Structural characterization of the carbohydrates of the rat ovarian luteinizing hormone/chorionic gonadotropin receptor

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The numbers and types of oligosaccharide present on the rat ovarian luteinizing hormone (LH)/chorionic gonadotropin (CG) receptor were determined by treating radiolabelled purified receptors with glycosidases and examining the changes in electrophoretic mobility and number of radiolabelled bands on SDS/PAGE. The purified receptor was also transferred to nitrocellulose after SDS/PAGE and probed with digoxigenin-labelled lectins. The following conclusions were drawn: (1) the rat ovarian LH/CG receptor contains at least two complex-type N-linked

oligosaccharide chains, of which one is biantennary and the rest multiantennary. (2) The N-linked chains terminate in either unsubstituted galactose or sialic acid linked α 2-3 or α 2-6 to the penultimate galactose. (3) The N-linked oligosaccharides also contain internal poly(*N*-acetyl-lactosamine) sequences and fucose-linked α 1-6 to the proximal *N*-acetylglucosamine. (4) No O-linked carbohydrate moieties are present on the receptor molecule.

INTRODUCTION

The action of pituitary luteinizing hormone (LH) and its placentally produced agonist, chorionic gonadotropin (CG), is initiated by binding to specific LH/CG receptors on the gonadal target cells [1]. This triggers the characteristic physiological responses via activation of adenylate cyclase [1]. Recent cloning of the cDNAs for the LH/CG receptor from several species [2–6] has shown that the receptor belongs to the superfamily of G-protein-coupled receptors. The large extracellular domain of the receptor contains six potential sites for N-linked glycosylation, but not all of them seem to be used [7]. Previous reports have indicated that the receptor contains sialylated complex-type N-linked oligosaccharides [7–11], but no detailed structural information is yet available.

The functional significance of the oligosaccharides of the LH/CG receptor is largely unknown. Removal of the carbohydrates from the mature membrane-inserted LH/CG receptor does not impair its ability to bind hormone [7,8,12] or to transmit the signal to adenylate cyclase [13]. On the other hand, inhibition of N-glycosylation by tunicamycin leads to loss of human (h)CG binding to intact LH/CG receptor-expressing cells [12]. In addition, Liu et al. [14] showed recently that alteration of one of the consensus sequences for N-linked glycosylation impaired the ability of the rat LH/CG receptor to be expressed at the plasma membrane, although hormone binding was not affected.

In view of the potential importance of the carbohydrates in functional activation and intracellular transport of the LH/CG receptor, the present study was undertaken to define further the structure of its carbohydrate chains, employing glycosidases and digoxigenin-labelled lectins. The results presented here indicate that the rat ovarian LH/CG receptor carries at least two complex-type N-linked oligosaccharides, both bi- and multiantennary, containing fucose, sialic acid and internal poly(*N*-acetyl-lactosamine) groups.

MATERIALS AND METHODS

Chemicals and hormone preparations

Peptide-*N*-glycosidase F (PNGase F, EC 3.5.1.52 of *Flavobacterium meningosepticum* from *Escherichia coli*), endoglycosidase H (endo H, EC 3.2.1.96, of *Streptomyces plicatus* from *E. coli*), endo- β -galactosidase (EC 3.2.1.103, from *Bacteroides fragilis*) and α -mannosidase (EC 3.2.1.24, from *Canavalia ensiformis*) were obtained from Boehringer-Mannheim (Mannheim, Germany). Endoglycosidase F₂ (endo F₂, EC 3.2.1.96, from *Flavobacterium meningosepticum*) and *O*-glycosidase (EC 3.2.1.97, from *Diplococcus pneumoniae*) were from Genzyme (Cambridge, MA, U.S.A.) and neuraminidase (EC 3.2.1.18, from *Vibrio cholerae*) was from Calbiochem (San Diego, CA, U.S.A.). The digoxigenin-labelled lectins (Table 1) and the glycan differentiation and detection kits were from Boehringer-Mannheim. Pregnant-mare serum gonadotropin (PMSG, 2820 i.u./mg) and hCG (5600 i.u./mg), which were used to induce pseudo-pregnancy, were obtained from Diosynth (Oss, Netherlands). Na¹²⁵I was from Amersham (Amersham, Bucks., U.K.) and the equipment and reagents for electrophoresis and blotting were from Bio-Rad (Richmond, CA, U.S.A.). All other reagents were of analytical grade and obtained from commercial suppliers.

