Carbohydrate structures in the β-subunit of human chorionic gonadotropin play a dominant role in hormonal activity

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1. INTRODUCTION

Human chorionic gonadotropin (hCG) is a glycoprotein hormone containing 30% carbohydrate. It consists of two non-identical polypeptide chains, designated as α- and β-subunits. The α-subunit is common among other glycoprotein hormones including LH, FSH and TSH, whereas the β-subunit dictates the specific hormonal activities. The assignment of β-subunit as the functional specific unit derives largely from the hybridization studies between the two subunits of interhormones and interspecies, in which the specific hormonal activity of such hybride always follows the β-subunit present. Studies based on chemical and enzymatical modifications of carbohydrate moieties in hCG have revealed that the terminal sialic acid residues are important in prolonging the plasma circulatory life of the hormone, and that successive exoglycosidase treatments of asialo-hCG reduce its ability to stimulate the production of cAMP and steroids in gonadal cells [1–5]. However, the role of the carbohydrate structures of each subunit in the essential hormonal activities of hCG has not been investigated. We therefore addressed the following questions in an attempt to clarify the role of carbohydate moieties in the subunits:

Abbreviations: (A) and (A-HF) desialylated and HF-deglycosylated modifications, respectively

(i) How does the modification of one subunit affect hormonal activity?
(ii) Can the removal of galactose residues from the asialo-molecules regenerate the in vivo biological activity?
(iii) Do the carbohydrate structures in α- and β-subunits have a different role?

Here, we present modifications of α- and β-subunits by desialylation with neuraminidase, and followed by treatment with anhydrous HF in the presence of anisole [6,7]. Four representative recombinants from modified and intact subunits were prepared. Evaluation of their biological activities has revealed that these recombinants were 28–46% as active as the native hCG in a receptor binding assay. In an in vivo bioassay, the β-modified recombinants were completely inactive whereas the α-modified recombinants preserve 2% [α(A):β] and 10% [α(A-HF):β] of the potency of hCG. Our results suggest that the carbohydrate structures in the β-subunit play a dominant role in the determination of in vivo biological activity.

2. MATERIALS AND METHODS

2.1. *Hormone preparation*

Purified hCG (biological potency, 11600 IU/mg) and its α- and β-subunits were lot CR119 which were obtained from the Center of Population Research, National Institute of Child Health and Human Development. The α- and β-subunit preparations contained <0.2–0.05% of un-

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dissociated and/or reassociated hCG as determined by an in vivo bioassay.

2.2. *Carbohydrate modifications of hCGα and hCGβ subunits*

2.2.1. Desialylation

The preparation of desialylated hCG subunits was carried out as follows. To a solution of each subunit (lot CR119, 50 mg) in 0.5 M potassium acetate (pH 6.0, 2 ml) was added 0.55 ml of the enzyme solution (neuraminidase, EC 3.2.1.18, from *Clostridium perfringens*, 92 μg of type X, Sigma, St. Louis) dissolved in the same buffer. The reaction mixture was incubated for 2 h at 37°C. Purification and removal of the enzyme were achieved by Sephadex G-100 gel chromatography using a series of 4 connecting columns (1.6 × 343 cm) pre-equilibrated with 0.1 M NH_4HCO_3 (pH 7.9). The fractions containing subunits were pooled and twice lyophilized to give a white powder (~40 mg). Quantitative analysis of sialic acid as in [8] indicated the complete desialysation for both subunits.

2.2.2. HF/Anisole treatment

Desialylated subunits (20 mg each) obtained above were treated with anhydrous HF (10 ml) for 1 h at 0°C in the presence of anisole (1 ml) as scavenger. Purification was carried out by the Sephadex G-100 gel chromatography as mentioned above for asialo-subunits. It yielded a white powder (~10 mg) after lyophilization.

2.3. *Recombinations of subunits*

Two subunits (3 mg each) were dissolved in 0.1 M NH_4HCO_3 (pH 7.9, 300 μl). The mixture was incubated for 24 h at 4°C, and lyophilized in a high vacuum rotatory concentrator (Savant). The residue was dissolved in 0.1 M NH_4HCO_3 (150 μl), and purified by HPLC using one TSK 2000SW and two 3000SW columns (Beckman Instrument, Calif.) in series. The fractions showing receptor binding activity were pooled and twice lyphilized to yield 3–4 mg of each recombinant.

2.4. *Bioassays*

Receptor binding activity was determined using a rat testis homogenate and ^{125}I-labeled hCG (lot CR121) as tracer [9]. Statistical analyses were performed by the computer program in [10].

