Characterization of Lactogen Receptor-binding Site 1 of Human Prolactin*

(Received for publication, January 16, 1996, and in revised form, March 18, 1996)

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Prolactin (PRL) binds to two molecules of PRL receptor (PRLR) through two regions referred to as binding sites 1 and 2. Although binding site 1 has been generally assigned to the pocket delimited by helix 1, helix 4, and the second half of loop 1, the residues involved in receptor binding have not yet all been precisely identified. In an earlier alanine-scanning mutational study, we identified three major binding determinants in loop 1 of human PRL (hPRL) (Goffin, V., Norman, M. & Martial, J. A. (1992) Mol. Endocrinol. 6, 1381-1392). Here we focus on the two other regions that form binding site 1, namely helices 1 and 4. Putative binding residues, selected on the basis of a three-dimensional model of hPRL constructed in this laboratory, were mutated to alanine, and recombinant hPRL mutants produced in Escherichia coli were tested for their ability to bind to the PRLR and to stimulate Nb2 cell proliferation. We thus identified nine single mutations (three in helix 1 and six in helix 4) whose effect was to reduce both binding and mitogenic activity by more than half as compared with wild-type hPRL, indicating the functional involvement of the corresponding residues. Adding these to the three binding determinants identified in loop 1, we now propose a complete picture of PRLR-binding site 1 of hPRL. As we earlier hypothesized, the binding site 1 determinants of hPRL differ from those of human growth hormone, a hPRL homolog.

Both receptors belong to class I of the newly described cytokine receptor superfamily (7-9). These receptors are all activated by clustering of two or more membrane subunits (for reviews, see Refs. 10-13). On the one hand, receptor activation can result from the hetero-dimerization of different subunits, such as a ligand-specific binding subunit (the α-chain) and a common signal transducer subunit (the β-chain). On the other hand, activation of some receptors can also result from the homodimerization of two identical binding components. This has been reported for the receptors of erythropoietin (14, 15), granulocyte colony-stimulating factor (16), GH (17, 18), and PRL (19-22).

The mechanism of activation of the human (h) GHR by hGH has been extensively studied, and a sequential dimerization model was proposed by Wells and co-workers in 1992 (17). According to their model of activation, the hormone first binds to its receptor through a set of amino acids forming the so-called binding site 1. The complex composed of one molecule of receptor and one molecule of hormone (H1-R1) remains inactive until it associates with a second (and identical) receptor molecule to yield an active H1-R2 complex. Receptor clustering leads to interactions between both receptors as well as between the hormone and the second receptor molecule (18). Binding of the two receptor molecules is thus sequential, and the set of amino acids of hGH interacting with the second hGHR molecule is called binding site 2. Interestingly, a sequential two-site model has also been hypothesized for interleukin-4, another four-helix bundle cytokine (25).

Mutational (23, 24) and crystallographic (18) studies of hGH have led to the identification of the amino acids belonging to both binding sites. Binding site 1 of hGH involves residues of helices 1 and 4 and of the second half of the long loop (loop 1) joining helices 1 and 2. On the other hand, binding site 2 is formed by residues belonging to the facing sides of helices 1 and 3 and a few residues in the small N-terminal loop. Due to the numerous structural and functional similarities between the PRL-PRLR and GH-GHR systems, we hypothesized earlier that the sequential receptor dimerization model described for the hGHR might also apply to activation of the PRLR (21, 26, 27). In agreement with this assumption, we showed that steric hindrance introduced in the helix 1/helix 4/loop 1 pocket (binding site 1) (28) or in the helix 1-helix 3 interface (binding site 2) of hPRL (21) is detrimental to activity. While these studies clearly indicate the general location of both binding sites on hPRL, not all residues involved in receptor binding have been identified. With respect to binding site 1, segment 58-74 (loop 1) has been characterized through systematic alanine-scanning mutagenesis (26). To the best of our knowledge, however, no systematic mutational study has yet focused on helices 1 and 4, so these helical segments, strongly suspected of containing several residues critical for tight receptor binding, remain essentially uncharacterized. To date, Arg-177 is the only amino

* This work was supported in part by Grants PAI P3-044 and PAI P3-042 from the Services Fe´de´raux des Affaires Scientifiques, Techniques, et Culturelles. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a fellowship from the Fonds pour la Formation à la Recherche dans l’Industrie et dans l’Agriculture.

