with an EC_{50} of 134 ± 25 ng/ml, whereas the dose-

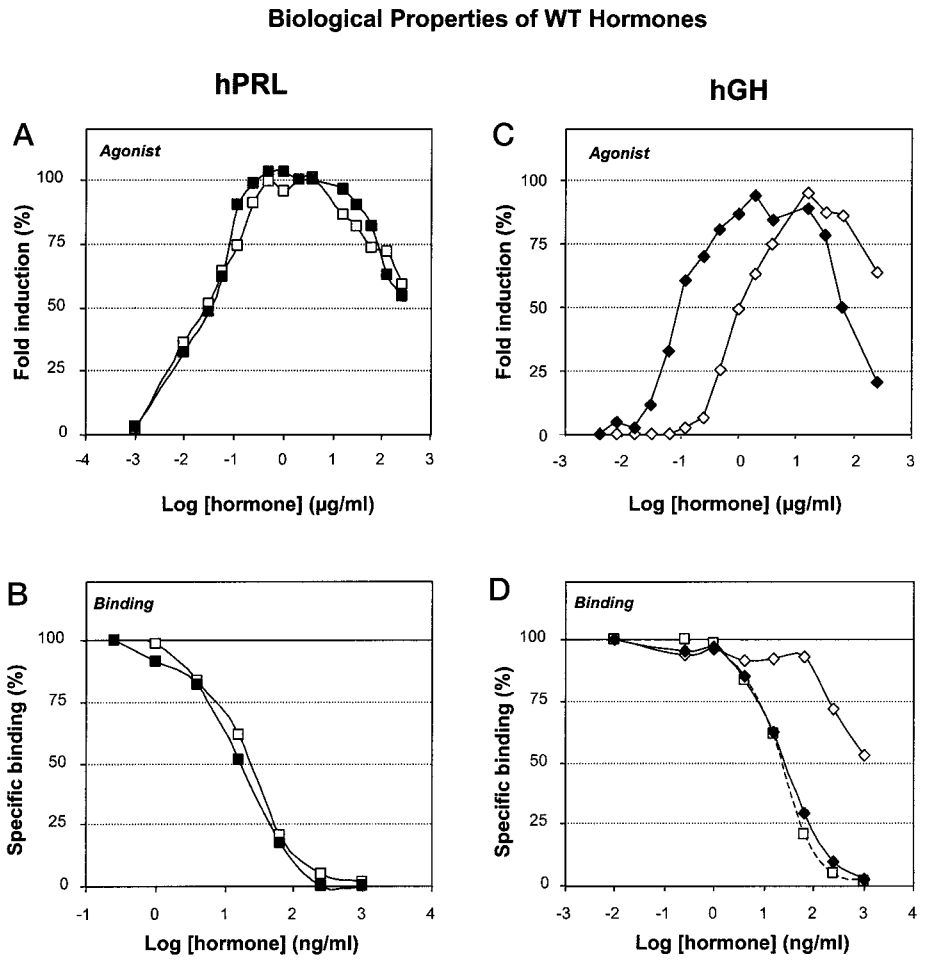

FIG. 1. **Effect of zinc on biological properties of WT hPRL and hGH.** *Panels A* and *C*, agonistic activity. Cells stably expressing the hPRLR and the LHRE-luciferase reporter gene (clone HL5) were stimulated for 18–24 h by increasing concentrations of WT hPRL (*A*) or WT hGH (*C*), in the absence of exogenous zinc (*open symbols*) or in the presence of 25 μM $ZnSO_4$ (*filled symbols*). Data are expressed as percentage of maximal activity achieved by WT hPRL. *Panels B* and *D*, competitive binding. Both hPRL (*B*) and hGH (*D*) were tested for their ability to compete with iodinated WT hPRL for binding to the human PRLR, in the presence of 1 mM EDTA (*open symbols*) or of 25 μM $ZnCl_2$ (*filled symbols*). Data are given as percentage of specific binding of WT hPRL. In *panel D*, the reference curve obtained with WT hPRL is represented (*dotted curve*) as a control. The figure represents one typical experiment, which is representative of at least three experiments performed in duplicate. Figure shows hPRL without (□) or with (■) 25 μM zinc, and hGH without (◇) or (◆) with 25 μM zinc.

response curve was expectedly displaced by ~1 log unit toward the higher concentrations without addition of zinc (EC_{50} = 1.42 ± 0.25 μg/ml; Fig. 1*C*). Both dose-response curves are bell-shaped, as reported by many investigators using various bioassays (22, 38, 39). However, self-antagonism was repeatedly more marked in the presence than in the absence of zinc. In binding experiments (Fig. 1*D*), the IC_{50} of hGH in the absence of zinc is 44-fold less than that of hPRL, in agreement with its lower bioactivity. Addition of zinc (25 μM) increases the affinity of hGH for the hPRLR by 55 ± 14-fold, making hGH as potent as hPRL (Fig. 1*D*). In conclusion, the results obtained using hGH validate the experimental procedures we used to investigate the effect of zinc on hPRL analog activity.

Effect of Zinc on Biological Properties of D183E hPRL—In the absence of zinc, the agonistic activity of the single mutant D183E was shifted to the right by more than 1 log unit compared with WT hPRL, and maximal response required up to 10–20 μg/ml of the analog, with an EC_{50} ~1.8 μg/ml, *i.e.* 20–40-fold higher than WT hPRL (Fig. 2*A*). An incremental increase of zinc concentration shifted the dose-response curve to the left, and the concentration of D183E leading to maximal activity dropped to less than 5 μg/ml (EC_{50} = 290 ng/ml) in presence of 25 μM zinc, which, however, remains ~6-fold higher than that of WT hPRL. We tested the antagonistic properties of D183E hPRL (and of other analogs) by competing a fixed concentration of WT hPRL (0.5 μg/ml) with increasing concentrations of each of these mutants. According to the fact that binding site 2 of D183E hPRL is not impaired, this analog failed to display any antagonistic activity irrespective to the presence of zinc (Fig. 2*B*). In binding studies, the IC_{50} value of D183E mutant was 7.7 ± 1.8-fold higher than that of WT hPRL in the presence of 1 mM EDTA, and 5.1 ± 2.4 in the presence of 25 μM zinc (Fig. 2*C*).

