Disruption of Potential Sites for N-Linked Glycosylation Does Not Impair Hormone Binding to the Lutropin/Choriogonadotropin Receptor if Asn-173 Is Left Intact*

Xuebo Liu†, David Davis†, and Deborah L. Segaloff†

From the Department of Physiology and Biophysics, The University of Iowa College of Medicine, Iowa City, Iowa 52242

The rat lutropin/choriogonadotropin receptor (rLHR) is a G protein-coupled receptor, the large extracellular domain of which binds human choriogonadotropin (hCG) with high affinity. Within the extracellular domain are six potential sites for N-linked glycosylation. Although several studies have attempted to determine if N-linked carbohydrates are necessary for hormone binding, the results have been in apparent disagreement. In this study we have used site-directed mutagenesis to singly and collectively alter the consensus sequences for N-linked glycosylation in the rLHR. In particular, we examined the binding activity in both intact cells as well as detergent-solubilized extracts so that the effects on trafficking to the plasma membrane could be determined. In addition, we independently examined the effects of substituting a particular Asn versus Thr or Ser residue within a given glycosylation consensus sequence.

Our data suggest that substitution of Asn-173 with Gln results in both a decreased ability of the receptor to be expressed on the plasma membrane as well as a vastly decreased binding affinity of the receptor for hCG. However, if the consensus sequence for N-linked glycosylation at Asn-173 is altered by substitution of Thr-175 with Ala (instead of Asn-173 to Gln), the resulting receptor binds hCG with high affinity although it is still impaired in its ability to be expressed at the plasma membrane. Furthermore, if all consensus sequences for N-linked glycosylation are mutated collectively while maintaining Asn-173 (by substituting Thr-175 with Ala instead of Asn-173 to Gln), the resulting deglycosylated receptor, although not expressed on the plasma membrane, binds hCG with high affinity. These results resolve the apparent discrepancies in the literature on the role of N-linked carbohydrates of the rLHR on the binding of hormone by showing that they are not required for high affinity binding but that Asn-173 is involved, either directly or indirectly, in the high affinity binding of hormone.

The rLHR is a member of a large family of G protein-coupled receptors that span the plasma membrane seven times. However, unlike the β-adrenergic and rhodopsin receptors, which typify this class of receptors, the rLHR has a large (341 amino acids) extracellular amino-terminal domain (1). In contrast to other G protein-coupled receptors such as the adrenergic receptors, which bind ligands through interactions with amino acids within the transmembrane helices (2–5), the large extracellular domain of the LH/CG receptor is both necessary and sufficient for binding hCG with high affinity (6–9). The extracellular domain of the rLHR, as well as the closely related follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) receptors, has been found to be composed of repetitive leucine-rich repeats (1, 10–12) and to contain several potential sites for N-linked glycosylation (1).

Although it is known that the rLHR is glycosylated (13, 14), it is not yet known which of the six potential sites for N-linked carbohydrate attachment are in fact glycosylated. Furthermore, it is not known what role(s) these N-linked carbohydrates play in the biological properties of the receptor. To examine whether putative carbohydrates are responsible for high affinity binding of hCG to the rLHR, various techniques including enzymatic removal of N-linked carbohydrates from purified rLHR or from membranes or cells expressing rLHR (13–15), treatment of cells with tunicamycin B (15), and alteration of the glycosylation consensus sequences by site-directed mutagenesis (16) have been employed to remove these carbohydrates. In general, the conclusions from N-glycanase treatments of the rLHR suggest that N-linked carbohydrates are not essential for high affinity binding (14, 15); however, one report suggests otherwise (13). On the other hand, tunicamycin treatment of rLHR-expressing cells led to the loss of hCG binding to intact cells (15). Similarly, recent studies utilizing site-directed mutagenesis have shown a complete loss of hCG binding activity to either intact cells or detergent solubilized cells expressing a rLHR in which Asn-173 was substituted with Gln (16). These results implicate potential carbohydrate at Asn-173 as absolutely essential to hormone binding and suggest, therefore, that a fully deglycosylated rLHR would similarly not bind hCG.

Given, therefore, that there was no clear consensus in the literature concerning the biological role(s) of the N-linked carbohydrates on the rLHR, we undertook the following studies. In this report we describe the results of mutagenesis of potential glycosylation sites singly and collectively within the rLHR on the binding activity of intact cells as well as detergent-solubilized extracts of cells expressing these mutants. Our results clearly resolve the discrepancies reported in the literature regarding the role of N-linked carbohydrates of the LHR by showing that they are not required for high affinity binding of hormone and that Asn-173 appears to be involved.

