Glycoconjugate Journal (1992) **9**:279–283

GLYCOPINION

Editor: RAYMOND A. DWEK

The anterior pituitary gland controls most of the endocrine functions of our bodies through the secretion of several hormones which can all be glycosylated and which display remarkable polymorphism. The classical pituitary glycoprotein hormones are the gonadotropins (LH and FSH) and TSH but, more recently, a glycosylated form of PRL (G-PRL) has also been identified. These glycoproteins are structurally the most complex informative polypeptides present in the circulation, and are the main regulators of the reproductive axis (LH, FSH and PRL) or of thyroid function (TSH). Their measurement in blood has long been the daily clinical basis for the diagnosis of endocrine disorders and the search for pituitary tumours. However, major discrepancies have been noted between the immunological assessment of these molecules and their biopotency, as reflected by the activity of the corresponding endocrine axis. Multiple molecular variants have been described for gonadotropins and TSH as well as for PRL, which accounts for the large variation in their biological properties. Several studies have identified uncommon clinical situations associated with the presence of hyperactive or inactive hormones, indicating that the polymorphism of these glycoproteins should be under physiological control in the normal state, even though the molecular basis for this phenomenon is not yet fully understood.

Over the past two decades extensive research in this field has elucidated the processes for the biosynthesis and secretion of these hormones, analysed their variations in bioactivity under normal and pathological conditions and, more recently, succeeded in producing recombinant molecules. This review, by Professor Catherine Ronin, is based on the hypothesis that glycosylation is the key to the molecular polymorphism of pituitary hormones, and it poses a number of important questions:

- Is glycosylation the major mechanism by which nature creates diversity among the pituitary hormones, allowing differential release and delivery of active products to their target organs?
- Is glycosylation a means of controlling biopotency?
- Is terminal sialic acid responsible for masking biological activity in luteinizing hormone?
- Do changes in sulfation or sialylation control hormone storage, secretion or trafficking?
- How do glycosylated and nonglycosylated prolactin differ in their functional activity? Why does the ratio alter during pregnancy?
- Why are immunoassays for human pituitary hormones notoriously difficult to standardize?
- Will x-ray diffraction data clarify the manner in which oligosaccharide chains alter the conformation of the active domains of pituitary hormones?

Glycosylation of pituitary hormones: a necessary and multistep control of biopotency

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Abbreviations: LH, Luteinizing hormone; FSH, follicle stimulating hormone; TSH, thyroid stimulating hormone; PRL, prolactin; CG, chorionogonadotropin; TRH, thyrotropin releasing hormone; GnRH, gonadotropin releasing hormone; h, human; b, bovine; cAMP, cyclic AMP; CHO, carbohydrate; Man, mannose; GlcNAc; *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Sial, sialic acid.

Together with the placental hormone hCG, pituitary gonadotropins and TSH are heterodimers formed by noncovalent association of distinct α- and β-subunits. A single gene encodes the α-subunit and, as a result, the amino acid sequence of all four hormones is identical within an animal species [1]. The pituitary β-subunits arise from separate genes, differ in primary sequence and confer on the heterodimers their immunological and biological specificity. However, there is a high degree of homology among them, particularly in the regions which are believed

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to interact with the α-subunit. The conserved position of the disulfide bonds within the β-sequences as well as the common α-subunit suggests an extensive similarity in the 3-dimensional structure of these molecules. Such structural homology has been responsible for extensive cross-reactivity among antibodies and, therefore, immunoassays of glycoprotein hormones have lacked specificity over many years. The microheterogeneity of these glycoproteins also precluded their crystallization in the native states, but informative x-ray data on deglycosylated hCG might be soon available. *N*-Glycosylation sites are two in the α-subunit, one in LH-β and TSH-β but two in FSH-β. Differences in the CHO composition of pituitary glycoprotein hormones suggests that there is significant variation in oligosaccharide structure. Uniquely, they may be sulfated as a SO_4-4GalNAc β1-4 GlcNAc β1-2 Man sequence, sialylated as a Sial α2-3(6) Gal β1-4 GlcNAc β1-2 Man containing oligosaccharide, or both sialylated and sulfated [2]. Sulfated CHO chains predominate in bLH and bTSH, while sialylated oligosaccharides are found in FSH of several animal species including man. In contrast, 2LH and hTSH contain both. The sulfotransferase is oligosaccharide-specific [3], i.e., requires only available GalNAc residues to act. The GalNAc-transferase, however, appears to be specific for a Pro-Leu-Arg tripeptide sequence [4], accounting for a preferential transfer of sulfate on bLH or bTSH, compared with FSH of either human or bovine origin.

