Evolutionary Conservation of the Sulfated Oligosaccharides on Vertebrate Glycoprotein Hormones That Control Circulatory Half-life*

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Stephen M. Manzella‡§, Shylaja M. Dharmesh‡, Mary C. Beranek‡, Penny Swanson¹, and J. U. Baenziger|| From the ‡Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110 and the §School of Fisheries, University of Washington, Seattle, Washington 98195

The circulatory half-life of the mammalian glycoprotein hormone lutropin is controlled by its unique Asn-linked oligosaccharides, which terminate with the sequence SO₄₋₄-GalNAcβ₁,4GlcNAc. A cluster of basic amino acids essential for recognition of the α subunit by the glycoprotein hormone N-acetylgalactosaminyltransferase is located within two turns of an α helix (Mengeling, B. J., Manzella, S. M., and Baenziger, J. U. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 502-506). The amino acids within this region are virtually invariant in the α subunits of all vertebrates, indicating that the recognition determinant utilized by the N-acetylgalactosaminyltransferase has been conserved in species ranging from teleost fish to mammals. We demonstrate that the glycoprotein hormone N-acetylgalactosaminyltransferase and the N-acetylgalactosamine-4-sulfotransferase responsible for the synthesis of these unique sulfated oligosaccharides are expressed in the pituitaries of vertebrates ranging from teleost fish to mammals. Furthermore, we show that Asn-linked oligosaccharides terminating with SO₄₋₄-GalNAcβ₁,4GlcNAc are present on the α and β subunits of the salmon glycoprotein hormone GTH II. Asn-linked oligosaccharides terminating with SO₄₋₄-GalNAcβ₁,4GlcNAc are unique structural features of the glycoprotein hormones that have been conserved during vertebrate evolution, suggesting they are critical for the expression of hormone biologic activity.

Even though extensive structural diversity and heterogeneity are characteristic of the oligosaccharides found on glycoproteins, there are instances in which highly distinctive oligosaccharide structures are present on specific glycoproteins or types of glycoproteins from different animal species. The presence of such characteristic oligosaccharide structures indicates that the glycopolys bearing them have biologic functions that are dependent on the structural features of these oligosaccharides. We (1-3) and others (4) have demonstrated that Asn-linked oligosaccharides terminating with SO₄₋₄-GalNAcβ₁,4GlcNAc are characteristic of the glycoprotein hormone lutropin (LH) and thyrotropin from a number of mammalian species, whereas oligosaccharides terminating with sialic acid-Galβ₁,4GlcNAc, commonly found on many glycoproteins, predominate on follitropin (2, 3). Oligosaccharides bearing terminal β₁,4-linked GalNAc₄₄SO₄ are recognized by a receptor in hepatic endothelial cells, resulting in the rapid removal of glycoproteins bearing these structures from the blood (5-7). The short circulatory half-life of LH in conjunction with its stimulated release from gonadotrophs by gonadotropin-releasing hormone produces a periodic rise and fall in circulating LH levels, which is thought to be essential for optimal activation of the ovarian LH receptor and fertilization. Thus, in mammals the presence of terminal GalNAc₄₄SO₄ is associated with a highly specific function.

The presence of terminal GalNAc₄₄SO₄ reflects the activity of a glycoprotein hormone N-acetylgalactosaminyltransferase and a GalNAc₄₄sulfotransferase, which we have shown are expressed in the anterior lobe of the pituitary from a number of mammals (8-11). In addition to the oligosaccharide acceptor, the N-acetylgalactosaminyltransferase recognizes a protein determinant within the α subunit of the glycoprotein hormones (8, 12). All of the information required for recognition of the α subunit by the GalNAc-transferase is contained within a 23-amino acid glycopeptide fragment (10). The recognition determinant consists of a cluster of basic amino acids that are contained within two turns of an α helix (13) A number of studies have demonstrated that the region of the α subunit, which includes the N-acetylgalactosaminyltransferase recognition determinant, also contains residues that are critical for combination with the β subunits (14, 15) and for binding to and activation of the LH/CG-receptor (16). Thus, a number of distinct functions are dependent on interactions of different proteins with the same region of the α subunit.

