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All-or-none *N*-glycosylation in primate follicle-stimulating hormone β-subunits

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Abstract

Human FSH exists as two major glycoforms designated, tetra-glycosylated and di-glycosylated hFSH. The former possesses both α- and β-subunit carbohydrates while the latter possesses only α-subunit carbohydrate. Western blotting differentiated the glycosylated, 24,000 M_r hFSHβ band from the non-glycosylated 21,000 M_r FSHβ band. Postmenopausal urinary hFSH preparations possessed 75–95% 24,000 M_r hFSHβ, while pituitary hFSH immunopurified from 21- to 43-year-old females and 21–43-year-old males possessed only 35–40% 24,000 M_r hFSHβ. The pituitary hFSH from a postmenopausal woman on estrogen replacement was 75% 21,000 M_r hFSHβ. Other immunopurified postmenopausal pituitary hFSH preparations possessed 50–60% 21,000 M_r hFSHβ. Gel filtration removed predominantly 21,000 M_r free hFSHβ and reduced its abundance to 13–22% in postmenopausal pituitary hFSH heterodimer preparations. A major regulatory mechanism for FSH glycosylation involves control of β-subunit *N*-glycosylation, possibly by inhibition of oligosaccharyl transferase. Two primate species exhibited the same all-or-none pattern of pituitary FSHβ glycosylation.

Keywords: *N*-Glycosylation; All-or-none pattern; Follicle-stimulating hormone

1. Introduction

Follicle-stimulating hormone (FSH) is classically composed of two dissimilar glycoprotein subunits, each decorated with two N-linked oligosaccharides (Bousfield et al., 2006). The α-subunit, which is common to FSH, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and, in some species, chorionic gonadotropin (CG), is glycosylated in the C-terminal half of the molecule at Asn^{52} and Asn^{78}. The former Asn residue is located in loop αL2, the portion of the α-subunit embraced by the β-subunit seatbelt loop (Lapthorn et al., 1994; Wu et al., 1994; Tegoni et al., 1999; Fox et al., 2001). The αL2 oligosaccharide is essential for signal transduction (Matzuk et al., 1989; Bishop et al., 1994; Flack et al., 1994) and may enhance heterodimer stability (Bousfield et al., 2004). The Asn^{78} oligosaccharide is located in a cleft between the αL3 and αL1 loops (Lapthorn et al., 1994; Wu et al., 1994; Tegoni et al., 1999; Fox et al., 2001). Oligosaccharides attached to Asn^{78} participate in β-subunit folding and stabilization (Strickland et al., 1985; van Zuylen et al., 1997, 1998; Erbel et al., 2000). Both α-subunit oligosaccharides are aligned along the long axis of the FSH molecule (Fox et al., 2001). The hormone-specific β-subunit is glycosylated in the amino terminal βL1 loop at two sites, Asn^{7} and Asn^{24}. Oligosaccharides attached to these residues are located in parallel β strands and project out from the narrow axis of the molecule. As N-linked oligosaccharides can span as much as 30 Å, these two oligosaccharides effectively double the narrow diameter (30–35 Å) of this elliptical molecule. The FSHβ oligosaccharides determine metabolic clearance rates, as a recombinant hFSH mutant engineered to

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eliminate both hFSHβ *N*-glycosylation sites was cleared from the circulation 10 times more rapidly than wild-type recombinant hFSH. The rapidly cleared, double mutant FSH was inactive in the classical *in vivo* Steelman Pohley assay (Bishop et al., 1995).

We refer to hFSH glycosylated at all four *N*-glycosylation sites as tetra-glycosylated hFSH. A novel hFSH glycoform, designated di-glycosylated hFSH, which possessed only α-subunit oligosaccharides was reported by our laboratory (Walton et al., 2001). Initially, di-glycosylated hFSH appeared to represent a minor variant in hFSH preparations. However, it subsequently appeared in every hFSH isoform preparation isolated by chromatofocusing, a procedure extensively used to characterize gonadotropins under different physiological conditions (Dahl et al., 1988; Grotjan et al., 1990; Ulloa-Aguirre et al., 1995). Moreover, a pituitary hFSH isoform fraction consisting of exclusively di-glycosylated hFSH was significantly more active *in vitro* than isoform fractions that were mixtures of di- and tetra-glycosylated hFSH. Although it was tempting to speculate that the ratio of the two glycoforms contributed to differences in biological activity associated with different FSH isoform preparations (Ulloa-Aguirre et al., 1995), the apparent low abundance of di-glycosylated hFSH made this idea seem unlikely (Walton et al., 2001).

Because di-glycosylated and tetra-glycosylated hFSH represented defined glycoforms that could be identified by Western blotting techniques, we began to look for evidence indicating physiological regulation of their abundance. Individual pituitary glands were the most readily accessible source of gonadotropins in which glycoform ratios could be measured with the sensitivity of existing human-specific antibodies. Two pituitaries, one from a 43-year-old woman and one from a 27-year-old man possessed more non-glycosylated hFSHβ than glycosylated hFSHβ, suggesting more di-glycosylated hFSH was expressed in young adults and that glycoform abundance in pooled pituitary gonadotropin preparations reflected an obvious age bias in postmortem tissue collections. Accordingly, we set out to compare hFSH derived from pituitaries of young adult women with that derived from postmenopausal pituitaries. Our hypothesis was feedback from the functioning ovary inhibited hFSHβ glycosylation. We predicted hFSH from cycling women would possess more di-glycosylated hFSH than tetra-glycosylated hFSH. As human pituitaries were difficult to obtain, the present study describes hFSH glycoform abundance measured in pooled pituitary extracts, postmenopausal urinary gonadotropin preparations, and individual pituitary glands from three primate species. Western blot analysis of hFSH preparations revealed that di-glycosylated hFSH was more abundant than tetra-glycosylated hFSH in humans aged 21–43.

