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Recognition by the glycoprotein hormone-specific *N*-acetylgalactosaminetransferase is independent of hormone native conformation

(glycosyltransferase/oligosaccharide/pituitary/gonadotropin)

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ABSTRACT **Some members of the glycoprotein hormone family [luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and free α subunit] bear unique asparagine-linked oligosaccharides with the terminal sequence SO_4-GalNAcβ1,4GlcNAcβ1,2Manα, whereas other members [human chorionic gonadotropin (hCG) and follicle-stimulating hormone (FSH)] bear predominantly oligosaccharides terminating in the sequence sialic acidα-Galβ1,4GlcNAcβ1,2Manα. We previously identified an *N*-acetylgalactosaminetransferase (GalNAc-transferase) in bovine pituitary membranes that specifically recognizes the α subunit peptide and adds GalNAc to the synthetic intermediate $GlcNAc_2Man_3GlcNAc_2$. In the current study we demonstrate that bLH, hCG, hCGβ, hCGα, and FSHα are recognized by the pituitary GalNAc-transferase *in vitro*, whereas oFSH, hFSH, and hFSHβ are not (b-, h-, and o- indicate bovine, human, and ovine). The apparent K_m values for addition of GalNAc to oligosaccharides on hCGα and hCGβ, 13.0 and 6.2 μM, respectively, are not altered by reduction and alkylation. Thus, recognition of the peptide determinant does not require maintenance of native tertiary structural features. In the presence of the recognition determinant the K_m for addition of GalNAc to the intermediate $GlcNAc_2Man_3GlcNAc_2$ is reduced from 1.2–2.6 mM to <13 μM. hFSH is not efficiently recognized by the GalNAc-transferase due to the absence of the recognition marker on hFSHβ and some degree of masking of the recognition marker on the α subunit when combined with FSHβ. Since recognition is directed at primary and possibly secondary structural features, it should be possible to determine which regions of the α and β subunits are responsible for the specificity of the GalNAc-transferase.**

Glycosylation is the most complex of commonly encountered post-translational modifications. Although large numbers of different asparagine-linked oligosaccharides arise from a common synthetic precursor by "processing," few examples of unique structures confined to specific proteins or families of proteins have been described. Furthermore, little is known about the mechanisms by which synthesis of specific structures is regulated. One example of protein-specific synthesis of unique oligosaccharides by modification of a common synthetic intermediate was the addition of phosphate to oligomannose-type oligosaccharides on lysosomal enzymes (1). Recognition of lysosomal enzyme oligosaccharides by the phosphotransferase is dependent on the maintenance of tertiary structure, making definition of the precise features involved difficult (2).

We have described a further instance in which unique oligosaccharide structures are found on a family of glycoproteins, the glycoprotein hormones (3–5). Each member of the glycoprotein hormone family is a dimer consisting of an α and a β subunit. The α subunit arises from a single gene and has the same amino acid sequence for each hormone. The β subunits arise from different genes and differ in their amino acid sequences, even though they are highly homologous (3, 6–8). Luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and free α subunits bear oligosaccharides terminating with the sequence SO_4-GalNAcβ1,4GlcNAcβ1, 2Manα, whereas human chorionic gonadotropin (hCG) and follicle-stimulating hormone (FSH) bear oligosaccharides terminating with the sequence sialic acidα-Galβ1,4GlcNAcβ1, 2Manα (4, 5, 9, 10). These structures, nonetheless, arise from a common intermediate in the synthetic pathway for asparagine-linked oligosaccharides (11, 12). Since asparagine-linked oligosaccharides terminating with GalNAc and sulfate are present on only a small fraction of the glycoproteins synthesized by the anterior pituitary (i.e., the glycoprotein hormones), the pituitary *N*-acetylgalactosaminetransferase (GalNAc-transferase) must specifically recognize the synthetic intermediate $GlcNAc_2Man_3GlcNAc_2$ (see refs. 11 and 12 for structures) when it is present on glycoproteins such as LH and TSH but not when it is present on other glycoproteins. The GalNAc-transferase also discriminates among closely related glycoproteins such as LH and FSH, which are both synthesized in the same cell, the gonadotroph, and have α subunits with the identical amino acid sequence (3). We recently established that a GalNAc-transferase is present in pituitary membranes, which adds GalNAc to the $GlcNAc_2Man_3GlcNAc_2$ synthetic intermediate with an apparent K_m of 25 μM when it is present on free α subunit and an apparent K_m of 1 mM when it is present on other glycoproteins such as transferrin (12). Thus, the pituitary GalNAc-transferase recognizes features encoded within the peptide of the α subunit. Since LH and FSH differ in the extent of GalNAc addition, the β subunit must also have an impact on recognition of α,β-dimeric forms of the hormones by the GalNAc-transferase.

