Human chorionic gonadotropin (hCG) is a heterodimeric member of a family of cystine knot-containing proteins that contain the consensus sequences Cys-X_{1}-Gly-X_{2}-Cys and Cys-X_{2}-Cys. Previously, we characterized the contributions that cysteine residues of the hCG subunit cystine knots make in folding, assembly, and bioactivity. Here, we determined the contributions that noncysteine residues make in hCG folding, secretion, and assembly. When the X_{1}, X_{2}, and X_{3} residues of hCG-α and -β were substituted by swapping their respective cystine knot motifs, the resulting chimeras appeared to fold correctly and were efficiently secreted. However, assembly of the chimeras with their wild type partner was almost completely abrogated. No single amino acid substitution completely accounted for the assembly inhibition, although the X_{2} residue made the greatest individual contribution. Analysis by tryptic mapping, high performance liquid chromatography, and SDS-polyacrylamide gel electrophoresis revealed that substitution of the central Gly in the Cys-X_{1}-Gly-X_{2}-Cys sequence of either the α- or β-subunit cystine knot resulted in non-native disulfide bond formation and subunit misfolding. This occurred even when the most conservative change possible (Gly → Ala) was made. From these studies we conclude that all three “X” residues within the hCG cystine knots are collectively, but not individually, required for the formation of assembly-competent hCG subunits and that the invariant Gly residue is required for efficient cystine knot formation and subunit folding.

The cystine knot motif defines a superfamily of dimeric proteins and appears to function as a structural scaffold that stabilizes the 3-loop structures of the individual subunits (1).

As shown in Fig. 1, the cystine knot consists of three disulfide (S-S)^1 bonds; two of these bonds bridge adjacent polypeptide strands, creating a ring that includes the intervening polypeptide backbone, and the third bond penetrates this ring (1, 2). The cystine knot is common to a biologically diverse set of dimeric proteins including transforming growth factor-β, vascular endothelial growth factor, platelet-derived growth factor, and human chorionic gonadotropin (hCG) (1–4). Additionally, more than 30 other proteins are predicted to contain this motif (2). The functional importance of the cystine knots of hCG (5–9), transforming growth factor-β1 (10), and platelet-derived growth factor (11) is evident from studies where cysteine residues within the knot were mutated, thus preventing a particular S-S bond from forming. Disruption of the cystine knot disulfides results in the synthesis of nonfunctional proteins that are usually degraded intracellularly. Thus, a more detailed understanding of the functional components of cystine knots will help to understand how members of this emerging protein family fold and assemble into biologically active molecules.

The subunits of hCG are prototypes for the cystine knot growth factor family (1). Heterodimeric hCG forms when hCG-β assembles with the common glycoprotein hormone α-subunit (GPH-α). GPH-α also assembles with luteinizing hormone β-subunit, thyroid-stimulating hormone β-subunit (TSH-β), and follicle-stimulating hormone β-subunit to form luteinizing hormone, TSH, and follicle-stimulating hormone β-subunit, respectively (12). Luteinizing hormone-β, TSH-β, and follicle-stimulating hormone β also contain the consensus residues required to form a cystine knot, but the actual presence of the knot has not yet been confirmed by structural studies.

Previously, we described the folding pathways of both hCG-β (9, 13–15) and GPH-α (6, 16) using S-S bond formation as an index of folding. The methods established in our laboratory for the folding of these two subunits now allow us to determine the importance of noncysteine residues of the cystine knot in hCG subunit folding, secretion, and assembly. Fig. 1 illustrates the location of the cystine knot motifs for both subunits of hCG, as well as the noncysteine knot disulfides (17, 18). The 8-residue ring of the cystine knot consists of four cysteines that form two S-S bridges, a Gly residue common to all 8-membered cystine knot rings, and three nonconserved residues (termed X_{1}, X_{2}, and X_{3}). Thus, the consensus sequences for this motif can be

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The abbreviations used are: S-S, disulfide; hCG, human chorionic gonadotropin; GPH-α, glycoprotein hormone α-subunit; TSH, thyroid-stimulating hormone; DTT, dithiothreitol; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; WT, wild type; HPLC, high performance liquid chromatography.
Creating the so-called “seat-belt.”

In this section, the spatial arrangement of cystine knots in hCG-β and hCG-α subunits is described. The noncystine knot S-S bond with Cys7 (Fig. 1) in hCG-β and Cys3 (Fig. 1) in hCG-α facilitates efficient assembly and (ii) the presence of the central invariant Gly residue in both cystine knots was determined using various amino acid substitutions. Furthermore, the role of the intervening Gly residue in folding, secretion, and assembly by employing a chimeric strategy where the residues within the hCG-β L1, L2, and L3 positions vary among hCG-α (19) and CysGlyCysGly (CGGC) was defined as C-X2-C-X-C and C-X-C (3, 19).