Taken together, these data indicate, first, that the D183E mutation *per se* is detrimental to the basal biological properties of hPRL; second, that the biological properties of this mutant are sensitive to the concentration of zinc; and third, that the addition of this divalent ion is not sufficient to rescue full affinity/agonistic activity compared with WT hPRL.

Effect of Zinc on Biological Properties of G129R hPRL—We previously reported that the agonistic activity of the analog carrying the single G129R mutation (impaired site 2) never exceeds 2–3% of WT hPRL activity, and this occurs at 1–10 μg/ml hormone (24). This observation was confirmed in the present study (Fig. 3*A*). In addition, we show that this residual activity is not influenced by zinc concentration (Fig. 3*A*, *inset*). As already reported (24), G129R competes WT hPRL with an IC_{50} 5–10-fold higher than the concentration of hPRL in the assay, *i.e.* ~5 μg/ml in our conditions. This effect was not modified by the addition of zinc (Fig. 3*B*), in agreement with the zinc-insensitivity of the agonistic activity of this analog (Fig. 3*A*). The affinity of G129R analog (Fig. 3*C*) was slightly lower than that of the zinc-sensitive D183E mutant (Fig. 2*C*), with an IC_{50} value ~10-fold higher than that of WT hPRL. Again, zinc failed to have any significant effect on binding (Fig. 3*C*).

Effect of Zinc on Biological Properties of D183E/G129R hPRL—The combination of D183E and G129R substitutions confers to this double hPRL mutant the ability to recover partial agonistic activity in the absence of zinc, to reach 20–40% of WT hPRL activity at 250 μg/ml (Fig. 4*A*). Compared with the dose-response curve of the G129R mutant, which is

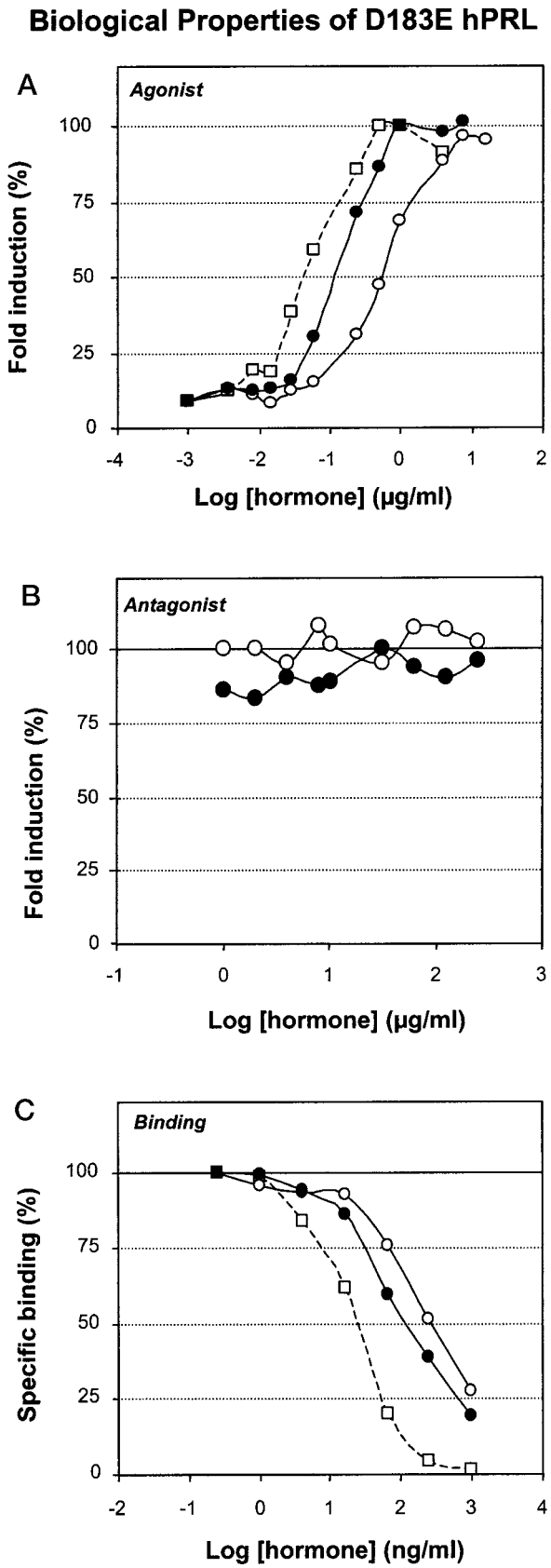

FIG. 2. **Effect of zinc on biological properties of D183E hPRL (site 1 mutant).** *Panel A*, agonistic activity; *panel B*, antagonistic activity; *panel C*, competitive binding. In *panels A* and *C*, the reference curve of WT hPRL is represented (*dotted curve*) as a control. Experiments were performed and are presented as explained in the legend to Fig. 1. Figure shows WT hPRL without zinc (□) and D183E hPRL without (○) or with (●) 25 μM zinc.

