Modulation of High Affinity Hormone Binding

HUMAN CHORIOGONADOTROPIN BINDING TO THE EXODOMAIN OF THE RECEPTOR IS INFLUENCED BY EXLOOP 2 OF THE RECEPTOR*

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The luteinizing/hormone receptors are seven-

The lutropin/choriogonadotropin receptor is a seven-transmembrane receptor and consists of two major domains of similar size, an extracellular exodomain and a membrane-associated endodomain which includes 3 exoloops. The uniquely large exodomain is responsible for high affinity hormone binding whereas receptor activation occurs at the endodomain. However, little is known about the relationship between the exodomain and endodomain. It was reported that hormone binding to the exodomain was improved when the endodomain was truncated. This result suggests that hormone binding to the endodomain was influenced by the endodomain. To test this hypothesis, amino acids of exoloop 2 were examined by Ala substitutions. The binding affinity was enhanced by some Ala substitutions but attenuated by others. These results indicate that exoloop 2 influences the hormone binding to the exodomain. Particularly, the high affinity hormone binding at the exodomain is constrained by a group of amino acids, Ser484, Asn585, Lys588, Ser490, and Ser499. Computer modeling suggests these residues may be positioned on one side of exoloop 2. It also influences the affinity for cAMP induction and the maximal cAMP production in distinct ways, in addition to its influence on the hormone binding affinity. The distinct ways of influencing these functions are sometimes in conflict and compromised to attain the maximal affinity for cAMP induction. As a result, the exodomain attains the maximal affinity for hormone binding when the endodomain is truncated and cAMP induction is disengaged.

EXPERIMENTAL PROCEDURES
Mutagenesis and Functional Expression of LH/CG Receptors—Mutant LH/CG-R cDNAs were prepared in pSELECT vector using the Altered Sites Mutagenesis System (Promega), sequenced, subcloned into pcDNA3 (Invitrogen) as described (8), and sequenced again to verify mutagenesis. In one of mutants, a stop codon was introduced immediately after the Gly477 codon to produce the truncated exodomain. Arg-Gly-Gly-Mutant LH/CG receptor constructs were transfected into human embryonic kidney 293 cells by the calcium phosphate method. Stable cell lines were established in minimum essential medium containing 10% horse serum and 500 μg/ml Geneticin (G-418).

125I-hCG Binding and Intracellular cAMP Assay—Stably transfected cells were assayed for 125I-hCG binding in the presence of 150,000 cpm of 125I-hCG (9) and increasing concentrations of cold hCG. hCG, batch CR 127, was supplied by the National Hormone and Pituitary Program.

Nontransfected cells did not show specific binding of hCG. For intracellular cAMP assay, ~50,000 cells were washed twice with Dulbecco’s modified Eagle’s media and incubated in the media containing isobutylmethylxanthine (0.1 μg/ml) for 15 min. Increasing concentrations of hCG were then added and the incubation was continued for 45 min at 37°C. After removing the media, the cells were rinsed once with fresh media without isobutylmethylxanthine, lysed in 70% ethanol, freeze-thawed in liquid nitrogen, and scraped. After pelleting cell debris at 16,000 × g for 10 min at 4°C, the supernatant was collected, dried under vacuum, and resuspended in 10 μl of the cAMP assay buffer which was provided by the manufacturer (Amersham). cAMP concentrations were determined with an 125I-cAMP assay kit (Amersham) following the manufacturer’s instructions and validated for use in our laboratory.

All assays were carried out in duplicate and repeated 4–6 times. Means and standard deviations were calculated and analyzed by Student’s t test to determine the statistical significance (p) of the differences.