hCG (10900 i.u./mg) was radioiodinated with Na¹²⁵I by the chloramine- τ method [27] as described in [7]. The specific radioactivity of the preparations was 60–70 Ci/g, as determined by self-displacement assay [28]. The fraction of the labelled hormone derivatives capable of binding to an excess of ovarian membranes was 40–55%.

Animals

Immature 27-day-old female Sprague-Dawley rats were rendered pseudo-pregnant by sequential injections of PMSG (40 i.u.) and hCG (25 i.u.), according to the method described by Parlow [29].

Abbreviations used: AAA, *Aleuria aurantia* lectin; CG, chorionic gonadotropin; Con A, *Canavalia ensiformis* lectin; DSA, *Datura stramonium* lectin; endo H, endoglycosidase H; endo F₂, endoglycosidase F₂; GNA, *Galanthus nivalis* lectin; L-PHA, *Phaseolus vulgaris* lectin; LH, luteinizing hormone; MAA, *Maackia amurensis* lectin; PMSF, phenylmethanesulphonyl fluoride; PMSG, pregnant-mare serum gonadotropin; PNA, *Arachis hypogaea* lectin; PNGase F, peptide-*N*-glycosidase F; RCA-120, *Ricinus communis* lectin; SNA, *Sambucus nigra* lectin.

Table 1 Specificity of digoxigenin-labelled lectins

Lectin (Organism)	Specificity	References
GNA (<i>Galanthus nivalis</i>)	Man α 1-3Man	[15]
Con A (<i>Canavalia ensiformis</i>)	Man α 1-6(Man α 1-3)Man	[16,17]
MAA (<i>Maackia amurensis</i>)	NeuAc α 2-3Gal β 1-4GlcNAc/Glc	[18,19]
SNA (<i>Sambucus nigra</i>)	NeuAc α 2-6Gal/GalNAc	[20]
AAA (<i>Aleuria aurantia</i>)	Fuc α 1-6GlcNAc	[21]
DSA (<i>Datura stramonium</i>)	Gal β 1-4GlcNAc	[22,23]
RCA-120 (<i>Ricinus communis</i>)	Gal β 1-4GlcNAc	[24]
L-PHA (<i>Phaseolus vulgaris</i>)	Gal β 1-4GlcNAc	[25]
PNA (<i>Arachis hypogaea</i>)	Gal β 1-3GalNAc	[26]

The luteinized ovaries were collected 7–9 days after hCG injection and stored at -70°C .

Purification of rat ovarian LH/CG receptor

Crude rat ovarian membranes were prepared as described previously [7] and the LH/CG receptor was purified by one cycle of affinity chromatography on hCG–Affigel 10, according to the method described by Keinänen et al. [30].

Radioiodination of purified rat ovarian LH/CG receptor

The purified LH/CG receptor was dialysed against 0.1 M sodium phosphate, pH 7.0, and radioiodinated by the chloramine-T method [27] as described in [30]. Briefly, to 1 pmol of purified receptor (100 μl) 5 μl (0.5 mCi) of Na^{125}I and 5 μl of chloramine-T solution (10 mg/ml of 10 mM PBS, containing 140 mM NaCl, pH 7.4) were added. After 1 min of incubation at room temperature with constant shaking, 100 μl of sodium metabisulphite solution (1.2 mg/ml of PBS) was added. The radioiodinated receptor was separated from free ^{125}I by gel filtration on Sephadex G-50, using PBS containing 0.1 % (v/v) Triton X-100 as eluent, and resolved under non-reducing conditions by SDS/PAGE on a 7.5 % (w/v) polyacrylamide slab gel. The 85000–95000- M_r band was excised and the ^{125}I -labelled receptor was eluted electrophoretically using a Bio-Rad Model 422 electro-eluter. The elution buffer was 50 mM NH_4HCO_3 /0.1 % (w/v) SDS. The electro-eluted radioiodinated receptor was evaporated to dryness in aliquots, using a Savant SpeedVac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) and stored at -70°C .

