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Sequence-Function Relationships Within the Expanding Family of Prolactin, Growth Hormone, Placental Lactogen, and Related Proteins in Mammals*

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I. Introduction

PRL PL, and GH are homologous proteins that are thought to have arisen from a common ancestral gene by two successive tandem duplications (Refs. 1–5; for review, see Ref. 6). PRL and GH are mainly secreted by the anterior pituitary of all vertebrates. The divergence of the PRL and GH lineage from the common ancestral gene has been located some 400 million years ago, which is in good agreement with the presence of distinct PRL and GH in fish (2, 3, 6, 7). PL is uniquely observed in mammals and is secreted in the placenta by syncytiotrophoblastic cells (for reviews, see Refs. 8–10). Initially, PLs were proposed to have evolved from the common PRL/GH precursor gene (1). Further cloning and analysis of PRL, GH, and PL genes from different species (chromosome carrier, gene size, splicing sites, sequence identity; see Refs. 6, 7, 11, and 12) have led to a reconsideration of this hypothesis, and it is now assumed that primate PLs evolved from the GH lineage while nonprimate PLs arose from the PRL lineage.

PRL, GH, and PL share several structural and biological features (for review, see Ref.12). They are all constituted of 190–200 residues and the molecular weight of mature proteins is ~22,000–23,000. To date, only the three dimensional (3D) structure of two members of the PRL/GH/PL family has been determined by x-ray diffraction: porcine GH (pGH; Ref. 13) and human (h) GH [unbound affinity-matured hGH (14) or wild type hGH complexed to hGH- or hPRL-binding proteins (15, 16)]. The 3D structure of these proteins is very similar and is composed of four anti-parallel α-helices (four-helix bundle) connected in the unique "up-up-down-down" fashion (13). Based on the amino acid sequence similarities within the PRL/GH/PL family, several authors (13, 15, 17, 18) have suggested that the structure described for GH may serve as the global folding model for all the members of this family. This is in good agreement with different studies aimed at predicting the 3D structure of hGH (19), bovine (b) GH (20) or hPRL (Ref. 21 and Fig. 1).

A number of different biological activities have been reported for the proteins of this family. GH is usually linked to physiological processes related to growth and morphogenesis (for reviews, see Refs. 22 and 23), whereas PRL is mainly involved in phenomena such as lactation, reproduction, osmoregulation, and immunomodulation (for reviews, see Refs. 24–27). Depending on species, PL exerts PRL-like and/or GH-like effects and acts at both fetal and maternal levels (for reviews, see Refs. 7, 8, 10, 28). The biological activities of PRL, GH, and PL are mediated by specific membrane receptors found in several tissues (reviewed in Refs. 29–31). Classically, the GH or somatogen receptor (GHR) has been presented as the specific receptor for GH (32, 33), whereas the PRL or lactogen receptor (PRLR) has been considered specific for PRL and PL (34–37). However, these

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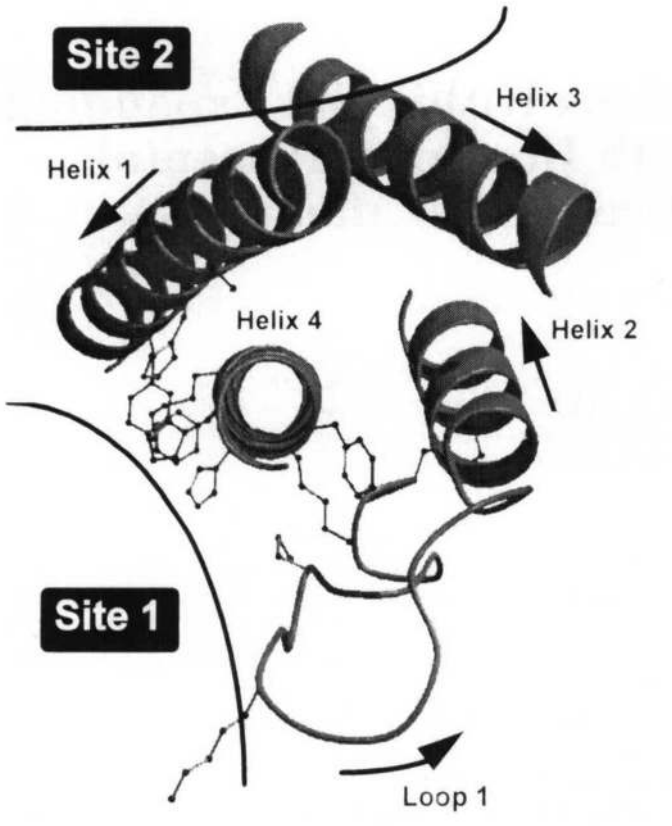

FIG. 1. Folding and global location of binding sites 1 and 2 of hPRL. The theoretical 3D structure of hPRL (21) has been modeled using the crystallographic coordinates of porcine GH (13). The four-helix bundle scaffold is assumed to be shared by all members of the PRL/GH/PL family. *Arrows* indicate the N- to C terminus orientation of helices (helix 4 points toward the viewer). Global location of binding sites 1 and 2 of hPRL are indicated. Binding site 1 is composed of helix 1, loop 1, and helix 4 and side chains of the binding determinants identified by mutational studies are represented (III.A.2.C). Binding site 2 is located around the cleft defined by helices 1 and 3.

TABLE 1. References and code numbers in the Swissprot databank of all sequences aligned in Figs. 3–6

Hormone	Reference	Code in Swissprot
Human		
hPRL	Cooke *et al.*, 1981 (5)	P01236
hGH	Martial *et al.*, 1979 (73)	P01241
hPL	Shine *et al.*, 1977 (74)	P01243
Bovine		
bPL	Schuler *et al.*, 1988 (75)	P09611
bPRP-I	Schuler and Hurley, 1987 (64)	P05402
bPRP-II	Kessler *et al.*, 1989 (66)	P12401
bPRP-III	Kessler *et al.*, 1989 (66)	P12402
bPRP-IV	Yamakawa *et al.*, 1990 (67)	P19159
bPRP-V	Tanaka *et al.*, 1989 (68)	P18917
bPRP-VI	Tanaka *et al.*, 1991 (69)	
		(PIR: S14722)
Rodent		
mPL-I	Colosi *et al.*, 1987 (48)	P18121
rPL-I	Robertson *et al.*, 1990 (49)	P21702
rPL-Iv	Robertson *et al.*, 1991 (50)	P34207
		(PIR: A38666)
mPL-II	Jackson *et al.*, 1986 (52)	P09586
rPL-II	Duckworth *et al.*, 1986 (53)	P09321
hamPL-II	Southard *et al.*, 1989 (54)	P14059
mPLF	Linzer *et al.*, 1985 (55)	P04095
mPRP	Linzer and Nathans, 1985 (57)	P04769
*rdec*PRP	Roby *et al.*, 1993 (58)	
		(GenBank: L06441)
rPLP-A	Duckworth *et al.*, 1986 (59)	P09320
rPLP-B	Duckworth *et al.*, 1988 (61)	P24800
rPLP-C	Deb *et al.*, 1991 (63)	P33579
		(GenBank: M76537)

For some, access numbers in PIR or GenBank databanks are also indicated. *Numbers in parentheses* refer to references found in the list.

restrictive specificities are not always respected. For example, primate GHs are able to bind to the lactogen receptor with high affinity (38, 39), whereas recent studies have shown that some ungulate PLs [bPL and ovine (o) PL] bind to the somatogen receptor (40–43). On the other hand, a separate receptor specific for PLs has been reported and characterized in ovine fetal liver and bovine endometrium (44–47), but attempts to clone a PL receptor have been unsuccessful, and little is known about its functional implication.

On the basis of their structural features [location and type of the intron/exon splice sites, chromosome location, nucleic acid and amino acid sequences, (predicted) length of mature protein, etc.], several recently cloned cDNAs have been proposed as new members of the PRL/GH/PL gene family (see Table 1): mouse (m) PL-I (48), rat (r) PL-I (49), rPL-I *variant* (rPL-I*v*; see Refs. 50 and 51), mPL-II (52), rPL-II (53), hamster (ham) PL-II (54), mouse proliferin (mPLF; see Ref. 55), mouse proliferin-related protein (mPRP; see Refs. 56 and 57), rat decidual PRL-related protein (r*dec*PRP; see Ref. 58), rat PRL-like protein-A (rPLP-A; see Refs. 59 and 60), rPLP-B (61), rPLP-C (62, 63), bPRL-related protein-I (bPRP-I; see Refs 64 and 65), bPRP-II and bPRP-III (66), bPRP-IV (67), bPRP-V (68), bPRP-VI (69), and somatolactin (70–72). If one excepts rodent PLs, which are potent lactogens, the ability of these PRPs to exert classical GH/PRL bioactivity remains unexplored. To understand the actual role of these newly identified proteins at the physiological level, their mechanism of action needs to be clarified at the molecular level. The initial event leading to expression of the biological activity of these circulating proteins is likely their interaction with a membrane receptor. If the ability of PRPs to bind (or not) to somatogen and/or lactogen receptors could be predicted from analysis of their amino acid sequence, this could help in elucidating their biological properties.

On the basis of conserved structural features, the lactogen and somatogen receptors have been linked to a recently defined family of receptors called the cytokine (or hematopoietic) receptor superfamily (76, 77). Class 1 cytokine receptors include receptors for erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-2 (β chain), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9 and IL-11, IL-13, IL-15, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, oncostatin M, the signal transducer gp130, and the closely related leptin receptor (OBR; see Ref. 78); the class 2 cytokine receptor subfamily is composed of receptors for interferons (IFN) and IL-10 (for reviews, see Refs. 18, 29, 77, and 79–88). Structural similarities between class 1 cytokine receptors reside mainly in their extracellular domain (ECD) and the membrane-proximal region of the cytoplasmic domain. In the ECDs, conserved motifs consist of two pairs of

disulfide-bonded cysteines and a five-residue sequence Trp-Ser-X-Trp-Ser, termed "WS motif." Structural determination [GHR (15); PRLR (16)] or prediction (17, 83, 89–91) of the ECD of class 1 cytokine receptors reveals a common scaffold composed of two β-sheets each containing seven β-strands. Interestingly, the ligands of those class 1 receptors whose 3D structure has been determined [GH (13–15); IL-2 (92); IL-4 (93, 94); IL-5 (95); GM-CSF (96); LIF (91)] or predicted (Refs. 19–21; for review, see Ref. 84) all share the four-helix bundle scaffold (Fig. 1). Moreover, although IFNγ contains six α-helices, its soluble high-affinity receptor also folds in two domains of seven β-strands (97). The structural similarities between ligands (cytokines) and ligand-binding domains of the class 1 cytokine receptors raise the question of the specificity of the hormone-receptor interactions. First, although the global scaffolds are conserved (four-helix bundle for cytokines, two β-sheet sandwiches for the receptors), the main chain structures are obviously not strictly identical (superposable) at the atomic level, and the overall conformation of the interacting proteins must, at least in part, direct the specificity. Second, as such protein-protein interactions classically involve a large number of residues on both molecules, the binding capability of each cytokine must be linked to the presence in its amino acid sequence of the key residues guiding the specificity of the hormone-receptor interaction. Obviously, this is also true for the receptor with which it interacts.

In 1986, Nicoll and colleagues (12) concluded their outstanding review aimed at linking structural features to biological properties within the PRL/GH/PL family by predicting the usefulness of the site-directed mutagenesis approach for such purpose. Ten years later, mutational studies have indeed allowed the identification of some features involved in the biological functions of cytokines. For PRL/GH/PL proteins, as for most of the cytokines, however, the picture still remains incomplete. Extensive studies performed by several groups at Genentech have considerably highlighted the molecular basis of hGH functions (Refs. 14–16, 39, 98–108; for reviews, see Refs. 109–111). The Liège laboratory has been involved for many years in the cloning and recombinant bacterial expression of several PRL and GH proteins (2, 4, 5, 73, 112–115), which permitted the initiation of studies on structure-function relationships of hPRL (21, 116–120a), while others focused on bPRL (121–123). Data on PLs remain more fragmentary since mutational data are only available for a few residues (41, 42, 124–127).

The present review is aimed at summarizing the currently available data concerning the mutational studies performed on proteins belonging to the PRL/GH/PL family and at cross-correlating this information with the respective biological properties of different members of this hormonal family. As such a sequence-function correlation is based on amino acid sequence comparisons, *Section II* describes the alignment used for the present study and emphasizes the limits of the method. In the next part (*Section III*), we have attempted to define, at the molecular/residue level, the rules that lead PRL, GH, and PL to bind (or not to bind) to the lactogen and somatogen receptors through their binding site 1 (see below). The data obtained by site-directed mutagenesis studies have been summarized and serve to define what we refer to as the "binding mechanisms." In the fourth section, we focus on the existence and location of a second binding site in these hormones. Although still less well documented than binding site 1, current data regarding binding site 2 also support correlations between some features of the primary structures and binding activities. In the last part of this review (*Section V*), we have examined the PRPs, for most of which, if one excludes proliferin and proliferin-related protein (128, 129), no biological function or specific receptor has yet been clearly reported. By cross-correlating their amino acid sequence with the above determined "binding mechanisms," we attempt to understand (or, sometimes, to predict) their ability to bind (or not) to each type of known receptor.

The biological and physiological aspects of the PRL/GH/PL proteins have been well documented through excellent reviews (8, 10, 22–25, 28, 130–133). Similarly, recent reviews have focused on the influence of specific molecular features with the biological properties of these hormones, such as the occurrence of oligomerization, deletions, deamidation, glycosylation, or phosphorylation (134–136). Therefore, none of these themes has been reconsidered in the present work, which focuses mainly on hormone-receptor interactions at the molecular/residue level. Finally, the interaction between PLs and the putative specific PL receptor remains totally uncharacterized. In this review, therefore, discussions concerning PLs will only consider their ability to bind to the lactogen and somatogen receptors, which are thus far the only interactions documented from mutational studies of PLs.

II. Sequence Alignment

When protein sequences are aligned, gaps are introduced within the sequences to increase the number of matching residues and, thereby, maximize homology. The degree of similarity between the aligned sequences of the PRL/GH/PL proteins has been used to decipher their genetic origin (1–3, 6, 7).

When sequences are aligned with the aim of further cross-correlation with biological properties, however, alignments must be considered more carefully. In particular, a one-residue gap within an α-helix modifies the register of the helix and implies a 100° rotation of the residues before and after the gap with respect to each other. Thus, if a sequence identity (or disparity) between two homologous proteins is aimed at being linked to the conservation (or loss) of a biological property, one must be sure that the aligned residues are actually at topologically equivalent positions in the folded proteins that are compared. In other words, comparisons at the primary structure level must be applicable at the tertiary structure level.