The residues at the X1, X2, and X3 positions vary among cystine knot-containing proteins and their functional importance is largely unknown. The sequence containing X1 and X2 in all four glycoprotein β-subunits is CAGYC. The equivalent sequence in GPH-α is CGMGC, where the Cys at the X2 position forms a noncystine knot S-S bond with Cys7 (Fig. 1). In this report, we investigated the contribution of hCG X1, X2, and X3 residues in folding, secretion, and assembly by employing a chimeric strategy where the residues within the hCG-β and GPH-α cystine knots were interchanged individually or collectively. Furthermore, the role of the intervening Gly residue in both cystine knots was determined using various amino acid substitutions. We report that: (i) there is a subunit-specific complement of three “X” residues, all of which are needed for efficient assembly and (ii) the presence of the central invariant Gly is an absolute requirement for efficient folding, cystine knot formation, and hCG assembly.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293T cells (20) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, and penicillin (100 units/ml)/streptomycin (100 μg/ml) (Life Technologies, Inc.).

**Site-directed Mutagenesis**—Mutations were made using a “megaprimer” polymerase chain reaction methodology (21) with Pfu polymerase (Stratagene). The GPH-α polymerase chain reaction products were cloned into pECDNA (Invitrogen) and the hCG-β polymerase chain reaction products were cloned into pGEM (9). DNA sequencing confirmed incorporation of desired mutations. Plasmid DNA was purified using the Maxi Plasmid Kit (Qiagen) according to the manufacturer's protocol and used for transient transfection as described below.

**Transient Transfection**—293T cells (1.9 × 10⁶) were plated into 60-mm plastic dishes and grown overnight to 70–80% confluency. Plasmid DNA was precipitated as described previously (6). For coexpression of hCG-β and GPH-α, both plasmids were included in the precipitation. To obtain comparable expression levels in coexpression studies, a 40:1 GPH-α to hCG-β ratio of plasmid was used. The resulting precipitate was added dropwise to the dishes and agitation gently to mix. To ensure a uniform precipitate exposure, one large-scale precipitation was distributed equally among all dishes. Cells were incubated for 2 days at 37 °C and used for metabolic labeling.

**Metabolic Labeling with [35S]Cysteine**—Transiently transfected 293T cells were pulse-labeled for the times indicated in the text with [35S]Cysteine (~110 Ci/mm; PerkinElmer Life Sciences), at a concentration of 100–150 μCi/ml, in serum-free medium lacking cysteine (9). For experiments using dithiothreitol (DTT), the DTT was added with the [35S]Cysteine at a final concentration of 2.0 mM. Pulse incubations were carried out as described previously (13) and cells were incubated for the chase times indicated; the chase medium was saved for secretion studies. Cells were harvested by rinsing with cold phosphate-buffered saline and immediately lysed in 2.5 ml of phosphate-buffered saline containing detergents (1.0% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS), protease inhibitors (20 mM EDTA and 2 mM phenylmethylsulfonyl fluoride), and 5 mM N-ethylmaleimide (NEM), pH 7.0, or 50 mM iodoacetate, pH 8.0, to trap free thiols in folding intermediates that contained unformed S-S bonds. NEM was used for GPH-α because it results in efficient alkylation of GPH-α thiols and better separation of folding intermediates by HPLC (6). Similarly, iodoacetate was used for hCG-β because it efficiently alkylates β-subunit thiols and facilitates mapping of hCG-β tryptic peptides (13, 14). Cell lysates were incubated for 10–20 min at room temperature in the dark, followed by disruption through a 22-gauge needle (5 times) and centrifuged for 1 h at 100,000 × g.

**Immunoprecipitation of hCG Subunits from Cell Lysates and Chase Media**—Immunoreactive forms of GPH-α or hCG-β were immunoprecipitated with polyclonal antibodies specific for each respective subunit (6, 22). Immunoprecipitations were carried out at 4 °C for 16–20 h with rotation in the dark. Immune complexes were precipitated with protein A-Sepharose (Sigma) and prepared for SDS-polyacrylamide gel electrophoresis (PAGE) or reversed-phase HPLC as described below.