125I-hCG Binding to Solubilized LH/CG Receptor—Transfected cells were washed twice with ice-cold 150 mM NaCl, 20 mM HEPES, pH 7.4 (buffer A). Cells were scraped on ice and collected in buffer A containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 10 mM EDTA) and pelleted by centrifugation at 1,300 × g for 10 min. Cells from a 10-cm plate were resuspended in 0.6 ml of buffer A containing 1% Nonidet P-40, 20% glycerol, and the above protease inhibitors (buffer B), incubated on ice for 15 min, and diluted with 5.4 ml of buffer A containing 20% glycerol plus the protease inhibitors (buffer C). The mixture was centrifuged at 100,000 × g for 60 min. The supernatant (500 μl) was mixed with 150,000 cpm of 125I-hCG...
FIG. 1. hCG binding to solubilized LH/CG receptor and the exodomain of the receptor. The full-length LH/CG receptor and the exodomain were separately expressed in 293 cells, solubilized in Nonidet P-40, and used for $^{125}$I-hCG binding in the presence of increasing concentrations of nonradioactive hCG (A) and Scatchard analysis (B) was plotted against specific binding. Experiments were repeated 4–6 times in duplicate, and mean and S.D. were calculated as presented in the table section of the figure. In addition, the statistical significance of the each mutant data was analyzed twice for different purposes. First to determine the statistical significance ($p$ values) of repeat data for each, mutants were analyzed by Student’s $t$ test. In addition, the values for the exodomain were compared with the corresponding values of the wild type receptor using ANOVA with 95% confidence. The result indicates that the $K_d$ values of intracellular receptor are higher than those of extracellular receptors. Therefore, the binding affinity of intracellular receptors is worse than that of the corresponding extracellular receptors. Furthermore, it indicates that the extracellular or intracellular location of the exodomain alone is not responsible for the different $K_d$ values.

Alternatively, the different $K_d$ values of the truncated intracellular exodomain and the extracellular exodomain of the wild type receptor may come from the interaction with the endodomain. For example, the exodomain of the wild type receptor may interact with the endodomain which in turn influences the hormone binding affinity whereas the truncated intracellular exodomain does not have any exodomain to interact with. Such an influence by the endodomain on the exodomain is likely to occur at the extracellular portion of the endodomain, including the three exoloops. To determine such a region(s) and amino acid residues, the 20 amino acids of exoloop 2 were individually substituted with Ala to produce 20 substitution mutants. They are Ser$^{484}$, Asn$^{485}$, Tyr$^{486}$, Met$^{487}$, Lys$^{488}$, Ser$^{489}$, Ile$^{490}$, Cys$^{492}$, Leu$^{493}$, Pro$^{494}$, Met$^{495}$, Asp$^{496}$, Val$^{497}$, Gly$^{498}$, Ser$^{499}$, Thr$^{500}$, Leu$^{501}$, Ser$^{502}$, and Gly$^{503}$. The resulting mutants are LH/CG-R$^{S484A}$, LH/CG-R$^{N485A}$, LH/CG-R$^{Y486A}$, LH/CG-R$^{M487A}$, LH/CG-R$^{K488A}$, LH/CG-R$^{N485A}$, LH/CG-R$^{L489A}$, LH/CG-R$^{N489A}$, LH/CG-R$^{I491A}$, LH/CG-R$^{I491A}$, LH/CG-R$^{S484A}$, LH/CG-R$^{S484A}$, LH/CG-R$^{R485A}$, LH/CG-R$^{R485A}$, LH/CG-R$^{R485A}$, LH/CG-R$^{R485A}$, LH/CG-R$^{R485A}$, and LH/CG-R$^{R485A}$. (Figs. 2 and 3).

For the convenience of data presentation and analysis, exoloop 2 amino acids are divided into two groups, 11 upstream (Fig. 2) and 9 downstream amino acids (Fig. 3).

Effects of Ala Substitution for Upstream Amino Acids of Exoloop 2—Among 11 mutant receptors with Ala substitution for the upstream amino acids, 6 were surface-expressed in reasonable receptor concentrations, 12,100–64,800 receptors/cell (Fig. 2). $K_d$ values of these surface-expressed receptors were diverse in the range of 250 to 840 pM. LH/CG-R$^{445A}$, LH/CG-R$^{446A}$, and LH/CG-R$^{449A}$, displayed lower $K_d$ values, indicating that their hormone binding affinities are better than that of the wild type receptor. On the other hand, the $K_d$ value of LH/CG-R$^{449A}$ was 2-fold higher, an indication for a low binding affinity. In contrast to these surface-expressed mutants, hCG binding to the cells transfected with the other 5 mutants was marginal or not detected. For example, hCG bound to LH/CG-R$^{443A}$ on intact cells with a normal affinity but the receptor concentration was <10% of the concentration of the wild type receptor $^{125}$I-hCG binding to intact cells was hardly detectable for LH/CG-R$^{443A}$, LH/CG-R$^{446A}$, LH/CG-R$^{449A}$, and LH/CG-R$^{449A}$.

