Site Specificity of the Chorionic Gonadotropin N-Linked Oligosaccharides in Signal Transduction*

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The role of the human chorionic gonadotropin (hCG) N-linked oligosaccharides in receptor binding and signal transduction was analyzed using site-directed mutagenesis and transfection studies. hCG derivatives with alterations at individual glycosylation sites were expressed in Chinese hamster ovary cells. Receptor binding studies showed that absence of any or all of the hCG N-linked oligosaccharides had only a minor effect on the receptor affinity of the derivatives. Similarly, absence of the N-linked oligosaccharides from the β subunit or a single oligosaccharide from Asn-78 of α had no effect on the production of cAMP or on steroidogenesis. However, the absence of carbohydrate at Asn-52 of α decreases both the steroidogenic and cAMP response. Furthermore, absence of this critical oligosaccharide unit on α unmask differences in the two N-linked oligosaccharides on β; the β Asn-13 oligosaccharide but not the β Asn-30 oligosaccharide plays a more important role in steroidogenesis. Dimers containing deglycosylated β subunit and an α subunit lacking either the Asn-52 oligosaccharide or both oligosaccharides fail to stimulate cAMP or steroid formation. Moreover, these derivatives bind to receptor and behave as competitive antagonists. The use of site-directed mutagenesis was critical in uncovering site-specific functions of the hCG N-linked oligosaccharides in signal transduction and reveals the importance of the Asn-52 oligosaccharide in this process.

The glycoprotein hormones chorionic gonadotropin (hCG),1 lutropin, follitropin, and thyrotropin are a family of heterodimeric proteins which share a common α subunit but differ in their hormone-specific β subunit (1). These hormones function through adenylate cyclase-linked receptors which in the case of the gonadotropins leads to increased steroidogenesis. Although it is widely held that receptor specificity is determined by the β subunit (2, 3), modifications of either α or β can disrupt receptor binding (1, 4, 5), and free α or β subunits are essentially inactive (6). However, cross-linking studies show that it is predominantly the α subunit that interacts with the receptor (7, 8). Furthermore, several groups (9–11) have also shown that all immunoreactive sites on the α subunit but not all of the sites on the β subunit are blocked upon binding of the hormone to its receptor. These data suggest that the α subunit is “sandwiched” between the β subunit and receptor and may play a more active role than the β subunit in receptor binding. However, Keutmann et al. (12) have shown that a single peptide domain of CGSβ can bind to the receptor and stimulate steroidogenesis at high concentrations, implying that β domains may play an important role in signal transduction.

The oligosaccharides on the glycoprotein hormones have been implicated in several actions including the maintenance of intracellular stability, secretion, assembly, receptor binding, steroidogenesis, and modulation of plasma half-life (for review, see Refs. 1 and 4). Chemical or enzymatic treatments to remove all (13–17) or part (18, 19) of the N-linked oligosaccharide chains on hCG (two N-linked units on both α and β) reveal that progressive removal of carbohydrate from the hormone decreases activity but has little effect on receptor binding. Similar results have also been observed with other members of the glycoprotein hormone family (20–22). Several groups (13, 20, 22) have also suggested that the oligosaccharides on the common α subunit rather than the β subunits may play a more dominant role in signal transduction. However, there are several drawbacks associated with the use of chemical or enzymatic treatments to remove N-linked oligosaccharides. First, these treatments may damage the protein backbone and may also affect the O-linked oligosaccharides. Second, removal of the N-linked carbohydrate is incomplete. Third, chemical or enzymatic treatments cannot be used to study individual glycosylation sites since oligosaccharide units are removed indiscriminately.

To investigate the role of the individual N-linked oligosaccharides on hCG, we have employed oligonucleotide-directed mutagenesis and transfection studies. The major advantage of site-directed mutagenesis is that individual glycosylation sites on multiglycosylated proteins can be targeted for analysis. Earlier mutagenesis studies from our laboratory have shown site-specific intracellular functions of the two N-linked chains on α (23). Using CHO transfected clones secreting mutant hCG dimers which lack the N-linked oligosaccharides at individual sites on α or CGSβ or both, we analyzed the role of the individual N-linked oligosaccharides in receptor binding and steroidogenesis. These studies showed that absence of the α or CGS N-linked oligosaccharides has little effect on receptor interactions of the mutant hormones. However, by using the mutagenesis approach, we have identified site-specific functions of the N-linked oligosaccharides on both the α and CGSβ subunits in signal transduction.

