Lutropins appear to contact two independent sites in the extracellular domain of their receptors

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Human chorionic gonadotropin (hCG) and bovine lutropin (bLH), a hormone chemically more similar to most mammalian lutropins than hCG, interact with the extracellular domains of their gonadal lutropin receptors (LHRs). These portions of the rat and human LHRs are 85 % identical and both receptors bind hCG with high, albeit not identical, affinity. However, at least 1000-fold more bLH is required to inhibit binding of radiolabelled hCG to the human LHR than to the rat LHR, a phenomenon that proved useful for identifying regions of the extracellular domain that contact lutropins. Previous studies using truncated receptors and lutropin/follitropin receptor chimaeras localized most, if not all, high-affinity ligand contacts to the N-terminal three-fifths of the rat LHR extracellular domain. We report here that 10-fold more bLH was needed to inhibit binding of labelled hCG to rat/human LHR chimaeras containing the N-terminal three-fifths of the human LHR extracellular domain than to the

rat LHR. Unexpectedly, 100-fold more bLH was required to inhibit binding of labelled hCG to chimaeras containing the C-terminal one-fifth of the human LHR extracellular domain than to the rat LHR. The ability of the C-terminal portion of the human LHR extracellular domain to inhibit bLH binding suggests this region of the receptor also contacts the ligand even though it is not needed for ligand binding. The extracellular domains of all the glycoprotein hormone receptors are thought to be horseshoe-shaped, a consequence of their leucine-rich repeat motifs. Portions of the ligand that become located within the cavity created by the concave surface of the horseshoe would have the opportunity to contact residues in the C-terminal portion of the extracellular domain. Changes to the ligand or receptor that influence this interaction would be expected to alter binding and confound efforts to identify residues in key ligand–receptor contacts.

INTRODUCTION

The actions of follitropin (FSH), lutropin (LH) and, in some species, chorionic gonadotropin (CG), on gonadal receptors are required for fertility. These gonadotropins and the other member of the glycoprotein hormone family, thyrotropin (TSH), are composed of a conserved α -subunit and a hormone-specific β -subunit that enables them to distinguish LH, FSH and TSH receptors [1]. Both hormone subunits have similar architectures and consist of three large loops held together by a cysteine knot [2,3]. The β -subunit has an additional loop, known as the seat-belt [2], which surrounds the second α -subunit loop to stabilize the heterodimer. The seat-belt is largely responsible for the influence of the human CG (hCG) β -subunit on receptor-binding specificity [4–6], probably through an influence on hormone conformation. Homodimeric hCG analogues built from α -subunit loop 2 and β -subunit loops 1 and 3 have hormonal activity even in the absence of the seat-belt [7].

Receptors for these hormones have large N-terminal extracellular domains containing several leucine-rich repeats [8–10]. Aside from the LH receptor (LHR), which binds LH and CG, most receptors recognize only one ligand with very high affinity. Two observations suggested that receptor residues in the N-terminal three-fifths of the extracellular domain control ligand binding. First, truncated receptors containing only this portion of the rat LHR bound hCG with high affinity [11]. Second, substitution of rat LHR residues 93–170 for their counterparts in the rat FSH receptor (FSHR) created an LH/FSH receptor

chimaera that had 10000-fold higher affinity for hCG than the rat FSHR [5]. The extracellular domain is coupled to conserved transmembrane domains composed of seven hydrophobic α -helices that have relatively little influence on hormone binding [11,12].

