The Role of the Asparagine-linked Oligosaccharides of the α Subunit in the Secretion and Assembly of Human Chorionic Gonadotropin

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Abstract. Human chorionic gonadotropin (hCG) is a member of a family of heterodimeric glycoprotein hormones that have a common α subunit but differ in their hormone-specific β subunit. Site-directed mutagenesis of the two asparagine-linked glycosylation sites of hCGα was used to study the function of the individual oligosaccharide chains in secretion and subunit assembly. Expression vectors for the α genes (wild-type and mutant) and the hCGβ gene were constructed and transfected into Chinese hamster ovary cells. Loss of the oligosaccharide at position 78 causes the mutant subunit to be degraded quickly and <20% is secreted. However, the presence of hCGβ stabilizes this mutant and allows ~45% of the subunit in the form of a dimer to exit the cell. Absence of carbohydrate at asparagine 52 does not perturb the stability or transport of the α subunit but does affect dimer secretion; under conditions where this mutant or hCGβ was in excess, <30% is secreted in the form of a dimer. Mutagenesis of both glycosylation sites affects monomer and dimer secretion but at levels intermediate between the single-site mutants. We conclude that there are site-specific functions of the hCGα asparagine-linked oligosaccharides with respect to the stability and assembly of hCG.

HUMAN chorionic gonadotropin (hCG)[1] is a member of the glycoprotein hormone family which includes lutropin, follitropin, and thyrotropin. These hormones are heterodimers consisting of an α subunit that is common among the four hormones, and a β subunit that gives each its unique biological activity (5, 36, 53). The hCGα subunit contains two asparagine (Asn)-linked oligosaccharide chains (13, 23), whereas the hCGβ subunit contains two Asn-linked and four serine O-linked carbohydrate units (3, 22, 24).

Several studies have addressed the role of the Asn-linked oligosaccharide chains on the gonadotropins. Chemical or enzymatic methods have been used to remove all or part of the oligosaccharide chains to obtain a better understanding of carbohydrate function. While desialylation of hCG causes it to be rapidly cleared via the hepatic route (32), its effects on biological activity in vitro are not clear (1, 33, 51). Removal of carbohydrate internal to sialic acid or complete deglycosylation converts hCG into an antagonist; it binds more tightly to its receptor, but shows decreased biological activity in vitro (6, 21, 25, 33). Although the role of the oligosaccharide side-chains in folding and assembly of the hCG subunits in vivo is unknown, it has been postulated that these oligosaccharides are important for correct disulfide bond formation (48). Combination of hCG subunits apparently occurs when the oligosaccharides are removed chemically (21, 25) or enzymatically (16). However, one problem with these methods is that incompletely deglycosylated derivatives are generated; in addition, such procedures can cause denaturation and/or dissociation of the subunits, and therefore alter the hormone in other ways not related to carbohydrate removal.

The most common method to prevent Asn-linked glycosylation is with tunicamycin which blocks the synthesis of the lipid-carbohydrate carrier complex (49). The problems with this approach are that individual carbohydrate units on a multiglycosylated protein cannot be examined, and indirect effects of tunicamycin could affect secretion of the glycoprotein. We have used oligonucleotide-directed mutagenesis to investigate the role of the individual Asn-linked oligosaccharides on hCGα in secretion and assembly in vivo. Using DNA-mediated transfer techniques, we have transfected vectors containing wild-type and mutant hCGα genes into Chinese hamster ovary (CHO) cells and selected lines expressing the various subunits. We have also co-transfected the hCGβ construct with the wild-type and mutant hCGα genes to examine assembly in vivo. Using site-directed mutagenesis of the hCGα glycosylation sites, removal of a single oligosaccharide enhances degradation whereas loss of the

1. *Abbreviations used in this paper*: Asn, asparagine; CHO, Chinese hamster ovary; Endo F, Endoglycosidase F; Endo H, Endoglycosidase H; hCG, human chorionic gonadotropin; Thr, threonine.

