Mutants of Human Choriogonadotropin Lacking *N*-Glycosyl Chains in the α-Subunit. 1. Mechanism for the Differential Action of the N-Linked Carbohydrates†

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ABSTRACT: Analogs of human choriogonadotropin (hCG) lacking *N*-glycosyl chains at α52Asn and α78Asn were purified from the culture media of insect cells by immunoaffinity chromatography using a monoclonal antibody column. As previously reported, while analogs lacking carbohydrate at α52Asn and α78Asn had similar receptor binding activities compared with the wild type recombinant hCG (hCGwt), they differed in their signal transduction properties. The mutant lacking carbohydrate at α78Asn had 20% less cAMP-stimulating activity than hCGwt, but the absence of glycosylation at α52Asn resulted in the reduction of cAMP accumulation by 90−95%. A similar effect of the mutations was observed on the stimulation of steroidogenesis. Circular dichroism spectra of the two mutants showed significant differences. The mutant lacking carbohydrate at α52Asn had a much higher negative mean residue ellipticity (MRE) at 200 nm and a lower negative MRE at 220 nm than that lacking carbohydrate at α78Asn and hCGwt. The dissociation rates of the α52Asn and α78Asn carbohydrate deficient mutants at pH 3 and room temperature, measured by using 1-anilino-8-naphthalenesulfonate, were 9.4×10^{-5} and 3.8×10^{-5} s^{-1}, respectively, as compared with 1.5×10^{-5} s^{-1} for hCGwt. The results of both CD measurements and dissociation studies strongly suggest that the absence of carbohydrate at α52Asn results in conformational changes in the mutant which might explain the loss in its signal transduction function. This is further supported by indirect evidence from two other lines of experimentation. Unlike the mutant lacking carbohydrate at α78Asn, the one lacking carbohydrate at α52Asn cross-reacted with the two subunit specific monoclonal antibodies, anti-hCGα and anti-hCG*β*, which normally did not cross-react with the native or the hCGwt. Also, polyclonal anti-hCG*β* but not anti-hCGα was able to restore the cAMP-producing activity of the α52Asn carbohydrate deficient mutant. From all the data taken together, it appears that the loss of second messenger-producing activity of hCG with the absence of the glycosyl chain at α52Asn was probably due to a conformational change in the heterodimer rather than due to the loss of the α52Asn−carbohydrate−receptor interaction.

Human choriogonadotropin (hCG)[1] belongs to a family of heterodimeric glycoprotein hormones including lutropin (LH), follitropin (FSH), and thyrotropin (TSH) which are comprised of two noncovalently bonded subunits designated α and *β* (Pierce & Parsons, 1981). Their biological functions are mediated by G-protein-linked receptors involving cAMP and IP_3 as second messengers (Davis et al., 1986, 1987; Gudermann et al., 1992; Gupta et al., 1995). The common α-subunit is shared by all the hormones and is postulated to be primarily responsible for signal transduction, while the *β*-subunit is responsible for specificity (Pierce & Parsons, 1981). Both subunits in the heterodimeric form are necessary for their biological function. hCG has four N-linked carbohydrates, two in the α-subunit at Asn residues 52 and 78 and two in the *β*-subunit at Asn residues 13 and 30. In addition, the *β*-subunit has four O-linked carbohydrate chains at Ser residues 121, 128, 132, and 138 (Kessler et al., 1979a,b; Endo et al., 1979). The N-linked carbohydrates have been shown, by their removal chemically with HF or trifluoromethanesulfonic acid (TFMS) (Manjunath & Sairam, 1982; Sojar & Bahl, 1987; Kalyan & Bahl, 1983) or with enzymes including endo- and exoglycosidases (Moyle et al., 1975; Thotakura & Bahl, 1987), to be involved in signal transduction. These studies also led to the finding that the carbohydrate in the α-subunit played a dominant role in the signal transduction of hCG (Sairam & Bhargavi, 1985). Using site-directed mutagenesis, it was further shown that the carbohydrate chain at the 52nd position was more critical in signal transduction than the one at the 78th position (Matzuk et al., 1989; Matzuk & Boime, 1988). While the loss of the former resulted in the loss of signal transduction by the hormone, the loss of the latter had no significant effect (Matzuk et al., 1989; Matzuk & Boime, 1988). The receptor binding in either mutant remained unaltered. The molecular mechanism underlying the differential action of the two N-linked carbohydrates is not clearly understood. In this work, we have prepared two carbohydrate mutants of hCGα

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; hCG, human choriogonadotropin; hCGwt, wild type recombinant hCG; hCGα52Gln*β*wt and hCGα80Gln*β*wt, recombinant hCG mutants lacking carbohydrate at α52Asn or α78Asn, respectively; LH, FSH, and TSH, lutropin, follitropin, and thyrotropin, respectively; MOI, multiplicity of infection; PI, postinfection; RIA, radioimmunoassay; RRA, radioreceptor assay.

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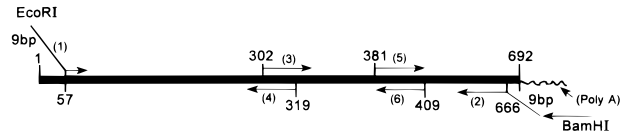
lacking *N*-glycosyl chains at α52Asn and α78Asn by site-directed mutagenesis and have coexpressed them with the wild type hCG*β* in a baculovirus expression system (Chen & Bahl, 1991a). The mutants were purified in adequate amounts by immunoaffinity chromatography and were studied by circular dichroism. Their dissociation kinetics at low pH using a fluorometric reagent, 1-anilino-8-naphthalenesulfonate (ANS), and receptor binding and signal transduction properties were also studied. The mutant lacking carbohydrate at α52Asn showed significant differences in the circular dichroism spectrum and in its dissociation kinetics at low pH with respect to those of the mutant lacking carbohydrate at α78Asn and the wild type hCG. Highly specific monoclonal antibodies against α- and *β*-subunits which did not react significantly with the hCGwt were found to cross-react with hCGα52Gln*β*wt, while the mutant hCGα80Gln*β*wt lacking glycosylation at α78Asn like hCGwt showed no cross-reactivity with any of the antibodies. Polyclonal antibodies against hCG*β* but not hCGα were able to restore the loss of signal transduction activity of the hCG analog lacking carbohydrate at position 52 in the α-subunit in mouse Leydig cells. All these observations strongly suggest that the loss of biological activity of the heterodimer with mutation at α52Asn to Gln was due to a conformational change in the heterodimer caused by the loss of the carbohydrate. Thus, it appears that the function of the carbohydrate chain at α52Asn is the maintenance of the conformational integrity of the heterodimer rather than direct participation in the receptor binding.

