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Glycosylation of G-protein-coupled receptors for hormones central to normal reproductive functioning: its occurrence and role

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Many hormones that are central to normal reproductive functioning mediate their physiological effects by activating a receptor which belongs to the large family of G-protein-coupled receptors (GPCR). Members of this family of receptor proteins are usually glycosylated on extracellular domains. In recent years the role of this glycosylation in cell surface expression/protein folding, ligand recognition and receptor–effector coupling has been investigated. This review summarises current knowledge of the role of glycosylation in the functioning of the receptors for gonadotrophin-releasing hormone (GnRH), luteinizing hormone/human chorionic gonadotrophin (LH/HCG), follicle stimulating hormone (FSH), oxytocin (OT) and vasopressin (AVP).

Key words: glycosylation/glycohormone receptors/gonadotrophin-releasing hormone receptor/oxytocin receptor/vasopressin receptor

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Introduction

The physiological actions of many hormones and neurotransmitters are mediated by receptors which generate their second messengers via coupling to G-proteins. A large number of these G-protein-coupled receptors (GPCR) have now been cloned and have been shown to share a characteristic architecture comprising a bundle of seven α-helical transmembrane (TM) domains linked by extracellular and intracellular loops (Figure 1). All these receptors must bind their natural agonists and couple to a G-protein. This conservation of function no doubt underlies the preservation of the TM7 motif and the conservation of certain characteristic residues throughout this family of receptor proteins. The TM helical bundle provides the binding site for small ligands such as the biogenic amines acetylcholine and noradrenaline. The binding site for larger ligands, such as peptides, involves additional interactions with the extracellular domains (Strader *et al*., 1995; Wheatley, 1998). In common with most membrane proteins, GPCR are usually glycosylated. Characteristically, GPCR possess at least one glycosylation site in their *N*-terminal domain (Figure 1), although exceptions do occur. For example, the A_2 adenosine receptor lacks sites in the *N*-terminus but they are present in the second extracellular loop, whereas the α_{2b} adrenergic receptor does not contain any putative glycosylation sites (Libert *et al*., 1989). In recent years, the occurrence of oligosaccharides and their role in GPCR action has been extensively studied. This article reviews glycosylation and its role for those GPCR central to reproductive functioning.

The review is divided into four basic sections. A short initial section gives details of the glycosylation process. This is followed by an explanation of the techniques most commonly employed to probe receptor glycosylation. A summary of current understanding of the role of glycosylation for individual receptors is detailed in the third section followed by concluding comments.

Glycosylation of receptors

Protein glycosylation is a complex multi-step process and for more details the reader is directed to the review by Kornfeld and Kornfeld (1985). Oligosaccharides linked to the nitrogen in the side-chain amide of asparagine residues (*N*-linked

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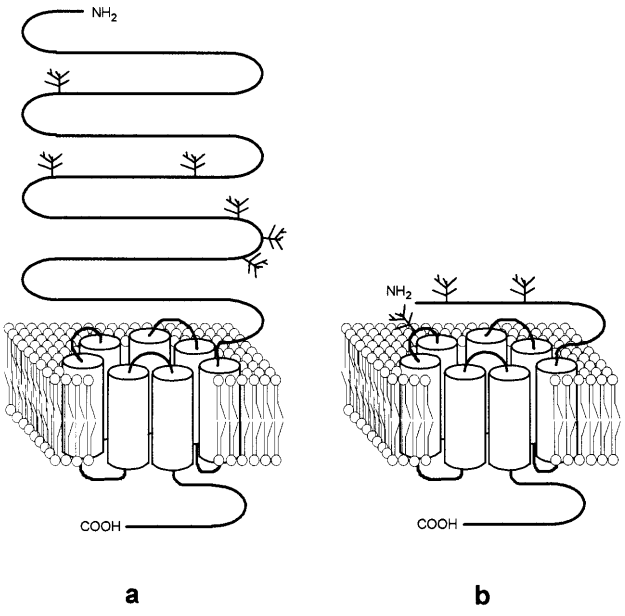

Figure 1. Schematic representation of G-protein-coupled receptors (GPCR) assembled in a plasma membrane. (**a**) A glycohormone receptor and (**b**) A 'classical' GPCR. In each case the seven α-helical transmembrane domains are represented as cylinders traversing the lipid bilayer. Glycosylation is indicated by the branched structures attached to the main polypeptide chain. The amino-, and carboxy-, terminii are labelled.