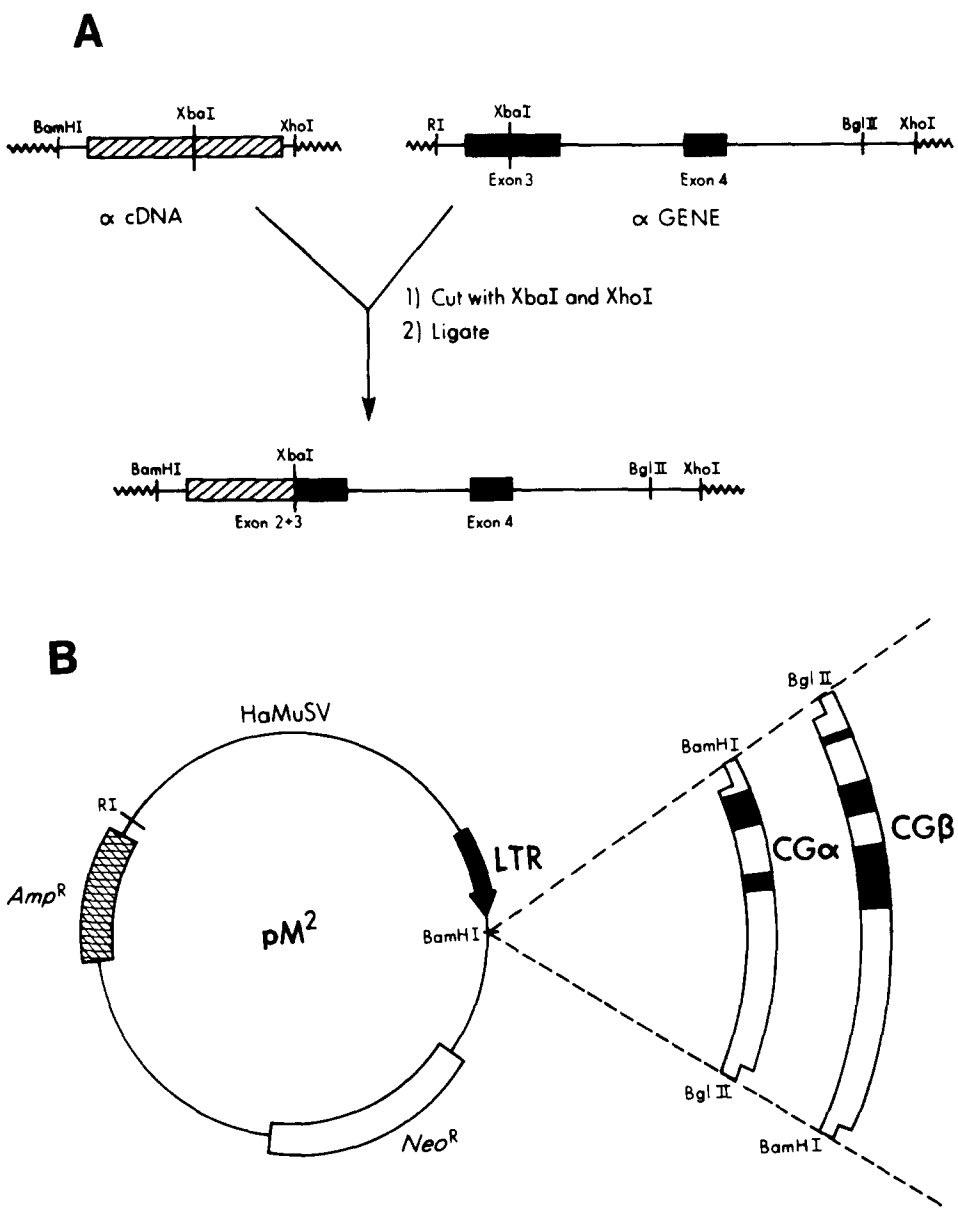


Figure 1. Engineering of hCGα gene and hCG expression vectors. *A* shows the construction of an hCGα minigene. The hCGα cDNA protein coding sequence (*crosshatched box*) is bordered by two unique restriction enzyme sites, Bam HI and Xho I. The third and fourth exons (*solid boxes*) of the hCGα gene were isolated on one Eco RI (*RI*)-Xho I fragment. The Xba I-Xho I fragment from the hCGα gene was inserted into the Xba I-Xho I sites of the hCGα cDNA-containing plasmid (4, 38), resulting in a 2.4-kb minigene maintaining the correct reading frame of the coding sequence. This α minigene construct contains two coding regions (*Exon 2+3* and *Exon 4*), a single intron and polyadenylation signals 3′ to exon 4. This intron contains the identical splice signals which are in the original hCGα gene. The construction of the expression vectors $pM^2CG\alpha$ and $pM^2CG\beta$ (*B*) are described in Materials and Methods and reference 29. In brief, hCGβ and hCGα genes (α-WT, αΔAsnl, αΔAsn2, αΔAsn[1+2], αΔThr2, and αΔThr[1+2]) were inserted into the single Bam HI restriction enzyme site in pM^2 downstream of the Harvey murine sarcoma virus (*HaMuSV*) long terminal repeat (*LTR*) (11) (*arrow*). pM^2 is a pSV2neo derivative (47) which contains the Amp^R gene (*crosshatched box*) and the Neo^R gene (*open box*) for selection with the neomycin analogue, G418. Coding regions of the hCGα and hCGβ genes are denoted by the dark boxes.

other oligosaccharide alters the secretion of dimer. The loss of both oligosaccharides affects both secretion and dimerization but at intermediate levels between the single-site mutants. We conclude that there are site-specific intracellular functions of the two Asn-linked oligosaccharide chains on hCGα.

Materials and Methods

Enzymes used to prepare vectors were purchased from New England Biolabs, Beverly, MA; Bethesda Research Laboratories, Gaithersburg, MD; or Boehringer Mannheim Diagnostics, Inc., Houston, TX. Klenow fragment was a gift from Dr. John Majors, Washington University, St. Louis, MO. The DNA vector, M13 UM20 (30), was obtained from International Biotechnologies, Inc., New Haven, CT. Oligonucleotides used for the site-directed mutagenesis were prepared by the Washington University Sequencing Facility, St. Louis, MO. Cell culture media and reagents were prepared by the Washington University Center for Basic Cancer Research, St. Louis, MO. Dialyzed calf serum and the neomycin analogue, G418, were purchased from Gibco Laboratories, Grand Island, NY. FCS was purchased from Hazelton Systems, Inc., Aberdeen, MD; and Pansorbin, a *Staphylococcus aureus* membrane preparation, from Behring Diagnostics, American Hoechst Corp., San Diego, CA. [^{35}S]Cysteine (>1,000 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. Endoglycosidase H (Endo H) (lot No. 0019) was obtained from Miles Laboratories, Naperville, IL, and Endoglycosidase F (Endo F) was a gift from Dr. Jacques Baenziger, Washington University, St. Louis, MO. Tunicamycin was purchased from Calbiochem-Behring Corp., La Jolla, CA. All other reagents were purchased from Sigma Chemical Company, St. Louis, MO.

Gene and Vector Constructions

Construction of the hCGα minigene is described in the legend to Fig. 1. This recombinant was constructed because of the much lower protein expression of the cDNA in other constructs (data not shown) and because the hCGα gene was too large to insert into pM^2. pM^2 was constructed by ligating an Eco Rl-Bam Hl fragment from pDVXR (obtained from C. Hardy and M. Botchan, University of California, Berkeley, CA) containing the Harvey murine sarcoma virus long terminal repeat (ll) into the Eco Rl and Bam Hl sites of pSV2neo (47). $pM^2CG\beta$ was constructed by inserting a Bgl II-Bam Hl fragment containing the hCGβ gene (37) into the single Bam Hl site in pM^2 (see Fig. 1 *B* and reference 29). $pM^2CG\alpha$ and the mutant de-

rivatives, pM²αΔAsn1, pM2αΔAsn2, pM²αΔAsn(1+2), pM²αΔThr1, pM²αΔThr2, and pM²αΔThr(1+2) were constructed by inserting Bam-Bgl II fragments containing the wild-type hCGα (Fig. 1 *A*) and the mutant hCGα genes (not shown) into the compatible Bam H1 site in pM². The mutant genes were derived from mutagenesis of the hCGα fusion gene that had been inserted into M13 UM20 (see below).

Site-directed Mutagenesis

The Bam HI-Xho I fragment containing the hCGα fusion gene, as described in Fig. 1, was ligated into M13 UM20 and the single-stranded viral recombinant DNA was isolated for mutagenesis. Mutant 22-mer oligomers, 5′-GGTGACGTCCTTTTGCACCAAC-3′ and 5′-CTTAGTGGAGCGGGATATG-3′, were synthesized for mutagenesis of the Asn^{52} and Asn^{78} codons, respectively; and mutant 26-mer oligomers, 5′-GTGGACTCTGAGGCCACGTTCTTTTG-3′ and 5′-CAGTGGCACGCCGCATGGTTCTCCAC-3′, were synthesized for mutagenesis of the corresponding Thr^{54} and Thr^{80} codons, respectively. The mutagenesis procedure used was identical to that described by Seeburg et al. (44) except for the following modifications. The temperature of the annealing reaction was begun at 10°C above the calculated T_m and allowed to cool to 10°C below the T_m before placing the reaction on ice. The DNA synthesis reaction on the recombinant template was incubated overnight at 16°C followed by transformation of competent *Escherichia coli* K12 JM109 cells. Hybridization and washing in tetramethylammonium chloride were as described in Wood et al. (55); the wash temperature for the 22-mers was 61°C and for the 26-mers was 67°C. All mutant phage were further purified and rescreened to ensure that there was no wild-type phage contamination before sequencing. The integrity of the gene was rechecked by restriction enzyme analysis. The mutation frequency achieved was consistently >5%.

DNA Transfection and Clone Selection

Plasmids pM²CGα, pM²αΔAsn1, pM²αΔAsn2, pM²αΔAsn(1+2), pM²αΔThr1, pM²αΔThr2, and pM²αΔThr(1+2) were transfected alone or co-transfected with pM²CGβ into CHO cells by a modification of the calcium phosphate method (17, 35, 45). Cells were selected for insertion of the plasmid DNA by growing in culture medium containing 0.25 mg/ml of the neomycin analogue, G418. Transfected colonies resistant to G418 were harvested ~11 d after transfection and subsequently screened for the expression of hCGα (wild-type and mutant), or hCG dimer (wild-type and mutant) by immunoprecipitation of metabolically labeled cells (see below).