Enzymic digestion of ^{125}I -labelled and native ovarian LH/CG receptors

The ^{125}I -labelled ovarian LH/CG receptor was treated with glycosidases by adding 5 μl of enzyme (final concentrations, unless otherwise indicated in the Figure legends: α -mannosidase, 2.5 units/ml; endo H, 20 munits/ml; neuraminidase, 10 munits/ml; endo- β -galactosidase, 20 munits/ml; O-glycosidase, 25 munits/ml; endo F₂, 4 munits/ml and PNGase F, 2 units/ml) to 50 μl of receptor solution (10000 c.p.m.) and the mixture was incubated for the indicated period of time at 30°C . The reaction was stopped by adding 50 μl of $2\times$ SDS sample buffer [2 % (w/v) SDS, 125 mM Tris/HCl (pH 6.8), 10 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol and 0.001 % (w/v) Bromophenol Blue] and

heating the tubes at 95°C for 2 min. The incubation buffer for neuraminidase and O-glycosidase treatments was 50 mM sodium phosphate, pH 6.0, 0.5 % (v/v) Triton X-100 and 0.2 mM phenylmethanesulphonyl fluoride (PMSF). The O-glycosidase buffer also contained 10 mM D-galactonic acid γ -lactone. The α -mannosidase buffer contained 50 mM sodium acetate (pH 4.5), 0.5 % (v/v) Triton X-100, 5 mM EDTA and 0.2 mM PMSF, and that of endo- β -galactosidase, 50 mM sodium acetate (pH 5.8), 0.5 % (v/v) Triton X-100, 1 mM EDTA and 0.02 % (w/v) BSA. The endo H and endo F₂ buffers contained 50 mM sodium acetate (pH 5.0), 0.5 % (v/v) Triton X-100, 0.1 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol, 50 mM EDTA, 0.2 % (w/v) BSA (endo H) and 0.2 mM PMSF and that of PNGase F, 50 mM sodium phosphate (pH 7.0), 0.5 % (v/v) Triton X-100, 0.1 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol, 50 mM EDTA, 0.2 mM PMSF and 10 mM 1,10-phenanthroline.

The purified, native LH/CG receptor was dialysed before PNGase F treatment against 0.1 mM sodium phosphate, pH 7.0/0.5 % (v/v) Triton X-100/5 mM EDTA/0.2 mM PMSF/10 mM 1,10-phenanthroline. To 300 μl of receptor (7.5 pmol), 15 μl (3 units) of PNGase F or buffer (control) was added, and the mixture was incubated at 30°C for 2 h. The hormone-binding characteristics of the control and PNGase F-treated receptors were determined as described previously [7], except that samples were incubated with the hormone for 20 h at 4°C . A sample containing 500 fmol of receptor was subjected to ligand or immunoblotting after stopping the enzyme reaction with an equal volume of SDS sample buffer lacking 2-mercaptoethanol. The rest was precipitated with trichloroacetic acid [final concentration of 10 % (v/v)]. Soybean trypsin inhibitor (100 $\mu\text{g}/\text{ml}$) was added as a carrier, and the pellet was washed twice with ice-cold ethanol/diethyl ether (1:1) and solubilized in SDS sample buffer lacking 2-mercaptoethanol. Samples containing 2.5 pmol of control or PNGase F-treated receptors and 1.5 pmol of untreated receptor were subjected to glycan detection and lectin blotting experiments respectively.