That changes in sulfation or sialylation may control hormone storage and secretion is an important issue that has not yet been investigated thoroughly because of the lack of a gonadotroph or thyrotroph permanent cell line. While core glycosylation is required for proper folding and assembly of α- and β-subunits during biosynthesis [5], carbohydrate processing and terminal glycosylation appear to be under the control of several endocrine factors that regulate intracellular trafficking and release of these hormones. Since LH and FSH are synthesized within the same pituitary cells, it has been proposed that sulfate/Sial signalling is important in the sorting of gonadotropins into separate secretory granules [2]. The hypothalamic regulatory peptide GnRH was shown to produce a dramatic change in the nature of bioactive LH isoforms secreted by rat hemilobes as it decreased their sulfate/Man ratio [6]. Extensive studies carried out with rodents further demonstrated that both TSH core and terminal glycosylation is under physiological control, especially that of thyroid hormones and the hypothalamic factor TRH [7]. In euthryoid animals, TSH CHO branching and sialylation were shown to increase during development from the fetus to the adult. Ontogenesis of the thyroid axis is accompanied by an enhancement of the sialic acid content in TSH and a concomitant decrease in sulfation. In adult animals, stereotaxic lesions of the hypothalamus, mimicking central hypothyroidism, proved to increase biantennary structures on TSH in contrast to multiantennary CHO chains observed in primary hypothrydoidism. Concomitantly, the sulfate/Man ratio was altered in both α- and β-subunits of TSH, suggesting that terminal glycosylation is equally regulated in the two subunits. In patients with idiopathic central hypothyroidism, we have prelimimary evidence that changes in both core and terminal glycosylation occur in serum TSH which are not fully reversed by chronic treatment with TRH [8].

PRL is also encoded by a single gene, synthesized as a prehormone in the lactotroph cells of the pituitary and secreted as a mature polypeptide that can be *N*-glycosylated at a single position [9]. Only human, ovine and porcine PRLs contain the consensus sequence at this location, whereas the bovine polypeptide has an aspartic acid in place of the asparagine residue and is not glycosylated. Murine PRL has other sites elsewhere in the sequence and can be also glycosylated. However, the amount of G-PRL in the pituitary is highly variable among animal species, in the range 8–50% with about 15% in man; the detailed oligosaccharide structures have not been elucidated so far. Variable amounts of G-PRL are secreted by human prolactinomas in culture, accounting for up to 20% of the total hormone secreted [10]. Nonglycosylated (NG) PRL is clearly the major secretory product of pituitary lactotroph cells, although in plasma, G-PRL has been detected in considerable quantities in humans and pigs [9]. Blood concentration of G-PRL shows significant variation with the physiological state of the individual, especially during pregnancy [11]. However, good clinical investigation of subjects with varying NG/G ratio is still lacking.

It has been well established that gondatropins, TSH or PRL exist in multiple forms differing in bioactivity and glycosylation. The number and relative abundance of each isohormone depends upon the isolation techniques, the source of biological material and the physiological status of the donor. Isohormones of LH and FSH have been shown to differ noticeably in the pituitary, serum and urine, as well as according to age and sex. Isoelectric or chromatofocusing identified six to ten components for both hormones within the pH ranges 6.4–3.8 for FSH and 9.8–7.9 for LH [12] of mammalian species including man. Up to a dozen of forms were also detected for intrapituitary TSH, although over a wider range of pIs (8.3–4.8) [13]. At least four human G-PRLs about neutral pI (7.6–7.1) could be isolated using similar techniques (Hoffmann, T., Gunz, G., Jaquet, P. and Ronin, C., unpublished). The common feature to this molecular heterogeneity is that none of these isoforms proved to be equally potent. Differences in both immunoreactivity and bioactivity have been noted by most investigators but the bioactive to immunoactive (B/I) ratios vary greatly among laboratories [14]. Major discrepancies between the concentrations measured by the two assays have been well documented for gonadotropins, especially between crude and purified hormone preparations from the pituitary and urine and among blood samples under normal and pathological conditions [15]. This problem is

particularly troublesome for the clinical investigation of patients who may present with biological features that correlate poorly with their endocrine disorder.