Homologues of the glycoprotein hormones have been identified in vertebrates from mammals to teleost fish (17). In mammals, follitropin is responsible for follicular development, LH drives oocyte maturation and ovulation, and thyrotropin regulates thyroid function (18). Similar functions have been attributed to glycoprotein hormone homologues in other vertebrate species. Features of both the common α subunit and the hormone-specific β subunits have been conserved in vertebrate glycoprotein hormones including locations of the Cys and Asn glycosylation sites (17, 18). Furthermore, crystallographic studies of human chorionic gonadotropin have revealed that both the α and β subunits have a cysteine-knot motif like that of GalNAc, N-acetylgalactosamine; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; GGnM-MCO, GalNAcβ₁,4GlcNAcβ₂Man₄a₁-O(CH₂)₈-COOH₂; S4GnM-MCO, SO₄₋₄GalNAcβ₁,4GlcNAcβ₂Man₄a₁-O(CH₂)₈-COOH₂; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
found on nerve growth factor, transforming growth factor-β, and platelet-derived growth factor (19, 20). The residues that are critical for recognition of the α subunit by the β1,4-acetylgalactosaminyltransferase fall within a region of the α subunit that is virtually invariant among vertebrate species. Since the recognition determinant utilized by the mammalian N-acetylgalactosaminyltransferase is present within the α subunits of virtually all vertebrate species, we wished to determine if the glycoprotein hormone N-acetylgalactosaminyltransferase (8), the GalNAc-4-sulfotransferase (9), and the sulfated oligosaccharide structures, which we have described on LH, thyrotropin, and the free, uncombined β subunits of mammalian species (2, 3), are also conserved. The presence of these same sulfated oligosaccharides on glycoprotein hormones of other vertebrates would strongly support the biologic importance of these structures, which have been found to control the circulatory half-life of LH in mammals (6, 7).

MATERIALS AND METHODS

Transfase Assays—Pituitaries were dispersed in 5 volumes (vol/wt) of buffer A (25 mM Tris, pH 7.5, 250 mM sucrose, 1 mM EDTA, 0.15% Triton X-100) and sonicated with a Branson sonifier for 2×10-s pulses at a setting of 5. The homogenates were then clarified by sedimentation at 2000 × g for 10 min, and the supernatants were used at 8°C until used.

Transfer of GalNAc by the glycoprotein hormone N-acetylgalactosaminyltransferase (GalNAc-transferase) to different oligosaccharide acceptors on human chorionic gonadotropin (hCG) and transferrin (Trf), which do and do not contain the GalNAc-transferase recognition motif, respectively, was compared using the assay previously described (11). Each 50-μl transferase reaction consisted of 25 mM HEPES (pH 7.5), 0.1% (w/v) Triton X-100, 10 mM ATP, 15% (v/v) glycerol, 10 mM MnCl2, protease inhibitors (5.75 milli-trypsin inhibitor units of aprotinin, 166 mg/ml glycerol, 0.1% (w/v) Triton X-100, 10 mM ATP, 166 mg/ml glycerol, protease inhibitors (5.75 milli-trypsin inhibitor units of aprotinin, 1 μg each of leupeptin, antipain, pepstatin, and chymostatin), 1 mM UDP-GalNAc, 200 ng of agal-hCG or 420 ng of agal-Trf (hCg and Trf, which had been lyophilized and stored in 0.1 M NaHCO3 at pH 8.5–9.0, were dissolved in 1.5-mI microfuge tubes. The homogenates were dialyzed against 2000 × g for 3 min. The reaction was then brought to a final concentration of 50 mM NaF, 0.4% Nonidet P-40, 1 mM EDTA, pH 8.0, and the glycosidase was added by digestion with 0.1 μg/ml of Pronase E in 126 mM NaCl, 62.5 mM Tris, pH 6.8. Reactions were clarified by sedimentation at 2000 × g for 10 min, and the supernatants were used at 8°C until used.