SDS/PAGE and electroblotting

SDS/PAGE was performed according to the method described by Laemmli [31], using 4 % (w/v) polyacrylamide stacking gels and 7.5 % (w/v) polyacrylamide separating gels. Relative molecular mass markers (SDS-6H, Sigma) detected by staining with Coomassie Brilliant Blue [32] or silver [33] were used to calibrate the gels. For ligand and lectin blotting analyses and for glycan detection, the proteins, resolved by SDS/PAGE under non-reducing conditions, were transferred electrophoretically from the gels on to nitrocellulose (Schering, Germany) (100 V, 60 min, Bio-Rad Mini-Trans Blot apparatus). The nitrocellulose-bound proteins were analysed by probing them with either ^{125}I -labelled hCG, a polyclonal anti-receptor serum [34] or digoxigenin-labelled lectins (Table 1), according to previously published procedures [30,36,37]. In glycan detection analysis, nitrocellulose-bound glycoproteins were detected by enzyme immunoassay after oxidation of adjacent groups in sugars, followed by covalent binding of spacer-linked digoxigenin, according to the method described by the manufacturer (Boehringer-Mannheim). The electroblotted M_r markers were detected by staining with Ponceau S [0.2 % (w/v) in 3 % (w/v) aqueous trichloroacetic acid] for 5 min. For the detection of radioactivity, the dried gels or nitrocellulose sheets were exposed at -70°C for 1–21 days, using Fuji X-ray film (Fuji Film, Japan) with an intensifying screen (Kodak X-Omatic, Eastman Kodak, Rochester, NY, U.S.A.). The relative quantities of the labelled bands on the autoradiograms were analysed by densitometric scanning using

an image-analysis processor M2 (Imaging Research, Brock University, Ontario, Canada).

Miscellaneous

Protein was assayed by the method of Bradford [38] with BSA as standard.

Figure 1 Radiolodination of purified rat ovarian LH/CG receptor

The LH/CG receptor from pseudo-pregnant rat ovaries was purified by one cycle of affinity chromatography on hCG-Affigel 10 and radiolodinated by the chloramine-T method as described in the Materials and methods section. Samples were subjected to SDS/PAGE [7.5% (w/v) polyacrylamide slab gel]. Lane 1, the silver-stained purified receptor; lane 2, the non-reduced crude ^{125}I -labelled receptor; lane 3, the reduced crude ^{125}I -labelled receptor; and lane 4, the reduced electro-eluted ^{125}I -labelled receptor. M_r markers are indicated on the right (from the top: myosin, β -galactosidase, phosphorylase b, BSA and egg albumin).

Figure 2 Treatment of ^{125}I -labelled rat ovarian LH/CG receptor with exo- and endo-glycosidases

The electro-eluted ^{125}I -labelled LH/CG receptor was treated with exo- and endo-glycosidases at 30 °C as described in the Materials and methods section. Lane 1, untreated control; lane 2, α -mannosidase (2.5 units/ml, 16 h); lane 3, endo H (20 munits/ml, 16 h); lane 4, neuraminidase (10 munits/ml, 1 h); lane 5, endo- β -galactosidase (20 munits/ml, 4 h); lane 6, endo F_2 (4 munits/ml, 16 h); lane 7, neuraminidase (10 munits/ml, 1 h) and O -glycosidase (25 munits/ml, 16 h); lane 8, O -glycosidase (25 munits/ml, 16 h). All reactions were stopped by adding 2 $\times$ SDS sample buffer, followed by electrophoresis on SDS/7.5% (w/v) polyacrylamide gels. The enzyme effects shown are maximal, as was revealed by changing the incubation times or the amounts of enzyme, and the results are representative of numerous similar experiments.