Cell Culture

The CHO cells were maintained in medium I (Ham's F12 medium supplemented with penicillin [100 U/ml], streptomycin [100 μg/ml], and glutamine [2 mM]) containing 5% (vol/vol) FCS at 37°C in a humidified 5% CO_2 incubator. Transfected clones were maintained in the above culture medium supplemented with 0.25 mg/ml active G418.

Metabolic Labeling

On day 0, cells were plated into 12-well dishes (350,000 cells/well) in 1 ml of medium I supplemented with 5% FCS. For continuous labeling experiments, cells were washed twice with cysteine-free medium II (medium I supplemented with 5% dialyzed calf serum) and labeled for 7 or 8 h in 1 ml of cysteine-free medium II containing 50 μCi/ml [^{35}S]cysteine (>1,000 Ci/mmol). For pulse-chase experiments, the cells were washed twice and preincubated for 1.5 h with cysteine-free medium II, followed by a 20-min pulse-labeling in cysteine-free medium II containing 100 μCi/ml [^{35}S]cysteine. The labeled cells were then washed twice with medium II containing 1 mM unlabeled cysteine and incubated in this chase medium for the indicated times.

Immunoprecipitation

Medium and cell lysate samples were prepared, immunoprecipitated, and treated with endoglycosidases as described (10). Polyclonal antisera against hCGα and hCGβ were prepared in our laboratory and were also a gift from Drs. Steven Birken and Robert Canfield, Columbia University College of Physicians and Surgeons, New York, NY. The antiserum used for each experiment is described in the text. Each antiserum had been titered and was added in severalfold excess to ensure complete immunoprecipitation.

PAGE

The hCGα, hCGβ, and hCG dimer immunoprecipitates were resolved on 15% SDS-polyacrylamide gels by the method of Laemmli (26). Gels were soaked for 10 min in 1 M sodium salicylate, dried, and autoradiographed with preflashed film as described (10). Autoradiographs were scanned with an Ultroscan XL laser densitometer (LKB Instruments, Inc., Gaithersburg, MD).

Results

Site-directed Mutagenesis of hCGα

The recognition signal for Asn-linked glycosylation of proteins is the tripeptide Asn-X-Thr/Ser (18, 19, 28). The hCGα subunit contains two glycosylation sites at Asn 52 and 78. Oligonucleotide-directed mutagenesis (56) was chosen to examine the functional importance of the individual glycosylation sites in secretion and subunit assembly. Mutagenesis of either the Asn or the Thr amino acids in the Asn-X-Thr recognition sequence in the hCGα gene would be sufficient to prevent transfer of the carbodydrate to the α subunit (18, 19, 28). We chose to convert the Asn to aspartic acid residues because they are isoteric and have similar hydrogen bond characteristics (see reference 31). The 5′-AAC-3′ triplet coding for Asn 52 was converted to 5′-GAC-3′ coding for aspartic acid (designated αΔAsn1). A similar change was made at position 78 in a different clone (designated αΔAsn2) and a third mutant gene was constructed in which both sites were mutated (designated αΔAsn[1+2]). The Thr residues in these triplets were converted to alanine residues to assess that the results from the Asn mutants were due to loss of an oligosaccharide and not to alterations in the protein sequence (see reference 43). These mutants are designated αΔThr1, αΔThr2, and αΔThr(1+2) to denote changes of Thr^{54}, Thr^{80}, or both Thr's, respectively, to alanine residues. After mutant selections, we sequenced the mutated regions to verify that the changes were correct and that there were no other sequence alterations (Fig. 2 A) (42). The wild-type (designated α-WT) and mutant hCGα proteins are depicted for ease of representation in Fig. 2 *B*. Construction of these mutants allowed us to examine the association of the individual hCGα Asn-linked oligosaccharides to the secretion, stability, and assembly of the intact dimer.

Expression of hCGα Glycosylation Mutants

To analyze the synthesis of the wild-type and mutant proteins, we inserted these genes into a eukaryotic expression vector, pM², and transfected the resultant plasmids into CHO cells. Stable clonal cell lines expressing α-WT, αΔAsn1, αΔAsn2, and αΔAsn(1+2) were isolated. These cell lines were labeled in the presence of [^{35}S]cysteine for 7 h, and media and cell lysate were analyzed for immunoprecipitable hCGα by SDS-PAGE (Fig. 3). Except for αΔAsn(1+2), the intracellular ("lysate") hCGα forms migrated faster than the corresponding extracellular ("medium") form. This upward shift in molecular mass upon secretion of wild-type hCGα (intracellular, 23 kD; extracellular, 28 kD) is due to terminal processing of the Asn-linked chains on α-WT (data not shown) and is most likely responsible for the smaller shifts of the secreted forms of αΔAsn1 (intracellular, 20 kD; extracellular, 22 kD) and αΔAsn2 (intracellular, 20 kD; extracellular, 23.5 kD). The change in molecular mass is not

