Metabolism of gonadotropins: comparisons of the primary structures of the human pituitary and urinary LH β cores and the chimpanzee CG β core demonstrate universality of core production

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Abstract

The gonadotropins are a family of closely related heterodimeric glycoprotein hormones homologous in structure to disulfide-knot growth factors. Metabolic proteolytic processing *in vivo* of this disulfide cross-linked region results in urinary excretion of a residual highly stable core structure. The primary structure of the pituitary form of the hLH β core was reported earlier, but it has proved difficult to isolate the urinary core, although antibodies to the pituitary core demonstrated its presence. By conventional and immunoaffinity methods, the urinary core has been isolated and its structure determined by both chemical and mass spectrometric methods. The urinary hLH β core is the same as the pituitary-extracted hLH β core, β 6–40 disulfide bridged to β 55–93, except that the

pituitary core is more heterogeneous containing also β 49–93. These findings imply a dual origin of urinary cores, both directly from a secreting tissue and by kidney processing of circulating hormone. We also found that pregnant chimpanzees excrete a CG β core with a primary structure identical to that of the human CG β core of pregnancy. In conclusion, gonadotropin core generation and urinary excretion of nearly identical gonadotropin metabolites is common among primates. Although possible biological functions of these core fragments remain unproven, they have diagnostic utility because of their stability and abundance.

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Introduction

The gonadotropins are a family of closely related heterodimeric glycoprotein hormones, present in mammals, which are important to the correct functioning of the reproductive system (Pierce & Parsons 1981). The reproductive physiology, structure and function, receptor interactions and biosynthesis of these hormones have been studied in detail, but much less information is available concerning their metabolic processing. In mammals, the glycoprotein hormones consist of a common α subunit, encoded by a single α gene, non-covalently bound to a receptor-target-specific β subunit (Pierce & Parsons 1981). The solved crystal structure of human chorionic gonadotropin (hCG) showed that each of the gonadotropin subunits is densely disulfide bridged, resembles the other in structure, and also has structural homology to the disulfide-knot growth factor proteins (Lapthorn *et al.* 1994, 1995, Wu *et al.* 1994, Isaacs 1995). The disulfide-knot feature leads to protease generation of disulfide-bridged core fragments (the main urinary metabolites of the gonadotropins), which retain significant immunogenic features that render them measurable in many assays for

intact subunits (Birken *et al.* 1987, 1988, 1993, 1996, Akar *et al.* 1988). The hypothesis of this study was that urinary excretion of such stable and discrete glycoprotein core metabolic fragments of similar or identical structures is a common degradative pathway in both humans and non-human species. The non-human species examined to substantiate this hypothesis is the chimpanzee.

The first naturally generated and urine-excreted gonadotropin core fragment to be studied was the hCG β core fragment (hCG β cf) (Birken *et al.* 1988, 1996, Blithe *et al.* 1988), discovered more than 27 years ago by virtue of its cross-reaction with antisera to the β subunit of hCG (Schroeder & Halter 1983). Highly specific assays were developed to the hCG β cf and applied in a diagnostic mode to various clinical situations including certain hCG-secreting cancers for which the core, the molar concentration of which in urine greatly exceeds that of heterodimeric hormone or free subunit, provided a more sensitive assay for the presence of circulating hCG or hCG β (O'Connor *et al.* 1994, Birken *et al.* 1996, Cole *et al.* 1996b, Cole 1997, 1998). Extensive studies of the compartmental distribution of the hCG β cf and its concentration throughout pregnancy were conducted by

de Medeiros *et al.* (1992a, b), who concluded that hCG β cf increased in parallel to hCG throughout pregnancy, but at a greater molar ratio, indicating possible production sites additional to that of kidney alone. As hCG and human luteinizing hormone (hLH) are highly homologous in structure, it was predicted that an hLH core was likely to be present (Iles *et al.* 1992, 1999, Neven *et al.* 1993). The hLH β core fragment (hLH β cf) was isolated not from urine, but from pituitary (Birken *et al.* 1993, 1996). The development of a specific assay that could discriminate between the hCG and hLH cores permitted new diagnostic applications of the measurement of such core fragments (O'Connor *et al.* 1998, 1999).

The proteolytic processes within the kidney and the pituitary and other tissues leading to production of gonadotropin core molecules appear to be widespread, at least among primates. The current report describes the structure of the chimpanzee CG β core, the first report of a non-human gonadotropin core structure, along with most of the structure of its parent chimpanzee CG hormone. This β subunit core was found to be exactly homologous to the hCG β core, leading to the proposal that core excretion is a generalized metabolic pathway for gonadotropins within primates, and perhaps within most mammals.

Materials and Methods

Antibody reagents

Monoclonal antibodies used in this report were all produced at Columbia University: B505 to hLH β cf (O'Connor *et al.* 1998), A109 and A105 to hCG α , B107 to hCG, B210 to hCG β cf (Krichevsky *et al.* 1991). The production and application of antibodies to hCG, its subunits and metabolites were reviewed earlier (O'Connor *et al.* 1988, 1994).

Purification of the urinary LH β core

Procedure for urine concentration Large samples of postmenopausal urine collected by volunteers into 24 h 4 liter collection bottles were transported to the laboratory within 1–2 days of collection (they had been kept cold or frozen) and then 1 g/l sodium azide was added. Urine was thawed at room temperature (40–60 liter), its pH adjusted to 7.2–7.5, and then it was allowed to form a precipitate overnight at 4 °C. The settled precipitate region on the bottom of the 55 liter carboy was centrifuged and then particulate matter was removed by use of a Millipore Pellicon 0.45 μ m particle exclusion filter at 4 °C. The filtrate was next concentrated in a 3000 mol. wt cut-off Pellicon ultrafilter cassette (two tandem 0.5 m², Catalog No. P2 PLBCC05). Using this method, each 40–60 liter quantity of urine was reduced to 0.5–1.0 liter.