Studies reported here were performed during efforts to identify receptor residues that contact the ligand. They relied on the observations that the human and rat LHRs have similar amino acid sequences but different abilities to bind non-human mammalian LHs such as bovine LH (bLH) [13,14]. The rat LHR binds hCG 5–10-fold better than the human LHR and about 10–30-fold better than most non-primate mammalian LHs including rat LH. In contrast, the human LHR is more discriminating and binds hCG at least 1000-fold better than most other mammalian LHs. We assumed that residues of the human LHR that enabled it to distinguish hCG and bLH were likely to participate in hormone contacts and attempted to identify them by monitoring the ability of bLH to bind several rat/human LHR chimaeras. Here we show that substitution of a few human LHR-specific residues into the C-terminal fifth of the rat LHR extracellular domain reduced its ability to bind bLH 100-fold. This revealed that the C-terminal portion of the rat LHR extracellular domain can have a substantial inhibitory influence on hormone–receptor interaction even though it is not needed for ligand binding [11]. Earlier studies suggested that receptor-bound ligands might project into the space created by the concave surface of the receptor extracellular domain [15]. The finding that the C-terminal portion of the extracellular domain

Abbreviations used: CG, chorionic gonadotropin; hCG, human CG; LH, lutropin; bLH, bovine LH; FSH, follitropin; TSH, thyrotropin; LHR, LH receptor; FSHR, FSH receptor.

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can dramatically influence ligand binding supports the idea that this orientation of the ligand enables it to contact the C-terminal portion of the extracellular domain.

EXPERIMENTAL

Highly purified bLH was obtained from Dr. John Pierce (UCLA, Los Angeles, CA, U.S.A.). Highly purified urinary hCG (CR121 and CR127) was obtained from Dr. Robert Canfield and Dr. Steven Birken (Columbia University, New York, NY, U.S.A.). The cDNA encoding the human LHR was obtained from Dr. Aaron Hsueh (Stanford University, Palo Alto, CA, U.S.A.) and the cDNA for the rat LH receptor was obtained by PCR amplification of a rat ovarian library [16].

The amino acid sequences of the chimaeras are described in Figures 1 and 2. Most rat/human LHR chimaeras were prepared by site-directed mutagenesis using an overlap-extension strategy [17], modified as described previously [15,18]. The remainder were made by swapping homologous endonuclease restriction fragments. All PCR-derived mutants were confirmed by dideoxy-DNA sequencing.

Receptor chimaeras were expressed in COS-7 cells using methods described previously [5,19]. Because the affinity of bLH for the human LHR and most rat/human LHR analogues was low, it was not possible to use ¹²⁵I-bLH to monitor receptor binding. Therefore, we measured the interactions of hCG and bLH with the rat LHR, human LHR and receptor chimaeras by monitoring the ability of bLH to compete for binding of ¹²⁵I-hCG, a tracer that bound well to COS-7 cells, which had been transfected with each of the receptor analogues. Further, to maximize the concentration of bLH that could be obtained in each assay, we kept the volume of the incubation relatively small (0.25 ml). Under these conditions, in the absence of unlabelled inhibitor, approx. 20 % of the biologically active radiolabel became bound to the receptors. Methods for radio-iodinating hCG to a specific activity of 40–50 µCi/µg have been described

[20]. This procedure labelled only the α-subunit of the heterodimer as has been noted earlier [21]. Although not tested in these studies, we found previously that approx. 65 % of this material is biologically active, as defined by being capable of binding to an excess number of LH receptors. ¹²⁵I-hCG (approx. 0.07 µCi, 1.5 ng) was mixed with unlabelled hCG or bLH and the mixture added to intact cells in physiological salt solutions (120 mM NaCl/4.75 mM KCl/2 mM CaCl₂/1.2 mM Na₂HPO₄/1.2 mM MgSO₄) containing 20 mM Hepes buffer (pH 7.4) and 1 mg BSA per ml. The latter was added to prevent non-specific adsorption of the hormones to the surfaces of the plastic incubation tubes. Under these conditions, the ligands bind only to surface-expressed receptors. After 1 h at 37 °C, the reaction mixture was diluted by addition of 2 ml of ice-cold 150 mM NaCl solution containing 2 mg of BSA. Finally, the cells were separated from the mixture by centrifugation at 1000 *g* (10 min) at 4 °C, the supernatant was aspirated, and radiolabel bound to the cell pellet quantified in a gamma counter.