Using the multiple alignment algorithm PILEUP provided with the University of Wisconsin Genetics Computing Group software (137, 138), we have tested several gap and gap length penalty combinations for aligning 22 protein sequences of the extended PRL/GH/PL family (Refs. 5, 48–50, 52–55, 57–59, 61, 63, 64, 66–69, and 73–75; see Table 1). If one excepts the N- and C terminus tails, which are highly divergent in size within the PRL/GH/PL family, especially in

the PRPs, median penalty combinations (2.0 or 3.0 weight per gap, 0.2 or 0.3 weight per residue within the gap) gave rise to almost identical alignments. The 2.0 per gap/0.2 per residue weight combination was selected for the present analysis (see below).

Several sequence alignments have been reported for the PRL/GH/PL family (as for example Refs. 6, 56, 71, 101, 113, 121, 126, and 139–141; for review, see Ref. 12). Depending on the alignment algorithms used, gaps were not always introduced at identical positions within the sequences compared. Therefore, the cross-correlation between amino acid sequences and biological properties is likely to achieve different conclusions depending on the alignment algorithm used.

Within the three regions delimiting binding site 1 [involving helix 1, helix 4, and loop 1 (Fig. 1); see *Section III*], however, no discrepancy occurs between previously published alignments and the one reported here with the 2.0/0.2 penalty combination. This correlates with the global folding of these proteins, since secondary structure elements (helices 1 and 4) are usually conserved between homologous proteins, whereas the second half of loop 1 (Cys58-Gln74) is bordered on its N terminus by a structural constraint ubiquitous in the PRL/GH/PL family: the "large" disulfide bridge linking Cys58 to Cys174 of helix 4 (hPRL numbering). This observation strengthens the likelihood that the primary structure similarities within binding site 1 can be transferred at the tertiary structure level.

The most divergent region between PRL, PL, and GH lies in the vicinity of residues 110–160 (PRL numbering), corresponding roughly to helix 3 and the long loop 3, joining helices 3 and 4 (21). As binding site 2 of hGH and hPRL has been linked to a region including helix 3 (Refs. 15, 21, and 118; Fig. 1), the likelihood that an erroneous sequence-function correlation in the area of binding site 2 might be directly influenced by sequence alignment had to be considered. As discussed below (*Section IV*), the probable accuracy of our alignment has been assessed by data obtained from mutational studies of these hormones.

III. Binding Site 1

Within a family of homologous proteins, it is usually assumed that a common biological function, such as a binding specificity for a determined receptor, is mediated by identical (or conservatively substituted) residues located at topologically equivalent positions. Based on this assumption, several studies aimed at cross-correlating sequence identity with a conserved biological function have been reported for the PRL/GH/PL proteins (12, 121, 125, 140). In many cases, residues predicted to be functionally important could be mutated without significantly affecting bioactivity (98, 121, 125, 140). Sequence comparison, used as a unique tool for elucidating the structural features responsible for the binding specificities within this family, has thus appeared inappropriate.

New data on the sequence-structure-function relationships within the PRL/GH/PL family have appeared in the literature. The exhaustive studies of the binding of hGH to both somatogen (15, 98, 142) and lactogen (16, 99) receptors have highlighted the molecular basis of these hormone-receptor interactions. The first identified binding site on hGH (later called binding site 1) has been linked to a region delimited by helices 1 and 4 and loop 1, connecting helices 1 and 2 (98, 99). We recently showed that hPRL also possesses a binding site very similar to that described for hGH and located on the same face of the protein (Refs. 21, 116, 117, and 120; for review, see Ref. 31; Fig. 1). This proposal is in agreement with results obtained by mutational analysis of bPRL (122, 123).

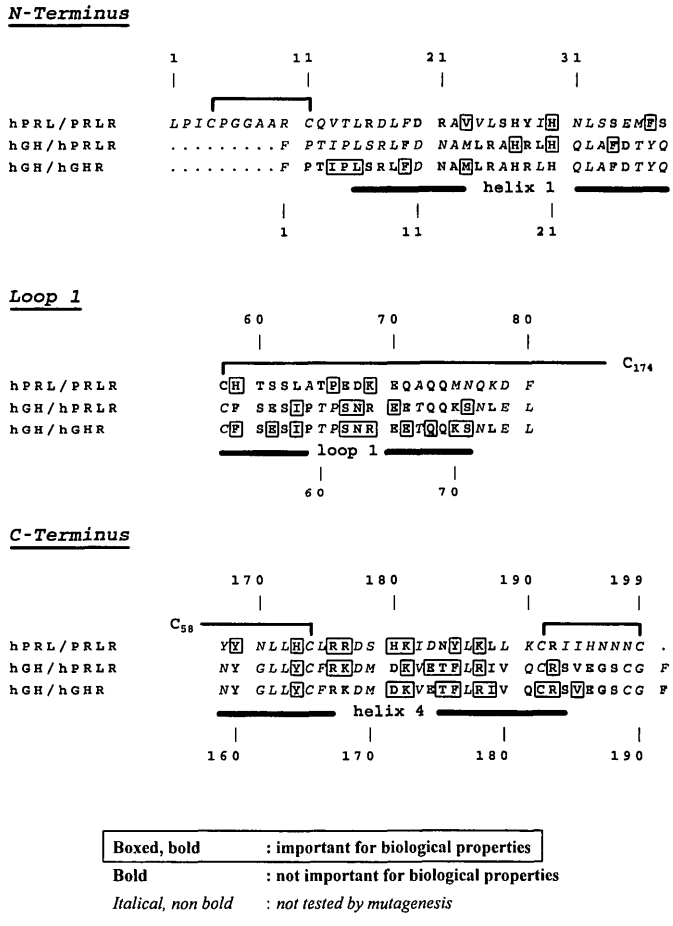

FIG. 2. Comparison of the binding site 1 determinants of hGH and hPRL. The three regions constituting the binding site 1 of hPRL and hGH (N terminus, second half of loop 1, and C terminus) are aligned. Location of helix 1, loop 1, and helix 4, as defined for hGH (15), is indicated. Residue numbering *above and below* the sequences refer to hPRL and hGH, respectively.

Residues involved in the three different hormone-receptor interactions (hPRL/PRLR; hGH/hPRLR; hGH/hGHR) are compared. Data for the hPRL/PRLR interactions have been obtained in the Liège laboratory (116, 120); results from studies on bPRL were added when no data were available for hPRL (R177, K187; see Refs. 122 and 123). Data for the hGH/hGHR and hGH/hPRLR interactions are from Cunningham and Wells (Refs. 98 and 99, respectively) and were obtained using the ECDs of the receptors (binding proteins).

Residues reducing the binding by more than 2-fold (hGH, hPRL) or Nb2 mitogenic effect (bPRL) are in *bold and boxed*; those whose mutation does not alter biological property are in *bold*. Residues not tested by mutagenesis are in *italic and light-face*. Although R21 and Y28 were reported to be functionnally important for bPRL (122, 123), their involvement in binding site 2 is anticipated, and they are thus not considered as binding site 1 determinants (see *Section III.A.2.c*).

TABLE 2. Qualitative binding abilities of GH (primate and nonprimate), PRL and PL (primate, ungulate, and rodent) to somatogen (GHR) and lactogen (PRLR) receptors

Receptors	GH		PRL	PL		
	Primate	Nonprimate		Primate	Ungulate	Rodent
Somatogen (GHR)		+[a]	−	±	+	−
		1.a	*1.b*	*1.c*	*1.d*	*1.e*
Lactogen (PRLR)	++	−	+	++	+	
	2.a	*2.b*	*2.c*	*2.d*	*2.e*	

This table must be regarded as a mnemonic device aimed at guiding the reader through *Section III*. Each of the 10 hormone-receptor interactions are separately discussed in subsections whose numbering corresponds to Table 2 (III.A.1.a, etc.).

[a] ++, very strong affinity; +, strong affinity; ±, low affinity; −, no affinity.

Closer analysis of the above mentioned studies within the PRL/GH/PL family led us to propose that, contrary to the general assumption, "a similar binding potency does not necessarily result from an identical mechanism at the molecular and/or residue level" (116). In other words, sequence disparity within a region predetermined as functionally important for one protein does not necessarily prevent functional similarity for a homologous protein. This hypothesis is mainly based on two experimental observations. First, as far as binding site 1 is concerned, we have demonstrated that the residues involved in the binding of hGH or hPRL to the lactogen receptor are neither identical nor located at topologically equivalent positions (Refs. 21, 116, and 120; Fig. 2). Second, whereas hGH requires zinc chelation for binding to the lactogen receptor with high affinity, hPRL binding to the same receptor is independent of metal chelation (39). We have thus proposed that the binding of PRL/GH/PL proteins to the lactogen receptor can occur through at least two types of mechanisms, differing by their respective requirements at the residue level (Fig. 2).

This hypothesis led us to reconsider the way sequence comparisons within the PRL/GH/PL family were to be regarded with the aim of determining which hormone possesses the appropriate residues for binding to a defined type of receptor. In the following section, we have summarized the available data concerning the binding of hPRL (116, 117, 120), bPRL (121–123), hGH (99), hPL (126), or mPL-II (124, 125) to the lactogen receptor and of hGH (98) or bPL (41, 42, 127) to the somatogen receptor. These data have served to define what we named the "binding mechanisms." When no data are reported for a particular hormone-receptor interaction or when the binding potency is well established but the binding residues are not yet identified, we have formulated some hypotheses based on the other documented interactions for their association with a particular "binding mechanism."

A. Sequence-function correlation

Binding of PRL/GH/PL proteins to the lactogen or somatogen receptors is not necessarily an "all or nothing" phenomenon (PRL binds to the former, but not to the latter); it can also vary on the affinity scale (as for example, the affinity of hPL for the somatogen receptor is weaker than that of hGH but significantly higher than that, unmeasurable, of hPRL). These qualitative binding potencies within the PRL/GH/PL family are summarized in Table 2. This table presents 10 different hormone-receptor interactions, each of which is separately considered in the subsections (1.a, 1.b, 2.a, 2.b, etc.) referred to in Table 2. Some of the information currently available for PRL/GH/PL proteins was obtained using recombinant GH- and PRL-binding proteins (BP) instead of membrane receptors to test the effect on binding of point mutations in the ligands. Although the primary structure of these binding proteins are completely identical to the ECD of the corresponding membrane-bound receptors, one cannot exclude that the hormone-binding mechanisms show specific characteristics depending on whether soluble or cell-anchored receptors are used. However, it appears that one of the most striking differences between soluble and membrane-bound receptors is related to the stoichiometry of the ligand-receptor complexes (receptor dimerization), which thus involves phenomena occurring at binding site 2 (see *Section IV.A.4*). Therefore, as far as binding site 1 is concerned, it is likely that mutational studies performed using binding proteins can be extended to full length receptors. Data discussed in the 10 following subsections are summarized in Table 3, which appears at the end of *Section III.A.*

1. Binding to the somatogen receptor.

a. GHs. Experimental data: Cunningham and Wells (98) have identified 25 residues in hGH (referred to as "hGH-like binding determinants" in Table 3) whose mutation to alanine reduces the binding affinity for the hGHBP by more than 50% (Figs. 2 and 3). They also identified one residue, Glu174, that naturally alters the hormone receptor interaction, since its mutation to Ala increases the binding affinity by more than 4-fold (98).

Binding mechanism: Nonprimate GHs are unable to bind to the human somatogen receptor. Cunningham and Wells (98) have suggested that mutation of at least 10 of the 25 binding determinants identified in hGH (Ile4, Met14, Ser62, Asn63, Arg64, Lys70, Tyr164, Asp171, Phe176, Ile179; Fig. 2) could explain this binding inability. In agreement, Souza and colleagues (143) proposed that species specificity resides mainly in the sole substitution of Asp171 for His in all nonprimate GH, which is incompatible with the presence of Arg43 in the hGH receptor (15, 143). Conversely, primate GHs can bind to the nonprimate somatogen receptors with high affinity (41, 144). One can reasonably assume that at least some of the 10 above-mentioned amino acids are specific binding determinants for the GH-somatogen receptor interactions in primates, whereas the 15 remaining residues (referred to as "GH-like binding determinants" in Table 3) are more typical of GH-somatogen receptor interactions in general. Nevertheless, one cannot exclude that somatogen binding of nonprimate GHs involves residues other than these 15 binding determinants, identified on hGH alone.