In this study we have further explored the specificity and properties of the pituitary glycoprotein hormone-specific GalNAc-transferase to determine if β subunits, like the α subunit, are recognized by the GalNAc-transferase and if they modulate recognition of the α subunit. We have also examined whether maintenance of native tertiary structure is required for recognition.

MATERIALS AND METHODS

Preparation of Substrates. hCG, hFSH, ovine FSH (oFSH), and their isolated subunits were obtained from the National

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Abbreviations: LH, luteinizing hormone; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; CG, chorionic gonadotropin; mU, milliunit(s); CHO, Chinese hamster ovary; h-, human; o-, ovine; b-, bovine.

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Hormone and Pituitary Program. Recombinant bovine LH (bLH) expressed in Chinese hamster ovary cells (bLH/CHO) was purified as described (13). A glycopeptide bearing a dibranched complex-type oligosaccharide was prepared by exhaustive Pronase digestion of bovine IgG and subsequent purification on Con A. J chain was purified from a human IgM myeloma protein as described (14). Substrates (2 mg/ml in 10 mM sodium cacodylate, pH 6.0) were digested with 8.3 milliunits (mU) of clostridial neuraminidase (Sigma type X) and 0.625 mU of diplococcal β-galactosidase (15) for 72 hr and subsequently heated to 65°C for 10 min to inactivate the β-galactosidase. Proteins were quantitated using the bicinchoninic acid protein assay (16). The glycopeptide prepared from bovine IgG was quantitated by the phenol/sulfuric acid assay (17). The integrity of each substrate was confirmed by SDS/PAGE under reducing and nonreducing conditions (13).

Reduction and Alkylation. Substrates (1 mg/ml) were reduced with dithiothreitol (10 mg/ml) in 0.5 ml of 1 M Tris, pH 8.0/6 M guanidine hydrochloride/1 mM EDTA at 37°C for 4 hr. Alkylation was carried out with either 2-bromoethylamine (10 mg/ml), iodoacetamide (24 mg/ml), or iodoacetic acid (24 mg/ml).

GalNAc-Transferase and Galactosyl-Transferase (Gal-Transferase) Assays. Assays were performed as described (12). Bovine pituitary membranes, prepared as described (12), were the source of GalNAc-transferase. Purified bovine Gal-transferase (3 mU per assay, Sigma) was used to determine the levels of Gal acceptor activity—i.e., terminal GlcNAc.

RESULTS

Specificity of the Pituitary GalNAc-Transferase. Members of the glycoprotein hormone family were compared as substrates for the addition of GalNAc to their oligosaccharides (Fig. 1). With the exception of oFSH and hFSH, the oligosaccharides on each of the substrates examined terminate exclusively with sialic acid and Gal (oFSH and hFSH contain 40% and 7% sulfated structures, respectively) (3, 5, 13). Substrates were prepared by removal of terminal sialic acid and Gal with neuraminidase and β-galactosidase. The presence of terminal GlcNAc was confirmed by incubation of each substrate with UDP-[^{3}H]Gal and highly enriched Gal-transferase. In each instance the amount of [^{3}H]Gal incorporated was proportional to the number of oligosaccharides present on the glycoprotein. The substrates were then compared as acceptors for addition of GalNAc by the pituitary GalNAc-transferase (Fig. 1).