The abilities of hCG and bLH to compete with ¹²⁵I-hCG for binding to the receptors and receptor analogues were described by the relationship:

$$b = B[T]/\{[T] + K_T + K_T([I]/K_I)^c\}$$
 (1)

where *b* is the molar concentration of bound ¹²⁵I-hCG, *B* is the total molar concentration of binding sites, [*T*] (tracer) is the molar concentration of free ¹²⁵I-hCG (estimated by subtracting the bound tracer from the total amount of biologically active tracer), *K_T* is the dissociation constant of ¹²⁵I-hCG for the receptor, *K_I* is the dissociation constant of unlabelled inhibitor [i.e. hCG (*K_H*) or bLH (*K_L*)] for the receptor, [*I*] is the molar concentration of free unlabelled ligand, and *c* is a unitless slope factor. Curve fitting was performed using the commercially available package Prism (Graph Pad Software, Inc., San Diego, CA, U.S.A.). The slopes of the displacement curves obtained using bLH were approx. −0.7 regardless of the receptor or

Figure 1 Structures of the extracellular domains of the receptor chimaeras used in these studies

Chimaeras are named for the regions derived from the human LHR (black bars) or the rat FSH receptor (checked bar). Numbers at the top refer to amino acid number. IC₅₀ values were calculated with the four-parameter logit equation in Prism. As described in the text, IC₅₀ ratios shown underestimate the differences in affinities of bLH for the human and chimaeric LHR analogues relative to that of the rat LHR (rLHR). Dissociation constants were calculated based on the model as described in Experimental. To conserve the limited amounts of bLH available, binding to r/hLHR(47:123) and r/hLHR(124:201) was determined in experiments in which the highest concentration was 1.3 µM (10 µg/0.25 ml). At this level we did not observe sufficient displacement of ¹²⁵I-hCG to calculate meaningful IC₅₀ values and the values illustrated for these analogues are minimum lower limits. Only one study was performed with 13 µM bLH (100 µg/0.25 ml) and the value shown for the relative potency of bLH in human LHR assays was calculated from the experiment illustrated in Figure 5. Calculation of *K_L* requires knowledge of *K_H*, a value not determined for all analogues, and the assumption that there is a single class of binding sites. As a result, these values are likely to be only relative approximations. Asterisks indicate that two *K_L* values were calculated for these LHR chimaeras using values of *K_H* for the rat and human LHR. The lower 95% confidence limit is that obtained with *K_H* for the binding of hCG to the rat LHR and the higher 95% confidence limit is that calculated assuming *K_H* for binding to the human LHR.

$$\{(K_{\text{I,x}})(K_{\text{T,r}})(K_{\text{T,x}} + [\text{T}])\} / \{(K_{\text{I,r}})(K_{\text{T,x}})(K_{\text{T,r}} + [\text{T}])\} \quad (2)$$

When $[T]$ is much smaller than K_{Tx} or K_{Tr} , this reduces to K_{Lx}/K_{Lr} and the ratio of the IC_{50} values is proportional to the ratio of the dissociation constants. In contrast, when $[T]$ is large the ratio of the IC_{50} values is proportional to $(K_{Lx}/K_{Lr})(K_{Tr}/K_{Tx})$. Because smaller concentrations of hCG were needed to block binding of labelled hCG to the rat LHR than to the human LHR and most chimaeras, K_{Tr} is less than or equal to K_{Tx} and the ratio of the IC_{50} values underestimates the actual difference in the dissociation constants. The facts that: (i) IC_{50} values can be obtained without regard to the model of binding; and (ii) that significant differences between these values would be obtained only if there were equal or larger differences in the abilities of the receptor analogues to bind bLH, suggested that use of IC_{50} ratios would be the most conservative approach to examining our data.

