Functional Glycosylation Sites of the Rat Luteinizing Hormone Receptor Required for Ligand Binding*

Ran Zhang, Huiqing Cai, Naheed Fatima, Ellen Buczko, and Maria L. Dufau‡

From the Section on Molecular Endocrinology, Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892-4510

The contribution of N-linked glycosylation to the ligand binding activity of the rat luteinizing hormone receptor (LHR) was studied in wild-type and mutant LHR expressed in mammalian (COS1) cells and overexpressed in insect (SF9) cells. The binding affinities of the holoreceptor and its truncated splice variant (form B) lacking the transmembrane domain were equivalent in both cell lines. Tunicamycin-treated transfected SF9 cells expressed a carbohydrate-free LH receptor that lacked hormone binding activity. Functional carbohydrate chains essential for binding activity were localized to glycosylation sites at Asn-173 and Asn-152. Glycosidase treatment of the double mutant N173Q/N152Q revealed the presence of at least one additional carbohydrate chain at Asn-269, Asn-277, or Asn-291 that does not contribute to hormone binding. Asn-77 was not glycosylated, but its mutation to Gln reduced hormone binding. LHR expressed in insect cells contained only high mannose carbohydrate chains, and those located at Asn-173 and Asn-152 were sufficient for high-affinity hormone binding. Enzymatic cleavage of glycosyl chains indicated that only the proximal N-acetylglucosamine residue, which is common to high mannose and complex carbohydrate forms, is necessary for acquisition of the high affinity conformation of the receptor. The carbohydrate chains of the LHR appear to be involved in intramolecular folding of the nascent receptor rather than in its interaction with the hormone.

The LHR is a glycoprotein present in the cell membrane of gonadal cells, with six potential N-linked glycosylation sites (Fig. 1) and N-linked carbohydrate (CHO) chains of the complex type (1–3). A functional role for N-linked carbohydrates in a high affinity hormone binding has not yet been established, and is subject to some controversy. Conflicting reports on the effects of deglycosylation (2, 4) and site-directed mutagenesis (5, 6) of the mature holoreceptor on hormone binding activity may be a function of the original receptor state. N-Linked carbohydrate chains of the rat ovarian LH receptor have previously been shown to be essential for high affinity hormone binding by a number of different methods, including site-directed mutagenesis of the Asn of the putative glycosylation sites (5), tunicamycin treatment (7), and enzymatic deglycosylation of solubilized receptors (2). These procedures have not resolved the question of whether the reported carbohydrate requirement of the receptor is based on a direct interaction with the hormone or is related to the conformation of the ligand binding site.

In order to study the importance of post-translational glycosylation on LHR binding activity, we have evaluated both the membrane-bound LHR holoreceptor (form A) (3) and its high-affinity hormone-binding splice variant, form B, which lacks the transmembrane and cytoplasmic domains (8). These receptors and their mutated forms were expressed in Baculovirus-infected insect cells and in the mammalian COS-1 cells. It is noteworthy that glycoproteins in insect cells have been shown to carry N-linked carbohydrates of the high mannose type that are trimmed to shorter core structures (Man₃GlcNAc₂) (9). In contrast, the mammalian cell, which has been shown to express high-affinity LHR (8), carries a full complement of trimming and complex glycosidases in its post-translational machinery (2). The present experiments demonstrate a post-translational glycosylation requirement that is satisfied by high mannose carbohydrate chains. Previous studies have shown that mutation of only three of the six Asn of the LHR putative glycosylation sites (Asn-77, Asn-152, and Asn-173) reduced hormone binding activity, and this could not be attributed to changes in LHR transport to the cell surface (5). In this study, we demonstrate by direct substitution of Asn and Ser/Thr of these putative glycosylation sites that only Asn-152 and Asn-173 carry carbohydrate chains that are required for hormone binding activity. In addition, the importance of the proximal N-acetylglucosamine of the carbohydrate chain for the high affinity conformation of the LHR was demonstrated.