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1 The abbreviations used are: PRL, prolactin (prefixes "h" and "b" indicate human and bovine, respectively); PRLR, PRL receptor; hPRLbp, human PRL-binding protein; GH, growth hormone; GHR, GH receptor; hGHbp, human GH-binding protein.

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acid within these helical segments to have been unambiguously
identified as very important for the mitogenic activity of bovine
PRL (bPRL) toward Nb2 cells (29).

Growth hormones, and presumably PRL, are composed of four α-helices and adopt the “four-helix bundle” fold (18, 27, 30, 31). In these proteins, the hydrophobic faces of amphipathic helices form the hydrophobic core (18, 27, 30). Therefore, mutational analysis within α-helices must be conducted with caution since any mutation affecting the hydrophobic core can alter the global folding pattern, as reported for some bPRL mutants (32). To date, no crystallographic structure has been reported for PRL; this has prevented any structure-based mutational study. Therefore, we have recently developed a three-dimensional model of hPRL (27), constructed on the basis of the crystallographic coordinates of porcine GH, the first elucidated structure for a protein of the PRL/GH family (30). On the basis of these data, we selected 7 residues in helix 1 and 10 residues in helix 4 whose side chain orientations were compatible with an involvement in the pocket of binding site 1 (see Fig. 1). Although meeting this criterion, Arg-177 was not considered again in the present study since its importance has been demonstratated by others (29). The 16 remaining amino acids were individually mutated to alanine, and the effect of each mutation was examined by measuring the binding and mitogenic activity of the hormone mutants. In agreement with our hypothesis, both helical regions contain several residues that are required for the hormone’s biological potency. Linking the present study with our previous analysis of loop 1 (26) and with structural data now available for hPRL (27), we can provide a complete picture of receptor-binding site 1 of hPRL.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany), Amersham International (Buckinghamshire, United Kingdom), Life Technologies, Inc., and Eurogentec (Seraing, Belgium). 10DO-GEN was purchased from Sigma, and carrier-free Na125I was obtained from Amersham International. Amphoteries (pH range of 5–7) and pl protein markers were from Pharmacia (Uppsala). Oligonucleotides were from Eurogentec. Culture media and sera were purchased from Life Technologies, Inc.

Methods

Oligonucleotide-directed Mutagenesis

All mutated hPRL cdNAS (33) were constructed as previously reported (21, 26) by the oligonucleotide-directed mutagenesis method of Sayers et al. (34). The vector used was a single-stranded M13. We used the oligonucleotide-directed mutagenesis system of Boehringer Mannheim and strictly followed the manufacturer’s instructions. Clones containing the expected mutation were identified by DNA sequencing; the oligonucleotide-directed mutagenesis system of Boehringer Mannheim (Mannheim, Germany), Amersham International (Buckinghamshire, United Kingdom), Life Technologies, Inc., and Eurogentec (Seraing, Belgium). 10DO-GEN was purchased from Sigma, and carrier-free Na125I was obtained from Amersham International. Amphoteries (pH range of 5–7) and pl protein markers were from Pharmacia (Uppsala). Oligonucleotides were from Eurogentec. Culture media and sera were purchased from Life Technologies, Inc.

Production and Purification of Proteins

Recombinant native hPRL and hPRL mutants were overproduced in 500-ml cultures of Escherichia coli B12L(DE3) cells and purified as described previously (35). Purity was assessed by SDS-polyacrylamide gel electrophoresis according to Laemmli (37).

Quantification of Proteins

Proteins were quantified physically by weighing the lyophilized powder on a precision balance (Electrobalance, Cahn 26) and chemically by the Bradford method (36). The disparity between weight and chemical measurements never exceeded 20%.

Isoelectrofocusing

The isoelectric point of the hPRL mutants was estimated by isoelectrofocusing (pH range of 5–7) as described previously (21).