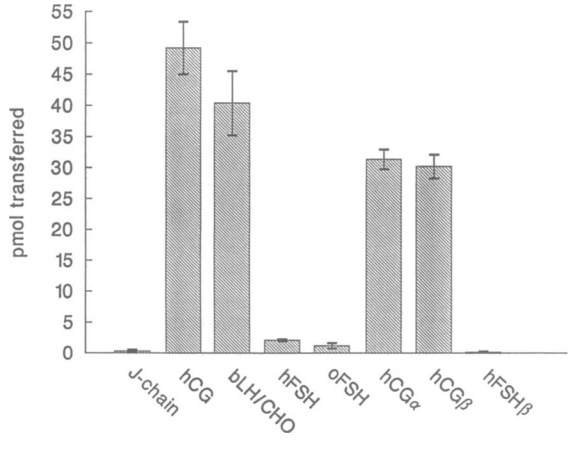


FIG. 1. Comparison of glycoproteins as substrates for the GalNAc-transferase. Each glycoprotein was digested with neuraminidase and β-galactosidase to produce oligosaccharides with structure $GlcNAc_2Man_3GlcNAc_2$. Assays contained 4 μM substrate, 720 μM UDP-GalNAc, and bovine pituitary membranes containing 385 μU/ml of GalNAc-transferase activity. The average ± SEM of three determinations is shown for each protein. Each glycoprotein was also tested as an acceptor for Gal addition using purified bovine milk galactosyltransferase to confirm the correct oligosaccharide intermediate was present. J chain contains one asparagine-linked oligosaccharide, whereas hCG, hFSH, and oFSH contain four, and bLH/CHO contains three. The isolated subunits hCGα, hCGβ, and hFSHβ each contain two asparagine-linked oligosaccharides.

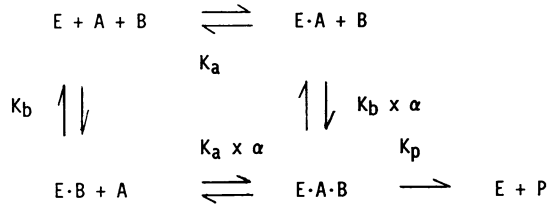
hCG and bLH/CHO were excellent acceptors for GalNAc addition, whereas J chain, bearing the identical oligosaccharide structure, did not incorporate significant amounts of GalNAc. The levels of GalNAc incorporation seen with J chain were similar to those seen with a glycopeptide with only an asparagine residue (data not shown), indicating the peptide portion of J chain had neither a positive nor a negative impact on recognition. In contrast to hCG and bLH/CHO, neither hFSH nor oFSH was a good acceptor for addition of GalNAc, incorporating only slightly more GalNAc than J chain.

As we observed previously, the free α subunit derived from hCG was an acceptor for GalNAc addition. Isolated hCGβ was also an acceptor for GalNAc addition, displaying levels of GalNAc incorporation similar to the free α subunit. In contrast, the β subunit of hFSH did not incorporate significantly greater amounts of GalNAc than J chain. Even at concentrations of 10 and 32 μM, hFSHβ did not incorporate significant levels of GalNAc (0.43 and 1.23 pmol transferred, respectively).

Since the isolated α and β subunits of hCG were both recognized by the GalNAc-transferase, one or more recognition sites for the transferase must be encoded within the peptide of each subunit. The studies described did not allow us to ascertain whether the recognition sites on the α and β subunits were utilized by the GalNAc-transferase when the subunits were combined to form dimeric species. However, hCG and bLH were acceptors for GalNAc addition, whereas hFSH and oFSH were not efficiently recognized by the GalNAc-transferase. This was true despite the presence of the common α subunit with its recognition marker. hFSHβ, unlike hCGβ, showed no evidence of recognition by the GalNAc-transferase at any of the substrate concentrations used. The properties of FSH and hFSHβ supported our previous hypothesis that FSHβ does not contain the recognition marker and may mask the recognition marker on the associated α subunit (3).