**SDS-PAGE and Quantitation of [35S]Cysteine-labeled Subunits**—Radiolabeled folding forms that adsorbed to protein A-Sepharose beads were eluted as described previously (13). Briefly, protein A-Sepharose beads containing immunopurified subunits were resuspended in two concentrated SDS gel sample buffer (125 mM Tris-HCl, pH 6.8, containing 2% SDS, 20% glycerol, and 40 μg/ml bromphenol blue). For reducing conditions, β-mercaptoethanol was included at a final concentration of 2%. Samples were boiled for 5 min, loaded on polyacrylamide gradient slab gels (5–20%), and run by the method of Laemmli (23). Gels were stained by vacuum on filter paper and exposed to a phosphorscreen (Molecular Dynamics). The phosphorscreen was scanned on a Molecular Dynamics ImageQuant (version 5.0) volume report. To determine the percentage of GPH-α that had combined with hCG-β in Figs. 3 and 7, the following formula was used: [100/12 (amount of hCG-β that immunoprecipitated with GPH-α) + (amount of GPH-α in anti-β immunoprecipitation)/total GPH-α]. For the α-C31Y mutant, 8/12 was used instead of 10/12 because this mutant has only 8 cysteine residues.

**Reversed-phase HPLC Analysis**—Radiolabeled folding intermediates that adsorbed to protein A-Sepharose beads were prepared as described previously (13). Briefly, protein A-Sepharose beads/antibody-antigen immunocomplexes were washed three times with phosphate-buffered saline containing detergents (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) followed by four washes with phosphate-buffered saline lacking detergents. Immunocomplexes were pelleted between washes by centrifugation for 1 min at 2000 × g. To dissociate the Sepharose/antibody/antigen interactions, immunocomplexes were treated with 6 μM guanidine HCl, pH 3.0 (sequential grade; Pierce), for 16–20 h while rotating at room temperature. 100 μg of myoglobin was added as a carrier. The guanidine eluates were injected onto a Vydac 300-Å C4 reversed-phase column equilibrated with 0.1% trifluoroacetic acid and eluted using an acetonitrile gradient as described previously (14). Fractions were collected in 1-min intervals and quantitated by scintillation counting. Samples were stored at −20 °C until further characterization.

**Tryptic Digestion and Reversed-phase HPLC Analysis of Tryptic Pep...**
tides—HPLC fractions from C4 reversed-phase HPLC representing hCG-β folding intermediates ρ1 or ρ2 were pooled, concentrated, and digested for 16–20 h in silanized polypropylene tubes containing 100–200 μg of myoglobin, 0.03% trypsin (Sigma), 5 mM CaCl2, and 50–150 mM Tris-HCl, pH 8. Digestions were continued for 2 h with two additional aliquots of 25 μg of trypsin (0.06% final concentration) (13, 14). hCG-β tryptic peptides were separated on a Vydac C4 reversed-phase column as described previously (14). Amino acid sequencing was used previously to identify the peptide(s) in each peak (14).

Amino Acid Analysis Procedure to Determine S-S Bond Content—A modified protocol, similar to the one determined in the S-S bonding pathways for potato carboxypeptidase inhibitor and human epidermal growth factor (24, 25) was used. [35S]cysteine-containing folding forms isolated from reversed-phase HPLC were dried in vacuo and hydrolyzed as described previously (6). Quantitation of [35S]cysteine and succinyl-[35S]cysteine (hydrolysis product of NEM-Cys) was performed using a modification of the method described by Cohen and Michael (26). Hydrolysates were resuspended in 10 μl of 10 mM HCl. To this, 70 μl of 0.2% formic acid, pH 8.8, was added. Derivatization of amino acids was performed by adding 30 μl of 6-aminooquinonyl-N-hydroxysuccinimidyl carbamate (8 mg/ml in anhydrous acetonitrile). Samples were dried in vacuo. Before injection, samples were resuspended in 110 μl of buffer A (140 mM sodium acetate, 1% trypsin, pH 4.9). Derivatized amino acids were separated by HPLC as described previously (6) using buffer A and buffer B (60% acetonitrile in water). The column was eluted at 1.0 ml/min at 30 °C and 1-ml fractions were collected and quantitated by scintillation counting. Recovery of [35S]cysteine represented S-S bonded cysteine residues, whereas succinyl-[35S]cysteine represented cysteine residues of unformed S-S bonds. The percentage of [35S]cysteine and succinyl-[35S]cysteine was calculated by dividing the counts/min recovered for each species by the total counts/min recovered. Fully folded [35S]cysteine-labeled hCG-β was used as a positive control for [35S]cysteine content.

RESULTS

Swapping of hCG-β and GPH-α Cystine Knot Motifs—The only conserved sequences in cystine knot-containing proteins that have an 8-membered ring are the C-X1-G-X2-C and C-X2-C sequences (3, 19). The contribution that the X residues make toward attaining a native conformation is unknown. Furthermore, it is not known whether cystine knot sequences are protein-specific, or whether the residues of the knot motif are functionally interchangeable. To address this, we used a chimeric strategy wherein cystine knot residues of GPH-α and hCG-β were swapped singly or collectively.