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1 The abbreviations used are: hCG, human chorionic gonadotropin; CHO cells, Chinese hamster ovary cells; LH, luteinizing hormone (lutropin); WT, wild-type.
EXPERIMENTAL PROCEDURES

Materials—Enzymes used in the preparation of DNA vectors and constructs were purchased from Bethesda Research Laboratories and New England Biolabs. Cell culture reagents were obtained from the Washington University Tissue Culture Facility with the exception of G418 and horse serum (Gibco) and fetal calf serum (Sigma). Monoclonal antibodies B107 and B109 and purified hCG standard (CR119) were the gift of Drs. Krichevsky, Birken, and Canfield (Columbia University, New York, NY). The MA-10 Leydig tumor cells were the gift of Dr. Mario Ascoli (Population Council, New York, NY). Radioimmunoassay kits for the determination of cAMP and progesterone were supplied by Du Pont-New England Nuclear and Immuchem Corp. (Carson, CA), respectively. Isotubutimethylxanthine, theophylline, and bovine serum aliquots were purchased from Sigma. All other reagents are as described (23, 26).

DNA Transfection and Clone Selection—CHO cells co-transfected with pM'CGa and pM'CGPAAsn2, pM'aAAsn1, and pM'aAAsn2, pM'CGPAAsn1(1+2) and pM'aAAsnThr1, pM'aAAsnThr2, and pM'aAAsnThr(1+2) have also been described previously (25). CHO cells co-transfected with pM'CGpAAsn1(1+2), pMCGpAAsn1, pM'aAAsn1(1+2), pM'CGpAAsnThr1, pM'CGpAAsnThr2, and pM'CGpAAsnThr(1+2) have also been maintained in medium I (Ham's F-12 medium supplemented with 10% fetal calf serum) (28) and 1'1-hCG (CR119).

All binding data are reported as percentage of control bound (5-10%; 35,000 cpm). Assays of the bioactivity of wild-type and mutant hCG derivatives was determined with a radioligand receptor assay using intact MA-10 cells (23) and 1'1-hCG (CR119). On day 0, 400,000 MA-10 cells were plated into 6 × 35-mm dishes in Waymouth MB752/1 (pH 7.4) supplemented with 15% horse serum and gentamycin (40 μg/ml) (2 mM), 5% fetal calf serum (v/v), and G418 (0.25 mg/ml)) at 37 °C in a humidified 5% CO2 incubator. For hormone collection, cells secreting wild-type and mutant hCG were plated and grown to confluency in medium I except that a minimal essential medium was substituted for Ham's F12, fetal calf serum was increased to 10% (v/v), and the G418 was deleted. After 48 h of incubation, medium was collected, centrifuged to remove cell debris, and concentrated several fold with Amicon centripreps. HCG wild-type and mutant dimers were quantitated using two different radioimmunoassays using dimer-specific monoclonal antibodies B107 and B109 (29) and CR119 hCG as the standard. Each hCG radioimmunoassay was repeated three times.

Receptor, cAMP, and Progesterone Assays—The receptor binding activity of recombinant wild-type and mutant hCG derivatives was determined with a radioligand receptor assay using intact MA-10 cells (23) and 1'1-hCG (CR119). The MA-10 Leydig tumor cells were the gift of Drs. Krichevsky, Birken, and Canfield (Columbia University, New York, NY). The MA-10 Leydig tumor cells were the gift of Dr. Mario Ascoli (Population Council, New York, NY). Radioimmunoassay kits for the determination of cAMP and progesterone were supplied by Du Pont-New England Nuclear and Immuchem Corp. (Carson, CA), respectively. Isotubutimethylxanthine, theophylline, and bovine serum aliquots were purchased from Sigma. All other reagents are as described (23, 26).

RESULTS AND DISCUSSION

hCG Glycosylation Mutants—The recognition signal for N-linked glycosylation of proteins is the tripeptide sequence Asn-X-Thr/Ser (24, 25). The common α subunit contains two glycosylation sites at asparagines 52 and 78 while CGβ has two sites at positions 13 and 30 (Fig. 1). We have previously used oligonucleotide-directed mutagenesis to generate α and CGβ mutants with alterations at one or both N-linked sites to study the role of these oligosaccharides in secretion and assembly of hCG (23, 26). Co-transfection of these wild-type and mutant α and CGβ subunit genes in CHO cells and selection of stable cell lines producing mutant hCG dimers allowed us to examine the role of individual asialoglycosidase units in receptor binding and steroidogenesis. Our earlier studies of the intracellular role of the N-linked oligosaccharides on α (23) and β (26) utilized mutations in both the Asn and Thr of the consensus sequences to ensure that absence of the oligosaccharide and not a change in the protein structure was responsible for the effects seen (see Ref. 27). Since both sets of mutants manifest comparable intracellular effects, we utilized the α and β asparagine mutants for the biologic activity studies in some cases verified asparagine mutant findings with threonine mutants. Since both threonine and asparagine mutants of the β chain combined similarly with wild-type α (26), we generated several new cell lines in which addition of 0.34 ml of 0.2 M HClO4 and an additional centrifugation step. The supernatant was assayed for cAMP using a radioimmunoassay kit supplied by Du Pont-New England Nuclear.