Structural Analyses

Circular Dichroism—Lyophilized proteins were resuspended in 50 mM NH4HCO3, pH 8, at a concentration ranging from 300 to 500 μg/ml. Spectra were recorded with a CD6 dichrograph (Instruments SA-J O-BIN YVON, Longjumeau, France) linked to a personal computer for data recording and analysis (dichrograph software, Instruments SA-J OBIN YVON). For each protein, four spectra recorded between 195 and 260 nm were averaged. Measurements were performed in a 0.1-cm path length quartz cell. The helicity was calculated at 222 nm according to Chou et al. (38).

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

Nb2 Cell Culture and in Vitro Bioassay

The bioactivity of the hPRL mutants was estimated by their ability to stimulate growth of lactogen-dependent Nb2 lymphoma cells (39). The procedure used (40) has been previously detailed (21, 26). Briefly, cells were cultured in Fisher’s medium containing 10% horse serum and 10% fetal calf serum. 24 h before the bioassay, the cells were synchronized in culture medium containing only 1% fetal calf serum. Bioassays were performed in fetal calf serum-free Fisher’s medium (starvation medium). Various amounts of hPRL samples diluted in starvation medium (from 25 to 100 μl) were added to 2.5 ml of cells (1–2 × 105 cells/ml) plated in 6-well Falcon plates. Nb2 cells were counted with a Coulter counter (Coulter Electronics Ltd., Harpenden, Hertfordshire, United Kingdom) after 3 days. Two to four experiments were performed in duplicate for each mutant. The ED50 value (amount of hormone needed to achieve half-maximal cell growth) was calculated, and the relative bioactivity of each mutant with respect to native hPRL was estimated as the ratio of the native versus mutant ED50 values.

Binding Experiments

Binding of hPRL mutants to the lactogen receptor was performed as reported earlier (21, 26, 28). Briefly, homogenates from 3 × 107 Nb2 cells were incubated for 16 h at 25°C with 30,000–50,000 cpm 125I-hPRL in the presence of increasing amounts of unlabeled native hPRL or hPRL analog (final reaction volume of 0.5 ml). The assay was terminated by addition of 0.5 ml of ice-cold buffer (0.025 mM Tris-HCl, 0.01 mM MgCl2, pH 7.5) followed by centrifugation (5 min, 11,000 × g). The supernatants were removed carefully, and the radioactivity of the pellet was analyzed in a γ-counter. Each mutant was tested at least three times in duplicate. Specific binding was calculated as the difference between radioactivity bound in the absence (B0, maximal binding) and in the presence (nonspecific binding) of 2 μg of unlabeled native hPRL. In the different experiments, nonspecific binding never exceeded 20% of maximal binding. Data are presented as percentages of specific binding. Competition curves were analyzed using the LIGAND PC program (41). The relative binding affinity of each mutant was estimated as the ratio of the native versus mutant IC50 values.
RESULTS

Structure-based Design of the Mutational Study

Residues to be mutated were selected on the basis of a three-dimensional model of hPRL constructed in our laboratory (27), which is to date the only atomic structure available for any PRL. Selection was based on two criteria. First, residues located on the exposed faces of the helices 1 and 4 were listed. Then, among this set of amino acids, we considered only those whose side chain orientations were compatible with an involvement in the binding site 1 pocket. The side chains of the 16 residues that were studied by mutagenesis are colored in green (helix 1) and blue (helix 4).

TABLE I

Structural analysis of hPRL mutants

The helical content of each alanine substitution mutant was measured by circular dichroism and calculated at 222 nm according to Chen et al. (38). The apparent molecular mass of each mutant was estimated by gel filtration as described under “Experimental Procedures.” The calculated apparent molecular masses are presented.

| hPRL mutants | Helicity | Apparent molecular mass |
|--------------|----------|-------------------------|
| Native hPRL  | 46.1     | 21.0                    |
| Helix 1      |          |                         |
| R16A         | 44.3     | 20.7                    |
| V23A         | 41.3     | 20.9                    |
| S26A         | 45.7     | 20.9                    |
| H30A         | 41.7     | 21.5                    |
| S34A         | 41.1     | 21.0                    |
| F37A         | 42.7     | 21.0                    |
| S38A         | 45.0     | 21.3                    |
| Helix 4      |          |                         |
| Y169A        | 41.9     | 20.0                    |
| H173A        | 46.7     | 22.2                    |
| R176A        | 45.1     | 23.6                    |
| H180A        | 40.5     | 21.1                    |
| K181A        | 50.6     | 22.2                    |
| K181E        | 46.6     | 23.6                    |
| N184A        | 40.4     | 21.1                    |
| Y185A        | 45.8     | 21.1                    |
| L188A        | 48.4     | 21.1                    |
| R192A        | 39.0     | 23.6                    |
| V23A/H30A/F37A | 43.4   | 23.6                    |

The production and purification yields were similar to that of native hPRL during renaturation.