Aqueous stable serine protease inhibitor (AEBSF; Calbiochem product 101500) concentrate (10 mg) was added (700 ml at a time) to the urine concentrate, which was gel filtered to remove salts and hydrophobic components, using a process-scale Sephadex G-25 column (10 liter gel) in 0.05 M ammonium bicarbonate containing 0.002 M EDTA. The column eluate was pooled just up to the salt peak, so that small molecules would also be captured and lyophilized. The protein was dissolved at a concentration of 50 mg/ml in 0.15 M ammonium bicarbonate with another 10 mg AEBSF and then gel filtered on a Pharmacia Hi Resolution Superdex 200 (60/600) column in 0.1 M ammonium bicarbonate containing 0.02 M EDTA. Five pools were lyophilized: I=high mol. wt; II=50–70 kDa; III=25–50 kDa; IV=8–25 kDa; V=region of less than 8 kDa, including some of the salt region.

Antibody extraction of the hLH β cf Antibody B505 (that had been generated to the pituitary hLH β cf) was immobilized (5 mg/ml) on BioRad Affigel 10 using the method of the manufacturer. Postmenopausal urine pool IV containing hLH β cf as determined by immunoassay, was resuspended in 0.1 M ammonium bicarbonate with the following mixture of protease inhibitors: 250 μ g/ml solution of AEBSF (Calbiochem product 101500); EST (Calbiochem 330005, 1 mg/ml in ethanol and added at 50 μ l/ml to the incubation mixture); E-64 (Calbiochem 324890, solution of 1 mg in 2.8 ml of water, diluted 1:100 in incubation mixture); pepstatin (Calbiochem 516482, 1 mg in 1.45 ml of methanol and used at 1:1000 dilution into the mixture); leupeptin (Calbiochem 108975, dissolved at 1 mg in 200 μ l of water and added at 2 μ l per ml of incubation mixture). There was already adequate EDTA present because it was in the gel filtration column buffer. This mixture was rotated overnight at 4 °C with the immunoaffinity gel in a small polypropylene column. The next day the column was washed with 10 column volumes of PBS, 2 column volumes of 0.2 M glycine-HCl, pH 5, and then the protein was eluted with 2 column volumes of 2 M acetic acid, following which the column was rapidly returned to neutral pH by washing with PBS. Both the 0.2 M glycine-HCl wash and the main eluate of 2 M acetic acid were reduced to 200–400 μ l in a Savant Speed Vac (Farmingdale, NY, USA) and then separated by reverse-phase HPLC on a Pharmacia Smart System (Pharmacia, Piscataway, NJ, USA), as described below.

HPLC procedures

The LH β urinary core was purified on the Pharmacia Smart System using the μ RP C2/C18 3.2/2.0 column loaded and eluted with a 0.1% trifluoroacetic acid (TFA) (A) and 0.1% TFA/100% acetonitrile (B) gradient system as follows: flow rate 240 μ l/min; 10 min, 0% B; 40 min,

Figure 1 Separation of immunoaffinity extracts of partially purified preparations of the urinary hLH β core (A) and similar separation of the pituitary hLH β core on reverse phase HPLC (B). AUFS, absorbance units full scale.

50% B; 45 min, 80% B. Fractions 240 μ l in volume were collected.

Reduction and alkylation of LH β cores

Fractions 20+21 were pooled from two reverse-phase HPLC runs (Fig. 1), and dried separately in 500 μ l Eppendorf tubes. The contents of each vial were re-suspended in 50 μ l 4 M guanidine, 0.2 M Tris-HCl, pH 8.0. Next, dithiothreitol (DTT) was freshly dissolved at 10 mg/ml in 1 M Tris, pH 8.5 and 10 μ l of the DTT solution (100 μ g) was added at time 0. The solution was incubated for 1 h at 55 $^{\circ}$ C, then a second 10 μ l of another freshly made 10 mg/ml DTT solution was added and the vial incubated for an additional 1 h, after which 2 mg iodoacetamide from a 40 mg/ml solution in the 1 M Tris, pH 8 buffer (50 μ l of a 40 mg/ml solution) was added. The solution was incubated at ambient temperature for 15 min (alkylation step) and then 40 μ l β -mercaptoethanol were added (to use up excess alkylating reagent) and the

mixture was incubated for 5 min, desalted, and separated on the Pharmacia Smart System RP C2/C18 system reverse-phase using the TFA/acetonitrile system described under the HPLC methods section.

Deglycosylation of hLH β f alkylated peptides

Complete removal of the single glycan group present on hLH β 6–40 was accomplished by digestion with recombinant N-glycanase (product E-5006 provided by Oxford Glycosystems) according to the manufacturer's directions. After overnight deglycosylation, the reaction products were separated on the same Smart System column and gradient described above and then subjected to Zip-Tip desalting, and analyzed on matrix assisted laser desorption ionization (MALDI) as described below.

Chimpanzee studies

Collection of chimpanzee urine and blood Urine and blood samples were obtained from three pregnant chimpanzees by members of the Lemsip facility of New York University (courtesy of Dr Jim Mahoney) in Tuxedo, New York. Blood and urine were both collected under sterile conditions according to approved animal treatment procedures of the Lemsip facility and New York University. Urine was collected by catheter (30 ml) under sterile conditions at the same time as the blood samples (4 ml). Blood was centrifuged and the separated sera were frozen. The urine was centrifuged to remove any sediment and was frozen. These materials were shipped frozen to our laboratory for analysis.

Immunochemical analysis of chimpanzee serum and urine Chimpanzee serum or urine was size-fractionated by gel filtration on Pharmacia Superose 12 columns (two in tandem, 10 mm $\times$ 24 cm) in 0.1 M ammonium bicarbonate at a flow rate of 0.5 ml/min. Serum (2 ml) was particle-filtered using Millipore 25 mm 0.2 μ m Durapore filters. Urine specimens were first concentrated 4 times in a Savant Speed Vac (8 ml reduced to 2 ml) before particle filtration. Injections of serum or urine (1.8 ml) were made into the tandem Superose 12 columns and 0.5 ml fractions were collected.