FIG. 1. Wild-type and mutant α (top) and CGβ (bottom) subunits. The common α subunit and CGβ both contain two N-linked glycosylation sites. Using site-directed mutagenesis, we have previously (23, 26) mutated the asparagine (Asn) codons of the glycosylation consensus sequences in theα subunit (top) to aspartic acid (Asp) and have changed the Asn codons of CGβ (bottom) to glutamine (Gln). The α mutants lacking carbohydrate via an asparagine (αAsn1, αAsn2, and αAsnThr(1+2)) change are shown, and the CGβ glycosylation mutants are similarly designated. Mutants αAsn(1+2) and βAsn(1+2) lacking N-linked oligosaccharides at both sites are also referred to as αAAsn (deglycosylated α) and βAAsn (deglycosylated β), respectively.
the β asparagine mutants were co-expressed with the α asparagine mutants.

**LH/hCG Receptor Binding of Glycosylation Mutants**—To assess the ability of the mutant hCG derivatives to bind to the LH/hCG receptor, we analyzed the binding of the mutant hormones to the LH/hCG receptor on the surface of a murine Leydig tumor cell line, MA-10 (Ref. 28; Fig. 2). In the radioligand receptor assays, a comparison of hCG produced in CHO cells with its mutant derivatives showed that absence of N-linked carbohydrate on α, CGβ, or both has little effect on the binding of the mutant hormones to the murine LH/hCG receptor. In all cases, there was >90% variation in the affinity of the mutant versus wild-type hormones for the receptor (Table I). Mutant dimers lacking sugar at site I (αΔAsn1), site II (αΔAsn2), or both sites (αΔAsn1(1+2)) of the α subunit (Fig. 2) or any of the other mutants analyzed have similar affinities for the receptor compared to CG-WT (Table I). Thus, as reported by others (13–17), the N-linked oligosaccharides on hCG play a minor role in binding to the LH/hCG receptor and there are no major differences in receptor affinity of mutants lacking oligosaccharides at the individual glycosylation sites.

**Steroidogenesis Activity of hCG Mutants**—These mutant hCG derivatives were examined next for their ability to stimulate MA-10 cell steroidogenesis. Absence of oligosaccharides from both sites of the β subunit when combined with wild-type α had little effect on the dose-response curves or maximum progesterone produced compared to CG-WT (Fig. 3a). Similar findings were seen when either site I or site II was absent on the β subunit and combined with wild-type α (Table I), and therefore the absence of sugar on CGβ at the first, second, or both positions does not affect steroidogenesis.

Analysis of the α subunit oligosaccharides reveals differences in the roles of the two carbohydrate units in signal transduction. Whereas absence of the oligosaccharide at site II of α (αΔAsn2) does not affect steroidogenesis (Fig. 3b), absence of carbohydrate at site I of α (αΔAsn1) shifts the dose-response curve (EC50) ~5-fold and causes a 30% decrease in the maximum progesterone produced (Fig. 3c, αΔAsn1/β). This difference is likely due to the absence of the oligosaccharide at these sites, since a threonine change at α site II does not alter steroidogenesis whereas a threonine change at the first site has a greater effect on receptor binding and steroidogenesis (data not shown). Absence of both oligosaccharides on α when combined with β-WT shifts the dose-response curve further (~6-fold) and also decreases (~40%) the maximum progesterone produced (Fig. 3c, dga/β). These studies also confirm earlier glycosidase studies (13) which showed that absence of carbohydrate on CGa has more of an effect than absence of sugar on CGβ. However, using the mutagenesis approach, we identified a site-specific difference between the two oligosaccharides on α in signal transduction: the oligosaccharide at site I but not site II is critical for steroidogenic activity. In contrast, other investigators (16, 17) using chemical methods to deglycosylate the subunits of hCG saw decreased activity with deglycosylation of either subunit, suggesting that these treatments were altering the β subunit elsewhere (perhaps at the O-linked sugars) or affecting the combination of these chemically treated β subunits with an α subunit. Furthermore, the asparagine to glutamine mutagenic change was not responsible for this enhanced activity of the deglycosylated β mutants, since mutant β subunits with threonine to asparagine alterations at the first, second, or both sites exhibited identical behavior (data not shown).