* These studies were supported by National Institutes of Health Grant HD-22196 (to D. L. S.). Services and facilities were also provided by the University of Iowa Diabetes and Endocrinology Research Center Grant DK-25295. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

† These authors contributed equally to this work.

§ Recipient of National Institutes of Health Research Career Development Award HD-00968. To whom all correspondence and reprint requests should be sent: Dept. of Physiology and Biophysics, The University of Iowa College of Medicine, Iowa City, IA 52242. Tel.: 319-335-7860; Fax: 319-335-9925.

1 The abbreviations used are: rLHR, rat lutropin/choriogonadotropin receptor; LHR, lutropin/choriogonadotropin receptor; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; hCG, human choriogonadotropin; LH/CG, lutropin/choriogonadotropin.
either directly or indirectly, in maintaining the high affinity binding of the rLHR.

MATERIALS AND METHODS

Construction of Glycosylation Mutants of the rLHR cDNA—A PstI-XhoI fragment of the rLHR, encompassing the region coding for the extracellular domain of the receptor, was excised from pCLHR (1) and subcloned into pSELECT (Promega). This was then used as a template for oligonucleotide-directed mutagenesis to create all the mutants discussed except rLHR(T154A) and rLHR(T175A). Mutagenesis in the pSELECT system was done following manufacturer’s instructions, except that annealing temperatures were increased to 70—85 °C and incubation time for DNA synthesis was increased to 90—120 min. rLHR(T154A) and rLHR(T175A) were constructed by the polymerase chain reaction (17, 18) using as a template pDLHR, which is a derivative of pDLHR in which a unique SalI restriction site was created by substituting guanine 1266 with cytosine in such a way that the coding sequence remains unchanged. The wild type and mutant rLHR receptors were all sluced into the expression vector pcDNA/I-neo (Invitrogen). The identity and fidelity of all mutants both before and after subcloning into the expression vector were confirmed by dyeoxy sequencing.

Transfections—COS-7 (ATCC CRL 1651) and 293 (ATCC CRL 1573) cells were maintained in a humidified 5% CO2 atmosphere. The extracellular domain of the rLHR has six potential sites for N-linked glycosylation identified by the presence of the Asn-X-Thr/Ser consensus sequence (25). To determine whether carbohydrates play a role in the high affinity binding of hCG to the rLHR, we initially created rLHR mutants by site-directed mutagenesis in which the glycosylation consensus sequence at each of these potential sites was altered by substituting the Asn residue with Gln. These manipulations were designed to create constructs in which the potential glycosylation sites at residues 77, 152, 173, and 291 were individually altered whereas the sites at residues 269 and 277 were simultaneously altered. In addition, a mutant rLHR lacking all potential N-linked glycosylation was created by substituting the Asn residue with Gin. These manipulations were designed to create constructs in which the potential glycosylation sites at residues 77, 152, 173, and 291 were individually altered whereas the sites at residues 269 and 277 were simultaneously altered.

Data shown are the mean ± S.E. of three individual experiments done in 293 cells. Within a given experiment determinations were performed in duplicate.

Table I

| Receptor                  | 125I-hCG bound to the cell surface | 131I-hCG bound to soluble extracts | % receptor at the cell surface |
|---------------------------|-----------------------------------|-----------------------------------|-------------------------------|
|                           | ng/10⁶ cells                       | ng/10⁶ cells                       |                               |
| rLHR                      | 1.70 ± 0.20                       | 2.36 ± 0.43                       | 76 ± 7                        |
| rLHR(N77Q)                | 0.84 ± 0.19                       | 1.17 ± 0.32                       | 79 ± 8                        |
| rLHR(N152Q)               | 0.83 ± 0.09                       | 1.42 ± 0.22                       | 61 ± 7                        |
| rLHR(N173Q)               | 0.03 ± 0.02                       | 0.17 ± 0.05                       | 11 ± 8                        |
| rLHR(N269,277Q)           | 2.39 ± 0.76                       | 3.87 ± 1.07                       | 63 ± 7                        |
| rLHR(N291Q)               | 2.53 ± 0.94                       | 3.62 ± 0.20                       | 68 ± 3                        |
| rLHR(N77,152,173,269,277,291Q) | 0.03 ± 0.01                     | 0.10 ± 0.03                       | 25 ± 4                        |
| rLHR(T154A)               | 1.77 ± 0.18                       | 2.41 ± 0.15                       | 73 ± 6                        |
| rLHR(T175A)               | 0.15 ± 0.03                       | 1.00 ± 0.23                       | 15 ± 2                        |
| rLHR(N77,152,269,277,291Q;T175A) | 0.03 ± 0.01                 | 0.77 ± 0.07                       | 4 ± 1                         |