2. Materials and methods

2.1. Hormone preparations

Crude human glycoprotein hormone extracts, designated GTN1–GTN4, were obtained from Dr. Anne Hartree (Cambridge University, Cambridge, UK) (Walton et al., 2001). Human gonadotropin preparations derived from postmenopausal urine (Pergonal) were purchased from Serono Inc. (Rockland, MD) (Findley et al., 1988). Five individual human pituitary glands used during the initial phases of the study were obtained from the National Disease Research Interchange (Philadelphia, PA) and the Co-operative Human Tissue Network (Nashville, TN). Several pituitaries used in later stages of the study were derived from autopsies performed by Dr. Rance on three women aged 21–27 and four women aged 70–77. Pituitaries were collected from individuals known not to have diseases or treatments that would affect the hypothalamic-pituitary-gonadal axis. One exception was a gland obtained from a postmenopausal female receiving estrogen. Frozen monkey pituitary glands were the generous gifts of Dr. Cindy Bethea, Oregon National Primate Research Center, Beaverton, OR, and Dr. Tony Plant, University of Pittsburg School of Medicine, Pittsburg, PA. Dr. A.F. Parlow and the NIDDK National Hormone and Pituitary Program provided purified hFSH and hLH reference preparations NIDDK-hFSH-SIAFP-B-3 and NIDDK-hLH-B-SIAFP-2, respectively.

2.2. Hormone isolation

Individual pituitary glands were acetone-extracted overnight at 4 °C, then air-dried overnight at 25 °C, and stored at −20 °C. Each pituitary was homogenized in 3-ml aliquots 0.02 M sodium phosphate buffer, pH 7.0, along with 100 μl Sigma–Aldrich (St. Louis, MO) general-purpose protease inhibitors, using a Teflon-glass homogenizer. Homogenization was repeated twice with 3-ml aliquots of phosphate buffer. Pooled extracts were centrifuged at 20,000 × *g* in a Sorvall (Heraus, Norwall, CT) RC-26 centrifuge and the clear supernatant applied to an anti-hFSHβ monoclonal antibody column. Initial affinity chromatography procedures employing immobilized monoclonal antibody 46.3 H6.B7 were carried out as previously reported (Walton et al., 2001). In later experiments, the wash buffer was changed to 0.1 M sodium phosphate, 0.3 M sodium chloride, pH 7.0, and the elution buffer was 0.1 M glycine–HCl, pH 2.7, containing 0.5 M NaCl (Weiner et al., 1991). The hFSH samples were concentrated and dialyzed using Centricon P-10 centrifugal ultrafiltration cartridges and protein in the retained fraction recovered by evaporation in a Savant SpeedVac. Dried proteins were reconstituted in 20–50 μl 0.01 M sodium phosphate buffer, pH 7.0. Immunopurified hFSH preparations were characterized by Western blotting. Selected samples were quantified by radioligand assay and characterized by automated Edman degradation and mass spectrometry.

2.3. Radioligand assay

An institutional animal care and use committee approved all animal procedures. Receptor-binding assays were performed using 25 mg rat testis homogenate tissue/tube and 2.5 ng of ^{125}I-eFSH tracer (Nguyen et al., 2003). The chloramine T technique was used for iodination, producing specific activities of 30–50 μCi/μg. Duplicate assay tubes were incubated for 2 h at 37 °C in a shaking water bath, then centrifuged, the supernatant aspirated, and the pellet counted in a Packard (Meriden, CT) Cobra II gamma counter. The counting efficiency was >74%. Data were analyzed using Prism v4.0 (GraphPad Software Inc., San Diego, CA).

2.4. Western blotting

Samples were prepared for SDS-PAGE on 15% polyacrylamide mini-slab gels under reducing conditions and electroblotted to Bio-Rad (Hercules, CA) Immobilon-P (Walton et al., 2001). Membranes were blocked with 5% non-fat dry milk dissolved in 150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 0.05% Tween 20, and incubated overnight with anti-hFSHβ monoclonal antibody RFSH20 diluted 1:1000 (Walton et al., 2001). The next day, the membranes were washed and incubated with 1:1000 diluted anti-mouse antiserum conjugated to horseradish peroxidase for 1 h, washed, treated with Amersham (Piscataway, NJ) ECL Plus chemiluminescence reagent for 1 min and exposed to X-ray film for varying periods of time. The density of the bands on the X-ray film was measured with a Bio-Rad (Hercules, CA) GelDoc 1000 and density analysis carried out using the Bio-Rad software package Quantity One v 4.5.1. In later experiments, a Bio-Rad VersaDoc 4000 measured the chemiluminescence directly.

2.5. Mass spectrometry

Immunopurified hFSH preparations were analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry using a Hewlett-Packard MALDI-TOF MS. Further analysis involved reverse-phase HPLC coupled to a Micromass Q-TOF-2. Samples were applied to a 1 mm × 5 mm C-18 reverse-phase column equilibrated with 0.1% formic acid in water. The chromatogram was developed with a gradient from 5% to 80% acetonitrile containing 0.1% formic acid over 45 min. Column effluent was monitored by UV absorbance followed by on-line mass spectrometry. MALDI-MS analysis of macaque FSH samples was performed using a Waters-Micromass (Manchester, UK) TofSpec 2E reflectron time-of-flight mass spectrometer fitted with a N_2 (337 nm, 3 ns pulse) laser and a delayed extraction ion source. The matrix employed was sinapinic acid.

2.6. Edman degradation

Samples consisting of 2 μl immunopurified hFSH or macaque FSH were applied to glass fiber disks that had been coated with Biobrene and precycled through three rounds of Edman chemistry. Automated Edman degradation was carried out in an Applied Biosystems (Foster City, CA) model 492 Procise sequencer using the pulsed-liquid automated Edman procedure implementing the manufacturer's recommended method.