Peptide Recognition by the GalNAc-Transferase Is Not Destroyed by Reduction and Alkylation. The studies summarized in Fig. 1 indicated that native forms of hCG, bLH, and their subunits were recognized by the pituitary GalNAc-transferase. To determine if recognition required maintenance of tertiary structure, we compared the incorporation of GalNAc onto hCG oligosaccharides prior to and following reduction and alkylation. The α and β subunits of hCG contain 10 and 12 cysteine residues, respectively, each of which forms intra-chain disulfide bonds (6, 7). As a result, reduction and alkylation severely disrupt the tertiary structures of hCGβ and hCGα. Alkylation of hCG with either iodoacetamide or bromoethylamine reduced the incorporation of GalNAc to levels equal to 25–50% of the amount obtained with native hCG when tested at a single substrate concentration. In contrast, alkylation with iodoacetic acid reduced incorporation of GalNAc to <5% of that obtained with native hCG, even though there was no reduction in the level of Gal incorporated by Gal-transferase. Thus, introduction of neutral or basic substituents by way of iodoacetamide and ethylamine, respectively, did not destroy recognition by the GalNAc-transferase, whereas introduction of an acidic group by way of iodoacetic acid abolished recognition. Furthermore, GalNAc was incorporated into isolated, reduced, and alkylated hCGα and hCGβ, indicating that maintenance of native tertiary structure *per se* is not essential for recognition of either subunit by the GalNAc-transferase.

The extent to which recognition by the GalNAc-transferase was altered by the disruption of tertiary structure was assessed by determining the apparent K_m for native and for reduced and alkylated substrates. Since the GalNAc-transferase is a bireactant enzyme, we have represented GalNAc addition to oligosaccharide acceptors as a bireactant reaction in rapid equilibrium in the equation below:



Saturation curves were constructed for oligosaccharide acceptors at different concentrations of UDP-GalNAc and for UDP-GalNAc at different concentrations of acceptor. Lineweaver–Burk plots derived from saturation curves for hCG are illustrated in Fig. 2*A*. The family of lines derived at the different UDP-GalNAc concentrations intersected below the x axis at the K_a for hCG, indicating the apparent K_a for hCG increased as the concentration of UDP-GalNAc increased. The same result was obtained with all glycoproteins and glycopeptides tested. Furthermore, the association constant for UDP-GalNAc, K_b, also increased with increasing concentrations of acceptor.

The apparent K_m for either substrate is defined as the substrate concentration that gives a velocity one-half of maximal when the concentration of the other substrate is at saturation. Thus, the K_m for hCG = $K_a \times \alpha$ and the K_m for UDP-GalNAc = $K_b \times \alpha$, and the constant α is a measure of the influence one substrate has on the binding of the other. Values for $\alpha > 1$ indicate that binding of one substrate results in an increased association constant for the other (described above). When binding of one substrate does not influence binding of the other $\alpha = 1$ and $K_m = K_a$ (18). Fig. 2*B* illustrates a plot of [1/apparent V_{max}], derived from the Lineweaver–Burk plots, versus 1/[hCG] to determine the apparent K_m for hCG from the x intercept. The apparent K_m for UDP-GalNAc was determined in the same fashion.

The apparent K_m was determined for each of the substrates presented in Tables 1 and 2. All of the substrates examined had positive α values. In the case of hCG and bLH/CHO, α values fell in the range of 10–20, whereas α = 3.4 for the glycopeptide isolated from bovine IgG. Since the catalytic event is assumed to be rate limiting, the apparent K_m represents the association constant for substrate with the GalNAc-transferase and is the most direct measure of peptide and oligosaccharide recognition by the GalNAc-transferase. Absence of the peptide recognition site increased the apparent K_m by 100-fold or more (Table 2, hCG versus J chain) but did not significantly alter the catalytic rate, since the V_{max} was similar for oligosaccharide acceptors associated with peptide recognized by the GalNAc-transferase and for oligosaccharides not associated with this peptide recognition marker—i.e., hCG and J chain, respectively.