RESULTS

Characterization of the carbohydrates of rat ovarian LH/CG receptor using glycosidases

To study the structure of the carbohydrate chains of the rat ovarian LH/CG receptor using glycosidases, the receptor was first purified from pseudo-pregnant ovaries by one-step affinity chromatography on hCG-Affigel 10. Visualization of the polypeptides after SDS/PAGE by silver staining (Figure 1, lane 1) or autoradiography of the radiolabelled receptor (Figure 1, lane 2) revealed the presence of a major 90000- M_r polypeptide under non-reducing conditions and minor bands in the 30000–60000- M_r range. Reduction of the polypeptides by 2% (v/v) 2-mercaptoethanol retarded the electrophoretic mobility of the 90000- M_r receptor by about 2000 (Figure 1, lane 3), indicating that it contains internal disulphide bonds. The iodinated LH/CG receptor was electro-eluted from the SDS/polyacrylamide gel (Figure 1, lane 4), and used for enzymic deglycosylation experiments, because of the minor 30000–60000- M_r bands, which might have complicated interpretation of the results. Identification of the 90000- M_r protein as the hormone-binding unit of the receptor was carried out by transferring the purified receptor preparation to nitrocellulose after SDS/PAGE, followed by incubation with ^{125}I -hCG (ligand blotting) and autoradiography (see Figure 4, lanes 3 and 5).

The types of oligosaccharide present on the LH/CG receptor were determined by subjecting the electro-eluted ^{125}I -labelled receptor to exo- and endo-glycosidase treatments as described in the Materials and methods section. After the enzyme treatments, changes in the mobility and number of ^{125}I -labelled bands were examined on SDS/polyacrylamide gels.

To determine whether the LH/CG receptor contains mannose-rich or hybrid-type N-linked oligosaccharides, it was subjected to α -mannosidase and endo H treatments (Figure 2, lanes 2 and 3). The former enzyme can remove terminal α -linked mannose residues [39], whereas the latter is able to digest mannose-rich and hybrid-type N-linked oligosaccharides [40]. Treatment with α -mannosidase (2.5 units/ml) or endo H (20 munits/ml) for 16 h failed to decrease the M_r value of the 92000- M_r receptor, whereas under similar conditions they were fully able to digest carboxypeptidase Y, as revealed by silver staining of the SDS/PAGE-resolved protein (results not shown). These findings indicate that the receptor contains only complex-type N-linked glycans.

To characterize the N-linked complex-type oligosaccharides, the ^{125}I -labelled receptor was first subjected to neuraminidase treatment (Figure 2, lane 4). The *Vibrio cholerae* enzyme (10 munits/ml for 1 h) decreased the apparent M_r value of the receptor to about 81000, indicating the presence of sialic acid linked either α 2-3 or α 2-6 to galactose [41]. The ^{125}I -labelled receptor was also examined for the presence of poly(*N*-acetyl-lactosamine) sequences by subjecting it to endo- β -galactosidase (Figure 2, lane 5), an enzyme known to cleave repeating Gal β 1-4GlcNAc sequences [42]. Indeed, treatment with this enzyme at a concentration of 20 munits/ml for 4 h decreased the M_r of the receptor to about 86000.

The presence of O-linked oligosaccharides was evaluated by subjecting the ^{125}I -labelled LH/CG receptor to O -glycosidase treatment, both before and after sialic acid removal (Figure 2, lanes 7 and 8). This enzyme can remove unsubstituted O-linked Gal β 1-3GalNAc disaccharides [43]. Treatment of the receptor with O -glycosidase (25 munits/ml) for up to 16 h, or sequential treatment with neuraminidase and O -glycosidase, were not able to increase the electrophoretic mobility of the receptor. Chemical O-linked deglycosylation with sodium hydroxide (0.1 M) and

Figure 3 PNGase F treatment of ^{125}I -labelled rat ovarian LH/CG receptor

The electro-eluted ^{125}I -labelled LH/CG receptor was incubated: (a) with (lanes 2–7) and without (lanes 1 and 8) PNGase F (2 units/ml) for the indicated period of time at 30 °C, or (b) with (lanes 2–8) and without (lanes 1 and 9) the indicated amount of PNGase F for 2 h at 30 °C. Reactions were stopped by adding 2 × SDS sample buffer, followed by electrophoresis on SDS/7.5% (w/v) polyacrylamide gels, and autoradiography. The results are representative of three similar experiments.

sodium borohydride (0.4 M) for 2–24 h at room temperature, a treatment known to decompose O-glycosidic bonds via β -elimination [44], also failed to support the existence of O-linked glycans associated with the receptor (results not shown).