The 35S-labeled SGGnM-MCO assay product was treated with 1.0 N HCl for 16 h at 4°C. After washing in 20 mM Tris, pH 7.5, 0.5% Tween 20, 0.1% bovine serum albumin, the blot was incubated with 0.4 μg/ml streptavidin-peroxidase (Sigma) for 1 h followed by chemiluminescence reagent (DuPont NEN) and exposed to film.

In Vitro Incorporation of [35S]SO4—Partially purified bovine submaxillary gland GalNAc-sulfotransferase was incubated with [35S]PAPS and potential glycoprotein substrates under the same conditions used for the GalNAc-4-sulfotransferase assay described above, except that GGMN-MCO and unlabeled PAPS were omitted (25). Reactions were stopped by addition of an equal volume of sample buffer (10% glycerol, 5% mercaptoethanol, 2% SDS, 0.003% bromphenol blue, and 62.5 mM Tris, pH 6.8). The samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and visualized by autoradiography.

Characterization of G TH β1 Asn-linked Oligosaccharides—Asn-linked oligosaccharides were released from 5 mg of GTH II β by digestion with peptide N-glycosidase F and labeled by reduction with B[3H]H2, as described (2). The 3H oligosaccharides were separated from other degradation products by gel filtration on Sephadex G-10 in 126 mM NH4HCO3, 10 mM MnCl2, 0.1 M NaHCO3 prior to analysis by HPLC as described (9, 24).

RESULTS

The Glycoprotein Hormone GalNAc-transferase and GalNAc-4-sulfotransferase Are Expressed in the Pituitaries of All Vertebrates—The sequence of the region of the human α subunit between two sets of double cysteines (Cys31–Cys60) is illustrated in Fig. 1. We recently provided evidence (13) that the residues PLR and KK, which are found within two turns of an α helix (19, 20), are critical for recognition of the α subunit by the glycoprotein hormone GalNAc-transferase. Alignment with α subunits from species representing other vertebrate classes (Fig. 1) reveals that this entire region, including the residues critical for recognition by the glycoprotein hormone GalNAc-transferase, are highly conserved. Based on mutagenesis studies (13), substitution of either Ala or Met for the Leu within the Pro-Leu-Arg sequence would not be expected to alter recogni-
tion by the GalNAc-transferase significantly. Thus, the α subunits from all vertebrates have the potential to be selectively modified by addition of β1,4-linked GalNAc-4-SO₃ to their Asn-linked oligosaccharides. Furthermore, since the number and location of the Asn-linked oligosaccharides on the α subunits have been conserved, the spatial relationship of the oligosaccharides to the recognition determinant should also remain the same on the α subunits from the different vertebrate classes. We therefore examined pituitary extracts from representatives of each class of vertebrate for glycoprotein hormone:GalNAc-transferase and GalNAc-4-sulfotransferase activities with the same properties as those we have previously described in mammalian pituitary extracts (9–11).

GalNAc-transferase activity with the appropriate specificity was detected in pituitary extracts from representatives of each vertebrate class (Table I). GalNAc was added to oligosaccharide acceptors on hCG, which contains the recognition motif, but not Trf, which does not contain the recognition motif, at the equimolar concentrations of acceptor protein. Thus, a GalNAc-transferase, which specifically modifies the oligosaccharides on glycoprotein hormones, is present in extracts from all the pituitary examined. GalNAc-4-sulfotransferase activity was detected in the same extracts (Table I), indicating that GalNAc added to oligosaccharide acceptors would likely be further modified by sulfate addition.

The specific activities (pmol/mg/h) of the GalNAc-transferase and GalNAc-4-sulfotransferase differed by as much as 6-fold among the extracts from different classes of vertebrates. At least three cell types (gonadotrophs, thyrotrophs, and corticotrophs) express GalNAc-transferase and sulfotransferase in mammalian pituitaries (1–3, 11, 24); furthermore, the levels of both transferase activities in gonadotrophs are modulated by the hormonal state of the animal (28). As a result, differences in specific activity may reflect the proportion of cells expressing GalNAc-transferase and GalNAc-4-sulfotransferase, the levels within specific cell types, hormonal state, and/or other factors. Nonetheless, the levels of both transferases are sufficient to account for the presence of oligosaccharides terminating with GalNAc-4-SO₃ on one or more glycoproteins synthesized within the pituitaries of all vertebrate classes.