Preparation of material for primary sequence analysis of chimpanzee chorionic gonadotropin Chimpanzee pregnancy urine was collected from cages equipped with bottom screens, filtered to remove debris, centrifuged, and extracted using the kaolin-acetone procedure of Albert (1956). Some extracts were further purified by absorption and elution from Con A Sepharose (Matsuura & Chen 1980, Udagawa *et al.* 1998). Various ion exchange procedures (both cation and anion methods) and gel filtration studies indicated most of the chorionic gonadotropin had undergone peptide bond cleavages and was widely dispersed on these columns. The

material prepared for structural studies was purified by immunoaffinity chromatography on insolubilized A109, an anti-hCG α subunit monoclonal antibody bound to cyanogen bromide-activated Sepharose at a concentration of 5 mg of antibody per ml of gel using the procedure described by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ, USA). Crude chimp CG powder was dissolved in PBS and loaded onto the A109 column, washed extensively with PBS (10 column volumes) and eluted with 1 M, 2 M and 3 M acetic acid. The 1 M and 2 M eluates contained the bulk of the chimp CG. These batches were further separated on gel filtration on Superose 12 columns as described above with the separation monitored by liquid-phase radioimmunoassay for dimeric hCG (B107) and for α subunit (A105) and for the β COOH-terminal peptide (CTP) region of hCG.

Purification of chimp CG β core The chimp CG β core was partially purified from chimpanzee pregnancy urine (sterile urine and well-preserved) by gel filtration as described above and then extracted by immunoaffinity chromatography using immobilized B210 antibody columns eluted with 2 M acetic acid as described elsewhere (Birken *et al.* 1988), and lyophilized.

Separation of subunits of chimpanzee CG Purified chimp CG was dissolved in 50 μ l 8 M urea, 0.4 M NH_4HCO_3 , pH 8.0. Reduction and alkylation were carried out by adding 5 μ l 0.04 M DTT and maintaining the solution at 50 °C for 1 h, cooling to room temperature, and adding 5 μ l 0.08 M iodoacetamide. The solution was allowed to react in the dark for 30 min and was diluted to 250 μ l with 0.1% TFA before HPLC separation. Subunits were separated on a Hewlett-Packard model 1090 HPLC using a Brownlee RP 300 column (2.1 mm $\times$ 22 cm) eluted with (A) 0.07% TFA and (B) 0.066% TFA–80% acetonitrile as follows: 0–10 min, 0% B; 10–80 min, 0–98% B. The flow rate was 200 μ l/min and the eluant was monitored at 210 nm. Purity and concentration of selected fractions were determined by amino acid analysis on a Beckman 6300A amino acid analyzer.

Tryptic digestion of chimpanzee subunits for structural studies Reduced and alkylated chimp CG α (8.2 μ g) and CG β (7.0 μ g) were digested with 0.3 μ g trypsin in 100 μ l NH_4HCO_3 , pH 8.0 at 37 °C for 20 h. Peptides were separated by HPLC on a Vydac C18 column (The Separations Group, Hesperia, CA, USA) using the conditions described above for separation of subunits.

N-terminal chemical sequencing

Reduced and alkylated subunits and tryptic peptides were sequenced on an Applied Biosystems model 470A protein sequencer (Foster City, CA, USA).

Structural analysis of chimp CG β core The purified core molecules were sequenced in a PE Biosystems Procise sequencer for 10 cycles. In addition, the core was reduced with DTT, alkylated with iodoacetamide, separated from reductant and salts by use of a Millipore C18 zip tip, and subjected to analysis on a PE Voyager DE RP MALDI mass spectrometer.

MALDI mass spectrometric analysis

Peptides to be analyzed were desalted through a Millipore C18 Zip Tip and either eluted with 50% acetonitrile, dried, and redissolved in matrix solution, or eluted from the Zip Tip with matrix solution. Matrix solution was 4-hydroxy- α -cyanocinnamic acid (10 mg/ml) dissolved in 50% acetonitrile–0.1% TFA with insulin (mol. wt 5734.59) and angiotensin I (mol. wt 1297.51) as internal standards. Mass spectrometric analysis was performed on a PerSeptive Voyager DE RP MALDI mass spectrometer operated in linear mode.

Results

Purification of the urinary LH β core

Isolation of the urinary hLH β cf proved difficult. Its low concentration, even in postmenopausal urine, and the presence in the urinary concentrate of proteases that attacked the insolubilized antibody and the non-specific co-purification of other urinary proteins confounded many attempts at purification. The addition of protease inhibitors to all steps of the separations, and the use of the high-sensitivity columns available with the Pharmacia Smart System overcame these difficulties. Figure 1 shows the reverse-phase HPLC pattern of the acid eluate from the B505 immunoaffinity column (see Methods). The presence of proteases that tended to cleave antibody fragments from the immunoaffinity column even in the presence of an excess of a protease-inhibitor cocktail is still apparent with the elution of mouse immunoglobulin fragments. The hLH β cf elutes consistently in fractions 20–21 from this column as a broad, flat peak. Immunoassay of all column fractions with the B505 immunometric assay for pituitary hLH β cf indicated that this was the only such fragment elution position from the column; other urinary proteins such as the defensins and granulins tend to elute just before the core peak. Chemical sequence analysis of this peak indicated that its peptide content displayed the two same amino termini as the hCG β cf, residues 6 and 55. No other amino termini were detected. In contrast, the pituitary hLH β cf displayed a third amino terminus in addition to those two, residue 49, indicating that the pituitary β core was a mixture of molecules composed of either β 6–40 linked to β 49–93 or β 6–40 linked to β 55–93 (Birken *et al.* 1993).

Figure 2 Reverse-phase HPLC separations of the products of reduction and alkylation with iodoacetamide of hLH β cores (panel A, urinary core and panel B, pituitary core). Identical conditions were employed for the separations. Polypeptide chains identified by Edman sequencing and mass spectrometry. AUFS, absorbance units full scale.