Table II

| Receptor                  | 125I-hCG bound to the cell surface | 131I-hCG bound to soluble extracts | % receptor at the cell surface |
|---------------------------|-----------------------------------|-----------------------------------|-------------------------------|
|                           | ng/10⁶ cells                       | ng/10⁶ cells                       |                               |
| rLHR                      | 1.70 ± 0.20                       | 2.36 ± 0.43                       | 76 ± 7                        |
| rLHR(N77Q)                | 0.84 ± 0.19                       | 1.17 ± 0.32                       | 79 ± 8                        |
| rLHR(N152Q)               | 0.83 ± 0.09                       | 1.42 ± 0.22                       | 61 ± 7                        |
| rLHR(N173Q)               | 0.03 ± 0.02                       | 0.17 ± 0.05                       | 11 ± 8                        |
| rLHR(N269,277Q)           | 2.39 ± 0.76                       | 3.87 ± 1.07                       | 63 ± 7                        |
| rLHR(N291Q)               | 2.53 ± 0.94                       | 3.62 ± 0.20                       | 68 ± 3                        |
| rLHR(N77,152,173,269,277,291Q) | 0.03 ± 0.01                     | 0.10 ± 0.03                       | 25 ± 4                        |
| rLHR(T154A)               | 1.77 ± 0.18                       | 2.41 ± 0.15                       | 73 ± 6                        |
| rLHR(T175A)               | 0.15 ± 0.03                       | 1.00 ± 0.23                       | 15 ± 2                        |
| rLHR(N77,152,269,277,291Q;T175A) | 0.03 ± 0.01                 | 0.77 ± 0.07                       | 4 ± 1                         |

Table III

| Receptor                  | 125I-hCG bound to the cell surface | 131I-hCG bound to soluble extracts | % receptor at the cell surface |
|---------------------------|-----------------------------------|-----------------------------------|-------------------------------|
|                           | ng/10⁶ cells                       | ng/10⁶ cells                       |                               |
| rLHR                      | 1.70 ± 0.20                       | 2.36 ± 0.43                       | 76 ± 7                        |
| rLHR(N77Q)                | 0.84 ± 0.19                       | 1.17 ± 0.32                       | 79 ± 8                        |
| rLHR(N152Q)               | 0.83 ± 0.09                       | 1.42 ± 0.22                       | 61 ± 7                        |
| rLHR(N173Q)               | 0.03 ± 0.02                       | 0.17 ± 0.05                       | 11 ± 8                        |
| rLHR(N269,277Q)           | 2.39 ± 0.76                       | 3.87 ± 1.07                       | 63 ± 7                        |
| rLHR(N291Q)               | 2.53 ± 0.94                       | 3.62 ± 0.20                       | 68 ± 3                        |
| rLHR(N77,152,173,269,277,291Q) | 0.03 ± 0.01                     | 0.10 ± 0.03                       | 25 ± 4                        |
| rLHR(T154A)               | 1.77 ± 0.18                       | 2.41 ± 0.15                       | 73 ± 6                        |
| rLHR(T175A)               | 0.15 ± 0.03                       | 1.00 ± 0.23                       | 15 ± 2                        |
| rLHR(N77,152,269,277,291Q;T175A) | 0.03 ± 0.01                 | 0.77 ± 0.07                       | 4 ± 1                         |
TABLE II

| Receptor                        | K_d (nM) |
|--------------------------------|----------|
| rLHR                           | 0.188 ± 0.023 |
| rLHR(N77Q)                     | 0.170 ± 0.027 |
| rLHR(N152Q)                    | 0.341 ± 0.046 |
| rLHR(N173Q)                    | ≥20      |
| rLHR(N269,277Q)                | 0.219 ± 0.023 |
| rLHR(N291Q)                    | 0.236 ± 0.049 |
| rLHR(N77,152,173,269,277,291Q) | 0.374 ± 0.119 |
| rLHR(T154A)                    | 0.483 ± 0.024 |
| rLHR(N77,152,269,277,291Q;T175A)| 0.525 ± 0.021 |

*a Data shown are the mean ± S.E. of three individual experiments done in 293 or COS-7 cells. Within a given experiment determinations were performed in duplicate.