MATERIALS AND METHODS

Cells. Sf9 insect cells, a clonal isolate of *Spodoptera frugiperda* obtained from ATCC, were used as the host for the Baculovirus Autographa Californica nuclear polyhedrosis virus (AcMNPV). The cells were cultured (Summers & Smith, 1988) at 27 °C in Grace's medium (Gibco, pH 6.2) supplemented with 3.3 g/L lactalbumin hydrolysate and 3.3 g/L yeastolate (Difco). The Grace's complete medium contained 10% fetal bovine serum (FBS), 50 units/mL penicillin, 100 *μ*g/mL streptomycin, 2.5 *μ*g/mL fungizone, and 0.1% surfactant, pluronic F-68 (complete). High-Five cells were obtained from Invitrogen and were grown in serum free medium (Ex-Cell 405, JRH Biosciences). MA-10 Leydig cells were kindly provided by M. Ascoli.

Antibodies. All monoclonal and polyclonal antibodies used in this study were prepared in this laboratory (Bahl et al., 1976; Thotakura & Bahl, 1985a,b). The monoclonal anti-hCG*β* antibody, B17, had broad specificity reacting with both hCG*β* and hCG. After its purification on DEAE Affi-Gel Blue (Thotakura & Bahl, 1985a), it was conjugated to Affi-Gel 10 (Pierce Chemical Co.) according to the manufacturer's directions, and the resulting adsorbent was used for the purification of hCG and hCG analogs. The monoclonal anti-hCG*β* antibody, B158, was highly specific for the *β*-subunit and did not cross-react significantly with intact hCG (Thotakura & Bahl, 1985b). It was purified in the same manner as B17 and was utilized for the preparation of immunoaffinity adsorbent for further purification of hCG and hCG analogs from the contaminating hCG*β* subunit. The monoclonal anti-hCGα antibody, A2D4, was highly specific for hCGα and had negligible cross-reactivity with native hCG (Thotakura & Bahl, 1985a). The polyclonal antibodies against hCG, hCGα, and hCG*β* were used for all radioimmunoassays and Western immunoblotting. The latter two were also employed in signal transduction studies.

Scheme 1[a]



[a] The numbers in parentheses represent the primer numbers as described in the text, and the other numbers represent the number of the nucleotide in the hCGα cDNA sequence.

Mutagenization and Construction of the Transfection Vectors. cDNA for hCGα from BeWo cells cloned in pGEM-3Z (Shen & Bahl, 1990) was used for mutagenization by two sequential PCRs using Ultma DNA Polymerase (Perkin-Elmer). For each mutation, a set of forward 5′ end and reverse 3′ end primers was synthesized for the first PCR step. Similarly, for the second PCR step, a set of gene specific primers containing *Eco*RI and *Bam*HI restriction sites were synthesized for directional cloning as shown in Scheme 1. The primers used were synthesized by the Oligonucleotide Facility at the State University of New York at Buffalo and are as follows: (1, forward) CGC GGA ATT CTG CAA AAA GCC CAG AGA AAG GAG CGC CAT GGA TTA CTA CAG, (2, reverse) CGC GGA TCC GCA GTG GAA CAA GCT, (3, forward) GTC CAA AAG CAA GTC ACC TCA GAG TCC, (4, reverse) GGT GAC TTG CTT TTG GAC CAA CAT CGT C, (5, forward) TGG AGA ACC ACC AAG CGT GCC ACT GCA GTA, and (6, reverse) GCA GTG GCA CGC TTG GTG GTT CTC CAC TTT G. The products of the second PCR, the mutated DNA fragments, were cloned at the unique *Eco*RI−*Bam*HI sites in the polylinker of the PVL-1392 expression vector for the insect cells (Shao et al., 1996). In this vector, the insert is under the control of the baculovirus polyhedrin gene promoter. These constructs were sequenced to confirm the desired mutation and also to check for any unwanted mutation as a consequence of PCR.

Transfection and Selection of the Recombinant Virus. The recombinant plasmids containing the mutated hCGα cDNAs were cotransfected with the linearized wild type AcMNPV DNA using the MaxBac Baculovirus Expression System Kit (Invitrogen). The transfection was mediated by cationic liposomes according to the directions provided in the manufacturer's manual. After 3 days of incubation, the medium containing the recombinant virus was collected and plated on 1% low-melting agarose (Sigma). The isolated recombinant plaques were transferred to wells containing 2.5×10^5 Sf9 cells per milliliter of Grace's complete medium per well in a 24-well plate. After 3 days, the medium was tested for the expression of hCGα by RIA using [^{125}I]hCGα and anti-hCGα polyclonal antibody. The positive samples were incubated for an additional 3−4 days until all cells were lysed. This virus stock (P1 stock) was used for the preparation of high-titer virus by infecting 2×10^6 Sf9 cells in a 25 cm^2 flask with 0.5 mL of P1 stock. The flask was incubated until all cells were lysed, and 2% of this P2 virus stock was used as the inoculum for obtaining large quantities of the recombinant virus by infecting 2×10^6 Sf9 cells/mL in Spinner flasks.

Expression and Purification of the Recombinant Proteins. Insect High-Five cells were used to express the recombinant

hCG analogs by coinfection of each of the hCGα mutants with the wild type hCG*β* virus. The MOI of the α-subunit was kept around 5 and that of hCG*β* around 4. Coinfection was carried out in Spinner flasks in serum free medium (JRH), and the cells were incubated for 60 h at 27 °C. The cell viability at the time of harvest was approximately 70−80% as determined by trypan blue exclusion. The infected cells were cooled on ice following the addition of protease inhibitors phenylmethanesulfonyl fluoride (PMSF) at 1 mM and *N*-ethylmaleiamide (NEM) at 5 mM. Sodium azide at a final concentration of 0.02% was also added to protect against any microbial growth during purification. Cells were pelleted at 4 °C by centrifugation for 10 min at 4000 rpm using a GSA rotor in a Sorvall Centrifuge. The supernatant was recentrifuged at 100000*g* in a Beckmann ultracentrifuge for 30 min. The supernatant was concentrated to 100−200 mL by ultrafiltration using a PM 10 membrane (Amicon) with an exclusion limit of 10 000. The concentrated medium was then applied to the immunoaffinity B17 column (1 × 12 cm) at a flow rate of 10 mL/h with a peristaltic pump. The medium was passed twice through the column for maximal recovery. The column was washed with 50 mM Tris-HCl buffer at pH 8.0 with an approximately 10× bed volume or until the absorbance at 280 nm of the effluent was <0.001. The bound protein was eluted with 50 mM glycine hydrochloride (pH 2.4) containing 140 mM NaCl. Five 7.5 mL fractions were collected in tubes containing 1 M Tris-HCl at pH 8.5 so that the pH of the effluent was immediately brought to 7.5. Fractions 2−4 were pooled and were further purified by passing through the hCG*β* specific monoclonal antibody (B158) column (1 × 3 cm).