The Hormone-Specific GalNAc-Transferase Does Not Require Maintenance of Native Tertiary Structural Features for Recognition. The kinetic parameters of hCG dimer, hCGα, and hCGβ as well as their reduced and carboxamidomethylated derivatives were determined (Table 1) to establish the extent to which recognition by the GalNAc-transferase required maintenance of tertiary structural features. Dimer hCG, hCGβ, and hCGα had similar apparent K_m values, which were significantly lower than the apparent K_m obtained for J chain (2.6 mM) or a glycopeptide (1.2 mM). Reduction and carboxamidomethylation did not significantly alter the

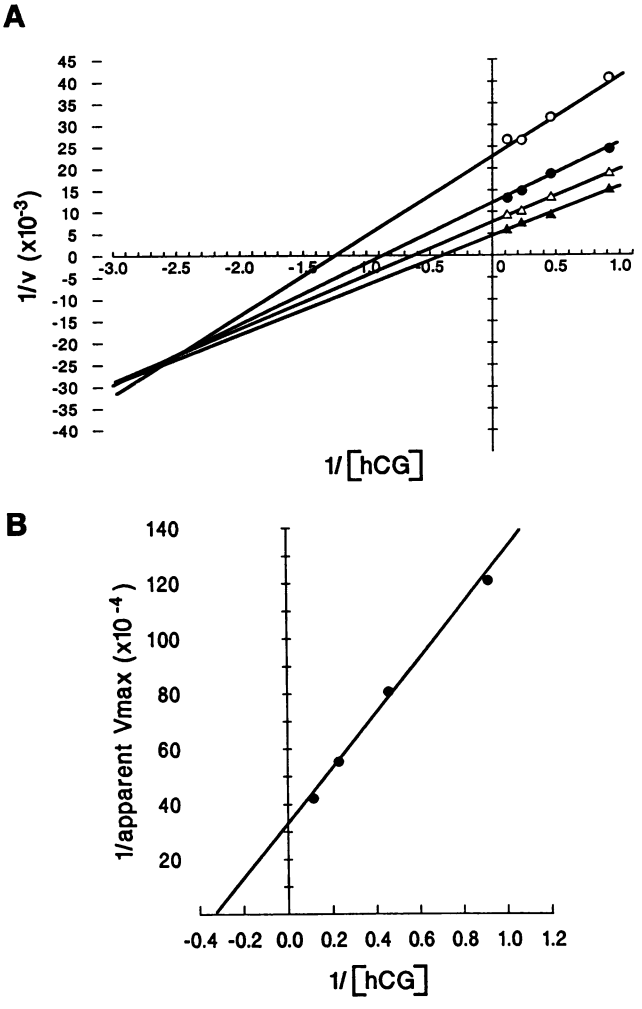


FIG. 2. Determination of GalNAc-transferase kinetic parameters. (*A*) Double-reciprocal plots of saturation curves for GalNAc addition to hCG at different concentrations of UDP-GalNAc. GalNAc-transferase assays were conducted as described in the legend to Fig. 1 at hCG concentrations of 1.1, 2.2, 4.4, and 8.8 μM. The assays were performed at UDP-GalNAc concentrations of 90 μM (○), 180 μM (●), 360 μM (△), and 720 μM (▲). (*B*) Determination of the apparent K_m for hCG. The reciprocal of the apparent V_{max} versus the reciprocal of the hCG concentration was plotted. The apparent V_{max} values for hCG were derived from plots of $1/v$ versus 1/[UDP-GalNAc] at different concentrations of hCG. The apparent V_{max} for hCG was determined from the y intercept and the apparent K_m for hCG was determined from the x intercept.

apparent K_m for either hCGβ or hCGα; however, the apparent V_{max} was reduced by roughly 50% and accounted for the reduction in catalytic efficiency. Thus, reduction and alkylation did not cause a detectable alteration in recognition of hCGβ or α subunit (hCGα) by the GalNAc-transferase.