Structural analysis of the urinary hLH β cf

Figure 2 displays the reverse-phase HPLC patterns of reduced, carboxyamidomethylated hLH β cfs: panel A is the urinary hLH β cf and panel B shows the pituitary hLH β cf, both alkylated by the same procedure and run conditions in succession on the same column and gradient. Each of these peaks was subjected to chemical amino-terminal sequence analysis, which identified the peptide components as shown in Fig. 2. In addition, each major peak of non-glycan-containing peptide shown in Fig. 2 was subjected to MALDI analysis, which confirmed identification of hLH β 55–93 in both urinary (m/z 4540·86) and pituitary hLH β (m/z 4540·82) cores on the basis of the $[M+H]^+$ -calculated average as 4541·32 (Fig. 3). MALDI analysis also confirmed identification of hLH β 49–93 in the hLH β pituitary core structure (observed $[M+H]^+$ =5186·7 atomic mass units (AMU) compared with a calculated $[M+H]^+$ of 5187·12).

It is clear that the urinary hLH β cf is more homogeneous than is the pituitary hLH β cf. The urinary core contains only two chains, whereas the pituitary core is a mixture of molecules containing two chains with two different amino termini.

The glycan-containing alkylated chain of pituitary hLH β (see Fig. 2) was examined by MALDI before and after deglycosylation. Before deglycosylation, the 6–40 chain of the pituitary core was measured as two predominant peaks of 4937·00 and 5098·89; after deglycosylation, only a single main peak was measured, as 4061·35 (calculated average $[M+H]^+$ =4060·75), which was a loss of a glycan of 875·65 (inferred glycan mass of 875·8 AMU) and a glycan of 1037·54 (inferred glycan mass of 1038·0 AMU) (Fig. 4). The first glycan corresponds to a structure of two *N*-acetyl glucosamines, two mannose and one fucose,

Figure 3 Comparison of the molecular weights of the pituitary (pit) and the urinary hLH β core non-glycan peptides. MALDI spectra of purified reduced and alkylated hLH β core peptides as isolated from reverse-phase HPLC (according to the procedure used to generate Fig. 2). Molecular weight standards were S1 (angiotensin I, 1297·51) and insulin (S2, 5734·59). Ions were $[M+H]^+$ except for doubly charged S2 as indicated on the spectra.

Figure 4 Comparison of the molecular weights of the glycan-containing pituitary hLH β core peptide before and after complete removal of the glycan moiety. MALDI spectra of purified, reduced and alkylated hLH β 6–40 (isolated as in Fig. 2) before (upper) and after (lower) treatment with *N*-glycanase. S1 is standard angiotensin I (1297.51) and S2 is insulin (5734.59), as described in the legend to Fig. 3.

whereas the greater-mass glycan may represent the same structure with one additional mannose. The mass of the urinary 6–40 chain after deglycosylation could not be visualized, probably because of lower available quantities for the deglycosylation step, but its elution position and N-terminus were exactly the same as those of the pituitary core 6–40.

Purification of chimpanzee CG

The chimpanzee CG isolated from batches of pregnancy urine collected from cages of pregnant chimpanzees proved to be significantly damaged by protease activity. Immunoassays of gel filtration profiles of this material indicated that less than 10% of the β CTP region remained present on the heterodimeric molecule. Sequence analyses indicated internal peptide bond cleavages. Attempts at purification using ion exchangers were not fruitful, because of widespread dispersion of the chimp CG resulting from these peptide bond cleavages. Consequently, immunoaffinity chromatography was used. Immobilized anti-hCG α antibody A109 was used, with best results for extraction of heterodimeric chimpanzee CG from kaolin–acetone chimp pregnancy urine concentrates. The immunoaffinity extract was gel filtered and monitored with a dimer-directed radioimmunoassay (B107) to select the heterodimeric chimp CG pool. This material was used for primary sequence analysis, but was nearly completely devoid of the β CTP region.

Discovery and isolation of the chimpanzee CG β core

As collection of pregnancy urine from the bottom of cages of pregnant chimps led to extensively degraded forms of

chimp CG, urine was collected from three pregnant chimpanzees by use of a catheter and the urine was stored frozen under sterile conditions. Likewise, blood was collected in sterile format from the same chimpanzees at the same time. The urine and serum were both subjected to gel filtration separation under the same conditions, followed by immunoassay of each fraction. Figure 5 shows gel filtration separations of the urine and serum of a typical pregnant chimpanzee. The fractions were analyzed by three liquid-phase radioimmunoassays, one for hCG (B107), the second for hCG β cf (B210), and the third for the hCG β CTP region (R525) (O'Connor *et al.* 1994). These assays for the human hormone forms are quite sensitive in measurement of the chimpanzee hormones. The serum separation shows only the chimp CG and no β core, whereas the urine gel filtration shows two peaks, one of CG and a second of β core in the same size position as one would observe hCG β cf. As the urine was collected and stored frozen under sterile conditions, the observed chimp CG β core (present at 5 times the concentration of chimp CG by these immunoassays) was produced naturally in the urine and not generated by *in vitro* proteolytic activity. It is also noted that the serum sample demonstrates that the two immunoassays, one for heterodimeric hormone and one for the β CTP region, both measure the same quantity of CG on a molar basis in the same fractions. This proves that circulating chimpanzee CG contains the CTP region and that it has immunoreactivity similar to that of the analogous region of hCG.