Analytical Techniques. SDS−PAGE and Western Blotting. The purity of the hCG analogs was checked by SDS−PAGE at 4 °C under nonreducing conditions using the Laemmli (1970) discontinuous gel system with a 5% stacking gel at pH 6.8 and a 12% running gel at pH 8.8. The bands were visualized by silver staining (Sambrook et al., 1989). For immunoblotting, the protein bands were transferred to a nitrocellulose membrane using a semidry trans-blot apparatus (Bio-Rad) for 20 min at 15 V, and the blotting was carried out in Tris-HCl-buffered saline (pH 7.5) containing 5% nonfat dry milk. Anti-hCG was used at a 1:10000 dilution, and the bands were visualized by using alkaline phosphatase-linked goat anti-rabbit IgG (Sambrook et al., 1989).

Radioimmuno- (RIA) and Radioligand Receptor Assays (RRA). Antigens were radio-iodinated to a specific activity of 30 mCi/mg with the chloramine T method (Pandian & Bahl, 1977). For RIA and RRA, approximately 30 000 cpm were used per tube. RIA were performed using ^{125}I-labeled hCG, hCG*β*, or hCGα and a polyclonal anti-hCG 1:100000 dilution and anti-hCG*β* or anti-hCGα antibodies and a 1:50000 dilution (Bahl et al., 1976). All solutions for the RIA were prepared in phosphate-buffered saline (PBS) containing 0.1% BSA. Incubation was carried out at 4 °C overnight with shaking in 0.3 mL using different concentrations of unlabeled antigens, corresponding diluted antibodies, and approximately 35 000 cpm (approximately 0.3 ng) of ^{125}I-labeled antigen. The amount of [^{125}I]antigen−antibody complex was determined by precipitation with 10% polyethylene glycol (PEG 8000, Sigma) in the presence of 0.08% *γ*-globulin (Sigma) as a carrier at 4 °C for 30 min. The mixture was centrifuged, and after the supernatant was aspirated, the pellets were counted to obtain the amount of the bound ^{125}I-labeled ligand. When monoclonal anti-hCGα antibody, A2D4, or anti-hCG*β* antibody, B158, was used in the RIA, the primary antigen−antibody complex was further treated with a second antibody, goat anti-mouse IgG at a 1:1000 dilution, before precipitation with *γ*-globulin and PEG as described above. The binding activity of hCG and hCG analogs was examined by RRA using [^{125}I]hCG and crude rat ovarian membrane LH/CG receptor prepared from superovulated ovaries (Sojar & Bahl, 1989). The incubation mixture (300 *μ*L) contained the unlabeled hCG, the crude membrane receptor, and 35 000 cpm of [^{125}I]hCG. The amount of receptor preparation used was that which precipitated approximately 30% of the total [^{125}I]hCG and was predetermined for each batch of the membrane receptor suspension. The [^{125}I]ligand−receptor complex was precipitated as described above for RIA.

Determination of cAMP and Progesterone. The MA-10 cell line of cultured Leydig tumor cells was used for these assays (Ascoli, 1981). The cells were maintained in Waymouth's medium (Gibco) containing 10% fetal bovine serum, 20 mM HEPES, 50 units/mL penicillin, and 100 *μ*g/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C (Thotakura & Bahl, 1985a). The cells were plated at 5 × 10^5 cells per 50 mm plate and were allowed to grow for 2 days. After 2 days, the medium was changed, and on the third day, the cells were washed three times with Waymouth medium containing 0.1% BSA (medium A). One milliliter of a solution with various concentrations of hCGwt or its analogs was added to each plate, and the plates were incubated for 1 h in the presence of 0.5 mM isobutylmethylxanthine (IBMX, Sigma). To determine the effect of anti-hCGα and anti-hCG*β* on the induction of cAMP by hCGwt or its mutants, 1 mL samples containing 100 ng/mL hCGwt or hCG analogs were added (Chen & Bahl, 1991b) to Leydig cells (5 × 10^5 cells per 50 mm plate). After incubation for 30 min at 37 °C, the medium containing hCGwt or hCG analogs was aspirated and the cells were washed with medium A to remove any unbound hormone. Then, 1 mL of anti-hCGα or anti-hCG*β* antibodies diluted in medium A containing 1 mM IBMX was added to each plate. The plates were incubated on ice for 3 h and were then transferred to a 37 °C incubator for 30 min. For the determination of cAMP, the cells were washed three times with ice cold PBS and were lysed on the plate with 0.5 mL of 0.1 N HCl containing 5 mM EDTA and 1 mM IBMX (lysis solution). The estimation of cAMP was carried out by cAMP binding assay using [^{3}H]cAMP and cAMP binding protein provided in the assay kit (Amersham). For the progesterone determination, the cells were grown on plates as described for the cAMP assay, and after treatment with various concentrations of hCG (Chen & Bahl, 1991b), they were incubated for 4 h at 37 °C. The medium was collected and briefly centrifuged, and 100 *μ*L of the supernatant was used for the progesterone estimation by RIA using [^{125}I]progesterone and anti-progesterone antibodies supplied in the assay kit (ICN).

Circular Dichroism of hCG Mutants. CD measurements were made on a JASCO 500 spectropolarimeter. The instrument was calibrated with 0.06% (w/v) camphorsulfonic acid-d_{10}. Experiments were performed at room temperature using a quartz cuvette with a path length of 1 mm. The final concentration of the samples was 0.4 mg/mL in 25 mM phosphate buffer at pH 7.5. The baseline spectrum obtained

under identical conditions using a buffer blank was subtracted from the sample spectra. To improve the signal-to-noise ratio, an average of four scans was taken to plot the spectra. The error bars are shown to represent the noise level in the lower-wavelength range due to the absorption of carbohydrate functional groups.

Effect of pH on the Dissociation Kinetics of hCGα52Glnβwt and hCGα80Glnβwt. ANS was purchased from Molecular Probes Inc. and was used without further purification. The fluorescence measurements were performed on an SLM AMINCO 8000 series spectrofluoremeter. The data were acquired in the slow kinetics mode, with 400 and 460 nm as the excitation and emission wavelengths, respectively. The excitation and emission slits were 4 nm. The stock solution (1 mg/mL) of the probe was prepared in 25 mM phosphate at pH 7.5, and 4 μL was added to 1.5 mL of the protein solution. The binding of the probe to the protein was ensured by incubating a 150 μg/mL protein solution with 4 μL of the probe at pH 7.4. The dissociation was initiated by lowering the pH to 3 by the addition of 0.5 M glycine buffer at pH 2.4. The curve fitting was done using the curve fit options in Cricket Graph. The fluorescence intensities of the ANS bound to the proteins taken in the first 24 s were normalized for the comparison of the spectrum.

