Metabolism of gonadotropins: comparisons of the primary structures of the human pituitary and urinary LHβ cores and the chimpanzee CGβ core demonstrate universality of core production

S Birken, M A Gawinowicz, Y Maydelman and Y Milgrom

Departments of Medicine and Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, New York, New York 10032, USA
(Requests for offprints should be addressed to S Birken, Department of Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, 630 W 168th St, New York, New York 10032, USA; Email: sb18@columbia.edu)

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 hLHβ 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 hLHβ core is the same as the pituitary-extracted hLHβ core, β6–40 disulfide bridged to β55–93, except that the pituitary core is more heterogeneous containing also β49–93. 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 CGβ core with a primary structure identical to that of the human CGβ 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, Isacs 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 hCGβ core fragment (hCGβcf) (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 hCGβcf 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 hCGβ (O’Connor et al. 1994, Birken et al. 1996, Cole et al. 1996b, Cole 1997, 1998). Extensive studies of the compartmental distribution of the hCGβcf and its concentration throughout pregnancy were conducted by
de Medeiros et al. (1992a, b), who concluded that hCGβcf 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 hLHβ core fragment (hLHβcf) 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 CGβ 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 hCGβ 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 hLHβcf (O’Connor et al. 1998), A109 and A105 to hCGα, B107 to hCG, B210 to hCGβcf (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 LHβ 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 µm 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 hLHβcf Antibody B505 (that had been generated to the pituitary hLHβcf) was immobilized (5 mg/ml) on BioRad Affigel 10 using the method of the manufacturer. Postmenopausal urine pool IV containing hLHβcf as determined by immunoassay, was resuspended in 0·1 M ammonium bicarbonate with the following mixture of protease inhibitors: 250 µg/ml solution of AEBSF (Calbiochem product 101500); EST (Calbiochem 330005, 1 mg/ml in ethanol and added at 50 µl/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); leupeptin (Calbiochem 516482, 1 mg in 1·45 ml of methanol and used at 1:1000 dilution into the mixture); pepstatin (Calbiochem 516482, 1 mg in 50 µl/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 µl of water and added at 2 µl 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 µl 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 LHβ urinary core was purified on the Pharmacia Smart System using the µRP 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 µl/min; 10 min, 0% B; 40 min,
Reduction and alkylation of LHβ cores

Fractions 20+21 were pooled from two reverse-phase HPLC runs (Fig. 1), and dried separately in 500 µl Eppendorf tubes. The contents of each vial were re-suspended in 50 µl 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 µl of the DTT solution (100 µg) was added at time 0. The solution was incubated for 1 h at 55 °C, then a second 10 µl 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 µl of a 40 mg/ml solution) was added. The solution was incubated at ambient temperature for 15 min (alkylation step) and then 40 µl 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 hLHβsf alkylated peptides

Complete removal of the single glycan group present on hLHβ6–40 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.

Immunochromatographic 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 × 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 µm 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 CGβ core** The chimp CGβ 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 µl 8 M urea, 0·4 M NH₄HCO₃, pH 8·0. Reduction and alkylation were carried out by adding 5 µl 0·04 M DTT and maintaining the solution at 50 °C for 1 h, cooling to room temperature, and adding 5 µl 0·08 M iodoacetamide. The solution was allowed to react in the dark for 30 min and was diluted to 250 µl 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 × 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 µl/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 CGα (8·2 µg) and CGβ (7·0 µg) were digested with 0·3 µg trypsin in 100 µl NH₄HCO₃, 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 CGβ 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 LHβ core**

Isolation of the urinary hLHβcf 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 hLHβcf elutes consistently in fractions 20–21 from this column as a broad, flat peak. Immunassay of all column fractions with the B505 immunometric assay for pituitary hLHβcf indicated that this was the only such fragment elution position from the column; other urinary proteins such as the defensins and granulins tended 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 hCGβcf, residues 6 and 55. No other amino termini were detected. In contrast, the pituitary hLHβcf 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 β6–40 linked to β49–93 or β6–40 linked to β55–93 (Birken et al. 1993).
Structural analysis of the urinary hLHβcf

Figure 2 displays the reverse-phase HPLC patterns of reduced, carboxamidomethylated hLHβcf: panel A is the urinary hLHβcf and panel B shows the pituitary hLHβcf, 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 hLHβ55–93 in both urinary (m/z 4540:86) and pituitary hLHβ (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 hLHβ49–93 in the hLHβ 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 hLHβcf is more homogeneous than is the pituitary hLHβcf. 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 hLHβ (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,
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-chimp CGα 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 CGβ 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 hCGβcf (B210), and the third for the hCGβ 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 hCGβcf. As the urine was collected and stored frozen under sterile conditions, the observed chimp CGβ 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
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 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 CGβ core**

Chemical sequence analysis of the chimpanzee CGβ core revealed identical amino termini of β residues 6 and 55, as in human CG core. Both human and chimpanzee hCGβcf were reduced and alkylated and subjected to MALDI analysis. The β55–92 peptide of thechimp CG was m/z 4384-25, whereas the same peptide from the 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 hCGβcf and the chimp CGβcf, 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 hCGβcf 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 (Lapthorn 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 (hCGβcf)
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 hCGβcf, 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 hLHβcf, has been shown to be potentially useful in menopausal studies (Birken et al. 1996, 1999) and also for possible differentiation of a cancer marker, hCGβcf, 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 hCGβcf may indicate the presence of a malignancy in a postmenopausal woman, whereas the presence of LHβ 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 LHβ 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 hLHβcf several years ago, this assay was based on development of antibodies to the hLHβcf 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 LHβ 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 hLHβcf. The structure of the pituitary hLHβcf 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 hLHβcf, but may indicate carbohydrate differences also.

Comparative HPLC patterns of the alkylated chains of the urinary and the pituitary hLHβcf would lead one to propose a precursor–product relationship, as the pituitary core contains a mixture of molecules with either β49–93 or β55–93 linked to β6–40 whereas the urinary hLHβcf contains only two peptides, β55–93 linked to β6–40. 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 hCGβcf. 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 hCGβcf is composed of hCGβ–40 disulfide bridge to hCGβ55–92. In contrast, the LHβcf is composed of the same analogous first peptide hLHβ–40, but is disulfide-bridged to hLHβ55–93 (Birken et al. 1993, 1996, O’Connor et al. 1998). Thus the LHβcf second peptide extends through a cysteine residue, in contrast to that of the hCGβcf. The observed glycans on the LHβ–40 structure represent known glycans on pituitary LHβ subunit with some trimming down to the mannose core, as are the major glycan structures of the hCGβ 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 mannosae and the other with two mannosae.

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

There is also evidence that hCGβcf 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). hCGβcf-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 LHβcf is from circulating LH, because of the pattern of peaking of LHβcf 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 β117. Both amino acid substitutions are in non-critical areas of the molecule. The β117 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 hCGβ subunit analyzed in detail by Policastro et al. (1983) indicated that β117 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 α50 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 bioactivity. 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 CGβcf with a structure that mimics that of the human hCGβcf 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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