RESULTS AND DISCUSSION

hCG is 10–30 fold more potent than most mammalian LHs in rat LHR binding assays [14,22,23], an observation that we confirmed using bLH (Figure 3). We also confirmed the observation that concentrations of bLH greater than $1 \mu M$ were needed to inhibit binding of ^{125}I -hCG to the human LHR [13,14]. The low affinity of bLH for the human LHR could be caused by the absence of receptor residues needed for bLH binding, the presence of residues that inhibit bLH binding, or both. Based on the idea that residues in the N-terminal three-fifths of the extracellular domain appeared sufficient for high-affinity binding [11], we focused on this portion of the receptor first. We compared the bLH and hCG binding abilities of rat/human LHR chimaeras containing human LHR residues throughout the entire region (i.e. residues 1–201) and in two subdomains (i.e. 90–123 and

124–201). As a 'negative control', we studied binding of these ligands to a chimaera with human LHR residues in the remainder of the extracellular domain (i.e. residues 202–341).

Every rat/human LHR chimaera bound ^{125}I -hCG. Because the binding assays were performed under conditions that did not disrupt the cells, this showed that the receptor analogues were expressed at the cell surface, an indication that each folded similarly to its progenitors, the rat and human LHRs. Approx. 4–6 fold more hCG was needed to inhibit binding of ^{125}I -hCG to the human LHR than to the rat LHR (Figure 3, left panel), indicating that, under physiological conditions, the affinity of the rat LHR for hCG is greater than that of the human LHR. Twice as much hCG was needed to inhibit binding of ^{125}I -hCG to chimaeras containing all or parts of human LHR residues 1–201 than to the rat LHR (Figure 3). Approx. 3–6 fold more hCG was needed to inhibit binding of ^{125}I -hCG to chimaeras containing human LHR residues 202–341 than to the rat LHR (results not shown). This suggested that residues in different parts of the extracellular domain reduced the ability of the human LHR to bind hCG relative to the rat LHR. However, because differences in the amounts of hCG needed to block binding of ^{125}I -hCG to the rat and human LHRs were small, we did not try to identify specific amino acids that contributed to this effect.

Human LHR residues 1–201, the N-terminal three-fifths of the extracellular domain, influenced the ability of the chimaeras to bind bLH (Figure 3, right panel), but by a much smaller amount than we had expected. Only 10-fold more bLH was needed to inhibit binding of ^{125}I -hCG to the chimaera in which this whole region was derived from the human LHR [i.e. residues 1–201, r/hLHR(1:201)]. (Chimaeras are named on the basis of their content of human LHR residues, the remainder of the chimaera is derived from the rat LHR.) This indicated that human LHR

Figure 3 Influence of human LHR residues 1–201 on binding to rat/human LHR chimaeras

r/hLHR(1:201) refers to a chimaera containing human LHR residues 1–201 and rat LHR residues throughout the remainder of the molecule. LHR/FSHR(202:341) refers to a chimaera in which rat LHR residues 202–341 were replaced with their rat FSHR counterparts. Values are means $\pm$ S.E.M. of triplicates. These panels illustrate data from a single experiment that was typical of three separate studies. In this experiment, the total amount of radiolabelled hCG added to each tube was 110 000 c.p.m. and the amount of ^{125}I -hCG bound measured in the absence of unlabelled hormone (maximum binding) was: 15 000 (rat LHR), 11 000 (human LHR), 10 000 (LHR/FSHR chimaera) and 9000 c.p.m. (rat/human LHR chimaera) above the blank (2000 c.p.m.). Curves were fitted using Prism.

Figure 4 Influence of human LHR residues 90–123 on bLH binding

To study the influence of human LHR amino acids between residues 90 and 123, we made substitutions that converted rat LHR amino acids Leu90 to Arg and Thr98 to Ile (L90R/T98I); Thr115 to Lys and Leu116 to Phe (T115K/L116F); and Ile122 to Val and Ser123 to Phe (I122V/S123F). The amounts of bLH needed to half-maximally inhibit binding of ^{125}I -hCG to the rat LHR and these rat LHR analogues were not statistically different.