RESULTS

Expression and Purification of hCG Mutants. To express the hCG analogs lacking the glycosyl chain at α52Asn or α78Asn in the α-subunit, the mutated hCGα cDNAs were cloned in the expression vector pVL-1392 for the insect cells. The mutated fragment was cloned in an orientation which allowed the insert to be under the control of the strong polyhedrin promoter. The scheme for mutagenization of the common α-subunit of the glycoprotein hormones (Scheme 1) and the construction of the transfer vectors and the primers used are described in detail in Materials and Methods. Although N-glycosylation could be blocked at both sites individually by changing either the Asn or the Thr to Gln of the recognition triplet sequence, Asn-X-Thr/Ser, in this study, we chose to block glycosylation by mutating α52sn to Gln or α80Thr to Gln rather than α54Thr or α78Asn to Gln because the expression levels were higher in the mutants in which α52Asn and α80Thr were replaced with Gln. The two mutants thus prepared lacking glycosylation at either α52Asn or α78Asn are designated hCGα52Glnβwt and hCGα80Glnβwt, respectively.

The recombinant viruses containing the hCGα cDNAs were generated by the transfection of insect cells with transfer vectors containing wild type or mutated hCGα cDNA and AcMNPV linear DNA using cationic liposomes. Five to ten plaques from each construct were screened for the expression of hCGα mutants by RIA using [^{125}I]hCGα and anti-hCGα antibodies and by Western immunoblotting. The recombinant viruses giving the highest expression levels of hCGα mutants were subsequently used for the expression of the heterodimers. The High-Five cells were coinfected with the recombinant viruses and wild type hCGβ virus at an MOI of about 5. The expression levels of the mutants ranged between 5 and 6 μg/mL, compared with 20 μg/mL for the wild type. Hormone concentrations were determined by RIA using [^{125}I]hCG and anti-hCG with hCGwt as the standard.

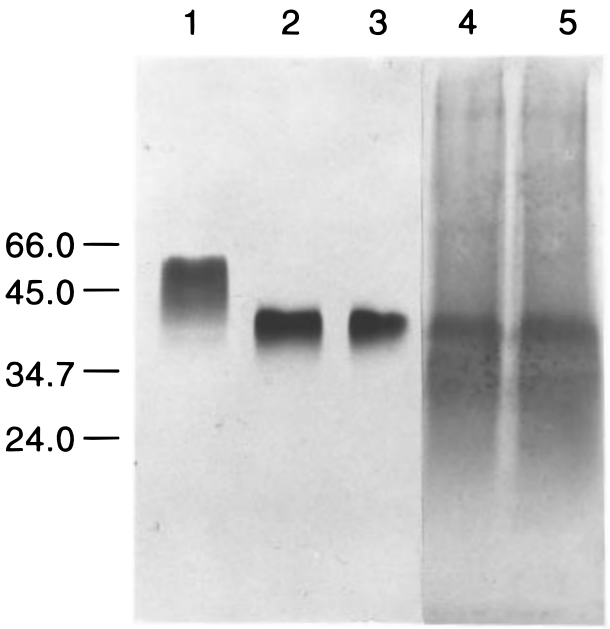


FIGURE 1: SDS−PAGE and immunoblotting of the purified hCG analogs under nonreducing conditions. Electrophoresis was performed in a 12% polyacrylamide gel at 4 °C. Immunoblots were obtained after transferring the protein bands to a nitrocellulose membrane. The protein bands were visualized by using rabbit anti-hCG and goat anti-rabbit IgG-conjugated alkaline phosphatase (lanes 1−3, silver staining; and lanes 4 and 5, immunoblotting): lane 1, hCGwt; lanes 2 and 4, hCGα52Glnβwt; and lanes 3 and 5, hCGα80Glnβwt.

The dose−response curves of the mutants were all parallel to the standard curve (data not shown), indicating the identity of the epitopes in the mutants and the wild type hCG. The viability of the cells 24 h PI was >95% and dropped to 70% at 60 h PI as measured by trypan blue. All of the mutant was found to be secreted in the medium when measured 24 h PI, at which time the viability of the cells was almost complete.

For the purification of the two hCG mutants, 500−1500 mL cultures of High-Five cells were used. About 60 h PI, the medium was collected and processed as described above. hCGwt and its two analogs were purified in a single step by immunoaffinity chromatography on an anti-hCGβ column using a monoclonal antibody (B17) against hCGβ (Thotakura & Bahl, 1985b). Any contamination with the β-subunit was removed by immunoaffinity chromatography on subunit specific monoclonal anti-hCGβ (B158). All hCG analogs were examined for homogeneity and molecular size by SDS−PAGE at 4 °C under nonreducing conditions. The bands were visualized by silver staining and by Western immunoblotting. Under nonreducing conditions, only a single predominant band was observed on SDS−PAGE in both mutants with molecular masses of about 43 kDa, compared with 45 kDa for the wild type hCG (Figure 1). The dissociation of hCGwt or the mutants into subunits under low-temperature conditions was undetectable.

Receptor Binding and Signal Transduction Properties of the hCG Mutants Lacking Carbohydrates at α52Asn and α78Asn. The receptor binding activity of the mutants was tested by the inhibition of binding of [^{125}I]hCG to the insoluble rat ovarian membrane receptor. A comparison of hCGwt with its mutant analogs, produced in the insect cells, showed that the absence of N-linked carbohydrates at position 52 or 78 resulted in a slight increase (5−10%) in