MATERIALS AND METHODS

Design of LHR Form A and B Wild-type and Mutant DNA Constructs

The full-length rat ovarian LH/CG receptor (LHR form A) cDNA and the alternate splice variant form B cDNA (lacking the first 266 bp of exon 11) were subcloned into the human cytomegalovirus promoter-driven expression vector, pCMV4 (a gift from Dr. D. Russell, University of Texas Southern Medical Center) as described previously (8). LHR mutants were constructed using the recombinant circle polymerase chain reaction (10). Synthetic oligonucleotides (Midland Certified Reagent Co., Midland, Texas) were designed to replace Asn-77, Asn-152, or Asn-173 with Gln and Ser-79, Ser-154, or Thr-175 with Ala. WT and mutant form A cDNAs were subcloned into the SmaI site of baculovirus transfer vector pVL1392, and form B was subcloned into the EcoRI site of pVL1393 (Pharmlingen, San Diego, CA), for infection of insect cells. The orientation of the WT and mutant form A and form B constructs was determined by restriction enzyme analysis, and mutations were verified by DNA sequencing.

Expression of WT and Mutant LHR in Mammalian COS1 and Insect Cells

WT and mutant LHR cDNAs in pCMV4 expression vectors were transfected in mammalian COS1 cells by the lipofectAMINE (LifeTech-
pressed membrane-bound receptor activity was determined by 125I-hCG
labeling and binding assay (3). The recombinant virus titers were
amplified by reinfection of Sf9 cells with recombinant virus (108 plaque-
forming units/mg of enzyme protein). The reaction mixture was
incubated from 3 to 24 h at 37 or 22°C. Samples were aliquoted for
immunoblots (25 μg) or ligand blots (100 μg).

Preparation of Detergent-solubilized Extracts of Transfected COS1 Cells and Sf9 Cells

COS1 Cells—48 h after transfection with WT or mutant LHR cDNAs, COS1 cells were harvested, homogenized, and solubilized as described previously (8). Protein concentration was determined by BCA protein assay (Pierce).

Sf9 Cells—48 h after transfection with WT or mutant LHR cDNAs, Sf9 cells were harvested in 50 strokes at 1800 rpm of a tissue grinder (Potter-Elvehjem with PTFE pestle, Thomas Scientific, Swedesboro, NJ); glycerol and Nonidet P-40 were added to final concentrations of 20 and 1%, respectively. Extract was rotated for 1 h at 4°C, and diluted to a final Nonidet P-40 concentration of 0.1%. The solubilized mixture was centrifuged, and protein concentration was determined as described above.

Preparation of Detergent-solubilized Extracts of Transfected COS1 Cells and Sf9 Cells

COS1 Cells—48 h after transfection with WT or mutant LHR cDNAs, COS1 cells were harvested, homogenized, and solubilized as described previously (8). Protein concentration was determined by BCA protein assay (Pierce).

Sf9 Cells—48 h after transfection with WT or mutant LHR cDNAs, Sf9 cells were homogenized in 50 strokes at 1800 rpm of a tissue grinder (Potter-Elvehjem with PTFE pestle, Thomas Scientific, Swedesboro, NJ); glycerol and Nonidet P-40 were added to final concentrations of 20 and 1%, respectively. Extract was rotated for 1 h at 4°C, and diluted to a final Nonidet P-40 concentration of 0.1%. The solubilized mixture was centrifuged, and protein concentration was determined as described above.

125I-hCG binding to LHR

Intact COS1 cells or solubilized extracts from transfected COS1 or insect Sf9 cells were used for 125I-hCG binding assay as described previously (8). Binding was performed using displacement assays by incubating intact cells or solubilized extracts with 125I-hCG and increasing concentration of unlabeled hCG. Nonspecific binding was determined in samples containing an excess of the unlabeled hCG (1 μg). The binding parameters (Kd and binding capacities [Q]) were analyzed from Scatchard plots and saturation curves using a nonlinear model curve fitting program (12). All experiments were performed at least 3 times in triplicate. Statistical analysis for all studies were performed using analysis of variance (Supernova, Berkeley, CA) and Past Hoc Fisher LSD tests at the 99% significance level.

Enzymatic Deglycosylation of WT and Mutant LHR Receptor Using Endo H, Endo F, and N-Glycanase

Enzymes

Endo-β-N-acetylglucosaminidase H (Endo H (EC 3.2.1.96), 40 units/mg of enzyme protein), peptide-N-glycosidase F (N-glycanase (EC 3.5.1.52), 25,000 units/mg of enzyme protein), and Endo-β-N-acetylglucosaminidase (Endo F (EC 3.2.1.96), 1400 units/mg of enzyme protein) were obtained from Boehringer-Mannheim.