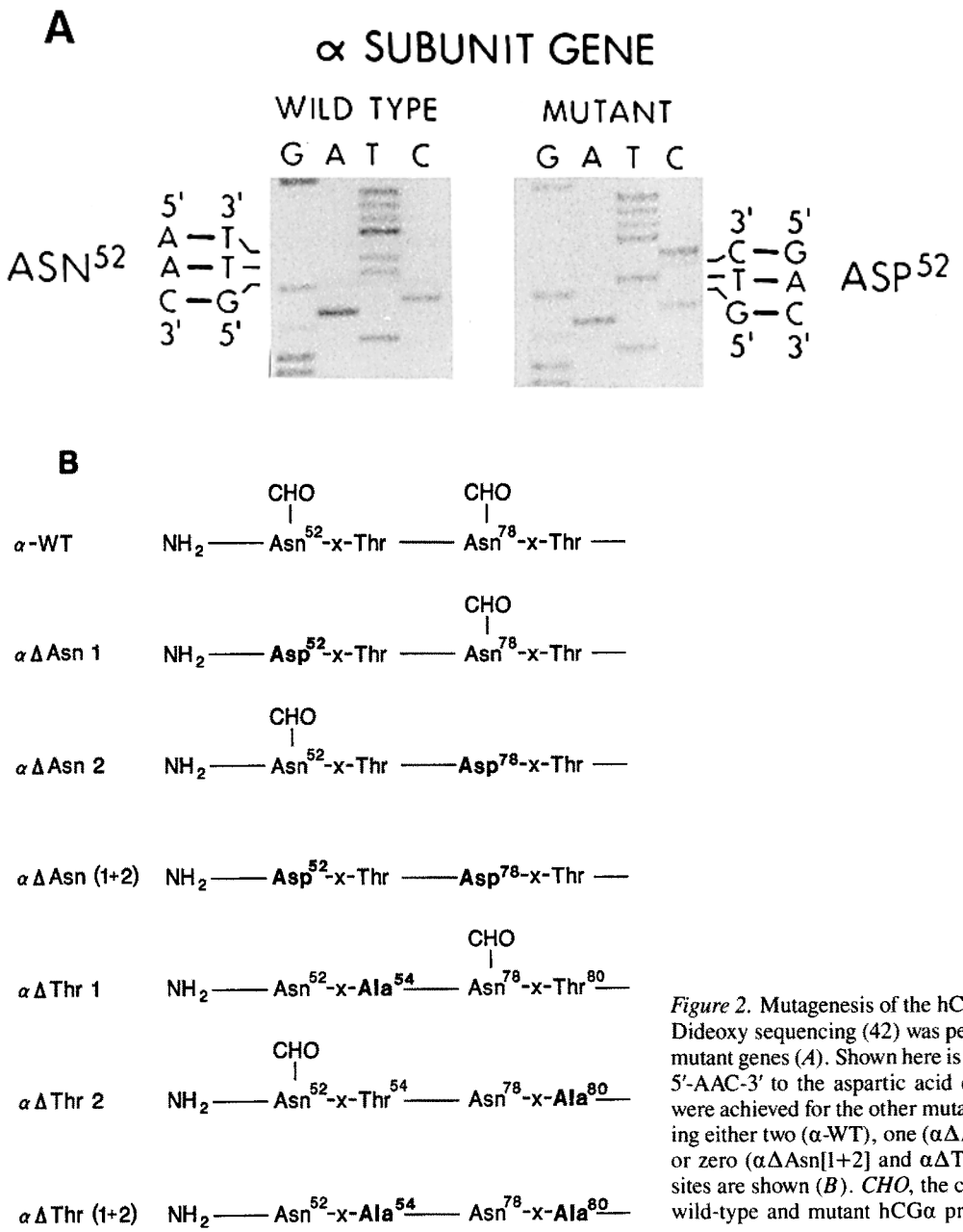


Figure 2. Mutagenesis of the hCGα Asn-linked glycosylation sites. Dideoxy sequencing (42) was performed on both the wild-type and mutant genes (*A*). Shown here is the conversion of the Asn 52 codon 5'-AAC-3' to the aspartic acid codon 5'-GAC-3'. Identical results were achieved for the other mutations. The hCGα proteins containing either two (α-WT), one (αΔAsn1, αΔAsn2, αΔThr1, αΔThr2), or zero (αΔAsn[1+2] and αΔThr[1+2]) Asn-linked glycosylation sites are shown (*B*). *CHO*, the carbohydrate moiety attached to the wild-type and mutant hCGα proteins.

seen upon secretion of the mutant αΔAsn(1+2) (15 kD) since it is devoid of Asn-linked oligosaccharides (data not shown).

Wild-type hCGα (α-WT) is expressed at high levels and secreted very efficiently into the medium with severalfold more extracellular than intracellular material present. αΔAsn1 is also secreted in large amounts (with more material present in the medium than the lysate), but at a level lower than α-WT. However, unlike α-WT and αΔAsn1, αΔAsn2 and αΔAsn(1+2) are not secreted into the medium in substantial amounts. Removal of the glycosylation sites and not the alteration in the amino acid is presumably responsible for these changes since the Thr→Alanine mutants give identical results (see below).

To document that the Asn-linked oligosaccharides on the secreted α forms were complex in structure, and that the changes in the mobility of the mutated proteins were not due to proteolysis, we treated the wild-type and the two mutants, αΔAsn1 and αΔAsn2, with Endo H (50) and Endo F (12). Endo H cleaves high mannose, noncomplex, and hybrid-type oligosaccharides, whereas Endo F cleaves, in addition, complex oligosaccharides. Immunoprecipitable hCGα from the media of clones expressing α-WT, αΔAsn1, and αΔAsn2 was treated with Endo H or Endo F, and the products resolved on SDS-PAGE gels (Fig. 4). Scanning densitometry revealed that <5% of the α-WT, αΔAsn1, and αΔAsn2 were sensitive to Endo H. Wild-type and mutant material were completely sensitive to Endo F, and the digested material migrated at approximately the same molecular mass as deglycosylated wild-type α (15 kD). Thus, >95% of the secreted material is resistant to Endo H but sensitive to Endo

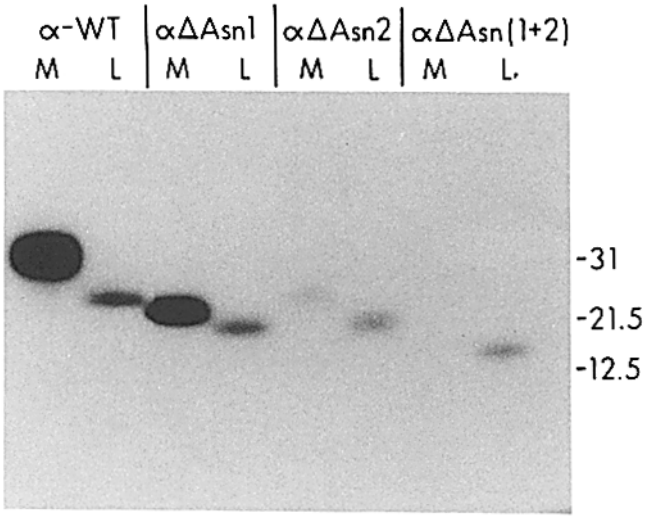


Figure 3. Expression of wild-type and Asn-linked hCGα glycosylation mutants in CHO cells. CHO clones expressing native hCGα (*α-WT*) and Asn-linked glycosylation mutants (*αΔAsn1*, *αΔAsn2*, and *αΔAsn[1+2]*) were labeled for 7 h with 50 μCi/ml [^{35}S]cysteine. Aliquots of medium (*M*) and cell lysate (*L*) were immunoprecipitated with hCGα antiserum, and analyzed as described in Materials and Methods. Molecular mass standards (carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 21.5 kD; cytochrome C, 12.5 kD) are shown at the right.

F, showing that the mutant and native subunits are processed to complex-type oligosaccharides and that their presence in the medium is due to passage through the normal secretory pathway and not due to cell lysis. Also, the Endo F data show that changes in mobility of the mutant subunits is due to the absence of oligosaccharide rather than proteolysis.

Secretion Kinetics of hCGα Mutants

As described above, the level of secretion of the mutants differed from wild-type α and could be attributed to lower levels of expression, increased proteolysis, and/or a block in transport or secretion. To distinguish between these possibilities, pulse–chase experiments were performed on clones expressing α-WT and the individual mutants (both Asn and Thr mutants). Confluent cells were pulse labeled with [^{35}S]cysteine for 20 min, chased with unlabeled cysteine for the indicated times, and the intracellular ("lysate") and extracellular ("medium") hCGα forms were immunoprecipitated with a polyclonal hCGα-specific antiserum (Fig. 5). In Fig. 5 *A*, the kinetics of disappearance of the intracellular α-WT is the same as that seen for the appearance of α-WT in the medium ($t_{½}$ = 85 min). Scanning densitometry confirms that there is >95% recovery in the medium of α-WT. These results were comparable to pulse–chase studies of wild-type hCGα secreted from C127 mouse mammary tumor cells transfected with hCGα (10).

The pulse–chase kinetics of αΔAsn1 (Fig. 5 *B*) and αΔThr1 (Fig. 5 *C*) show identical rates of recovery from the cells, and their secretion into the medium ($t_{½}$ = 85 min) was similar to wild-type hCGα (compare with Fig. 5 *A*). Furthermore, >95% of αΔAsn1 and αΔThr1 accumulated in the medium but, unlike wild-type α, these mutant subunits migrate at a more homogeneous position on the gel (see Discussion) and the secreted forms show less of an upward shift (only 2 kD). Thus, the absence of the oligosaccharide at Asn 52 by mutation of either the Asn or Thr residues does not affect either its secretion rate nor its recovery in the medium. Since αΔAsn1 and α-WT show no differences in secretion or degradation rates, the different secretion levels of αΔAsn1 and α-WT seen in Fig. 3 must be attributed to clonal variation in expression.