residues in the N-terminal three-fifths of the extracellular domain contributed relatively little to the ability of the human LHR to distinguish between hCG and bLH. Multiple human LHR amino acids between residues 1 and 201 accounted for the influence of this region on bLH–receptor interactions. Thus, 6.6-fold and 2.1-fold more bLH were required to inhibit binding of ^{125}I -hCG to chimaeras containing human LHR residues between amino acids 90–123 and 124–201 than to the rat LHR (Figure 1). The small loss in bLH potency caused by the four unique human LHR residues between 90 and 123 appeared due to an interaction between these residues, and not to any single one. Consequently, the amounts of bLH needed to inhibit binding of radiolabelled hCG to rat LHR analogues containing only two human LHR residues in this region, namely L90R/T98I, T115K/L116F and I122V/S123F, were similar to those needed to inhibit binding of hCG to the rat LHR (Figure 4). Together, these observations showed clearly that human residues in the N-terminal three-fifths of the LHR extracellular domain have a role in its ability to distinguish bLH and hCG. However, these human LHR residues cannot be entirely responsible for the inability of the human LHR to interact with bLH since at least 100-fold more bLH was required to inhibit binding of ^{125}I -hCG to the human LHR than to this chimaera.

The presence of human LHR residues in the remainder of the rat LHR extracellular domain, a part of the rat LHR not needed for hCG binding [8], had a greater influence on bLH binding than residues in any other part of the receptor. As summarized in Figure 1, approx. 100-fold more bLH was required to inhibit binding of ^{125}I -hCG to the chimaera containing human LHR residues in the C-terminal end of its extracellular domain [i.e., residues 202–341, r/hLHR(202:341)] than to the rat LHR. This

Figure 5 Influence of human LHR C-terminal residues on binding of bLH to rat/human LHR chimaeras

Several portions of the human LHR extracellular domain C-terminal arm influence binding of bLH to rat/human LHR chimaeras. These data were taken from a single experiment representative of at least three studies in which we monitored the ability of bLH to compete with ^{125}I -hCG for binding to cells expressing rat LHR, human LHR and rat/human LHR chimaeras. Values are means $\pm$ S.E.M. of triplicates. The total amount of radiolabelled hCG added to each tube was 120 000 c.p.m. and the maximum radiolabel bound for each receptor analogue varied between 13 000 and 15 000 c.p.m. over the blank (approx. 1000 c.p.m.). The lines were calculated by fitting the data to the competitive-binding equation using Prism as described in the text. The slope factor for each curve except that of the human LHR was determined by first fitting the lines with a four-parameter logistic curve. The value for the curve describing the interaction of bLH with human LHR was assumed to be -0.7 .

was 10-fold more bLH than needed to inhibit binding of ^{125}I -hCG to the N-terminal three-fifths of the extracellular domain [i.e., human LHR residues 1–201, r/hLHR(1:201)]. Nonetheless, human LHR residues in both regions were required for the entire difference in the abilities of the rat LHR and human LHR to bind bLH. This was confirmed by the observation that analogues having human LHR residues 48–341 and 124–341 were less active than those containing human LHR residues 201–341 or 1–201 (Figure 1).

To localize residues in the C-terminal portion of the extracellular domain that interfered with bLH activity, we compared the ability of bLH to prevent binding of ^{125}I -hCG to chimaeras containing human LHR residues 202–341, 265–341, 289–341 and 310–341 (Figures 1 and 5). There was no difference in the amount of bLH needed to inhibit binding of ^{125}I -hCG to chimaeras containing human LHR residues 202–341 and 265–341 [i.e., r/hLHR(202:341) and r/hLHR(265:341)]. Thus, none of the human LHR-specific amino acids between residues 202 and 264 (Figure 2) appeared to contribute to the low potency of bLH in human LHR assays. Instead, human-specific LHR residues that interfered with bLH binding were distributed throughout the remaining fifth of the extracellular domain (i.e. residues 265–341). This was seen by comparing the relative amounts of bLH needed to inhibit binding of ^{125}I -hCG to the rat LHR and rat/human