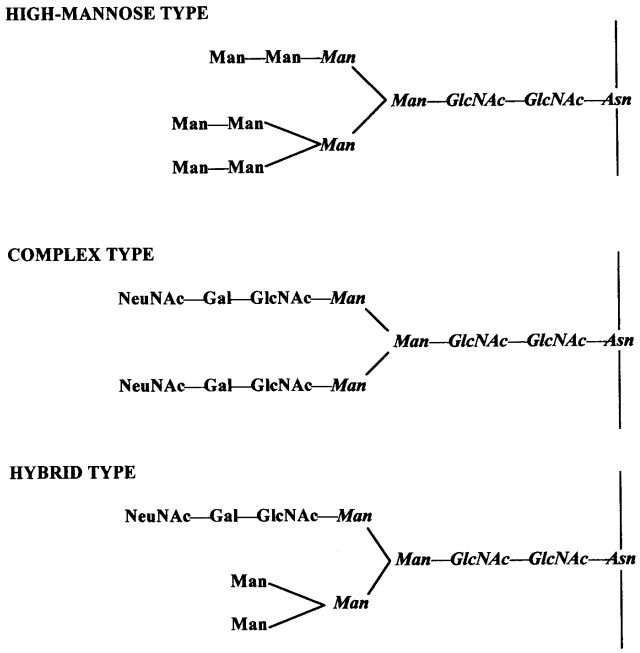

Figure 2. Structures of *N*-linked oligosaccharides. In each case, the pentasaccharide core structure present in all asparagine-linked glycans is indicated by italics.

glycosylation) exist in three categories termed high-mannose, complex and hybrid (Figure 2). All of these *N*-linked glycans share a common pentasaccharide core structure of:

Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc—Asn

This is because the first step of *N*-glycosylation is the co-translational transfer of $Glc_3Man_9GlcNAc_2$- from the lipid carrier dolichol pyrophosphate oligosaccharide onto the nascent protein by oligosaccharide transferase in the lumen of the rough endoplasmic reticulum (ER). The glycan attachment site is the Asn residue in the consensus sequence Asn-Xaa-Thr/Ser. An aspartyl residue at the Xaa position seems to prevent glycosylation as does a prolyl juxtaposed to the Thr/Ser (Bause, 1983; Gavel and von Heijne, 1990). The Asn-Xaa-Thr/Ser acceptor site must be correctly orientated and accessible for glycosylation to occur. Furthermore, as this modification occurs co-translationally, the period during which glycosylation is possible may be brief. Consequently, not all consensus sites are glycosylated. As the glycoprotein is moved through the smooth ER and the Golgi apparatus, the oligosaccharide is trimmed and elaborated. The initial step of this oligosaccharide processing is the removal of the three glucosyl residues which results in a high-mannose type chain. Complex oligosaccharides contain additions to the core glycan which include galactosyl, fucosyl, sialyl (NeuNAc) and GlcNAc residues (Figure 2). Sometimes a glycoprotein contains an hybrid oligosaccharide which contains one branch of high-mannose and one complex type. It is also possible for the glycan to possess more than two oligosaccharide chains. These are referred to as tri-, or tetra, antennary structures for three and four branches respectively.

A separate class of glycosylation involves the glycan linking to the hydroxyl of Ser or Thr via an *N*-acetylgalactosamine (GalNAc). This is classified as *O*-linked glycosylation. This glycan can also include galactosyl, fucosyl, NeuNAc and GlcNAc residues.

Strategies for establishing that a receptor is glycosylated and for determining its functional significance

Analysis by SDS–PAGE

The electrophoretic mobility of glycosylated peptides are anomalous when analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoreseis (SDS–PAGE). Consequently, they migrate with an apparent molecular weight greater than the peptide molecular weight. This effect is not merely due to the additional mass contributed by the oligosaccharide. Protein migration is affected by the carbohydrate interfering with the SDS-polypeptide association and by the effect of the glycan moieties on the movement of the denatured protein through the separating gel. In addition, glycoproteins appear as a diffuse band on SDS–PAGE, probably due to heterogeneity in the glycan modification or differences in the association of SDS and individual protein

chains. Nevertheless, this difference in the electrophoretic mobility of a peptide when it is glycosylated, compared with when it is de-glycosylated, constitutes a useful assay for establishing that a consensus glycosylation site is actually modified. In addition, the decrease in apparent molecular weight of a protein following deglycosylation may be associated with a concomitant shift from a diffuse band to a well-focused band.

Disruption of glycosylation by site-directed mutagenesis

As the consensus site for *N*-linked glycosylation is -Asn-Xaa-Ser/Thr-, putative glycosylation sites can be ablated in two ways: (i) mutating the Asn to which the oligosaccharide chain attaches; or (ii), mutating the Ser/Thr residue which fulfils an important hydrogen bonding role during transfer of the precursor oligosaccharide to the Asn by the glycosyltransferase (Hortin and Boime, 1980; Bause and Legler, 1981).

