Evidence for a conformational change in deglycosylated glycoprotein hormones

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Removal of long-chain asparagine-linked carbohydrates leads to loss of receptor-effector coupling in gonadotropin target tissue This implies a direct interaction between carbohydrate and cell membrane components To examine other mechanisms by which carbohydrate could activate post-receptor events, we have used sequence-specific and conformation-specific α-subunit radioimmunoassays as probes for conformational changes in deglycosylated choriogonadotropin and follitropin Immunoreactivity of either hormone was enhanced 8–13-fold after removal of carbohydrate by anhydrous hydrogen fluoride Removal of sialic acid alone had little effect on reactivity Based on the specificity of the antisera, the effect could be localized to a region in the amino-terminus remote in the linear sequence from actual sites of carbohydrate attachment The results suggest that a conformational change in the α-subunit could account, at least in part, for the observed effects of deglycosylation on glycoprotein hormone action

Carbohydrate *Glycoprotein* *Follitropin* *Choriogonadotropin* *Conformation* *Membrane receptor*

1. INTRODUCTION

There is now extensive evidence that carbohydrate side-chains are essential for the target-organ actions of the several glycoprotein hormones: lutropin (LH), follitropin (FSH), choriogonadotropin (hCG) and thyrotropin (TSH). Several laboratories have shown that receptor binding is unchanged or even enhanced, while post-receptor events such as adenylate cyclase activation or steroid production are markedly diminished after deglycosylation of the hormone by enzymatic or chemical means [1–6].

The mechanism by which these side-chains exert their effects on biological activity have not yet been established. Although the results to date point to a direct role for carbohydrate in receptor/cyclase 'coupling', it is also possible that subtle conformational changes in the peptide chain might account, at least in part, for the observed effects of carbohydrate removal.

We have used immunoassays based on region-specific antisera to α-subunit to provide evidence that a conformational change does in fact occur consequent to chemical deglycosylation of both hCG and human follitropin

2 MATERIALS AND METHODS

hCG was purchased in partially purified form from Organon Ltd (Oss, The Netherlands) and purified by gel filtration and ion-exchange chromatography [6,7]. α-Subunit was separated by urea/DEAE chromatography and gel filtration [7]. Purified human FSH (hFSH; preparation AFP-4161B) was obtained from the National Hormone and Pituitary Program (Baltimore, MD). Reduced, carboxymethylated (RCXM) hormones and subunits were prepared by treatment with dithiothreitol/guanidine hydrochloride followed by iodoacetic acid as described [6].

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2.1. *Deglycosylation*

Aliquots (2–5 mg) of whole hCG, α-subunit, or hFSH were treated with anhydrous HF without scavenger for 1 h at 0°C in a Kel-F distillation apparatus (Protein Research Foundation, Osaka, Japan) [3,6]. Modified hCG and hFSH were separated from cleaved carbohydrate by gel filtration on Sephadex G-100 (Pharmacia, Piscataway, NJ) eluted with 0.2 M ammonium bicarbonate buffer, pH 8 7 [6]. α-Subunit was separated using Sephadex G-25 with 0.1 M acetic acid. Desialylated hCG and α-subunit were prepared by treatment with neuraminidase (Worthington, Freehold, NJ) at pH 5.0 for 1 h at 37°C [7].

2.2 *Radioimmunoassays*

Assays for native hCG and its subunits and for hFSH were carried out by a double-antibody technique described by Faiman and Ryan [8], using radioiodinated native hormone as tracer. The respective antisera employed are detailed in table 1. Sequence-specific immunoassays from RCXM α-subunit were done as described by Keutmann et al. [9], using 3 amino-terminal-directed antisera: nos 101, 110 and 118 RCXM-α was used as antigen, labeled tracer and reference standard The conformation of the α-subunit was also probed using a monoclonal antibody (no.A101), raised against native hFSH and reacting predominantly with the α-subunit in associated native hFSH (Hojo, H. and Ryan, R.J., in preparation). Details of the assay are given in section 3 and the legend to fig.4.