LHR chimaeras containing human LHR residues 265–341, 289–341 and 310–341 [i.e., h/rLHR(265:341), h/rLHR(289:341) and h/rLHR(310:341)]. Compared with the sequences of receptors that bind bLH (i.e. rat, bovine and porcine LHR, Figure 2), chimaera r/hLHR(310:341) contains only three unique human LHR residues (Glu310, Pro319 and Arg320). However, 6-fold more bLH was required to inhibit binding of ^{125}I -hCG to this chimaera than to the rat LHR (Figures 1 and 5). Since residue 310 is negatively charged in both receptors, Pro319 and Arg320 would be expected to make the largest contribution to the ability of this human LHR region to distinguish bLH. Chimera r/hLHR(289:341) contains only five additional unique human LHR residues (Val289, Lys292, Ser297, Met298 and Leu299), yet 5-fold more bLH was required to inhibit binding of ^{125}I -hCG to this chimaera than to the h/rLHR(310:341) chimaera (Figures 1 and 5). Chimera r/hLHR(265:341) contains only two additional unique human LHR residues, namely His272 and Ser275, yet 4-fold more bLH was needed to inhibit binding of ^{125}I -hCG to this chimaera than to r/hLHR(289:341) (Figures 1 and 5). These observations suggested each of these regions made a similar small contribution to the specificity of the human LHR.

Key contacts in antigen–antibody [24] or ligand–receptor complexes [25–29] have been identified by a combination of mutagenesis and crystallography. Unlike the relatively small (i.e. 4–6-fold) effects of replacing rat LHR residues with their human LHR counterparts, substitution of key contact residues usually alters protein–protein interaction at least 10-fold and often 100-fold or more. The small differences in binding of the type that we observed were nearly always associated with mutations of residues other than those needed for high affinity interactions. Since no single human LHR residue had this much influence on bLH binding, we concluded that additional homologue-scanning mutagenesis would not enable us to distinguish human LHR residues that participated in ligand contacts from those that influenced receptor conformation.

The ability of hCG to bind truncated LHR showed that residues in the C-terminal region of the extracellular domain are not required for receptor binding [11]. Nonetheless, it is conceivable this region might contain residues essential for bLH binding to the rat LHR that are missing in the human LHR. Truncated receptors are not expressed on the plasma membrane and it could be argued that their interactions with ligands differ substantially from those receptors and receptor analogues that are expressed at the cell surface. We have found that the chimaera in which rat LHR residues 202–341 are replaced with their counterparts from the rat FSHR, a molecule that does not bind LHs, is expressed on the cell surface and binds hCG with high affinity [5]. Because the sequences of the rat LHR and FSHR are so different in the C-terminal two-fifths of their extracellular domains (Figure 2), it seemed unlikely that this chimaera would contain residues needed for ligand contacts in this region. We observed that only 10-fold more bLH was sufficient to inhibit binding of ^{125}I -hCG to this chimaera [LHR/FSHR(202:348)] than to the rat LHR (Figures 1 and 3). This implied that rat LHR-specific residues in this region make only a minor positive contribution, if any, to binding of bLH. The finding that this chimaera has much greater ability to recognize bLH than a chimaera that contains only 10 unique human LHR residues in this region [r/hLHR(265:341)] strongly suggests that the human residues in this region of rat/human LHR chimaeras influence specificity by suppressing ligand binding.

These observations showed that the N-terminal three-fifths and C-terminal fifth of the extracellular domain influence ligand binding independently. Residues in the N-terminal three-fifths of

the extracellular domain contribute contacts essential for high-affinity binding and ligand specificity. Residues in the C-terminal portion of the extracellular domain participate in contacts that appear primarily to inhibit ligand binding, thereby exerting an influence on binding specificity.