Prevention of glycosylation by tunicamycin

Tunicamycin is a nucleoside antibiotic from *Streptomyces lysosuperificus*. This inhibits the formation of GlcNAc-PP-dolichol generated by the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol pyrophosphate. Consequently, tunicamycin inhibits the first step in the lipid-linked saccharide pathway which synthesises $Glc_3Man_9GlcNAc_2$ and therefore tunicamycin effectively blocks glycosylation (Elbein, 1984). Tunicamycin is often employed to prevent *N*-glycosylation of recombinant receptors expressed by cells in culture.

Removal of the glycan by glycosidases

The susceptibility of receptors to specific glycosidases has been used extensively to establish that receptors are glycosylated and also to analyse the nature of the glycan chain. In addition, this information can also indicate the extent of oligosaccharide processing, and by implication maturation, of receptor constructs in recombinant expression systems. The most commonly employed glycosidases are cited below and their cleavage sites are presented in Figure 3.

Peptide: N-glycosidase F

PNGaseF; Peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase; EC3.5.1.52) hydrolyses all common types of *N*-linked glycan chains (Tarentino *et al.*, 1985).

Endoglycosidase H

EndoH; Endo-β-acetylglucosaminidase H; EC 3.2.1.96) cleaves high mannose and hybrid-type glycan chains. It does not cleave complex *N*-linked oligosaccharides. Consequently, the degree of oligosaccharide processing and receptor maturation can be ascertained by differences in susceptibility to EndoH and PNGaseF.

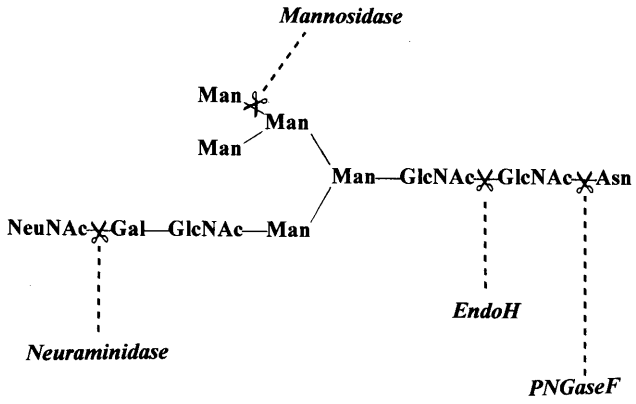

Figure 3. Cleavage sites of glycosidases commonly employed to study *N*-glycosylation. Cleavage sites of glycosidases (in italics) are shown. EndoH catalyses the cleavage of the bond indicated, in high-mannose, but not complex chains. In the interest of clarity the glycosidic bond configurations are not shown.

Neuraminidase

Sialidase (acylneuraminyl hydrolase; EC 3.2.1.18) cleaves terminal *N*-acetylneuraminic acid (sialic acid).

O-glycosidase

Endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97) hydrolyses *O*-linked glycan chains.

Glycosylation of GPCR central to normal reproduction

Gonadotrophin-releasing hormone receptor

Gonadotrophin-releasing hormone (GnRH) is a hypothalamic decapeptide which has a central role in the regulation of the reproductive system. GnRH acts on gonadotrophs in the pituitary to control the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) which subsequently regulate gonad activity. This pivotal physiological function of GnRH has allowed GnRH agonists and antagonists to be employed for therapeutic intervention in disease states of the reproductive system, such as precocious puberty, hirsutism and certain carcinomas. In addition, pulsitile administration of GnRH has been used clinically to treat infertility and hypogonadotrophic hypergonadism (Karten and Rivier, 1986; Barbieri, 1992). The effects of GnRH are mediated by specific GnRH receptors (GnRHR) expressed by gonadotrophs. These GnRHR are coupled to phosphoinositidase C via the G-proteins $G_{q/11}$, thereby increasing intracellular calcium and regulating gonadotrophin exocytosis (Hsieh and Martini, 1992; Tse and Hille, 1992).

The GnRHR was first cloned from the αT3-1 gonadotroph cell line by Tsutsumi *et al.* (1992) and has since been cloned from mouse (Reinhart *et al.*, 1992), rat (Kaiser *et al.*, 1992), sheep (Illing *et al.*, 1993), human (Kakar *et al.*, 1992) and cow (Kaker

et al., 1993). In general terms the sequences of the cloned GnRHR exhibit the characteristic structural motifs of GPCR described earlier. However, they are unusual within the GPCR family of proteins in two respects. First there is a complete lack of a *C*-terminal tail intracellular domain which probably underlies the lack of rapid agonist-induced desensitisation observed for GnRHR. Second, the conserved Asp and Asn usually found in TM2 and TM7 respectively of GPCRs (Baldwin, 1993) are reversed such that the TM2 helix contains the Asn and the TM7 helix the Asp. This discovery has provided insight into constraints within the helical packing for the whole GPCR family of proteins (Zhou *et al.*, 1994).