In contrast to the kinetics of αΔAsn1, αΔThr1, and α-WT, mutagenesis of the second glycosylation site (αΔAsn2 and αΔThr2) markedly affects the behavior of the protein (Fig. 5, *D* and *E*). Densitometric scanning of the autoradiograph indicates that the intracellular αΔAsn2 and αΔThr2 subunits disappear with similar kinetics as α-WT, αΔAsn1, and αΔThr1 ($t_{½}$ = 80 min). However, the amount recovered in the medium is much lower than αΔAsn1, αΔThr1, or α-WT; only 19 and 17% of the synthesized αΔThr2 and αΔAsn2, respectively, are recovered. Thus, loss of carbohydrate at position 78 by either the Thr or Asn alteration affects the stability of the hCGα subunit resulting in an enhanced degradation rate. Moreover, in contrast to αΔAsn1 and αΔThr1, the extracellular form of αΔAsn2 and αΔThr2 is heterogeneous like α-WT (see Discussion).

The secretion kinetics of the deglycosylated mutants αΔAsn(1+2) and αΔThr(1+2) (Fig. 5, *F* and *G*) present a different pattern from that seen above. Unlike the fast disappearance from the cell of the wild-type or single mutated subunits, the double mutants are secreted from the cell very slowly ($t_{½} \approx$ 6 h) and <50% can be recovered in the medium even after 10 h. We also examined the secretion kinetics of the wild-type α from cells treated with tunicamycin, an inhibitor of N-linked glycosylation (Fig. 5 *H*). In this case, as seen with double mutants, this deglycosylated α form is secreted slowly ($t_{½} \approx$ 3 h) and it is also recovered quantitatively in the medium. Thus, both the tunicamycin deglycosylated α and the mutant α subunits are slower to exit the cells; the small differences in the rates and extent of secretion may be due to indirect effects of tunicamycin on the cell or subtle effects of the amino acid substitutions.

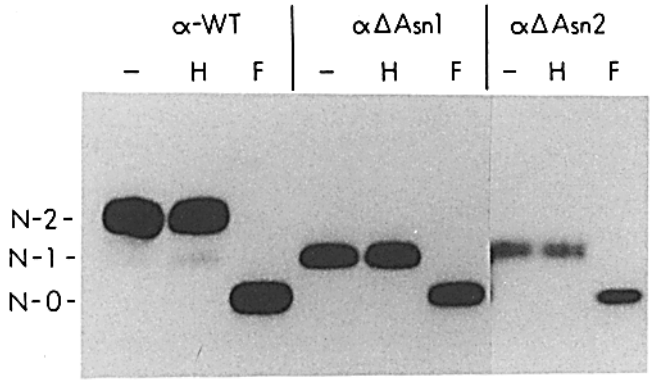
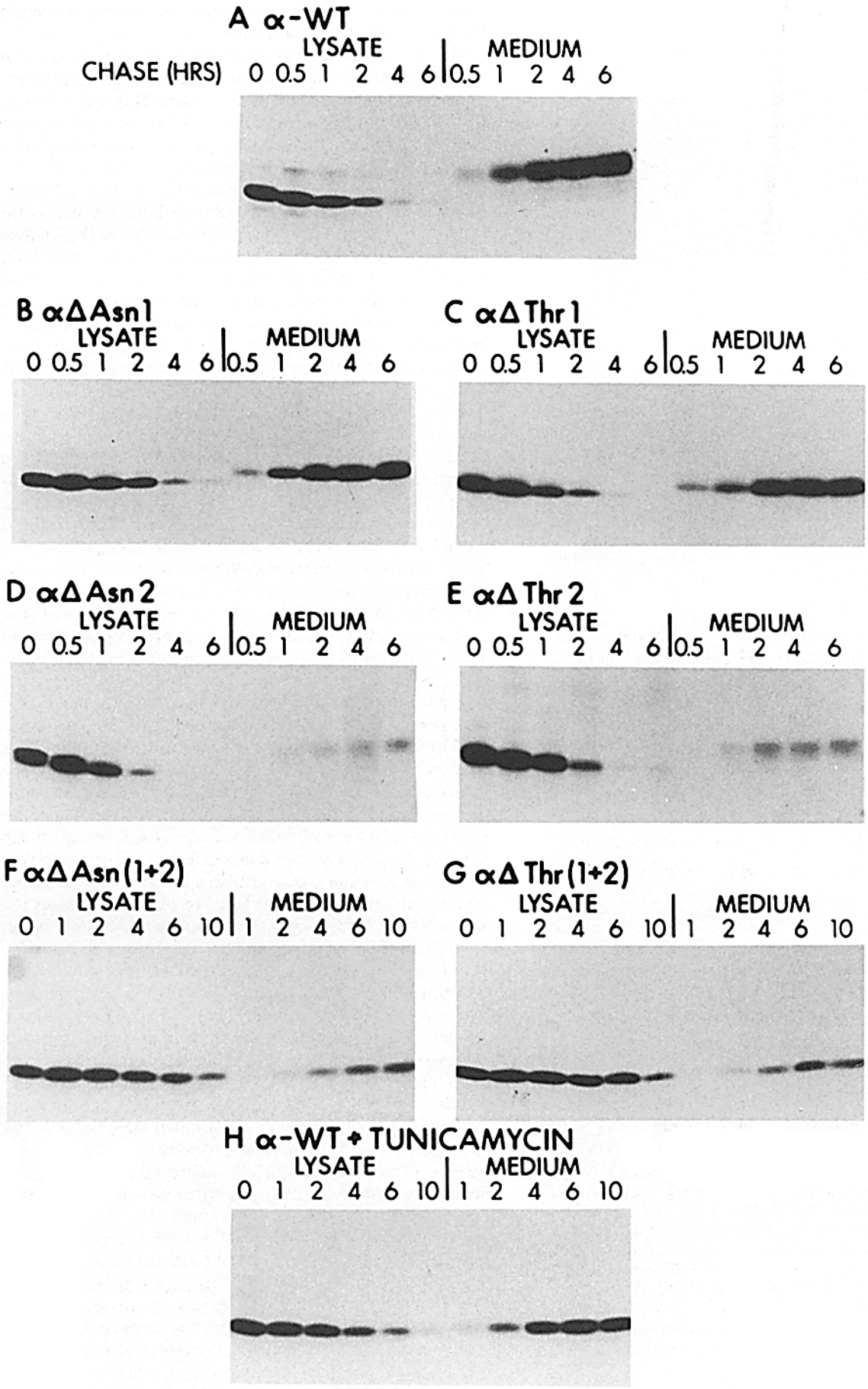


Figure 4. Endoglycosidase treatment of hCGα wild-type and monoglycosylated mutant subunits. Clones expressing α-WT, αΔAsn1, and αΔAsn2 were labeled for 7 h and aliquots of media were immunoprecipitated with hCGα antiserum before digestion with Endo H (*H*), Endo F (*F*), or no enzyme (–). Electrophoresis and autoradiography were performed as described in Materials and Methods. The migration of the doubly glycosylated, monoglycosylated, and deglycosylated subunits are indicated by *N-2*, *N-1*, and *N-0*, respectively.