Structural analysis of chimpanzee CG

The partial sequences of chimp CG α and β subunits (except for the β CTP, which was missing) were

Figure 5 Demonstration of the presence of a chimpanzee CG β core molecule in the urine of a pregnant animal (upper panel), but its absence from the animal's blood (lower panel), exactly similar to core appearance in human pregnancies. Gel filtration (Superdex 12) of a concentrate of urine from a pregnant chimpanzee (upper panel) as compared with serum from the same animal chromatographed under the same conditions. Each column fraction (0.5 ml fractions) was immunoassayed for heterodimeric hCG (B107), for hCG β core (B210) and for hCG β CTP (R525), all by liquid-phase radioimmunoassay.

determined by automated Edman sequencing of the intact reduced and alkylated subunits and their tryptic fragments (Fig. 6). In the α subunit, 85 of 92 amino acids were positively identified, whereas 96 of 122 amino acids were identified in the CG β subunit. Of those left undetermined, three sites in the β subunit and two in the α corresponded to glycosylation sites in the human hCG sequences and produced blank cycles in the sequence. Only one residue in each subunit differed from the human sequence: in subunit α , V is substituted for Q at position 50; in β , V replaces D at 117.

Structural analysis of the chimpanzee CG β core

Chemical sequence analysis of the chimpanzee CG β core revealed identical amino termini of β residues 6 and 55, as in human CG core. Both human and chimpanzee hCG β cf were reduced and alkylated and subjected to MALDI analysis. The β 55–92 peptide of the chimp CG was m/z 4384.25, whereas the same peptide from the

Figure 6 Comparison of primary sequences of human and chimpanzee CG. (A) Comparison of alpha subunits. The difference between human and chimp sequences is in **bold** typeface. (B) Comparison of the beta subunits. Undetermined residues in chimp are indicated as x or, for known homologous glycosylation sites, as *.

human core was m/z 4384.64 (calculated m/z is 4385.11 for the $[M+H]^+$ ion). Figure 7 compares the MALDI patterns obtained after reduction and alkylation of the hCG β cf and the chimp CG β cf, performed under the same conditions of chemical alkylation and the same MALDI conditions. In both cases, the glycan-containing component of the cores cannot be visualized. When the alkylated glycan-containing peptide chain of the hCG β cf was first separated on reverse-phase HPLC and then subjected to enzymic deglycosylation, MALDI analysis indicated the expected molecular weight. There was not enough chimpanzee CG for this type of procedure.

Discussion

Excretion of gonadotropin subunit core molecules into urine is a result of proteolytic digestion of the densely disulfide-bridged knot motif of the β subunit structure discovered upon solution of the crystal structure of hCG (Laphorn *et al.* 1994, 1995, Wu *et al.* 1994, Isaacs 1995). The presence of this core disulfide motif holds most of the peptide structure together even in the presence of multiple peptide bond cleavages (Birken *et al.* 1987). The precise identities of the proteases that produce the core fragments are not known, and exact duplication of production of the fragments from hCG has not yet been possible to achieve *in vitro*. One question we sought to address in this report was whether core generation is one of the general metabolic degradation patterns of the glycoprotein hormones and can be observed in non-human species. We found that one such core fragment of identical structure (hCG β cf)

Figure 7 Comparative MALDI spectra of reduced and alkylated chimp CG and hCG β core chains. Under the conditions employed, only the non-glycan chains were ionized and are the identical molecular weights proving the identity of these portions of the two cores from two different species.

can be measured and isolated from another mammal (chimpanzee). We chose to look for this fragment in this species on the basis of our structural analysis of chimpanzee CG, which indicated its nearly complete identity to hCG, making it likely that proteolytic processing would be identical. As core molecules are generally present in urine in much greater molar concentrations than dimeric gonadotropins or free subunits, measurement of such core molecules may increase assay sensitivity for gonadotropins in non-humans, such as for very early pregnancy diagnosis, just as has been the case for humans (O'Connor *et al.* 1994).

There is a report of possible important functions of gonadotropin core molecules (Albini *et al.* 1997). Although the biological functional significance, if any, of the core fragments is not yet proven, these urinary metabolites, in particular the hCG β cf, have diagnostic applications in cancer tests (Lee *et al.* 1991, Cole *et al.* 1996b, O'Connor *et al.* 1999) and abnormal pregnancies (Cole 1995, Cole *et al.* 1996a). Furthermore, the presence of the core in urine is definitive proof of the presence of circulating hCG, and has been used to provide such evidence when certain blood tests for hCG prove misleading (Cole 1998, Cole *et al.* 1999).

Measurement of the urinary metabolite of hLH, the hLH β cf, has been shown to be potentially useful in menopausal studies (Birken *et al.* 1996, 1999) and also for possible differentiation of a cancer marker, hCG β cf, from a natural gonadotropin metabolite in postmenopausal women (Iles *et al.* 1999, O'Connor *et al.* 1999). For example, the presence of significant quantities of hCG β cf may indicate the presence of a malignancy in a postmenopausal woman, whereas the presence of LH β core would be normal (Birken *et al.* 1993, Neven *et al.* 1993, Birken *et al.* 1996, O'Connor *et al.* 1998, 1999). Before

the development of a specific LH β core assay, it would not have been possible to discriminate between the two very similar core molecules.

Although we had developed a specific immunoassay for the urinary hLH β cf several years ago, this assay was based on development of antibodies to the hLH β cf isolated from a pituitary extract (O'Connor *et al.* 1998, 1999). The pituitary version of the core is used as the assay standard. Until now, the structure of the urinary core had not been elucidated. Isolation of the urinary LH β core fragment proved to be elusive, because of its relatively low concentration and tendency to co-purify with other urinary proteins and peptides. We now report the isolation and structural analysis of the urinary hLH β cf. The structure of the pituitary hLH β cf appears to suggest that is a precursor to that of the urinary core, and may indicate a contribution of pituitary secretion of core into the urinary core pool (Birken *et al.* 1999). It was observed earlier that the pituitary and urinary forms of the cores eluted in slightly different positions on reverse-phase HPLC (O'Connor *et al.* 1998). That observation may be attributable to the peptide structural difference between pituitary and urinary hLH β cf, but may indicate carbohydrate differences also.