Reaction conditions

Reducing, Denatured Conditions—Cell extracts (125 μg of protein/10 μl) of Sf9 cells transfected with WT and mutant LHR receptor cDNAs were suspended in 10 μl of buffer (Endo H, 100 mM sodium acetate, pH 5.5; Endo F, 150 mM sodium phosphate, pH 7.4; N-glycanase, 200 mM sodium phosphate, 10 mM 1,10-phenanthroline hydrox ydate, pH 8, 6) containing 1% SDS, 0.2 mM β-mercaptoethanol and boiled for 5 min. Subsequently, 20 μl of buffer containing Nonidet P-40 (1.25% for N-glycanase, and 1.5% for Endo F) was added with 20 μl of enzyme (200 units of Endo H, 1 unit of Endo F, 4 units of N-glycanase). The reaction mixture was incubated from 3 to 24 h at 37 or 22°C. Samples were aliquoted for immunoblots (25 μg) or ligand blots (100 μg).

Nonreducing, Nondenaturing Conditions—Reaction mixture and incubation were same as above without SDS or β-mercaptoethanol in the buffer, and samples were not boiled prior to digestion.

Western Blot Analysis of Wild-type and Mutant LH/CG Receptor

25 μg of protein from detergent-solubilized Sf9 cells transfected with WT and mutant LHR form B cDNAs or from enzyme deglycosylation reactions were boiled for 5 min in sample buffer (final concentration, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, 0.02% bromphenol blue) and subjected to 10% SDS-PAGE electrophoresis analysis under reducing conditions. Following transfer to nitrocellulose membrane (Bio-Rad), the LH/CG receptor was probed with a rabbit monoclonal antibody against the N-terminus of the rat LH/CG receptor (1:1000 dilution) for 1 h at 22°C. The membranes were washed and developed with the enhanced chemiluminescence detection kit (ECL, Amersham Corp.). Negative controls are Sf9 cells transfected with pVL1393 vector without LH/CG receptor form B cDNA.

Ligand Blot

100 μg of protein from cell extract or enzymatic digestion were added to sample buffer, electrophoresed on 10% SDS-PAGE, and transferred electrophoretically to nitrocellulose. Binding of hCG to WT and mutant receptor was performed by incubation of the blots with 125I-hCG (106 cpm/ml) for 16 h at 4°C in the absence or presence of excess unlabeled hCG (1 μg/ml). The blots were processed and autoradiographed as described previously (13).
Functional Glycosylation Sites in the LH Receptor

RESULTS

LHR Expression from Mammalian COS1 and Insect Cell Lines—Transfection of the membrane-bound LH holoreceptor (form A) into either the mammalian COS1 or the baculovirus-infected insect Sf9 cell line results in the expression of high affinity receptors. Scatchard analysis of the wild-type receptors expressed in COS1 cells (8) and in insect cells revealed that their gonadotropin binding affinities (K_a) were equivalent in both cell lines but somewhat lower than that observed in particular ovarian membrane fractions (Table I) (15). Hormone binding activity was detectable on the surface of the intact COS1 cell, while in the insect cell, LH holoreceptor binding activity was only recovered following detergent solubilization (Table I). Overexpression of the form A LHR was achieved in the insect cell where wild-type form A LHR was expressed in the insect cell at levels of 129-fold greater than that observed in the transfected COS1 cell and 30-fold greater than that found in ovarian membranes (Fig. 2).

The wild-type LHR soluble splice variant form B (Ref. 8, Fig. 1) that is overexpressed in insect cells at levels of 107 ± 3 pmol/mg of protein, shows similar high hCG hormone binding affinities when expressed from insect or COS1 (8) cells (Table I). Form B LHR expressed in insect cells was utilized in a number of subsequent experiments as a simplified prototype of the high affinity LHR form A hormone binding domain.