The GnRHR from all five species cited above possesses conserved consensus *N*-glycosylation sites at Asn^{18} and Asn^{102}. The GnRHR from mouse and rat each have an additional site at Asn^{4}. Studies on the role of glycosylation in GnRHR function which pre-dated the cloning of the receptor were contradictory. For example, it was reported that treatment with tunicamycin or sialidase decreased receptor expression but did not change binding affinities (Schvartz and Hazum, 1985). In contrast, the effect of neuraminidase, or periodate, on ligand binding suggested that glycosylation was a prerequisite for high affinity binding of agonists and antagonists (Hazum, 1982; Keinan and Hazum, 1985).

The availability of the receptor cDNA allowed the role of carbohydrate in GnRHR function to be addressed directly. Davidson *et al.* (1995) used site-directed mutagenesis to individually ablate the putative glycosylation sites (Asn^{4}, Asn^{18}, Asn^{102}) in the mouse receptor. In each case, the canonical site was disrupted by an Asn→Gln substitution. The photoaffinity analogue [^{125}I]-[D-Lys-*N*-azidobenzoyl]GnRH was used to label wild-type and mutated GnRH receptors expressed in COS-1 cells. Subsequent analysis by SDS–PAGE revealed that the wild-type GnRHR migrated as a diffuse band, typical of glycoproteins, between 55–75 kDa. Both the N4Q and N18Q GnRHR mutants migrated with an apparent molecular weight of 45–65 kDa. This faster migration is entirely consistent with a decrease in glycosylation (see previous section). Interestingly, the migration of the N102Q mutant receptor on SDS–PAGE was indistinguishable from the wild-type receptor indicating that although Asn^{102} is a putative *N*-glycosylation site, it is not actually utilized. This non-glycosylation has subsequently been shown to have important functional ramifications as the Asn side-chain is critical for high affinity binding of GnRH analogues with a *C*-terminal glycinamide (Davidson *et al.*, 1996a). If Asn^{102} was glycosylated, this role in ligand binding would be compromised. All of the Asn→Gln mutant constructs bound GnRH with normal affinity and stimulated inositol 1,4,5 trisphosphate (IP_3) production when activated by agonist. However, the N4Q and N18Q mutant receptors exhibited reduced expression, with B_{max} values only 46% and 39% of wild-type respectively. GnRHR-induced IP_3 production by the N4Q and N18Q mutant receptors was ~55% of wild-type,

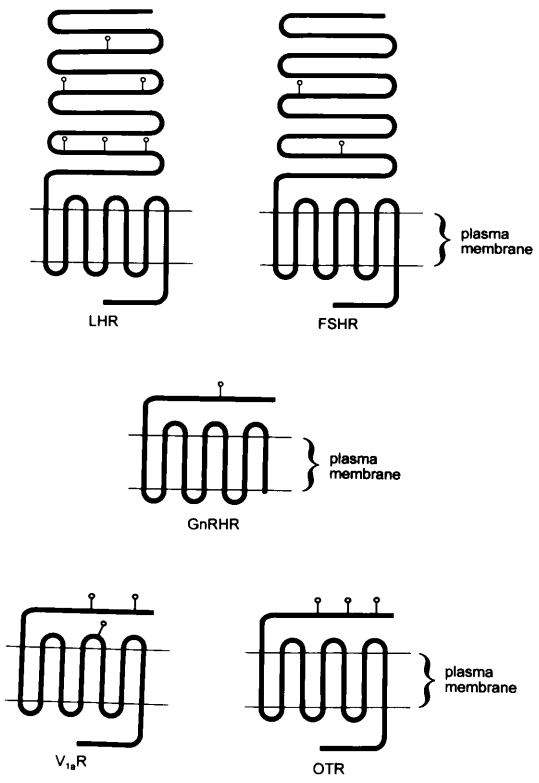

Figure 4. The position of glycosylation sites present on G-protein-coupled receptors (GPCR) central to reproductive functioning. In each case glycosylation sites are indicated by ().

which is consistent with the decreased receptor number observed and did not reflect a decrease in receptor–G-protein coupling *per se*. Consistent with the lack of glycosylation of Asn^{102}, the N102Q receptor construct did not exhibit perturbed receptor glycosylation nor exhibit reduced IP_3 production.