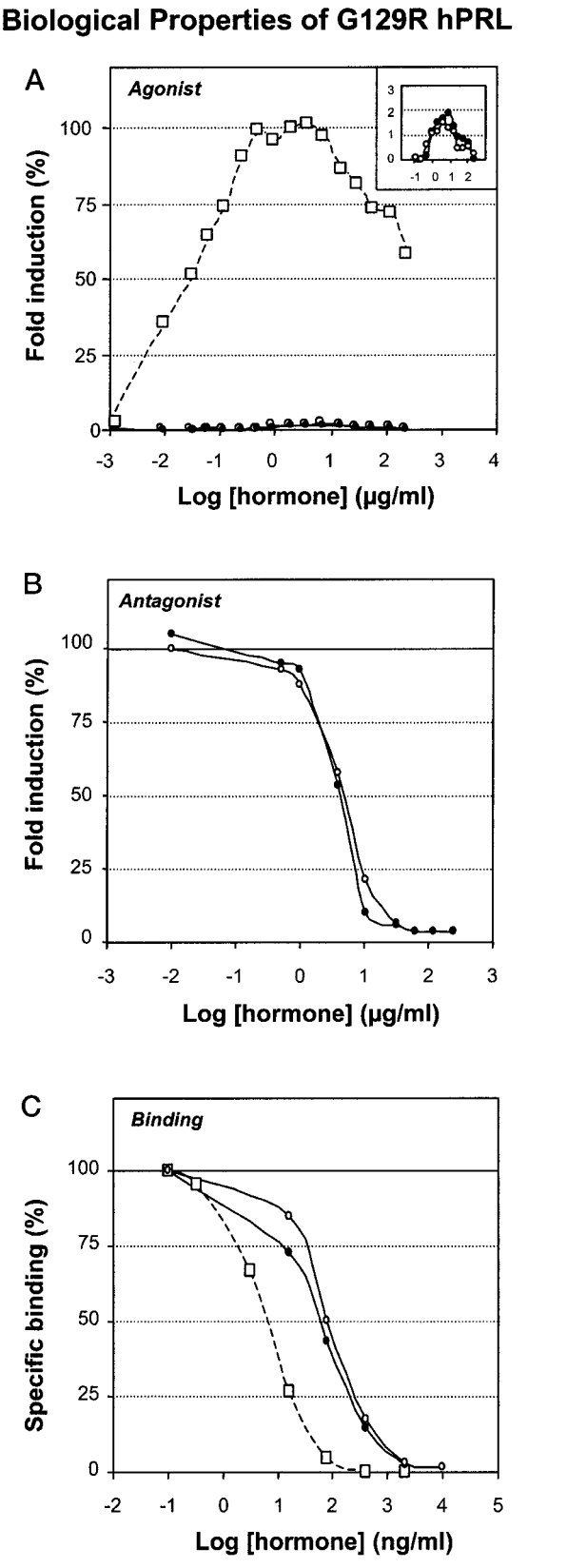

FIG. 3. **Effect of zinc on biological properties of G129R hPRL (site 2 mutant).** *Panel A*, agonistic activity; *panel B*, antagonistic activity; *panel C*, competitive binding. In *panel A*, the *inset* shows the residual activity of G129R; note that the *y* axis of the *inset* ranges from 0 to 3% of maximal activity. In *panels A* and *C*, the reference curve of WT hPRL is represented (*dotted curve*) as a control. Experiments were performed and are presented as explained in the legend to Fig. 1. Figure shows WT hPRL without zinc (□) and G129R hPRL without (○) or with (●) 25 μM zinc.