3. Results

We compared the relative abundance of glycosylated and non-glycosylated hFSHβ in two common sources of purified hFSH preparations: pooled human pituitary extracts (Hartree, 1975) and postmenopausal urinary gonadotropin preparations (Reichert, 1967). Western blot analysis of 20–40 μg samples of four pooled human pituitary extracts revealed that the majority of the hFSHβ present was the M_r 24,000, glycosylated glycoform (Fig. 1). The little M_r 21,000 non-glycosylated glycoform

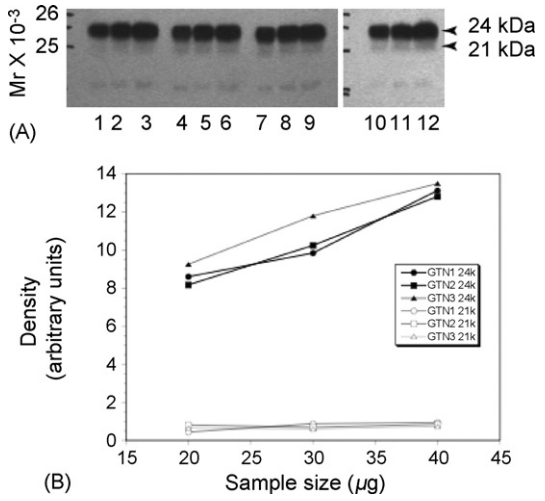

Fig. 1. Relative abundance of hFSHβ isoforms in four batches of crude human pituitary extract glycoprotein fractions (GTN) by Western blotting using the anti-FSHβ monoclonal antibody RFSH20. In all four preparations, the M_r 24,000 band represented >95% of the immunoactivity of 30–50 μg samples. (A) X-ray film showing hFSHβ immunoactivity. GTN extracts 1–3 were examined in the same gel. A second gel was necessary to accommodate GTN4 samples. Lanes 1–3: 20 μg, 30 μg, and 40 μg, respectively of GTN1; lanes 4–6: 20 μg, 30 μg, and 40 μg, respectively of GTN2; lanes 7–9: 20 μg, 30 μg, and 40 μg, respectively of GTN3; lanes 10–12: 20 μg, 30 μg, and 40 μg, respectively of GTN4. SDS-PAGE was performed on reduced samples using 15% polyacrylamide gels. (B) Densitometric analysis of the GTN 1–3 bands in lanes 1–9.

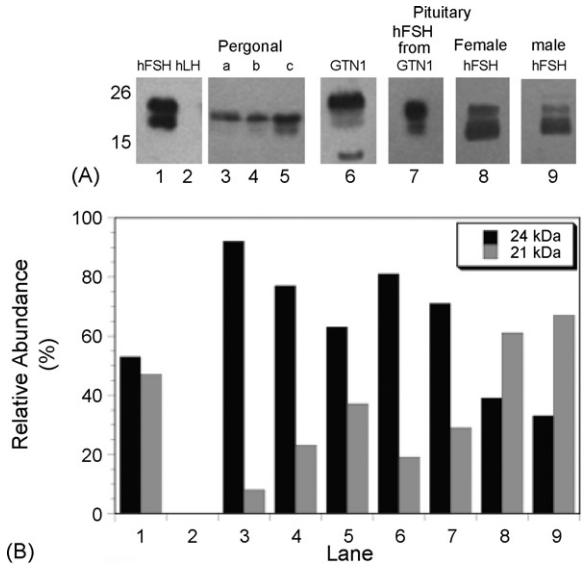

Fig. 2. Relative abundance of hFSHβ isoforms in various FSH preparations, as indicated by Western blotting. (A) Analysis of hFSHβ isoforms using the anti-FSHβ monoclonal antibody RFSH20. Lane 1: 1 μg NIDDK-hFSH-SIAFP-B-3; lane 2: 1 μg NIDDK-hLH-B-SIAFP-2; lane 3: Pergonal lot 'a'; lane 4: Pergonal lot 'b'; lane 5: Pergonal lot 'c'; lane 6: 60 μg GTN1 from a previous report (Walton et al., 2001); lane 7: immunopurified hFSH obtained from human pituitary extract GTN1; lane 8: hFSH immunopurified from the pituitary gland of a 43-year-old female; lane 9: hFSH immunopurified from a 27-year-old male. (B) Relative abundance estimated from densitometric analysis of the X-ray films.

visible remained below the linear detection range of X-ray film (Fig. 1B). The M_r 24,000 glycoform was also much more abundant than the M_r 21,000 hFSHβ glycoform in three commercially available urinary hFSH preparations, comprising 75–95% of the hFSHβ subunit (Fig. 2, lanes 3–5).

We immunopurified hFSH from one of the crude pituitary preparations using a monoclonal antibody, 46.3 H6.B7 (Fig. 2, lane 7). This was comparable to the 24% relative abundance reported for the M_r 21,000 hFSHβ glycoform following conventional hFSH isolation from the same source (Walton et al., 2001). A 60 μg sample of crude pituitary extract possessed 15% M_r 21,000 hFSHβ glycoform (Fig. 2, lane 6). In striking contrast to the foregoing, when we examined hFSH preparations immunopurified from a 27-year-old male pituitary and a 43-year-old female pituitary gland, the preparations possessed 60–65% non-glycosylated, M_r 21,000 hFSHβ glycoform (Fig. 2, lanes 8 and 9).