Glycosylation of both sites is required for normal receptor levels of the rodent GnRHR, as deletion of either site individually is reflected in a decreased B_{max}. The importance of glycosylation of both Asn^{4} and Asn^{18} was reinforced by recombinant expression studies with the human GnRHR. This has only one glycosylation site at Asn^{18} (Figure 4) and expression in COS-1 cells gave low receptor levels. However, an 80% increase in GnRHR expression was observed when an additional *N*-glycosylation site was engineered into the human receptor (S4N) at the locus of the second consensus site in the rodent GnRHR (Davidson *et al.*, 1996b).

Overall, these data establish that glycosylation has an important role in GnRHR expression, and/or stability of the receptor, at the plasma membrane.

LH receptor

The effects of the large pituitary glycoprotein luteinizing hormone (LH) are mediated by human chorionic gonadotrophin HCG/LH receptors (HCG/LHR) which also bind the related placental glycoprotein agonist, HCG.

Activation of the receptor stimulates adenylyl cyclase via the G-protein G_S, thereby raising intracellular cAMP which subsequently stimulates steroid biosynthesis and secretion (Dufau, 1998). When the HCG/LHR was cloned (McFarland *et al*., 1989) it was found to have the characteristic features of a GPCR. In addition, the HCG/LHR possessed an extended *N*-terminus comprising 341 amino acids with leucine-rich repeats. This elongated *N*-terminus (Figure 1) is a characteristic of the receptors for the glycoprotein hormones HCG/LH, FSH (Sprengel *et al*., 1990) and thyroid stimulating hormone (TSH) (Nagayami *et al*., 1989). As such these three receptors represent a subfamily of GPCR proteins. Interestingly, in each case this *N*-terminal domain of the receptor alone is sufficient for high affinity binding of the glycohormone (Xie *et al*., 1990; Seetharamaiah *et al*., 1994; Davis *et al*., 1995).

Six consensus sites for *N*-glycosylation exist within the large *N*-terminal extracellular domain of the HCG/LHR at Asn^{77}, Asn^{152}, Asn^{173}, Asn^{269}, Asn^{277} and Asn^{291} (Figure 4). Given the importance of this region to receptor–ligand interaction, the role of glycosylation in HCG/LHR function has been extensively investigated. Exposing [^{125}I]-affinity-labelled, purified HCG/LHR to PNGaseF under limiting conditions generated a deglycosylated receptor of 62 kDa from the native 92 kDa receptor. As the fully deglycosylated receptor was produced via only 1–3 intermediates, it was concluded that not all six putative glycosylation sites were used (Petäjä-Repo *et al*., 1991; Petäjä-Repo, 1994). However, even if all six sites were utilized, it is unlikely that deglycosylation by PNGaseF and SDS–PAGE analysis would be sufficiently sensitive to reveal all the partially glycosylated intermediate states of the HCG/LHR. Nevertheless, a similar conclusion was reached by others employing a combination of site-directed mutagenesis and tunicamycin treatment to disrupt the glycosylation (Zhang *et al*., 1995). This latter study indicated that Asn^{77} was not glycosylated, that both Asn^{152} and Asn^{173} were glycosylated and that at least one of the three remaining consensus sites at Asn^{269}, Asn^{277} and Asn^{291} was utilized. As an alternative strategy to establish how many of the potential sites are glycosylated, Davis *et al*. (1997) engineered a series of HCG/LHR constructs in which all but one consensus site had been disrupted by mutation. Each of the six members of the series had a different *N*-glycosylation consensus site retained. For each HCG/LHR construct, enzymatic deglycosylation by PNGaseF treatment resulted in an apparent reduction in mass on SDS–PAGE, indicating that all six putative glycosylation sites of the HCG/LHR are indeed glycosylated (Figure 4).

The nature of the carbohydrates of the HCG/LHR has been elucidated using specific endo-, and exo-, glycosidases. It was demonstrated that the oligosaccharide chains were susceptible to cleavage by PNGaseF (as described above; Petäjä-Repo *et al*., 1991), and by neuraminidase (Rajaniemi *et al*., 1996). In contrast, EndoH, α-mannosidase and *O*-glycosidase (which cleave high mannose/hybrid *N*-linked sugars, mannose residues from high mannose oligosaccharides and *O*-linked oligosaccharides respectively) did not modify the HCG/LHR carbohydrate chains (Petäjä-Repo, 1994; Rajaniemi *et al*., 1996). Consequently, the HCG/LHR contains only *N*-linked complex oligosaccharide some of which terminate in sialic acid.

