Vol. 122, No. 3, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 16, 1984 Pages 1260-1267

AN OLIGOSACCHARIDE OF THE O-LINKED TYPE DISTINGUISHES THE FREE FROM THE COMBINED FORM OF hCG α SUBUNIT

Laurence A. Cole*, Fulvio Perini*,
Steven Birken+ and Raymond W. Ruddon*

Departments of Internal Medicine and Pharmacology*,
University of Michigan Medical School, Ann Arbor, MI 48109.

Department of Medicine+, College of Physicians and Surgeons
of Columbia University, New York, NY 10032

Received June 22, 1984

SUMMARY: JAR malignant trophoblast cells produce a free α subunit in addition to an α combined with β subunit as hCG. The free α is larger by gel chromatography and SDS-PAGE than combined α and is unable to associate with β subunit to form hCG. A tryptic fragment, representing amino acid residues 36-42, derived from free α was larger than the corresponding fragment from combined α. After neuraminidase treatment, the fragment from free α bound peanut lectin agarose, which is specific for Galβ1-3GalNAc as found in O-linked oligosaccharides. The fragment also contained Gal and GalNAc (and a lesser amount of GlcNAc) as determined by glycosidase sensitivity and amino sugar analyses. Removal of this tryptic fragment ablated the size difference between free and combined α subunits.

INTRODUCTION: hCG is a glycoprotein hormone composed of two dissimilar subunits, α and β, joined non-covalently. hCGα[1] contains two N-linked oligosaccharides, while hCGβ contains four O-linked in addition to two N-linked oligosaccharides. The hCG dimer is produced by the trophoblast in pregnancy and by trophoblastic neoplasms (1). In addition to αβ dimer, these cells secrete free α and free β (1-3). Whereas the free β secreted by JAR trophoblastic cells is similar in size to β contained in dimer (M_r = 36,000) and associates with hCGα to form hCG (2), the free α (M_r = 24,000) is larger than combined α (M_r = 22,000) and is unable to associate with hCGβ to form hCG (1,2,4). Thus, free α may have a regulatory role in dimer formation. Here we compare the two forms of α subunit and show that one tryptic peptide of the free form contains an O-linked type of oligosaccharide that accounts for the size difference between the free and combined forms.

[1] Abbreviations: hCG-α, α component of hCG; free α, free α-subunit; hCGβ, β component of hCG; free β, free β subunit; PNA-agarose, peanut agglutinin linked to agarose; M_r, relative molecular mass as determined by SDS polyacrylamide gel electrophoresis; PBS, phosphate buffered saline, pH 7.5; RIA, radioimmunoassay; DPCC, diphenylcarbamyl chloride; GalNAc, N-acetylgalactosamine; GalNAc-ol, N-acetylgalactosaminitol; GlcNAc, N-acetylglucosamine; GalN, galactosamine; GlcN, glucosamine.

0006-291X/84 $1.50

MATERIALS AND METHODS: Highly purified standard hCGα and hCGβ (batch CR123) were kindly provided by the Hormone Distribution Program, NIADDKD. Asialo hCGβ tryptic COOH-terminal peptide fragments were prepared as previously described (5). RIAs of standard hCG preparations and hCGβ tryptic peptide fragments, gel filtration on Bio-Gel P100, and amino acid and amino sugar analyses were all carried out as previously described (4-8).

Samples containing 1 mg protein in 1 ml PBS were digested with 10 µl DPCC treated trypsin (Sigma; 3 mg/ml in 0.001 M HCl) for 2 hr at 25°C. Reactions were terminated by heating 3 min at 100°C. For neuraminidase and β-galactosidase digestions, pH was adjusted to 5.0 by the addition of sodium citrate. Neuraminidase digestions were carried out as previously described (7). Digestions with bovine testis β-galactosidase (gift from Dr. I. Goldstein of The University of Michigan) were carried out for 24 hr at 37°C. One unit of enzyme was added to start reactions and a second unit was added at 12 hr. Gel filtration on a 1 x 110 cm column of Bio-Gel P4 (200-400 mesh) was carried out in 0.1 M NH_4HCO_3, pH 7.8. Samples, 1 ml, plus markers (0.25 mg Vitamin B_{12} and 0.1 mg ^{3}Tyr) were applied to the columns, which were eluted at a rate of 4 ml/hr; 1 ml fractions were collected.