Differential Recognition of LH, hCG, and FSH by the Pituitary GalNAc-Transferase. The results presented in Fig. 1 indicated that there were significant differences in the ability of the GalNAc-transferase to transfer GalNAc to the identical oligosaccharide on different peptides. J chain, hFSH, bLH/CHO, and hCG bear predominantly complex-type oligosaccharides terminating in sialic acid and Gal. Following removal of terminal sialic acid and Gal, the apparent K_m for addition of GalNAc to oligosaccharide acceptors was 3.9 μM for bLH/CHO and 4.4 μM for hCG (Table 2). The apparent K_m for addition of GalNAc to the same oligosaccharide acceptor on J chain was 2.6 mM. As a result, the catalytic efficiencies for addition of GalNAc to hCG and bLH/CHO

Table 1. Kinetic parameters for native and reduced/carboxamidomethylated (RCM) hCG and hCG subunits

Substrate	Apparent K_m, μM		Apparent V_{max}, pmol/hr per μg of membranes	Relative catalytic efficiency, V_{max}/K_m (protein)
	Protein	UDP-GalNAc		
hCG	4.4	810	0.98	517
hCG_α	13.0	720	1.22	218
hCG_β	6.2	470	0.70	262
hCG_α (RCM)	7.0	630	0.51	169
hCG_β (RCM)	9.3	730	0.44	110
Glycopeptide	1200	1300	1.08	2

Assays were conducted as described in the legend to Fig. 1. Lineweaver–Burk plots such as those shown in Fig. 2 were used to determine the V_{max} and apparent K_m values for hCG and UDP-GalNAc at four different concentrations of UDP-GalNAc and hCG, respectively. These were replotted (1/apparent V_{max} versus 1/[UDP-GalNAc] or 1/[hCG]) to obtain the apparent K_m from the x intercept and the apparent V_{max} from the y intercept at saturation. Catalytic efficiencies are given relative to J chain (see Table 2).

were 517- and 542-fold greater, respectively, than for addition of GalNAc to $GlcNAc_2Man_3GlcNAc_2$ on J chain.

hFSH and hFSHβ, like J chain, were not recognized by the pituitary GalNAc-transferase, whereas the free α subunit was recognized (Fig. 1). The apparent K_m for hFSH, determined from saturation curves, was 66 μM (Table 2). Although 10-fold greater than the apparent K_m obtained for hCG and bLH/CHO, this value was significantly lower than the K_m for addition of GalNAc to J chain oligosaccharides. The apparent V_{max} for hFSH (0.46 pmol/hr per μg of membrane protein) resulted in a calculated catalytic efficiency only 16-fold greater than that for J chain (Table 2). Since there was no evidence for a recognition marker on FSHβ, recognition of dimer FSH must reflect the contribution of the α subunit. The apparent K_m of 66 μM may reflect addition of GalNAc to oligosaccharides on α subunit that has dissociated from FSHβ during the assay. Alternatively, the GalNAc-transferase may recognize the α subunit peptide while in the form of dimeric FSH but with a significantly greater K_m than when it is in the form of free α subunit.

The asparagine-linked oligosaccharides on hFSH terminate in sialic acid and Gal; however, a larger proportion of the oligosaccharides on hFSH are tribranched than on either hCG or bLH/CHO (5). GalNAc added to tribranched structures would not be detected in our assay, which relies on binding of dibranched oligosaccharides by Con A. We therefore determined the levels of Gal and GalNAc that could be transferred to J chain, hCGα, hFSHα, hFSH, and hFSHβ using purified Gal-transferase and pituitary GalNAc-transferase. The amount of GalNAc incorporated was normalized to the amount of Gal incorporated to assess exclusively differences in acceptor activity for oligosaccharides that are dibranched (Fig. 3). Seven-fold more GalNAc was added to dibranched acceptors on hFSHα than on hFSHβ and 3-fold more than on hFSH dimer. Nearly twice as much GalNAc was added to dibranched acceptors on hCGα than on hFSHα, indicating that differences in the oligosaccharide acceptor on these two forms of α subunit influence the levels of incorporation seen. Nonetheless, the reduced incorporation of GalNAc into hFSH dimer as compared to free FSHα added further support to the hypothesis that the association of hFSHβ with α results in masking of the GalNAc-transferase recognition site on the α subunit (3).