In our previous report [7], it was shown that PNGase F, an enzyme that is capable of removing all types of complex-type N-linked oligosaccharides via hydrolysis of the *N*-acetylglucosaminyl-asparagine bond [45], increased the electrophoretic mobility of the rat ovarian membrane-bound LH/CG receptor in two phases. In the present report, however, time-course experiments showed that incubation of the ^{125}I -labelled receptor with PNGase F formed a 62000- M_r product via 1–3 ill-defined intermediates (Figure 3a). As dose-response experiments also resulted in several intermediates in addition to the final 62000- M_r product (Figure 3b), it is apparent that the LH/CG receptor contains more than two carbohydrate chains. Interestingly, endo F_2 , an enzyme that cleaves biantennary complex-type chains [46], was also able to decrease the M_r of the ^{125}I -labelled receptor to about 87000 (Figure 2, lane 6), suggesting that one of the oligosaccharide chains is biantennary, the rest containing three or more antennae.

The complete absence of oligosaccharides on the 62000- M_r

Figure 4 Deglycosylation of purified rat ovarian LH/CG receptor with PNGase F

The affinity-purified LH/CG receptor (25 pmol/ml) was treated with (lanes 2, 4, 6 and 8) and without (lanes 1, 3, 5 and 7) PNGase F (10 units/ml) for 2 h at 30 °C and transferred electrophoretically from SDS/polyacrylamide gels on to nitrocellulose as described in the Materials and methods section. The nitrocellulose-bound proteins were probed using a glycan detection method (lanes 1 and 2), with ^{125}I -hCG, in the absence (lanes 3 and 4) and presence (lanes 5 and 6) of an excess of unlabelled hCG, or with a polyclonal anti-receptor serum (lanes 7 and 8). The 158000- M_r band in lanes 3 and 7 may represent a dimeric form of the LH/CG receptor.

receptor polypeptide was confirmed by subjecting the electroblotted receptor to a chemical glycan detection reaction (Figure 4, lanes 1 and 2). In this method, provided by Boehringer-Mannheim, adjacent hydroxyl groups in the carbohydrate chains are oxidized to aldehyde groups by mild periodate treatment and digoxigenin is then covalently attached to these aldehydes. The labelled glycoproteins are subsequently detected in enzyme immunoassay using a digoxigenin-specific antibody conjugated to alkaline phosphatase. Although the 62000- M_r LH/CG receptor species was not detected by this method (Figure 4, lane 2), it was still recognized by receptor antibodies (Figure 4, lane 8) and it was able to bind ^{125}I -hCG (Figure 4, lane 4). Scatchard plots [47] of the equilibrium binding data revealed that both the binding affinity and the number of binding sites were unaltered after PNGase F treatment. Analysis of three independent experiments showed that the K_d values were 0.12 ± 0.02 nM and 0.18 ± 0.06 nM and the B_{max} values were 367 ± 89 pmol/mg of protein and 349 ± 107 pmol/mg of protein for the control and enzyme-treated receptor preparations respectively. In contrast, densitometric scanning of the autoradiograms showed that ^{125}I -hCG binding to the electroblotted receptor was reduced by about 60% after deglycosylation (Figure 4, lane 4).

Characterization of the carbohydrates of rat ovarian LH/CG receptor using digoxigenin-labelled lectins

To verify the findings obtained with exo- and endo-glycosidases and to delineate further the structure of the carbohydrate chains of the rat ovarian LH/CG receptor, purified trichloroacetic acid-precipitated receptor was transferred to nitrocellulose after SDS/PAGE and probed with various digoxigenin-labelled lectins (Table 1). The bound lectins were detected by using an anti-digoxigenin antibody conjugated to alkaline phosphatase.