As three more male pituitaries were available, we immunopurified hFSH from each gland and determined the relative abundance of both hFSHβ glycoforms. Western blotting of the additional pituitary hFSH preparations along with the two isolated earlier indicated that all hFSH preparations were composed of 60–65% non-glycosylated M_r 21,000 hFSHβ glycoform (Fig. 3A and B). MALDI-MS analysis of the same hFSH preparations was consistent with the existence of two hFSHβ forms, along with an overlapping series of intermediate mass hFSHβ ions that partially obscured the hFSHβ ions (Fig. 3C–H). The masses of the smaller hFSHβ glycoform ranged from *m/z* 12,224 to *m/z* 12,476.4, while those of the larger hFSHβ glycoform ranged from *m/z* 16,460.8 to *m/z* 17,497.0. The α-

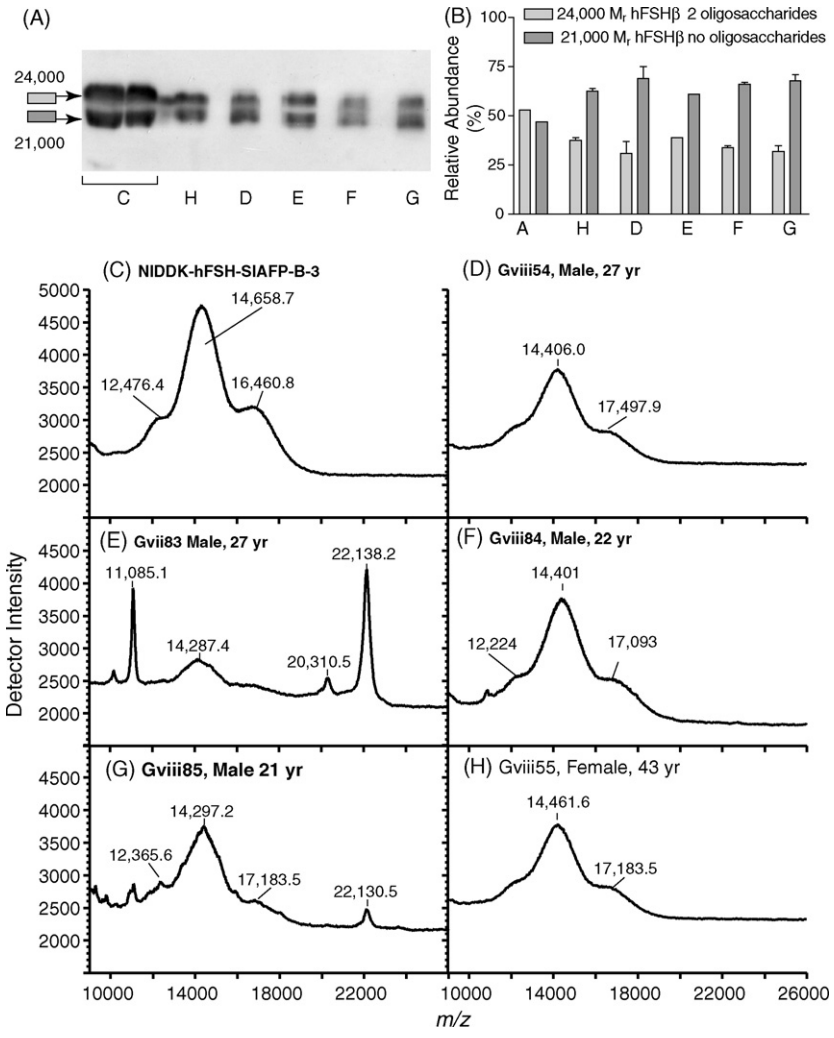

Fig. 3. Analysis of individual pituitary hFSH preparations. (A) Relative abundance of hFSHβ glycoforms from individual human pituitaries by Western blotting using the anti-FSHβ monoclonal antibody RFSH20. Lane-identifying letters correspond to mass spectrometry panels, below. (A) Western blot showing immunoreactive 24,000 M_r and 21,000 M_r hFSHβ bands. Lane C: 1 μg and 2 μg samples NIDDK-hFSH-SIAFP-B-3; lane H: hFSH from 43-year female (a sample from the same gland was shown in (A)); lane D: hFSH from 27-year male (a sample from the same gland was shown in (A)); lane E: hFSH from different 27-year-old male; lane F: hFSH from 22-year-old male; lane F: hFSH from 21-year-old male. (B) Relative abundance of 24,000 M_r and 21,000 M_r hFSHβ glycoforms calculated from band density obtained from two Western blots involving the same samples as in panel A (mean ± S.D., $n = 2$). (C–H) MALDI-MS analysis of hFSH preparations. (C) NIDDK-hFSH-SIAFP-B-3. (D) Purified hFSH from pituitary gland of 27-year-old male. (E) Purified hFSH from pituitary gland of second 27-year-old male. (F) Purified hFSH from pituitary gland of 22-year-old male. (G) Purified hFSH from pituitary gland of 21-year-old male. (H) Purified hFSH from pituitary gland of 43-year-old female.

subunit masses ranged from *m/z* 14,287.2 to *m/z* 14,461.6. The MALDI-MS spectrum for a 27-year-old male hFSH preparation was dominated by an *m/z* 22,138.2 ion that was identified as human growth hormone (Fig. 3E). Automated Edman degradation produced the sequence: Phe-Pro-Xxx-Ile-Pro-Leu-Ser-Arg-Phe-, matching the known sequence of Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-Phe- for the GH amino terminus. Tandem liquid chromatography–mass spectrometry readily identified hGH in this fraction and in another possessing much less GH. However, we were unable to detect hFSH subunits using this procedure even when the hFSH reference preparation was examined (data not shown).

Human FSH was immunopurified from seven female pituitary glands. Radioligand receptor-binding studies indicated recoveries of 11–290 μg hFSH from 70- to 77-year-old female pituitaries and 96–320 μg hFSH from 21- to 27-year-old female pituitaries. Western blot analysis of hFSH derived from three 21- to 27-year-old female pituitaries provided consistent estimates of ∼65% for the M_r 21,000 hFSHβ glycoform (Fig. 4). Analysis of four 70–77-year-old female pituitaries provided more variable results. A 70-year-old female pituitary from an individual receiving estrogen replacement therapy provided only 11 μg hFSH and possessed 75% non-glycosylated M_r 21,000 hFSHβ. Two pituitaries from 71- and 72-year-old females possessed equal amounts of both glycoforms, while hFSH from a 77-year-old female possessed 60% M_r 21,000 hFSHβ.