To determine whether any of these mutant receptors were trapped inside the cells or defective in hCG binding, stably transfected cells were solubilized in Nonidet P-40 and assayed for hormone binding. As shown in Fig. 2, C and D, all five mutants in the detergent solution bound hCG ($p^1 < 0.05$) and the receptor concentrations were not significantly different from the concentrations of the wild type receptor ($p^1 < 0.05$).
These results indicate that the five mutant receptors were expressed but not efficiently transported to the cell surface. The \( K_d \) value of LH/CG-RS484A was 2-fold lower than that of the wild type receptor, an indication of a 2-fold improved affinity for hCG binding. In contrast to the improved binding affinity, LH/CG-RP494A showed a 2-fold lower affinity as the \( K_d \) value was 2-fold higher. The other three mutants in solution showed slightly higher \( K_d \) values. These results indicate that the Ala substitutions somewhat impacted the hormone binding affinity of the mutant receptors, in addition to impairing their expression on the cell surface.

In contrast to the diverse \( K_d \) values, EC\(_{50}\) values for cAMP induction by most of surface-expressed mutant receptors except LH/CG-RV489A are 3–5-fold higher than the EC\(_{50}\) value of the wild type receptor (Fig. 2E). This is a significant reduction in the affinity for cAMP induction and indicates that these Ala substitutions impaired, but did not improve, the affinity for cAMP induction. In contrast to this normal binding with poor cAMP induction, LH/CG-RV489A bound hCG with a 2-fold lower affinity yet induced cAMP normally (\( p < 0.001 \)).

**Ala Substitution for Downstream Amino Acids of Exoloop 2—** All 9 mutant receptors with Ala substitution for the downstream amino acids were surface-expressed and the receptor concentrations were reasonable, being in the range of 12,200–37,000/cell (Fig. 3). Their affinities for hormone binding were generally similar to that of the wild type receptor. However, the \( K_d \) value of LH/CG-RS499A was lower (280 pM) than that of the wild type receptor. This result indicates that the S499A substitution improved the binding affinity as did the S484A, K488A, and S490A substitutions. In contrast to the improved binding affinities, the \( K_d \) values of LH/CG-RM495A and LH/CG-RS502A were higher, 840 and 630 pM, respectively. All of the mutants were capable of inducing cAMP and the EC\(_{50}\) values for cAMP induction were either similar to or slightly higher, up to 3-fold, than that of the wild type receptor. The maximum cAMP induction levels were similar to or lower than that produced by the wild type receptor.

**Effect of Surface Receptor Concentrations—** Surface concen-

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**TABLE I**

Comparison of receptors present on the plasma membrane and those trapped in the cell

| Receptor | Binding to intact cells | Binding in Nonidet P-40 solution |
|----------|-------------------------|---------------------------------|
| Receptor | \( K_d \) (pM) | Receptor | \( K_d \) (pM) |
| P562F    | 775 ± 80 c | 1,307 ± 64 |
| P463F    | 563 ± 70 a | 1,192 ± 41 |
| P591F    | 648 ± 25 b | 1,498 ± 92 |

**Exoloop 2 of LH/CG Receptor**

Several LH/CG receptors with Pro to Phe substitution. P463F, P562F, and P591F, show varying levels of surface and intracellular expression (11). The extracellular concentration of receptors present on the plasma membrane was determined from the receptors which \(^{125}\)I-hCG bound to intact cells. The total receptor concentration was determined with \(^{125}\)I-hCG binding to receptors present in Nonidet P-40 solution of whole cells. The intracellular concentration of receptors present in cells was estimated by substracting the extracellular receptor concentration from the total receptor concentration. R stands for receptor.