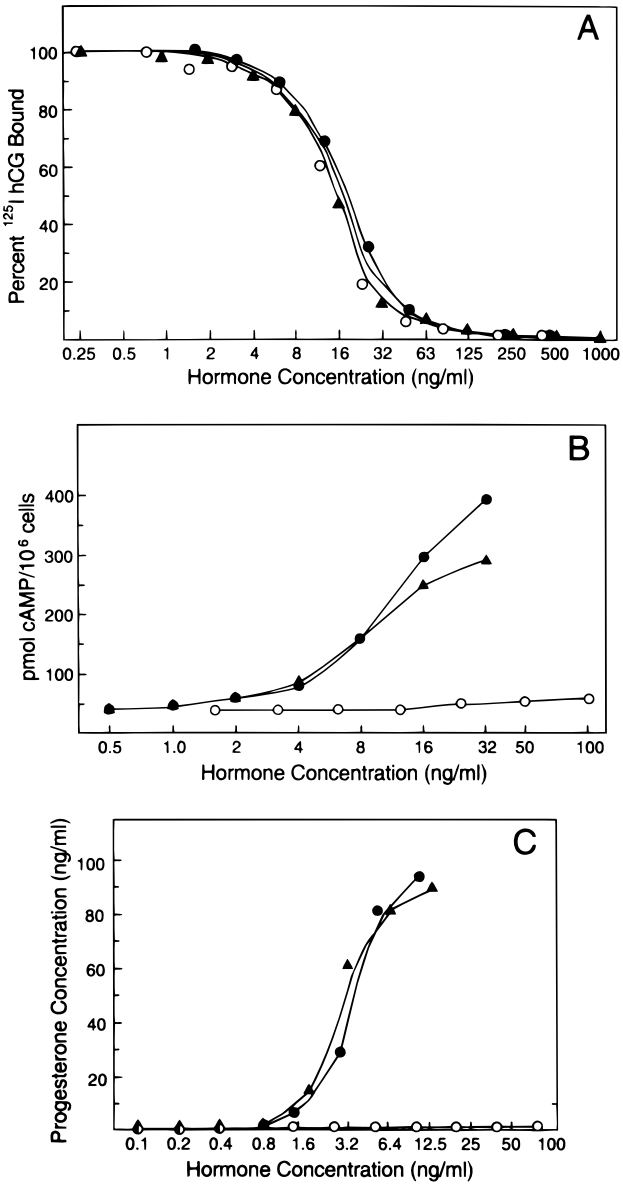


FIGURE 2: Biological properties of the hCG analogs. (A) Radioligand receptor assay of the hCGwt and its analogs. Binding activity was measured by the competition of binding of [^{125}I]hCG to the crude rat ovarian membrane receptor preparation with hCG analogs: hCGwt (●), hCGα52Gln*β*wt (○), and hCGα80Gln*β*wt (▲). (B) Stimulation of cAMP by hCG analogs in mouse Leydig MA-10 cells. The cells were incubated with various concentrations of hCGwt and its analogs at 37 °C for 1 h, and the cAMP produced was estimated by cAMP binding assay using [^{3}H]cAMP and cAMP binding protein: hCGwt (●), hCGα52Gln*β*wt (○), and hCGα80Gln*β*wt (▲). (C) Steroidogenesis of the hCGwt and its analogs in Leydig MA-10 cells. The cells were exposed to various concentrations of hCGwt and its analogs. The production of progesterone was measured in the medium by radioimmunoassay using [^{125}I]-progesterone and anti-progesterone antibodies: hCGwt (●), hCGα52Gln*β*wt (○), and hCGα80Gln*β*wt (▲). The fluctuation within and between experiments in the case of binding and progesterone determinations was about 10% or below, while that of the cAMP determinations was about 10−15%.

the binding of the mutant hormones to the LH/hCG receptor relative to hCGwt (Figure 2A).

The cAMP- and progesterone-stimulating properties of the carbohydrate analogs of hCG were measured by estimating the amounts of cAMP and progesterone accumulated in the MA-10 Leydig cells as described in Materials and Methods. The results of cAMP determinations are presented in Figure

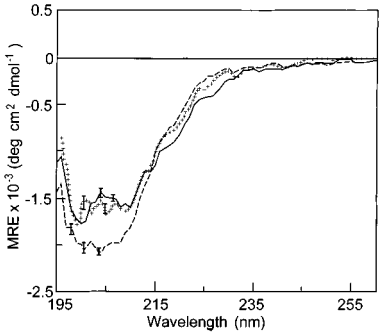


FIGURE 3: CD spectra of hCG and its mutants in 25 mM phosphate buffer at pH 7.5. The CD spectra were acquired in the range of 260−195 nm. The path length of the quartz cuvette used was 1 mm. The concentration of the protein used was typically in the range of 0.4 mg/mL. The bars in the figure represent the noise level due to the contribution of the carbohydrate absorption in the lower-wavelength range. The qualitative estimation of the secondary structure, ordered to random coil (aperiodic structure), was performed using the ellipticity values at 220 and 200 nm: hCGwt (+++), hCGα52Gln*β*wt (---), and hCGα80Gln*β*wt (−).

2B. The dose−response curves of the hCG analogs showed variation when compared to that of hCGwt. The absence of glycosylation at α78Asn affected by mutation of either α78Asn to Gln (data not shown) or α80Thr to Gln resulted in the reduction of total cAMP accumulation by 20−30%. However, the loss of glycosylation at position α52Asn had a much more drastic effect on the cAMP accumulation. The activity of hCGα52Gln*β*wt was reduced by 90−95%. A similar effect was observed in the case of hCGα54Gln*β*wt (data not shown).

The dose−response curves (Figure 2C) of the two hCG mutants showed considerable variation in their steroidogenic activities when they were compared to that of hCGwt. The differences were also observed between the residues Asn or Thr mutated for the same glycosylation site. Although changing α80Thr to Gln caused slight activation (Figure 2C), mutating the α78Asn to Gln showed an approximate 10% decrease in steroidogenesis (data not shown). The absence of glycosylation at the 52nd position because of replacement of either α52Asn (Figure 2C) or α54Thr with Gln(data not shown) almost completely inhibited the production of progesterone.

Circular Dichroism Studies of the Carbohydrate Deficient hCG Mutants. To compare their secondary structures, the CD spectra of hCGwt, hCGα52Gln*β*wt, and hCGα80Gln*β*wt were recorded under identical conditions (Figure 3). The spectra are the average of four scans, and the noise level is shown by error bars. The qualitative secondary structural estimations were performed on the basis of the mean residue ellipticity (MRE) values at particular wavelengths. The estimation of the ordered structures such as α-helix and *β*-sheet is made at 220 nm, whereas random coil (aperiodic structure) is observed at 200 nm (Johnson, 1990). The mutant, hCGα52Gln*β*wt lacking carbohydrate at α52Asn, at 220 nm was found to have a lower negative MRE than hCGα80Gln*β*wt or hCGwt, indicating that the former had less ordered structure. This is consistent with the observed MRE values at 200 nm which is the most negative for hCGα52Gln*β*wt. It may be pointed out that the contribution of the polysaccharides to the secondary structure is small, between 1 and 5%, in the wavelength range of 185−250 nm (Merz, 1988). Similar results were obtained when the

Table 1: Percentages of Secondary Structural Elements[a] in the hCG Mutants Lacking *N*-Glycosyl Chains in hCGα

protein	β-sheet	aperiodic conformation	others (α-helix, β-like turns, SS bonds + aromatic residues)
hCGα52Glnβwt	24	44	32
hCGα80Glnβwt	31	31	38
hCGwt[b]	33	33	34