**K_d** values for these mutants were estimated from Fig. 1.

![Graph](image_url)

**Fig. 1.** Binding of 125I-hCG to cells expressing the wild type rLHR, rLHR(N77,152,173,269,277,291Q), or rLHR(N173Q). Detergent-soluble extracts of 293 cells expressing the wild type rLHR (●), rLHR(N173Q) (●), or rLHR(N173Q) (▲) were incubated with increasing concentrations of 125I-hCG as outlined under "Materials and Methods."

rLHR(N77,152,173,269,277,291Q), probably because of a very low affinity of these mutant receptors for the ligand. Fig. 1 supports this contention, illustrating a marked shift to the right in the 125I-hCG binding curves of these two mutants relative to the wild type rLHR, indicating a reduction of at least 100-fold in the binding affinities of these two mutants. Since rLHR(N173Q) is the only mutant that alone resulted in a major loss of high affinity binding, it would seem to indicate that substitution of Asn-173 with Gln would also be responsible for the similar loss in high affinity binding seen with the rLHR(N77,152,173,269,277,291Q) mutant.

Although one possible cause for the small decrease in binding affinity to the rLHR(N152Q) mutant and the significant decrease in binding affinities to the rLHR(N173Q) and rLHR(N77,152,173,269,277,291Q) mutants might be the lack of carbohydrate chains at residues 152 and 173, respectively, another possibility might be the substitution of a critical amino acid. To address the possibility that an amino acid important in hormone binding, the mutants rLHR(T154A) and rLHR(T175A) were constructed, where the glycosylation consensus sequences were altered by substituting Thr-154 and -175 with Ala rather than substituting Asn-152 and -173 with Gln. Since there is an absolute requirement for the hydroxyl groups of either Ser or Thr to serve as hydrogen bond donors during the glycosylation of an Asn residue (26), substitution of the Ser or Thr residues within a glycosylation sequence has been shown to prevent glycosylation at the corresponding Asn residues (26-28). Therefore, the mutants rLHR(T154A) and rLHR(T175A) should again encode for rLHRs, which should not be N-glycosylated at residues 152 or 173. One would predict comparable decreases in high affinity binding between the Asn to Gln mutants and the Thr to Ala mutants if the putative carbohydrates at Asn-152 and -173 were indeed essential to rLHR function. As shown in Table II, both rLHR(T154A) and rLHR(N152Q) still bound hCG with 2-3-fold reduced affinities, suggesting that this small decrease in binding affinity may be due to a loss of carbohydrate at residue 152. In contrast, whereas rLHR(N173Q) bound hCG with an affinity too low to accurately determine, rLHR(T175A) bound hCG with a high affinity, reduced only 2-3-fold relative to the wild type rLHR. These data suggest that the marked decrease in binding affinity in rLHR(N173Q) is due primarily to substitution of the Asn-173 residue per se and not to the loss of a putative carbohydrate at that site. As with rLHR(N173Q), however, rLHR(T175A) was still primarily trapped intracellularly (Table I).

One would predict, therefore, that if a fully deglycosylated rLHR were made in which glycosylation at Asn-173 was prevented by the substitution of Thr-175 instead of Asn-173, this deglycosylated receptor would bind hCG with high affinity. Indeed, as shown in Table II, rLHR(N77,152,269,277,291Q; T175A), unlike rLHR(N77,152,173,269,277,291Q), binds hCG with a high affinity. As shown in Table I, however, this mutant was still not properly expressed at the plasma membrane.

**DISCUSSION**

The LHR has a large extracellular domain that has been shown to be necessary and sufficient for high affinity binding to hCG (6-9). Located within this region are six potential sites for N-linked glycosylation represented by the consensus sequence Asn-X-Thr/Ser (25). Although it is known that the rLHR is glycosylated (13, 14), it is not yet known which of the six potential sites are glycosylated or what role these carbohydrates play in the proper functioning of the rLHR. When soluble extracts of cells expressing the wild type rLHR or rLHRs in which potential glycosylation sites were altered were assayed for hCG binding affinities, we observed a slight decrease in binding affinity with rLHR(N152Q), a significant decrease with rLHR(N77,152,173,269,277,291Q), and normal binding affinities for the substitutions of Asn residues 77, 269, 277, and 291 (see Table II). However, when we substituted Ala for Thr-175 (see rLHR(T175A)), destroying the 173-175 glycosylation consensus sequence while maintaining the Asn-173 residue, we observed high affinity hCG binding. Although, for technical reasons, it has not yet been possible to determine whether Asn-173 of rLHR is actually glycosylated, we would expect, based upon the results of others (25-28), that if Asn-173 is actually glycosylated then substitution of either Asn-173 or Thr-175 would be equally effective in preventing such glycosylation. As such, our data suggest that a putative carbohydrate at Asn-173 is not essential for high affinity binding, while indicating an important role for Asn-173 in the high affinity binding of hCG by the rLHR. Thus, a fully deglycosylated rLHR in which Asn-173 is left intact (see rLHR(N77,152,269,277,291Q; T175A)) has only a 2-3-fold lower affinity for hCG relative to the wild type rLHR (see Table II). These data, taken together with the data on rLHR(N173Q) and rLHR(T175A), suggest that N-linked glycosylation of the rLHR is not essential for high affinity binding.
binding of hCG, but that Asn-173 is somehow critical for this function.