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|----------|----------------|----------|----------------|
| P562F    | 775 ± 80 c | 1,307 ± 64 |
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| Receptor | Binding On Cells | Binding In Solution | cAMP Synthesis |
|----------|------------------|---------------------|---------------|
| Receptor | \( K_d \) (pM) | Receptor | \( K_d \) (pM) | EC\(_{50}\) (pM) | Max (nmol/10^6 Cells) |
| P562F    | 775 ± 80 c | 1,307 ± 64 |
| P463F    | 563 ± 70 a | 1,192 ± 41 |
| P591F    | 648 ± 25 b | 1,498 ± 92 |

**Fig. 2. Ala scan of upstream residues of exoloop 2.** The upstream 11 amino acids of exoloop 2, from Ser\(^{464}\) to Pro\(^{494}\), were individually substituted with Ala and the resulting mutant receptors were stably expressed on human 293 cells. The cells were assayed for hormone binding and hCG-dependent CAMP induction. After experiments were repeated 4–6 times in duplicate, the statistical significance of the each mutant data was analyzed. The \( p^1 \) values (the statistical significance of repeats) are presented as: \( a \) for \( p^1 < 0.001 \); \( b \) for \( p^1 < 0.01 \); and \( c \) for \( p^1 < 0.05 \). ND indicates not detectable.

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trations of mutant receptors shown in Figs. 2 and 3 were diverse. To determine whether levels of surface receptor concentrations had any effect on hormone binding, cells were transiently transfected with varying concentrations of receptor plasmids and selected for those expressing 40,000 receptors/cell (Table II). Cells were assayed for hormone binding to intact cells. The $K_d$ values (Table II) for the wild type receptor LH/CG-R N485A and LH/CG-R M487A, LH/CG-R K489A, and LH/CG-R D496A were similar to the $K_d$ values of the same receptors which were expressed at diverse levels (Figs. 2 and 3). These results show that the surface receptor concentration did not impact the hormone binding affinity. To test whether receptor transport to the surface membrane might have had any effect on the hormone binding affinity, cells expressing the receptors were solubilized and assayed for hormone binding to solubilized receptors (Table II). The results show that the $\% K_d$ values of receptors were similar to those of receptors expressed on the cell surface, an indication for no significant effect of receptor transport on hormone binding.

Double Ala Substitutions for Nearby Asp and Glu—Ionic amino acids of the LH/CG receptor have been implicated for important roles (8, 12–14). However, the D496A substitution and E498A substitution individually had marginal effects on surface expression, hCG binding, and cAMP induction. Therefore, both Asp496 and Glu498 were replaced with Ala to produce a double substitution mutant, LH/CG-R D496A/E498A. These double substitutions substantially attenuated the surface expression of the mutant and reduced the affinity for cAMP induction. Remarkably, the hormone binding affinity improved by more than 2-fold, underscoring the importance of their potential role to attenuate the high affinity hormone binding of the natural receptor. It is not clear whether these two nearby anionic residues play an important and mutually substitutable role in the hormone binding affinity.

Verification of Mutagenesis—Our site-directed mutagenesis requires a synthetic oligonucleotides with a mutant sequence and furthermore, does not involve polymerase chain reaction. After mutagenesis, the mutant and flanking sequences are verified by sequencing. In addition, the same sequence is confirmed once more after a mutant cDNA is subcloned into the expression vector. Therefore, it is highly unlikely that a mutant cDNA might have undergone an unintended mutation(s) during the mutagenesis and subcloning. To confirm this, mutant cDNAs were reverted to the wild type cDNA which was in turn used to transfect cells. All of the revertants behaved the same as the wild type receptor in surface expression, hormone binding, and cAMP induction (data not included), indicating that there were no mutations other than the intended Ala substitutions.

**TABLE II**

| Binding On Cells | cAMP Synthesis |
|------------------|----------------|
| Kd (pM)          | Receptor/Cell  | EC$_{50}$ (pM) | Max (fmol/10$^5$ Cells) |
| Wild type        | 70 ± 12 b      | 45,100 ± 6,200 | 860 ± 90                 |
| N485A            | 152 ± 54 c     | 51 ± 7 b       |                           |
| M487A            | 125 ± 44 c     | 79 ± 9 a       |                           |
| K489A            | 204 ± 75 b     | 55 ± 6 a       |                           |
| D496A            | 166 ± 53 a     | 70 ± 10 a      |                           |
| E498A            | 220 ± 90 a     | 83 ± 13 a      |                           |
| S484A            | 81 ± 16 a      | 73 ± 11 a      |                           |
| S490A            | 77 ± 29 b      | 70 ± 17 a      |                           |
| S499A            | 135 ± 62 c     | 60 ± 5 a       |                           |
| D496A/E498A      | 191 ± 78 b     | 63 ± 9 a       |                           |
|                  | 200 ± 53 b     | 51 ± 3 a       |                           |