A GH variant (hGH-V) that displays 13 differences with pituitary hGH (also referred to as hGH-N) at the residue level has been isolated from human placenta (145, 146). However, these amino acid substitutions affect only one of the 25 binding determinants identified in hGH-N (Glu66 replaced with Lys), in agreement with the observation that hGH-V is as potent as hGH-N for binding to the somatogen receptor (147,

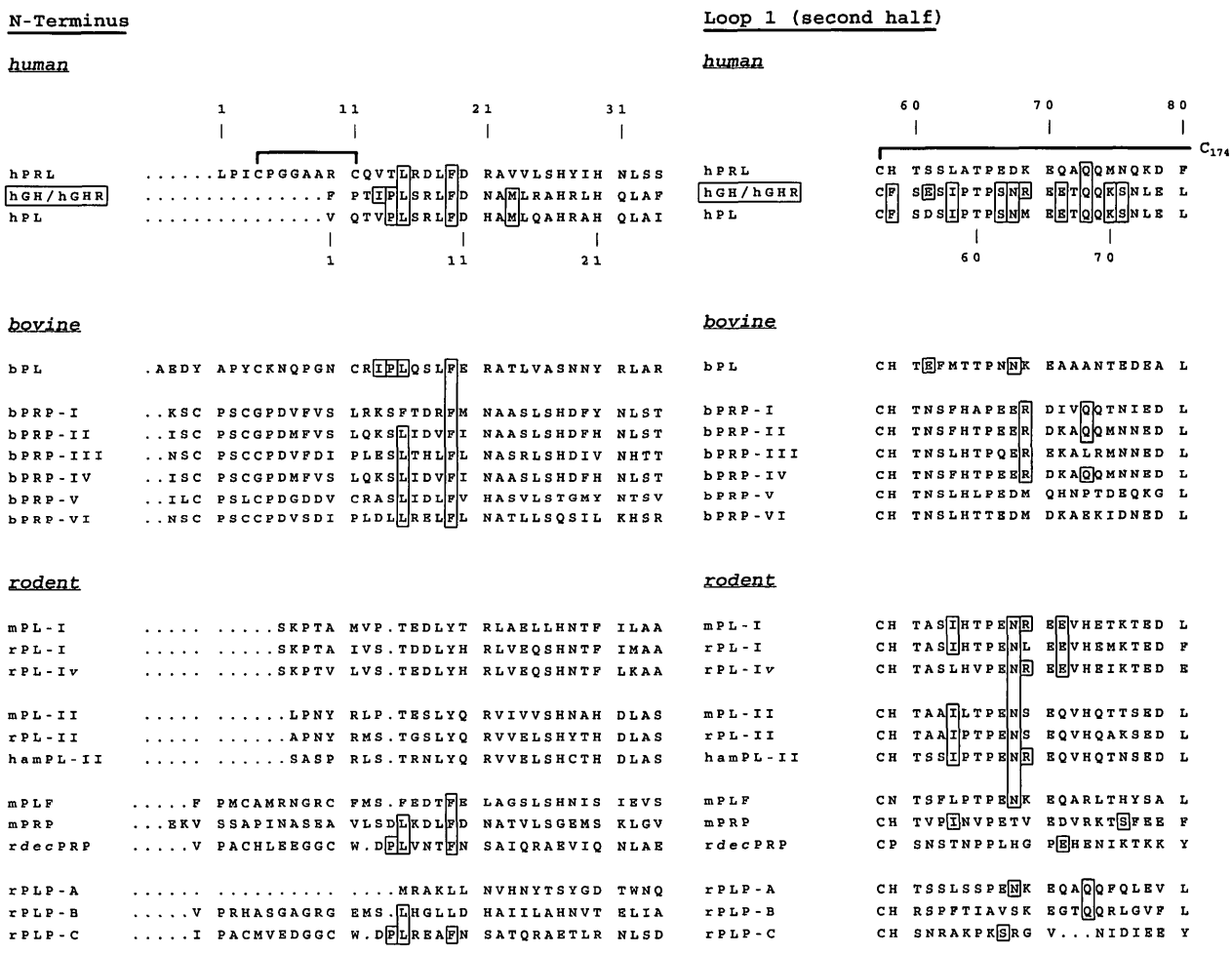

FIG. 3. Conservation within several members of the PRL/GH/PL family of the binding site 1 determinants for the hGH/hGHR interaction. Sequences of the three segments constituting PRL/GH binding site 1 (see Fig. 2) have been aligned using the PILEUP multiple alignment algorithm (see *Section II*). Residue numbering *above and below* the sequences refer to hPRL and hGH, respectively.
In the *upper panels*, residues important for the binding of hGH to the hGHBP (98) are *boxed*. Whenever conserved in other proteins from human, bovine, or rodent families (lower panels), they are also *boxed*. Proteins aligned (see references in Table 1) are: *Human family*: hPRL, hGH, hPL. *Bovine family*: bPL and bPRP I-VI; the nomenclature used is from Schuler *et al.* (228). *Rodent family*: rPL-I, rPL-Iv, rPL-II, mPL-I and mPL-II, hamPL-II, mPLF, mPRP, r*dec*PRP, rPLP-A, rPLP-B, and rPLP-C.

148). This strongly suggests that the interactions of pituitary and placental hGH with the somatogen receptor involve the same mechanism.

b. PRLs. Experimental data: PRLs do not bind to the somatogen receptor. Of the 25 binding determinants of hGH (98), only six are conserved in hPRL (Leu15, Phe19, Gln73, Lys181, Cys191, Arg192; Fig. 3; Table 3). By introducing eight point mutations in the hPRL sequence, Cunningham *et al.* (102) have engineered a hGH-like hPRL showing only a 6-fold reduced affinity for the hGHBP with respect to hGH. Seven of these mutations involve the introduction of hGH-binding determinants at homologous positions in hPRL (positions 62, 63, 66, 171, 175, 176, and 178, hGH numbering). The eighth mutation is the substitution of Ala for Asp183. This residue is indeed homologous to Glu174 in hGH, which naturally alters the hGH-somatogen binding (see *Section IIIA.1.a* above; see Ref. 98).

Binding mechanism: One can thus assume that PRLs do not bind to the somatogen receptor because they lack the typical hGH-binding determinants. Nevertheless, the additional introduction of other hGH-binding determinants into the hPRL sequence did not show the expected cumulative effect on binding affinity (102). This suggests that, apart from the natural mutations of binding determinants, the lack of binding of PRLs to the somatogen receptor also results from the inhibitory effect of other residues. In the context of the lactogen receptor, we and others have clearly demonstrated such an effect for Asp183 (102) and for three residues within loop 1 [Gln74 (116), Gln71 and Gln73 (V. Goffin, unpublished results)], whose mutation to Ala increases receptor affinity by 1.5 to 2.5-fold.

c. Primate PLs. Experimental data: hPL and hGH share 85% of identical residues (126). Despite this very high similarity, the affinity of hPL for the hGHBP is 2300-fold less than hGH (126). Among the 25 binding determinants of hGH, only four are mutated in hPL (Val4, Asp56, Met64, Met179; Fig. 3). When these positions are mutated to their hGH counterparts (with the exception of Met64, replaced with a Lys instead of an Arg, as in hGH) and Glu174 mutated to Ala (see *Sections III.A.1.a.* and *III.A.1.b*), the binding affinity for the somato-

FIG. 3 (*continued*).

gen-binding protein is roughly identical to that of wild type hGH (126).

Binding mechanism: The weak affinity of hPL for the somatogenic binding protein thus seems to result from the natural mutation of somatogen-binding determinants in hPL. The fact that fewer mutations need to be introduced in hPL than in hPRL to engineer somatogen binding (five *vs* eight, respectively) can probably be correlated with the more recent divergence of the primate GH/PL lineages compared with the separation of ancestral GH/PRL lineages (6, 7).

d. Ungulate PLs. Experimental data: Contrary to hPL, ovine and bovine PLs show somatogen potency (Refs. 41, 42, 127, 149, and 150; for reviews, see Refs. 10 and 151). This observation was unexpected since nonprimate PLs are assumed to have evolved from the PRL lineage (6, 7), which is not somatogenic. The amino acids involved in the interaction have not yet been identified. Vashdi-Elberg and colleagues (127) recently reported that deletions within the N terminus of bPL modify its somatogen properties; however, as we anticipate those mutations to affect binding site 2 rather than site 1, these data will be discussed in a later section (*Section IV.A.3.b*).

Binding mechanism: If we consider the 25 binding determinants identified in hGH (*Section III.A.1.a*; Fig. 2; see Ref. 98), only 10 are present in bPL (Ile4, Pro5, Leu6, Phe10, Glu56, Asn63, Lys172, Thr175, Cys182, Arg183 (hGH numbering); Fig. 3; Table 3). Ungulate PLs bind with high affinity to primate as well as nonprimate GH receptors (10, 149), suggesting that somatogen binding of these PLs does not require residues specific for hormone-receptor interactions in primates. We have suggested that GH binding to nonprimate somatogen receptor could be mediated by 15 binding determinants, ubiquitous in GHs (see *Section III.A.1.a*; Table 3). Of these, only eight residues are conserved in bPL (the above mentioned residues with the exception of Ile4 and Asn63). Moreover, Vashdi and co-workers (41) have shown that bPL binds to the rat somatogen receptor 5-fold more tightly than hGH. Interestingly, the position topologically equivalent to Glu174 of hGH is occupied by a Ser in bPL, *i.e.* a smaller and uncharged amino acid that could favor a tighter hormone-receptor interaction. Tyr174 in oPL, although also uncharged, would have the opposite steric effect.

These observations raise three possibilities. First, these eight (or 10) hGH-like binding determinants are intrinsically sufficient to assure somatogen binding of bPL. By comparison with hPRL, which contains six of these 15 typically GH-binding residues and is however not somatogenic, this hypothesis appears improbable. Second, as formulated above (*Section III.A.1.a*), binding to nonprimate somatogen receptors could require more residues than the 15 proposed binding determinants; the presence of such additional binding residues in the bPL (and ungulate PLs in general) sequence could explain its (their) somatogen binding. Third, the somatogen binding of bPL could result from a mechanism totally different at the molecular/residue level from that described for hGH and involve a totally different set of amino acids.

Sequence analysis alone cannot prove or disprove any of these hypotheses. In the latter proposal, a similar biological property shared by GHs and bPL, namely somatogen binding, would occur through two different mechanisms having different requirements at the residue level. It should also be noticed that protein sequences of ovine and bovine PLs are much more divergent than usually observed for proteins from closely related species, suggesting that the rapid rate of evolution results form adaptative rather than neutral mutations (152). This raises the possibility that, even within ungulates, PL somatogen binding might occur through different mechanisms. Further study of ungulate PLs by site-directed mutagenesis should highlight the actual mechanism of this unexpected hormone-receptor interaction. Cross-correlation between GH binding determinants (Fig. 3) and oPL/bPL sequence comparisons should help elucidate which binding mechanism is used by these ungulate PLs for somatogen binding.

e. Other PLs. Experimental data: It has been suggested by Vashdi and co-workers (41) that nonprimate PLs other than bovine (or ovine) might also bind to the somatogen receptor. To the best of our knowledge, no study has confirmed this hypothesis. In contrast, we have shown that rPL-I and rPL-II do not bind to the transfected rGH receptor (153).

Binding mechanism: Among six rodent PLs (mPL-I, rPL-I, rPL-I*v*, hamPL-II, mPL-II, and rPL-II; Fig. 3; see Ref. 141), only four hGH-binding determinants are ubiquitous (Asn63, Lys172, Cys182, and Arg183), and three others are highly

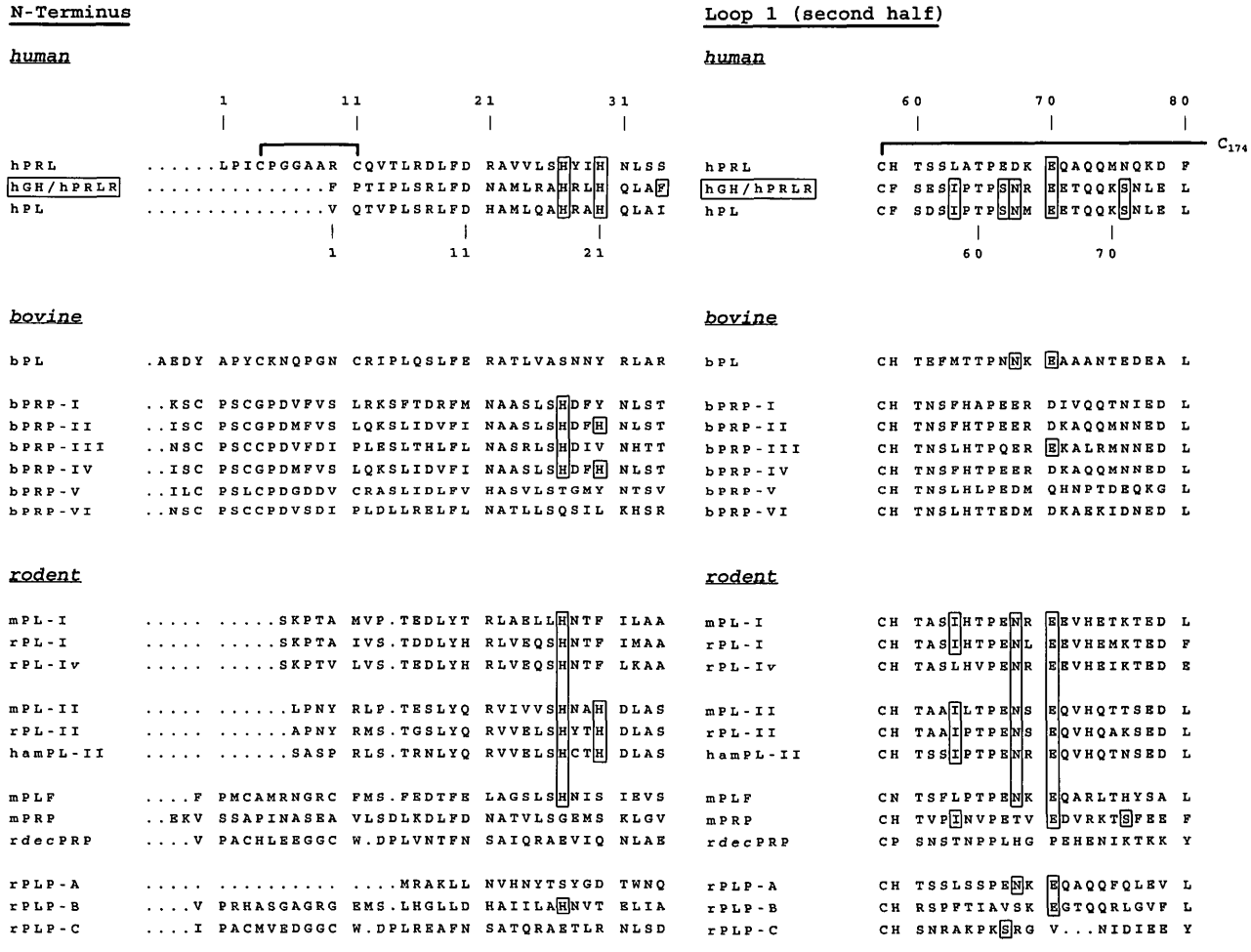

FIG. 4. Conservation within several members of the PRL/GH/PL family of the binding site 1 determinants for the hGH/hPRLR interaction. Sequences of the three segments constituting PRL/GH binding site 1 (see Fig. 2) have been aligned using the PILEUP multiple alignment algorithm (see *Section II*). Residue numbering *above and below* the sequences refer to hPRL and hGH, respectively.
In the *upper panels*, residues important for the binding of hGH to the hPRL binding protein (99) are *boxed*. Whenever conserved in other proteins from human, bovine, rodent families (lower panels), they are also *boxed*. See legend to Fig. 3 for more details.

conserved (Ile58, Arg64, and Glu66; hGH numbering). Moreover, the position equivalent to Glu174 in hGH, a residue that naturally reduces the affinity (*Section III.A.1.a*), is occupied by an Asp, much more similar to the Glu of hGH than to the Ser of bPL. Based on our hypotheses, it is likely that rodent PLs do not bind to the somatogen receptor, in agreement with our observations (153).