Swapping of hCG cystine knot motifs was accomplished by site-directed mutagenesis at the X1, X2, and X3 positions to match the residue(s) of the other subunit. Three single GPH-α mutants (α-M29A, α-C31Y, and α-H83Q) and a GPH-α mutant containing all three mutations (termed αGPH-α) were constructed for hCG-β. Cys7, which normally pairs with Cys31 to form a S-S bond, was also converted to Ala so that no unpaired thiols remained. Cys7, which normally pairs with Cys31 to form a S-S bond, was also converted to Ala so that no unpaired thiols remained. Removal of S-S bond 7–31 does not affect GPH-α folding, secretion, combination with hCG-β or hCG bioactivity (6, 7).

TABLE I

| GPH-α and hCG-β cystine knot chimeras |
|--------------------------------------|
| **GPH-α variant** | **C-x1-G-x2-C**a | **C-x3-C**b |
| α-WT | C-M-G-C-C | C-H-C |
| α-M29A | C-M-G-C-C | C-H-C |
| α-C31Y | C-M-G-C-C | C-H-C |
| α-H83Q | C-M-G-C-C | C-H-C |
| αβ-knot | C-M-G-C-C | C-H-C |
| **hCG-β variant** | **C-x2-G-x3-C**c | **C-x3-C**d |
| β-WT | C-A-G-Y-C | C-Q-C |
| β-A35M | C-M-G-Y-C | C-Q-C |
| β-Y37A | C-A-G-A-C | C-Q-C |
| β-Q89H | C-A-G-Y-C | C-Q-C |
| βα-knot | C-M-G-A-C | C-Q-C |

* Residues 28–32 in GPH-α.
* Residues 82–84 in GPH-α.
* Residues 34–38 in hCG-β.
* Residues 88–90 in hCG-β.
* Cys’, which pairs with Cys1 to form a S-S bond, was also converted to Ala so that no unpaired thiols remained. Removal of S-S bond 7–31 does not affect GPH-α folding, secretion, combination with hCG-β or hCG bioactivity (6, 7).

Swapping of cysteine knot residues was performed by adding 30 μl of 6-aminooquinonyl-N-hydroxysuccinimidyl carbamate (8 mg/ml in anhydrous acetonitrile). Samples were dried in vacuo. Before injection, samples were resuspended in 110 μl of buffer A (140 mM sodium acetate, 1% trypsin, pH 4.9). Derivatized amino acids were separated by HPLC as described previously (6) using buffer A and buffer B (60% acetonitrile in water). The column was eluted at 1.0 ml/min at 30 °C and 1-ml fractions were collected and quantitated by scintillation counting. Recovery of [35S]cysteine represented S-S bonded cysteine residues, whereas succinyl-[35S]cysteine represented cysteine residues of unformed S-S bonds. The percentage of [35S]cysteine and succinyl-[35S]cysteine was calculated by dividing the counts/min recovered for each species by the total counts/min recovered. Fully folded [35S]cysteine-labeled hCG-β was used as a positive control for [35S]cysteine content.

function of hCG Cystine Knot Residues

The six single mutants (three GPH-α and three hCG-β), two triple mutants (αβ-knot and βα-knot), and wild type (WT) subunits were analyzed by SDS-PAGE (14, 22). No significant differences in folding were observed for any of the mutants (data not shown), suggesting that these cystine knots may be interchangeable. To test this further, we determined the efficiency of subunit secretion. Secretion was assayed by pulse labeling transiently transfected 293T cells for 10 min, followed by a 10-min or 8-h chase. The immunopurified cell lysates and media were analyzed by SDS-PAGE (Fig. 2A), and the bands were quantitated as described under “Experimental Procedures.” The percent secretion for the GPH-α and hCG-β chimeras are shown in Fig. 2, B and C. Consistent with previous studies, about 80% of WT GPH-α and hCG-β were secreted by 8 h. Furthermore, swapping of single residues or the entire cystine knot did not significantly affect subunit secretion of GPH-α or hCG-β (Fig. 2). This efficient secretion is another indicator that these subunits folded to a native or native-like conformation, since misfolded or incompletely folded hCG subunits are generally retained intracellularly and degraded (5, 6).