Of the nine lectins tested, six were able to recognize the non-reduced 90000- M_r ovarian receptor (Figure 5). Neither *Gallanthus nivalis* lectin (GNA) nor *Canavalia ensiformis* lectin (Con A) was able to bind to the receptor, indicating that it does not contain mannose-rich or hybrid-type N-linked oligosaccharides. The low affinity of Con A towards biantennary complex-type chains [48] may explain its inability to bind to the receptor,

Figure 5 Probing of purified rat ovarian LH/CG receptor with digoxigenin-labelled lectins

The affinity-purified LH/CG receptor (15 pmol) was precipitated with 10% trichloroacetic acid and the precipitated proteins were transferred electrophoretically from SDS/polyacrylamide gels on to nitrocellulose as described in the Materials and methods section. The nitrocellulose sheet was cut to strips, which were then probed with the indicated digoxigenin-labelled lectins. The bound lectins were detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase.

although the sensitivity of the receptor to endo F_2 (see Figure 2, lane 6) indicates the presence of biantennary oligosaccharides. Binding of lectins from *Maackia amurensis* (MAA), *Sambucus nigra* (SNA) and *Aleuria aurantia* (AAA) to the receptor indicates that the complex-type N-linked oligosaccharides contain sialic acid linked either α 2-3 or α 2-6 to galactose and fucose linked α 1-6 to the proximal N-acetylglucosamine. However, not all of the antennae of the N-linked chains terminate in sialic acid, as *Datura stramonium* lectin (DSA) and *Ricinus communis* lectin (RCA-120) were able to bind to the receptor. These lectins recognize terminal galactose residues linked β 1-4 to N-acetylglucosamine [23,24]. The strong positive reaction obtained with DSA is consistent with the susceptibility of the ^{125}I -labelled receptor to endo- β -galactosidase (see Figure 2, lane 5). DSA is known to bind to unbranched [22,23] and branched [49] poly(*N*-acetyl-lactosamine) sequences. On the other hand, *Phaseolus vulgaris* lectin (L-PHA) also showed a strong positive reaction, although peripheral poly(*N*-acetyl-lactosamine) sequences are known to abolish interaction with this lectin [25]. This discrepancy can be explained by the presence of α 2-3-linked sialic acid, which significantly enhances interaction of oligosaccharides with L-PHA [25]. Finally, *Arachis hypogaea* lectin (PNA) was not able to recognize the receptor, not even when the sialic acids were removed with neuraminidase (results not shown). This indicates that the LH/CG receptor has no Gal β 1-3GalNAc-containing oligosaccharides [26] which are typical of O-linked carbohydrates.

DISCUSSION

This investigation has provided information concerning previously unrecognized structural features of the carbohydrate units on the LH/CG receptor. The results indicate that the rat ovarian LH/CG receptor has more than two complex-type N-linked carbohydrate chains, both biantennary and multi-antennary. Fucose is linked α 1-6 to the proximal N-acetylglucosamine and the antennae contain either terminal unsubstituted galactose or sialic acid linked α 2-3 and α 2-6 to galactose. Some of the antennae also contain poly(*N*-acetyl-lactosamine) sequences. Moreover, the LH/CG receptor does not appear to contain O-linked carbohydrate moieties.

There are only a few examples of receptors that are known to contain O-linked carbohydrates, namely, receptors for low-density lipoprotein [50], interleukin-2 [51], von Willebrand factor and thrombin (platelet glycoprotein 1b; [52]), platelet-derived growth factor [53] and insulin receptor [54]. The LH/CG receptor does not appear to be an exception. The absence of O-linked oligosaccharides was demonstrated by several independent methods. O-Glycosidase was not able to digest the receptor, either before or after sialic acid removal, which is consistent with previous findings [8,9,11]. Similarly, PNA did not recognize the receptor on nitrocellulose, either before or after neuraminidase treatment. However, given the rather extreme site-specific cleavage requirements of O-glycosidase [43] and the binding specificity of PNA [26], these negative results must be interpreted with caution. Nevertheless, the resistance of the ^{125}I -labelled receptor to mild alkaline and reducing agents, conditions which decompose O-glycosidic bonds via β -elimination, supported the conclusion that all of the carbohydrate moieties on the receptor are linked to asparagine residues. In addition, the absence of carbohydrates on the PNGase F-treated 62000- M_r receptor species was shown by a glycan detection method.