Tunicamycin Treatment of Wild-type LHR form B—To investigate the role of N-linked glycosylation in the synthesis of the high affinity LHR, form B-infected insect cells expressing the spliced variant were treated with tunicamycin, which prevents synthesis of dolichyl N-acetylgalcosamine pyrophosphate, the initial step in N-linked glycosylation in the endoplasmic reticulum. In western immunoblots, anti-LHR(36–51) antibody-specifically recognized the nonglycosylated LHR expressed from tunicamycin-treated cells (Fig. 3, left, lanes a and b). The nonglycosylated form B LHR exhibits a reduction in molecular weight of approximately 6 kDa in comparison with the wild-type LHR of 37.8 ± 0.9 kDa (n = 10) (Fig. 3, left, lanes b and c). This difference corresponds to the absence of N-linked carbohydrate chains. Ligand blots showing the binding of labeled hCG to the form B LHR reveal that the tunicamycin-treated receptor does not bind labeled hCG (Fig. 3, right, lane b), whereas the wild-type form B LHR does bind the labeled hormone (Fig. 3, right, lane a), and this binding is abolished in the presence of excess cold hCG (Fig. 3, right, lane d). Thus, ligand and Western blots show that tunicamycin treatment abolishes LHR hormone binding, without reducing LHR protein levels (Fig. 3, left, lanes b and c). Neither tunicamycin treatment, nor the absence of N-linked carbohydrates affected steady state levels of the receptor molecule in comparison with untreated controls. This experiment demonstrates that synthesis of the high affinity hormone binding domain in the LH receptor requires the post-translational addition of N-linked carbohydrate chains.

Identification of Glycosylation Sites on the LHR—Initial studies indicated that three of the six potential glycosylation sites on the LHR are of functional importance to hormone binding activity (Asn-77, Asn-152, and Asn-173) (13) (Fig. 1 and Ref. 5). The relevance of these reductions in binding activity to the elimination of carbohydrate chains at these glycosylation positions was investigated by mutation of the Asn or Ser/Thr of the consensus site Asn-Xaa-Ser/Thr, and SDS-gel electrophoresis. Mutation of either the Asn or Ser/Thr position should result in similar changes in hormone binding activity if the CHO chain is the only factor in binding activity (see below). Substitution of either Asn-173 to Gln or Thr-175 to Ala in the form A holoreceptor abolished hormone binding activity when measured from the surface of intact COS1 cells (Fig. 4, left). This loss of activity could not be attributed to deviations in transport to the cell surface since the same effect was noted in soluble fractions prepared from COS1 cells (Fig. 4, right). Similarly, in the insect cell, the detergent extracted form A and form B LHR mutants Asn-173 → Gln and Thr-175 → Ala exhibited no detectable hormone binding activity (Fig. 5). To assess whether the loss of hormone binding activity of Asn-173 → Gln and Thr-175 → Ala was related to defects in protein synthesis, radiolmmunoassay analysis of expressed wild-type and mutant LHR protein was performed, and protein levels of the Asn-173 → Gln and Thr-175 → Ala mutants were not significantly different from wild-type. Thus, loss of binding activity of the mutant LHRs was solely attributed to defects in hormone binding rather than reduced protein levels (Table II).

The equivalent changes in hormone binding activity between the Asn-173 and Thr-175 mutant form B LHRs indicate that the deviation in hormone binding activity could be related to the absence of a carbohydrate chain. Immunoblots of the mutant Asn-173 → Gln or Thr-175 → Ala show equivalent reductions in apparent molecular mass of about 2 kDa from the wild-type molecular mass (37.8 ± 0.9 kDa (n = 10) to 35.5 ± 0.3 kDa (n = 7)), indicating that position 173 is glycosylated (Fig. 6, left, lanes 1, 4, and 5). Ligand blots of the Asn-173 → Gln and Thr-175 → Ala mutant LHRs confirm that these receptors cannot bind hCG (Fig. 6, right, A, lanes 2 and 3). These studies demonstrate that the glycosylation position at Asn-173 is of major importance to hormone binding activity in both the mammalian and insect cell, and serves a basic hormone binding function that does not involve transport or protein stability.
Since tunicamycin treatment reduced the molecular mass of the form B LHR expressed from the insect cell by approximately 6 kDa, and mutation of Asn-173 decreased the molecular weight of form B LHR by only 2 kDa, additional glycosylation positions that carry carbohydrate chains on the form B LHR were predicted to exist. Our previous studies showed that mutation of the putative glycosylation position at Asn-152 significantly reduced hormone binding activity close to nonspecific levels (5). Immunoblots of the form B Asn-152→Gln and Ser-154→Ala LHR mutant proteins showed similar reductions in molecular mass of approximately 2 kDa in comparison with the wild-type form B LHR of 38 kDa, indicating that the glycosylation position at Asn-152 also carries a carbohydrate chain (Fig. 6, left, lanes 1, 2, and 3). Mutation of the Asn-152 in the form B LHR, expressed from insect cells, resulted in a total loss of hormone binding activity (Table II), but mutation of Ser-154→Ala exhibited only a 60% reduction in hormone binding activity (Table II). These results were verified in ligand blots where the Asn-152→Gln mutant exhibited no hCG binding (Fig. 6B, lane 2), whereas, the Ser-154→Ala mutant exhibited reduced but visible binding (Fig. 6B, lane 3). Scatchard analyses and radioimmunossay of mutant and WT receptors indicate that the reduction of S154A binding activity is due to an increase in the number of inactive nonbinding recep-