Structural Characterization of hPRL Mutants

Since all residue changes reported in this study affect regular secondary structures (α-helices), each mutant was first structurally characterized by circular dichroism and chromatography on a molecular sieve to assess its proper folding. The isoelectric point was also determined for each mutant.

Isoelectric Point—The major isofrom of purified recombinant hPRL exhibits a pl of 6.2 (21, 35). Introduction or removal of charged residues alters the net protein charge. Accordingly, the pl values of hPRL mutants R16A, R176A, K181A, K181E, and R192A were 0.2–0.3 units lower than normal (data not shown), thus confirming at protein level the presence of the mutations.

Circular Dichroism—hPRL has an α-helix content of 45 ± 5% (21, 26, 28) as determined by circular dichroism. In agreement with the spectrum of native hPRL, all mutants produced in this study displayed the typical curve of all α-proteins, with two minima at 222 and 208 nm and a maximum at 195 nm. The calculated helicities are reported in Table I. They are all in the range of 45 ± 5%, suggesting no significant alteration of the overall secondary structure content.

Apparent Molecular Mass—The apparent molecular mass of each mutant was estimated from its retention time on a high pressure molecular sieve. The calculated apparent molecular masses are reported in Table I. In agreement with the theoretical molecular mass of hPRL (23 kDa), the estimated molecular masses of all the mutants are in the range of 22 ± 2 kDa; they correlate with the data obtained by CD analysis.
genic activity of these mutants toward Nb2 cells was reduced by 66\% of the reference value, respectively (Fig. 3).

The relative mitogenic potency of each mutant was estimated as the amount of native hPRL required to produce half-maximal proliferation of Nb2 cells (ED_{50}). Each mutant was tested in at least three independent experiments. The relative mitogenic potency of each mutant was estimated as the amount of native versus mutant hPRL required to produce half-maximal proliferation of Nb2 cells (ED_{50}). Each mutant was tested at least three times in duplicate. A typical experiment is presented.

Since no significant alteration of the global structure was detected by either procedure, we conclude that any alteration of the biological properties (see below) reflects the functional involvement of the mutated residue rather than an unexpected effect of the mutation on protein folding.

Biological Analysis of hPRL Mutants

We have previously reported that recombinant native hPRL stimulates Nb2 cells as efficiently as pituitary-purified hPRL, with half-maximal growth at \(-200\) pg of hPRL/ml (ED_{50}) (26). Therefore, recombinant wild-type hPRL was used as a reference for estimating the bioactivity of all the hPRL mutants. Nb2 cells contain \(-12,000\) PRL receptors/cell (43). As described earlier (21, 26, 28), Nb2 cells were also used in binding assays of hPRL mutants. Typical Nb2 cell proliferation and binding curves are represented in Fig. 2. Binding and cell proliferation data are summarized in Fig. 3.

When alanine was substituted for Val-23, His-30, or Phe-37 of helix 1, binding of hPRL to the Nb2 PRLR decreased significantly, with the affinity dropping to only 26 \pm 5, 30 \pm 10, or 21 \pm 6\% of the reference value, respectively (Fig. 3A). The mitogenic activity of these mutants toward Nb2 cells was reduced accordingly (26 \pm 9\% for V23A, 39 \pm 1\% for H30A, and 44 \pm 5\% for F37A). A less marked affinity change was observed when alanine replaced Arg-16 (101 \pm 19\%), Ser-26 (48 \pm 15\%), Ser-34 (66 \pm 5\%), or Ser-38 (52 \pm 12\%); this tallies with the mitogenic potency of these mutants (73 \pm 12\% for R16A, 46 \pm 3\% for S26A, 97 \pm 23\% for S34A, and 94 \pm 7\% for S38A) (Fig. 3A). These data suggest that Val-23, His-30, and Phe-37 are binding determinants of hPRL. By comparison with mutations in loop 1 (26) or in helix 4 (see below), the helix 1 mutations have but a limited effect since the most effective among them, F37A, results in a 5-fold reduction of binding. We thus constructed a hPRL mutant carrying the three most effective mutations, namely V23A/H30A/F37A. Although this variant displayed lesser biological activity than any of the single mutants (15 \pm 1\% mitogenic activity), the effects of these mutations did not appear to be additive, indicating a limited involvement of helix 1 in binding site 1 of hPRL.