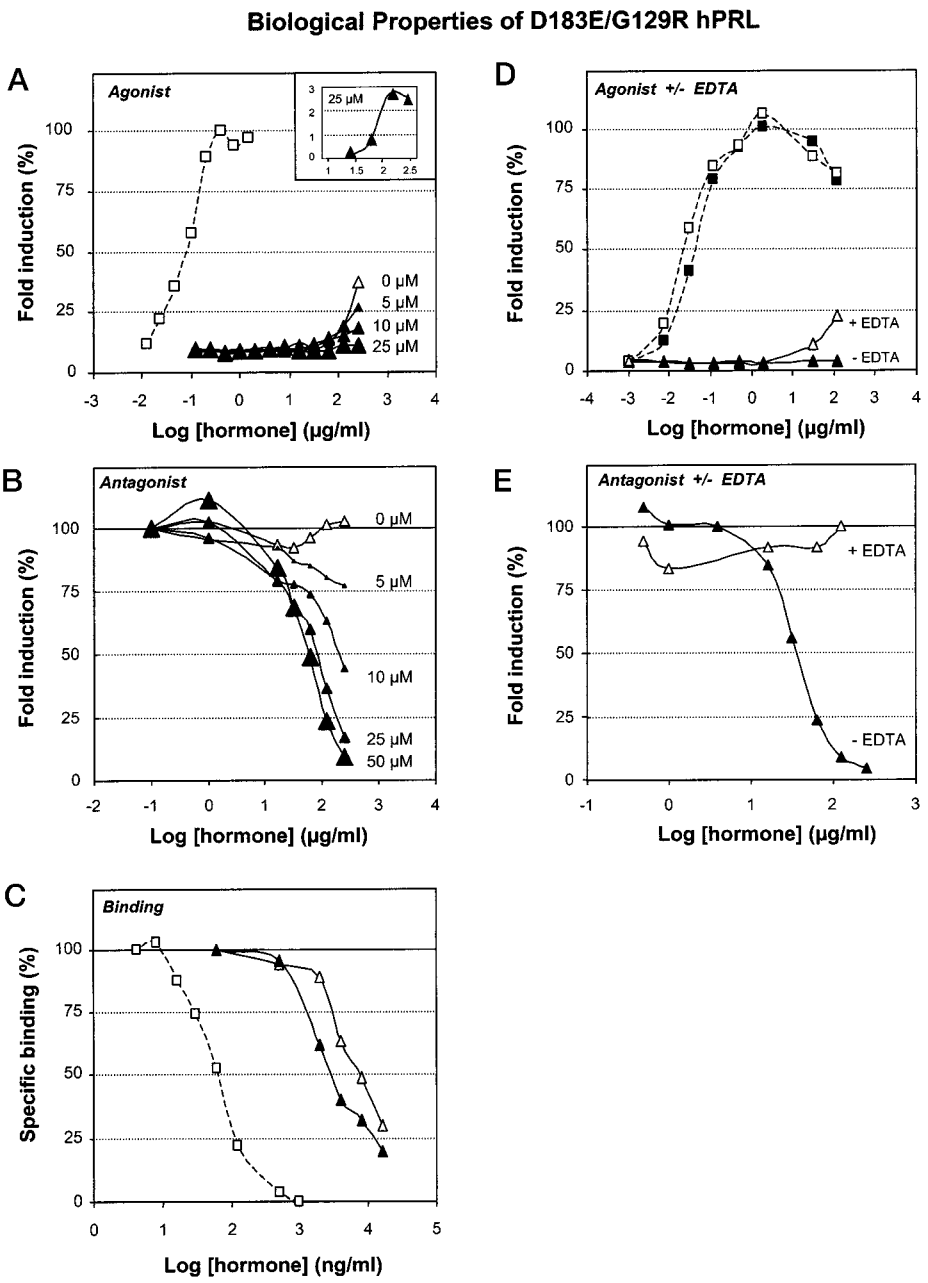

FIG. 4. **Effect of zinc on biological properties of D183E/G129R hPRL (mutant of sites 1 and 2).** *Panels A* and *D*, agonistic activity; *panels B* and *E*, antagonistic activity; *panel C*, competitive binding. In *panels A* and *B*, successive increases of zinc concentrations (0, 5, 10, and 25 μM) have been tested as indicated, and the potential of 0.1 mM EDTA to reverse the effect of 25 μM $ZnSO_4$ is shown in *panels D* and *E*. The *inset* in *panel A* shows the residual activity of D183E/G129R in the presence of 25 μM $ZnSO_4$; note that the *y* axis of *insets* ranges from 0 to 3% of maximal activity. In *panels A*, *C*, and *D*, the reference curve of WT hPRL is represented (*dotted curve*) as a control. *Panels A–C* show WT hPRL without zinc (□) and D183E/G129R hPRL without (△) or with 5 μM to 25 μM zinc (▲, with size of the symbol proportional to zinc concentration). *Panels D* and *E* show hPRL + 25 μM zinc without (■) or with 0.1 mM EDTA (□), and D183E/G129R hPRL + 25 μM zinc without (▲) or with 0.1 mM EDTA (△).

centered on the 1–10 μg/ml hormone concentration (Fig. 3*A*, *inset*), the dose-response curve of the D183E/G129R mutant is thus markedly shifted toward the higher concentrations (Fig. 4*A*). In contrast to the D183E mutant, whose agonistic activity is partially rescued by zinc (Fig. 2*A*), the activity of the D183E/G129R analog is progressively decreased upon iterative addition of zinc (Fig. 4*A*). At 25 μM zinc, the maximal activity achieved by the double mutant is similar to that of G129R hPRL (2–3% of hPRL activity; Fig. 4*A*, *inset*), but is obtained at much higher concentrations (compare *insets* of Figs. 3*A* and 4*A*). In order to confirm the role of zinc in these experiments, we analyzed the effect of 0.1 mM EDTA on the bioactivity of the D183E/G129R mutant in the presence of 25 μM zinc. We observed that EDTA is able to reverse the effect of 25 μM zinc, since ion chelation allowed the recovery of some agonistic activity (Fig. 4*D*), which was similar to that observed without addition of zinc (Fig. 4*A*). The absence of any modification of hPRL activity, in the absence or in the presence of zinc (Fig. 1*A*) or of zinc and EDTA (Fig. 4*D*), confirms that this divalent ion acts specifically on the bioactivity of D183E/G129R analog.

The antagonistic activity of the double mutant D183E/G129R was directly dependent on zinc concentration (Fig. 4*B*). In the absence of zinc or at low ion concentrations (5 μM), this analog failed to abolish the effect of WT hPRL, even at concentrations as high as 250 μg/ml. Successive increases of zinc led to a progressive increase of antagonistic properties with IC_{50} values ranging from 160 ± 47 μg/ml (10 μM zinc), to 60.3 ± 16 μg/ml (25 μM zinc) and 41.8 ± 16 μg/ml (50 μM zinc). As observed for agonistic activity (Fig. 4*D*), low concentration of EDTA (0.1 mM) confirmed the ion dependence of the antagonistic effect, since it completely reversed the effect of 25 μM zinc on the antagonistic properties of the D183E/G129R analog (Fig. 4*E*). Taken together, the behavior of the double mutant was thus totally unexpected since zinc has an opposite effect on the biological properties of this analog compared with that observed on the single D183E mutant.

The double mutant D183E/G129R has much less affinity for the hPRLR than any of the other analogs analyzed in the present study, since its IC_{50} value is ~2 logs (116-fold) higher than that of WT hPRL in the absence of zinc (Fig. 4*C*). Addition

of 25 μM zinc increases its affinity for the hPRLR by 2.3 ± 0.3-fold (IC_{50} value 50-fold higher than WT hPRL).

Effect of Various Divalent Ions on the Biological Properties of D183E hPRL—We have shown that both hPRL mutants carrying the D183E mutation are zinc-sensitive, whereas WT hPRL and the G129R analog are not. To assess the specificity of zinc with respect to other divalent ions, we tested Mn^{2+}, Mg^{2+}, Ni^{2+}, Ca^{2+}, and Cu^{2+} (25 μM each) on the biological properties of D183E analog. For each cation, a standard curve of WT hPRL was performed separately as a control to detect any side effect of the ions on cell response. The activity of WT hPRL was not significantly affected by any of the ion added (data not shown). With the exception of zinc, none of the divalent ions tested was able to enhance the bioactivity of D183E mutant (Fig. 5*A*). In binding studies, the IC_{50} value of hPRL was neither affected by any of the ions tested (data not shown). With respect to the affinity of the D183E, only minor changes of binding affinity were observed, as reported above for zinc (Fig. 2*C*). However, manganese and copper ions appeared as effective as zinc to slightly enhance the affinity of the analog (by ~1.5-fold), whereas magnesium, calcium and nickel had no effect compared with EDTA (Fig. 5*B*). Taken together, these data indicate that zinc is the only divalent cation that has an effect on both binding and agonistic activity of D183E hPRL analog.