**Table I**

Binding affinities ($K_a = 10^9 M^{-1}$) of rat LH receptor expressed in COS1 and insect cells and LH receptor from the rat ovary

| LHR species | COS1 cells | Insect cells | Ovary |
|-------------|------------|--------------|-------|
|             | Surface $^a$ | Soluble $^a$ |        | Surface $^a$ | Soluble $^a$ |        |
| Form A      | 3.84 ± 0.69 | 2.27 ± 0.25  | ND$^d$ | 2.18 ± 0.28  | 3.57 (8)    | 13.0 |
| Form B      | ND         | 3.57 (8)    |        | ND$^d$       | 2.74 ± 0.33 | 2.5  |

$^a$ hCG binding to surface of intact cells.

$^b$ Soluble, detergent-solubilized particulate fraction (COS1), whole cell homogenates (insect), detergent solubilized ovarian membrane (15).

$^c$ hCG binding to particulate ovarian preparations (15) mean ± S.E.

$^d$ ND, not detectable.
carbohydrate chains at other glycosylation positions.

Carbohydrate Composition of Essential Carbohydrate Chains—Localization of the carbohydrate requirement to Asn-173, and Asn-152 in the LHR expressed from either mammalian COS1 or the insect cell suggests that a specific complex carbohydrate composition in the mammalian cell may not be essential, since the insect cell lacks the complex glycosidases of the mammalian cell (9). To determine whether the LHR expressed from the insect cell carries only high mannose chains, the receptor was treated with the glycosidase Endo H that cleaves between the two core N-acetylgalactosamines of a high mannose chain, and the reduction in molecular weight was compared with LHR digestion by N-glycanase that cleaves both high mannose and complex carbohydrate chains at the amino acid Asn. The Endo H- and N-glycanase-digested LHR migrated to identical positions on an SDS gel (31.5 ± 0.4 kDa n = 9, 31.5 ± 0.5 kDa, n = 6, respectively) when visualized by antibody in an immunoblot (Fig. 7, top panel). The contribution of the proximal N-acetylgalactosamine that remains after Endo H treatment was not visible on an SDS gel. The wild-type high affinity receptor expressed from the insect cell is glycosylated only with high mannose chains.

Effect of Enzymatic Deglycosylation on Hormone Binding of Form B—To determine if the high mannose carbohydrate chains of the receptor expressed from the insect cell functions exclusively in the initial folding of the nascent receptor, or also in the renaturation of the detergent solubilized processed receptor, we studied whether or not the deglycosylated soluble receptor was able to renature to an active hormone binding configuration following SDS-gel electrophoresis in ligand blots (Fig. 8). Treatment of the wild-type form B LHR with Endo H under SDS denaturing and reducing conditions (see "Materials and Methods"), followed by SDS-gel electrophoresis and 125I-labeled hCG ligand blotting, resulted in a decrease in the receptor molecular mass of 6 kDa and renaturation of the LHR to a high affinity binding conformation (Fig. 8). Similar results were obtained with Endo F, which cleaves at the same position as Endo H, giving a molecular mass of 31.6 ± 0.2 (n = 4) under reducing, denaturing, or nonreducing, nonrenaturing conditions (Fig. 8).