[a] The percentages of secondary structural elements were obtained using CCA. The deconvoluted curves were assigned to the corresponding secondary structures using the Automatic Spectral Assignment option in the CCA software. The decovolution values are the mean of two CCA analyses performed with two different CD experiments. The reference data set containing 30 proteins was used for the deconvolution. The contributions due to the absorption of carbohydrate functional groups were not considered in the secondary structural calculations. [b] Wild type lyophilized recombinant hCG. The β-sheet content of the wild type recombinant hCG without prior lyophilization was much higher, about 52 ± 4% (Shao et al., 1997). See the text for details.

quantitative estimation of the secondary structures was performed using convex constraint analysis (CCA; Perczel et al., 1992). The percentages of the secondary structural elements calculated from CCA are given in Table 1. Obviously, the mutant hCGα52Glnβwt had a lower β-sheet (24%) and a higher random coil or aperiodic structure (44%) content, compared with hCGα80Glnβwt (31 and 31%) and hCGwt (33 and 33%). The wild type recombinant hCG employed in these studies was a previously lyophilized sample. Shao et al. (1997) observed that, when hCGwt was used without prior lyophilization, it showed a much higher content of the β-sheet structure, about 52 ± 4%, compared with 33% for the lyophilized sample. It may be noted that the two carbohydrate mutants were not lyophilized. The helical content in the hCG structure is quite low, between 1 and 5%, as noted previously (Merz, 1988; Merz et al., 1973) and is consistent with the recent X-ray diffraction data (Wu et al., 1994; Lapthorn et al., 1994). The component curves of the deconvoluted spectra had a little contribution from the "α-helical like" spectrum. Despite a smaller α-helical content, it was still detectable in the component curves and the data are presented with "other contributions".

Dissociation of Carbohydrate Deficient hCG Mutants at Low pH. The rates of dissociation of the hCG mutants lacking an *N*-glycosyl chain at α52Asn or α78Asn were studied by ANS fluorescence as described earlier (Merz, 1988; Aloj et al., 1973) . The undissociated hCG binds to the probe stronger than the free subunits. Therefore, the dissociation kinetics can be monitored by following the fluorescence intensity of the hCG−ANS complex. At time 0 and pH 7.4, the fluorescence intensity of the ANS bound to hCGwt and to the mutants, hCGα52Glnβwt and hCGα80Glnβwt, was normalized to about 1 (Figure 4), although the fluorescence intensity of ANS bound to hCGwt was higher than that of the mutants. The quantum yield in the absence of the hormone was much lower than that taken in the presence of the protein, irrespective of the state of glycosylation. The dissociation kinetics of the hCGwt and the carbohydrate deficient hCG mutants at pH 3.0 are shown in Figure 4. The dissociation of hCGwt was much slower than that of hCGα52Glnβwt or hCGα80Glnβwt. The dissociation rates of the two mutants, hCGα52Glnβwt and hCGα80Glnβwt, calculated from the slope of the curves (Figure 4), were 9.4×10^{-5} and 3.8×10^{-5} s^{-1}, respectively, compared

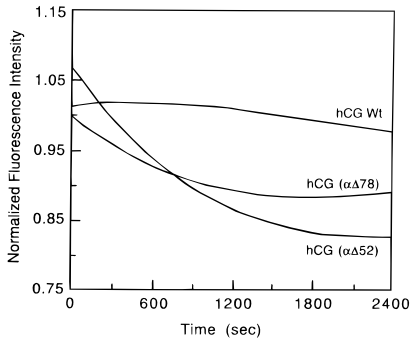


FIGURE 4: Dissociation kinetics of the two mutants, hCGα52Glnβwt and hCGα80Glnβwt, and hCGwt at pH 3.0. The fluorescence intensity of the hormone−ANS complex was observed at 24 °C at various time intervals in the slow kinetics mode. The emission was observed at 460 nm and the excitation at 400 nm. The slits were 4 nm.

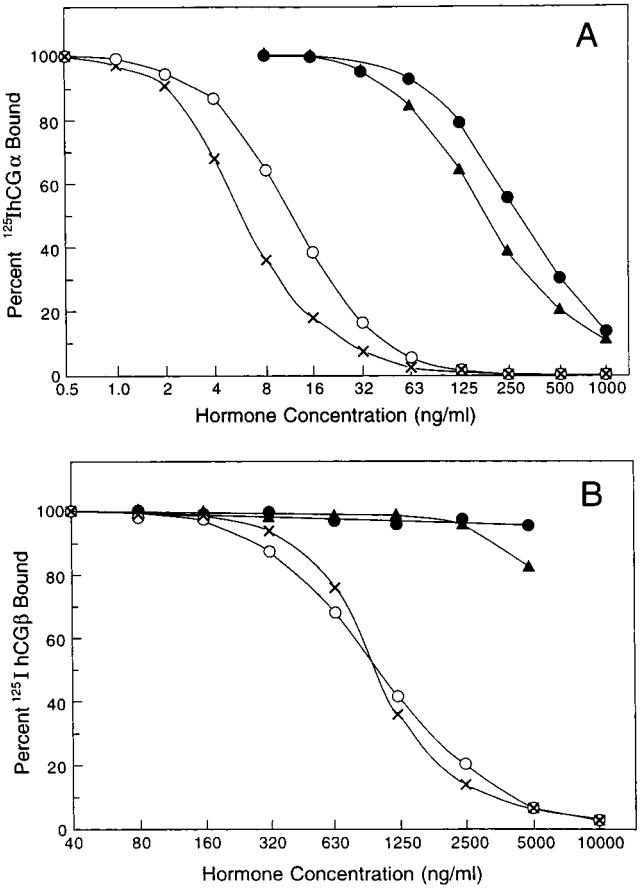


FIGURE 5: Radioimmunoassays of hCGwt, hCGα52Glnβwt, and hCGα80Glnβwt using the monoclonal anti-hCGα antibody, A2D4 (A), and the monoclonal anti-hCGβ antibody, B158 (B): native hCGα or native hCGβ (×), hCGwt (●), hCGα52Glnβwt (○), and hCGα80Glnβwt (▲) .

with 1.5×10^{-5} s^{-1} for hCGwt. These results are qualitatively consistent with those of the SDS−PAGE analysis of the hCGwt and the mutant samples incubated at pH 3.0 for different time intervals (data not shown).