2. Binding to the lactogen receptor.

a. Primate GHs. Experimental data: Cunningham and Wells (99) have determined 17 binding determinants for binding of hGH to the lactogen-binding protein (hPRLBP; Fig. 4; Table 3). Among this set of residues, some (10) are also somatogen-binding determinants (Fig. 3), but for most of them, their respective importance for binding to either receptor differs (99). These authors have demonstrated that the lactogen binding of hGH requires the presence of Zn^{2+} ions: the absence of zinc ions reduces the hGH affinity for the ECD of the lactogen receptor by 4 orders of magnitude (39). Among the 17 binding determinants, five have a direct effect on zinc chelation: His18, His21, and Glu174 (together with His188 on the receptor) were initially proposed to coordinate one Zn^{2+} ion, and Arg167 and Lys172 were thought to influence the shape of the zinc-binding site (39). The 12 remaining residues are assumed to be involved in direct interactions with the receptor. Recent determination of the crystal structure of hGH bound to hPRLBP (16) mostly confirmed mutagenesis data, with a restriction for residues involved in zinc chelation. It appears indeed from this 3D structure analysis that His21 does not mediate ion chelation, but rather acts by maintaining Glu174 in the appropriate conformation for zinc binding. The fourth residue involved in zinc chelation is actually Asp217 on the receptor (16).

Binding mechanism: Zinc mediation is the major molecular event for the binding of hGH to hPRLBP since EDTA drops the affinity of the interaction by 8000-fold, whereas the most effective residue substitution on hGH, if one excludes those altering the metal-chelation, only reduces the affinity by 25-fold (39). In agreement with this observation, we refer to this zinc-dependent hormone-receptor interaction as "hGH-like."

These observations lead to two comments. First, the lactogenic activity of hGH (and primate GHs in general) de-

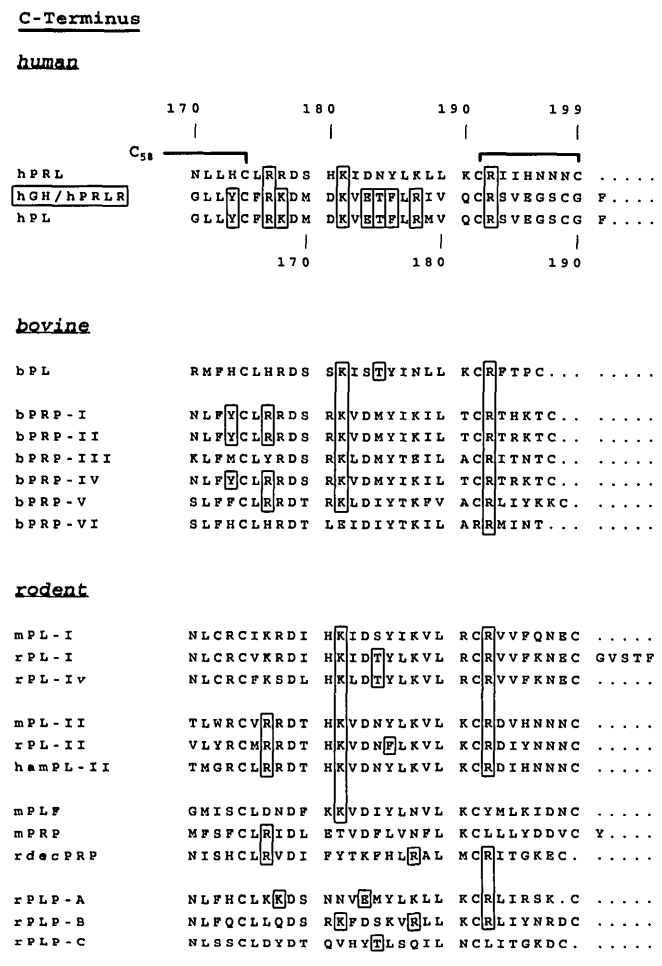

FIG. 4 (*continued*).

pends on the serum concentration of free Zn^{2+} ions, which is assumed to approximate the dissociation constant ($Kd \sim 0.4\ \mu M$) for Zn^{2+} binding to the hGH-hPRLBP complex (39). Moreover, since binding studies are often performed in buffers containing millimolar concentrations of salts such as $MgCl_2$, it is likely that the amount of contaminant zinc ions is sufficient to ensure lactogen binding of primate GHs. Second, despite its inhibitory effect on somatogen binding (98), Glu174 is an absolute requirement for zinc-mediated lactogenic binding. This could explain why this residue has been conserved through evolution despite its negative effect on somatogen binding.

b. Nonprimate GHs. Experimental data: Nonprimate GHs do not bind to the lactogen receptor. In all nonprimate GHs, His18 is mutated into a Gln. Cunningham and co-workers (39) have suggested that, as a consequence of this mutation, these nonprimate GHs are not able to coordinate Zn^{2+} and, thereby, to bind to the lactogen receptor through the "hGH-like" mechanism.

Binding mechanism: The replacement of His18 with Gln cannot be the only reason, however. Despite the mutation of His18 (to Arg), the placental variant hGH-V (145, 146) binds with a significant affinity (30-fold reduced compared with hGH) to the rPRLR (147, 148, 154). Among the 12 binding determinants of hGH that are not linked to the zinc mediation (see *Section III.A.2.a*), only two are mutated in hGH-V (Phe25 and Glu65); thus, lactogen binding can still occur. Otherwise, nonprimate GHs possess only six of these 12 binding determinants (Ile58, Lys168, Thr175, Arg178, Arg183). In addition to the inability to chelate zinc ions, as proposed by Cunningham and colleagues (39), we suggest that nonprimate GHs are unable to bind to the lactogen receptor because their amino acid sequence intrinsically lacks most of the required binding determinants (Table 3).

c. PRLs. Experimental data: Contrary to hGH, data available for binding site 1 of PRLs have been obtained using membrane-bound PRLR (from rat Nb2 cells). By scanning the 58–74 region of hPRL by site-directed mutagenesis, the Liège group has identified three major binding determinants that reduce binding to the Nb2 PRLR by 50% or more (His59, Pro66, and Lys69). In helices 1 and 4, also predicted as part of binding site 1 (21, 117), the same laboratory has recently identified nine other binding determinants: Val23, His30, and Phe37 on helix 1, and Tyr169, His173, Arg176, His180, Lys181, and Tyr185 on helix 4 (120). Moreover, Luck and associates (122, 123) have characterized several residues of bPRL selected by sequence comparison, among which Arg177 and, to a lesser extent, Lys187 can be considered as binding determinants of bPRL since their alanine substitution reduces by at least 50% the mitogenic activity of the corresponding bPRL analogs on Nb2 cells. Since both residues are ubiquitous in mammalian PRLs (12), they are presumably binding determinants for all these species also. Arg21 and Tyr28, although functionally important for bPRL (122, 123), are assumed to lie within binding site 2 (21, 118) and, therefore, are not included in the list of amino acids defining binding site 1. The distribution of binding site 1 determinants along primary and tertiary structures of hPRL are represented in Figs. 5 and 1, respectively.

Binding mechanism: From mutational analysis of loop 1 (region 58–74), we demonstrated several years ago that the binding residues of hPRL are neither of an identical type nor at topologically equivalent positions to those of hGH (116). In agreement, our most recent results with respect to helical regions (120) show that the distribution of lactogen-binding determinants within hPRL and hGH sequences differs (Fig. 2). Moreover, contrary to hGH, lactogen binding of hPRL does not depend on zinc (39). Finally, the dissociation constants (*Kd*s) obtained by Cunningham and co-workers for binding to the hPRLBP are very different for each hormone: $Kd_{hPRL} = 2.1$ nM; $Kd_{hGH} = 0.033$ nM in the presence of Zn^{2+}; $Kd_{hGH} = 270$ nM in the presence of EDTA.

The molecular mechanisms by which hGH and hPRL bind to the lactogen receptor differ in their residue and metal requirements. In contrast to the "hGH-like" binding mechanism, we have used "PRL-like" to describe the way PRLs interact with the lactogen receptor (Table 3).

d. Primate PLs. Experimental data: Binding to the lactogen receptor is a typical PRL-like property. However, a mutational study of hPL (126) has shown that the lactogen binding of hPL shares more similarities with the lactogen binding of hGH than that of hPRL. First, the binding affinity of hPL for the hPRLBP is dependent on zinc (126) and roughly identical to that of hGH ($K_d \sim 46$ pM *vs.* 33 pM), *i.e.* 2 orders of magnitude higher than that of hPRL measured in the same assay ($K_d \sim 2.1$ nM). Second, among the 17 binding deter-

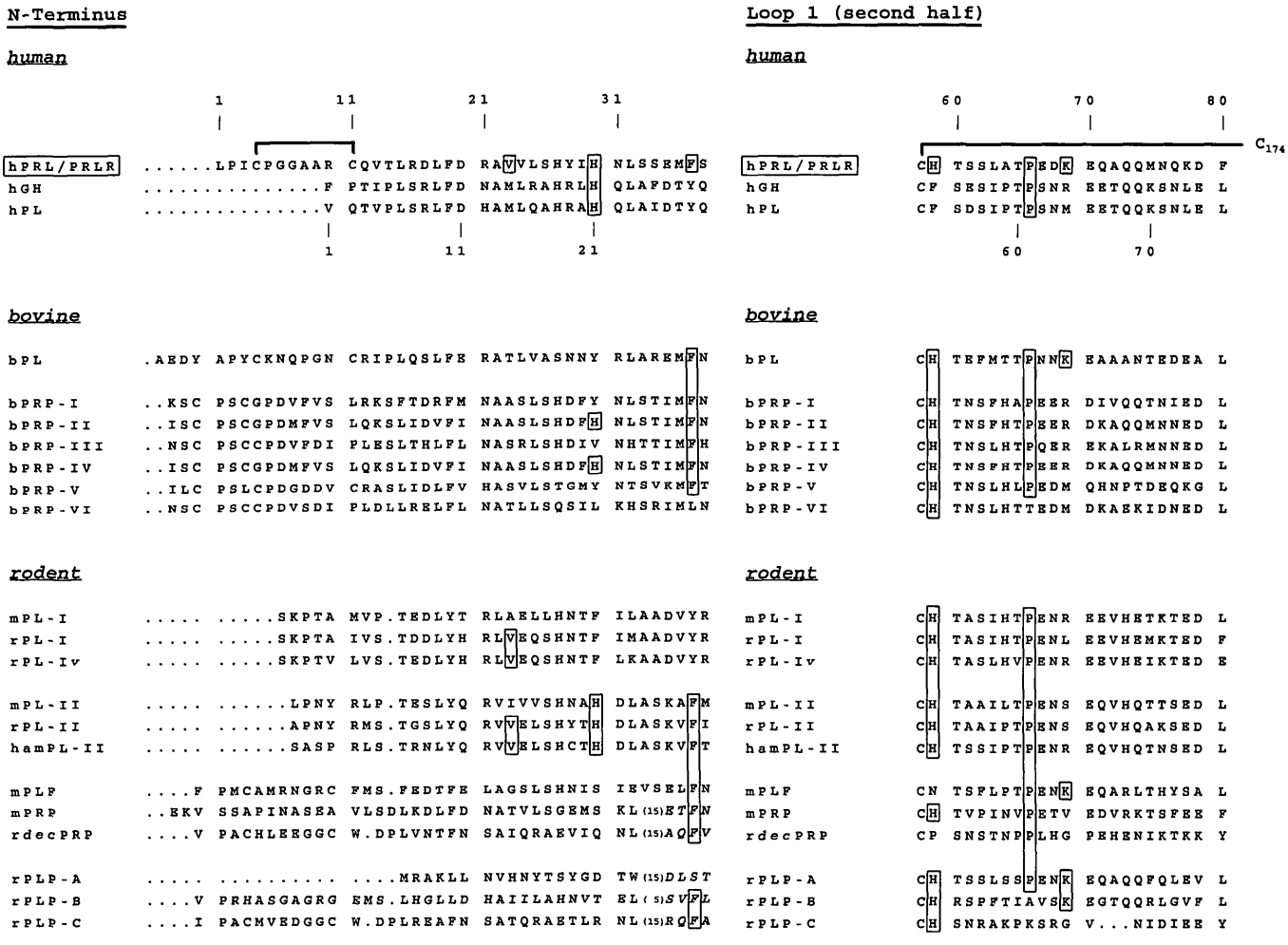

FIG. 5. Conservation within several members of the PRL/GH/PL family of the binding site 1 determinants for the hPRL/PRLR interaction. Sequences of the three segments constituting PRL/GH binding site 1 (see Fig. 2) have been aligned using the PILEUP multiple alignment algorithm (see *Section II*). Residue numbering *above and below* the sequences refer to hPRL and hGH, respectively.
In the *upper panels*, residues important for the binding of PRL to the PRLR are *boxed*. Experimental data obtained by site-directed mutagenesis are from studies on hPRL (116, 120) or, when not available, on bPRL (R177 and K187; see Refs. 122 and 123). Whenever conserved in other proteins from human, bovine, or rodent families (lower panels), they are also *boxed*. Five rodent sequences (*rdec*PRP, rPLP-A, rPLP-B, rPLP-C and mPRP) display a five- to 15-residue insertion in the N-terminus with respect to all other sequences aligned (number indicated in *brackets*). Consequently, the downstream sequence (in which the conserved Phe37 is found) is likely to have no topological equivalence at the tertiary level and is therefore shown in *italics*.

minants of hGH (99), only Phe25 is mutated to Ile in hPL (yet, this Ile25 is also a binding determinant for hPL; see Ref. 126). Third, while Lys69 in hPRL is essential for binding (116), its homolog in hPL (Met64) can be mutated without effect (126).

Binding mechanism: In agreement with Lowman and coworkers (126), we conclude that the binding of hPL to the lactogen receptor is "hGH-like," and not "PRL-like"; this was unexpected.

e. Nonprimate PLs. Experimental data: PLs (PL-I and -II) are known to bind to the lactogen receptor (125, 155–160). Nevertheless, very few data are available concerning the residues involved in the binding of nonprimate PLs to the lactogen receptor. Davis and Linzer (125) defined Arg14, Arg169, and Lys179 as important residues for the lactogenic properties of mPL-II. Effective mutations anticipated to alter binding site 2 of bPL (42, 127) will be discussed in a later section (*Section IV.A.3.b*).

Binding mechanism: Correlation of sequence alignments with the documented hormone-receptor interactions described above allows some hypotheses.