In helix 4, six mutations were found to significantly alter binding: Tyr-169 (18 \pm 7\%), His-173 (30 \pm 4\%), Arg-176 (20 \pm 3\%), His-180 (8 \pm 1\%), Lys-181 (3.7 \pm 1.2\%), and Tyr-185 (23 \pm 5\%). Accordingly, the mitogenic activities of these mutants were also markedly reduced: 9.1 \pm 2.4\% for Y169A, 13 \pm 1.5\% for H173A, 5.2 \pm 0.5\% for R176A, 6.2 \pm 2\% for H180A, 1.3 \pm 0.2\% for K181A, and 8.1 \pm 1.4\% for Y185A (Fig. 3B). When Asn-184, Leu-188, or Arg-192 was replaced with alanine, the effect was weak (mitogenic activity ranged from 63 to 104\%), although binding of the L188A analog was more significantly reduced (34 \pm 4\%). Tyr-169, His-173, Arg-176, His-180, Lys-181, and Tyr-185 were thus identified as binding determinants within helix 4. To evaluate the importance of a positive charge at position 181 (Lys-181 is the strongest binding determinant of hPRL), we replaced this Lys residue with Glu. The biological activity of the K181E mutant was \sim 10 times lower than that of the K181A mutant, indicating that a positive charge is required at this position.

DISCUSSION

Structure-based Prediction of Putative Binding Residues—A few years ago, Luck et al. (29, 32, 44) reported the effects of point mutations on the mitogenic activity of bPRL. Since no structure was available for any PRL at that time, the residues to be mutated were selected mainly on the basis of sequence comparisons between members of the PRL/GH family. In many cases, point mutations either proved ineffective (44) or were assumed to affect biological properties solely as a result of altered global protein folding (32). Consequently, only a very few residues, such as Arg-177, could be clearly identified as functionally required for bPRL bioactivity (29).

To circumvent the lack of a three-dimensional structure of PRL, we have recently constructed a three-dimensional model of hPRL (27) based on the crystallographic structure of porcine GH, the first structure of a member of the PRL/GH family that has been determined experimentally (30). Thanks to this model, we were able to formulate hypotheses concerning the interaction between PRL and its receptor, notably with regard to the location of both binding sites and to the residues that form them (27).

Analysis of sequence-structure-function relationships in PRL led us to propose that binding site 1 involves the pocket delimited by helix 1, helix 4, and loop 1 (26–28). In agreement, mutational analysis of loop 1 clearly demonstrated the involvement of this region in bioactivity (26). To confirm our hypothesis, we decided to further characterize binding site 1 by scanning the two remaining regions, namely helices 1 and 4. As reported earlier, helix 1 is involved in both binding sites since residues facing helix 3 are involved in binding site 2, whereas residues facing helix 4 are predicted to be part of binding site 1.
Therefore, we chose in this study to mutate only those residues that point toward the binding site 1 pocket. Helix 4, on the other hand, is at the center of binding site 1; there were more residues in this region (10 residues) to be investigated in order to assess their involvement in the protein’s biological properties.

Structural and Biological Analysis of hPRL Mutants—Because all the selected residues are predicted to be located on exposed faces of helices, they should not affect protein folding. Accordingly, our structural analyses of the various mutants failed to detect any significant alteration of the global protein conformation. Although CD analysis and estimation of the apparent molecular masses are probably not sufficiently sensitive methods for detecting subtle and local structural changes, it should be stressed that by combining these methods, we have previously been able to identify misfolded hPRL mutants and to eliminate them from our study (21, 26). Moreover, we know of no reported structurally disruptive alanine substitution of any residue located on an exposed face of PRL or GH, with the sole exception of the cysteines involved in disulfide bonds (21, 24, 26, 42).

