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# The Minute Structural Difference between the Hormone hCG and the Autocrine Hyperglycosylated hCG

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## Abstract

**Introduction:** In 1997 I discovered hyperglycosylated hCG, a separate and independent molecule to the hormone hCG. The structure of hyperglycosylated hCG was also examined, it was a molecule varying from hCG by just 3 or 4 small sugar side chains, or 2.8% of molecular weight. While the hormone hCG binds a luteinizing hormone (LH)/hCG hormone receptor, hyperglycosylated hCG and its β-subunit are autocrines binding and antagonizing a TGF-β-II receptor. Here structural differences between the two molecules are investigated.

**Methods:** Nicking or cleavage of the hormone hCG and the autocrine hyperglycosylated hCG, and dissociation of subunits were carefully investigated using sequence analysis.

**Results:** Research showed that hyperglycosylated hCG was much more rapidly nicked or cleaved at β47-48 than the hormone hCG. And that nicked hCG was much more rapidly dissociated into subunits than non-nicked hCG.

**Discussion:** A model was generated. As proposed, hyperglycosylated hCG is first rapidly nicked or cleaved at β47-48 and then rapidly dissociated. The nicked hyperglycosylated hCG β-subunit antagonizes the TGF-β-II receptor. In contrast, the endocrine hCG is blocked from nicking, which limits dissociation, only intact hCG binds the LH/hCG hormone receptor.

**Keywords:** HCG; Hyperglycosylated hCG; TGF-β-II; Leukocyte elastase; Dissociation

## Introduction

Two major molecules, the hormone human chorionic gonadotropin (hCG) and the autocrine hyperglycosylated hCG both share the same 92 amino acid α-subunit and 145 amino acid β-subunit hCG sequences. Yet are two completely separate or very different molecules [1,2]. The hormone hCG and the autocrine hyperglycosylated hCG also share the same four N-linked oligosaccharides but different O-linked sugar structures [2,3]. The hormone hCG binds a joint hCG/luteinizing hormone (LH) receptor, and has zero binding of a transforming growth factor-β-II (TGF-β-II) receptor. The autocrine hyperglycosylated hCG, by contrast, binds and antagonizes a TGF-β-II receptor [4-6] and has zero binding of a hormone hCG/LH receptor. How can this be? How can the receptor binding specifics be so different when the molecules are virtually the same?

The only structural difference between the hormone hCG and the autocrine hyperglycosylated hCG is in the four short O-linked sugar side chains on the β-subunit C-terminal peptide [2,3]. The hormone hCG has four type one structures "NeuAc-Gal-(NeuAc)-GalNAc-" and "NeuAc-Gal-GalNAc-", while the autocrine hyperglycosylated hCG has three or occasionally four type two structures "NeuAc-Gal-GlcNAc-(NeuAc-Gal)-GalNAc-" and "NeuAc-Gal-GlcNAc-(Gal)-GalNAc-" [2,3]. The difference between the two types of structure, three galactose (molecular weight 162) and three N-acetylglucosamine residues (molecular weight 203), is minuscule compared to the molecular weight of hyperglycosylated hCG (39,149 daltons), it is just 2.8% of the molecular weight.

How can this miniscule structural difference, 2.8% of the molecular weight, have such a major effect on the product? The hormone which binds an LH/hCG receptor and the autocrine which binds a TGF-β-II receptor, with no cross-reactivity.

The hormone hCG primarily drives creation and maintenance of hemochorial placentation, the human fetal feeding system in pregnancy [1,7]. In addition it manages progesterone production in early pregnancy [8,9] and drives cytotrophoblast cell differentiation [10], attenuates pregnancy implantation [11], suppresses contractions during pregnancy [12,13] and suppresses maternal macrophage rejection during pregnancy [14,15]. The hormone hCG also supplements LH during the menstrual cycle [16,17] and promotes organ development in the fetus during gestation [18,19].

The autocrine hyperglycosylated hCG controls pregnancy implantation at the start of gestation [20,21]. It controls deep implantation of hemochorial placentation at the end of the first trimester of pregnancy [22], controls placenta growth during pregnancy [23] and controls malignancy by all human cancer cells [24,25].

Two extremely different molecules, a hormone that promotes growth and differentiation, and an autocrine that drives malignancy and invasion. Both molecules having the same amino acid sequence, the same N-linked oligosaccharides and being seemingly virtually identical in structure. How can this be? This is a very rare oddity of biochemistry that has confused and confounded readers for many

years. It is all examined experimentally and carefully explained for the first time here.