Finally, we assayed for the ability of the GPH-α cystine knot chimeras to assemble with β-WT and, conversely, for the ability of hCG-β cystine knot chimeras to assemble with α-WT. To do this, 293T cells coexpressing both subunits were pulse-labeled and chased for 8 h. Unassembled GPH-α and intact hCG αβ dimer were first precipitated from the collected medium with a polyclonal α-antiserum. This step was followed by a second precipitation with a polyclonal β-antiserum to recover any excess, unassembled hCG-β. There was a dramatic decrease in the ability of αβ-knot and βα-knot to combine with β-WT and α-WT, respectively (Fig. 3, A and B). Furthermore, this decreased combination was not due to altering any other residue, as all of the single mutations were significantly less deleterious than the triple mutations. However, the single mutations at the X2 position (α-C31Y and β-Y37A) did decrease assembly to a level intermediate to that of WT and the triple mutants. Co-transfection of αβ-knot and βα-knot together resulted in <5% assembly (data not shown). Taken together, these data indicate...
that the noncysteine residues within the GPH-α and hCG-β cystine knots contribute to an assembly-competent conformation in a subunit-specific manner. Furthermore, this contribution appears to be a function of the set of all noncysteine residues, as opposed to arising from the contribution of a single residue.

The Central Gly Residue in the hCG-β Cystine Knot Is Critical for Folding, Secretion, and Assembly—As alluded to above, the central Gly residue in hCG-β is conserved among all known cystine knots that contain an 8-membered ring structure (1, 2). A mutation resulting in the conversion of the central Gly residue in hCG-β to Arg in TSH-β alters folding, secretion, and/or assembly (28), G36N was chosen because Asn has a smaller neutral side chain in comparison with the positively charged Arg, and G36A was chosen because it is the most conservative change possible; however, Ala in most cases can adopt the required positive torsion angle only under conditions of unfavorable steric hindrance (29). 293T cells expressing WT or cystine knot chimeras (panel A) or WT hCG-β and GPH-α (WT or cystine knot chimeras) (panel B) were pulse-labeled with [35S]cysteine and chased for 8 h. The medium was collected and immunoprecipitated with polyclonal antibody to GPH-α. This immunoprecipitation pulls down unassembled and assembled GPH-α and co-precipitates hCG-β combined with GPH-α. Shown above each bar is a representative SDS-PAGE gel. The remaining unassembled hCG-β was immunoprecipitated with anti-β to ensure that there was excess hCG-β such that it was not a limiting factor in assembly (data not shown). In some cases, a portion of the total GPH-α was present in the anti-β immunoprecipitation and was included in calculating the percentage of combined GPH-α. Quantitation was performed as described under “Experimental Procedures” and is shown in the bar graphs for WT subunits (solid bars), composite chimeras (open bars), and single mutants (shaded bars). In both A and B, the results are displayed as the percentage of total secreted GPH-α that had dimerized with hCG-β. Each bar represents the mean ± S.D. of at least three experiments.

β-G36N, and β-G36R. Mutation to Arg was chosen because this mutation is observed in the naturally occurring TSH-β mutant (28), G36N was chosen because Asn has a smaller neutral side chain in comparison with the positively charged Arg, and G36A was chosen because it is the most conservative change possible; however, Ala in most cases can adopt the required positive torsion angle only under conditions of unfavorable steric hindrance (29). 293T cells expressing β-WT, β-G36N, β-G36R, or β-G36A were pulse-labeled with [35S]cysteine and chased for 0, 5, 15, 30, 60, 120, or 480 min. Fig. 4A shows the progression of β-WT from pβ1 (the earliest detectable folding intermediate) to pβ2 to mature β. At chase times ≥60 min, β-WT was detectable in the media as mature, secreted β. Fig. 4B shows that most of β-G36A did not progress beyond pβ1 and was not secreted. However, a pβ2-like species of β-G36A was isolated by reversed-phase HPLC (Fig. 4C). This species was termed ‘‘pβ2-like’’ since it eluted from HPLC at a similar time to that of WT pβ2 (13, 14). Folding and secretion data for β-G36N and β-G36R subunits yielded similar results to those shown in Fig. 4 (not shown).
Tryptic digestion of fully folded native hCG-β (i.e. all native S-S bonds formed) produces [35S]cysteine-labeled peptides, all of which are linked by S-S bonds (13, 14). If a particular S-S bond has not yet formed in a given intermediate, then digestion with trypsin to release peptides not linked by S-S bonds from the otherwise S-S linked core. The resulting mixture of peptides and S-S linked peptides were separated by C18 reversed-phase HPLC. A, trypsic map of WT pβ1. B, trypsic map of β-G36A pβ1. C, trypsic map of WT pβ2. D, trypsic map of β-G36A pβ2-like. The identities of the peaks (13, 14) are: peak 1α and 1b, peptide 96–104; peak 2, peptides 69–74 and 105–114; peak 3, peptide 87–94; peak 4, peptide 9–20; peak 5, S-S linked peptides. Peaks marked with an asterisk are peaks that appear to represent S-S-linked peptides connected by non-native S-S bonds (13, 14).