Previous studies on site-directed mutagenesis of the rLHR had suggested that N-linked glycosylation was essential for high affinity binding of hCG because intact cells expressing rLHR(N173Q) did not bind any detectable hCG (16). It was suggested, therefore, that a lack of carbohydrate at Asn-173 would predict a loss of binding activity of a fully deglycosylated rLHR (16). Our data show that either the fully deglycosylated receptor or one in which Asn-173 has been mutated are not properly expressed at the plasma membrane. Furthermore, by assaying the detergent-soluble extracts of cells, it has been possible to show that the reduction in binding affinity due to substitution of Asn-173 is due to an alteration in that amino acid per se as opposed to the diarurpong of N-linked glycosylation at that position.

Although the studies published on tunicamycin-treated rLHR-expressing cells would seem to contradict our results by suggesting that a glycosylated LH/CG receptor is essential for high affinity binding (15), it is possible that the lack of binding observed to tunicamycin-treated intact cells reflected a decreased ability of the deglycosylated receptor to be transported properly to the plasma membrane. Indeed, as observed consistently in our lab with the fully deglycosylated rLHR(N77,152,269,277,291Q; T175A), expression at the plasma membrane is greatly reduced while high binding affinity is observed intracellularly (see Tables I and II). Enzymatic removal of the rLHR carbohydrates also generally supports the conclusion that a fully deglycosylated receptor binds hormone with high affinity (14, 15). One report, however, suggests that enzymatically deglycosylated rLHR no longer binds hCG (13). One possible explanation for this discrepancy may be that in the latter study the lengths of the incubations with glycodiesase were far longer than in the other studies where binding activity was preserved. With these considerations in mind, as well as the data from the studies presented herein, it appears rather conclusive that N-linked glycosylation of the LHR is not required for hormone binding.

Within the three glycoprotein hormone receptors, the only consensus sequence for potential N-linked glycosylation that is conserved among them is the one corresponding to Asn-173 of the rLHR. Studies by Russo et al. (29) on the human TSH receptor have shown that substitution of the corresponding Asn with Gln does not impair the binding affinity of the receptor for TSH. However, studies from our laboratory on the rFSH receptor have yielded results similar to those observed with the rLHR, in that substitution of the corresponding Asn in the rFSH receptor to Gln results in a receptor with a drastically reduced binding affinity, whereas substitution of the corresponding Thr to Ala within the consensus sequence does not have a deleterious effect on binding affinity.2 Another consequence of substitutions of either Asn-173 or Thr-175 in the rLHR is to cause the mutated receptors to remain trapped intracellularly (see Table I). Therefore, fully deglycosylated receptors in which either of these two sites were altered resulted in a lack of expression of these receptors at the plasma membrane. One possible interpretation for these data is that Asn-173 is normally glycosylated and that the carbohydrate at this site is necessary for the proper trafficking of the receptor to the plasma membrane. Although studies are underway to determine the sites of N-linked carbohydrate attachment to the rLHR, we do not yet know if this site is indeed glycosylated. It should be pointed out, however, that a lack of carbohydrate at this site in these mutant receptors is not the only possible explanation for their being trapped intracellularly. Indeed, we have found that numerous other mutations of the rLHR, some of which also are single amino acid substitutions, result in receptors that are not properly expressed on the plasma membrane (30). Therefore, the lack of expression of rLHR(N173Q) and rLHR(T175A) at the plasma membrane may reflect the alteration of those amino acids per se and not necessarily the deletion of a potential glycosylation site.

In summary, our data help to resolve seeming discrepancies in the literature concerning the role of N-linked carbohydrates in the binding activity of the rLHR by showing that the fully deglycosylated receptor, although not expressed at the plasma membrane, does bind hCG with high affinity and by showing that Asn-173 is important, either directly or indirectly, in maintaining the high affinity binding activity of the receptor. Current studies are underway to determine which of the six potential sites are glycosylated and to determine what role, if any, each of the N-linked carbohydrates has on the ability of hormone to stimulate adenylcyclase.

Acknowledgments—We thank Dr. Mario Ascoli for helpful discussions and for critically reading the manuscript, Kimberly Collison and Julie Jacquette for expert technical assistance, and Dr. Haiyun Wang for the preparation of PDLHr.

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