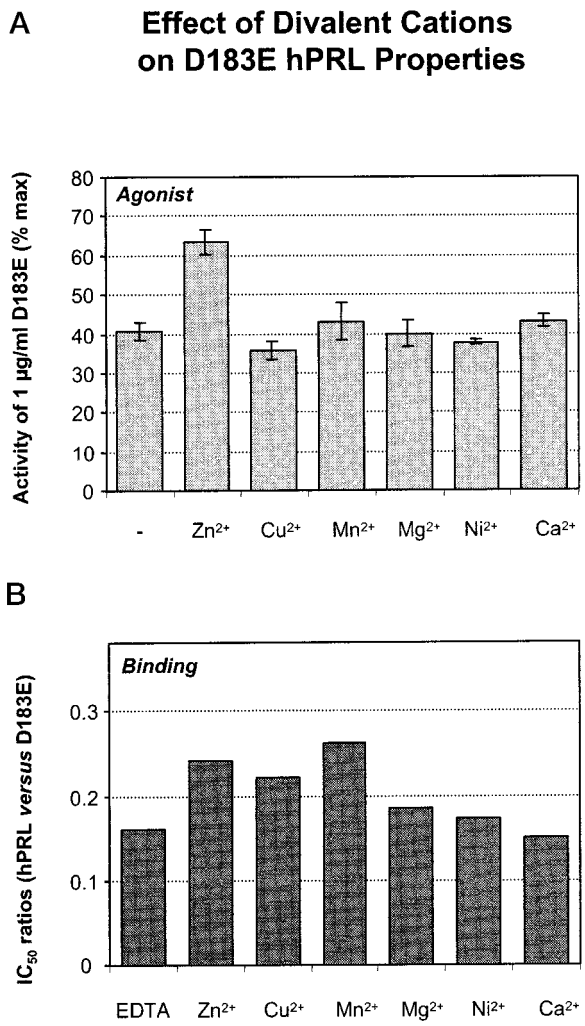

FIG. 5. **Effect of various divalent cations on binding and bioactivity of the D183E hPRL.** Six divalent cations were tested for their ability to modify the activity of the D183E hPRL analog in the LHRE-luciferase bioassay (*panel A*) or its binding to the hPRLR (*panel B*). Experiments were performed as described in legend to Fig. 1. For each cation, a standard curve using WT hPRL was performed as control and the biological properties of D183E mutant are expressed with respect to those reference curves. WT hPRL biological properties were not significantly altered by any of the cations tested. *Panel A*, the activity obtained by a fixed concentration of 1 μg/ml D183E (in the linear part of the agonistic curve) is expressed as the percentage of maximal -fold induction achieved by WT hPRL in the presence of the divalent cation indicated. The experiment represented was performed in triplicate and is representative of three independent experiments. Only zinc cations increase the D183E bioactivity. *Panel B*, the ratio of the IC_{50} values of WT hPRL *versus* D183E is indicated for each divalent cation. As reported in Fig. 2, Zn^{2+} slightly enhances the affinity of D183E for the hPRLR. Cu^{2+} and Mn^{2+} were as potent, whereas the effect of Mg^{2+}, Ni^{2+}, and Ca^{2+} was undistinguishable from that of EDTA.

DISCUSSION

Zinc Does Not Interfere with Receptor Binding of hPRL—Although hPRL binds zinc (18–20), it does not affect either its binding affinity for the hPRLR or its bioactivity (15, 22). This differs from the observations made with hGH (15). Two explanations can be proposed. Zinc binding by hPRL could involve amino acids located in a region distinct from either of the two known receptor binding sites (12), thereby explaining the absence of interference between zinc binding and receptor binding. Alternatively, the zinc binding pocket of hPRL might be similar to that of hGH and located within receptor binding site 1, but the ion coordination would not affect the interaction between hPRL and its receptor. Two observations argue in favor of the latter hypothesis. First, Sun and colleagues (20) have shown that mutation of binding site 1 residue $His\text{-}27_{hPRL}$ affects the interaction of hPRL with zinc (but not its bioactivity), suggesting that the zinc binding sites of hPRL and hGH are located within the same region of the hormone, *i.e.* receptor binding site 1. Second, comparison of the three-dimensional structures of hGH·hPRLBP (crystallographic data; Ref. 16) and rabbit (rb)PRL-rbPRLBP (theoretical model; Ref. 40) complexes allows to speculate why zinc binding by PRL does not interfere with receptor binding (Fig. 6). In the human GH·PRLBP complex (Fig. 6*A*), the side-chain extremities of the four zinc coordinating residues ($His\text{-}18_{hGH}$, $Glu\text{-}174_{hGH}$, $Asp\text{-}187_{hPRLBP}$, $His\text{-}188_{hPRLBP}$) are distant from each other by less than 4 Å (16), which allows ion coordination. Since PRLs are predicted to adopt a four-α-helix bundle fold (8, 40) very similar to that described for GHs (14, 41), residues topologically equivalent in aligned sequences of GHs and PRLs are anticipated to be placed similarly in the folded proteins. From the PRL/GH sequence alignments that were used for modeling human and rabbit PRL three-dimensional structures, $His\text{-}18_{hGH}$ and $Glu\text{-}174_{hGH}$ are topologically equivalent to His-27 and Asp-183 in PRLs, respectively (Fig. 6). Due to its shorter side chain, $Asp\text{-}183_{rbPRL}$ is 4.65 Å distant from $Asp\text{-}187_{rbPRLBP}$ (Fig. 6*B*), compared with 3.14 Å between $Glu\text{-}174_{hGH}$ and $Asp\text{-}187_{hPRLBP}$ in the hGH·hPRLBP complex (Fig. 6*A*). Moreover, the side chain of $His\text{-}188_{rbPRLBP}$ in the rabbit PRL·PRLBP complex (Fig. 6*B*) is oriented in an opposite direction compared with the homologous histidine in human PRLBP complexed to hGH (Fig. 6*A*). Hence, this residue points outside the putative zinc binding site and is ~10 Å away from $His\text{-}27_{rbPRL}$. We thus hypothesize that two of the putative zinc coordinating residues within rbPRL ($Asp\text{-}183_{rbPRL}$) and its receptor ($His\text{-}188_{rbPRLBP}$) are presumably too far from the two others ($His\text{-}27_{rbPRL}$ and $Asp\text{-}187_{rbPRLBP}$) to allow an interaction network involving one zinc ion. Although these speculations are based on the rabbit model, the similar conformation of the putative zinc binding pocket in rabbit (Fig. 6*B*) and human (Fig. 6*C*) PRLs suggests that structural hypotheses made from the former can be extended to the latter.