Scheme 1 indicates that mannose residues are not required for renaturation of the mature denatured receptor and that any high mannose function occurs prior to the formation of the processed receptor. In contrast, N-glycanase treatment completely abolished ligand binding, although the receptor was visualized by antibody, and deglycosylated to 32 kDa (Fig. 8, top panel, bottom panel lane 3). These experiments indicate that the proximal N-acetylgalactosamine linked to Asn, which is retained on the Endo H- or Endo F-treated LHR but not the N-glycanase-treated LHR, is essential for the renaturation of the high affinity hormone binding receptor following electrophoresis on an SDS-denaturing gel.

**DISCUSSION**

These studies have demonstrated that N-linked carbohydrate chains, specifically those attached to the two glycosylation sites Asn-173 and Asn-152, are essential for the assembly of a high affinity hormone binding site within the extracellular domain of the LH receptor. Mutation of either Asn-173 or Thr-175 within the glycosylation consensus sequence Asn-Xaa-Thr in the form A or form B LHR proteins abolished binding activity (Table II). This loss of activity was correlated with the loss of a carbohydrate chain in immunoblots in which the mutant LHR proteins showed equivalent reductions in molecular mass of approximately 2 kDa (Fig. 6). The carbohydrate chain at Asn-173 is essential for binding activity, but it is not the only carbohydrate chain required for production of the high affinity form of the receptor.
The present study is of particular relevance because other researchers have described retention of hormone binding activity with the Thr-175 → Ala mutant protein (6), and attributed the loss of activity by Asn-173 → Gln LHR to the substitution of Asn-173 for Gln, rather than the loss of a carbohydrate chain. However, in our experiments, no hormone binding activity was observed after tunicamycin treatment, where the expressed LHR retained the native amino acids Asn-173 and Asn-152 without the carbohydrate chains (Fig. 3), confirming the importance of the N-linked carbohydrate chain rather than the Asn-173 for LHR activity.

Our studies demonstrate that high mannose carbohydrate chains are sufficient for the formation of the high affinity hormone binding domain of the form B LHR expressed from insect cells. In contrast, the carbohydrate chains of the high affinity rat ovarian LHR are not high mannose but are rather of the complex type (2). This suggests that either carbohydrate residues common to both the high mannose and complex chains are central to the formation of the hormone binding domain, or that carbohydrate function occurs in the mammalian cell prior to processing of the high mannose chains to the complex form, as has been reported with the mannose 6-phosphate receptor (17).

Monoglucose high mannose chains have been implicated in folding of nascent proteins (18) as well as with the chaperone protein calnexin interaction (19) prior to entry into the Golgi. However, the ability of the denatured mature LHR to refold spontaneously during electrophoretic transfer to nitrocellulose in ligand blots (Ref. 2, Fig. 6) indicates that chaperone proteins and processing enzymes in the endoplasmic reticulum are not necessary for the formation of the hormone binding domain. The high mannose (Fig. 6) or complex carbohydrates (2) of the mature LHR are equally capable of refolding the receptor protein. Total elimination of the N-linked CHO chain by mutation of the Asn-173 glycosylation site, tunicamycin treatment, or N-glycanase treatment yields mature receptors that exhibit no hormone binding activity and are incapable of renaturation following electrophoresis (Figs. 3, 6, and 8). In the purified mature ovarian LHR, renaturation was prevented by deglycosylation with N-glycanase in one study (2), although other studies with the membrane-bound and partially purified LHR indicate that deglycosylation of the mammalian LHR does not impair hormone binding (4, 20). None of these studies performed the deglycosylation reaction on the SDS-denatured and reduced LH receptor, suggesting that the discrepancy may have arisen from incomplete deglycosylation. This was also suggested by reported differences in the molecular masses of the deglycosylated receptor (59 versus 62 kDa, Refs. 2 and 20).