Monoclonal antibody 46.3H6.B7 binds both hFSH heterodimer and hFSHβ subunit. To address the question of whether or not immunopurified hFSH preparations also included hFSHβ subunit, we performed gel filtration on hFSH isolated from

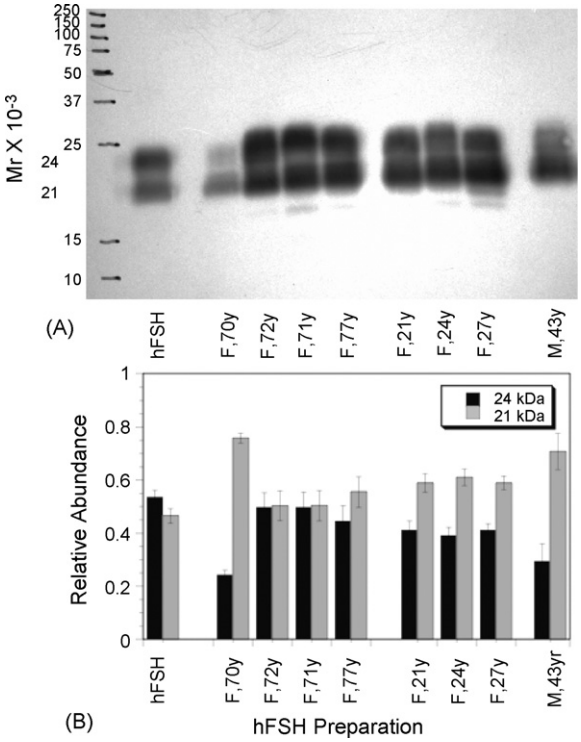

Fig. 4. Relative abundance of hFSH glycoforms in hFSH preparations derived from young and old women as indicated by FSHβ-specific Western blotting. Representative results from three gels. (A) X-ray film showing 24,000 M_r and 21,000 M_r hFSHβ bands. (B) Relative abundance of each hFSHβ glycoform determined from band density. Gender and age of samples are indicated.

21- and 72-year-old individuals (Fig. 5). Two peaks emerged and Western blot analysis of both fractions indicated the larger peak was comprised of both subunits, indicating hFSH, while the smaller peak was comprised of only hFSHβ subunit. Both hFSHβ glycoforms were present in the former fraction, while the latter consisted of largely non-glycosylated hFSHβ. We subjected the rest of the hFSH preparations to gel filtration and obtained similar chromatograms. Protein recoveries were too low for four of the samples to be included in the Western blot analysis. Comparison of the remaining hFSH samples showed a pattern consistent with our original hypothesis that a substantial amount of hFSH in cycling women was di-glycosylated hFSH while the majority in postmenopausal women was tetra-glycosylated hFSH.

We also examined FSH isolated from individual female macaque pituitaries. In pituitaries derived from ovariectomized female rhesus, the rhFSHβ migrated as two major bands with the same relative mobility as hFSHβ subunit bands (Fig. 6A). The FSHβ patterns from ovariectomized, estrogen/progesterone-treated female pituitaries were the same as ovariectomized monkey FSHβ pattern, consisting of two bands with electrophoretic mobilities similar to the hFSHβ M_r 24,000 and M_r 21,000 bands. Mass spectrometry of two samples confirmed that the changes in electrophoretic mobility reflected changes in mass (Fig. 6B and C). Automated Edman degradation revealed the presence of PTH-Asn associated with both Asn^7 and Asn^{24}, indicating that carbohydrate had not been attached to the M_r 21,000 FSHβ subunit glycoforms (Fig. 7). However, the very broad distribution of α-subunit ions largely obscured the FSHβ ions.

FSH preparations isolated from three intact female Japanese macaque (jm) pituitaries were compared with jmFSH from three ovariectomized females (Fig. 8). A broad pattern of immunoreactive jmFSHβ bands was associated with jmFSH isolated from intact animals and the amounts of jmFSH in each extract varied. This was altered into a largely two-band pattern in FSH isolated from ovariectomized animals. C-terminal FSHβ fragment bands in lanes 3–5 indicated limited degradation in these preparations (Fig. 8, lanes 3–5 lowest arrowhead).

4. Discussion

We have encountered two patterns of partial pituitary FSHβ *N*-glycosylation (Walton et al., 2001; Bousfield et al., 1996). In the horse pituitary, 50% of purified eFSHβ preparations lacked carbohydrate attached to Asn^7 (Bousfield et al., 1996). Purified human pituitary FSH was comprised of 24% M_r 21,000 hFSHβ that lacked both N-linked oligosaccharides (Walton et al., 2001). Recombinant, insect cell-expressed hFSHβ was partially glycosylated at Asn^{24} (Fox et al., 2001), while recombinant hFSH derived from CHO cells appeared to possess both β-subunit glycans (Xing and Moyle, 2003; Gervais et al., 2003). The all-or-none pattern of FSHβ glycosylation appeared to occur in two other primate species, while electrophoretic mobilities for oFSH and pFSH subunit bands were consistent with the eFSH pattern of one or two β-subunit *N*-glycans (Walton et al., 2001; Gordon et al., 1989). The differences in human FSHβ glycosylation observed when hFSH was expressed in different cell lines most likely reflected tissue-specific differences in glycoprotein processing. The physiological significance of both patterns of FSHβ glycosylation remain speculative, although all-or-none primate FSHβ glycosylation most likely impacts clearance from the circulation (Walton et al., 2001).