PNA-agarose lectin affinity chromatography was carried out as follows: Samples (1 ml) were applied to 0.7 x 25 cm columns, which were equilibrated with PBS. The columns were first washed with 72 ml PBS, and then with 30 ml PBS containing 0.2 M lactose (15 ml/hr, 2 ml fractions collected).

JAR malignant trophoblast cells were maintained in culture as previously described (4). JAR cells (70% confluent in 150 cm^2 flasks) were cultured 20 hr in Leu-free media containing 10% dialyzed fetal bovine serum and a mixture of [^{3}H]Leu, [^{3}H]Pro and either [^{3}H]Man or [^{3}H]GlcN; total radioactivity 5 mCi/flask (2). Radioactive free α and hCGα were purified from the media by methods similar to those previously described (2). Briefly, hCGβ antiserum linked to Sepharose was used to extract hCG dimer + free β from the media. After repeating this adsorption step to insure complete removal of dimer, free α was extracted by adsorption with hCGα antiserum linked to Sepharose. Bound components were eluted with guanidine hydrochloride, desalted and lyophilized. Free α preparations were taken up in PBS and further purified by gel filtration on Bio-Gel P100. Free α eluted as a single peak prior to the position of standard hCGα. Aliquots of free α preparations migrated as single bands on SDS polyacrylamide gels (M_r = 24,000). hCG + free β preparations were taken up in 1 ml of 4 M guanidine hydrochloride, pH 4; dimer was dissociated by incubation for 30 min at 37°C, and then subunits were separated by gel filtration on Bio-Gel P100. The hCGα components eluted as a single peak in the position of standard hCGα. Aliquots of hCGα preparations migrated as single bands on SDS polyacrylamide gels (M_r = 22,000). As a carrier protein, myoglobin, 1 mg/ml, was added to free α and hCGα preparations.

RESULTS AND DISCUSSION: As previously reported, PNA specifically binds asialo O-linked oligosaccharides of the structure Galβ1-3GalNAc (9) and binds asialo hCG through such O-linked oligosaccharides on the β subunit (6). As indicated in Table 1, PNA-agarose binds neuraminidase digested standard hCGβ and JAR hCGβ but not the subunits also treated with β-galactosidase, which cleaves the lectin determinant. Binding to PNA-agarose was the first indicator that JAR free α, but not hCGα, contains an O-linked oligosaccharide. Neuraminidase treated hCGα from JAR cells was unbound on PNA-agarose, but 75% of the similarly treated free α preparation was retarded (eluted with column buffer later than the unbound component) by PNA-agarose. The interaction of free α with PNA-agarose was ablated by β-galactosidase treatment. That free α was

Table 1. PNA-agarose affinity chromatography[a]

Substrate	% Unbound	% Retarded	% Bound
Standard hCG preparations			
hCG	94		6
hCGβ	95		5
hCGα	100		
Neuraminidase digested standard hCG preparations			
hCG			100
hCGβ			100
hCGβ, also β-galactosidase digested	97		3
hCGα	100		
Neuraminidase digested standard hCGβ tryptic COOH-terminal peptide fragments			
134-145 with 1 O-linked oligosaccharide		5	95
123-133 with 2 O-linked oligosaccharides			100
123-145 with 3 O-linked oligosaccharides			100
133-145 synthetic pentadecapeptide	100		
Neuraminidase digested JAR biosynthetically-labeled preparations			
hCGβ	3		97
hCGβ, also β-galactosidase digested	100		
free β	4		96
hCGα	94		6
free α	25	75	
free α, column equilibrated with 0.2 M lactose	100		
free α, also β-galactosidase digested	100		
Neuraminidase digested JAR α-subunit tryptic peptides (from Fig. 1B)			
hCGα pool 1	98		2
2	100		
3	100		
free α pool 1	95		5
2	100		
3'	22		78
3', also β-galactosidase digested	100		