## Materials and Method

### Nicking of hCG and hyperglycosylated hCG

The commercial enzyme human leucocyte elastase (Sigma-Aldrich, St Louis MO), catalogue E1508, has been proven to nick or cleave hCG at β47-48 as occurs in human blood [26-28].

Human leucocyte elastase action on the endocrine hCG and on hyperglycosylated hCG was examined. Experiments were conducted with 0.25 units enzyme in 60 µl 0.1 M Tris-HCl with 0.5 M NaCl, 0.1% w/w Brij 35, pH 8.5 containing 10 mM phenylmethane sulfonyl fluoride and 5 nmol hormone hCG, batch P1, not nicked or 5 nmol hyperglycosylated hCG batch C7, not nicked. Eleven micro test tubes were assembled of each mixture, and incubated for 1.0, 1.33, 1.67, 2.0, 4.0, 8.0, 12, 16, 21, 24 and 30 h. After incubation, the samples were frozen at -70°C and then tested in the service laboratory at Columbia-Presbyterian Medical Center by Edman Degradation sequence analysis. The percentage of nicked hCG molecules, having a sequence starting at Val β48, cleaved at β47-48 (sequence: Val-Leu-Pro-Ala) was determined as was the absence of other nicking demonstrated by sequence analysis (having an N-terminal sequence Val-Leu-Gln-Gly if cleaved at β43-44, having a sequence Leu-Gln-Gly-Pro if cleaved at β44-45, the two other cleavage sites).

### Dissociation of non-nicked and nicked hCG

Stability of hyperglycosylated hCG standard (batch C7) and of 100% nicked hyperglycosylated hCG standard (batch C5) was investigated. C7 and C5 were dissolve in normal male serum (hCG-free) (Sigma), catalogue S2145. The normal male serum was preserved with 5x penicillin-streptomycin-fungizone. The final volume was 1.0 ml. Sample in quadruplicate were incubated at 37°C and aliquots, 0.02 ml removed at 16, 22, 29, 40, 70, 120, 200, 400, 700 and 1000 h for intact hCG measurement. Dissociation was measured using the 2119 microtiter plate immunometric assay, a test measuring only intact or only non-dissociated hCG. The assay used monoclonal antibody 2119; this is an anti α-subunit antibody. This was the capture antibody. 4001-peroxidase was tracer antibody, this is a peroxidase labelled anti-core β-subunit polyclonal antibody. Procedures are those published previously [1]. The quantity of hCG detected by the 2119 assay was used to determine the concentration of intact or non-dissociated hCG.

## Results

Nicking or cleavage in the β39-58 amphipathic loop of the hormone hCG and the autocrine hyperglycosylated hCG was investigated. Nicking or cleavage in the β39-58 loop can be performed by the protease human leukocyte elastase [26-28]. This enzyme cleaves the hormone hCG, hCG dissociated β-subunit, hyperglycosylated hCG and hyperglycosylated hCG dissociated β-subunit in the β47-48 in the β39-58 loop [26-28].

| Sample | Incubation time (h) | Sequence analysis (% sequence Val-Leu-Pro) |
|---|---|---|
| hCG, batch PI, not nicked, 5 nmol | 1.0 h | 0% nicked |
| hCG, batch PI, not nicked, 5 nmol | 1.33 h | 0% nicked |
| hCG, batch PI, not nicked, 5 nmol | 1.67 h | 0% nicked |
| hCG, batch PI, not nicked, 5 nmol | 2.0 h | 0% nicked |
| hCG, batch PI, not nicked, 5 nmol | 4.0 h | 9% nicked |
| hCG, batch PI, not nicked, 5 nmol | 8.0 h | 20% nicked |
| hCG, batch PI, not nicked, 5 nmol | 12 h | 28% nicked |
| hCG, batch PI, not nicked, 5 nmol | 16 h | 52% nicked |
| hCG, batch PI, not nicked, 5 nmol | 21 h | 89% nicked |
| hCG, batch PI, not nicked, 5 nmol | 24 h | 95% nicked |
| hCG, batch PI, not nicked, 5 nmol | 30 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 1 h | 43% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 1.33 h | 74% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 1.67 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 2.0 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 4.0 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 8.0 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 12 h | 100% nicked |

| | | |
|---|---|---|
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 16 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 21 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 24 h | 100% nicked |
| Hyperglycosylated hCG, batch C7, not nicked, 5 nmol | 30 h | 100% nicked |

**Table 1:** Nicking of hCG and hyperglycosylated hCG, 0.58 nmol, by incubation with human leukocyte elastase, 0.25 units at 37°C in 0.2 ml Tris-HCl, 0.5 M NaCl, 0.1% Brij, pH 8.5.