This study confirms the assumed involvement of both helices 1 and 4 in binding site 1 of hPRL. As previously observed for hGH (Refs. 24, 45, and 46; for review, see Ref. 47), binding site 1 is centered on helix 4 since this segment contains not only the greatest number of binding determinants, but also those whose replacement with alanine is the most detrimental to both binding and mitogenic activity. The most effective mutations in helix 1 (V23A and F37A) cause only a 5-fold reduction of the biological activity, compared with the 100-fold decrease in
binding when Lys-181 is replaced with alanine. Even when the three most effective mutations in helix 1 are combined (V23A, H30A, and F37A), the mitogenic activity is diminished only 6-fold; this suggests a limited involvement of this helical segment in the interaction with the receptor. Considering that a 2-fold reduction of both binding and mitogenic activity reflects a significant functional involvement of an amino acid, we have identified 12 residues (referred to as "binding determinants") in receptor-binding site 1 of hPRL (Figs. 4 and 5): Val-23, His-30, and Phe-37 in helix 1 (this work); His-59, Pro-66, and Lys-69 in loop 1 (26); and Tyr-169, His-173, Arg-176, His-180, Lys-181, and Tyr-185 in helix 4 (this work). Furthermore, Luck et al. (29), using the same bioassay, found that mutating Arg-177 in bPRL drastically alters the bioactivity of the hormone (reducing it to 1.1% of the reference value). Since an Arg residue is found at this position in all PRLs, it is most likely that this residue is also a major binding determinant of hPRL.

Some hPRL mutants, such as S26A, S34A, S38A, and L188A, display lesser binding, but normal to slightly altered mitogenic activity. This might reflect the "spare receptor" phenomenon, in which maximal biological activity occurs at submaximal receptor occupancy. In the Nb2 system, maximal cell growth has been reported to occur at 35% of maximal binding (42). Alanine substitution of Lys-187 is reported to halve the mitogenic activity of bPRL, but since this position remained almost insensitive to other mutations (replacement with Leu, Asn, or Arg) (29), it seems unlikely that Lys-187 is a major determinant of receptor binding. Finally, Luck et al. (29, 32) reported that mutation of Arg-21 or Tyr-28 to various amino acids reduces the effect on Nb2 cells by a factor of 2-5. Our three-dimensional structural model of hPRL (27) suggests that the side chains of both these residues point outside binding site 1; we anticipate that they belong to binding site 2, which involves the facing sides of helices 1 and 3 (21, 27). For these various reasons, we do not consider any of the residues just mentioned to belong to binding site 1. Fig. 4 shows the spatial distribution of the 13 determinants of binding site 1 of hPRL. Although all potential binding determinants were selected on the basis of our three-dimensional model, we cannot rule out the involvement of other residues not tested in this study, although it does appear unlikely.

Fig. 4. Distribution of binding determinants on hPRL. The three-dimensional model of hPRL (27) is shown with binding site 1 facing the viewer (see Fig. 1 for details). The side chains of the residues identified as strong determinants of hPRL binding to the PRLR are colored in green (helix 1), blue (helix 4), and red (loop 1). The side chain of Arg-177 (29) is also represented.

Fig. 5. Distribution of the determinants on hPRL for binding to the PRLR and on hGH for binding to the PRLR and GHR. Amino acid sequences forming binding sites 1 of hPRL and hGH are aligned. Numbers above and below the sequences correspond to hPRL and hGH, respectively. The first line represents the binding determinants on hPRL for binding to the PRLR (Ref. 26 and this study). Identification of Arg-177 as an important residue is from Luck et al. (29). The second line refers to binding determinants on hGH for binding to the PRLR, identified by Cunningham and Wells (42) using the hPRLbp; and the third line represents the determinants on hGH for binding to the GHPR, identified by means of the hGHbp (24).
that replacing Arg-177 with an alanine or a glutamate also decreases bPRL bioactivity to 1.1 and 0.3% of the reference value, respectively. Finally, Clarkson and Wells (46) proposed, on the basis of an energy analysis of the hGH-hGHbp interface, that electrostatic interactions might contribute to determining the binding specificity; this is also possible in the case of PRL (see below).