[a]Standard hCG and hCGβ tryptic peptide preparations were made as described in "Materials and Methods". JAR biosynthetically-labeled α-subunit tryptic fragments were separated on Bio-Gel P4 as shown in Fig. 1B. Pools were lyophilized then taken up in 0.1 M sodium acetate buffer, pH 5, and neuraminidase digested. Samples were applied to PNA-agarose columns, eluted and fractions collected as described in "Materials and Methods". Fractions containing standard hCG preparations and hCGβ tryptic peptides were quantitated by RIAs, and those containing JAR preparations by their radioactivity. The percent unbound is that component in the void volume, the percent retarded is that component eluted with PBS somewhat later than the void volume (2-6 column volumes) and the percent bound is that component only eluted with 0.2 M lactose.

retarded rather than bound (i.e., eluted with a specific inhibitor) by the lectin suggested a weak interaction.

We examined the known amino acid sequence of standard hCGα (1) for a potential site of O-glycosylation (Thr or Ser, commonly with at least one adjacent prolyl residue, 10). Such a site is the peptide Ala-Tyr-Pro-Thr-Pro-Leu-Arg (residues 36-42). As reported (11), this peptide is one of the

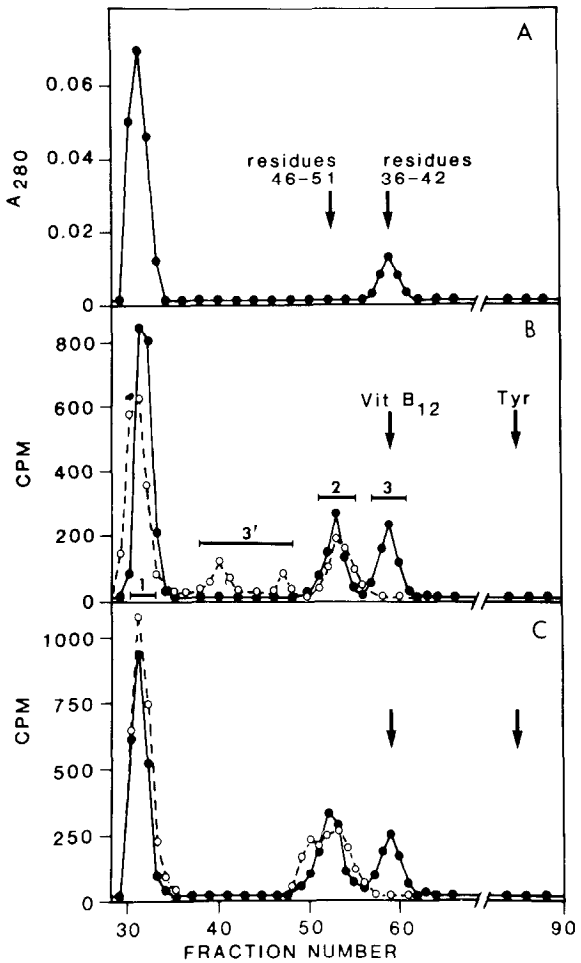

Fig. 1. Gel filtration on Bio-Gel P4 of tryptic digests of standard hCGα and JAR [^{3}H]Leu/Pro/GlcN biosynthetically-labeled α-subunit preparations. Panel A shows the tryptic digest of 2 mg standard hCGα. Fractions containing peptides with aromatic amino acids were monitored by their absorbance at 280 nm, other fractions by amino acid analyses. Panel B shows JAR biosynthetically-labeled hCGα (●——●) and free α (O-----O) tryptic digests. Samples were applied to columns together with elution markers, vitamin B_{12} and Tyr. In eluate fractions tryptic peptides were identified by their radioactivity and elution markers (arrows) by their absorbance at 280 nm. As illustrated, pools were prepared of the peak fractions of hCGα peptides (1, 2 and 3) and free α peptides (1, 2 and 3') for further studies. Panel C shows neuraminidase treated JAR biosynthetically-labeled hCGα (●——●) and free α (O-----O) tryptic digests.