Endocrine hCG batch P1 which is not nicked, and hyperglycosylated hCG batch C7, which is not nicked, were incubated with purified human leukocyte elastase, 0.25 units at 37°C in 0.2 µl Tris-HCl, 0.5M NaCl, pH 8.5, and incubated for 11 different time intervals (Table 1). As found, endocrine hCG batch P1 became 100% nicked after 30 h incubation at 37°C.

| Sample | Incubation time (h) | 2119 Intact hCG assay ng/ml (dissociated) |
|---|---|---|
| Hyperglycosylated hCG, C7 standard, 0% nicked | 200 h | 630 ± 120 (37% dissociated) |
| Hyperglycosylated hCG, C7 standard, 0% nicked | 400 h | 380 ± 115 (62% dissociated) |
| Hyperglycosylated hCG, C7 standard, 0% nicked | 700 h | 1.2 ± 0.33 (99.8% dissociated) |
| Hyperglycosylated hCG, C5 standard, 100% nicked | 16 h | 220 ± 45 (78% dissociated) |
| Hyperglycosylated hCG, C5 standard, 100% nicked | 22 h | 0.7 ± 0.11 (99.9% dissociated) |

**Table 2:** Dissociation of hyperglycosylated hCG in serum. Hyperglycosylated hCG standard C7 and standard C5, 1000 ng were dissolved in control male serum in quadruplicate, with 10x penicillin–streptomycin–fungizone antibiotic and incubated at 37°C until ~100% dissociated.

Hyperglycosylated hCG batch C7, in contrast, was 100% nicked after a very much shorter incubation, 1.67 h or in just 1 h 40 min at 37°C. Hyperglycosylated hCG batch C7 was nicked 100% 18-fold faster than endocrine hCG batch P1, (Table 1), yet the only structural difference is in O-linked oligosaccharides at the β-subunit C-terminal peptide [28]. In both cases, sequence analysis showed that cleavage at β47-48 was the only nicking that occurred. This is consistent with non-nicked hyperglycosylated hCG being very much more rapidly nicked than the hormone hCG.

The stability of hyperglycosylated hCG and nicked hyperglycosylated hCG were examined. Hyperglycosylated hCG standard, batch C7, not-nicked, and hyperglycosylated hCG standard batch C5, 100% nicked at β47-48 [2,3], were dissolved in normal male serum preserved with penicillin-streptomycin-fungizone in quadruplicate. Mixtures were incubated at 37°C (Table 2). Hyperglycosylated hCG standard C7 was 100% dissociated in 700 h, while hyperglycosylated hCG C5 standard was 100% dissociated in just 22 h. Nicked hyperglycosylated hCG was 100% dissociated 32x faster than non-nicked hyperglycosylated hCG standard (Table 2).

This supports the concept of extremely rapid nicking of hyperglycosylated hCG and extremely rapid dissociation of hyperglycosylated hCG following nicking (Figure 1).

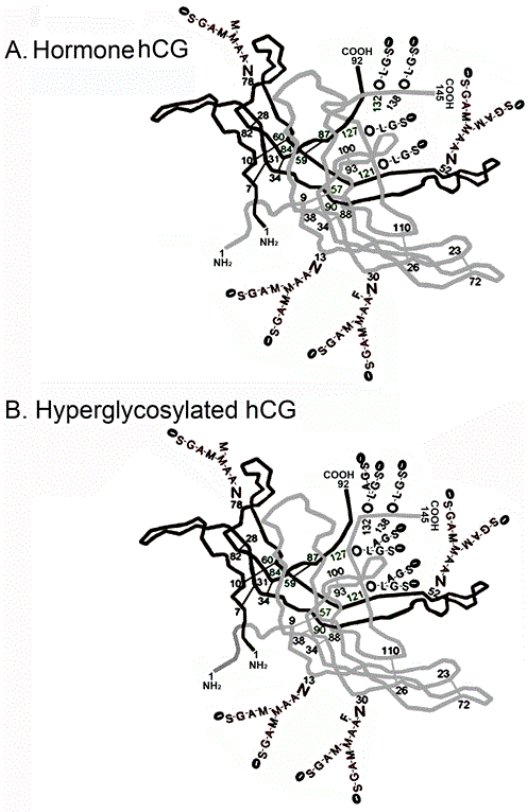


**Figure 1:** The three dimensional structures of the hormone hCG (panel A) and the autocrine hyperglycosylated hCG (panel B) as determined by Butler SA.