Both eFSH and hFSH preparations used to establish β-subunit glycosylation site occupancy were derived from pooled pituitaries combining individuals of unknown gender, age, and physiological state. In the present study, examination of hFSH isolated from both male and female individual human pituitaries indicated that the M_r 21,000, non-glycosylated hFSHβ glycoform comprised 40–75% of the hFSH preparations. MALDI-MS of several of these hFSH preparations confirmed the presence of both β-subunit glycoforms despite considerable overlap from α-subunit ions (Walton et al., 2001). Isolated α-subunit from pituitary hFSH isoform preparations produced a heterogeneous collection of ions covering a broad range of masses with the *m/z* value for each centroid ranging from 13,796 to 14,377 (Walton et al., 2001). In our previous study, separate ions for the full-length and truncated non-glycosylated pituitary hFSHβ isoforms were observed (Walton et al., 2001). As the present study employed a lower resolution instrument, these ion peaks overlapped. Nevertheless, the *m/z* values were consistent with the presence of non-glycosylated hFSHβ. Moreover, the relative peak heights were also in agreement with the Western blotting results. For example, hFSHβ in the pituitary hFSH reference preparation was comprised of more glycosylated than

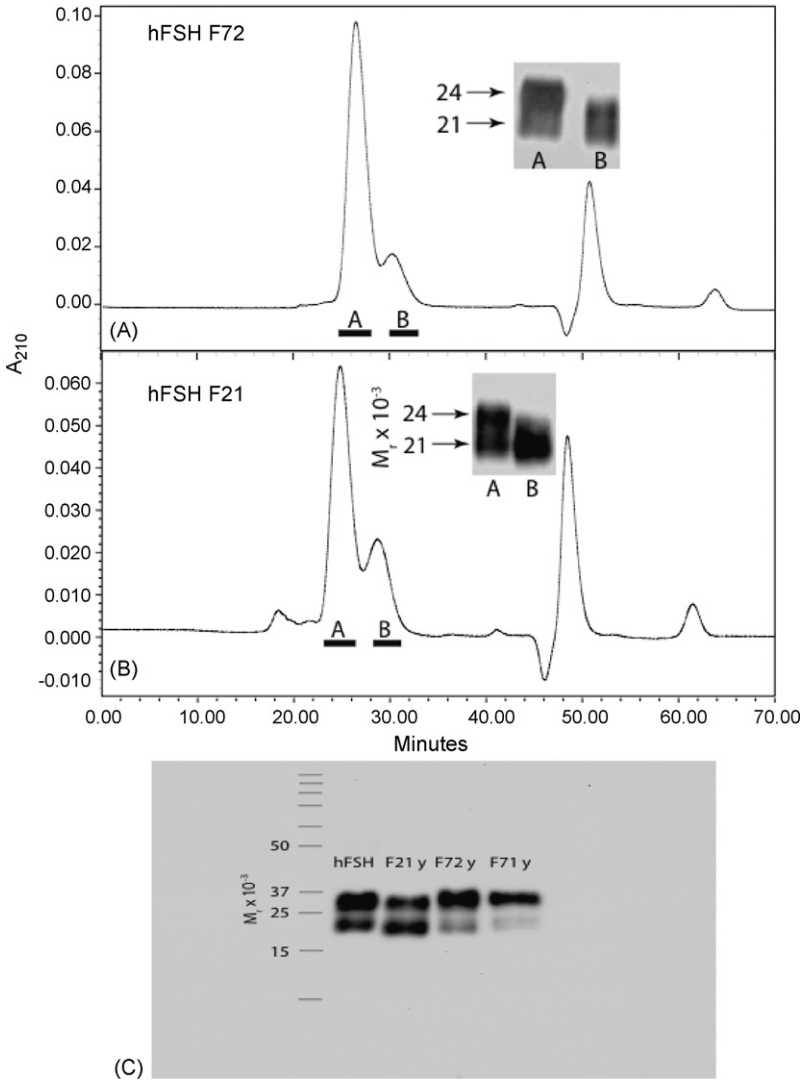

Fig. 5. Effects of gel filtration on hFSH glycoform abundance as indicated by hFSHβ-specific Western blotting. (A) Superdex 75 chromatogram of hFSH isolated from 72-year female pituitary. The solid bars show the portions of the chromatogram pooled to obtain the hFSH and hFSHβ fractions. The inset shows Western blot of fractions A and B, as indicated, using hFSHβ peptide monoclonal antibody P03. (B) Superdex 75 chromatogram of hFSH isolated from 21-year-old female pituitary. Inset shows Western blot as in panel A. (C) Western blot of Superdex 75-purified hFSH preparations probed with monoclonal antibody RFSH20. Di-glycosylated hFSH glycoform abundance: 21 years, 54%; 72 years, 22%; 71 years 13%.

non-glycosylated hFSHβ and the intensity of the *m/z* 16,460.8 species was greater than that of the *m/z* 12,476.4. In the case of hFSH isolated from individual pituitaries, the non-glycosylated hFSHβ ion peak intensities were greater than those of the glycosylated glycoform ions. Mass spectrometry results provided independent confirmation of Western blotting experiments that showed immunopurified hFSH preparations derived from individual pituitaries were comprised of a substantial amount of non-glycosylated FSHβ subunit.

Based on the low abundance of M_r 21,000 hFSHβ glycoform present in crude postmenopausal urine, in contrast with its substantially increased abundance in individual human pituitaries, we hypothesized that the greater abundance of the M_r 24,000 form in postmenopausal urine reflected the physiological changes associated with menopause. Reduced circulating estrogen levels associated with menopause were predicted to coincide with higher levels of the M_r 24,000 hFSHβ glycoform, just as ovariectomy increased the abundance of high MW rhFSH isoforms in rhesus monkey pituitaries (Peckham et al., 1973). A switch in expression of predominantly non-glycosylated hFSHβ in pituitary hFSH derived from cycling women to predominantly glycosylated hFSHβ in pituitary hFSH from elderly women contributed to the increased circulating hFSH concentrations in postmenopausal women. The greater abundance of M_r 24,000 hFSHβ glycoform in urinary FSH was consistent with this hypothesis, as hFSH possessing the M_r 21,000 hFSHβ glycoform should have been preferentially eliminated in urine, because the kidney provides the major clearance mechanism for exclusively sialylated glycoprotein hormones, such as hFSH (see Walton et al., 2001 and references therein). Recombinant hFSH mutants lacking either one or both β-subunit *N*-glycosylation sites were cleared more rapidly than wild-type recombinant hFSH (Bishop et al., 1995). The fact that non-glycosylated hFSHβ abundance was low in all three