products of trypsin treatment of unreduced standard hCGα. We used Bio-Gel P4 to separate and compare the tryptic fragments of unreduced standard hCGα and unreduced biosynthetically-labeled JAR hCGα and free α (Fig. 1). By absorbance at 280 nm, two peaks of standard hCGα fragments were detected, in the void volume and in tube 59. Determination of the Leu/Pro ratio by amino acid analysis was used to identify the disulfide cross-linked core and the peptides 36-42 and 46-51. JAR hCGα tryptic fragments eluted in the same

volumes as standard hCGα fragments, strongly suggesting that the compositions of the two JAR peptides corresponded to those of standard hCGα. One free α tryptic fragment eluted in the position of the disulfide cross-linked core, a second in the position of peptide 46-51, but no radioactivity was found in the position of peptide 36-42. Instead, two small peaks corresponding to peptides of larger molecular size were observed (Fig. 1B). As expected, treatment with neuraminidase failed to shift the elution positions of the hCGα fragments (N-linked oligosaccharides are in the disulfide cross-linked core, 11). In contrast, treatment of the free α tryptic digest with neuraminidase shifted the elution position of the two small peaks, with the appearance of an incompletely resolved peak to the left of peptide 46-51 (Fig. 1C). With β-galactosidase treatment the elution position of the incompletely resolved peak was shifted to a position between peptide 46-51 and hCGα peptide 36-42 (not shown). This indicated the presence of NeuAc and βGal on the tryptic peptide derived from free α. To further examine this peptide and to determine if it is responsible for the free α:PNA-agarose interaction (Table 1), pools were prepared from the Bio-Gel P4 peaks of JAR free α and hCGα tryptic fragments (Fig. 1). The pools were lyophilized, taken up in 0.1 M sodium acetate, pH 5, neuraminidase treated then applied to PNA-agarose. The neuraminidase treated free α pool 3' (the unique glycosidase-sensitive peaks) was the only component bound by PNA-agarose (Table 1). The treated pool 3' was bound rather than retarded by the lectin, similar to treated hCGβ tryptic glycopeptides containing 1, 2, or 3 O-linked oligosaccharides. That the free α glycoprotein is retarded by PNA-agarose but its tryptic fragment containing the carbohydrate determinant is bound suggests that the presence of the disulfide cross-linked core sterically hinders or lessens the affinity of the determinant for the lectin.

The PNA-agarose affinity chromatography suggested an oligosaccharide on free α of the structure Galβ1-3GalNAc. Sensitivity to neuraminidase and β-galactosidase showed that the structure is sialylated and contains βGal. To confirm the presence of GalNAc on free α pool 3' and to show that pool 3' was in fact a glycosylated form of the absent peak, 36-42 (Fig. 1B), amino acid analyses were carried out on the labeled pools (Table 2). Free α pool 3' and hCGα pool 3 (residues 36-42) contained the same ratio of Leu to Pro residues (1:1.9). This is the expected Leu to Pro ratio for standard hCGα residue 36-42 (1:2). No amino sugars were detected on the hCGα tryptic fragment; however, free α pool 3' contained labeled GalN (acid hydrolysis product of GalNAc) and lesser amounts of labeled GlcN (acid hydrolysis product of GlcNAc). Our results clearly suggest that free α peptide 36-42, unlike the corresponding hCGα peptide, contains an oligosaccharide of the composition NeuAc, βGal, GalNAc probably arranged as are the O-linked tetrasaccharides on

Table 2. Amino acid analyses[a] of JAR [^{3}H]Pro/Leu/GlcN-labeled hCGα and free α

Sample	Leu:Pro ratio	Leu:GlcN ratio	Leu:GalN ratio
hCGα pool 3 (residues 36-42)	1:1.9	1:0	1:0
free α pool 3'	1:1.9	1:0.39	1:0.71

[a]Pools of JAR free α and hCGα tryptic fragments separated on Bio-Gel P4 (Fig. 1B) were made. The pools, and JAR free α, hCGα and hCGβ preparations were lyophilized then acid hydrolyzed and subjected to amino acid analyses. The relative cpm/residue was determined, assuming 4 Leu, 7 Pro and 8 GlcNAc residues on hCGα or free α, and 4 GalNAc residues on hCGβ isolated from the same media preparation (1). Values are the ratio of Leu residues to Pro, GlcN and GalN (hydrolyzed GlcNAc and GalNAc) residues.

hCGβ (1) containing the PNA-agarose determinant, NeuAc-Galβ1-3 (NeuAc-)GalNAc-O-.