Salmon is representative of the lowest vertebrate class in which glycoprotein hormone homologues have been identified. We therefore characterized the GalNAc-transferase and sulfotransferase products produced by salmon pituitary extracts to verify that the salmon transferases synthesize the same structures as their mammalian counterparts.

Agal-hCG and UDP-[³H]GalNAc were incubated with salmon pituitary extract to produce a radiolabeled product for characterization. The [³H] label, which had been incorporated into hCG, was released upon digestion with peptide:N-glycosi-

| Vertebrate pituitary representative class | GalNAc-transferase | GalNAc-4-sulfotransferase |
|------------------------------------------|-------------------|--------------------------|
| hCG                                      | 146               | 14                       |
| Laboratory rat (Mammalia)               |                   |                          |
| Domestic chicken (Aves)                  | 230               | 28                       |
| Slider turtle (Reptilia)                 | 148               | 38                       |
| North American bullfrog                  | 72                | 17                       |
| (Amphibia)                               |                   |                          |
| Coho salmon (Osteichthytes)              | 41                | 16                       |

Evolutionary expression of vertebrate pituitary glycoprotein hormone:GalNAc-transferase and the GalNAc-4-sulfotransferase

| Vertebrate pituitary representative class | GalNAc-transferase | GalNAc-4-sulfotransferase |
|------------------------------------------|-------------------|--------------------------|
| hCG                                      | 146               | 14                       |
| Laboratory rat (Mammalia)               |                   |                          |
| Domestic chicken (Aves)                  | 230               | 28                       |
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| (Amphibia)                               |                   |                          |
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| Evolutionary expression of vertebrate pituitary glycoprotein hormone:GalNAc-transferase and the GalNAc-4-sulfotransferase |
|--------------------------------------------------------------------------------------------------------------------------|
| Vertebrate pituitary representative class                                                                                   |
| GalNAc-transferase                                                                                                          |
| GalNAc-4-sulfotransferase                                                                                                   |
| hCG                                                                                                                         |
| 146                                                                                                                         |
| Laboratory rat (Mammalia)                                                                                                  |
| 146                                                                                                                         |
| Domestic chicken (Aves)                                                                                                     |
| 230                                                                                                                         |
| Slider turtle (Reptilia)                                                                                                     |
| 148                                                                                                                         |
| North American bullfrog (Amphibia)                                                                                          |
| 72                                                                                                                          |
| Coho salmon (Osteichthytes)                                                                                                 |
| 41                                                                                                                          |
Fig. 2. The salmon pituitary GalNAc-transferase product binds to W. floribunda-agarose and is released by jack bean β-hexosaminidase. Agal-hCG oligosaccharides were labeled with 3H by incubating agal-hCG with UDP-[3H]GalNAc and an extract of salmon pituitary. The 3H oligosaccharides were released from hCG by digestion with peptide-N-glycosidase F and separated from other products by gel filtration on Sephadex G-15. Panel A, the 3H oligosaccharides were applied to W. floribunda-agarose in 20 mM Tris, pH 7.4, 150 mM NaCl. The column was eluted with 20 mM Tris, pH 7.4, 150 mM NaCl to remove unbound material and with the same buffer containing 50 mM GalNAc (arrow) to specifically elute bound material. Panel B, the 3H oligosaccharides were analyzed on Aminex HPX-87H column eluted with 0.01 N H2SO4 before (broken line) and following digestion with jack bean β-hexosaminidase (solid line). The elution positions of GlcNAc and GalNAc are indicated.