Comparative HPLC patterns of the alkylated chains of the urinary and the pituitary hLH β cf would lead one to propose a precursor-product relationship, as the pituitary core contains a mixture of molecules with either β 49–93 or β 55–93 linked to β 6–40 whereas the urinary hLH β cf contains only two peptides, β 55–93 linked to β 6–40. It seems that the proteolytic generating process went further to completion in the urinary form, to generate a more homogeneous end product, which is very similar to the urinary hCG β cf. It is not likely that the soluble proteases in urine itself are responsible for the more complete

cleavage of the urinary core material, because no other new cleavage sites were found in the urinary core.

The hCG β cf is composed of hCG β 6–40 disulfide bridge to hCG β 55–92. In contrast, the hLH β cf is composed of the same analogous first peptide hLH β 6–40, but is disulfide-bridged to hLH β 55–93 (Birken *et al.* 1993, 1996, O'Connor *et al.* 1998). Thus the hLH β cf second peptide extends through a cysteine residue, in contrast to that of the hCG β cf. The observed glycans on the hLH β 6–40 structure represent known glycans on pituitary hLH β subunit with some trimming down to the mannose core, as are the major glycan structures of the hCG β core (Blithe *et al.* 1989, Green & Baenziger 1988a, b, Stockell & Renwick 1992). We did not identify glycan-containing chains with their full sugar complement including either sialic acid or sulfate termini. However, there were a number of small ion peaks observed with MALDI, and it is possible that such structures were present but that the two predominant structures are mannose core glycans, one with all three mannoses and the other with two mannoses.

The putative precursor-product relationship between the pituitary and urinary hLH β cf led to the suggestion that some secretion of hLH β cf directly from the pituitary to the blood to the urine is possible. There is considerable evidence, based on clearance studies, that the hCG β cf is produced by kidney degradation of circulating hCG (Wehmann *et al.* 1984). A new study using infusion of recombinant hCG reinforces the origin of hCG β cf by kidney processing of circulating hCG, but also points to possible multiple pathways of production of hCG β cf in urine, as hCG β cf was only 12.2% of the amount of hCG excreted in urine whereas, in pregnancy, hCG β cf is present in much greater concentrations as compared with hCG (Norman *et al.* 2000).

There is also evidence that hCG β cf can be detected in both placental and pituitary tissue, raising the possibility of some direct secretion and rapid clearance of this core into the urine (Cole & Birken 1988, Hoermann *et al.* 1995, Udagawa *et al.* 1998). hCG β cf-like material has also been reported in molar fluid, and it is hypothesized that it is derived from macrophage processing of hCG (Iles *et al.* 1999, Khan *et al.* 2000). It is nevertheless likely that the major source of urinary hLH β cf is from circulating LH, because of the pattern of peaking of hLH β cf in urine 24–48 h after the LH surge during the menstrual cycle (Iles *et al.* 1992, 1999, Neven *et al.* 1993, O'Connor *et al.* 1998, Birken *et al.* 1999).

The primary structure of chimpanzee chorionic gonadotropin reported here demonstrates that it is nearly identical to that of hCG. Only two differences were noted, valine for glutamine at residue 50 in the α subunit and valine for aspartic acid at β 117. Both amino acid substitutions are in non-critical areas of the molecule. The β 117 substitution is in an unstructured area of the β subunit that was too mobile to be visualized in the crystal model of the structure. The multiple human genes for the

hCG β subunit analyzed in detail by Policastro *et al.* (1983) indicated that β 117 is a variable amino acid in humans (D to A variants noted) and so it is not surprising that this amino acid differs between human and chimpanzee, despite the close similarity of human and chimp proteins in general. The α 50 substitution in α subunit is glutamine in the human and Rhesus monkey, but proline in marmoset, dog, rabbit, rat and most other sequenced mammalian glycoprotein α subunits, as indicated by a BLAST search on the National Center for Biotechnology Information (NCBI) web site. This residue is not a critical area of structure of the molecule, and is unlikely to effect bio-activity. In the marmoset, the major primary structural differences from human resided in the β CTP portion (Simula *et al.* 1995). Unfortunately, most of the chimp CTP portion had been cleaved off the chimp CG that was derived from urine collections from cage bottoms. The chimp CG with its CTP portion intact was found to be present in urine obtained under sterile conditions by catheter (as shown by immunological measurement), but proved an inadequate quantity to permit CTP isolation and structural analysis. This portion of the structure has not been determined, but is likely to be very similar to the hCG CTP, on the basis of immunoreactivity with hCG CTP antibodies. The near identity of the primary structures of the two gonadotropins led us to explore the possibility that a β core similar to that of the human is excreted in the urine of pregnant chimpanzees.

It was necessary to use sterile urine (taken by catheter) in order to measure and isolate the chimpanzee core molecules because of the rapid proteolytic damage to molecules in urine collected by bottom-cage devices. Unless sterile urine were used, it would not be possible to distinguish between molecules excreted by the kidney and those generated later in urine by bacterial proteolysis. Human urine is generally nearly sterile upon collection directly into a receiving vessel, but this technique is not possible to accomplish with non-humans.

The hormonal patterns in the chimpanzee are known to be very similar to those of humans (Faiman *et al.* 1974, Hobson 1975, Reyes *et al.* 1975, Hobson *et al.* 1976, Kaplan & Grumbach 1978). In addition, there is evidence of structural similarities derived by immunological measurements between the chimpanzee and human CGs, including the presence of the β CTP region (Chen & Hogden 1976, Nixon *et al.* 1977). The finding of a chimpanzee urinary CG β cf with a structure that mimics that of the human hCG β cf so precisely, implies that such urinary core generation is a common occurrence among primates. Other mammals that do not manufacture CG-type molecules produce LH molecules, and LH urinary core generation is likely to also be common among these species, just as is found in humans. The question of whether such core generation has any functional significance or merely represents a natural metabolic catabolism of gonadotropins remains open. Certainly, in the case of

the LH cores, some of these molecules may circulate, because the core is found within the pituitary itself (Birken *et al.* 1993, 1996).