The structure of the rat LH/CG receptor polypeptide, deduced from its cDNA sequence, indicates that the extracellular domain of the receptor contains six potential N-glycosylation sites [3]. The present results, and those we published earlier [7], suggest that at least two of these sites are used. In our previous report, PNGase F-treatment increased the electrophoretic mobility of the membrane-bound rat ovarian LH/CG receptor in two clear phases, but in the present report, the M_r value of the reduced 92000- M_r ^{125}I -labelled receptor was decreased to about 62000 via one to three intermediates. Thus, more carbohydrate chains appear to be present than we previously thought.

All the N-linked oligosaccharide units on the LH/CG receptor appear to be of the complex type. This was demonstrated directly by the susceptibility of the entire receptor population to neuraminidase and to endo- β -galactosidase. On the other hand, the inability of α -mannosidase and endo H to digest the ^{125}I -labelled receptor, and that of GNA and Con A to recognize the purified receptor, provides indirect proof. The complex-type chains appear to carry varying numbers of antennae. The susceptibility of the ^{125}I -labelled receptor to endo F_2 indicates the presence of biantennary chains, but because the action of this enzyme did not increase the electrophoretic mobility of the receptor to the same extent as that of PNGase F some of the chains contain either three or more antennae. This is in contrast with the results reported by Minegishi et al. [11]. These investigators concluded that both the rat ovarian and testicular LH/CG receptors contain only biantennary complex-type oligosaccharides, based on the finding that the ^{32}P -labelled receptors were susceptible to endo-glycosidase F. This discrepancy can be explained by the presence of various enzyme activities in the earlier commercially available endoglycosidase F preparations [55,56].

The marked susceptibility of the ^{125}I -labelled LH/CG receptor to endo- β -galactosidase revealed that it contains repeating Gal β 1-4GlcNAc groups. These elongated carbohydrate chains are also present on mannose 6-phosphate [57], insulin [58] and epidermal-growth-factor [59] receptors, and, interestingly, those on the latter of these have been observed to be carriers of blood-group antigens. It is also interesting to note that all of these receptors are integral membrane proteins with a luminal/extracytoplasmic N-terminus carrying the ligand-binding site, analogously to the LH/CG receptor. The occurrence of poly(*N*-acetyl-lactosamine)-type chains on the LH/CG receptor is likely to account for the strong affinity of the receptor for wheat germ agglutinin, a property that has been used successfully in receptor

purification [60]. It has been shown that N-linked glycopeptides containing *N*-acetyl-lactosamine-repeating units bind to this lectin with a higher affinity than do conventional sialylated complex-type carbohydrates [61].

Removal of oligosaccharides from the native membrane-bound LH/CG receptor does not impair its ability to bind hormone [7,8] or to activate adenylate cyclase [13]. Similarly, the present results indicate that the deglycosylated receptor is able to bind ¹²⁵I-hCG, even when the oligosaccharides were removed after solubilization. Equilibrium binding data revealed that both the binding affinity and the maximum binding capacity were unaltered after PNGase F treatment. However, hormone binding to the nitrocellulose-bound receptor was reduced by about 60% after deglycosylation. It has been suggested that ligand blotting is based on the spontaneous renaturation of receptor molecules electroblotted on to nitrocellulose [62]. It is therefore conceivable that this renaturation after SDS/PAGE is impaired to some extent if the oligosaccharides are removed from the solubilized receptor. An alternative possibility is that the deglycosylated receptor does not retain its hormone-binding conformation during SDS/PAGE as efficiently as the intact receptor, but this seems unlikely.

The question of the exact sites to which the N-linked chains are attached on the LH/CG receptor will have to await more detailed glycosylation-site analysis, and the precise composition of the carbohydrate units will require direct isolation and characterization of these units. Further work is also required to determine whether these carbohydrate units are necessary for functional activation, for intracellular stability and for transport of the receptor to the cell surface.

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