Comparison of Binding Sites 1 of hPRL and hGH—It is usually assumed that homologous proteins exert a common activity through identical or very similar mechanisms. In the present context, hPRL and hGH might thus be expected to bind by the same mechanism to the lactogen receptor. The available data indicate otherwise. First, whereas tight binding of hGH to the hPRLbp requires mediation by a zinc ion, hPRL binding to the hPRLbp is zinc-independent (48). Second, as shown in Fig. 5, hGH and hPRL clearly appear to bind to the PRLR via different sets of amino acids (Refs. 26 and 42 and this work). For example, Lys-69, Tyr-169, and His-180 play a major role in hPRL binding, whereas their hGH counterparts (Arg-64, Tyr-160, and Asp-171) can be mutated without significantly affecting binding to the hPRLbp (Table II). In contrast, Ile-58, Ser-62, Glu-65, and Arg-183 are required in hGH, while their hPRL counterparts (Leu-63, Glu-67, Glu-70, and Arg-192) can be mutated without compromising receptor binding. Even when topologically equivalent residues are binding determinants for both hormones, they do not appear to be equally important (Table II). One of the rare similarities between binding of hGH and hPRL to the PRLR is the involvement of two basic residues: Arg-176 and Arg-177 in hPRL and their topological equivalents (Arg-167 and Lys-168) in hGH (see Fig. 5). In hGH, both residues are considered specificity determinants, meaning that they are crucial to hGH binding to the PRLR, but not to its binding to the GHR (42, 47, 49). As Arg-176 and Arg-177 in hPRL are also among the strongest binding determinants (Table II), one would expect these residues to be a characteristic requirement for PRLR binding, in agreement with the proposed role of charged residues in determining the binding specificity (46). The disruptive effect of mutating Arg-167 (in hGH), however, has been partly linked to an indirect alteration of the zinc-binding pocket conformation (Table II) (42), so the role of this arginine is likely to differ from that of Arg-176 (in hPRL) since receptor binding to PRL is zinc-independent (48). The same applies to His-30 in hPRL and its counterpart (His-21) in hGH since the former is a real binding determinant in hPRL, while the latter is involved only in zinc chelation in hGH (42, 49).

Among the features common to the interaction of hGH with the hGHbp (18) and with the hPRLbp (49) are the major contacts involving two Trp residues found in both receptors (Trp-104 and Trp-169 in the hGHbp and Trp-74 and Trp-139 in the hPRLbp (Refs. 18 and 49; for a review, see Ref. 47). In the hGH-hGHbp interaction, these Trp residues are buried in a hydrophobic environment formed by the alkyl portions of Lys-172 and Thr-175 surrounded by Asp-171 and Phe-176 (48), in keeping with earlier findings that these residues are among those accounting for the majority of the free energy of the hormone-receptor interaction (45). In hPRL, the topologically equivalent amino acids are Lys-181, Asn-184, His-180, and Tyr-185. With the exception of Asn-184, these residues are also strong binding determinants in hPRL (Table II), suggesting analogous interactions of helix 4 amino acids with Trp-74 and Trp-139 of the PRLR. As these two Trp residues are not found in the other cytokine receptors, it is likely that the network of hormone-receptor contacts involving these Trp residues is a characteristic feature of the interactions between PRL/GH hormones and their receptors.