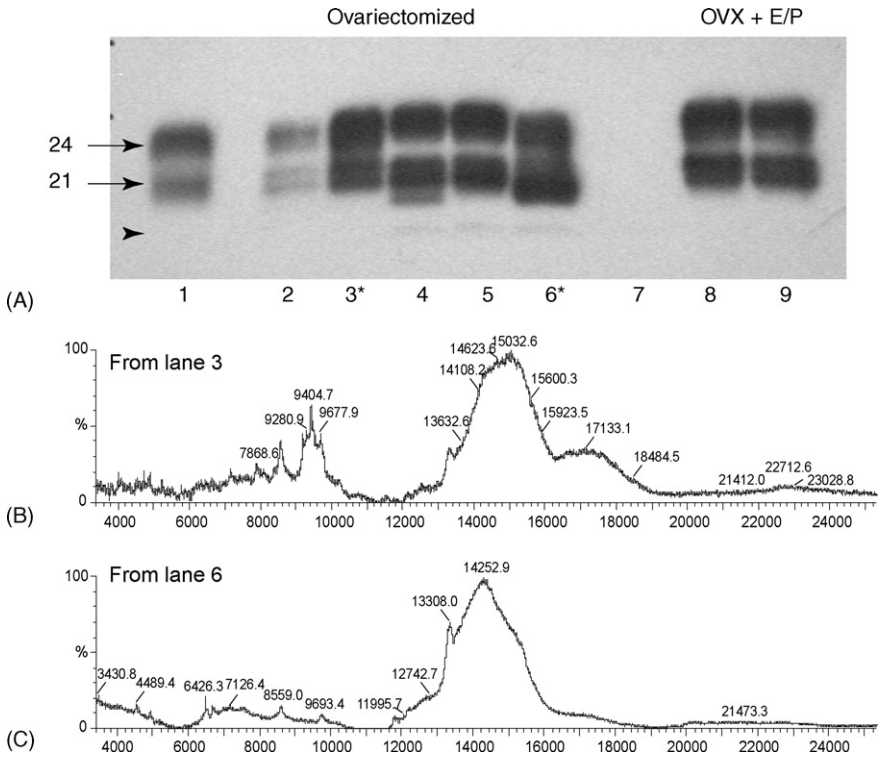

Fig. 6. Ovariectomized rhesus FSHβ isoforms. (A) Western blot of rhFSH isolated from individual female rhesus pituitaries. The primary antibody was RFSH20. Lane numbers with asterisks indicate those samples evaluated by MALDI-MS. Lane 1: hFSH; lanes 2–6: rhFSH isolated from individual ovariectomized female monkey pituitaries; lanes 7–9: rhFSH isolated from pituitaries of ovariectomized female monkeys treated with estrogen and progesterone. Note that the amount of rhFSH in lane 7 was below detectable limits. Mass spectrometry of rhesus FSH preparations. (B) MALDI-MS analysis of rhFSH preparation in lane 3. (C) MALDI-MS analysis of rhFSH preparation in lane 6. Note the absence of the *m/z* 17,133.1 species.

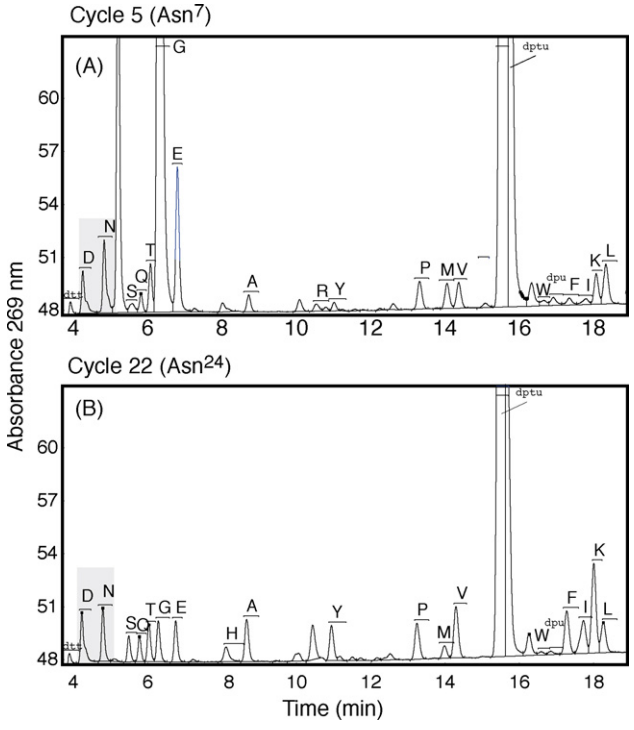

Fig. 7. Edman degradation of rhFSH preparation. (A) PTH-amino acid derivative chromatogram at sequencer cycle 5 corresponding to *N*-glycosylation site Asn^7 in the more abundant N-terminally truncated isoform. Note the presence of PTH-Asn, indicating oligosaccharide was never attached to this site. (B) Cycle 22, corresponding to Asn^{24} the second *N*-glycosylation site.

postmenopausal urinary gonadotropin preparations, suggested a corresponding low abundance in circulation. Direct analysis of hFSH serum samples necessary to address this issue, has not yet been possible. Western blotting of human serum samples subjected to the immunopurification procedure employed in these studies revealed only immunoglobulin bands, presumably of human origin (data not shown). Proteomic analysis of human serum samples exposed to anti-hFSH antisera immobilized on Biacore chips failed to find anything remotely resembling FSH (G.R. Bousfield and Dobrin Nedelkov, Intrinsic Bioprobes Inc., Tempe, AZ, unpublished data). Proteomic analysis probably failed due to inadequate FSH purification, which the data in Fig. 3E indicate is critical for characterization of FSH by mass spectrometry.