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References

- Akar AH, Wehmann RE, Blithe DL, Blacker C & Nisula BC 1988 A radioimmunoassay for the core fragment of the human chorionic gonadotropin beta-subunit. *Journal of Clinical Endocrinology and Metabolism* **66** 538–545.
- Albert A 1956 Purification of human chorionic gonadotropin. *Recent Progress in Hormone Research* **12** 227–296.
- Albini A, Paglieri I, Orenco G, Carlone S, Aluigi MG, DeMarchi R, Matteucci C, Mantovani A, Carozzi F, Donini S & Benelli R 1997 The beta-core fragment of human chorionic gonadotrophin inhibits growth of Kaposi's sarcoma-derived cells and a new immortalized Kaposi's sarcoma cell line. *AIDS* **11** 713–721.
- Birken S, Kolks MA, Amr S, Nisula B & Puett D 1987 Structural and functional studies of the tryptic core of the human chorionic gonadotropin beta-subunit. *Endocrinology* **121** 657–666.
- Birken S, Armstrong EG, Kolks MA, Cole LA, Agosto GM, Krichovsky A, Vaitukaitis JL & Canfield RE 1988 Structure of the human chorionic gonadotropin beta-subunit fragment from pregnancy urine. *Endocrinology* **123** 572–583.
- Birken S, Chen Y, Gawinowicz MA, Agosto GM, Canfield RE & Hartree AS 1993 Structure and significance of human luteinizing hormone-beta core fragment purified from human pituitary extracts. *Endocrinology* **133** 985–989.
- Birken S, Kovalevskaya G & O'Connor J 1996 Metabolism of hCG and hLH to multiple urinary forms. *Molecular and Cellular Endocrinology* **125** 121–131.
- Birken S, Santoro N, Maydelman Y, Kovalevskaya G, Lobo R, Freeman EW, Warren M, McMahon D & O'Connor J 1999 Differences in urinary excretion patterns of the hLH beta core fragment in premenopausal, perimenopausal, and postmenopausal women. *Menopause* **6** 290–298.
- Blithe DL, Akar AH, Wehmann RE & Nisula BC 1988 Purification of beta-core fragment from pregnancy urine and demonstration that its carbohydrate moieties differ from those of native human chorionic gonadotropin-beta. *Endocrinology* **122** 173–180.
- Blithe DL, Wehmann RE & Nisula BC 1989 Carbohydrate composition of beta-core. *Endocrinology* **125** 2267–2272.
- Chen HC & Hodgen GD 1976 Primate chorionic gonadotropins: antigenic similarities to the unique carboxyl-terminal peptide of HCGbeta subunit. *Journal of Clinical Endocrinology and Metabolism* **43** 1414–1417.
- Cole LA 1995 Down's syndrome screening using urine beta-core fragment test: choice of immunoassay. *Prenatal Diagnosis* **15** 679–680.
- Cole LA 1997 Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites. *Clinical Chemistry* **43** 2233–2243.
- Cole LA 1998 Phantom hCG and phantom choriocarcinoma. *Gynecological Oncology* **71** 325–329.
- Cole LA & Birken S 1988 Origin and occurrence of human chorionic gonadotropin beta-subunit core fragment. *Molecular Endocrinology* **2** 825–830.
- Cole L, Isozaki T, Palomaki G, Canick J, Iles R, Kellner L, Saller D & Cuckle H 1996a Detection of beta-core fragment in second trimester Down's syndrome pregnancies. *Early Human Development* **47** (Suppl) S47–S48.
- Cole LA, Tanaka A, Kim GS, Park SY, Koh MW, Schwartz PE, Chambers JT & Nam JH 1996b Beta-core fragment (beta-core/UGF/UGP), a tumor marker: a 7-year report. *Gynecological Oncology* **60** 264–270.
- Cole LA, Rinne KM, Shahabi S & Omrani A 1999 False-positive hCG assay results leading to unnecessary surgery and chemotherapy and needless occurrences of diabetes and coma. *Clinical Chemistry* **45** 313–314.
- Faiman C, Reyes RI & Winter JS 1974 Serum gonadotropin patterns during the perinatal period in man and in the chimpanzee. In *Endocrinologie Sexuelle de la Periode Perinatale*, pp 281–297. Eds MG Forest & J Bertrand. Paris: INSERM.
- Green ED & Baenziger JU 1988a Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. I. Structural elucidation of the sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. *Journal of Biological Chemistry* **263** 25–35.
- Green ED & Baenziger JU 1988b Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. II. Distributions of sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. *Journal of Biological Chemistry* **263** 36–44.
- Hobson BM 1975 Chorionic gonadotropin in the placenta of a chimpanzee (*Pan troglodytes*). *Folia Primatologica (Basel)* **23** 135–139.
- Hobson W, Coulston F, Faiman C, Winter JS & Reyes F 1976 Reproductive endocrinology of female chimpanzees: a suitable model of humans. *Journal of Toxicology and Environmental Health* **1** 657–668.
- Hoermann R, Spoettl G, Berger P & Mann K 1995 Immunoreactive human chorionic gonadotropin beta core fragment in human pituitary. *Experimental Clinical Endocrinology and Diabetes* **103** 324–331.
- Iles RK, Lee CL, Howes I, Davies S, Edwards R & Chard T 1992 Immunoreactive beta-core-like material in normal postmenopausal urine: human chorionic gonadotrophin or LH origin? Evidence for the existence of LH core. *Journal of Endocrinology* **133** 459–466.
- Iles RK, Javid MK, Gunn LK & Chard T 1999 Cross-reaction with luteinizing hormone beta-core is responsible for the age-dependent increase of immunoreactive beta-core fragment of human chorionic gonadotropin in women with nonmalignant conditions. *Clinical Chemistry* **45** 532–538.
- Isaacs NW 1995 Cystine knots. *Current Opinion in Structural Biology* **5** 391–395.
- Kaplan SL & Grumbach MM 1978 Pituitary and placental gonadotrophins and sex steroids in the human and sub-human primate fetus. *Clinical Endocrinology and Metabolism* **7** 487–511.
- Khan S, Katabuchi H, Araki M, Ohba T, Koizumi T, Okamura H & Nishimura R 2000 The molar vesicle fluid contains the beta-core fragment of human chorionic gonadotropin. *Placenta* **21** 79–87.
- Krichovsky A, Birken S, O'Connor J, Bikel K, Schlatterer J, Yi C, Agosto G & Canfield R 1991 Development and characterization of a new, highly specific antibody to the human chorionic gonadotropin-beta fragment. *Endocrinology* **128** 1255–1264.

- Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ & Isaacs NW 1994 Crystal structure of human chorionic gonadotropin. *Nature* **369** 455–461.
- Lapthorn AJ, Janes RW, Isaacs NW & Wallace BA 1995 Cystine nooses and protein specificity. *Nature Structural Biology* **2** 266–268.
- Lee CL, Iles RK, Shepherd JH, Hudson CN & Chard T 1991 The purification and development of a radioimmunoassay for beta-core fragment of human chorionic gonadotrophin in urine: application as a marker of gynaecological cancer in premenopausal and postmenopausal women. *Journal of Endocrinology* **130** 481–489.
- Matsuura S & Chen HC 1980 A simple and effective solvent system for elution of gonadotropins from concanavalin a affinity chromatography. *Analytical Biochemistry* **106** 402–410.
- de Medeiros SF, Amato F, Bacich D, Wang L, Matthews CD & Norman RJ 1992a Distribution of the beta-core human chorionic gonadotrophin fragment in human body fluids. *Journal of Endocrinology* **135** 175–188.
- de Medeiros SF, Amato F, Matthews CD & Norman RJ 1992b Urinary concentrations of beta core fragment of hCG throughout pregnancy. *Obstetrics and Gynecology* **80** 223–228.
- National Center for Biotechnology Information (NCBI) <http://www.ncbi.nlm.nih.gov>
- Neven P, Iles RK, Howes I, Sharma K, Shepherd JH, Edwards R, Collins WP & Chard T 1993 Substantial urinary concentrations of material resembling beta-core fragment of chorionic gonadotropin beta-subunit in mid-menstrual cycle. *Clinical Chemistry* **39** 1857–1860.
- Nixon WE, Chen HC & Hodgen GD 1977 Antigenic similarities to HCG subunits among chorionic gonadotropins of nonhuman primates. *Journal of Medical Primatology* **6** 195–202.
- Norman RJ, Buchholz MM, Somogyi AA & Amato F 2000 hCGbeta core fragment is a metabolite of hCG: evidence from infusion of recombinant hCG. *Journal of Endocrinology* **164** 299–305.
- O'Connor JF, Schlatterer JP, Birken S, Krichevsky A, Armstrong EG, McMahon D & Canfield RE 1988 Development of highly sensitive immunoassays to measure human chorionic gonadotropin, its beta-subunit, and beta core fragment in the urine: application to malignancies. *Cancer Research* **48** 1361–1366.
- O'Connor JF, Birken S, Lustbader JW, Krichevsky A, Chen Y & Canfield RE 1994 Recent advances in the chemistry and immunochemistry of human chorionic gonadotropin: impact on clinical measurements. *Endocrine Reviews* **15** 650–683.
- O'Connor JF, Kovalevskaya G, Birken S, Schlatterer JP, Schechter D, McMahon DJ & Canfield RE 1998 The expression of the urinary forms of human luteinizing hormone beta fragment in various populations as assessed by a specific immunoradiometric assay. *Human Reproduction* **13** 826–835.
- O'Connor JF, Kovalevskaya G & Birken S 1999 Interference of luteinizing hormone beta-core fragment in urinary gonadotropin assays. *Clinical Chemistry* **45** 2290–2292.
- Pierce JG & Parsons TF 1981 Glycoprotein hormones: structure and function. *Annual Reviews of Biochemistry* **50** 465–495.
- PolICASTRO P, Ovitt CE, Hoshina M, Fukuoka H, Boothby MR & Boime I 1983 The beta subunit of human chorionic gonadotropin is encoded by multiple genes. *Journal of Biological Chemistry* **258** 11492–11499.
- Reyes FI, Winter JS, Faiman C & Hobson WC 1975 Serial serum levels of gonadotropins, prolactin and sex steroids in the nonpregnant and pregnant chimpanzee. *Endocrinology* **96** 1447–1455.
- Schroeder HR & Halter CM 1983 Specificity of human beta-choriogonadotropin assays for the hormone and for an immunoreactive fragment present in urine during normal pregnancy. *Clinical Chemistry* **29** 667–671.
- Simula AP, Amato F, Faast R, Lopata A, Berka J & Norman RJ 1995 Luteinizing hormone/chorionic gonadotropin bioactivity in the common marmoset (*Callithrix jacchus*) is due to a chorionic gonadotropin molecule with a structure intermediate between human chorionic gonadotropin and human luteinizing hormone. *Biology of Reproduction* **53** 380–389.
- Stockell HA & Renwick AG 1992 Molecular structures of glycoprotein hormones and functions of their carbohydrate components. *Biochemical Journal* **287** 665–679.
- Udagawa A, Okamoto T, Nomura S, Matsuo K, Suzuki H & Mizutani S 1998 Human chorionic gonadotropin beta-core fragment is present in the human placenta. *Molecular and Cellular Endocrinology* **139** 171–178.
- Wehmann RE, Amr S, Rosa C & Nisula BC 1984 Metabolism, distribution and excretion of purified human chorionic gonadotropin and its subunits in man. *Annals d'Endocrinologie (Paris)* **45** 291–295.
- Wu H, Lustbader JW, Liu Y, Canfield RE & Hendrickson WA 1994 Structure of human chorionic gonadotropin at 2.6 Å resolution from MAD analysis of the selenomethionyl protein. *Structure* **2** 545–558.

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