DISCUSSION

The sulfated asparagine-linked oligosaccharides present on certain members of the glycoprotein hormone family are unique structures that have not to date been encountered on other glycoproteins. The vast majority of glycoproteins synthesized in the anterior pituitary bear oligosaccharides terminating with the sequence sialic acidα-Galβ1,4GlcNAcβ1, 2Manα rather than the sequence SO_4-GalNAcβ1,4GlcNAcβ1, 2Manα. We previously established that the identical processing intermediates are utilized in the synthesis of both types of oligosaccharide (11). Furthermore, we provided evidence that a determinant encoded within the peptide portion of the hormone α subunit accounts for the specific addition of GalNAc to $GlcNAc_2Man_3GlcNAc_2$ intermediate by the GalNAc-transferase (12).

Our current studies provide new insights into the characteristics of the recognition site(s) utilized by the pituitary GalNAc-transferase. The β subunit of hCG, like its α subunit, contains a protein structural element that is involved in recognition by the GalNAc-transferase. hCGβ and hCGα have similar apparent K_m values of 6.2 and 13 μM for GalNAc addition, respectively, whereas dimer hCG has an apparent K_m of 4.4 μM. In contrast, J chain and a glycopeptide bearing the identical oligosaccharide acceptor as hCGβ or hCGα have K_m values of 2.6 and 1.2 mM, respectively. Even though we have demonstrated that both subunits of hCG contain a protein recognition marker, we do not know if both are utilized in the dimer form of the hormone. Furthermore, hCGβ and hCGα each contain two asparagine-linked oligosaccharides (19). Thus, one or as many as four recognition sites may be required for addition of GalNAc moieties to the four oligosaccharides on hCG.

hCG, produced in placental trophoblasts, is not a physiological target for the GalNAc-transferase. Urinary hCG does not contain GalNAc on its asparagine-linked oligosaccharides because placental membranes do not contain detectable

Table 2. Kinetic parameters of bLH/CHO, hFSH, hCG, and J chain

Substrate	Apparent K_m, μM		Apparent V_{max}, pmol/hr per μg of membranes	Relative catalytic efficiency, V_{max}/K_m (protein)
	Protein	UDP-GalNAc		
J chain	2600	2200	1.12	1
hFSH	66	270	0.46	16
bLH/CHO	3.9	710	0.91	542
hCG	4.4	810	0.98	517

Kinetic parameters were determined as described in the legend to Table 1.

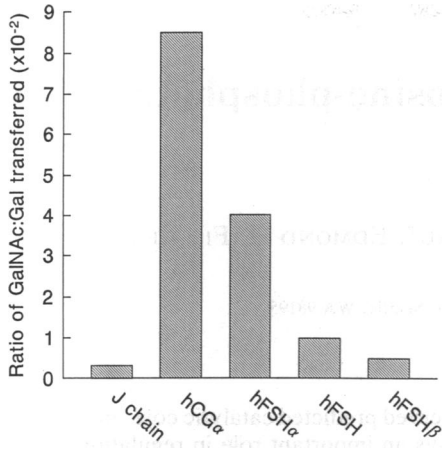


FIG. 3. Comparison of hFSH and hFSH subunits as substrates for the GalNAc-transferase. The level of Gal and GalNAc incorporation was determined for each substrate at a concentration of 4 μM using purified bovine milk Gal-transferase and bovine pituitary GalNAc-transferase, respectively. The ratio of GalNAc:Gal incorporation is presented to normalize for differences due to the presence of structures that may not be captured by the Con A step used for analysis of the product.