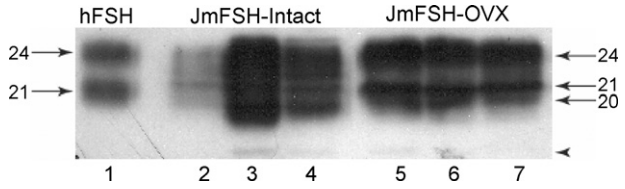

Fig. 8. Japanese macaque FSHβ isoforms. FSH was isolated from pituitaries removed from intact and ovariectomized females and analyzed by FSHβ Western blotting using monoclonal antibody RFSH20. Note the multiple immunoreactive FSHβ bands from intact pituitaries collapse to two major zones of immunoactivity following ovariectomy. In lane 3, an hFSHβ fragment is visible, indicating limited sample degradation. Lane 1: hFSH; lanes 2–4: intact female FSH; lanes 5–7: ovariectomized female FSH.

We predicted that pituitaries of postmenopausal females would possess more M_r 24,000 hFSHβ than those from cycling females. Increased pituitary content of a larger rhFSH glycoform was reported following ovariectomy in rhesus monkeys (Peckham et al., 1973). It now appears that the larger rhFSH isoform represented tetra-glycosylated FSH. The fact that it could be detected by as crude a technique as Sephadex G-100 gel filtration suggested a substantially greater structural alteration than merely increased oligosaccharide branch termination with sialic acid. Indeed, while estrogen replacement therapy in ovariectomized rhesus monkeys completely reversed the increase in FSH molecular size, neuraminidase digestion did not always achieve complete reversal (Peckham and Knobil, 1976). The greater expression of the non-glycosylated hFSHβ observed in pituitaries from presumably cycling women aged 21–24 was consistent with the hypothesis that estrogen inhibits FSHβ glycosylation. However, the higher than expected abundance of di-glycosylated hFSH isolated from pituitaries of women aged 70–77 was apparently inconsistent with this hypothesis. We had assumed that free FSHβ glycoform abundance in the pituitary would reflect its abundance in FSH preparations based on preliminary experiments with hFSH isolated from pooled pituitary extracts. This assumption proved incorrect for individual pituitaries, as free hFSHβ was largely the M_r 21,000 glycoform, while hFSH possessed both glycoforms. Following gel filtration, examination of a postmenopausal pituitary hFSH heterodimer fraction revealed di-glycosylated hFSH abundance was reduced to the low levels observed in postmenopausal urinary preparations. Thus, our hypothesis is correct and a shift in FSH glycoform abundance is associated with menopause.

Early studies of pituitary FSH heterogeneity indicated estrogen was responsible for altering FSH molecular size, which was assumed to be a consequence of changes in glycosylation (Peckham et al., 1973; Peckham and Knobil, 1976; Bogdanove et al., 1974). Changes in sialic acid were suspected because neuraminidase digestion sometimes produced the same changes in molecular size (Peckham and Knobil, 1976). The first direct evidence for estrogen regulation of pituitary glycosylation was reported by Damian-Matsumura et al. (1999) who demonstrated estrogen reduced α2,3-sialyltransferase expression in rat pituitaries. One of the pituitaries in the present study was derived from a postmenopausal women receiving estrogen replacement therapy. The high abundance of non-glycosylated hFSHβ was consistent with the hypothesis that estrogen inhibits FSHβ *N*-glycosylation at the level of oligosaccharyl transferase. In the present issue, Campo (2005) report differences in hFSH glycan branching under different physiological conditions. Thus, all phases of *N*-glycosylation beginning with transfer of the preformed high mannose precursor through glycan remodeling involving adding 1–4 branches to each oligosaccharide to addition of terminal residues, such as sialic acid appear to be regulated by gonadal feedback.

The correspondence between pituitary FSH glycoform and urinary FSH glycoform abundance is significant because it means that hFSH in urine samples can be used to assess pituitary and possibly circulating di-glycosylated and tetra-glycosylated hFSH glycoform abundance. This will permit clinical studies to determine whether di-glycosylated hFSH abundance declines progressively during the perimenopausal period or abruptly at the menopausal transition.

When we identified non-glycosylated hFSHβ, we speculated that the all-or-none pattern of hFSHβ glycosylation could be a general phenomenon in primates in contrast with partial FSHβ glycosylation in horses, pigs, and sheep. Supporting evidence has been obtained for two non-human primate species. Ovariectomy produced the same M_r 24,000 and M_r 21,000 bands observed for hFSH following Western blot analysis of both rhesus and Japanese macaque FSH preparations. Automated Edman degradation of rhesus FSH encountered PTH-Asn at cycles 5 and 7, which corresponded to Asn^7 in the truncated and full-length rhesus FSHβ isoforms, respectively. PTH-Asn was also detected at cycles 24 and 26, however, the data were less convincing due to the high background that accumulated after the substantially greater number of Edman degradation cycles. The presence of PTH-Asn derivatives in cycles corresponding to rhFSHβ glycosylation sites indicated that carbohydrate had not been transferred to the M_r 21,000 rhFSHβ glycoform in the endoplasmic reticulum. Significantly, all rhesus pituitary FSH isoforms are known to be secreted (Chappel et al., 1984), therefore, a substantial amount of di-glycosylated rhFSH glycoforms are present in the rhesus circulation. MALDI-MS was less suitable for rhesus FSH analysis because of greater overlap in the *m/z* values for M_r 21,000 FSHβ ions and the α-subunit ions. While the latter appeared to extend over a broader range of *m/z* values, confirming data in the form of broad α-subunit bands were not available because the α-specific antibodies available did not cross-react with the monkey α-subunits.

Pituitary hFSH consists of two glycoforms, di-glycosylated FSH and tetra-glycosylated FSH. The relative abundance of both glycoforms is physiologically regulated and can be measured by hFSHβ Western blot analysis provided heterodimeric hFSH preparations are measured. Menopause is associated with reduction in the abundance of di-glycosylated hFSH. Examination of urinary hFSH glycoform abundance will establish the timing of this event.

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