The precise role of the oligosaccharide moieties of the HCG/LHR is controversial, as data published by various laboratories appear to be mutually exclusive. For example, hormone binding was reported to be unaffected by enzymatic de-glycosylation of the mature receptor (Keinänen, 1988; Ji *et al*., 1990; Patäjä-Repo *et al*., 1993; Tapanainen *et al*., 1993) but to be decreased by disruption of three of the glycosylation sites (Asn^{77}, Asn^{152} and Asn^{173}) using site-directed mutagenesis (Zhang *et al*., 1991). This decreased ligand binding was not due to compromised expression of the receptor at the cell surface. These apparently conflicting data were reconciled however, when it was proposed that glycosylation has a role in intra-molecular folding of the nascent HCG/LHR but that carbohydrate is not required once the receptor has been folded into the active conformation and inserted into the plasma membrane (Zhang *et al*., 1995). In this regard, it is noteworthy that the oligosaccharide chains of the HCG/LHR are accessible to glycosidases in the hormone–receptor complex, which is indicative that they are not in intimate contact with the ligand (Patäjä-Repo *et al*., 1991). This contention is supported by recent computer modelling of the extracellular domain of the HCG/LHR which positions Asn^{77}, Asn^{152} and Asn^{173} along the outer circumference of the structure with no obvious involvement with hormone binding or signalling (Bhowmick *et al*., 1996).

The presence of an oligosaccharide moiety on Asn^{152} and Asn^{173} seems to be critical for the acquisition of high affinity hormone binding. This is particularly important for Asn^{173}, as an $Asn^{173}\rightarrow Gln^{173}$ substitution resulted in a total loss of HCG binding which was not due to impaired translocation of the HCG/LHR to the plasma membrane (Zhang *et al*., 1991). This observation encouraged extensive study of this glycosylation site by several groups. At one stage it was suggested that it was the Asn^{173} residue *per se* that was required for hormone recognition rather than any linked carbohydrate (Liu *et al*., 1993). However, after a period of confusion, when contradictory data were published by different groups studying the same glycosylation-defective mutation (T175A; Liu *et al*., 1993; Zhang *et al*., 1995), it was finally established that the non-glycosylated mutant receptor does not bind HCG (Zhang *et al*., 1995; Davis *et al*., 1997). Glycosylation of only Asn^{173} and Asn^{152} is sufficient for high affinity hormone binding. Although, *in vivo*, this modification would be with complex carbohydrate, it is clear that unprocessed high-mannose chains also permit normal HCG/LHR function. Indeed, it appears that it is only the proximal *N*-acetylglucosamine residue, common to both complex and high-mannose carbohydrate, which is the pre-requisite to high affinity hormone binding (Zhang *et al*., 1995)

The use of tunicamycin, rather than site-directed mutagenesis, to prevent glycosylation has so far generated contradictory

results. Consequently, tunicamycin pre-treatment has been reported to prevent hormone binding (Zhang *et al*., 1995) and to have no effect on hormone binding (Davis *et al*., 1997).

The published data to date have established that the carbohydrate chains of the HCG/LHR do not have a direct role in either ligand binding nor intracellular signalling. Glycosylation is possibly a prerequisite to the nascent receptor protein folding correctly and acquiring the active conformation (Dufau, 1998), although currently there is no consensus within the field (Davis *et al*., 1997).

FSH receptor

The anterior pituitary glycohormone, FSH, regulates gonadal cell function by binding to specific FSH receptors (FSHR) which stimulate adenylyl cyclase via activation of the G-protein G_S. The FSHR was first cloned from rat (Sprengel *et al*., 1990), later from human (Kelton *et al*., 1992), and found to possess an elongated *N*-terminus (Figure 1) in common with the HCG/LHR. This receptor is glycosylated as it binds to lectin (Dattatreyamurty *et al*., 1990). Comparison of FSHR cDNA sequences from a range of species identified three putative *N*-glycosylation sites in the extended *N*-terminal domain which are conserved in all species studied to date. A forth consensus glycosylation site is present in the human receptor but as yet, it is not known if it is utilized.

To address the role of the glycan moiety in FSHR function, radioligand binding studies were performed with testicular plasma membranes that had been pre-treated with glycosidases. FSHR susceptibility to neuraminidase and PNGaseF, but not *O*-glycosidase, indicated the presence of sialic acid-containing *N*-linked oligosaccharides (Dattatreyamurty and Reichert, 1992). It was also demonstrated that deglycosylation of the FSHR by PNGaseF did not affect the binding of [^{125}I]-FSH or receptor–G-protein coupling (Dattatreyamurty and Reichert, 1992).