In other studies, ligand blotting of nonglycosylated receptor expressed from Escherichia coli showed only hormone bound to high molecular weight aggregates, and none bound to the monomeric form of the truncated LHR (1–294) under nonreducing conditions (21). In the present investigation, wild-type form B LHR expressed from insect cells was capable of ligand binding to electroblotted receptor under reducing (Fig. 8) as well as nonreducing conditions (Figs. 6 and 8). N-Glycanase treatment of the SDS-denatured and reduced high mannose form B LHR yielded a nonbinding receptor (Fig. 8). Denaturation and reduction per se did not impair the refolding process, since Endo H treatment of the denatured LHR did not abolish hormone binding activity.

| Glycosidase | Glycanas | 
|-------------|---------|
| PNGase F     | N-Glyc | 
| Endo H      | N-Glyc | 
| Endo F      | N-Glyc |

**Scheme 1.** Glycosidase specificity of generic high mannose carbohydrate chain. x, y, mannose residues; z, fucose or H; ‾, a cleavage site (16). PNGase F = N-glycanase.
binding (Fig. 8). These experiments suggest an important role for the proximal N-acetylglucosamine residue attached to the Asn of the glycosylation site that is present in both complex and high mannose chains, in refolding of the mature receptor and perhaps folding of the nascent receptor. This was confirmed with Endo F-treated receptor even under non-denaturing/non-reducing conditions. These experiments do not preclude the possibility that mannose residues are important in the initial processing of LHR expressed from the insect or mammalian cell.

In summary, our studies indicate that putative glycosylation positions Asn-77, Asn-152, and Asn-173 are relevant to specific functions that lead to the formation of an active hormone binding domain. Of these, only Asn-152 and Asn-173 carry carbohydrate chains, and the nonglycosylated Asn-152 and Asn-173 LHR expressed from tunicamycin-treated cells exhibits no hormone binding activity (Fig. 3). The Asn-173/Thr-175 glycosyl chain is essential for acquisition of hormone binding activity. We have established that complex carbohydrate chains are not required for hormone binding in the insect cell and that any carbohydrate function may permit folding of the nascent protein to a high affinity conformation. In addition, the contribution of the proximal N-acetylglucosamine to renaturation of the high affinity binding site of the LHR was clearly established by selective cleavage of the carbohydrate chain.

REFERENCES
1. Kusuda, S., and Dufau, M. L. (1988) J. Biol. Chem. 263, 3046–3049
2. Minegishi, T., Delgado, C., and Dufau, M. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1470–1474
3. McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosenblit, N., Nikolici, K., Segaloff, D. L., and Seeburg, P. H. (1989) Science 245, 494–499
4. Keinanen, C. P. (1988) Biochem. J. 256, 719–724
5. Zhang, R., Tsai-Morris, C. H., Kitamura, M., Buczko, E., and Dufau, M. L. (1991) Biochem. Biophys. Res. Commun. 181, 804–808
6. Liu, X., Davis, D., and Segaloff, D. L. (1993) J. Biol. Chem. 268, 1513–1516
7. J. I., Slaughter, R. G., and Ji, T. H. (1990) Endocrinology 127, 494–496
8. Tsai-Morris, C. H., Buczko, E., Wang, W., and Dufau, M. L. (1990) J. Biol. Chem. 265, 19385–19388
9. Kuroda, K., Geyer, H., Geyer, R., Doerfler, W., and Klein, H. (1990) Virology 174, 418–429
10. Jones, D. H., and Howard, B. H. (1990) BioTechniques 8, 178–183
11. Catt, K. J., Ketelslegers, J. M., and Dufau, M. L. (1976) in Methods in Receptor Research (Blecher, M., ed) Vol. I, pp. 175–250, Marcel Dekker, NY
12. Ketelslegers, J. M., Knott, G. D., and Catt, K. J. (1975) Biochemistry 14, 3075–3083
13. Minegishi, T., Kusuda, S., and Dufau, M. L. (1987) J. Biol. Chem. 262, 17138–17143
14. Koh, Y. C., Buczko, E., and Dufau, M. L. (1993) J. Biol. Chem. 268, 16267–16271
15. Kusuda, S., and Dufau, M. L. (1986) J. Biol. Chem. 261, 16161–16168
16. Tarentino, A. L., and Plummer, T. H., J. r. (1994) Methods Enzymol. 230, 44–56
17. Hillie, A., Waheed, A., and von Figura, K. (1990) J. Cell Biol. 110, 963–972
18. Danielson, E. M. (1992) Biochemistry 31, 2266–2272
19. Hammond, C., Braakman, I., and Helenius, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 913–917
20. Petaja-Repo, U. E. (1994) Biochem. J. 298, 361–366
21. Chen W., and Bahl, O. P. (1993) Mol. Cell. Endocrinol. 91, 35–41