Taken together, these data indicate that the central Gly of the hCG-β CAGYC region is critical for the proper formation of S-S bonds and thus, is important for its folding and secretion. This also implies that the Gly → Arg mutation observed in congenital isolated TSH deficiency (28) results from improper folding of TSH-β, which prevents its assembly with GPH-α.

Mutation of the Invariant GPH-α Cystine Knot Gly Residue—The previous section demonstrated that the Gly residue in the CAGYC sequence of hCG-β is critical for proper folding. This implies that this Gly may be critical for the folding of other cystine knot-containing proteins as well. To test this, we made the equivalent G30A mutation in the CMGCC sequence of GPH-α.

Unlike hCG-β folding intermediates, GPH-α intermediates do not migrate differently on SDS-PAGE (6). However, GPH-α folding can be monitored by changes in reversed-phase HPLC elution times; unfolded GPH-α containing no S-S bonds and being more hydrophobic, elutes later than the native conformation and folding intermediates (6, 16). Moreover, HPLC elution position correlates with the formation of S-S bonds as GPH-α folds to its less hydrophobic conformation (i.e. earlier eluting species contain more S-S bonds than the later eluting, less folded species) (6, 16). Shown in Fig. 6 are the HPLC profiles generated for α-G30A after a 10-min pulse in the presence of DTT, followed by 0-, 5-, and 30-min chases after DTT removal. DTT was used in the pulse to delay the formation of S-S bonds until its removal during the chase (6). α-G30A folding did not generate a species that eluted at the position of native α-WT (Fig. 6, vertical dotted line). Additionally, a late eluting peak
Function of hCG Cystine Knot Residues

Amino acid analysis was used to determine the relative amount of S-S bond formation as described under “Experimental Procedures.” The folding forms listed below correspond to the intermediates referred to on the HPLC chromatograms in Fig. 6. Shown are the calculated percentages of S-S bond formation (S-S), unformed S-S bonds (NEM-Cys), and the calculated number of S-S bonds formed. Native α-WT represents the fully folded, assembly-competent conformation (6).

| Table II  | Quantitation of S-S content in α-G30A folding intermediates |
|-----------|--------------------------------------------------------------|
| α-WT      | S-S              | NEM-Cys | Number of S-S bonds |
| Native    | 99 ± 0.7         | 1.2 ± 0.7 | 4.9 (5)              |
| α-G30A    | 75 ± 3.2         | 25 ± 3.2 | 3.8 (5)              |
| α1        | 96 ± 1.3         | 3.5 ± 1.3 | 4.8 (5)              |
| α2        | 100 ± 0.5        | 0.7 ± 0.7 | 1.3 (5)              |

*a Mean ± S.D. of at least three experiments.
*b Calculated as percentage S-S × total possible disulfide bonds that could theoretically form. The number in parentheses denotes the possible number of S-S bonds that could form for each GPH-α variant.

To determine the efficiency of secretion for α-G30A, 293T cells expressing α-G30A or α-WT were pulse-labeled for 10 min with [35S]cysteine and chased for 10 min or 8 h. The immunopurified cell lysates and media containing GPH-α were analyzed by reducing SDS-PAGE and bands quantitated as described under “Experimental Procedures.” About 80% of α-WT present at 10 min was secreted into the medium by 8 h (Fig. 7A). In contrast, only about 40% of α-G30A was secreted (Fig. 7A). Furthermore, <10% of α-G30A remained in the cell after 8 h (data not shown), indicating that about 50% had been degraded. This is consistent with our previous reports (6, 16) demonstrating that mutant forms of GPH-α that do not migrate at the position of α-WT on HPLC (i.e. they contain a non-native conformation) are readily degraded.

To determine whether α-G30A could assemble with hCG-β, both subunits were coexpressed in 293T cells, pulse-labeled with [35S]cysteine for 20 min, and chased for 8 h. Immunopurified subunits were analyzed by SDS-PAGE, the bands were quantitated, and the percentage of GPH-α that had combined with hCG-β was calculated (see “Experimental Procedures”). Results of this analysis are shown in Fig. 7B. Only 30% of the secreted α-G30A combined with WT hCG-β, compared with 80% for α-WT. These results further demonstrate the importance of the central cystine knot Gly residue in protein folding and heterodimer formation.