Indeed, standardization of the immunoassays for human pituitary hormones is critically important, and several organizations have supplied purified preparations to the laboratories over the past twenty years. Among them, the National Hormone Pituitary Program of the National Institutes of Health (Bethesda, MD, USA) offered successive International Reference Preparations (IRP) to normalize human plasma measurements, first as urinary standards and, later on, from post-mortem pituitary extracts. Using radioimmunoassays with polyclonal antibodies, it was soon realized that endocrine manipulations or disorders may cause changes in the relative abundance of one or more isoforms that were not adequately quantified when expressed in terms of IRP and thus as International Units (IU). Quantitation of FSH in serum often showed nonparallelism of displacement curves compared with pituitary or urinary standards [16]. On a practical basis, concentration of serum LH, whether measured in ng ml^{-1} or mIU ml^{-1}, varied as much as threefold among six reference laboratories [17, 18]. When two-site assays were developed with monoclonal antibodies, marked discrepancies were again noticed among the kits, although desired specificity and sensitivity were achieved. Recent work has even reported that these differences were also present in recombinant LH [18], which implies that the hormone produced by recombinant technology might not be useful for overcoming the standardization problem. These examples illustrate vividly that as well as this difficulty, another key to sound estimations should reside in the choice of both the capture and tracer monoclonal antibodies. They do not recognize with the same efficiency all the isohormones present in the serum, probably because of loss of epitopes by altered glycosylation and/or conformational changes. In this regard, we found that, for hTSH also, most of the monoclonal antibodies directed to the β-subunit or the dimer do not bind the deglycosylated hormone [19]. Since antibody pairing may be different among immunoassays, it is conceivable that each combination may bind a limited number of isoforms, thereby giving varying hormone levels for the same sample.

Compared with those of gonadotropins, immunoassays of PRL may be more advanced. High molecular weight variants and fragments of PRL are present in the circulation, and most antibodies against PRL are able to recognize them, so that the amount of the more potent form, i.e., NG-PRL can be overestimated. In addition, G-PRL is three times less reactive than NG-PRL when seen by polyclonal antibodies [10] and may further contribute to the variation of PRL level. Very recently, a two-site immunoassay with monoclonal antibodies proved to be specific in measuring NG-PRL [20], which may improve rapidly the measurement of this hormone in various clinical situations. This also suggests that form-specific assays are likely to be within our grasp for glycoprotein hormones. A new generation of antibodies directed against glycoforms of known bioactivity should be valuable in diagnosis as well as in the therapeutic follow-up of several disorders of the reproductive or thyroid axis.

Besides variability in immunological behaviour, marked disparities in biopotency have been noted among pituitary glycoprotein hormones and these have not been elucidated yet. Here, too, studies rely on standardization of the bioassays by IRPs. When crude LH preparations are used as references heavily for both bioassay and radioimmunoassay, significantly higher B/I ratios are obtained compared with highly purified standards because of the presence of immunoreactive forms that possess little or no biological activity [18]. Measurement of serum LH based on testosterone production by rat interstitial cells proved to be exquisitely sensitive and allowed the intrinsic variability among isohormones to be demonstrated. Receptor binding capacity, cAMP and steroid production of 7 isoforms of intrapituitary hLH correlate poorly with the activities obtained by immunoassays: there is a dramatic decrease in the B/I ratio from the more alkaline to the more acidic forms. Furthermore, desialylation of LH isoforms with less biological activity induced an increase in receptor binding and testosterone production, as if terminal glycosylation unmasked functional domains in the protein [21]. Similarly, replacement of sulfate by sialic acid in recombinant bLH drastically reduced *in vitro* steroid release, suggesting that terminal sugars act on the intrinsic biopotency of LH [22].

Glycosylation, especially the sialic acid content, is widely admitted to correlate with the isoelectric point of isohormones [12]. When human serum specimens are assayed after separation by isoelectric focusing, it can be seen that the most bioactive fraction resides at pI = 8.5 in mid-cycle plasma, at pI = 7.5–8.5 in post-menopausal plasma, and at even lower pIs in the post-menopausal pituitary gland [18]. This may rather reflect a change in sialic acid or sulfate capping, since we found no significant changes in CHO branching of circulating LH between normal and menopausal women (Papandreou, M.J., Ronin, C., and Beck-Peccoz, P., unpublished). Similarly, assessments of FSH biopotency have been based on the granulosa cell aromatase activity and extensively used because *in vivo* assays suffered from low sensitivity. These assays exhibit unique parallel dose–response curves for FSH from pituitary and urinary sources [16]. Studies in rodents showed that B/I ratios of FSH also change with physiological conditions [23]: androgens increase it while estrogens decrease it. Isoelectric focusing of human pituitary FSH further suggests that the percentage of acidic FSH increases with age, presumably due to changes in sialic acid content [24]. In serum, an increased B/I ratio of FSH was observed in GnRH agonist-treated patients with prostate cancer and testosterone-treated normal men together with an augmented alkaline FSH isoform [16]. Conversely, a

decreased ratio occurred in post-menopausal women treated with a GnRH antagonist for which alkaline FSH was increased [25]. This analysis documented a naturally occurring antihormone in blood and, although no details on its molecular structure have been obtained so far, it has provided a basis for putative hormone antagonists in various physiopathological states.