Engineering a hGH-like Zinc Binding Site in hPRL—In addition to the two hGH residues involved in zinc coordination

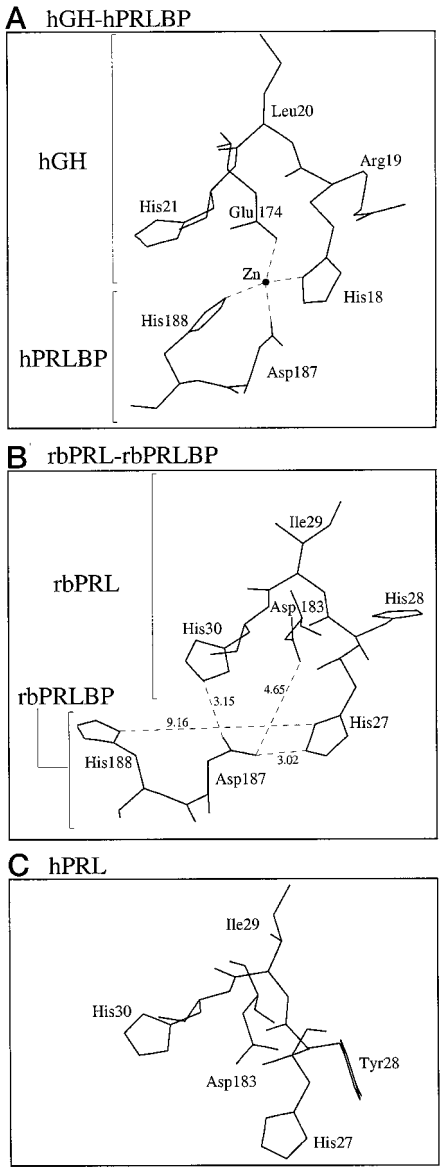

FIG. 6. **Zinc binding pocket in GH, PRL, and D183E analog: structural hypotheses.** Zinc mediation in the hGH/hPRLBP complex (*panel A*; Ref. 16) involves His-18$_{hGH}$, Glu-174$_{hGH}$, Asp-187$_{hPRLBP}$, and His-188$_{hPRLBP}$. His-21$_{hGH}$ does not participate in zinc coordination but pre-orients Glu-174$_{hGH}$ in a suitable conformation for zinc binding. The homologous residues in the rabbit PRL/PRLBP complex (*panel B*; Ref. 40) and, for the hormone only, in human PRL (*panel C*; Ref. 8), are represented. For clarity, residue numbering of hPRLBP has been used for rabbit PRLBP (actual numbers in rbPRLBP are Asp-189 and His-190). Substitution of Glu for Asp-183 in hPRL (D183E mutation) is assumed to shorten the distance between this residue and other potential zinc coordinants in order to allow zinc mediation. Since His-188$_{rbPRLBP}$ is oriented in an opposite direction to the homolgous histidine in hPRLBP and points ~10 Å away from His-27$_{rbPRL}$, we hypothesize that the zinc binding pocket of D183E hPRL analog involves His-30$_{hPRL}$ rather than His-188$_{hPRLBP}$ (see "Discussion").

(Fig. 6*A*), three residues have been shown to influence the structure of the zinc binding pocket: His-21$_{hGH}$, initially believed to be a zinc coordinating residue (15), Arg-167$_{hGH}$, and Lys-172$_{hGH}$ (15–17). These 3 amino acids are also conserved in aligned sequences of hGH and hPRL (8). Although any shift in sequence alignments can be misleading, the topological equivalence of these 5 residue pairs in folded hGH and hPRL is strengthened by the fact that they are located within the two most highly conserved regions within the PRL/GH family, namely helices 1 and 4 (8). Based on these sequence comparisons, the single mutation of Asp-183$_{hPRL}$ into Glu was thus anticipated to reconstitute in hPRL a hGH-like zinc binding pocket, with structural characteristics putatively conferring zinc sensitivity to the biological properties of hPRL. However, assuming that rabbit PRLBP is correctly modeled in this region, His-188$_{PRLBP}$ is in a conformation inappropriate for participating in the zinc binding pocket (Fig. 6*B*). Alternatively, it may be hypothesized that the fourth zinc coordinating residue in the D183E hPRL·hPRLR complex is His-30$_{hPRL}$ of the hormone instead of His-188$_{hPRLBP}$ of the binding protein (Fig. 6, *B* and *C*).

The D183E Mutation Is Detrimental to hPRL Biological Properties—The results obtained with the D183E hPRL were opposite to those expected, however, since the mutation *per se* decreases the binding affinity and the agonistic activity of this analog in the absence of zinc. At the hormone concentrations that we tested, maximal activity was achieved, in agreement with the fact that this analog is a site 1 mutant (43). The Asp to Glu substitution is highly conservative, and it is unlikely that the decrease of affinity results from a global alteration of protein folding since both circular dichroism and gel filtration chromatography were not affected for the D183E analog. Rather, we hypothesize a local steric effect due to the longer side chain of Glu compared with Asp. Interestingly, Glu-174$_{hGH}$, which is homologous to the engineered Glu-183$_{hPRL}$, is also detrimental to the binding of hGH to the hGHBP since its mutation into Ala increases the hormone affinity by 4.5-fold (42). It thus appears that a Glu at position 174$_{hGH}$/183$_{hPRL}$ prevents tight binding of these hormones to their specific receptors. In contrast, the interaction of hGH with the hPRLR requires Glu-174$_{hGH}$, since this is one of the four residues involved in the zinc coordination. In the presence of EDTA, however, Ala substitution of this amino acid has no effect on the residual affinity of hGH for the hPRLR (15), indicating that Glu-174$_{hGH}$ is not detrimental to this interaction. These observations are in agreement with our previous conclusion that the hPRL·hPRLR interaction is much more similar to the hGH-hGHR interaction than to the hGH·hPRLR interaction (8, 12), the latter being the only one involving zinc mediation.