**DISCUSSION**

The growth factor cystine knot superfamily of dimeric proteins contain similar structural topologies but lack significant sequence similarity other than the spacing of the six cysteine residues that form the three cystine residues of the knot (1). The importance of the cystine residues in producing functional proteins has been well documented (6, 7, 9–11, 16), but the role of the noncysteine residues located within these cystine knots is largely unknown.

All known growth factors containing a cystine knot motif have an 8-amino acid ring structure, with the exception of nerve growth factor, which contains a 14-membered ring. For the 8-residue rings, such as those found in GPH-α and hCG-β, the two sides of the ring contain 5- and 3-residues and are linked by two S-S bridges. Other than the cysteine residues, the only conserved residue of the ring is a Gly located at the central position of the 5-residue stretch, such that this sequence is termed C-X₇-G-X₃-C. Studies showing that this Gly residue is essential for producing functional TSH (28, 30) have been repeated in hCG-β using identical (Gly → Arg) and similar (Gly...
was evident from the non-native S-S bonds that had formed (Fig. 5), as well as the failure to efficiently convert to the pβ2 folding intermediate (Fig. 4B). The resulting non-native S-S bond formation and misfolding provides an explanation for why the central Gly residue is essential for hCG function.

Mutation of the equivalent Gly in GPH-α (α-G30A) gave similar results, although, the deleterious effects were less pronounced; 40% of α-G30A was secreted, 30% of which assembled with WT hCG-β. This result is consistent with a study that detected immunoreactive hCG when α-G30A and WT hCG-β were coexpressed in X. laevis oocytes (31). However, the mutant hCG heterodimer displayed no bioactivity in a murine testosterone production-based Leydig cells bioassay, suggesting that native hCG conformation was not attained (31).

A notable effect of the G30A mutation on GPH-α folding was that it slowed folding significantly. Following a 30-min chase, less than half of the α-G30A synthesized converted to the most folded form that contained five S-S bonds. This compares with a τ1/2 of about 90 s for WT GPH-α folding. Previously, we reported that disruption of the GPH-α cystine knot S-S bonds results in inefficient folding and secretion (6). The observation that α-G30A was inefficiently folded and secreted implies that the α-G30A mutation also interfered with formation of the GPH-α cystine knot.

There are several possible explanations for why mutation of the invariant Gly was more detrimental to hCG-β folding than that of GPH-α. First, the inherent flexibility of the GPH-α loop 2 (residues 33–58) (33) may allow for greater perturbation of the GPH-α cystine knot and permit closure of the cystine knot ring in a portion of the molecules by adopting the positive φ torsion angle needed at residue 30. Second, WT hCG-β folds at a much slower rate compared with GPH-α (τ1/2 = 5 min versus τ1/2 = 90 s, respectively) and, therefore, when the rate of hCG-β folding is slowed even further (as noted above for α-G30A), β-G36A may be more readily degraded before the subunit has time to fold. The latter possibility is based upon a recently proposed model (34, 35) that suggests that glycoproteins only have a limited amount of time to fold and exit the endoplasmic reticulum before being degraded.

The three other noncysteine residues of the hCG cystine knots are X1, X2, and X3 of the C-X1-G-X2-C and C-X3-C sequences. To understand the role(s) of these residues, we constructed chimeras in which the X residues of GPH-α and hCG-β were swapped individually or in combination, while leaving the central Gly unchanged. Folding and subunit secretion was not significantly affected in either the single or the triple mutants (αβ knot and βα knot) (Fig. 2). However, assembly of αβ knot and βα knot with their WT partners was decreased by about 90% (Fig. 3). The decrease in assembly was not due to any one particular substitution at the X1, X2, or X3 positions but, rather, was due to the combination of all three substitutions, suggesting that the set of all three X residues act in a subunit-specific manner.

The observation that αβ knot and βα knot are efficiently secreted but do not assemble adds to a growing body of evidence that suggests that determinants necessary for assembly and secretion of hCG subunits are different. Recently, we reported two examples of modified GPH-α subunits that are efficiently secreted but do not assemble with hCG-β (6); one modification changed residues in loop 2 necessary for combination while the other modification simultaneously removed both 7–31 and 59–87 S-S bonds. In addition, hCG-β mutants lacking the 93–100 or 26–110 S-S bonds are also efficiently secreted but do not assemble with GPH-α (5, 9). Thus, structural determinants needed for hCG assembly are not necessarily required for subunit secretion.

Function of hCG Cystine Knot Residues

was not necessarily required for hCG assembly.