Interaction of Carbohydrate Deficient Mutants of hCG with Antibodies. A change in the conformation in the heterodimer with the loss of glycosylation at α52Asn was also indicated by immunological studies using monoclonal antibodies that were specific to hCGα (A2D4) or hCGβ (B158) subunits (Figure 5) and did not significantly cross-react with the native hCG or hCGwt. To compare the

Table 2: Effect of Anti-hCGα and Anti-hCG*β* on the Signal-Transducing Properties of hCGα52Gln*β*wt and hCGα80Gln*β*wt[a]

antibody	hCGwt	hCGα52Gln*β*wt	hCGα80Gln*β*wt	anti-hCGα/*β* at a 1:250 dilution	cAMP[b] accumulation (pmol per 100 ng of protein)
anti-hCGα	+	−	−	+	2643
	−	+	−	+	152
	−	−	+	+	1923
	−	−	−	+	80
	−	−	−	−	80
anti-hCG*β*	+	−	−	+	4342
	−	+	−	+	4000
	−	−	+	+	5422
	−	−	−	+	80
	−	−	−	−	125

[a] To Leydig MA-10 cells (5×10^5 cells per 50 mm plate) was added 1 mL of samples containing 100 ng/mL hCGwt or its two mutants. After incubation for 30 min at 37 °C, the medium containing hCGwt or hCG mutants was washed with Waymouth medium containing 0.1% BSA (medium A) to remove any unbound hormone. Then, 1 mL of anti-hCGα or anti-hCG*β* antibodies diluted in medium A containing IBMX was added to each plate. The plates were incubated on ice for 3 h and then transferred to a 37 °C incubator for 30 min. cAMP determinations were made as described in the text. [b] Average of two experiments.

immunological properties of the fully glycosylated hCGwt or hCG glycosylated at either Asn78 (hCGα52Gln*β*wt) or Asn52 (hCGα80Gln*β*wt), the immunopotency of the hCG analogs was determined by RIA using the [^{125}I]hCGα tracer and the anti-hCGα monoclonal antibody, A2D4 (Figure 5A), or [^{125}I]hCG*β* and the anti-hCG*β* monoclonal antibody, B158 (Figure 5B). A2D4 seemed to recognize the hCGα epitope in the native hCG but only with an activity of 2% compared to that of the native hCGα subunit that was used for generating the antibodies (Thotakura & Bahl, 1985a). However, the heterodimer that was lacking the carbohydrate at α52Asn of the hCGα subunit had an immunopotency of almost 50% of that of the native hCGα, indicating that, in the mutant heterodimer, the epitope recognized by anti-hCGα was partly exposed. In the same RIA system, the immunopotency of hCGα80Gln*β*wt, on the other hand, was almost unaltered with an approximate value of 3.5% of that of native hCGα, a result similar to that of hCGwt. In the RIA using [^{125}I]hCG*β* and monoclonal anti-hCG*β*, the immunopotency of hCGα52Gln*β*wt was almost equal to that of native hCG*β*. A 50% inhibition of binding of the tracer was obtained by about 1 *μ*g of hCG*β* or hCGα52Gln*β*wt. However, at this concentration, the inhibition of binding by either hCGwt or hCGα80Gln*β*wt was less than 5% (Figure 5B), indicating that, while the epitope recognized by anti-hCG*β* (B158) was masked in hCGwt or hCGα80Gln*β*wt, it was fully exposed in hCGα52Gln*β*wt. These results can be reconciled on the basis of a conformational change in the heterodimer lacking carbohydrate at α52Asn. The loss of carbohydrate results in the exposure of the epitope(s) in the heterodimer recognized by the subunit specific monoclonal antibodies against native hCGα or native hCG*β*. The loss of carbohydrate at Asn78 does not seem to cause any conformational change as shown by its lack of cross-reactivity with specific monoclonal anti-hCGα or anti-hCG*β* antibodies.

Finally, the effect of polyclonal antibodies against hCGα or hCG*β* on the cAMP accumulation in mouse Leydig cells by hCG mutants lacking carbohydrate at α52Asn or α78Asn was investigated. Anti-hCGα failed to restore the cAMP-producing activity of hCGα52Gln*β*wt, while hCGwt or the hCGα80Gln*β*wt mutant lacking carbohydrate at Asn78 stimulated cAMP normally (Table 2). In contrast, anti-hCG*β* (Rebois & Liss, 1987) was able to restore the cAMP-producing activity of hCGα52Gln*β*wt to the same extent as that induced by hCGwt or hCGα80Gln*β*wt (Table 2). The antibodies by themselves did not have any effect on the basal level of cAMP in mouse Leydig cells. Although the effect of polyclonal antibodies against hCGα or hCG*β* on the restoration of the biological activity of the completely deglycosylated hCG has been previously reported (Rebois & Fishman, 1984), their effect on the hCG mutants lacking a specific carbohydrate chain has not been investigated. Furthermore, the lack of an effect with anti-hCGα as found in this study has not been previously observed.

DISCUSSION

Among the four *N*-glycosyl chains in hCG, the one present at α52Asn is important in its signal transduction function and thereby biological activity. The other three *N*-glycosyl chains, two in the *β*-subunit at *β*13Asn and *β*30Asn and one in the α-subunit at α78Asn, apparently have an insignificant role in the biological activity of the hormone (Matzuk et al., 1989). This result is not surprising in view of the recently reported crystal structure of hCG (Lapthorn et al., 1994; Wu et al., 1994). The *N*-glycosyl chain at α52Asn is the only one which is present at the interface of the α- and *β*-subunits. The other three are located on the outer face of the protein molecule. In this paper, we have prepared two analogs of hCG, one lacking an *N*-glycosyl chain at α52Asn and the other lacking an *N*-glycosyl chain at α78Asn. Both analogs bind to the receptor as well as the hCGwt (Figure 2A), indicating that the receptor binding site of the hormone is not affected by the replacement of either of the two glycosyl sites. This is consistent with the previous observations where deglycosylated hCG binds with the receptor with an equal or increased affinity (Kalyan & Bahl, 1983; Goverman et al., 1982). The site in the hormone responsible for the stimulation of the second messenger, however, is affected differently by the two glycosyl chains. While the absence of carbohydrate from α78Asn in hCG did not alter its signal transduction properties significantly, the removal from α52Asn almost completely abolished its ability to stimulate cAMP and progesterone (Figure 2B,C). This is in agreement with the previous studies (Sairam & Bhargavi, 1985; Matzuk et al., 1989) which demonstrated that carbohydrate at α52Asn was critical to the function of the hormone.