**DISCUSSION**

Influence of Exoloop 2 on Hormone Binding—Some Ala substitutions for exoloop 2 amino acids impacted the hormone binding affinity, noticeably enhancing or reducing the binding affinity whereas others did not have a significant effect (Fig. 4). To facilitate the comparison of these diverse effects, percent $K_d$ values for mutants were calculated by dividing their $K_d$ values with that of the wild type receptor (Table III and Fig. 4). Most noticeable were the S484A, K488A, S490A, S499A, and D496a/E498A substitutions. They improved the binding affinity up to 2-fold and these improvements were statistically significant ($p^2$...
<0.05 to $p^2<0.001$) according to ANOVA analysis. This is specific since the same Ala substitutions mostly attenuated, but never improved, the affinity for cAMP induction and other substitutions did not improve the binding affinity. It is interesting and could be significant that the extent of the improvement in the binding affinity of some of the mutant receptors is in the range of the enhanced binding affinity of the truncated exodomain as compared with the binding affinity of the extra-cellular exodomain of the wild type receptor. This correlation of the improvement and the extent of the improvement in the binding affinity after truncation of the endodomain and some Ala substitutions suggest that the high affinity hormone binding to the exodomain is influenced and furthermore, attenuated by the endodomain, including exoloop 2, of the wild type receptor. Exoloop 2 may interact with the exodomain or the exodomain-hormone complex to modulate the structure. If so, the exodomain may assume a structure more favorable for hormone binding in some mutants, including LH/CG-RS484A and LH/CG-RS484A/E498A. This is consistent with the recent suggestion that exoloop 2 contacts the hormone (15).

Structure of Exoloop 2—It is interesting to see that all of the three Ser to Ala substitutions in exoloop 2, S484A, S490A, and S499A, improved the binding affinity. It is not clear whether there is a correlation between the three Ser to Ala substitutions and the improved binding affinities and whether there is a structural relationship of the three residues. If there are, the three Ser residues and possibly some other residues including Lys488 (Fig. 4) might be arranged in the exterior of a structure. These residues may mark one side of an $\alpha$ helix or $\beta$-sheet with periodicities of 3.4 and 3.0, respectively. Similarly, S484A, K488A, and S490A substitutions resulted in lower than wild-type $K_d$ values and the residues might mark one side of a $\beta$-pleated sheet. However, in this case, there is an anomalous increased $K_d$ by the Y486A substitution and a decrease by the N485A substitution. With these observations, the structure of

![Table III](https://example.com/table3.png)

**Table III**

| Wild type | %Kd | %EC50 | %Max cAMP | %EC50 | %Max cAMP |
|-----------|-----|-------|-----------|-------|-----------|
| 100       | 100 | 100   | 1.0       | 1.0   |
| S484A     | 204 |
| N485A     | 128 | 25    | 0.20      | 2.72  |
| V489A     | 44  | 103    | 2.34      | 1.25  |
| K492A     | 74  |
| S490A     | 132 | 30    | 0.22      | 2.93  |
| I491A     | 82/84 | 19 | 0.23      | 2.53  |
| T500A     | 93  | 86    | 0.92      | 1.30  |
| E498A     | 82  | 42    | 0.51      | 2.57  |
| S499A     | 132 | 32    | 0.24      | 2.56  |
| V497A     | 82  | 34    | 0.41      | 2.50  |
| M495A     | 44  | 46    | 1.05      | 1.70  |
| K498A     | 82  | 42    | 0.51      | 2.57  |
| S499A     | 132 | 32    | 0.24      | 2.56  |
| N493A     | 100 | 24    | 0.24      | 4.67  |
| P494A     | 51  |
| M495A     | 44  | 46    | 1.05      | 1.70  |
| E498A     | 82  | 42    | 0.51      | 2.57  |
| S499A     | 132 | 32    | 0.24      | 2.56  |
| V497A     | 82  | 34    | 0.41      | 2.50  |
| M495A     | 44  | 46    | 1.05      | 1.70  |
| K498A     | 82  | 42    | 0.51      | 2.57  |
| S499A     | 132 | 32    | 0.24      | 2.56  |
| V497A     | 82  | 34    | 0.41      | 2.50  |
| M495A     | 44  | 46    | 1.05      | 1.70  |
| K498A     | 82  | 42    | 0.51      | 2.57  |
| S499A     | 132 | 32    | 0.24      | 2.56  |
| V497A     | 82  | 34    | 0.41      | 2.50  |
| M495A     | 44  | 46    | 1.05      | 1.70  |
| K498A     | 82  | 42    | 0.51      | 2.57  |
| S499A     | 132 | 32    | 0.24      | 2.56  |
exoloop 2 has been modeled to give a β-strand over the first seven residues of the loop followed by a short coil region and two turns of 310 helix. The loop has been constructed so that residues Ser484, Asn485, Lys488, Ser490, Asp496, and Ser499 have side chains close to each other. Therefore, all may have similar effects on the interactions of the loop with the local protein environment, but the exact nature of these interactions cannot be predicted with the available data. This simple model ignores the anomalies at Asn485 and Tyr486.