A similar observation was made for intrapituitary hTSH in which we recently described the presence of neutral isoforms poorly active in cAMP production and cell proliferation [13]. Their augmented secretion by pituitary adenomas in culture, however, did not correlate with the extent of hyperthyroidism in the patients before surgery, and thus whether or not such isoforms are inhibitory at the target cell is currently being tested. Also, recombinant forms of this hormone lacking the sulfate signal proved to be less active than pituitary TSH in stimulating cAMP production, although the binding properties of both hormones to the thyroid receptor were virtually identical [26]. Interestingly, the recombinant protein could recover full bioactivity after desialylation indicating that, asfor LH, terminal sugars modulate the bioactive conformation of the hormone. Regarding PRL, glycosylated variants are less potent in several *in vitro* bioassays [9, 10], suggesting for this hormone too that glycosylation may help to decrease biopotency. Owing to the multiple actions of this molecule on the mammary gland, the gonads and other cells, it is not known if this mechanism is involved in targeting the hormone to these tissues.

A better correlation between the *in vivo* and *in vitro* biopotency of individual glycoforms is clearly required to understand how these hormones act on their target organs. They may further serve as prototypes in designing molecules of therapeutic potential. The contribution of specific CHO determinants to the *in vivo* circulatory properties of hFSH and hTSH have now been thoroughly investigated. When expressed in Chinese Hamster ovary cells deficient in GlcNAc transferase I or incapable of sialylation, recombinant FSHs were indistinguishable from native FSH *in vitro* but were rapidly cleared from the circulation and thus were inactive *in vivo* [27] Thus, even though mannose-rich oligosaccharides are necessary for subunit assembly during biosynthesis, they must be converted to sialylated complex structures to maintain serum levels. Similarly, recombinant TSH appeared more sialylated than the native hormone, and had a lower metabolic clearance which was increased following neuraminidase treatment [26]. Thus, terminal glycosylation has a dual role in pituitary hormones: it controls both their intrinsic bioactivity and circulatory lifetime.

The respective contributions of sialic acid and sulfate to metabolic clearance have been addressed in part. Both desulfated pituitary and desialylated recombinant bLH were found to be active *in vivo* but cleared at a similar fast rate from the circulation. The sialylated hormone, however, showed a longer circulatory half-life which appeared to compensate for its reduced *in vitro* bioactivity compared with the sulfated hormone, so that both bLHs displayed the same *in vivo* biopotency as measured by ovulatory stimulation [28]. These findings demonstrate that a long circulatory half-life is not necessarily accompanied by elevated biopotency. In the case of either TSH and LH of human origin, a physiological control of their sulfate/Sial ratio may amplify the effect of a naturally pulsatile release of these glycoproteins in the circulation and also add pharmacological complexity in the delivery of individual isoforms to the target cells.

A critical step to understanding the structure–function relationships of glycoprotein hormones is currently being pursued by the mapping of the various functional domains of these proteins [29]. Synthetic peptides proved extremely useful for identifying the amino acid sequences involved in the interaction between the subunits, expressed as antigenic determinants or receptor binding sites, and those responsible for signal transduction in human LH, FSH and TSH. It is likely that both subunits are involved in the expression of multiple peptide areas that can be shared by different portions of the subunit to construct the bioactive surface of the hormone. This may include common sequences which can be expressed differentially among hormones or specific recognition sites which can be modulated by CHO chains [30]. It has long been recognized that chemically deglycosylated gonadotropins behaved as good antagonists *in vitro*, fully binding to the receptor but unable to stimulate the adenylate cyclase complex, and in this process the glycosylation of the α-subunit is essential [1]. We observed that, in hTSH, post-receptor events are indeed differentially affected by CHO removal, and proposed that signal transduction might be devoted to glycosylation-dependent domains distinct from the receptor recognition sites at the surface of the hormone [19]. It remains to determine whether or not each glycoform of the hormone can express a restricted set of such functional areas to modulate its intrinsic bioactivity.