More recently, the role of glycosylation in rat FSHR function has been investigated using a series of glycosylation-defective mutants (Davis *et al*., 1995). For each canonical *N*-linked glycosylation site two mutant receptors were engineered, one with an Asn→Gln substitution and the other with a Ser(Thr)→Ala substitution. As both of these residues are functionally implicated in the glycosylation process each of these mutations would disrupt oligosaccharide attachment to the Asn residue (see earlier section). The rational for this double construct strategy was to differentiate between effects being generated by substitution of an Asn residue *per se* and those effects resulting from removal of a glycosylation site. Using a combination of PNGaseF treatment and SDS–PAGE analysis to detect changes in the apparent molecular weight of the FSHR, it was demonstrated that Asn^{174} and Asn^{276} are glycosylated but Asn^{182} is not (Davis *et al*., 1995; Figure 4). The residue numbering cited starts with the first residue after the cleavage site of the 17 residue signal peptide.

Using mutant FSHR constructs expressed in HEK 293 cells, it was found that hormone binding was unaffected if the *N*-linked glycosylation at either Asn^{174} or Asn^{276} was disrupted. However, if both sites were ablated simultaneously, the FSH binding was lost. Likewise, if glycosylation of FSHR was prevented by tunicamycin treatment, no hormone binding was observed. In contrast, hormone binding was not affected when extensive PNGaseF digestion was used to remove the glycan moiety from the mature receptor. This latter observation is in agreement with the studies of others cited above (Dattatreyamurty and Reichert, 1992). These data indicate that at least one glycan moiety is required for correct folding of the nascent FSHR protein or for its appropriate insertion into the membrane. Once the receptor has been folded however, the *N*-linked oligosaccharide can be removed without detriment to the function of the receptor (Davis *et al*, 1995). When residues 1–346 of the *N*-terminal domain (FSHRt346) were expressed alone they bound FSH with wild-type affinity. As the FSHRt346 construct was sensitive to endoglycosidase H, it implied that the *N*-linked glycan moieties it contained comprised unprocessed mannose residues (Kobata, 1979; Trimble and Maley, 1984). Consequently, although high affinity FSH binding requires glycosylation of the nascent protein, there is not an obligation for these *N*-linked oligosaccharides to be fully processed (Davis *et al*, 1995).

The importance of these glycosylation sites for normal FSHR functioning in humans has recently been highlighted by the discovery of two naturally occurring inactivating mutations (Simoni *et al*., 1997). A genetic survey of pure ovarian dysgenesis in Finland revealed an autosomal recessive inheritance pattern. Further investigation identified the cause in nearly all cases as an Ala→Val substitution, juxtaposed to the glycosylation site at Asn^{174}, which resulted in a dysfunctional FSHR. In one woman the same glycosylation site was ablated by an Asn174Ile substitution. Again this mutation resulted in an FSHR with very little response to FSH (Gromoll *et al*., 1996).

It has, therefore, been established that glycosylation of the FSHR is critical for folding the nascent receptor into its active conformation but it does not have an important role in the function of the mature receptor.

Receptors for vasopressin and oxytocin

The neurohypophysial hormones [Arg^8]vasopressin (AVP) and oxytocin (OT) are both nonapeptides with an internal disulphide bond. They differ in their amino acid sequence at just two positions with Phe^3 and Arg^8 of AVP being substituted by Ile^3 and Leu^8 in OT. OT induces uterine contraction at parturition, milk ejection from the mammary gland (Soloff *et al*., 1979) and affects steroidogenesis in the testis (Pickering *et al*., 1989). In addition to the well-documented effects on blood pressure and urine volume, AVP also regulates steroidogenesis and stimulates seminiferous tubule contractility. The oxytocin receptor (OTR)

was initially cloned from human uterus (Kimura *et al*., 1992) and subsequently from a wide range of species (Ivell *et al*., 1997). The vasopressin receptor (VPR) was originally cloned from liver (Morel *et al*., 1992). A VPR has since been cloned from testicular myoid cells and shown to be of the V_{1a} subtype (Howl *et al*., 1995). The OTR and V_{1a} VPR exhibit ~45% amino acid homology and have related pharmacological profiles.

The OTR expressed by mammals has three canonical sites for *N*-linked glycosylation within the *N*-terminus (Figure 4) but there are only two such sites in rodents. When rat OTR was photoaffinity labelled by an [^{125}I]-radioiodinated ligand then treated with Endoglycosidase F there was a decrease in apparent molecular weight of the OTR, indicative that at least one of the glycosylation sites was utilized (Kojro *et al*., 1991). Disruption of the three *N*-linked glycosylation sites of the human OTR by site-directed mutagenesis at Asn^{8}, Asn^{15} and Asn^{26} by Kimura *et al.* (1997) individually, or in combination, indicated that the glycosylation status of the receptor did not influence receptor pharmacology.