The biological activity of hCG recombinants was evaluated by the uterine weight assay employing 20–21-day-old female Holtzman rats. After subcutaneous injections of test solution (0.5 ml) in 0.05 M phosphate–0.1 M NaCl buffer (pH 7.4) for 3 consecutive days, uteri were excised and weighed on the fourth day. The potency was calculated as in [11].

2.5. *Analytical methods*

The amino acid analyses were carried out by a Beckman Model 121MB analyzer after hydrolyses of the samples in constant-boiling HCl (24 h, 110°C). Glucosamine and galactosamine were also determined by the amino acid analyzer after hydrolysis in 3 M HCl at 100°C for 3 h. Neutral sugars (i.e., mannose, galactose and fucose) were determined by the procedure in [12], and sialic acid as in [8].

3. RESULTS

Desialylation of these subunits by neuraminidase treatment followed by Sephadex G-100 chromatography yielded preparations of purified asialo-hCGα and asialo-hCGβ. Table 1 shows that both asialo-subunits were completely free of sialic acid. Further deglycosylation was accomplished by the HF/anisole treatment. After purification by Sephadex G-100 gel chromatography, deglycosylated

Table 1

Monosaccharide compositions of the modified hCG subunits

Sugar	hCGα		hCGβ	
	Asialo	Asialo-HF	Asialo	Asialo-HF
NeuNAc	0	0 (0)	0	0 (0)
Gal	3.0	0 (0)	6.3	0 (0)
Man	5.6	1.6 (29)	5.2	1.5 (29)
Fuc	–	–	1.2	0 (0)
GlcNAc	7.3	3.0 (41)	5.6	2.6 (46)
GalNAc	–	–	2.8	2.1 (75)

Values are expressed as mol monosaccharide/mol protein, and those in parentheses represent % of monosaccharide remaining after the HF-treatment

hCGα and hCGβ were obtained. Table 1 shows their monosaccharide compositions. Based on the structure of carbohydrate moieties in native hCG, the HF treatment cleaves predominantly the saccharide linkages at the mannose residues of the Asn-linked moiety and at the galactose residue of the Ser-linked moiety. The absence of galactose in both subunits and fucose in the β-subunit after the HF-treatment is worth noting. Furthermore, no difference in amino acid compositions was detected between the native and desialylated or deglycosylated molecules of two subunits.

Four recombinants [α(A):β, α(A-HF):β, α:β(A) and α:β(A-HF)] were prepared and purified by the gel-HPLC chromatography. The representative chromatogram is shown in fig. 1. All recombinants showed a symmetrical peak and were well-separated from the excess α-subunits. In order to minimize any possible contaminants of the non-recombined subunits, only these earlier eluants exhibiting high receptor binding activity were collected. The amino acid analyses of all recombinants revealed that their compositions were in close agreement with the expected stoichiometry of the unmodified hCG.

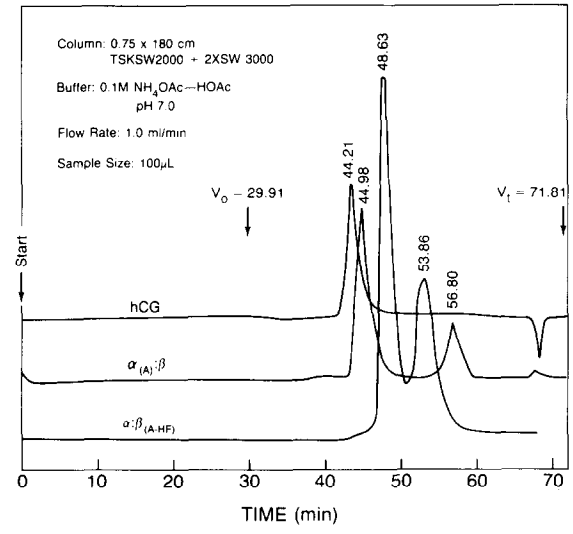


Fig. 1. High-performance gel chromatography of hCG and recombinants α(A)–β and α:β(A-HF). Ordinate is absorption at 225 nm. V_0 and V_t are void and total volume of columns, respectively. Elution volume (ml) for each component is indicated on the top of peak. Injected solution was ~2 mg protein/ml.

Table 2

Biological activity of hCG recombinants

hCG and analogues	Relative potency	
	Receptor binding assay	Uterine weight assay
hCG (lot CR119)	1.0	1.0
α(A):β	0.36 ± 0.04	0.019 ± 0.007
α(A-HF):β	0.28 ± 0.03	0.094 ± 0.010
α:β(A)	0.46 ± 0.05	n.d.
α:β(A-HF)	0.17 ± 0.02	n.d.