Peptide 36-42 contains a site for O-glycosylation, Thr 39. As recently reported (4), a portion of JAR hCGβ O-linked oligosaccharides also contain GlcNAc, seemingly as part of more complex structures of the same size as those found on fetuin, NeuAc-Galβ1-3(NeuAc-Galβ1-4GlcNAcβ-)GalNAc-O-. Our finding of a small amount of GlcN in free α pool 3' (Table 2) suggests that a portion of free α 36-42 peptides may also contain this larger alternative oligosaccharide. A larger GlcNAc-containing O-linked oligosaccharide and an hCGβ-like O-linked oligosaccharide as alternative structures on free α 36-42 could explain the finding of two peaks for this peptide in the eluate of Bio-Gel P4 (Fig. 1B). Several attempts were made to β-eliminate free α or its tryptic peptides and so release the oligosaccharide in question (4). Low yields were always obtained (about 40% GalNAc converted to GalNAc-ol) for the β-elimination of intact free α, although under the same conditions the β-elimination of hCGβ and of the incorporated standard ovine submaxillary mucin was complete (Peters, B.P. and Ruddon, R.W., unpublished observations), suggesting that steric factors block β-elimination of free α. Corless and Boime (Personal Communication) have recently found that the peptide backbone of the analogous tryptic fragment from the large free α of LH in bovine pituitary is unstable to alkaline borohydride. This could expose a free carboxyl terminal on the Thr attachment site of the oligosaccharide rendering it resistant to β-elimination (12). We are further investigating the β-elimination of free α, which will be the subject of a future communication.

We used gel filtration to determine if the O-linked oligosaccharide accounts for the size difference of free α and hCGα. Intact and trypsin digested free α and hCGα were chromatographed on Bio-Gel P100 (Fig. 2). Whereas free α eluted somewhat prior to hCGα, the disulfide cross-linked cores of both forms of α subunit co-eluted on Bio-Gel P100. This indicates that removal of peptide 36-42 containing the O-linked oligosaccharide ablates the