To analyze whether there is an interaction of the oligosaccharides on both α and CGβ, we analyzed mutants lacking sugars at various sites on α and CGβ for their ability to stimulate steroidogenesis. Comparison of mutants lacking N-linked sugar on α (dga) assembled with wild-type and mutant β subunits reveals that dga unMASKS apparent site-specific differences of the CGβ oligosaccharides. Comparison of the dga/β dimer mutant to mutant dga/βΔAsn2 in which the β lacks carbohydrate at site II suggests that the β site II oligosaccharide plays a minor role in steroidogenesis (data not shown). However, absence of sugar at site I of β when combined with deglycosylated α (dga/βΔAsn1) shifts the EC50 ~9-fold and reduces the maximum response to ~25% of CG-WT (Fig. 3d). These results are unexpected since these β mutants have similar steroidogenic responses when combined with α-WT (data not shown). A further shift in the EC50 (15-fold) and reduction in maximum steroid produced is also seen if both sugars on β are absent (Fig. 3d, dga/dgβ). Thus

### Table I

**Receptor affinity and maximum steroidogenesis of hCG derivatives**

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Receptor binding affinity (μg/ml)</th>
<th>Maximum progesterone % control ± S.E. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
<td></td>
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<tr>
<td>CG-WT</td>
<td>1.00</td>
<td>100%</td>
</tr>
<tr>
<td>αΔAsn1/β</td>
<td>0.75 ± 0.024</td>
<td>68.5 ± 2.4 (12)</td>
</tr>
<tr>
<td>αΔAsn2/β</td>
<td>1.24 ± 0.055</td>
<td>101.6 ± 1.9 (6)</td>
</tr>
<tr>
<td>dga/β</td>
<td>1.16 ± 0.055</td>
<td>57.6 ± 2.9 (10)</td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α/βΔAsn1</td>
<td>0.84 ± 0.003</td>
<td>103.6 ± 4.09 (5)</td>
</tr>
<tr>
<td>α/βΔAsn2</td>
<td>0.76 ± 0.005</td>
<td>99.8 ± 2.2 (5)</td>
</tr>
<tr>
<td>α/dgβ</td>
<td>1.02 ± 0.167</td>
<td>97.4 ± 2.3 (4)</td>
</tr>
<tr>
<td>C.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αΔAsn1/dgβ</td>
<td>0.83 ± 0.054</td>
<td>12.8 ± 3.1 (4)</td>
</tr>
<tr>
<td>αΔAsn2/dgβ</td>
<td>1.37 ± 0.183</td>
<td>98.7 ± 3.1 (7)</td>
</tr>
<tr>
<td>dga/dgβ</td>
<td>1.29 ± 0.005</td>
<td>12.3 ± 2.0 (7)</td>
</tr>
<tr>
<td>dga/βΔAsn1</td>
<td>1.27 ± 0.167</td>
<td>24.0 ± 3.6 (4)</td>
</tr>
<tr>
<td>dga/βΔAsn2</td>
<td>1.39 ± 0.151</td>
<td>50.9 ± 2.1 (5)</td>
</tr>
</tbody>
</table>

**Fig. 2.** Radioligand receptor assay of recombinant wild-type and mutant hCG derivatives. Binding activity was measured by displacement of 125I-hCG (CR119) from MA-10 cells (28). Cells were incubated ±15 h in 1 ml of assay medium containing 125I-hCG (100,000 cpm/ml) and varying concentrations of wild-type and mutant hCG derivatives. The amount of 125I-hCG bound to the cells was determined and is reported as percentage of control bound (5–10%); nonspecific binding was determined in the presence of 1 μg/ml unlabeled CR119 and accounted for <4% of the total bound. hCG derivatives used for this experiment are CG-WT (●); αΔAsn1/β (■); αΔAsn2/β (▲); αΔAsn1(1+2)/β (●, ■, ■); αΔAsn1/β (●, ■, ■). Relative binding of these mutants and others (not shown) compared to wild-type from two separate experiments is shown in Table I.
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Fig. 3. Steroidogenesis activity of wild-type and mutant hCG derivatives. MA-10 cells were prepared as described under "Experimental Procedures." Cells were incubated in 2 ml of assay medium containing varying concentrations of wild-type and mutant hCG derivatives. After 4 h, the medium was collected and stored at -20°C until assayed. Progesterone in the medium was determined by radioimmunoassay. All mutants were tested 22 times and compared to wild-type hCG produced from CHO cells.

this hCG derivative devoid of N-linked sugars on both subunits is a weak agonist although its binding to the LH/hCG receptor is not affected (Table I). Thus, the absence of carbohydrate on a unmaska site specificity of the CGβ oligosaccharides; the CG dimer containing deglycosylated α combined with the site I CGβ mutant is less steroidogenic than the corresponding analog lacking carbohydrate at β site II.