### Table II

| hGH | hGH-hGHbp | hGH-hPRLbp | hPRL-hPRL
|-----|-----------|------------|---------|
| Phe-10 | 17       | 100        | Phe-F19 |
| Met-14 | 45       | 26         | Val-23  |
| Ala-127 | 0.25    | 0.45       | Ser-26  |
| His-18 | 62       | <1 (72)    | His-27  |
| His-21 | 303      | 1 (135)    | His-30  |
| Phe-25 | 159      | 14         | Ser-34  |
| Tyr-28 | 21       | 21         | Phe-37  |
| Loop 1 |
| Phe-54 | 23       | 71         | His-59  |
| Glu-56 | 24       | 125        | Ser-61  |
| Ile-58 | 6        | 6          | Leu-63  |
| Phe-61 | 30       | 30         | Phe-66  |
| Ser-62 | 36       | 9          | Gly-67  |
| Asn-63 | 30       | 23         | Asp-68  |
| Arg-64 | 5        | 55         | Lys-69  |
| Glu-65 | 170      | 40         | Glu-70  |
| Glu-66 | 48       | 90         | Glu-75  |
| Gln-68 | 19       | 83         | Gln-73  |
| Lys-70 | 41       | 67         | Lys-75  |
| Ser-71 | 20       | 36         | Ser-71  |
| Helix 4 |
| Tyr-160 | 71     | 71         | Tyr-169 |
| Tyr-164 | 28     | 48         | Arg-176 |
| Arg-167 | 133    | <0.2 (3)   | Arg-176 |
| Lys-168 | 91      | 6          | Arg-177 |
| Asp-171 | 14     | 14         | Lys-180 |
| Lys-172 | 7       | <0.5 (3)   | Lys-181 |
| Glu-174 | 455    | <0.3 (77)  | Asp-183 |
| Thr-175 | 6       | 43         | Asp-184 |
| Phe-176 | 6       | 4           | Tyr-185 |
| Arg-178 | 2       | 14         | Lys-185 |
| Arg-183 | 48      | 38         | Arg-192 |
| Val-185 | 22      | 62         | Ile-194 |

Table II: Relative importance of binding determinants identified in hGH and hPRL

The binding affinities of mutants in which the binding determinants were mutated to alanine or glutamate were expressed as percentages of the affinity of the wild-type hormone. The first and last columns correspond to the aligned sequences of hGH and hPRL, respectively (see Fig. 5). All binding determinants for the three interactions hGH-hGHbp (second column; data from Cunningham and Wells (24)), hGH-hPRLbp (third column; data from Ref. 42), and hPRL-hPRL Nb2 (fourth column; data from Goffin et al. (26) and this study) are indicated. When no value is available for hPRL, results obtained for the bPRL mutants in the Nb2 proliferation assay are indicated and marked with an asterisk (29, 32, 44). The hGH-hPRLbp interaction is mediated by zinc chelation. In the third column, values in parentheses indicate the effects of mutations in the presence of EDTA, i.e. in the absence of zinc chelation (42). It appears that the effects of mutating His-18, His-21, Glu-174, and, to a lesser extent, Glu-167 are due mainly to alteration of zinc binding and/or pocket shape. Although binding of the L188A hPRL mutant is reduced by 33% (fourth column), this residue is not considered a strong determinant since its mitigating activity was reduced to a much lesser extent (see text).

Altogether, this study thus confirms our earlier hypothesis that hPRL and hGH bind to the PRLR via mechanisms with different requirements at the molecular/residue level (26, 27). In contrast, structural analysis of the binding sites (27) led us to propose that there is a closer parallel between the mechanisms by which hPRL and hGH bind to their respective receptors. These interactions do indeed share some common features, such as the non-involvement of zinc ions (48) and a similar distribution of strong binding determinants (for example, His-59, Lys-69, His-180, and Tyr-185 in hPRL and their respective equivalents (Phe-54, Arg-64, Asp-171, and Phe-176) in hGH) (see Table II). This work completes our picture of lactogen receptor-binding site 1 of hPRL. Combined with our previous work on binding site 2 (21), it provides a global view of the interaction between this important lactogenic hormone and its receptor.
Acknowledgments—We thank Drs. C. Houssier and A. Taquet for help with CD analysis. We thank Drs. R. Matagne and C. Grandfils for lending the Coulter counter apparatus and Dr. J. Smal for lending the γ-counter. We appreciate the critical reading of the manuscript by Dr. P. A. Kelly. We also thank M. Lion for technical assistance and G. Guillaume for constructing the mutated DNA for K181A and K181E. P. A. Kelly. We also thank M. Lion for technical assistance and G. Guillaume for constructing the mutated DNA for K181A and K181E. We are grateful to Drs. K. Hoffmann and J. Lamotte for help with the three-dimensional model figures.

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