Zinc mediation is characteristic of "hGH-like" lactogen binding. To the best of our knowledge, its involvement has never been documented for nonprimate PLs. In rodent and ungulate PLs, the position homologous to Glu174 in hGH is occupied by an Asp (rodents), a Ser (bPL), or a Tyr (oPL). Tyr or Ser cannot coordinate Zn^{2+} ions, but Asp could (161). Similarly, His18, the second zinc binding residue in hGH (16), is only conserved in rodent PLs (Fig. 4). Nevertheless, it is noteworthy that with an identical "His18-Asp174" sequence (His27-Asp183 in hPRL numbering), hPRL does not coordinate zinc. Finally, the three other residues that influence metal binding in hGH (His21, Arg167, Lys172; see Ref. 39) are not ubiquitous in rodents PLs (Fig. 4). Taken together, these observations suggest that lactogen binding of nonprimate (rodents and ungulates) PLs is not mediated by zinc and, therefore, is not "hGH-like" (Table 3).

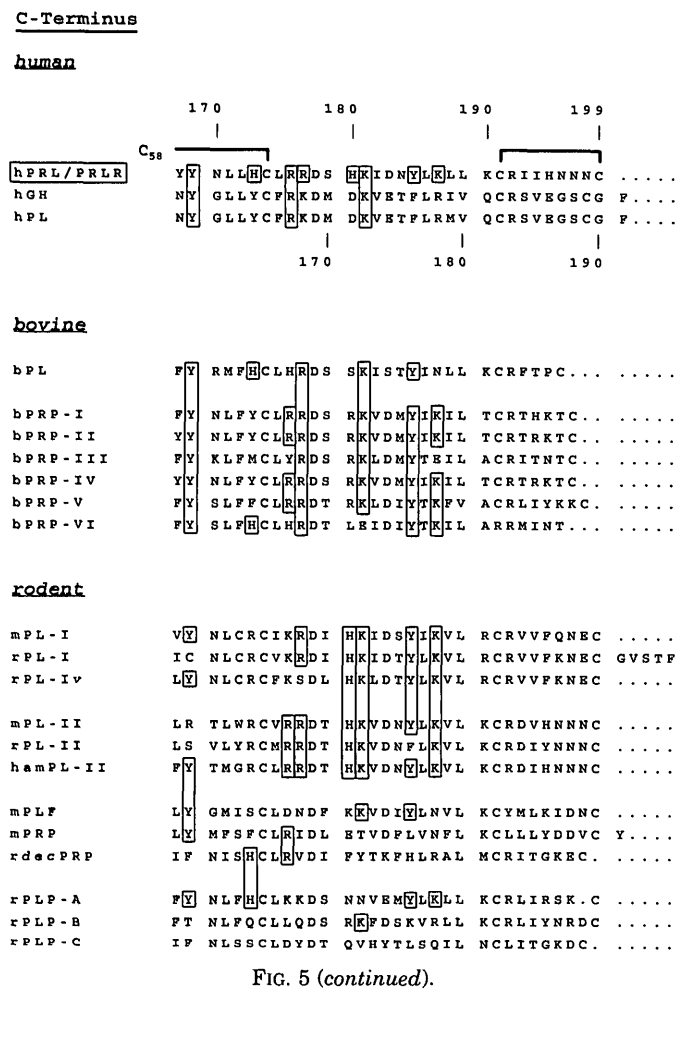

FIG. 5 (*continued*).

Davis and Linzer (125) have observed that mutation of Arg14, Arg169, or Lys179 reduces the lactogenic properties of mPL-II. These three amino acids are conserved in PRLs (Arg21, Arg177, and Lys187) and mutated in hGH (Asn12, Lys168, and Arg178). In bPRL, as in mPL-II, mutation of each of these three residues affects mitogenic activity on Nb2 cells (122, 123). Such observations suggest that the lactogen binding of nonprimate PLs could be "PRL-like" (Table 3). This would be in good agreement with the genetic origin of the nonprimate PLs, proposed to have arisen from the PRL lineage (6, 7). It also correlates with the weak lactogen properties of rPL-I*v* (50, 51) in which the position equivalent to Arg177, a major PRL-binding determinant, is occupied by a Ser (Fig. 5). Interestingly, substitution of Ser for Arg177 in bPRL reduces mitogenic activity by 95% (123). It should be noted, however, that conflicting results were recently obtained regarding the lactogenic activity of recombinant rPL-I*v* produced in Chinese hamster ovary (CHO) cells (162–164).

Whereas PLs, independently of their species origin, have been widely regarded as possessing "lactogenic" sequences based on their lactogenic properties (121, 125), it appears from the present study that this is partly inappropriate, since the binding mechanism (*i.e.* the binding residues) may be different for primate *vs.* nonprimate PLs.

B. Overview

Within a family of homologous proteins, it is usually assumed that sequence identity correlates with structural and/or functional similarities. Within the PRL/GH/PL family, distinct biological properties (lactogenic or somatogenic) have been widely used for predicting functionally important residues on the basis of amino acid sequence comparisons. As for example, Watahiki *et al.* (140) predicted that nine residues, common to 10 GHs (and for some, to hPL) and mutated in the other members of the family, were potentially involved in the properties of the GHs (Arg16, Leu20, His21, Ala24, Ser55, Ser79, Leu114, Leu124, Asp130; hGH numbering). However, none of these has been identified as binding determinants when tested by alanine-scanning mutagenesis in hGH (98, 99). Luck *et al.* (121) postulated that the binding residues typical of lactogen binding were those found in all lactogenic hormones (PLs, PRLs, and primate GHs) and mutated in all nonlactogenic hormones (nonprimate GHs). When the seven residues selected, based on this hypothesis, were replaced in bPRL with their bGH counterparts, however, no significant decrease in the lactogenic activity was observed. Davis and Linzer (125) also selected five residues in mPL-II on the basis of sequence comparison. Two of them, although typically found in lactogenic hormones, were mutated without affecting bioactivity.

These observations support the need for closer scrutiny of the information that can be deduced from sequence comparisons. It is now clear that the manner in which primate GHs, PLs, and PRLs bind to the lactogen receptor may result from a different mechanism at the molecular and residue levels (Table 3). Thus, it appears that sequence identities between all PRLs and/or PLs may not necessarily have implications at the functional level. For example, Ala substitution of Glu70 (hPRL numbering), a residue conserved in all lactogenic hormones (121), is detrimental to lactogen binding of hGH (99), but not of hPRL (116). Similarly, the (His27-X-X-His30) sequence expected to be a typical feature of lactogenic hormones (PRLs, PLs, and primate GHs; see Refs. 12 and 122), is only essential for the primate PLs and GHs since it is involved in zinc chelation ("hGH-like" binding mechanism).

Conversely, because of a different binding mechanism, some functionally important residues have been missed by sequence comparison, since their type, as well as their topological distribution within the binding site, are widely different (Fig. 2 for comparison). For example, Lys69, essential for hPRL (116), is mutated in primate GHs and primate PLs; moreover, in these hormones, this position is not essential for binding to the lactogen receptor (99, 126). These observations were recently confirmed by cross-correlation of the structures of hGH-hGHBP complexes (15) and hGH-hPRLBP complexes (Ref. 16; for review, see Ref. 109) with a hPRL 3D model that we constructed on the basis of the crystal structure of pGH (13, 21). Interaction of binding site 1 of hGH with either somatogen or lactogen receptors displays several differences (109). Since hPRL only binds to the lactogen receptor, one would anticipate its binding site 1 to show many more similarities with binding site 1 of the hGH to the lac-

TABLE 3. Activities and number of binding site 1 determinants of PRL/GH/PL to somatogen (GHR) and lactogen (PRLR) receptors: summary and hypotheses

Receptors	GH		PRL	PL		
	Primate (hGH)	Nonprimate (pGH)	(hPRL)	Primate (hPL)	Ungulate (bPL)	Rodent (mPL-II)
Somatogen						
Primate	+[a]	−	−	±	−(?)	−(?)
	25 hGH-like BD	15 hGH-like BD	6 hGH-like BD	21 hGH-like BD	10 hGH-like BD	6 hGH-like BD
Homologous (Same species)		+			+	−
		15 GH-like BD + others (?)			8 GH-like BD + others (?) (S174)	5 GH-like BD (D174)
Lactogen	++	−	+	++	+	+
	17 hGH-like BD	8 hGH-like BD	14 PRL-like BD	16 hGH-like BD	9 PRL-like BD (?)	10 PRL-like BD (?)
Zn^{2+} mediation	yes	no (H18Q)	no	yes	No (?)	No (?)

Each of the interactions listed in Table 2 and discussed in *Section III* are reported and characterized with respect to the number of binding determinants (BD) found in the different ligands (for each interaction, the exact number of BD refers to the representative hormone indicated in *brackets* at the top of the table). Experimental data are *underlined* and served to define binding mechanisms referred to as "hGH-like" for primate GHs, "GH-like" for any GHs, and "PRL-like" for PRLs. The remaining interactions have been derived from sequence comparisons (*Section III* for details).

[a] ++, Very strong affinity, +, strong affinity; ±, low affinity; −, no affinity; (?), hypothesis; *underlined*, experimental data.

togen receptor than to the somatogen receptor; surprisingly, the opposite is observed (21).

If mutational and structural studies of GHs, PRLs, and, to a lesser extent, PLs have considerably enhanced the understanding of the molecular bases of their receptor binding, it is likely that these data only provide part of the picture. Comprehensive studies of the binding energy of hormone-receptor interfaces have been recently reported for several four-helix bundle cytokines (100, 107, 165). With respect to the hGH-binding site 1 to the somatogen receptor, it was explicitly demonstrated that the binding energy is clustered around a small nucleus of residues centered on helix 4 (100, 107). Some residues, although involved in contacts with the receptor (15) or defined as important by mutagenesis (101), do not significantly contribute to the energy of the interaction. While sequence-structure-function correlation provides interesting data (Table 3), it now appears that the relative importance of residues identified as binding determinants by site-directed mutagenesis should be balanced by valuable structural/energetic studies.

IV. Binding Site 2

A. *Sequence-function correlation*

1. Receptor dimerization or oligomerization: a general rule in the cytokine receptor superfamily. Mutational (103), structural (15), and functional (104) studies have clearly demonstrated that hGH induces the dimerization of the somatogen receptor. This is achieved when one molecule of hormone, already bound to one receptor, binds to a second receptor molecule through another region of the hormone, called binding site 2. In hGH, binding site 2 is mainly delimited by residues belonging to helices 1 and 3 and the N terminus (Refs. 15 and 103; Fig. 1; for review, see Ref. 110). A similar binding mechanism is expected when hGH binds to the lactogen receptor (105). It is also clear that receptor dimerization is an absolute requirement for initiating signal transduction by both types of receptors, since GH analogs blocked at binding site 2 are unable to activate the membrane-bound receptors, and, in addition, are inactive in *in vitro* bioassays (104, 105, 166) as well as in transgenic mice expressing such mutants (167–172). Immunoblot analysis has further shown that activation of Jak2, the receptor-associated tyrosine kinase, occurs only after receptor dimerization of the PRLR (31, 173, 174). Finally, it has been proposed that the dimerized somatogen receptors are in a disulfide-linked form (175), although mutation of the free extracellular cysteine of the PRLR to serine has no effect on the functional activation of this receptor (176).

Apart from the above-mentioned studies, the ability of PRL/GH/PL to induce receptor dimerization (or oligomerization) has been indirectly investigated by studying the stoichiometry of hormone-binding proteins [*i.e.* ECD of the receptors] complexes. These studies, however, seem somewhat removed from the actual hormone-membrane-bound receptor interaction (127, 177). As for example, only 1:1 complexes (one hormone-one ECD) were obtained between hGH and the hPRLBP (16, 106), whereas functional bioassays strongly suggest the requirement of 1:2 complexes for expression of biological activity through the membrane-embedded PRLR (105). Similar confusing data were reported for bPL, which forms 1:1 complexes with the bGHBP (178), but 1:2 with hGHBP (Refs. 127 and 177 and references therein). Finally, a similar controversy has been observed for PRL: oPRL and rPRLBP form 1:2 complexes (179), whereas the same hormone only forms 1:1 complexes with rabbit and bovine PRLBP (177, 180). It appears that glycosylation pattern (181), species specificity (see *Section IV.A.4*), and other parameters may affect the observed stoichiometry of the hormone-binding protein

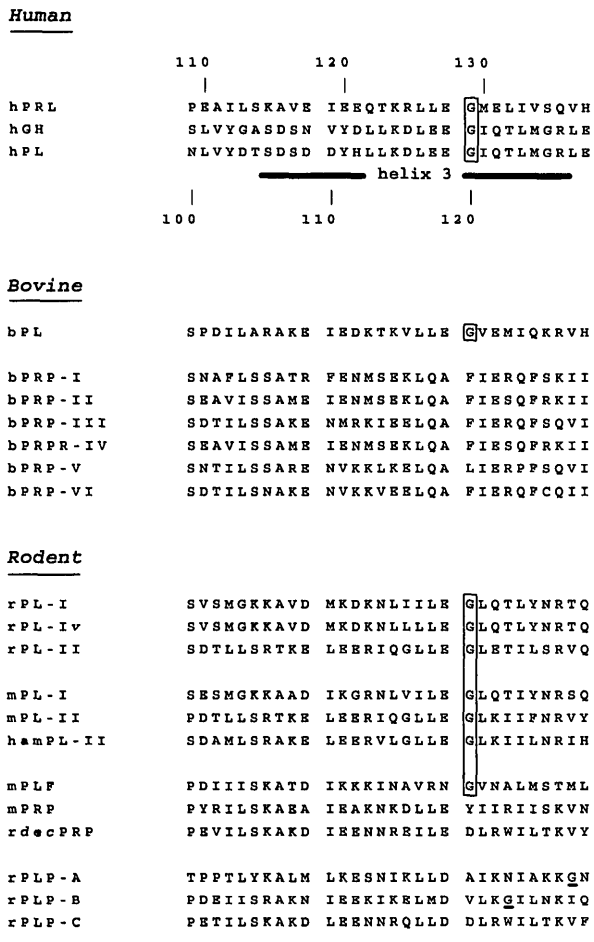

FIG. 6. Conservation within several members of the PRL/GH/PL family of the helix 3 glycine involved in binding site 2 of hGH and hPRL. Wherever aligned, the helix 3 Gly is *boxed*. In rPLP-A and rPLP-B, two glycine residues are also found in the putative helix 3 (Gly128 and Gly123 in hGH numbering, respectively). Although they are not topologically equivalent to the conserved PRL/GH/PL helix 3 Gly, these Gly residues are *underlined* since one cannot rule out the possibility that either their alignment has been missed by the algorithm used or that they have a similar role in maintaining a cleft in the putative helix 3.

complexes. Recent studies performed using a BIAcore apparatus have suggested that, in most cases, the 1:2 hormone-ECD complexes were missed by classic methods (*e.g.* gel filtration) due to extremely high dissociation kinetics (182). A similar discrepancy in apparent hormone-receptor stoichiometry between soluble binding protein or membrane-bound receptor has also been reported for EPO, another cytokine (183–185), although a recent study clearly demonstrated the EPO-induced dimerization of the EPO receptor ECD (186).