A α-WT
LYSATE
MEDIUM
CHASE (HRS) 0 0.5 1 2 4 6 0.5 1 2 4 6
B αΔAsn1
LYSATE
MEDIUM
0 0.5 1 2 4 6 0.5 1 2 4 6
C αΔThr1
LYSATE
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D αΔAsn2
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E αΔThr2
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F αΔAsn(1+2)
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G αΔThr(1+2)
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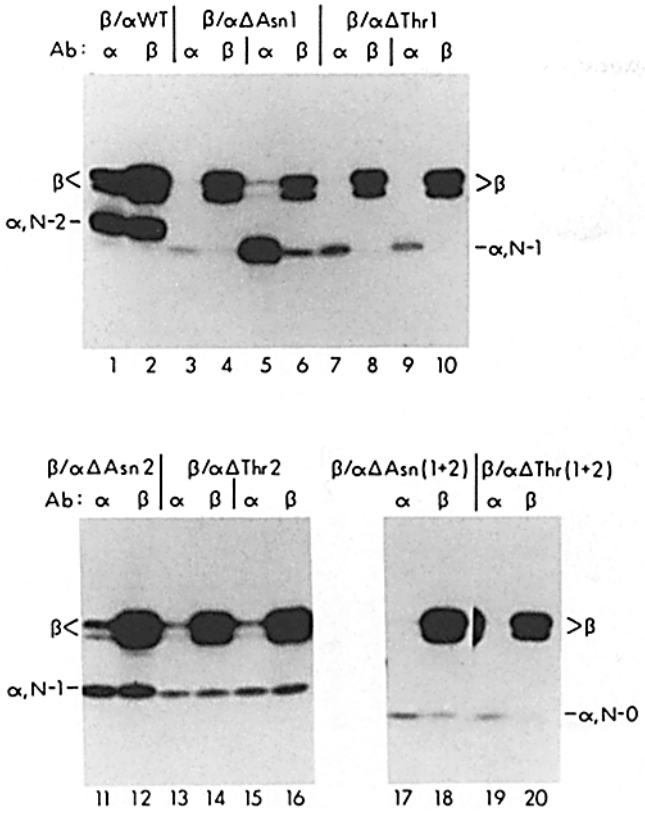

Figure 6. Assembly of hCGα mutants. Clones expressing α-WT and hCGβ (lanes *1* and *2*), αΔAsn1 and hCGβ (lanes *3* and *4*; lanes *5* and *6*), αΔThr1 and hCGβ (lanes *7* and *8*; lanes *9* and *10*), αΔAsn2 and hCGβ (lanes *11* and *12*), αΔThr2 and hCGβ (lanes *13* and *14*; lanes *15* and *16*), αΔAsn(1+2) and hCGβ (lanes *17* and *18*), and αΔThr(1+2) and hCGβ (lanes *19* and *20*) were labeled for 8 h. The media from each clone were divided into two aliquots and immunoprecipitated with hCGα or hCGβ antisera. Electrophoresis and autoradiography were as described in Materials and Methods.

Assembly of hCGα Mutants with hCGβ

To examine subunit assembly of the wild-type hCGβ subunit with hCGα and the various mutant hCGα subunits, pM²CGβ (see Fig. 1) was co-transfected with pM²CGα, single mutants, or double mutants. Clones expressing both subunits were then isolated and the ratio of hCGβ to hCGα was determined by immunoprecipitation with either hCGα- or hCGβ-specific antisera (Fig. 6). For each mutant, we isolated some clones synthesizing excess hCGβ subunit to ensure that the β subunit would not be limiting for dimer formation. Assembly of the various α forms with hCGβ was assessed by immunoprecipitation with either an hCGα-specific antiserum which recognizes both free hCGα and dimer, or with hCGβ-specific antiserum which recognizes both hCGβ and hCG dimer. Since hCGβ is in excess, there should be equal amounts of α subunit precipitated by both antisera if complete dimerization has occurred.

Immunoprecipitation of wild-type hCG dimer with either α or β antiserum (Fig. 6, lanes *1* and *2*) results in recovery of equal amounts of the α-WT subunit. This indicates that ≈100% of the α subunit is combined with the hCGβ in the dimer form. In all clones isolated, hCGβ exits as two forms which is due to the addition of one or two N-linked oligosaccharides (see reference 29). However, assembly of the glycosylation site I mutants (αΔAsn1 and αΔThr1) with the hCGβ subunit is much less efficient. Although hCGβ is severalfold in excess, <30% of the αΔAsn1 (clone 11-12, lanes *3* and *4*) and <10% of the αΔThr1 (clone 6-5, lanes *7* and *8*) can assemble into dimers since the total amount of α secreted (lane *3* or *7*) is greater than the dimer α (lane *4* or *8*). Clone 6-14 (lanes *9* and *10*) confirms that the lower amounts of assembly of the αΔThr1 mutant vs. the αΔAsn1 mutant is not peculiar to only one clone but is representative of all mutant αΔThr1 clones. Moreover, these data suggest that different amino acid replacements in this region appear to have a small affect unrelated to the absence of sugar. Even when a clone secretes much higher amounts of αΔAsn1 comparable to α-WT (clone 11-7, lanes *5* and *6*), <10% of the hCGβ is combined with this mutant or αΔAsn1 with hCGβ (compare subunits in lanes *5* and *6*). A similar situation is seen when a clone secreting high amounts of αΔThr1 is analyzed (data not shown). Absence of the oligosaccharide from position 52 seems to alter formation of hCG dimer even in the presence of excess β subunit which is unable to drive the dimerization reaction. Alternatively, loss of the oligosaccharide at this site may affect the stability of the α/β complex and cause intracellular dimer dissociation. In any case, the oligosaccharide at position 52 prevents secretion of intact hCG dimer by altering the assembly and/or stability of the dimer complex.

Analysis of clones secreting both hCGβ and glycosylation site II mutants (αΔAsn2 and αΔThr2) reveals that loss of the oligosaccharide of this position has no effect on combination. Equal amounts of the mutant subunits are immunoprecipitated by both the α and β antisera from clones 4-6 (lanes *11* and *12*), 7-10 (lanes *13* and *14*), and 7-17 (lanes *15* and *16*) indicating that ≈100% of the secreted α subunit is combined with hCGβ. Thus, even though loss of glycosylation site 78 causes increased degradation, it does not affect assembly with hCGβ.

Removal of both glycosylation sites from the α subunit (αΔAsn[1+2] and αΔThr[1+2]) has an intermediate effect on assembly with hCGβ. Analysis of clone 13-18 (lanes *17* and *18*) reveals that ≈65% of the total αΔAsn(1+2) secreted is in the form of dimer. Similarly, only ≈30% of the αΔThr-(1+2) is secreted as dimer from clone 27-9 (lanes *19* and *20*). These quantitative differences could be due to (*a*) the approximately twofold higher levels of hCGβ in clone 13-18 vs. clone 27-9, which could be driving more α to combine or

Figure 5. Kinetics of secretion of hCGα wild-type and Asn-glycosylation mutant subunits. CHO clones expressing α-WT (*A*), αΔAsn1 (*B*), αΔThr1 (*C*), αΔAsn2 (*D*), αΔThr2 (*E*), αΔAsn(1+2) (*F*), αΔThr(1+2) (*G*), and α-WT treated with tunicamycin (*H*) were pulse labeled for 20 min, chased for the indicated times in Medium III, immunoprecipitated with hCGα antiserum, and prepared as described in Materials and Methods. Tunicamycin (2 μg/ml) was present continuously in the preincubation, labeling, and chase media for the α-WT cells shown in *H*.