The true peptide weight of each hormone preparation was fixed by amino acid analysis, and relative potencies of deglycosylated vs native hormone or subunit were computed from the response curves as in [6]

3 RESULTS

3.1. *Chemical properties of deglycosylated hormones*

Consistent with our earlier findings, HF treatment removed 80% of long-chain, asparagine-linked carbohydrate from hCG, hFSH and α-subunit. Remaining carbohydrate was predominantly *N*-acetylglucosamine adjacent to asparagine residues (normally resistant to cleavage by HF), and 6–12% of mannose residues from the 'core' region of the chain. Detailed chemical analysis [6] showed the peptide chain to be intact after HF treatment

3 2 *Assays of hCG with sequence-specific antisera*

Immunoreactivity of hCG with antisera to native hormone or α-subunit was reduced by 30–60% after deglycosylation [6] (table 1a). By contrast, when tested in assays for RCXM–α-subunit, reactivity of whole hCG and hCG-α was markedly enhanced after deglycosylation (fig.1). Displacement curves for α-subunit were parallel, and the relative potencies of deglycosylated α-subunit were 8–13-fold higher than unmodified subunit (table 1b). Reactivity of unmodified whole hCG was typically weak, but assumed a slope parallel to α and RCXM-α with a similar increased

Table 1

Immunoreactivity of deglycosylated hCG and α-subunits

Hormone	Antiserum	Relative potency
(a) Assays with native antisera		
(Degly) hCG	hCG R2000	0 41
(Degly) hCG	hCG R2005	0 60
(Degly) hCG	hCG-α SA-6	0 69
(Degly) hCG	hCG-β R1-10	0 87
(b) Assays with RCXM-α antisera (deglycosylated hormones)		
(Degly) hCG	101	13 1
(Degly) hCG	110	8 0
(Degly) α	101	7 8
(Degly) α	110	10 8
(Degly) α	118	13.3
(Degly) RCXM-α	101	1 25
(Degly) RCXM-α	110	1 07
(c) Assays with RCXM-α antisera (desialylated hormones)		
(Asialo) hCG	101	1 40
(Asialo) hCG	110	1 00
(Asialo) hCG	118	1 13
(Asialo) α	101	1 71
(Asialo) α	110	1 49
(Asialo) α	118	1 40

Relative potency = activity ratio (treated hCG)/(untreated hCG) or (treated α)/(untreated α)

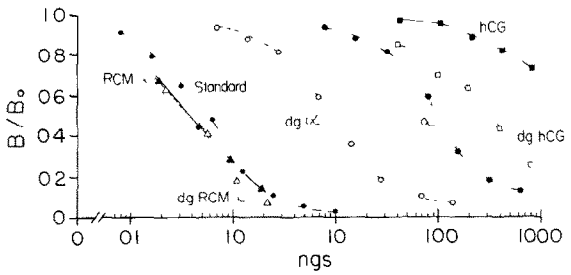

Fig 1. Displacement curves for hCG and α-subunit preparations in a radioimmunoassay with sequence-specific antibody (no 110). Abscissa shows peptide weight (ng) by amino acid analysis, and ordinate shows proportion of total bound ^{125}I-labeled RCXM–α tracer displaced by increasing doses of hormone. Immunopotency of both whole hCG and α-subunit were enhanced after deglycosylation, while no change was detected after deglycosylation of RCXM–α antigen.