As described in the *Introduction*, lactogen and somatogen receptors belong to the class 1 cytokine receptor subfamily. Receptor homodimerization has been described for the GHR (15), the G-CSF receptor (187), the EPO receptor (183, 184), and the PRLR (21, 108, 118, 120a, 174). Otherwise, hetero-oligomerization of multiple receptor subunits and/or signal transducer has been reported for all the other cytokine receptors. Activation of receptors for IL-3, IL-5, and GM-CSF requires partnering of ligand-specific α-chains with a common β-chain, called KH97 in human and AIC2B in mouse (187, 188). Receptors for IL-6 (189–191), ciliary neurotrophic factor (192–195), LIF (194, 196, 197), Oncostatin M (197), and IL-11 (198) associate with the common signal transducer gp130. It is currently unknown whether OBR, the recently cloned leptin receptor (78) that is closely related to gp130, is capable of initiating intracellular signaling or requires other transducers to form a functional receptor complex. Finally, receptors for IL-2 (199–201), IL-4 (90, 200, 202, 203), IL-7 (200), IL-15 (204), and possibly IL-9 and IL-13 (202), associate with the common IL-2Rγ chain (for reviews, see Refs. 79–84, 87, 88, 205). Receptor activation by membrane component clustering is thus a general rule within the cytokine receptor superfamily. Hormone-induced receptor homodimerization (or oligomerization) is therefore anticipated for the membrane receptors of all members of the PRL/GH/PL family.

2. The second binding site of PRL, GH and PL: importance of the helix 3 glycine. To date, the binding of a second site of hGH to the somatogen receptor (hGHBP) remains the only interaction that has been characterized by x-ray study. It is defined by the channel lying at the interface of helices 1 and 3 (Ref. 15 and Fig. 1). Mutational analysis of hGH (104, 105, 171), bGH (167–170, 172), hPRL (118, 120a) and hPL (108) have demonstrated the functional importance of a glycine residue carried by helix 3 (G119 in bGH, G120 in hGH and hPL, G129 in hPRL). In contrast to the residues classically defined as binding determinants by site-directed mutagenesis, which are assumed to act through an interaction of their side chain with the receptor, the role of the helix 3 glycine consists mainly in maintaining an empty space (cleft) within the helix 1-helix 3 interface to bury some receptor residues after the hormone-receptor interaction.

When the helix 3 Gly of human or bovine GHs is mutated in an amino acid with a larger side chain, such as an Arg, binding site 2 is sterically hindered, and these hormones are no longer able to induce dimerization of binding proteins (106). Since these GH analogs retain the ability to bind to the receptor through their binding site 1, they behave as antagonists (Refs. 104, 105, 166, and 168–172; Fig. 7). Similar residue substitution in hPL also leads to a lactogen receptor antagonist (108). In hPRL, replacement of Gly 129 with Arg does not completely abolish, but markedly reduces, the lactogen receptor-mediated mitogenic activity of the hormone on rat Nb2 cells (Ref. 118; see *Section IV.A.4*). A similar functional role of the glycine in helix 3 for GHs, PLs, and PRLs strongly suggests that this residue is topologically equivalent in the folded proteins. The alignment of these glycines in the multiple sequence alignment used for the present sequence-function study thus argues for the accuracy of that alignment (Fig. 6; *Section II*). It is noteworthy that the topological equivalence of the helix 3 glycine, which is deduced from recent functional data (104, 105, 108, 118, 120a, 170), was missed by several alignments previously reported (21, 66, 69, 101, 126).

Considering the functional data obtained with mutated GHs, PRLs, and PLs, the helix 3 glycine can thus be regarded as a "molecular marker" of a second binding site located around the helix 1-helix 3 interface as described for hGH (15).

That this glycine is ubiquitous in mammalian PRL, PL, or GH sequences (Fig. 6; see Ref. 12) suggests that the binding mechanism through this second binding site might share similar characteristics for these three proteins. Apart from helix 3 glycine, it appeared difficult to identify critical residues by classical substitution mutagenesis in hGH. Of 30 alanine substitutions in and around binding site 2 of hGH (103), only four affected dimerization of the hGHBP by more than 3-fold: Phe1, Ile4, Arg8 (all three in the N-terminal loop) and Asp116 (in helix 3). Phe1 is conserved in all GHs (12) and may represent a characteristic feature of the second binding site of GHs. The importance of Ile4 is controversial since transgenic mice expressing a I4A hGH variant[1] failed to exhibit a dwarf phenotype (171), as had been expected from the impaired ability of that hGH mutant to induce hGHBP dimerization (103). This suggests that this residue is not ubiquitously required for functionality of GH binding site 2 in all species (see *section IV.A.4*). As observed for hGH, alanine substitutions in the putative binding site 2 of hPRL had little effect (118) and, in agreement with the cleft-model of this binding site, small-to-large side chain mutations were required to alter biological properties.

Apart from Gly129, our study revealed the critical role of Ala22, located on helix 1, whose replacement with a Trp also markedly reduced the mitogenic activity of hPRL in Nb2 cells (118). There is currently no data available for the homologous position in hGH, which is also occupied by an alanine (Ala13), since the mutational strategy used by Cunningham and colleagues (103) is alanine-scanning. Interestingly, as observed for the helix 3 Gly, this helix 1 Ala is highly conserved among PRLs and GHs (Ref. 12 and Fig. 3). Moreover, studies using a BIAcore revealed that the rPRLR ECD can form 1:2 complexes with hGH, bPL, and oPRL, which all possess an alanine at position 22 (hPRL numbering), while only 1:1 complexes can be seen with the homologous hormone, rPRL, whose topologically equivalent residue is a valine (Ref. 182; see *Section IV.A.4* for discussion). These data suggest that the functional role of this helix 1 Ala could be similar to that of the helix 3 Gly, *i.e.* to maintain a cleft at the binding site 2. This hypothesis correlates with structural data, since helix 1 Ala is in the same environment as helix 3 Gly in hGH (15) and in the predicted structure of hPRL (21).

Data concerning the probable binding site 2 of nonprimate PLs remain fragmentary. The next subsection (*IV.A.3.b*) will discuss results obtained with ungulate PL mutants (oPL and bPL). To the best of our knowledge, no reported study has specifically investigated the second binding site of rodent PLs. As shown in Fig. 6, all have the characteristic helix 3 glycine, suggesting a possible GH- or PRL-like binding site 2. Interestingly, Ala22 (hPRL numbering) is replaced in rodent PLs (Fig. 3) by other aliphatic residues (Val or Leu). Although the ability of both rPL-I and rPL-II to stimulate the milk protein β-casein gene promoter is in the same range as oPRL (153), we would anticipate a lower affinity at the binding site 2 of rodent PLs compared with PRLs due to the larger side chains at position 22. Further mutational studies are obviously required.

3. Receptor specificity of the binding site 2. Primate GHs and ungulate PLs are particular in that they exhibit both lactogenic and somatogenic properties. It has been shown that binding site 1 of hGH is receptor-specific, meaning that the set of residues interacting with the receptor is not strictly identical whether the lactogen or the somatogen receptor is considered (Ref. 99; see *Sections III. A.1.a and A.2.a*; Fig. 2). The possibility that binding site 2 of primate GH and ungulate PL might also be receptor-specific needs to be addressed.

a. Primate GHs. The antagonistic properties of G120R hGH on both lactogen and somatogen human receptors (104, 105) indicate that this region is involved in the second binding site of the hormone, whatever the receptor considered. This does not preclude, however, a different involvement of residues surrounding the helix 3 glycine cleft in binding to each receptor type.

Deletion of the first seven N-terminal residues in hGH, accompanied by R8M and D11A substitutions, did not significantly affect the rat Nb2 or bovine mammary gland lactogen receptor-mediated properties of the hormone; in contrast, binding to and bioactivities mediated by somatogen receptors in human IM-9 lymphocytes were markedly reduced by this mutation (206, 207). As none of the deleted or substituted residues has been implicated in hGH binding site 1 to either the lactogen or somatogen receptors (98, 99), one might assume the differential effect of this mutation to be related to the second binding site. The N terminus of hGH has been clearly defined as part of the second binding site to the somatogen receptor (15, 103), correlating data obtained for binding of the seven-residue deletion hGH mutant on IM-9 cells (206). Cocrystallization of hGH and hPRLBP only gave rise to 1:1 complexes (16), however, and no structural data are currently available for assessing the poor, or even nonexistent, involvement of the seven N-terminal residues in the binding site 2 of hGH to the lactogen receptor. In this regard, alteration of lactogen binding of hGH required deletion of a larger segment. Precisely, deletion of the 13 N-terminal residues decreased the binding affinity of hGH for the lactogen receptor in Nb2 cells or lactating bovine mammary gland by 2 orders of magnitude (208). Moreover, this hGH variant displayed antagonistic properties on the lactogen receptor, arguing for an alteration of its binding site 2.

It is noteworthy that replacement, and not deletion, of the 13 first residues of hGH with the homologous amino acids from the nonlactogenic bGH did not affect lactogen binding (206). These data can be interpreted in two ways. On the one hand, some residues common to hGH and bGH within the 8–13 segment may be crucial determinants for hGH binding to the lactogen receptor. Candidates are Leu9, Phe10, Asn12, and Ala13 (for alignments, see Ref. 140). On the other hand, as helix 1 of hGH starts at residue 8 (15), deletion of the 13 N-terminal residue is likely to alter the global folding of the protein and, thereby, to prevent some of the remaining binding determinants from interacting with the receptor. Thus, replacement of the N-terminal tail by the homologous region from bGH could have a less disruptive effect on global fold-

[1] Throughout the manuscript, hormones carrying a single mutation are referred to using the following annotation: the residue found in the wild type protein (using the one-letter code for amino acids), the numbering of this residue within the mature protein sequence, and the substitution residue. For example, hPRL G129R represents a single mutation hPRL analog in which glycine 129 is replaced by arginine.

ing and, therefore, could be ineffective. Finally, it has been reported that replacement of a longer segment [1–23 (bGH)/24–191 (hGH)] decreases hGH lactogen binding and hGH-induced Nb2 cell proliferation by 2–3 orders of magnitude (209). Compared with the 1–13 (bGH)/14–191 (hGH) mutant, which retains full mitogenic activity on these cells, the additional 10-residue replacement only introduces three single mutations (Ser14→Val, His18→Gln, Arg19→His). As described earlier (Ref. 39; *Section III.A.2.a*), the "His18-X-X-His21" sequence is involved in zinc coordination, a major component in the lactogen binding of hGH. While replacement of this sequence by the nonprimate GH "X-His19-X-His21" sequence will prevent zinc coordination due to the 100° rotation of the first His, the decreased lactogen binding of this 1–23 (bGH)/24–191 (hGH) analog is likely to result from alteration of binding site 1 rather than binding site 2.

Taken together, these data show that hGH binding to lactogen or somatogen receptors is differentially affected by mutations. This suggests that residue requirements of binding sites 2 are, as already observed for binding site 1 (99), receptor-specific. On the basis of our hPRL structural model, we have recently suggested (21) that the second binding site of hPRL, and possibly of hGH, to the lactogen receptor is shifted toward the N terminus of helix 3/C-terminus of helix 1 region with respect to the binding site 2 of hGH to the somatogen receptor (15). The above mentioned experimental observations are in agreement with this proposal; however, additional mutagenesis studies are required to confirm our hypothesis.

b. Ungulate PLs. Since ungulate PLs (oPL, bPL) display somatogenic properties (10, 41, 42, 127, 150, 178), one would expect these hormones to possess the residues identified as binding determinants in the binding site 2 of hGH (15, 103). This does not seem true, however. Although both PLs have a helix 3 glycine (Fig. 6), they lack the binding determinants identified in the hGH tail (103; Phe1, Arg8, Asp116; the controversial Ile4 is conserved). Moreover, their N-terminal tail is PRL-like, *i.e.* 13 residues longer than hGH (Fig. 3), including the small N-terminal disulfide bridge.

One can thus postulate that mutation of the hGH-binding determinants combined with the elongated N terminus, presumed to sterically hinder binding site 2, weakens the ability of ungulate PLs to bind to a second somatogen receptor molecule. This is in agreement with the observation that deletion of the 13 N terminus residues in bPL did not affect (127), or even increased by 2-fold (42), the somatogenic activity of the hormone on rat hepatocytes or 3T3-F442A preadipocytes, respectively. In this regard, it has been reported that the affinity of bPL for the somatogen receptor is 5-fold greater compared with hGH, while its biological activity in 3T3-F442A preadipocytes is 6-fold lower (41). This apparent discrepancy might actually reflect a higher affinity at binding site 1 (see *Section III.A.1.d* for discussion) and, in contrast, a sterically hindered binding site 2 (see above). Such a weakened site 2 could also partly account for the versatility of bPL to form 1:1 or 1:2 complexes with the GHBP from different species (127, 177, 178, 210).

Contrary to what is observed for somatogen receptor-mediated properties, the N-terminal tail is an absolute requirement for bPL to exhibit lactogenic activity, since deletion of the 14 N-terminal residues decreases its mitogenic activity on Nb2 cells by nearly 3-fold (by comparison, it increases somatogenic activity by 2-fold; see Ref. 42). Moreover, ungulate PLs display the same cysteine pattern as PRL, including the N-terminal disulfide bridge. The interaction of ungulate PLs with the lactogen receptor through their binding site 2 is thus likely to share several features with that of PRLs. Mutations of Gly129 and Ala22 (hPRL numbering) in those PLs should help elucidate their binding mechanism.

These data strongly suggest that ungulate PLs interact with either somatogen or lactogen receptors through molecular mechanisms having different requirements at the residue level; this is reminiscent to what is observed with hGH whose binding site 1 (99) and presumably binding site 2 (*Section IV.A.3.*), are also receptor-specific.