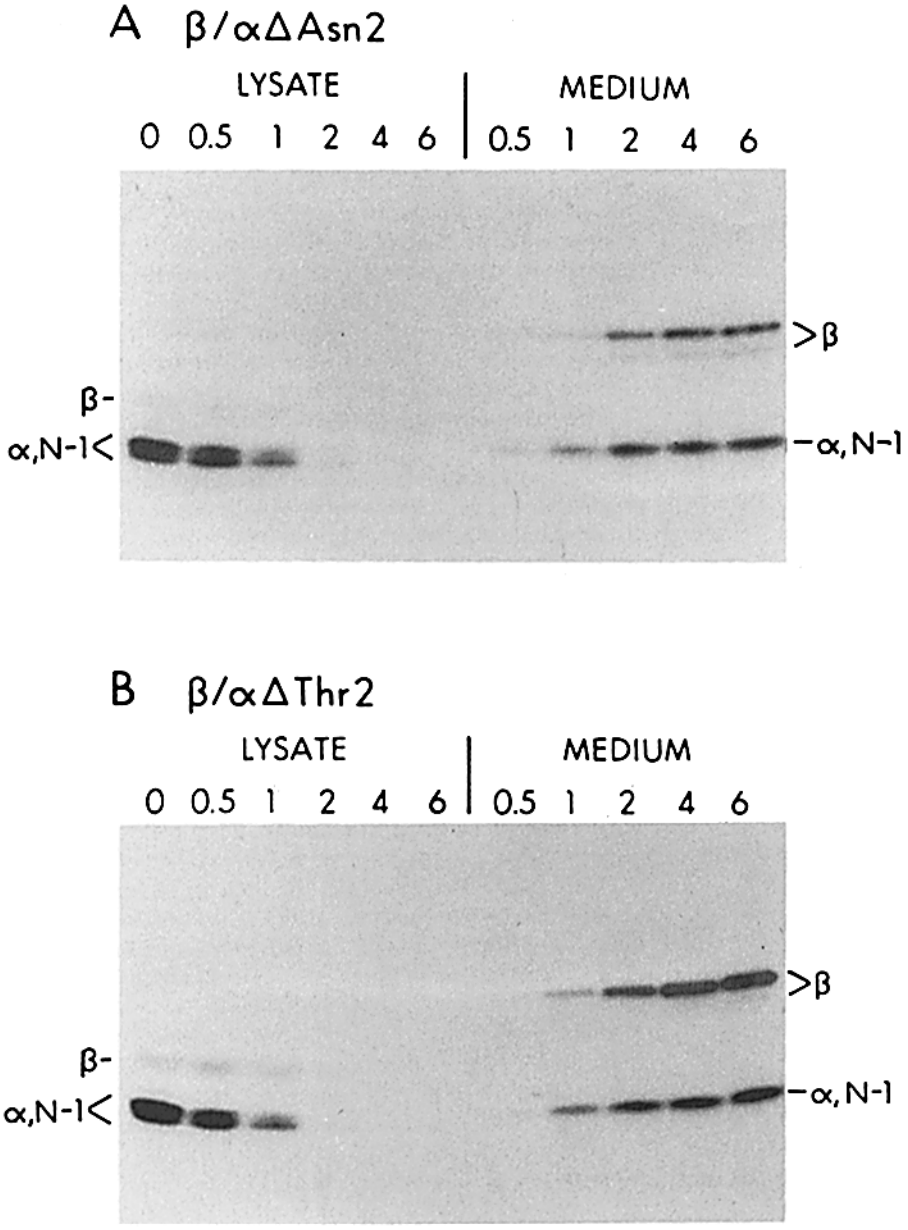

Figure 7. Kinetics of secretion and assembly of hCGα site II mutants with the hCGβ subunit. CHO clones expressing hCGβ and αΔAsn2 (*A*) and hCGβ and αΔThr2 (*B*) were treated as described in the legend to Fig. 5. The migration of the hCGα site II (*α,N-1*) and hCGβ (*β*) forms in the lysate and medium are shown.

(*b*) minor alterations in the protein backbone, as seen with the αΔAsn1 and αΔThr1 dimers discussed above. In both cases, though, the amount of hCGβ is severalfold in excess of the deglycosylated α mutants and is thus much higher than the ratio seen for wild-type (lanes *1* and *2*) where ≈100% combination has occurred. Thus, removal of both oligosaccharides alters assembly but to a lesser degree than loss of the oligosaccharide chain at position 52 alone. Treatment of dimer-secreting cells with tunicamycin also prevents assembly of the deglycosylated subunits, although in this case, lack of combination may also be due to loss of the two glycosylation sites on hCGβ and not to loss of the hCGα sites alone (data not shown).

Since αΔAsn2 and αΔThr2 efficiently combine with hCGβ, these clones were used to determine if the β subunit might stabilize or protect these mutant α subunits from degradation. Clones 4–6 (β/αΔAsn2) and 7–10 (β/αΔThr2) were subjected to pulse–chase analysis as described above, and both media and cell lysate samples were immunoprecipitated with an α-specific antiserum (Fig. 7). The disappearance of the intracellular forms of αΔAsn2 (Fig. 7 *A*, lysate) and αΔThr2 (Fig. 7 *B*, lysate) was identical and rapid ($t_{½}$ = 60 min). In both cases, hCGβ lacking its O-linked sugars is co-precipitated from the lysate with the mutant α forms although at much lower levels (Fig. 7, *A* and *B*, lysate). The appearance of the secreted αΔAsn2 and αΔThr2 (Fig. 7, *A* and *B*, medium) is similar ($t_{½}$ = 60 min), and is also accompanied by an equal amount of hCGβ as seen in Fig. 6. Unlike the secretion of free αΔAsn2 and αΔThr2 where <20% is secreted (Fig. 5, *D* and *E*), the presence of hCGβ

allows secretion of 42 and 49% of the synthesized αΔAsn2 and αΔThr2, respectively. Thus, the hCGβ stabilizes the site 78 mutants from degradation and allows greater than twofold more material to be secreted as dimer.

Discussion

Asn-linked oligosaccharides have been implicated in several physiologic functions, including the maintenance of intracellular stability, secretion, receptor binding and biological activity, and modulation of the plasma half-life (see references 2, 34). For gonadotropins, the role of the oligosaccharide chains in receptor binding and biological activity has been examined extensively using chemically or enzymatically treated hormone (1, 6, 21, 25, 33). Site-directed mutagenesis is an effective method to study the oligosaccharide sites on multiglycosylated proteins since individual glycosylation sites may have different functions. Here we changed both the Asn and Thr residues of the two glycosylation consensus sequences of hCGα. This control is critical in light of the findings of Santos-Aguado et al. (43) which show that different amino acid replacements in the glycosylation recognition sequence of HLA-A2 molecules, and not loss of carbohydrate, could affect secretion.