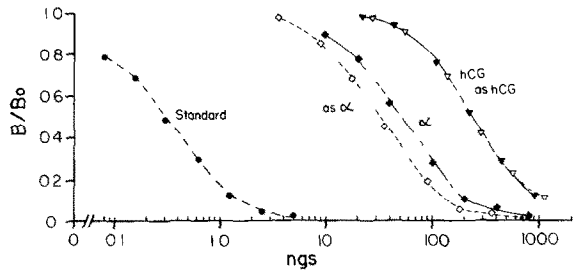

Fig 2. Dose-response curves for desialylated (As) and native hCG preparations in sequence-specific radioimmunoassay using antibody no 110 Increase in reactivity was minimal compared to more completely deglycosylated preparations (fig.1) Coordinates are labeled as in fig.1

potency after deglycosylation (fig.1). It should be noted that even after deglycosylation, the reactivities of the fully folded native hormones were about 100-fold less than the linearized RCXM-α antigen.

RCXM–α-subunit itself was tested before and after deglycosylation. The untreated peptide was, as expected, similar in reactivity to our purified RCXM–α standard, and no significant change in reactivity was observed following deglycosylation (fig.1, table 1b). Removal of terminal sialic acid from hCG or α-subunit effected a slight increase in immunoreactivity with the RCXM–α antisera, ranging from equivalent to 1.7-fold depending on the antiserum employed (fig.2, table 1c).

3.3. *Assays of hFSH with native and sequence-specific antisera*

Reactivity of deglycosylated hFSH was reduced to 42% of untreated hormone when tested with native hFSH polyclonal antiserum 12456 (fig.3a). Results of assays with RCXM–α antisera were comparable to those obtained with hCG; reactivity with As no.110 was enhanced 7-fold after

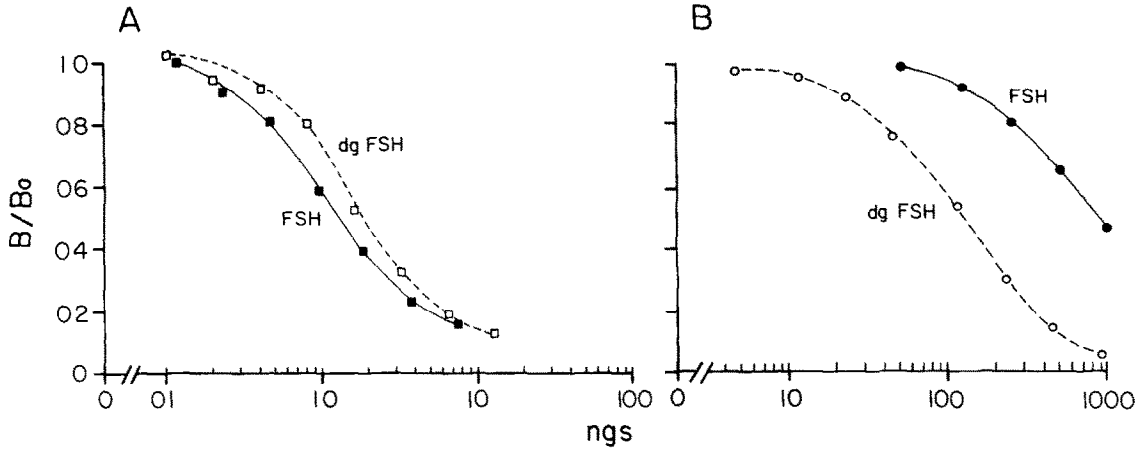

Fig 3. (A) Displacement curves for native human FSH in assay using polyclonal anti-hFSH antibody 12456, showing decrease in reactivity of hFSH after deglycosylation (B) Same preparations in sequence-specific assay using antibody no.110, showing increase in reactivity of deglycosylated hFSH

Table 2
Immunoreactivity of deglycosylated hFSH and α-subunits

Hormone	Antiserum	Relative potency
(a) Assay with polyclonal native antiserum		
(Degly) hFSH	hFSH 12456	0.42
(b) Assays with RCXM-α antisera		
(Degly) hFSH	101	12 0
(Degly) hFSH	110	7 0
(Degly) hFSH	118	9 0
(c) Assays with monoclonal antibody to α subunit of native hFSH		
(Degly) hFSH	A101	0 019
Native α	A101	0.003
(Degly) α	A101	0 025[a]
RCXM α	A101	<0 0007

[a] Potency (degly α)/(native α) = 7 5

Relative potency = activity ratio (degly hFSH or α-subunit)/(native hFSH)

deglycosylation (fig.3b) and similar increases were observed with As nos 101 and 118 (table 2b).