4. Receptor species specificity for binding site 2. The first evidence for the sequential model of hGH-induced somatogen receptor dimerization was provided by the observation that the hormone self-antagonizes at high concentrations (>100 nM) in hGH-dependent cell proliferation bioassays (Ref. 104; for review, see Ref. 111). At the molecular level, self-antagonism reflects the progressive disruption of active 1:2 complexes (one hormone, two receptors) for the formation of inactive 1:1 complexes in the presence of an excess of ligand (Fig. 7). In the case of hGH, this is due to the higher affinity of binding site 1 compared with that of binding site 2, which favors hormone interaction with the receptor through its binding site 1 in presence of an excess of ligand. Subsequently, this model of sequential receptor dimerization was also proposed to reflect activation of the lactogen receptor by hGH, hPRL, and hPL (105, 108).

In the Liège laboratory, we repeatedly failed to observe such self-antagonism of hPRL in the lactogen-dependent rat Nb2 cell proliferation bioassay, even at a concentration of 10 μM (Ref. 118 and Fig. 7). Although the Paris laboratory reported weak self-antagonism of oPRL at 100 μM in the same assay (173), this phenomenon remained much less obvious for PRLs than for hGH. Interestingly, we were able to observe self-antagonism (with IC_{50} around 1–3 μM) with hPRL mutants G129R and A22W whose binding site 2 is strongly hindered (118). Since the formation of inactive 1:1 complexes implies that the hormone binds to the receptor through one site preferentially, the one that has the higher affinity, the IC_{50} of self-antagonism (self-IC_{50}) can be regarded as the reflection of the difference in affinity between both sites. From our data, we have proposed that the affinities of both binding sites of wild type hPRL for the rPRLR are more or less equivalent or at least much less different than those of hGH (Fig. 7). From this model of receptor activation, the formation of 1:1 complexes will not be favored, or only very slightly, at high concentrations, since both binding sites of hPRL exhibit roughly identical affinities, which strengthens the stability of the complex. Accordingly, mutations within binding site 2 of hPRL (A22W, G129R) lower the affinity of this site, and a hGH-like situation is achieved, with a significant difference in binding affinity between both sites; therefore, self-antagonism is observed (Ref. 118 and Fig. 7).

In addition to the specific properties of the different hormones (affinity of each site), receptor dimer formation is also

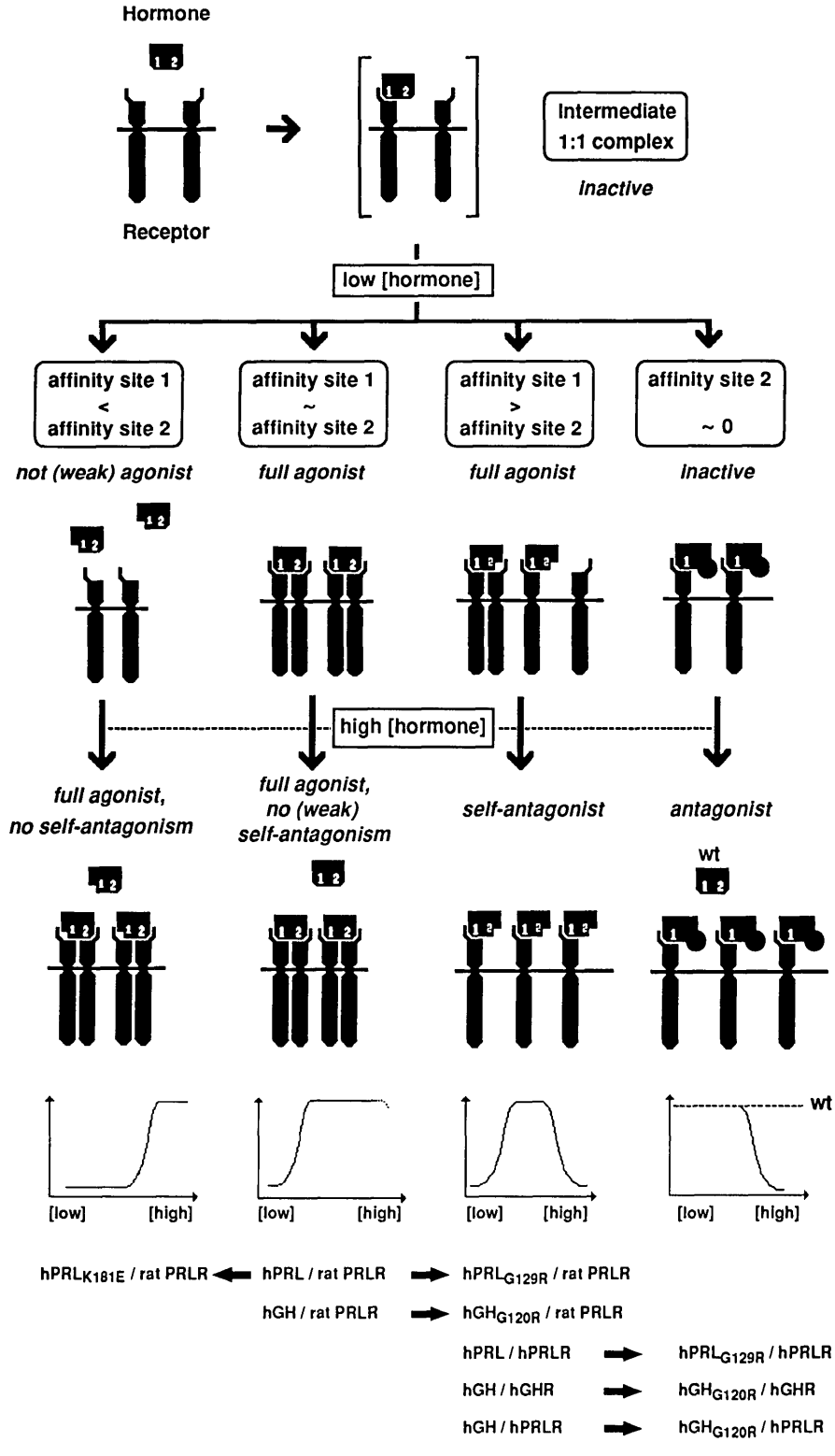

FIG. 7. Species specificity: models of hormone-receptor interaction based on the relative affinities of binding sites 1 and 2. Four models of hormone-receptor interactions are presented based on the relative affinities of binding sites 1 and 2. In the *lower part* of the figure, a typical curve is illustrated for each interaction, assuming that receptor activation requires dimerization (the X axis corresponds to the hormone concentration and the Y axis to any receptor-mediated bioactivity). For each curve, examples of particular hormone-receptor interactions are given. In a first step, the hormone (PRL, GH, or PL) binds to a receptor (PRLR or GHR) through its binding site 1 to form an inactive 1:1 complex. Then, four situations can be observed:

1. *The affinity of binding site 1 is weak* and receptor dimerization is achieved at high hormone concentrations, allowing sufficient formation of 1:2 complexes required for full agonistic activity. This is observed for hormone analogs mutated within site 1, such as K181E hPRL.
2. *The affinity of both binding sites is roughly identical* and full agonism is observed at low concentration due to sufficient dimer formation. At high concentration, no (or only weak) self-antagonism is observed, since high stability of 1:2 complexes does not favor the formation of 1:1 complexes. This case is illustrated by the interaction between rPRLR and hPRL.
3. *The affinity of binding site 1 is higher than the affinity of site 2.* In this case, full agonistic activity is also observed at relatively low concentration. At high concentrations, the higher affinity of binding site 1 favors the disruption of 1:2 complexes for 1:1 complexes and self-antagonism occurs. The curves obtained with hGH in hGHR-mediated bioassays or hGH G120R analog in the Nb2 proliferation assay in low zinc concentration follow this model.
4. Finally, if *the affinity of binding site 2 is nearly nil*, the hormone can not dimerize the receptor and fails to display any agonistic effect. Due to the ability to bind through site 1, such analogs behave as antagonists of wild type hormone when added in sufficient amounts (which depends on the affinity of first binding site). For example, G129R hPRL and G120R hGH antagonize the hPRLR (see *Section IV.A.4* for more details).

likely to depend on the species origin of the receptors themselves. As described above, the hGH G120R analog was first regarded as a full hGH antagonist. The antagonism of this variant on the somatogen receptor was confirmed in different bioassays using FDC-P1 cells transfected with a human somatogen/G-CSF hybrid receptor (104), human IM-9 cells (166, 171), or transgenic mice (171). Antagonism of the G120R hGH analog was also observed in lactogen bioassays such as proliferation of Nb2 cells or FDC-P1 cells transfected with the hPRLR (105). In all these experiments, no agonistic effect was observed for this G120R mutant, in agreement with its inability to induce receptor dimerization (Fig. 7). In a recent study, however, significant agonistic activity was reported for the G120R hGH analog in the rat Nb2 cell proliferation system (211). Although the discrepancy with the earlier report (105) could be partly explained by differences in zinc

concentrations used in the experiments (see *Section III.A.2.a* for the role of zinc), these data clearly demonstrated that replacement of Gly120 with Arg, which is sufficient to prevent hormone-induced dimerization of the lactogen receptor of human origin (105), cannot prevent dimerization of the lactogen receptor from rat (Ref. 211 and Fig. 7). This is reminiscent of our observations that site 2 mutants of hPRL (A22W, G129R) also retain mitogenic activity on rat Nb2 cells (118). In agreement with our earlier hypothesis (118), it thus appears that the affinity of the rPRLR for the binding site 2 of hPRL and hGH (and probably other members of the PRL/GH/PL family) is higher compared with that of the PRLR from human (and probably other) species. We recently confirmed this hypothesis using a transcriptional bioassay in which CHO cells or 293 fibroblasts were cotransfected with the PRLR from either rat (212) or human (36, 213) and a plasmid carrying the luciferase coding sequence under the control of PRL-responsive promoter sequences (120a). In such an assay, the agonistic activity of site 2 hPRL analogs is indeed higher for the rat than human PRLR. This also correlates with the observation that oPRL forms stable 1:2 complexes with rPRLR ECD (179) but only 1:1 with rabbit (177) and bovine (180) PRLR ECD. Moreover, we have shown that the interaction of rPRL with either human or rat PRLR also leads to bell-shaped curves, in agreement with a model of hormone-induced receptor dimerization (120a); compared with hPRL, however, self-antagonism occurs at lower concentrations, arguing for a lower receptor affinity of rPRL site 2 compared with the human hormone. Finally, such species specificities could also account for apparent controversial data about the poor ability of the I4A hGH analog to induce dimerization of the hGH binding protein (103) and the full agonistic properties of that variant on mouse somatogen receptors (171, 172).

The molecular basis of this species specificity is currently unknown since the interaction of lactogenic hormones with lactogen receptors at the binding site 2 interface has not been characterized by crystallographic analysis (16). Analysis of hormones from different species and/or carrying mutations within site 2 in different bioassays should provide important information, such as the relative affinity of both sites. Finally, conflicting results appearing within the last 2 years question the validity of the Nb2 proliferation assay, which emphasizes the agonistic properties of analogs elsewhere regarded as antagonists (105, 118, 120a, 211).

B. Overview

Mutagenesis data available for binding site 1 of GHs, PRLs, and, to a lesser extent, PLs clearly show that the interaction of those proteins with receptors can be seen as "variations on a theme." On the one hand (the "theme"), the regions of the proteins interacting with receptors involve a topologically equivalent side of the folded proteins (N terminus, loop 1, C terminus; Fig. 1). On the other hand (the "variations"), the residues within this patch that make direct contacts with residues of the receptors, are not strictly identical and appear to be both hormone- and receptor-specific (Fig. 2). The observations described in the above section lead to the same conclusions for binding site 2, although available data remain more fragmentary than for binding site 1.

The two-site model of receptor activation, first described for the hGH/somatogen receptor interaction (15, 103, 104), is likely to also reflect the interactions of hGH (105), hPRL (118, 173, 174), and hPL (108) with the lactogen receptor (for reviews, see Ref. 31, 110, and 111). Nonprimate PL-induced dimerization of the lactogen (and, for some, of the somatogen) receptor(s) is also assumed to occur. In this respect, the link between binding properties described in this review and hormonal activity is assumed to depend on the ability of the hormones to induce receptor dimerization. However, as one cannot rule out the possibility that activation of a given receptor could occur through different mechanisms depending on the ligand, specific studies aimed at investigating the existence and, thereby, the location of a second binding site on PLs must be carried out. The discussion and prediction reported in the above sections are aimed at directing such mutational analyses.

The natural occurrence of proteolytic fragments of PRL/GH proteins, such as 16K PRL or 1–43 and 44–191 hGH, has been reported (for reviews, see Refs. 135 and 214). Although these protein fragments, when purified from natural sources, first showed some classic lactogenic/somatogenic activities, recent studies using recombinant fragments clearly indicate the poor ability (44–191-hGH: see Ref. 215) or even the inability (16K hPRL: unpublished data; 1–43 hGH: see Ref. 215) to activate the PRLR or GHR. This is in good agreement with the absolute requirement of two intact binding sites for functional activation of the receptors and suggests that such modified hormones exert their biological properties through distinct receptors (215, 216).

Rodent PLs exhibit a highly variable cysteine pattern that is likely to have some effect on the global folding of the protein. For example, based on gel mobility, mPL-I lacks the large disulfide typical of PRL/GH proteins (for reviews, see Refs. 9 and 141). Moreover, rodent PLs lack the N terminus disulfide, which appears functionally important for the lactogenic properties of ungulate PLs (42, 127). As the N terminus is assumed to be part of binding site 2 (see above), this suggests that the interaction of rodent PLs with a second lactogen receptor, if it actually exists, might be different from that of ungulate PLs or PRLs. Similarly, Davis and Linzer (124) reported that disruption of the large disulfide in mPL-II (C51S mutation) did not affect binding to Nb2 lactogen receptors, although mitogenic activity in Nb2 cells was decreased. As both binding and activity on Nb2 cells of hPRL are drastically impaired by the homologous mutation (C58A; see Ref. 116), this might also reflect some important differences in the activation of the lactogen receptor depending on the ligand. Due to the lack of mutational data on rodent PLs, prediction of the residues involved in binding site 2 of these proteins would probably be misleading.

Recent data suggest that in addition to the general mechanisms of receptor activation, some features of the hormone-receptor interactions could result from species specificity. In this respect, it is worth noting that hormone self-antagonism and/or the 1:1 hormone-receptor ECD stoichiometry are/is almost always observed for homologous interactions (*i.e.* both ligand and receptor from the same species). The natural

occurrence of a second binding site of lower affinity could reflect a "physiological switch off" for abnormally high hormone concentrations (self-antagonism).