Zinc Enhances the Biological Properties of D183E hPRL Analog—Cunningham and colleagues (15) reported that zinc enhances the binding affinity of hGH for the hPRLBP by 8,000-fold compared with EDTA. In our hands, hGH affinity for the hPRLR was 55-fold higher in the presence of zinc than in the presence of EDTA. The fact that our binding experiments were performed on membrane-bound full-length hPRLR probably accounts for the lesser effect of zinc compared with the PRLBP, since the cytoplasmic tail of PRLR has been shown to influence the overall affinity of its ligands (44, 45). Under identical experimental conditions (25 μM zinc *versus* 1 mM EDTA), the effect of zinc is much less marked on the binding affinity of D183E hPRL analog than on hGH, and none of the other divalent cations tested appeared to be more efficient. Since the hGH-like zinc binding pocket is reconstituted in D183E hPRL, these observations suggest some structural differences at the interface of hGH·hPRLBP and D183E hPRL·hPRLBP complexes. The possible involvement of His-30$_{hPRL}$ instead of His-188$_{hPRLBP}$ in zinc binding site might be one explanation (see above).

Although zinc enhances only moderately the affinity of D183E hPRL, it does affect its biological properties to a much larger extent, since the ability of this analog to activate the hPRLR is shifted leftward by almost 1 log unit upon addition of 25 μM zinc. This effect is comparable to that observed for hGH. In addition to show the inefficiency of other divalent cations, we confirmed the specificity of zinc effect by analyzing the

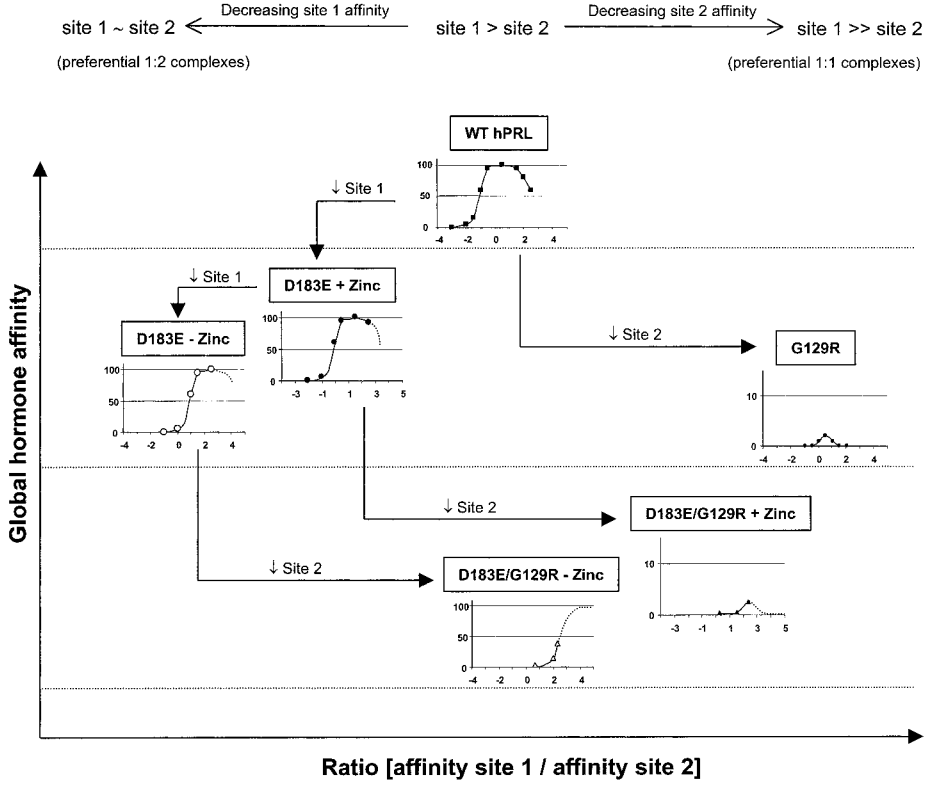

FIG. 7. **Schematic representation of hPRL analog dose-response curves according to the global hormone affinity and the relative affinities of binding sites 1 and 2.** The *y* axis represents an empirical scale of hormone global affinity (*horizontal dotted lines* correspond to affinity increments of 1 log unit). The Panels corresponding to each hPRL analog are positioned relative to the *y* axis according to their overall affinity (see "Results"). In each panel, experimental curves (Figs. 1–4) are schematized as *solid lines* for hormone concentrations that were tested (250 μg/ml) and as dotted lines when theoretically extrapolated. The *x* axis represents an empirical scale of increasing ratios of site 1 affinity/site 2 affinity. Moving toward the right along this axis corresponds to mutations (*e.g.* G129R) that lower the affinity at site 2 (hence, site 1/site 2 affinity ratio increases). Moving toward the left along the *x* axis corresponds to mutations (*e.g.* D183E) that lower the affinity at site 1 (hence, the site 1/site 2 affinity ratio decreases). We hypothesize that the amplitude of the biological response is, at least in part, directed by the relative affinities of binding sites 1 and 2. Following this model, the apparent discrepancy between binding and agonistic properties of the double D183E/G129R analog with respect to zinc concentrations can be understood.

bioactivity of the K181E analog, a site 1 mutant which also contains a non natural Glu in the same environment as the engineered Glu-183$_{hPRL}$ (27). We failed to detect any difference of the K181E dose-response curve whether zinc or EDTA was added (data not shown). Taken together, our results confirm first that the biological properties of the D183E mutant are zinc-sensitive and second, that this effect is specific to the mutation of Asp-183$_{hPRL}$ into Glu.

The Agonistic Properties of D183E/G129R hPRL Do Not Parallel Binding Affinity—The G129R mutation dramatically impairs the hPRL binding site 2. Hence, the G129R hPRL analog displays frankly reduced agonistic activity and acts as an antagonist (24). Unexpectedly, when the D183E mutation is introduced into this G129R hPRL analog, significant agonistic activity is recovered in the absence of zinc, despite the fact that the affinity of the double mutant is 1 log lower than that of the single G129R analog (Figs. 3*C* and 4*C*). The observation that, of two analogs carrying the same mutation impairing binding site 2 (Gly-129 into Arg), the one displaying the highest bioactivity is that displaying the lowest affinity clearly demonstrates that the agonistic properties of the hPRL analogs do not always parallel their global affinity. Even more paradoxically, whereas the affinity of the double mutant is slightly increased in the presence of zinc, its bioactivity is progressively decreased upon addition of this ion. Despite of the apparent discrepancy between binding affinity and agonistic properties, these results can be understood if one considers that the agonistic properties of the hPRL analogs are, at least in part, correlated to the relative affinities of each binding site. The models of hormone-receptor interaction that we propose are discussed in the next section and illustrated in Fig. 7.