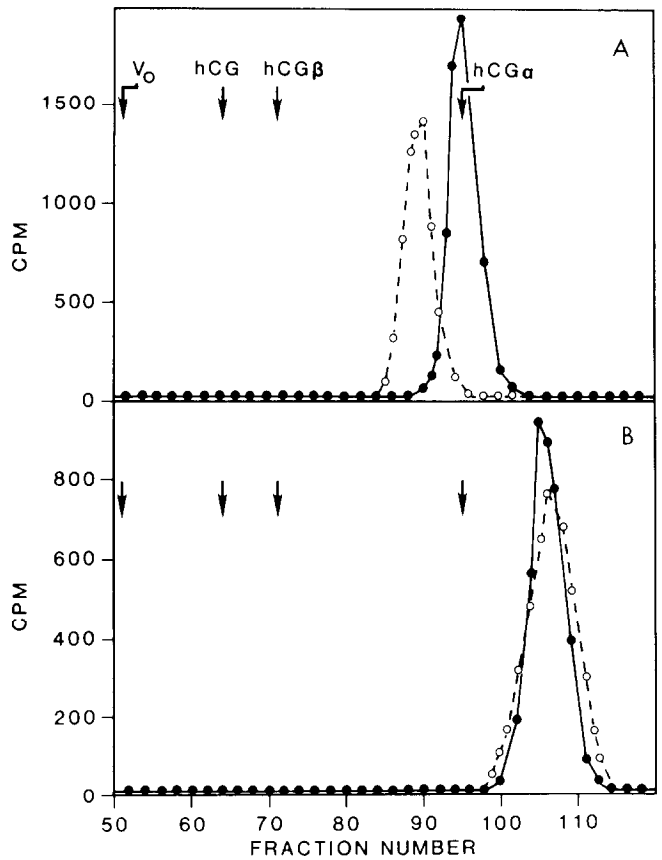

Fig. 2. Gel filtration on Bio-Gel P100 of JAR [^{3}H]Leu/Pro/Man biosynthetically-labeled hCGα and free α, and their disulfide cross-linked core tryptic fragments. Panel A, JAR hCGα (●—●) and free α (O----O). Panel B, tryptic digest of JAR hCGα (●—●) and free α (O----O); small peptides, residues 36-42 and 46-51 eluted in total volume (fraction 167, not shown). Arrows indicate the void volume and the elution positions of standard hCG, hCGα and hCGß.

free α vs. hCGα size difference (the third tryptic component, peptide 46-51, from free α co-eluted with that from hCGα on Bio-Gel P4, Fig. 1).

Our results using gel filtration, sizing of tryptic fragments, PNA-agarose affinity chromatography, β-elimination, and amino acid analysis indicate that an O-linked type of oligosaccharide, probably attached to Thr 39, predominantly accounts for the size difference between free α and hCGα. Free α is unable to combine with hCGα to form hCG (2). Parsons and Pierce (13) have examined the bovine pituitary free α and identified an O-linked oligosaccharide on Thr 43 (corresponds to hCGα Thr 39). They showed that bovine pituitary free α, which does not combine with bLH β-subunit, combined following the removal of the O-linked oligosaccharide. It seems likely therefore that the O-linked sugars on human trophoblast free α also block combination. Our future studies aim to examine O-glycosylation of α subunit and its possible role in regulating subunit combination and secretion.

ACKNOWLEDGMENTS: This work was supported by USPHS Grant CA-32949 from the National Cancer Institute and by Grant HD-15454 from the National Institute of Child Health and Human Development.

The authors thank Chris Corless and Dr. Irving Boime, Department of Pharmacology, Washington University of St. Louis, for allowing us to cite their unpublished results.

REFERENCES:

1. Hussa, R.O. (1981) Ligand Rev. 3, 1-43.
2. Cole, L.A., Hartle, R.J., Laferla, J.J., and Ruddon, R.W. (1983) Endocrinology 113, 1176-1178.
3. Cole, L.A., Kroll, T.G., Ruddon, R.W., and Hussa, R.O. (1984) J. Clin. Endocrinol. Metab. 58, 1200-1202.
4. Peters, B.P., Brooks, M., Hartle, R.J., Krzesicki, R.F., Perini, F., and Ruddon, R.W. (1983) J. Biol. Chem. 258, 14505-14515.
5. Birken, S., and Canfield, R.E. (1977) J. Biol. Chem. 252, 5386-5392.
6. Cole, L.A., Birken, S., Sutphen, S., Hussa, R.O., and Patillo, R.A. (1982) Endocrinology 110, 2198-2200.
7. Cole, L.A., and Hussa, R.O. (1981) Endocrinology 109, 2276-2278.
8. Perini, F., and Peters, B.P. (1982) Anal. Biochem. 123, 357-363.
9. Pereira, M.E.A., Kabat, E.A., Lotan, R., and Sharon, N. (1976) Carbohydrate Res. 51, 107-118.
10. Sadler, J.E. (1984) in Biology of Carbohydrates, Volume 2, V. Ginsburg and P.W. Robbins, eds., pp. 199-233, Wiley, New York.
11. Birken, S., Agosto, G., and Canfield, R. (1982) 64th Ann. Meet. Endocr. Soc., San Francisco (Abstr. 919).
12. Neuberger, A., Gottschalk, A., Marshall, R.D., and Spiro, R.G. (1972) in Glycoproteins, 2nd Edition, A. Gottschalk, ed., pp.450-490, Elsevier, Amsterdam.
13. Parsons, T.F., and Pierce, J.G. (1984) J. Biol. Chem. 259, 2662-2666.