As detailed above, activation of PRL/GH/PL receptor through homodimerization is supported by several reports. At the conclusion of this section, however, four comments have to be considered. First, the hormone-induced receptor dimerization, which appears to be required for efficient signal transduction, does not preclude the possible requirement of (an)other membrane component(s) that still remain(s) to be identified. Recent binding studies of lactogenic hormones on Nb2 cells suggest the existence of two receptor types differing by their affinity (217). While the low-affinity receptor has been identified as the cloned intermediate form of the PRLR (212), the "high-affinity and rapidly saturable" binding protein remains unidentified and could be another membrane signal transducer and/or affinity-converter. Involvement of G proteins has also been suggested (218).

Second, at least one report (219) suggests that some biological activities of GH, *e.g.* the antilipolytic (insulin-like) effect, do not require the integrity of the second binding site as defined from the 3D structure of the hGH-hGHBP complex (15). This raises the possibility that either some biological activities of GH could be mediated by nondimerized somatogen receptor or, conversely, that a region other than that defined as the second binding site of hGH (15) can lead to receptor dimerization.

Third, although it appears that receptor homo-, hetero- or oligomerization of cytokine receptors and/or signal transducers are required for signaling in a wide majority of cases, the specificity of the transmitted signal remains poorly understood. Study of chimeric receptors has suggested a role of the ECD in defining the phosphorylation pattern of cytoplasmic proteins (220, 221). This probably also reflects the functional role of some associated membrane transducers.

Finally, although the interaction of PRL/GH/PL proteins with membrane receptors appears to be the first step of the events leading to the specific expression of their biological effects, there are obviously several other parameters that modulate the biological response of target cells to hormone stimulation. For example, different PRLR isoforms have been isolated (for review, see Ref. 29) which, although they share an identical extracellular (binding) domain, show different abilities to transmit hormonal signals within the cells (222–226). Moreover, the relative levels of expression of somatogen and lactogen receptors vary depending on species, age, physiological state, organs, etc. (for review, see Ref. 29), so that the biological activity of a hormone will also depend on such parameters. For hormones that are able to bind to both receptor types, such as primate GHs or ungulate PLs, the ratio of PRLR *vs.* GHR will also direct their effect on target tissues. The very wide and species-specific spectrum of bioactivities of PLs over mammals probably reflects the numerous factors influencing hormone actions *in vivo*. For example, although all PLs exhibit high affinity for lactogen receptors, their effect at the fetal and/or maternal level differ among rodent, ruminant, and primate species (for reviews, see Refs. 7, 10, 28, and 227 and references therein). *In vivo*, this might be partly correlated with the highly variable PL concentrations found in fetal and maternal circulation (10), which range from less than 1 ng/ml (bovine) up to several micrograms per ml (primates, sheep, hamster). Even *in vitro*, however, striking differences in biological potencies have been reported between oPL and bPL (for review, see Refs. 10 and 227), such as the observation that oPL stimulates mammary gland secretions, whereas bPL does not. One of the most important parameters likely to direct PL functions *in vivo* is the presumed specific PL receptor, which has been suggested from several investigations (44–46), but remains currently unknown.

These observations emphasize that, if the interaction between a hormone and a receptor is an absolutely required initial step for PRL/GH/PL hormones to exert any biological effect, deciphering their binding mechanisms *in vitro* cannot, alone, provide full understanding of their activities *in vivo*.

V. PRL-Related Proteins (PRPs)

Several recently cloned proteins have been linked to the PRL/GH/PL family on the basis of their nucleic or amino acid sequences (Table 1). The physiological role of the PRPs still remains unknown, and their binding potencies are poorly characterized. Taking advantage of the binding mechanisms described above for the other members of the PRL/GH/PL family, we have attempted to analyze the (in)ability of these PRPs to bind to somatogen or lactogen receptors, on the basis of their amino acid composition.

Several alignments of PRPs have been reported (58, 66, 67, 69, 141). For our purpose, we have aligned six bPRPs (PRP I to VI following the nomenclature of Schuler *et al*; see Ref. 228) and six rodent PRPs (mPLF, mPRP, r*dec*PRP, rPLP-A, rPLP-B, and rPLP-C) together with the PL, GH, and PRL sequences analyzed in *Sections III and IV* of this review (Figs. 3–6 and Table 1). Due to the lack of mutational data, an exhaustive analysis of all reported sequences would be premature. The alignments reported in Figs. 3–6 must be considered as a first attempt to elucidate the molecular bases of the binding (in)abilities of these PRPs (Table 4).

A. The bovine PRP family

All bPRPs were identified by screening placental cDNA libraries and are predicted to be glycoproteins that contain Asn-linked oligosaccharide chains. The bPRPs show more amino acid sequence identities with PRLs (43–51%) than with GHs (21–24%) or even bPL (31–34%; see Refs. 227 and 229). The predicted sequence of the bPRPs is consistent with the conserved sequence residues forming the large disulfide loop, and all but one (bPRP VI) predict the cysteines forming the small loop near the carboxy terminus, indicative of a tertiary structure characteristic of the PRL/GH/PL family (69). Although sharing high sequence similarity with PRLs, purified bPRP-I was not found to bind to receptors for GH, PRL, or PL and is regarded as nonlactogenic (229, 230).

Yamakawa *et al.* (67) have pointed out four regions of high homology between bPRL-like proteins. Two of these contain segments involved in the binding site 1 of PRL/GH/PL, namely LD1 (second half of loop 1) and LD4 (second half of helix 4). As observed in Fig. 5, several PRL-binding determinants are indeed highly conserved in bPRPs. Some very

TABLE 4. Biological properties of PRPs from bovine and rodent families

Hormone	GHR binding	PRLR binding	PLR binding	Other properties
Bovine				
bPL	+ (240)	+ (241)	+ (46)	
bPRP-I	− (229)	− (229,230)	− (229)	
bPRP-II				
bPRP-III				
bPRP-IV				
bPRP-V				
bPRP-VI				
Rodent				
mPL-I	− (28)	+ (155,156)		
rPL-II	− (242)	+ (157,158)		
rPL-Iv		± (50,51)		
mPL-II	− (243)	+ (125,159)		
rPL-II	− (242)	+ (157)		
hamPL-II	− (244)	+ (160)		
mPLF	− (232)	− (125,232)		Mannose-6-P (232) Angiogenic (128) Uterotrophic (129)
mPRP	− (141)	− (233)		Antiangiogenic (128)
rdecPRP		± (236)		
rPLP-A	− (235)	− (235)		
rPLP-B	− (237)	± (237)		
rPLP-C	− (234)	− (234)		

Binding to either of the reported receptor types (GHR, PRLR, and PLR) for the PRL/GH/PL protein family is indicated whenever reported. Other reported bioactivities are summarized in the right column (see *Section V* for details). *Numbers in parentheses* refer to references found in the list.

important residues such as Lys69 (116) are lacking, however. Moreover, the N-terminal end is very divergent between PRL and PRPs. This can affect site 1 binding, since part of helix 1 is expected to be involved (117, 120). Similar observations seem to account for the lack of somatogen binding (Fig. 3). Out of the 25 binding site 1 determinants of hGH (101), only Phe10 and Arg183 are ubiquitous in the six bPRPs, while Leu6, Lys172, Ile179, and Cys182 are conserved in five bPRPs (Fig. 3).

Finally, whatever the receptor considered, the binding site 2 region of bPRPs appears very different from PRL and GH (Fig. 6). First, neither the crucial helix 3 glycine nor the helix 3 sequence are conserved in the PRP subfamily (67). Second, as the N terminus is involved in binding site 2 of PRL/GH/PL (see above), the absence of sequence consensus in this region is likely to prevent binding to a second receptor. Thus, evidence to date indicates that the bPRPs are orphan ligands awaiting the determination of specific receptors. In this respect, the specific PL receptor identified in bovine endometrium might be a candidate for such a receptor.

B. The rodent PRP family

An excellent review has been recently devoted to the structural analysis of the rodent PRL-like proteins (141). Two recently cloned rPRPs have been added in our alignment, r*dec*PRP (58) and rPLP-C (51, 62). All the rodent PRPs have sites for Asn-linked glycosylation, at varying positions, and glycosylated forms of the proteins have been purified.

The rodent PRP family exhibits a highly variable cysteine pattern (141). PLP-B alone shows the presence of the four cysteines involved in the two disulfide bridges which is a general feature of the PRL/PL/GH family (Ref. 141 and K. T. Shiverick, T. A. Medrano, P. J. Saunders, M. Edery, and P. A. Kelly, manuscript in preparation). PRL possess an additional disulfide at the N terminus (12), and PLF, PLP-C, and r*dec*PRP are assumed to share this feature with PRL (58). It may be noted that r*dec*PRP cross-reacts with anti-hPRL or anti-PLP-B antibodies, suggesting a similar folding for the three hormones (58). Conversely, mPRP and PLP-A are likely to lack the large disulfide bond (141, 231). Cysteine distribution, especially when leading to disulfide formation, influences protein folding. It is likely that significant structural alterations exist due to different cysteine distribution and glycosylation sites, which compromises binding predictions on the basis of their primary structure alone (for review, see Ref. 141).

In another approach, it appears that the three key "lactogen" amino acids (Arg14, Arg169, and Lys 179) identified in mPL-II by Davis and Linzer (125) are completely conserved in the rat and mPL-I and II but show little conservation among the rodents PRP (Fig. 5; in hPRL numbering, residues 21, 177, and 187). These amino acids reside in helix 1 and helix 4, respectively. Recombinant PLF, PRP, and PLP-C have none of the key residues and have not been found to have any lactogenic bioactivity (232–234). Recombinant PLP-A, which retains only one of the abovementioned lactogenic residues (Lys179), was not found to have bioactivity toward PRL- and GH- responsive cell lines, respectively (235). In contrast, r*dec*PRP (236) and rPLP-B (Ref. 237 and K. T. Shiverick, T. A. Medrano, P. J. Saunders, M. Edery, and P. A. Kelly, manuscript in preparation) retain none of these conserved amino acids, but have been reported to have some lactogenic activity. In this regard, it is noteworthy that the position equivalent to Lys179 is occupied by an Arg, *i.e.* an amino acid with similar physicochemical properties. Moreover, both proteins contain adjacent Arg and Lys residues which, although not topologically identical to the conserved positions from our alignment, may have similar role in maintaining some lactogenic activity. Thus, this approach to structural analysis may indicate some, albeit weaker, lactogenic activity, depending on which of the key amino acids are conserved. It should be noted that the binding site 2 region lacks the crucial helix 3 glycine in all rodent PRPs except PLF (Fig. 6), and the N terminus is quite variable as well (Fig. 5).

Finally, unpredicted new biological activities have recently been described for mPLF and mPRP. Jackson *et al.* (128) observed that PLF and PRP are positive and negative regulators of angiogenesis, respectively, in endothelial cells. The involvement of members of the PRL family in angiogenesis is not without precedent, since the 16K amino-terminal fragment of PRL can inhibit bovine capillary endothelial cell growth (216, 238, 239). A second study by Nelson *et al.* (129) found that PLF can bind to cells in the uterus and stimulate DNA synthesis. Although the receptors for PLF and PRP are unknown, the insulin-like growth factor II/mannose-6-phosphate receptor binds PLF (232) and may form the basis of a functional receptor complex.

VI. Conclusions

Our observation that a single biological property (lactogen receptor binding) can occur through different mechanisms, depending on which protein is concerned (primate PLs and GHs *vs.* PRLs and nonprimate PLs), is against the usually assumed cross-correlation between protein sequence and conserved function. This observation is probably true for both binding sites 1 and 2. Primate GHs and ungulate PLs exhibit both lactogenic and somatogenic properties. Although PRLR and GHR share similarities at the primary structure level and exhibit similar folding, it appears that the binding determinants of the ligands are receptor-specific. Thus, not only does the interaction of two homologous hormones (*e.g.* hPRL and hGH) with a single receptor (*e.g.* PRLR) occur through a different mechanism, but the binding of a single hormone (*e.g.* bPL) to homologous receptors (*e.g.* PRLR and GHR) also appears to involve different sets of binding determinants.

The case of ungulate PLs, able to bind to the somatogen receptor, remains unclear. Our study suggests a mutational analysis of the hormone focused on the eight "GH-like" binding determinants within binding site 1. Depending on the results, one of our suggested hypotheses should be selected. However, we expect a very specific binding mechanism that could be unique among the PLs. Little is known about the physiological role of PLs (for reviews, see Refs. 8, 10, 28, and 227). Although PLs are derived from a divergent lineage (primate *vs.* nonprimate), it is noteworthy that evolution has led to a convergence of their physiological roles. Nevertheless, their functional interactions at the molecular level is probably reminiscent of their genetic origin.

The present analysis highlights the limits of sequence comparison for predicting *ab initio* the molecular bases of a biological activity shared by several homologous proteins. This restriction could also be applied to other families of proteins. Once the relationship to one or another mechanism has been well established (*e.g.* "hGH-like" or "PRL-like" for lactogenic binding through binding site 1), however, sequence comparison remains a powerful predicting tool to design experiments aimed at deciphering the molecular bases of protein activity. The contacts between a hormone and its membrane receptor are typically protein-protein interactions involving a high number of residues (for hGH, see Refs. 15, 98, 99, and 107), with the consequence that the hormone-receptor binding is not an all-or-nothing process. Depending on the species, a hormone will bind more or less tightly to a defined receptor. It is assumed from the numerous mutational analyses mentioned above that the relative affinity of each hormone can, in some way, be related to the number of binding determinants that are present in its sequence. Because of the nonubiquity of these binding residues among the different species, it appears thus improbable that the residues functionally important for one type of interaction could be exhaustively predicted by sequence comparison alone. None of the sequence analyses previously reported pointed out the crucial role of the helix 3 glycine (121, 140). Such observations emphasize the need for systematic mutational studies paralleling sequence comparisons.

Although showing primary structure similarities with the PRL/GH/PL family, PRL-like proteins are probably quite functionally distant. They lack most of the residues identified as binding determinants for somatogen (Fig. 3) or lactogen binding (Figs. 4 and 5). Even if they are assumed to share the common cytokine four-helix bundle-folding pattern, their structure at the residue level is probably different, partly due to the distribution of cysteine residues and the resulting disulfide bridge distribution. While sequence-function relationships assume very similar folding between homologous proteins that are compared, better understanding of PRPs awaits mutational studies aimed at deciphering their functional features, characterization of their 3D structure and, last but not least, the identification of their specific receptors.

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