The Agonistic Properties of hPRL Analogs Are Correlated to the Relative Affinities of Binding Sites 1 and 2—It has been widely described that the bell-shaped curves obtained in PRLR-mediated (24) or GHR-mediated (28) bioassays reflect the dynamic profile of receptor dimerization with respect to hormone concentration. The slope up corresponds to the formation of active 1:2 complexes (1 ligand, 2 receptors), and the slope down (self-antagonism) reflects the preferential formation of inactive 1:1 complexes at high hormone concentrations. The self-antagonistic effect is due to the fact that these ligands have a binding site 1 of higher affinity compared with that of binding site 2 (12, 28). When both binding sites have similar affinities, the formation of 1:1 complexes is not favored at high hormone concentrations and self-antagonism is less marked, or even not observed, as we reported when hPRL activates the rat PRLR (24).

The G129R hPRL analog is modified at site 2. Its agonistic affinity is not shifted with respect to the hormone concentration, which reflects that the interaction of site 1 with the receptor is not altered. However, its maximal activity is much reduced compared with WT hPRL due to its impaired ability to induce the formation of receptor dimers (43). Since the relative affinity of both sites is even more unbalanced in favor of site 1 than in WT hPRL, a bell-shaped curve is also obtained (Fig. 7). In contrast, the D183E mutant has a reduced site 1 affinity, which induces a shift of the dose-response curve (slope up) toward the high concentrations, as previously reported for many other site 1 analogs (27). Since the site 2 of these mutants is not altered, they reach maximal activity, but this requires higher hormone concentrations (43). We hypothesize that self-antagonism (not observed experimentally) should be less marked for D183E than for WT hPRL since the affinity ratio of both sites is less unbalanced in favor of site 1 in the analog (Fig. 7). Since zinc slightly favors the interaction of the site 1 of D183E hPRL with the receptor, it shifts the curve back to the left.

Both binding sites 1 and 2 are impaired in the D183E/G129R analog, and, in the absence of zinc, the affinity at site 1 is as low as possible for this analog. Although the affinities of both sites 1 and 2 are altered, their ratio is much less unbalanced in favor of site 1 than in the single G129R. This favors to the formation of 1:2 complexes, which results in the recovery of partial agonistic activity. Whether hormone concentrations >250 μg/ml would lead to (sub)maximal activity can be speculated. Al-

though the addition of zinc moderately increases the overall affinity of D183E/G129R by acting on site 1, this balances the individual affinity ratio of each binding site in favor of site 1, as is the case for the single G129R analog. This leads to the preferential formation of 1:1 complexes, which consequently decreases the amplitude of the biological response in the presence of zinc (Fig. 7).

If such models of receptor activation are correct, they indicate that the ability of PRLR ligands to induce a biological response must take into account not only their global affinity, but also the relative affinities of their two binding sites.

Design of More Potent hPRL Antagonists—As a general observation, it appears much more difficult to design potent antagonists to the PRLR than to the GHR (24, 39). It has been hypothesized (46) that such a difference between the two receptors was due to the fact that the receptor-associated tyrosine kinase JAK2 (47) is constitutively bound to the PRLR (48) but not to the GHR (49). Hence, very transient formation of PRLR dimers might be sufficient to trigger signaling cascades, whereas in the case of the GHR, formation of more stable dimers might be required to recruit JAK2 prior to engagement of signaling molecules (46). Although this hypothesis is attractive, it awaits further demonstration.

The design of highly potent hPRL antagonists by impairing binding site 2 still remains to be achieved. Among 18 point mutations tested within hPRL site 2 (26), the most efficient is the G129R substitution. Although the agonistic activity of this analog is almost completely abolished, its antagonistic properties are relatively low due to its reduced affinity. If the hypothesis of Helman and colleagues is correct (46), the design of more potent hPRL antagonists should rather focus on a total inhibition of binding site 2 than on increasing site 1 affinity. Indeed, as demonstrated by the partial agonistic activity of the D183E/G129R analog, the G129R mutation is not as potent as initially believed for definitely blocking binding site 2 (24).

Walker and colleagues (50) recently reported that point mutation of Ser-179_{hPRL} into Asp (S179D mutation) generates a very potent hPRL antagonist when tested in the Nb2 bioassay. This unique report raises several questions, however. First, in contrast to all PRL or GH antagonists reported to date, this S179D hPRL analog acts in a non-competitive manner since increasing WT hPRL concentration can not overcome the inhibition. Second, since Ser-179_{hPRL} belongs to the fourth helix, this analog is the first antagonist involving a single mutation at binding site 1 (8, 27). Moreover, since Ser-179_{hPRL} side chain is predicted to be oriented toward the core of the protein (8), a direct effect on receptor binding is hardly anticipated. The authors proposed that the S179D substitution might affect the orientation of other regions of the protein, *e.g.* helix 3 or helix 4, which would thereby confer antagonistic properties to the analog. Alternatively, if the S179D mutation dramatically enhanced the affinity of the analog, which was not assessed by binding studies in the original report (50), we might hypothesize that the balance between the relative affinities of sites 1 and 2 is so unbalanced in favor of site 1 that only inactive 1:1 complexes could be formed. Elucidation of the mechanism of action of this S179D analog will require further study, including its characterization in other bioassays than the sole and non homologous rat Nb2 cell proliferation assay (24).

Conclusions—By engineering a hGH-like zinc binding pocket in hPRL, we have generated hPRL analogs that are clearly sensitive to zinc concentrations. Despite the high level of structural similarity between hGH and hPRL, the enhancing effect of zinc mediation on receptor binding that is demonstrated for the former could not be mimicked in the latter, due to the intrinsic detrimental effect of the D183E mutation. The double mutant D183E/G129R hPRL, which carries disturbing mutations within both binding sites, displayed an agonistic activity inversely proportional to its binding affinity with respect to zinc concentration. These unexpected findings indicate that other parameters than only the global hormone affinity influence the agonistic properties of hPRL analogs. We propose that the ratio of the individual affinities of binding site 1 and 2 is a parameter at least as important as the overall hormone affinity for directing biological responses.

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