3.4. *Assays with a conformationally dependent monoclonal antibody*

Monoclonal antibody no A101 recognized α-subunit in a manner highly specific for associated

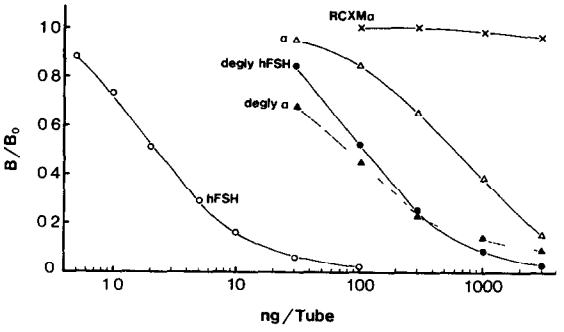

Fig 4 Dose-response curves for native and deglycosylated hFSH and α-subunit using a conformationally dependent monoclonal antibody Incubation mixtures contained 2 ng ^{125}I-labeled hFSH and 9 ng monoclonal antibody A no 101, incubated in 1 ml of 40 mM Tris-HCl, pH 7 4, for 24 h at 20°C and separated by the double antibody technique [8] Absolute (specific) binding was 30% Relative potencies are summarized in table 2c

hFSH; whole hFSH reacted 300-times greater than free α-subunit and 34-times greater than hCG (Hojo, H. and Ryan, R.J , in preparation). Reactivity with hFSH was markedly diminished after deglycosylation, to a potency only 2% of untreated hormone (fig.4; table 2c) Deglycosylated α-subunit was increased in reactivity by 7.5-fold over untreated α. Deglycosylated hCG was slightly (1.36-fold) increased in potency over native hCG.

4. DISCUSSION

The asparagine-linked long-chain carbohydrate on both subunits is clearly essential for coupling of receptor to post-receptor events in gonadotropin target tissues. The requirement for carbohydrate appears to occur at an early stage after receptor binding, since both the nucleotide-binding protein and adenylate cyclase enzyme in ovarian cells remain responsive to guanine nucleotides in the presence of deglycosylated hCG [10].

These observations imply a direct involvement of carbohydrate, either by influencing the conformation of the hormone-receptor complex or through interaction with a carbohydrate-binding moiety or lectin in the cell membrane. We have been unable to restore adenylate cyclase activity by simply adding back the cleaved carbohydrate fraction after deglycosylation. However, Calvo and Ryan [11] have shown that glycopeptides containing intact carbohydrate can inhibit activation of ovarian adenylate cyclase by native hCG. The effect is specific, since no inhibition was observed with glycopeptides or oligosaccharides from other sources. Carbohydrate binding to a membrane lectin component could, for example, promote receptor crosslinking as an initial step in cyclase activation [10,11].

As an alternative explanation, a conformational change affecting one or both subunits is important to consider, since both antigenic and receptor binding sites in the glycoprotein hormone molecule appear to be highly dependent on 3-dimensional structure. Direct measurements by circular dichroism (CD) [3,5,6] or ANS fluorescence [3] have not revealed major conformational changes in hCG after deglycosylation. Evidence for increased heat stability [12] and an enhanced rate of LH subunit reassociation as measured by tyrosine spectrum [13] suggested some alteration in confor-

mation. Although major conformational changes were not apparent by CD measurements of deglycosylated hCG [6], we have found differences in the CD spectrum between deglycosylated and intact hFSH (Charlesworth, C., Ryan, R.J. and Keutmann, H T , unpublished). Our additional evidence for a conformational change, based on a small but consistent decrease in immunoreactivity of hCG with native polyclonal antisera after deglycosylation, prompted us to examine the HF-treated preparations with region-specific and conformationally dependent antibodies

Antisera prepared against RCXM α-subunit generally have reacted only weakly with native hormone [9,14,15]. Our current results show that, while still substantially lower than RCXM-α-subunit itself, the reactivity of both α-subunit and whole hormone after deglycosylation is strikingly increased over that of the untreated native peptides. The findings suggest a change towards a more 'linear' conformation in the sequence region recognized by the antiserum.