One of the unanswered questions with regard to the signal transduction properties of hCG is concerned with how at the molecular level the carbohydrate at α52Asn affects the biological activity or signal transduction function. Does it

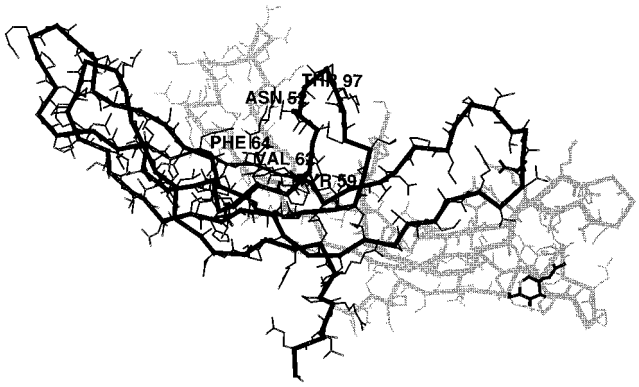


FIGURE 6: Carbohydrate chain in the α-subunit (dark gray) at Asn52 interacting with residues Tyr59, Val62, Phe64, Ala83, and Thr97 in the *β*-subunit (light gray). *β*83Ala is not shown in the figure (Lapthorn et al., 1994; Wu et al., 1994).

affect it by maintaining the conformation of hCG or by directly interacting with the lectin-like site in the hCG/LH receptor? According to the crystal structure of hCG (Lapthorn et al., 1994; Wu et al., 1994), the only carbohydrate that interacts at the subunit interface is on the α52Asn which contacts the *β*-subunit residues *β*59Tyr, *β*62Val, *β*64Phe, *β*83Ala, and *β*97Thr (Figure 6) of the determinant loop (Lapthorn et al., 1994). Conceivably, the loss of carbohydrate at α52Asn could diminish the interaction between the α- and *β*-subunits at this site and thereby result in a conformational change. Therefore, in this work, we have studied the conformational properties of the two mutants, lacking carbohydrate at α52Asn and α78Asn, and hCGwt and also the subunit affinity by determining the dissociation rates of the ANS−mutant complexes at low pH. The evidence, derived from the circular dichroism studies of the two hCG mutants, the dissociation kinetics of ANS−mutant complexes, and their interaction with the subunit specific monoclonal antibodies, indicates that the two analogs differed significantly in their conformations. The estimation of MRE values at 200 and 220 nm wavelengths combined with the analysis of the data by CCA (Perczel et al., 1992) showed that hCGα52Gln*β*wt had a significantly higher content of random coil or aperiodic structure and a lower content of the ordered structures, including *β*-sheet and α-helix, than hCGwt and the mutant hCGα80Gln*β*wt (Figure 3 and Table 1). The rates of dissociation of the mutant lacking carbohydrate at α52Asn at low pH and 24 °C were as much as 6 times higher than that of the hCGwt and about 3 times higher than that of the mutant devoid of carbohydrate at α78Asn (Figure 4). The weak subunit interaction in hCGα52Gln*β*wt was probably the result of a conformational change (Merz, 1988). Further evidence, showing the difference in the effects of the two glycosylation sites on the conformation of the heterodimer, comes from the differences in the cross-reactivities of hCGα52Gln*β*wt and hCGα80Gln*β*wt with two subunit specific monoclonal antibodies (Bahl et al., 1976; Thotakura & Bahl, 1985a), one specific for hCG*β* (B158) and the other specific for hCGα (A2D4). Both antibodies almost lacked cross-reactivity with the wild type hCG. However, hCGα52Gln*β*wt cross-reacted with both antibodies, whereas hCGα80Gln*β*wt like hCGwt had no significant cross-reactivity with either one of them (Figure 5). The data clearly indicate that there are conformational differences between the two mutants. Thus, the loss of signal-transducing function of hCG with the loss of carbohydrate at α52Asn might be due to a conformational change in the mutant, rather than due to the direct participation of the carbohydrate in binding to the lectin-like site present in the receptor as previously postulated (Braun et al., 1991). This is further supported by the fact that polyclonal anti-hCG*β* was able to restore the loss of cAMP accumulation by the mutation at α52Asn. It is further believed that the loss of the signal transduction function of hCG was not due to the replacement of the amino acid α52Asn with Gln since the replacement of α54Thr with Gln (data not shown) also yielded similar results. Although the mutants hCGα52Gln*β*wt and hCGα80Gln*β*wt have high mannose type carbohydrates at the glycosylated N-glycosylation sites and short O-linked oligosaccharides in the carboxy end of the *β*-subunit, these changes as reported previously (Chen & Bahl, 1991a; Matzuk et al., 1990) do not affect either receptor binding or signal transduction of the hormone and, therefore, do not affect the conclusions of the present studies.

On the basis of the above data, it is tempting to postulate that the first interaction of the heterodimeric hormone with the receptor is made through the *β*-subunit, presumably at the site proximal to or in the determinant loop (Lapthorn et al., 1994; Wu et al., 1994). The conformational change in the *β*-subunit that follows the initial interaction with the receptor is relayed to the α-subunit through the contact points between the α52Asn carbohydrate and the *β*-subunit. The signal from the α-subunit in turn is then passed onto the receptor, leading to the subsequent events in the signal transduction cascade. This hypothesis is consistent with the recently suggested "horseshoe" or "U" (Wu et al., 1994; Cosowsky et al., 1995; Moyle et al., 1995) shape structure for the extracellular domain of the rat LH receptor based on its analogy with the ribonuclease inhibitor (Kobe & Deisenhofer, 1993). One can visualize one arm of the receptor interacting with the *β*-subunit and the other arm with the α-subunit of the heterodimer with the α52Asn carbohydrate at the interface of the two subunits as a mediator of the signal. The binding of the α- and *β*-subunits of the heterodimer to two arms of the extracellular domain of the rat LH receptor would be analogous to the ligand-induced dimerization of the receptor, a mechanism which is considered to be a prerequisite to the activation of the effector, tyrosine kinase (Heldin, 1995). For example, PDGF is a homodimer in which each monomer binds to a single molecule of the receptor, thus resulting in the dimerization of the receptor (Welsh, 1994). The binding of EGF to its receptor is followed by the dimerization of the ligand−receptor complex (Hurwitz et al., 1991).

In summary, two mutants of hCG lacking a glycosyl chain at α52Asn or α78 in the α-subunit prepared by mutating α52Asn to Gln or α80Thr to Gln have been overexpressed in insect cells and have been purified to homogeneity in a single step by immunoaffinity chromatography in adequate amounts. The two mutants have different overall conformations. The conformational differences probably account for the differences in their signal transduction properties. While the carbohydrate at α52Asn is necessary, the one at α78Asn has a negligible effect on signal transduction. The data strongly imply that the carbohydrate at α52Asn maintains the function of the hormone by maintaining its conformational integrity.

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