Another method was developed for modeling nucleic acid loop structures (18) and incorporated the AMBER energy minimization (19). Crystal structure of the seven-helix bundle from bacteriorhodopsin (16) was used for anchoring the loop. Using a reduced-coordinates approach, random loop structures with the targeted pair of fixed ends were generated. Those loop structures with energies below a threshold were selected and subjected to a short run of energy equilibration using Metropolis Monte Carlo simulation (18) and then, 1,000 cycles of energy minimization using AMBER (19). It produced a structure with Ser484, Asn485, Lys488, Ser490, and Ser499 on one side of the loop surface (the lower models in Fig. 5). Therefore, it is possible that Ser484, Asn485, Lys488, Ser490, and Ser499 are coordinated to position themselves in a specific loop structure and to influence the hormone binding affinity. In contrast to the improved binding affinities of some of the substitutions, the affinity for cAMP induction is never augmented by the substitutions. Therefore, the hormone binding affinity and the maximal level of cAMP production seem to be compromised. Ala substitutions both positively and negatively impact the hormone binding affinity and the maximal level of cAMP. However, the affinity for cAMP induction is never augmented by the substitutions. For example, the hormone binding affinity for the exodomain reaches the maximum only when the endodomain is removed and cAMP induction is completely disengaged from the hormone binding.

Disulfide Bridge between Exoloops 1 and 2—The C492A substitution resulted in the loss of surface expression but not the high affinity hormone binding as found in other substitutions for Cys (20). It indicates the importance of Cys492 in surface expression but not in hormone binding. Cys residues corre-
sponding to Cys\textsuperscript{492} of exoloop 2 and Cys\textsuperscript{416} of exoloop 1 of the LH/CG-R have been implicated to form a disulfide bridge in the other seven TM receptors (21). If this is true for the LH/CG receptor, the disulfide may not be essential for hormone binding. Another possibility is that Cys\textsuperscript{492} of exoloop 2 and Cys\textsuperscript{416} of exoloop 1 may not form a disulfide or may form disulfides with other Cys residues of the exodomain. It will be of interest to see whether the putative disulfide plays a role in surface expression of the LH/CG receptor, as do disulfides of hCG in hCG processing (22).

Effects of Ala Substitutions on the Surface Expression—Some of the substitutions attenuated or blocked surface expression of the resulting mutants. As a result, most of LH/CG-R\textsubscript{S484A}, LH/CG-R\textsubscript{Y486A}, LH/CG-R\textsubscript{I491A}, LH/CG-R\textsubscript{C492A}, LH/CG-R\textsubscript{P494A}, and LH/CG-R\textsubscript{D496A/LH/CG-RE498A} were trapped in the cells. Therefore, these amino acids are crucial for surface expression of the receptor.

Conclusion—Our data demonstrate that exoloop 2 of the LH/CG-R distinctly influences the hormone binding affinity, the affinity for cAMP induction, and the maximal cAMP production, probably by the interaction of exoloop 2 with the exodomain and other parts of the endodomain such as TM 6 and TM 7. These distinct ways of influencing the functions are sometimes in conflict and compromises to attain the maximal affinity for cAMP induction. The high affinity hormone binding at the exodomain is constrained by some of the exoloop 2 residues, particularly, Ser\textsuperscript{484}, Asn\textsuperscript{485}, Lys\textsuperscript{488}, Ser\textsuperscript{490}, and Ser\textsuperscript{499}. As a result, the exodomain attains the maximal affinity for hormone binding when the endodomain is truncated and cAMP induction is disengaged.

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