The V_{1a} vasopressin receptor (V_{1a}R) has four putative *N*-glycosylation sites, Asn^{14} and Asn^{27} in the *N*-terminus, Asn^{198} and Asn^{333} in the second and third extracellular loops respectively. The receptor was shown to be glycosylated when expressed (Wheatley *et al*., 1997) but only three of the four putative sites are actually glycosylated (Figure 4). Asn^{333} is not glycosylated, probably due to the prolyl residue juxtaposed to Asn^{333} (S.R.Hawtin and M.Wheatley, unpublished data). In common with the OTR, neither the binding, nor the second messenger generation, was affected if the glycosylation sites at the *N*-terminus were ablated individually or simultaneously. Likewise mutation of Asn^{198} to Gln^{198} did not affect the receptors' properties (S.R.Hawtin and M.Wheatley, unpublished data). The fully de-glycosylated V_{1a} vasopressin receptor was however, expressed at markedly reduced levels (16±8% of the glycosylated receptor). These results are similar to the observations reported for the related V_2 subtype of VPR, which mediates the antidiuresis effect of vasopressin, and which is fully active when it is non-glycosylated (Innamorati *et al*., 1996).

For the neurohypophysial hormone receptors, glycosylation has no effect on ligand binding or cell signalling but it does have a role in expression and/or stability of the V_{1a} vasopressin receptor.

GPCR glycosylation in context

This review has focused on GPCR which are important to reproductive functioning. Although all the receptors discussed in this article are glycosylated, the role of this modification varies from receptor to receptor and is summarized in Table I. The role of glycosylation has also been investigated for many other GPCR. To date, the only GPCR reported to exhibit *O*-linked glycosylation are the PGE_2 and PGD_2 prostaglandin receptors (Morii and Watanabe, 1992). GPCR are related by sequence homology, structural motifs and mode of action but glycosylation does not fulfil a common role throughout the GPCR family of proteins. Consequently, it has been reported that oligosaccharide moieties are important for GnRHR and V_{1a}R expression and/or stability but not ligand binding (see above). Likewise, glycan chains are essential for correct folding/trafficking of the vasoactive intestinal peptide (VIP)-1 receptor (Convineau *et al*., 1996), the TSH receptor (Russo *et al*., 1991) and the FSH receptor (see above). For some GPCR, including the somatostatin receptor (Rens-Domiano and Reisine, 1991), rhodopsin (Kaushal *et al*., 1994), the β_2-adrenergic receptor (Rands *et al*., 1990), the TSH receptor (Russo *et al*., 1991) and the gastrin-releasing peptide receptor (bombesin BB_2 receptor; Kusui *et al*., 1994), glycosylation is important for high affinity ligand binding and/or receptor–G-protein coupling. For many GPCR however, glycosylation has no known function. This latter group includes the oxytocin receptor (see above), the histamine H_2 receptor (Fukushima *et al*., 1995), the m_2 muscarinic acetylcholine receptor (Van Koppen and Nathanson, 1990), the NK_1 receptor (Fong *et al*., 1992), the bombesin BB_1 (neuromedin B) receptor (Kusui *et al*., 1994), the A_{2a} adenosine receptor (Piersen *et al*., 1994) and the AT_2 angiotensin receptor (Servant *et al*., 1996).

In conclusion, although *N*-linked glycosylation of GPCRs is an almost universal phenomenon, its extent and influence on the mature protein's properties are variable and unpredictable. The existence of a consensus site detected by sequence analysis programmes does not indicate that the site is actually utilized by oligosaccharide transferase. Likewise, the demonstration that a consensus site for *N*-linked glycosylation is derived by glycan moieties does not necessarily indicate functional significance. Nevertheless, for most GPCR, glycosylation of at least one site is necessary for normal expression or function.

Table I. The role of glycosylation in the function of G-protein-coupled receptors (GPCR) central to normal reproduction

Receptor	Role of glycosylation
GnRHR	Expression and/or stability of the receptor. No effect on ligand binding or intracellular signalling.
HCG/LHR	Possible role in folding of the nascent receptor protein. No effect on ligand binding or intracellular signalling.
FSHR	Folding of the nascent receptor protein. No role in the mature receptor.
OTR	No effect on ligand binding or intracellular signalling. No known role.
V_{1a}R	Expression and/or stability of the receptor. No effect on ligand binding or intracellular signalling.

For each receptor cited the role of glycosylation is presented. GnRHR = gonadotrophin-releasing hormone receptor; HCG = human chorionic gonadotrophin; LHR = luteinizing hormone receptor; FSHR = follicle stimulating hormone receptor; OTR = oxytocin receptor; V_{1a}R = vasopressin receptor.

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