Fig. 3. Characterization of salmon pituitary sulfotransferase reaction product. The 35S-labeled product obtained upon incubation of a salmon pituitary extract with [35S]PAPS and GGnM-MCO was analyzed on CarboPac PA1 column as described (9). Panel A, [35S]SO4, GGnM-MCO; panel B, [35S]SO4, GGnM-MCO after treatment with alkali; and panel C, [35S]SO4, GGnM-MCO after partial acid hydrolysis. The elution positions of authentic standards are indicated: 1, SO4; 2, SO4,3-GGnM-MCO; 3, SO4,4-GGnM-MCO; 4, GlcNAc-3-SO4; 5, GalNAc-4-SO4; 6, GlcNAc-6-SO4; 7, GalNAc-6-SO4.
subunits of GTH II bear terminal β1,4-linked GalNAc-4-SO₄ and/or β1,4-linked GalNAc. These studies did not indicate what proportion of these oligosaccharides have this modification or their underlying structure.

A Major Fraction of the Asn-linked Oligosaccharides Present on Salmon GTH II β Subunit Terminates with β1,4-linked GalNAc or GalNAc-4-SO₄—Studies were undertaken to establish both the distribution and structural features of the Asn-linked oligosaccharides on GTH II β. This would allow us to determine if these structures are closely related to the sulfated oligosaccharides released by immobilized W. floribunda, indicating that removal of the sulfate exposed a β1,4-linked GalNAc. The linkage of the terminal GalNAc was confirmed by comparing the effects of jack bean and diplococcal β-hexosaminidase digestion of the neutral oligosaccharides produced by sulfatase digestion of S1. Digestion with jack bean β-hexosaminidase abolished binding by immobilized W. floribunda, whereas digestion with diplococcal β-hexosaminidase did not affect binding. We have previously shown that jack bean but not diplococcal β-hexosaminidase will release β1,4-linked GalNAc (I). The digestions were also monitored by ion suppression-amine desorption HPLC, which demonstrated that the oligosaccharide products comigrated with the appropriate standards prepared from mammalian glycoprotein hormones (34, 35). Based on the specificities of the glycosidases and comigration of digestion products with authentic standards bearing no charged residues (N0), one (N1), or two (N2) sialic acid residues; one (S1), two (S2), or three (S3) sulfate residues; one sulfate and one sialic acid residue (SN), one sulfate and two sialic acid residues (SN2), or two sulfates and one sialic acid (2N2).

**DISCUSSION**

Homologues of the glycoprotein hormones have been described in representatives of each class of vertebrate (17). The glycoprotein hormones are dimeric proteins consisting in each case of a common α subunit and a hormone-specific β subunit. Fish are the only known example in which two forms of α subunit differing in amino acid sequence are present (17). Recent crystallographic studies have revealed that both the α and β subunits of the glycoprotein hormones have cysteine knot motifs, placing them in a family of proteins that includes nerve growth factor, transforming growth factor-β, and platelet-de-
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Table II: Distribution and structure of the Asn-linked oligosaccharides on salmon GTH II β subunit

| Oligosaccharide | Name                   | % of Total |
|-----------------|------------------------|------------|
| Galβ4GlcNAcβ2Man3 | Manβ4GlcNAcβ4GlcNAcol | N0-A 13.6  |
| Galβ4GlcNAcβ2Man6 | Fuco6                 |            |
| Galβ4GlcNAcβ2Man3 | Manβ4GlcNAcβ4GlcNAcol | N0-B 6.4   |
| GalNAcβ4GlcNAcβ2Man6 | Manβ4GlcNAcβ4GlcNAcol | N1-A 23.8  |
| Galβ4GlcNAcβ2Man3/6 | Manβ4GlcNAcβ4GlcNAcol | N1-B 3.2   |
| Siaα2,3Galβ4GlcNAcβ2Man3/6 | Fuco6             |            |
| GalNAcβ4GlcNAcβ2Man3/6 | Manβ4GlcNAcβ4GlcNAcol | S1-A 27.3  |
| Siaα2,3Galβ4GlcNAcβ2Man3/6 | Manβ4GlcNAcβ4GlcNAcol | S1-B 1.7   |
| SO4-4GalNAcβ4GlcNAcβ2Man6 | Manβ4GlcNAcβ4GlcNAcol | N1-B 3.2   |
| GalNAcβ4GlcNAcβ2Man3/6 | Manβ4GlcNAcβ4GlcNAcol | S1-A 27.3  |
| SO4-4GalNAcβ4GlcNAcβ2Man6 | Manβ4GlcNAcβ4GlcNAcol | S1-B 1.7   |
| SO4-4GalNAcβ4GlcNAcβ2Man6 | Manβ4GlcNAcβ4GlcNAcol | S1-B 1.7   |

A number of structural features have been highly conserved among the glycoprotein hormone homologues including 1) the cysteines that form five and six disulfide bonds in α and β subunits, respectively, 2) the location and number of Asn-glycosylation sites, and 3) individual amino acids that are thought to be involved in dimer formation, receptor binding, and receptor activation.