levels of GalNAc-transferase activity when assayed *in vitro* (12). The high degree of sequence homology of hCGβ with LHβ presumably accounts for the retention of the recognition marker on hCGβ (6). Likewise, recombinant bLH produced by CHO cells bears predominantly dibranched complex oligosaccharides terminating with sialic acid and Gal because CHO cells do not express the GalNAc-transferase (13). Following removal of sialic acid and Gal, bLH/CHO is an excellent substrate for the GalNAc-transferase *in vitro*, with an apparent K_m of 3.9 μM for GalNAc addition. Due to the extensive sequence homology of hCGβ and LHβ, we expect that LHβ will also contain a recognition determinant(s), but this has not yet been formally demonstrated.

hFSH, in contrast to hCG and bLH/CHO, is not as efficiently recognized by the GalNAc-transferase. An apparent K_m of 66 μM was obtained; however, the catalytic efficiency is only 16-fold greater than that for J chain as compared to 542-fold for bLH/CHO. Only 7% of the oligosaccharides present on isolated hFSH contain GalNAc, indicating that it is not recognized efficiently by the GalNAc-transferase *in vivo* (5). For oFSH and bFSH the proportion of oligosaccharides bearing GalNAc is significantly greater, 40% and 13%, respectively (5). Our data demonstrate that the β subunit of hFSH does not contain a protein recognition marker for the GalNAc-transferase. Furthermore, since the α subunit is recognized by the GalNAc-transferase, association of the α subunit with FSHβ must also place constraints on recognition of the α subunit by the GalNAc-transferase to account for the low levels of GalNAc incorporation by FSH dimer *in vivo* and *in vitro*. These constraints may be less stringent for bFSH and oFSH than for hFSH since they contain significantly more GalNAc. Whether a 10-fold difference in the apparent K_m for GalNAc addition to LH and FSH can account for the differences seen *in vivo* remains to be addressed. Furthermore, since there may be significant dissociation of the FSH dimer into free α and β subunits prior to and during the transferase assay, the apparent K_m of 66 μM may be artificially low and reflect the presence of free α subunit. The situation *in vivo* is considerably more complex since the topological relationship of the GalNAc-transferase to other transferases such as Gal-transferase and GlcNAc-transferases III, IV, and V will also be important if they are competing for the same synthetic intermediate.

The kinetic properties of the GalNAc-transferase are also unusual for a glycosyltransferase. This is one of the first instances in which binding of the nucleotide sugar influences binding of the oligosaccharide target and vice versa. The apparent K_m for the hormone decreases as the concentration of UDP-GalNAc decreases, and the apparent K_m for UDP-GalNAc decreases as the concentration of hormone substrate is reduced. It is notable that the value of α is significantly larger for substrates such as hCG and LH, which contain the peptide recognition site, than for substrates that do not, such as the glycopeptide derived from bovine IgG. Little is currently known about regulation of nucleotide sugar levels within the lumen of the Golgi; however, the levels of LH and FSH within the Golgi are likely to vary significantly at different times within the hormonal cycle. The properties of the GalNAc-transferase may help assure GalNAc is added to the oligosaccharides of LH regardless of its concentration as it passes through the Golgi.

The apparent K_m for addition of GalNAc to the common synthetic intermediate $GlcNAc_2Man_3GlcNAc_2$ is increased by $\geq$100-fold when the appropriate recognition marker is absent. Since reduction and alkylation do not alter the apparent K_m for GalNAc addition to either hCGβ or hCGα, recognition by the GalNAc-transferase does not require maintenance of the tertiary structural features of the hormones. The loss of recognition following alkylation of reduced hCGβ or hCGα with iodoacetic acid rather than iodoacetamide or ethylamine suggests that amino acids essential for recognition can be identified by selective modification. In addition, since recognition may be dependent on features encoded in the primary amino acid sequence, it may be possible to localize and identify the amino acids and/or region mediating recognition by preparing proteolytic fragments of α and β subunits. Such fragments could be compared as acceptors and/or inhibitors of GalNAc addition.

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