Site-directed mutagenesis was used to study the two Asn-linked oligosaccharides on hCGα and their intracellular role in the secretion of intact dimer. Mutagenesis of the Asn-linked oligosaccharide at position 52 (αΔAsn1 and αΔThr1) did not affect the secretion of the mutant subunit; the kinetics of secretion and recovery in the medium of the monoglycosylated form was identical to wild-type hCGα. Mutagenesis of the oligosaccharide at position 78 (αΔAsn2 and αΔThr2) revealed that the subunit was rapidly degraded and <20% was secreted. Loss of this oligosaccharide may disrupt folding and/or disulfide bond formation, altering the stability of the peptide and rendering it more sensitive to proteases. Normally, the oligosaccharide at position 78 of α-WT is resistant to Endo F unless the subunit is denatured first (data not shown). Although we do not know the basis for this resistance, it is possible that the α polypeptide folds around this oligosaccharide to prevent the endoglycosidase from acting. This oligosaccharide might then be an important structural feature of the α subunit similar to immunoglobulin molecules where the single oligosaccharide has been visibly seen to lie in a groove and to play a major role in the interaction of the F_c and F_{ab} regions (46). An alternative explanation for this degradation is that removal of the carbohydrate unmasks some determinant that directs the position 78 mutants to another compartment (e.g., lysosomes) where it is degraded.

Removal of both glycosylation sites (αΔAsn[1+2] and αΔThr[1+2]) slows secretion fourfold but allows ≈50% of the completely deglycosylated subunits to be secreted. Although an additive effect might be expected upon removal of both glycosylation sites, an intermediate amount of material was secreted and at a slower rate. The data suggest an interdependence of sugar sites—the presence of only one oligosaccharide seems to affect the tertiary structure of the molecules more than no carbohydrate at all; differences in the folding of the single-site mutants (αΔAsn2 and αΔThr2) vs. the double-site mutants might account for the differential sensitivity to proteolytic degradation. Because tunicamycin prevents addition of all carbohydrates to multiglycosylated proteins, it is incapable of revealing this type of interdependence. This interdependence is an interesting finding in light of the mutagenesis study of the multiglycosylated VSV G membrane protein (27). The absence of the individual glycosylation sites of VSV G protein has no effect on the secretion (27), whereas loss of both sites causes aggregation of the deglycosylated protein (15, 27). Similarly, aggregation of the α double-site mutants might also account for the differential secretion rates of the single and double mutants but cannot explain the other differences. These findings for the α glycosylation mutants will be useful in understanding the role of the oligosaccharides on other multiglycosylated membrane and secretory proteins.

In pituitary and placenta, hCGα is secreted in excess of hCGβ. The uncombined α subunit is larger and more heterogeneous than dimer-derived α subunit (the form of α combined with β) (7, 20, 39, 40). Of major interest are the structural modifications responsible for the increased heterogeneity of free α (α*). Corless et al. (9) have shown that in C127 cells transfected with hCGα this heterogeneity resides on the Asn-linked oligosaccharides and is not due to the presence of an O-linked oligosaccharide unit as seen in cultured choriocarcinoma cells (8). The secreted α-WT and the site 78 mutants from CHO cells are more heterogeneous, whereas the secreted site 52 mutants are very homogeneous (Fig. 5). This heterogeneity is also seen when the α-WT is secreted from CHO cells defective in O-linked glycosylation confirming that the heterogeneity resides on the N-linked oligosaccharides (data not shown). Although we cannot exclude the possibility that mutagenesis of one site can affect terminal processing of the other site, the data imply that the oligosaccharide at position 52 is likely responsible for α* heterogeneity. These findings also suggest that the α oligosaccharides undergo site-specific carbohydrate processing.

In vivo assembly was analyzed by coexpression of the hCGα wild-type and mutant genes with the wild-type hCGβ gene. Whereas >95% of the secreted wild-type hCGα and site II mutant subunits combine with hCGβ, <30% of the site I mutants are secreted as dimer, and only ≈50% of the double mutants dimerize. Thus, loss of the oligosaccharide from position 52 alters assembly and/or stability of the complex. Since the double mutants are secreted slowly, they may have a greater chance of interacting with a β subunit, which accounts for the increased assembly compared to αΔAsn1 and αΔThr1. Differences in folding between the single- and double-site mutants might also account for the variations seen; loss of carbohydrate at position 52 may perturb the folding of the mutant subunit, and thereby alter α/β assembly without affecting proteolytic stability of this mutant. Alternatively, carbohydrate at position 52 may be present at the subunit/subunit interface and thus affect the interaction of the α and hCGβ subunits. Previous studies have shown that carbohydrate–protein interactions are likely to be associated with subunit interactions for the neuraminidase tetramer of influenza virus (52) and the influenza hemagglutin trimer (54) where carbohydrate has been visualized at subunit interfaces.

In the presence of hCGβ, site II mutants are stabilized and secreted as intact dimer. After combination, hCGβ might

stabilize these mutants by blocking or altering a determinant that normally causes their degradation. Alternatively, hCGβ may aid the folding of the α subunit. Gething et al. (14) have extensively analyzed the folding process in studies of the intracellular transport and assembly of influenza hemagglutinin molecules. Similar analyses will be critical in understanding (*a*) the role of hCGα oligosaccharides in the folding process and (*b*) why removal of each oligosaccharide does not have an additive effect on the stability and dimerization of the double mutants. Differences in folding of each mutant in the absence of carbohydrate could account for the variations in stability and assembly seen for these mutants.

These mutagenized forms of hCGα should also be useful for determining the association of specific oligosaccharides to biological activity of hCG. Sairam and Bhargavi (41) used chemical deglycosylation of bovine luteinizing hormone and follicle-stimulating hormone to examine the role of the oligosaccharides in the individual subunits. They showed that in contrast to the Asn-linked oligosaccharides on the β subunits, Asn-linked carbohydrates units on the α subunit were more critical for biological activity. Only one of the two Asn-linked oligosaccharides on hCGα may be necessary for biological activity. That the two oligosaccharides have different intracellular functions (glycosylation site 78 plays a role in the integrity of the hormone, while site 52 is important for assembly of intact hCG molecules) suggests site-specific functions of the two oligosaccharides on hCGα. Whether this is a common feature of other secretory glycoproteins bearing multiple glycosylation sites, whether this feature is unique to hCGα, will require mutagenesis of other glycoproteins.

We gratefully acknowledge Christine Kornmeier and Mark Spangler for expert technical assistance, and Carol Patterson and Barbara Baiter for careful preparation of the manuscript. We also thank Drs. J. Baenziger, M. Bielinska, C. Corless, S. Kornfeld, J. Merlie, S. Schlesinger, and A. Strauss for critical reading of the manuscript and helpful discussions. We also thank Drs. Wayne Barnes, Monty Krieger, and Richard Palmiter for technical advice.

This work was partially supported by grants from the National Institutes of Health (HD-20197) and the Monsanto Company. M. Matzuk is a participant in the Medical Scientist Training Program supported in part by grant HL-07275 from the National Heart, Lung, and Blood Institute and National Institute of General Medical Sciences grant GM07200.

Received for publication 19 October 1987, and in revised form 8 December 1987.

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