## Discussion

The research presented in this manuscript shows nicking of hyperglycosylated hCG by human leucocyte elastase, and the rapid dissociation splitting into an α-subunit and β-subunit, following nicking. Interestingly, even though the hormone hCG and the autocrine hyperglycosylated hCG are 97.2% structurally identical, leucocyte elastase cleaves hyperglycosylated hCG 18x faster that it cleaves the hormone hCG, furthermore hyperglycosylated hCG dissociates nicked hyperglycosylated hCG 32x faster than it dissociates the hormone hCG. It is concluded that hyperglycosylated hCG is

nicked and dissociated into separated subunits very much faster than the hormone hCG.

Intriguingly, hyperglycosylated hCG, which is rapidly nicked and dissociated, seemingly needs to be dissociated for TGF-β-II receptor action. Studies with hCG and cancer show that cancer cells produce a hyperglycosylated hCG free β-subunit, which retains the TGF-β-II receptor binding potential and activating the TGF-β-II receptor [4,6,29]. Studies show, that while the hormone hCG has no TGF-β-II activity, that dissociated hormone hCG β-subunit has full TGF-β-II biological activity [4,24,25]. Many researchers have used dissociated hormone β-subunit to stimulate cancer cells [4,6]. It is concluded that the TGF-β-II receptor binds a dissociated hCG β-subunit and not hyperglycosylated hCG [4].

The hormone hCG, in contrast, which is very slowly nicked and very slowly dissociated, must not be nicked or dissociated for maximal hormone LH/hCG receptor response [26-28,30]. The receptor only binds non-nicked intact hormone hCG.

It is established that the TGF-β-II receptor binds hyperglycosylated hCG dissociation products (nicked hyperglycosylated hCG β-subunit), and hyperglycosylated hCG free β-subunit as produced by non-trophoblastic neoplasms [4,24,25]. As such, the receptor contains a β-subunit-specific binding site.

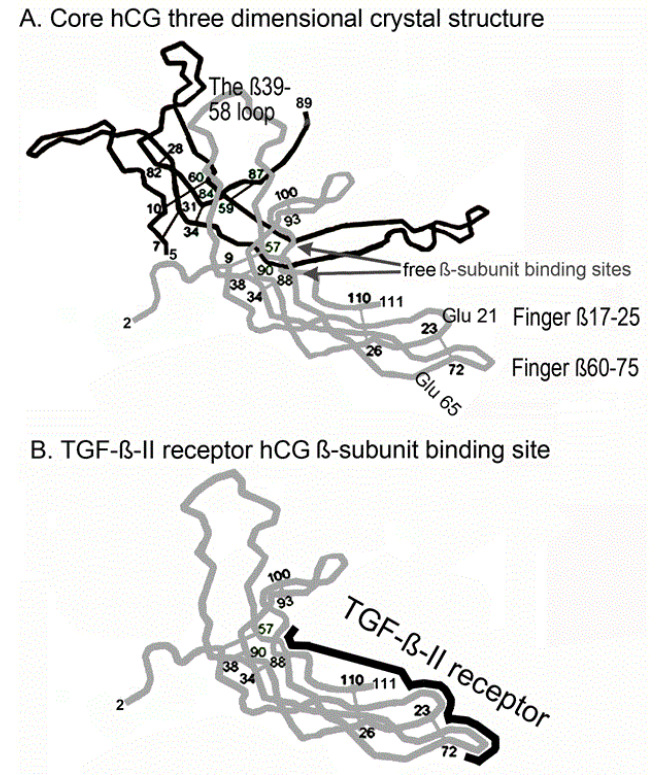


**Figure 2:** The three dimensional folding of hCG core molecules as shown by the crystal studies of Lustbader et al. [33], Wu et al. [34] and Lapthorn et al. [35] (Panel A). Panel B shows the proposed hCG β-subunit TGF-β-II binding site.

The TGF-β-II receptor binds two adjacent fingers on the TGF-β molecule terminating at Glu D55 and Glu E142 [31-33]. Examining the three-dimensional crystal structure of hCG as established by Lustbader et al. [34], Wu et al. [35] and Lapthorn et al. [36] analogous fingers terminating in Glu amino acids are found on both the hormone hCG and the autocrine hyperglycosylated hCG. This is β-subunit Glu residue β21 and Glu residue β65 on the adjacent fingers β17-25 and β60-75 (Figure 2). This is obvious TGF-β-II receptor binding site as bound by TGF-β and hyperglycosylated hCG free β-subunit.