During peer review, it was suggested that some of these observations might be due to heterogeneity in the tracer preparations that were employed, particularly since hCG and bLH inhibited the binding of radio-iodinated hCG to the rat LH receptor and receptor analogues with significantly different slopes (compare Figure 3, left and right panels). The preparations of hCG used to prepare the tracer were highly purified, but because hCG is a glycoprotein, it is almost certain that they contain some heterogeneity. Nonetheless, this does not explain the differences we observed in the ability of bLH to block binding of radio-labelled hCG to the rat LH receptor and the chimaeras. Thus, bLH blocked binding of the tracer to the rat LH receptor and the chimaeras with the same low slope (i.e. -0.7), regardless of their compositions. Further, an excess of either hormone inhibited binding of the tracer to the same low ‘blank’. A more likely explanation for this phenomenon is that it stems from receptor heterogeneity, as had been concluded from studies comparing the binding of hCG and ovine LH to rat testes homogenates [22]. Our data show that the composition of the receptor had a much greater influence on the binding of bLH than that of hCG (Figure 1). Thus, it might be expected that subtle variations in receptor structure caused by differences in its processing during synthesis and/or turnover would be more readily distinguished by bLH than hCG and explain the lower slope of the bLH dose response curves. The finding that the slope of the displacement curves was similar for all the chimaeric analogues studied suggests that both the rat and human receptors may be processed in the same fashion.

The influence of the C-terminal portion of the extracellular domain on ligand binding supports a model of hormone–receptor interaction based on the crystal structure of RNase inhibitor [30,31], the crystal structure of hCG [2,3], biochemical data summarized earlier [15] and the activity of homodimeric hCG analogues [7]. The model suggests that the ligand-binding domain of the LHR is horseshoe-shaped. A portion of this domain near the centre of its N-terminal half makes the primary high-affinity contact with a groove in the hormone created by the proximity of α -subunit loop 2 and β -subunit loops 1 and 3. The remainder of the hormone projects into the cavity created by the concave surface of the extracellular domain and occupies the space near its C-terminal side. The apparent proximity of C-terminal residues of the receptor extracellular domain and the hormone increases the probability that contacts between this portion of some receptors (i.e. human LHR) and ligands (i.e. bLH) can severely limit binding.

This notion readily accounts for differences in the abilities of hCG/bLH chimaeras to interact with the human LHR. During efforts to identify bLH residues that contact the human LHR, we observed that the heterodimer made by combining bLH α -subunit and hCG β -subunit bound human LHR nearly as well as hCG [32]. We also found that the hCG analogue (hCG β R95L) in which β -subunit seat-belt residue Arg95 was replaced by leucine, its bLH counterpart, bound human LHR like hCG [32]. These results showed that none of the residues in the bovine α -subunit disrupted human LHR binding and that the presence of Arg95 was not essential for high-affinity human LHR interactions. However, the presence of the bLH α -subunit and the hCG β R95L mutant β -subunit in the same molecule nearly abolished binding to human LHR [32]. Thus, the ability of this heterodimer to bind human LHR was reduced by subunit

interactions that distorted the conformation of this heterodimer relative to hCG. A similar phenomenon may explain the inability of bLH to bind human LHR.

The influence of LHR residues in the C-terminal fifth of the extracellular domain on ligand binding has important implications for strategies used to identify residues needed for high-affinity hormone–receptor interactions. Most current efforts to decipher hormone–receptor contacts have involved measurement of the effects of hormone and receptor mutations on the affinity of the hormone for the receptor. Any receptor mutation that increased or decreased the interaction of the C-terminal portion of the receptor extracellular domain with the ligand could disrupt or enhance binding, even though it is in a region of the protein outside the high-affinity hormone binding site. Likewise, any mutation in the ligand that increased or decreased its interaction with the C-terminal portion of the receptor extracellular domain might reduce or enhance receptor binding, even if the mutation does not disrupt the structure of the high-affinity contact site. Small changes in the structure of the hormone or receptor caused by binding of anti-hormone or anti-receptor antibodies could also change steric contacts between the ligand and receptor that would be expected to alter ligand–receptor binding. We anticipate that alternative approaches to the types of site-directed mutagenesis widely used will be required to identify receptor residues involved in high-affinity ligand–receptor interactions. At the very least, identifying hormone receptor contacts needed for high-affinity binding by mutagenesis may require the use of truncated receptor analogues that lack the potential inhibitory influence of the C-terminus, something beyond the scope of the current studies.

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