Since the steroidogenic activity of α/β is very weak but the α/β dimer elicits a normal response, we examined if one or both α oligosaccharides were necessary for achieving normal steroidogenesis when combined with β. Analysis of αAsn1/β (Fig. 3e) reveals that in the absence of the α site I sugar (site II present) this analog has weak steroidogenic activity (12%) comparable to α/β. Thus, the presence of a single oligosaccharide at site II of α cannot restore the steroidogenic potency of the hormone. However, analysis of αAsn2/β (Fig. 3f) shows that the presence of an oligosaccharide only at site I of α in this mutant (site II absent) restores maximal steroidogenesis. Thus, a mutant hCG containing deglycosylated β clearly demonstrates the site specificity of the α N-linked oligosaccharides and further emphasizes that the oligosaccharides at site I but not site II of α is a prerequisite for signal transduction in vitro (see Fig. 6).

Cyclic AMP Accumulation by Mutant hCG Derivatives—Since the hCG steroidogenic response is mediated through cAMP and since receptor binding is unaffected by the mutations, we examined if the differences in steroidogenic activity seen above were due to changes in adenylate cyclase activa-
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Fig. 4. Adenylate cyclase stimulation by wild-type and mutant hCG derivatives. Cells were prepared as described under “Experimental Procedures.” Cells were incubated for 45 min in 2 ml of assay medium containing 0.5 mM isobutylmethylxanthine and varying concentrations of mutant or wild-type hCG. Intracellular cAMP was determined (mean ± range) using a radioimmunoassay kit. All mutants were compared to wild-type (±2 times in their ability to stimulate adenylate cyclase.

Fig. 5. Competitive inhibition of adenylase cyclase activity by mutant hCG. Mutant hCG derivatives dga/dgβ or αΔAsn1/dgβ were tested as antagonists of wild-type hCG. MA-10 cells were incubated with varying concentrations of hCG and a constant amount (0.67 pmol/ml) of dga/dgβ or αΔAsn1/dgβ which were added simultaneously. Intracellular cAMP was assayed as described under “Experimental Procedures.”
the corresponding β subunits. We show here that a single oligosaccharide unit on hCG plays a critical role in adenylate cyclase activation and steroidogenesis. Only by using site-directed mutagenesis could such specific differences in glycosylation sites have been uncovered.

Similar to earlier studies, we have shown that occupancy of the LH/hCG receptor by some of these hCG glycosylation mutants is not sufficient for signal transduction to occur. Both glycoprotein subunits are necessary for receptor interaction and signal transduction (6–8, 12). Since absence of the bulky N-linked oligosaccharides does not alter hCG-receptor interaction, the α and β oligosaccharides are likely positioned on the outward face of the hCG when it is bound to the receptor. If this is so, how does absence of the oligosaccharide at site 1 of α affect signal transduction? Absence of the oligosaccharide from this site may cause a conformational change elsewhere in one or both of the subunits leading to a decrease in signal transduction without affecting receptor binding domains. This conformational change in the α and/or β subunits could be further exaggerated by absence of the β Asn-13 oligosaccharide. Several studies (29, 30) support this hypothesis that deglycosylation causes conformational changes resulting in decreased activity. These investigators showed that monoclonal antibodies to native hCG can convert the deglycosylated hCG into an active form, presumably due to a conformational change in which the deglycosylated hCG resembles native hCG.

Alternatively, several investigators (31, 32) have suggested that signal transduction at the hCG receptor occurs via receptor:receptor coupling (or aggregation) upon hCG binding. Calvo and Ryan (32) suggest that this interaction might occur via a hCG glycopeptide:oligosaccharide binding protein in which an hCG α oligosaccharide is directly involved. These observations are also consistent with our findings that the α sugars play a more important role in signal transduction and our findings would implicate the α glycopeptide containing Asn-52 as the major determinant. In any case, whether a direct role via an oligosaccharide:protein interaction or a conformational change in the hCG is responsible for the loss of activity of hCG, the studies presented here demonstrate that the oligosaccharide at asparagine 52 of α plays a major role in the signal transduction of hCG. These mutants may now be useful to further analyze hCG interactions at its receptor in vivo, and homogeneous preparations of these mutants may aid in further analyzing functionally important hCG regions by x-ray crystallography.

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REFERENCES