Figure 2 shows the three-dimensional crystal structure of hCG as proposed by Lustbader et al. [34], Wu et al. [35] and Lapthorn et al. [36]. This illustrates the proposed β-subunit specific binding site of the TGF-β-II receptor, adjacent to the TGF-β-II -binding fingers.

Lustbader et al. [34], Wu et al. [35] and Lapthorn et al. [36] all went about using X-ray crystallography to examine the three-dimensional structure of hCG. All found that they had to delete the N-linked and O-linked sugar side chains on hCG, the β-subunit C-terminal peptide and other peptide sequences before crystals could be made [33-35]. They made crystals from an hCG molecule comprising α-subunit residues 5-89 and β-subunit residues 2-111 (Figure 1) with no N- or O-linked oligosaccharides. This was just 50% of the molecule by molecular weight. This was just the root structure of the hormone hCG and the autocrine hyperglycosylated hCG and did not help resolve the difference between these two major macromolecules (Figure 2).

Butler [4] in England had problems with how an autocrine and a hormone emerged with one single identical amino acid sequence. To investigate this, he combined the established X-ray crystallography structures of the 50% root hCG structure [34-36] with a thermodynamic computer-model of the carbohydrate segments of hCG and the β-subunit C-terminal peptide of the hormone hCG and the autocrine hyperglycosylated hCG (not published). Figure 1 shows the complete three-dimensional structure of the hormone hCG and the autocrine hyperglycosylated hCG as proposed by Butler SA.

Intriguingly, the Butler [4] models explain why the hormone hCG and the autocrine hyperglycosylated hCG are so different. It explains the research findings shown here, why hyperglycosylated hCG is rapidly nicked and dissociated, and why the hormone hCG is not nicked and only slowly dissociated.

As shown by the models, the folding of the β-subunit C-terminal peptide on the hormone hCG, missing in the crystal structure [33-36], involves folding into the β39-58 loop blocking any nicking at β47-48 before terminating at β145. As dictated by the O-linked oligosaccharide differences, the C-terminal peptide on hyperglycosylated hCG did not involve such blocking (Figure 1). In many respects the research described here confirms the correctness of the Butler SA models and the Butler SA models confirm the correctness of this research (Figure 1).

These findings (Figure 1) are contrary to the published findings by Lustbader, by Wu and by Lapthorn [34-36], who predict without examining the C-terminal peptide β110-145, that this segment is independent of the molecules three-dimensional structure, protruding as a random non-folded region projecting away from the molecule. This projection is very wrong, the C-terminal peptide being an intricately folded part of the molecule that controls the molecules destiny.

The only physical difference found between the hormone hCG and the autocrine hyperglycosylated hCG is four O-linked oligosaccharides of type one on the hormone hCG, and of type two found on the autocrine hyperglycosylated hCG (Figures 1 and 2). The O-linked

oligosaccharide difference on the hormone hCG must force the β-subunit C-terminal peptide to fold into loop β39-58 blocking nicking.

The seemingly correct Butler three-dimensional structures (Figure 1), shows the tiny structural difference between the two molecules, yet clearly explain the biochemistry phenomena of these two independent, separate molecules. This 2.8% molecular weight sugar difference is at the root of the only difference between the two molecules (Figure 1). It explains how we have an autocrine that binds its TGF-β-II receptor as a cleaved and dissociated nicked hyperglycosylated hCG β-subunit. And how we have a hormone that only binds its LH/hCG hormone receptor as an intact α-, β-dimer. Two very different molecules. Unless someone can find problems with the Butler three dimensional structures, which is very doubtful, they should be used as complete structures models to illustrate for the structural difference between the hormone hCG and autocrine hyperglycosylated hCG molecules.

## Conclusion

In conclusion, we have two very different molecules, the hormone hCG (a non-nicked dimer) and the nicked/cleaved hyperglycosylated hCG β-subunit that binds the two very different receptors. Thus we explain this extreme biochemical oddity of how two molecules, and how an endocrine and an autocrine can both have identical amino acid sequences and just difference in the O-linked olugosaccharides. We have an intact hormone and a TGF-β-II autocrine of the cleaved dissociated molecule.

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