In summary, glycosylation of pituitary hormones may be regarded as a multipstep control of their bioactivity according to physiological need, starting with the assembly of the two subunits in a functional dimer, and the secretion of various glycoforms with distinct active conformations which produce ultimately different effects at the target organ. Hormone action is then a result of a variable combination of two key regulatory steps: at the receptor level, the availability of one or more isoforms is controlled by a selective release by the pituitary; and a defined circulatory half-life. Once bound, each isohormone exhibits a unique capability to induce specific transduction signals depending on the functional domains which are present at its surface. This hypothesis does not rule out the possibility that several glycoforms may be required to achieve dynamic regulation of the whole endocrine axis: the physiologically controlled

secretion of a slow-acting form may permit the basal activity of the target organ while a fast-acting form may surimpose a rapid stimulation to produce the desired biological effects according to the pituitary regulatory state.

It is hoped that a better understanding of the molecular basis for hormone polymorphism may provide the way to engineer these sophisticated glycoproteins by recombinant technology or synthetic peptide chemistry. Such analogs may be expected to produce the superagonists or antagonists which are currently needed in the management of fertility, thyroid autoimmunity or cancer.

References

1. Ryan R, Charlesworth MC, McCormick DJ, Milius RP, Keutmann HT (1988) *FASEB J* **2**: 2661–9.
2. Baenziger JU, Green ED (1988) *Biochim Biophys Acta* **947**: 287–306.
3. Smith PL, Baenziger JU (1988) *Science* **242**: 930–3.
4. Smith PL, Baenziger JU (1992) *Proc Natl Acad Sci USA* **89**: 329–33.
5. Weintrub BD, Stannard BS, Magner JA, Ronin C, Taylor T, Joshi L, Menezes-Ferreira MM, Gesundheit N (1986) *Recent Prog Hormone Res* **41**: 577–606.
6. Sardanons ML, Solano AR, Podesta EJ (1987) *J Biol Chem* **262**: 1149–55.
7. Weintraub BD, Wondisford FE, Steinfelder HJ, Gesunheit N, Gyves PW, Taylor T, De Cherney GS (1989) *Hormone Res* **32**: 22–4.
8. Papandreou MJ, Ronin C, Beck-Peccoz P (1991) 19th European Thyroid Association Meeting, *Annales d'Endrocinologie* **52**: 56 (Abst.).
9. Sinha YN (1992) *Trends Endocrinol Metab* **3**: 100–6.
10. Pellegrini I, Gunz G, Ronin C, Delori P, Jaquet P (1998) *Endocrinology* **122**: 2667–74.
11. Markow E, Lee DW, Hollingworth DR (1988) *J Clin Endocrinol Metab* **67**: 559.
12. Ulloa-Aguirre A, Espinoza R, Matsumura P, Chappel S (1988) *Human Reprod* **3**: 491–501.
13. Sergi I, Papandreou MJ, Medri G, Verrier B, Ronin C (1991) *Endrocrinology* **128**: 3259–68.
14. Chappel S (1990) *J Clin Endrocrinol Metab* **70**: 1494–5.
15. Dufau M, Valdhuis JD (1987) *Clinics Endocrinol Metab* **1** 153–76.
16. Dahl KD, Stone MP (1992) *J Andrology* **13**: 11–21.
17. Jaakola T, Ding YQ, Valavaara R, Rönnberg L, Hutaniemi I (1990) **70**: 1496–505.
18. Rosenfield R, Helke J (1992) *J Andrology* **13**: 1–10.
19. Papandreou MJ, Sergi I, Benkirane M, Ronin C (1991) *Mol Cell Endocrinol* **78**: 137–50.
20. Brue T, Benkirane M, Gunz G, Jaquet P (1992) *J Clin Endrocrinol Metab* in press.
21. Hattori M, Ozawa K, Wakabayashi K (1985) *Biochem Biophys Res Commun* **127**: 501–8.
22. Smith PL, Kaetzel D, Nilson J, Baenziger JU (1990) *J Biol Chem* **265**: 874–81.
23. Chappel SC, Ulloa-Aguirre A, Coutifaris C (1983) *Endocrine Rev* **4**: 179–211.
24. Wide L (1985) *Acta Endocrinol* (Copenhagen) **109**: 190–7.
25. Dahl K, Bicsak TA, Hsueh A (1987) **239**: 72–74.
26. Thotakura R, Desai R, Cole E, Pratt B, Weintraub BD (1991) **128**: 341–8.
27. Galway B, Hsueh A, Keene M, Boime I (1990) *Endocrinology* **127**: 93–100.
28. Baenziger JU, Kumar S, Brodbeck RM, Smith P, Beranek M (1992) *Proc Natl Acad Sci USA* **89**: 334–8.
29. Dias J (1992) *Trends Endocrinol Metab* **3**: 224–9.
30. Labbé-Jullié C, Sergi I, Darbon H, Ronin C (1992) *Endocrinology* in press.