The current studies demonstrate that yet another structural feature of the glycoprotein hormones is highly conserved through evolution from fish to mammals: the presence of Asn-linked oligosaccharides terminating with the sequence SO4-4GlcNAcβ1,4GlcNAcβ1,2Manα. We have determined that GalNAc-transferase and GalNAc-4-sulfotransferase activities with the same specificities and properties as the mammalian enzymes are expressed in representatives of each class of vertebrate. In addition, we have shown that salmon GTH II bears Asn-linked oligosaccharides with this terminal sequence. Taken together with the fact that the amino acids that we have shown are critical for recognition of the α subunit by the glycoprotein hormone GalNAc-transferase are conserved in the α subunits of each class of vertebrate, it is highly likely that individual members of the glycoprotein hormone family in each class of vertebrate bear Asn-linked oligosaccharides terminating with SO4-4GalNAcβ1,4GlcNAcβ1,2Manα.

The central region of the α subunit, a stretch of 30 amino acids between two sets of double cysteines (see Fig. 1) and the carboxyl-terminal region of the α subunit are highly conserved through evolution from fish to mammals (17). Various residues within the central region have been shown to be important for interaction with and activation of glycoprotein hormone receptors such as the LH receptor (16) and for dimer formation (14, 15). The remarkable degree of sequence conservation in this region may thus reflect the requirement to mediate a number of interactions with different β subunits and receptors. The requirement for recognition of specific basic residues within this region by the glycoprotein hormone GalNAc-transferase adds yet another restriction to the changes in amino acid sequence, which would be tolerated in this region.

We have previously shown that the presence of oligosaccharides terminating with SO4-4GalNAcβ1,4GlcNAcβ1,2Manα on LH plays an important role in mammals by reducing the circulatory half-life of LH in the blood (6). LH bearing oligosaccharides terminating with SO4-4GalNAcβ1,4GlcNAcβ1,2Manα binds by a SO4-4GalNAc-specific receptor, which is expressed on hepatic endothelial cells and removed from the circulation (5). This rapid removal is essential to produce the pulsatile rise and fall characteristic of circulating LH and is thought to be critical for maximal receptor activation. The presence of the sulfated oligosaccharides on glycoprotein hormones from all vertebrate classes suggests that these structures play a similar and critical biologic role in all vertebrate classes. Notably, Fontaine et al. (36) observed that the metabolic clearance rate for hCG, which bears oligosaccharides terminating with sialic acid-Gal, was 20 times slower than for carp GTH. If carp GTH, like salmon GTH, bears oligosaccharides terminating with SO4-4GalNAcβ1,4GlcNAcβ1,2Manα, this rapid rate of clearance could reflect the presence of a SO4-4GalNAc-specific receptor in the liver of fish similar to that found in mammals.

The presence of terminal SO4-4GalNAcβ1,4GlcNAcβ1,2Manα is characteristic of vertebrate glycoprotein hormones in species ranging from fish to mammals. Thus, expression of glycoprotein hormone biologic activity must at some juncture require the presence of oligosaccharides terminating with SO4-4GalNAcβ1,4GlcNAcβ1,2Manα. Our previous studies indicate the most likely role of these structures is to regulate the circulatory half-life of individual glycoprotein hormones through the GalNAc-4SO4 receptor; however, other equally critical functions are also possible. In addition, the presence of these same structures on other glycoproteins such as carbonic anhydrase...
synthesized in salivary glands (25) and proopiocortin (24) raises the possibility that these structures will serve other roles on other glycoproteins.

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