Studies with the conformationally dependent monoclonal antibody A no.101 also indicated a change in the conformation of hFSH-α. This antibody recognizes the α-subunit of hFSH better than the α-subunit in hCG and free α-subunit (see section 3). Deglycosylation strikingly diminished reactivity with the associated FSH α-subunit to only 2% of the untreated hormone, while recognition of the free subunit was enhanced. These effects appear specific for FSH because reactivity with hCG was not markedly altered after deglycosylation. The results also extend to deglycosylated subunit observations made previously that the conformation of the α-subunit is influenced by the presence and type of β-subunit [16].

We were able to rule out several other potential causes of enhanced immunoreactivity. Clips in the peptide chain as a result of HF treatment might produce reactive linear segments or altered conformation despite intact disulfide bridges However, we have shown by detailed chemical and sequence analyses [6] of these and other preparations that the peptide chain remains intact and undamaged after the deglycosylation procedure. Neuraminidase-treated hCG preparations were assayed since there is evidence that sialic acid alone can influence reactivity of hCG with anti-native antisera [17,18]. We found desialylation to enhance reactivity only slightly, if at all, with our RCXM-α antisera. Furthermore, since immunoreactivity of the RCXM-α antigen itself was unchanged after HF treatment, direct involvement of carbohydrates in antibody recognition was not a factor. Local changes close by the sites of carbohydrate attachment also are unlikely in view of a recent report [19] that the conformation of adjacent amino acids is not affected by glycosylation.

The 3 RCXM-α antisera used here were directed toward a segment close to the amino-terminus of the α-subunit [9]. This region is remote in the linear sequence from the sites of carbohydrate attachment at positions 52 and 78, although in the folded native subunit it may be looped into a position vicinal to one or both carbohydrates. The recognition site includes an aromatic residue (Phe) at position 17, shown by structure-function studies with ovine and porcine LH to be 'exposed' and perhaps accessible to the cell surface in the native hormone [20–22]. The cystine disulfide bridge between residues 11 and 35 in bovine LH-α (corresponding to cysteines 7–31 in hCG-α) has been selectively reduced and carbaminomethylated and the modified subunit reassociated with β-subunit [22]. The recombinant bound to receptors and the CD spectrum was not changed despite loss of the disulfide linkage. This offers a precedent for a molecule with altered conformation in the N-terminal region of α-subunit showing subunit-association, receptor-binding and CD properties similar to those found in the deglycosylated hormone.

The concept of a conformational change is supported by the results of Rebois and Fishman [23] who restored adenylate cyclase activity to Leydig cell incubates of deglycosylated hCG by addition of anti-hCG antisera Since antisera to α-subunit did not restore activity, the sequence region involved may be different from that identified in our studies. Although their findings suggest that the deglycosylated molecule can be reformed readily to an active conformation, the polyvalent antibody might also be activating adenylate cyclase by crosslinking or redistributing the hCG-receptor complexes [10,11,24].

Antisera to other regions of hCG and LH are in preparation for use as probes for conformational changes elsewhere in the molecule. While our present results are consistent with an influence of

carbohydrate on the conformation of the α-subunit, it remains to be determined whether the effects of deglycosylation can be attributed to this factor alone, to altered interaction between carbohydrate and membrane components, or both. Ultimately, since deglycosylation affects both receptor binding (enhanced) and cyclase activation (diminished), each mechanism may prove responsible for actions at separate stages of the target cell response.

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