→ Asn) mutations (31, 32). However, a lack of structural data and knowledge of the folding mechanisms for these subunits at the time of these observations failed to define why this Gly is critical. In light of more recent findings, including the hCG crystal structure (17, 18) and knowledge of GPH-α and hCG-β folding (6, 9, 14, 16), we can now address the mechanism by which specific residues within the hCG cystine knots contribute to hormone function. In particular, results from mutational analyses can be more precisely interpreted because we can distinguish between two general consequences of these mutations: (i) the mutation removes a key residue important for a direct subunit interaction; or (ii) the mutation causes global misfolding such that the protein cannot attain native structure.

The central Gly residue located between X1 and X2 is thought to be necessary because, in contrast with other amino acids, Gly can readily adopt a positive φ torsion angle, which allows it to avoid steric hindrance with the penetrating S-S bond of the cystine knot (1). The biological importance of this Gly can be inferred from several observations. First, a naturally occurring Gly → Arg mutation in TSH-β causes congenital isolated TSH deficiency (28). Second, mutation of the equivalent Gly in GPH-α prevents the production of functional hCG (31) and TSH (30). Third, mutation of this Gly to Arg or Asp in hCG-β results in undetectable levels of heterodimeric hCG being secreted from X. laevis oocytes (32).

In this report, we investigated the role of the invariant cystine knot Gly residue in the folding, secretion, and assembly of GPH-α and hCG-β, two prototypes of the growth factor cystine knot superfamily (1). Even the most conservative substitution possible, Gly → Ala, resulted in nearly 100% of hCG-β being misfolded and degraded intracellularly. The misfolding

Fig. 7. Secretion and assembly of α-WT and α-G30A. A, 293T cells expressing α-WT or α-G30A were pulse-labeled 10 min and chased for 10 min or 8 h. [35S]Cysteine-labeled GPH-α was immunoprecipitated from the 10-min cell lysate (representing intracellular GPH-α) and 8 h medium (representing secreted GPH-α). The immunopurified subunits were analyzed by reducing SDS-PAGE and quantitated. The percentage of GPH-α that remained in the cell lysate (data not shown), indicating that by 8 h the majority of the radiolabeled subunit had either been secreted or degraded intracellularly. B, 293T cells expressing α-WT or α-G30A and hCG-β were pulse-labeled with [35S]Cysteine and chased for 8 h. The percentage of GPH-α that had combined with WT hCG-β was determined as described in the legend to Fig. 3. Each bar represents the mean ± S.D. of at least three experiments.

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Our data suggest that the noncysteine residues within the hCG-β and GPH-α cystine knots are critical for intersubunit interactions that are necessary to form a stable dimer. This finding is further supported by an important feature of the hCG structure (17, 18). At the core of the dimer interface is a series of interchain β-sheets. Intimately involved in this β-sheet are the regions encompassing the C-X₆-G-X₆-C sequences of both subunits (residues 25–39 of GPH-α and 27–40 of hCG-β), which form a significant number of intersubunit hydrogen bonds. Thus, the simultaneous alteration of multiple X residues, as was done in α₅_knot and β₅_knot, could interfere with formation of this intersubunit β-sheet, whereas single changes might be less disruptive because most other intersubunit interactions remain intact.

The set of all three X residues within both hCG cystine knots are required for biological activity since they are necessary for dimer formation (Fig. 3) and only the hCG heterodimer is functional (12). Whether or not this region is important for receptor binding and signal transduction is unknown. A single-chain model that tethers assembly-incompetent subunits to their WT partner has been used successfully to address similar questions. GPH subunits containing cysteine mutations that disrupt the cystine knot, such that the free subunits alone cannot assemble with hCG-β, maintain in vitro biological activity when tethered to hCG-β (36, 37). These data suggest that the cystine knot region is necessary for heterodimer formation, but not for receptor binding and signal transduction. Thus, it seems likely that the intervening X residues may also not be directly involved in receptor binding and signal transduction.

A complete understanding of the common cystine knot motif must take into account all residues of the knot. Previous studies have focused primarily on the S-S bonds and some aspects of the central Gly residue (6, 8, 9, 28, 30–32). The data presented in this report provides evidence that the intervening X residues located between the cystine knot also make important contributions to hCG biosynthesis. Specifically, these residues appear to play a critical role in dimer formation rather than directly influencing individual subunit folding or secretion. Thus, most noncysteine residues within cystine knots may not be interchangeable because they appear to have subunit-specific functions. Future studies aimed at elucidating the role of analogous residues in other cystine knot proteins may determine how universal a role these residues play in the function of other members of the cystine knot superfamily.

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Functional Contributions of Noncysteine Residues within the Cystine Knots of Human Chorionic Gonadotropin Subunits
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