Values are expressed relative to mol hCG ± SE n.d., not detectable at a total dose of 3 μg/rat

Table 2 shows the radioligand–receptor binding activity of 4 recombinants. It is obvious that all recombinants displayed lower activity than hCG in competing with ^{125}I-hCG for binding to the rat testis homogenate. Desialylation of either α- or β-subunit in hCG reduced the binding potency to the extent of 36–46%. The HF treatment reduced further their potencies. The decreased binding activity of α:β(A-HF), compared with other recombinants, is most obvious. It may suggest that deglycosylation of the β-subunit causes a significant inactivation of the hormone binding process.

In the in vivo immature rat uterine weight assay, intact hCG (lot CR119) doubled uterine weight at 50 ng/rat. In parallel assays, both α:β(A) and α:β(A-HF) did not display any significant responses at 3 μg/rat. In contrast, the α(A):β species exhibited measurable activity at the same dose level, which was equivalent to a potency of 20 IU/mg or 2% of hCG (table 2). Replacement of α(A) by further deglycosylated α(A-HF) to form α(A-HF):β increased the bioactivity 5-fold over that of α(A):β, although their receptor binding activities were of the same order.

Collectively, these results indicate that the β-subunit does not tolerate modifications on the carbohydrate moieties whereas the α-subunit is relatively inert in maintaining the biological activities, when the subunit recombines with the intact counterpart. Furthermore, it is intriguing that the removal of galactose residues from the α-subunit causes the increase in the in vivo biological activity.

4. DISCUSSION

It has been recognized that asialo-hCG possesses insignificant in vivo biological activity [3,4]. On the other hand, it largely retains in vitro activities of receptor binding, cAMP and testosterone stimulations [1,2]. The cause of in vivo inactivation has been correlated with the marked decrease in plasma half-life due to the exposure of galactose residues at the termini of carbohydrate chains. Accordingly, a galactose-recognizing receptor in liver parenchymal cells quickly binds to asialo-hCG for metabolism and prevents the modified hormone from reaching its target organs [14]. The additional removal of galactose from asialo-hCG by β-galactosidase did not affect the receptor binding properties, but reduced stimulation of cAMP formation [4]. Similarly, the HF-deglycosylated hCG displayed higher testis receptor binding activity but at least 100-fold lower stimulation of cAMP and testosterone production in Leydig cell than those of the intact hCG [7]. The deglycosylatd hCG exhibited no in vivo biological activity as a result of modification in the carbohydrate moieties on both α- and β-subunits. Modifications of the galactose residues of asialo-hCG prolonged plasma half-life from 0.5 min to 10–17 min (49 min for intact hCG) in [15]. The in vivo ovarian uptake of those derivatives was substantially increased over that of asialo-hCG. However, they neither evaluated the in vivo biological activity nor directed at the question of how the modification of each subunit in hCG can affect the overall hormonal activity.

We have taken several precautions in the purification of both modified subunits and their recombinants. The recombinants were examined by HPLC, receptor binding activity and amino acid composition to ensure that they contained no free subunit. From the result of receptor binding experiments, all 4 recombinations exhibited 17–46% activity of hCG. The low activities of both α(A):β and α:β(A) appear to be in contradiction to the previous finding that asialo-hCG is as active as hCG in both receptor binding and steroidogenic activities in vitro [1,2]. However, the dissociated and subsequently re-associated hormone has displayed reduced in vivo biological activity as compared to that of the original hormone [13]. In addition, it is not yet known whether the reassociated hormone with both asialo-subunits will be as active as the asialo-hCG in vitro. Nonetheless, the complete absence of in vivo biological activity in α:β(A) and α:β(A-HF) is obvious. On the other hand, α(A):β and α(A-HF):β displayed some in vivo biological activity despite that all 4 recombinants showed nearly the same order of binding activity. Collectively, desialylation reduced significantly the in vivo biological activity of recombinants. The complete absence of activity in α:β(A) and residual activity in α(A):β indicates that the β-subunit is more sensitive to desialylation-related reduction of biological activity.

The increased activity of α(A-HF):β, as compared to α(A):β, reflects the fact that the absence of terminal galactose residues may cause the hormone to be relatively inert for the uptake by galactose receptor in liver. Unlike the case of α(A):β vs α(A-HF):β, α:β(A-HF) failed to regenerate the in vivo activity. These data further suggest that importance of carbohydrate structures differs between the α- and β-subunits, and that the β-subunit plays a dominant role in determining the hormonal activity of hCG. Perhaps the carbohydrate structure in the β-subunit carries the essential functional message necessary to convey the hormonal signal to the target cells.

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