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Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

I. THE GLYCOSYLATED END OF HUMAN α-SUBUNIT LOOP 2 IS THREADED THROUGH A β-SUBUNIT HOLE*

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Glycoprotein hormone heterodimers are stabilized by their unusual structures in which a glycosylated loop of the α-subunit straddles a hole in the β-subunit. This hole is formed when a cysteine at the end of a β-subunit strand known as the "seatbelt" becomes "latched" by a disulfide to a cysteine in the β-subunit core. The heterodimer is stabilized in part by the difficulty of threading the glycosylated end of the α-subunit loop 2 through this hole, a phenomenon required for subunit dissociation. Subunit combination *in vitro*, which occurs by the reverse process, can be accelerated by removing the α-subunit oligosaccharide. In cells, heterodimer assembly was thought to occur primarily by a mechanism in which the seatbelt is wrapped around the α-subunit after the subunits dock. Here we show that this "wraparound" process can be used to assemble disulfide cross-linked human choriogonadotropin analogs that contain an additional α-subunit cysteine, but only if the normal β-subunit latch site has been removed. Normally, the seatbelt is latched before the subunits dock and assembly is completed when the glycosylated end of α-subunit loop 2 is threaded beneath the seatbelt. The unexpected finding that most assembly of human choriogonadotropin, human follitropin, and human thyrotropin heterodimers occurs in this fashion, indicates that threading may be an important phenomenon during protein folding and macromolecule assembly in the endoplasmic reticulum. We suggest that the unusual structures of the glycoprotein hormones makes them useful for identifying factors that influence this process in living cells.

The glycoprotein hormones are essential for vertebrate reproduction and thyroid function. Each of the four human hormones is composed of non-covalently bound α- and β-subunits that are divided into three large loops (α1, α2, α3; β1, β2, β3)[1] by a cystine knot (1–3). The β-subunit also contains 20 additional residues that form a strand commonly known as the "seatbelt" because of its topology and its role in stabilizing the heterodimer (1). The seatbelt begins at the β-subunit cystine knot and its carboxyl-terminal cysteine is "latched" by a disulfide to a cysteine in loop β1. This creates a hole in the β-subunit that is bordered on one side by the core of the β-subunit and on the other side by the seatbelt. The α-subunit straddles this hole such that the glycosylated end of loop α2 must pass beneath the seatbelt through the β-subunit hole for the heterodimer to dissociate. This contributes to the stability of the heterodimer, which dissociates at low pH or in high concentrations of urea (4), but not in the presence of ionic detergents such as 0.1% sodium dodecyl sulfate. If the seatbelt were to be latched before the subunits combine, the glycosylated end of loop α2 would also need to pass through the β-subunit during heterodimer assembly. This would impede assembly, a notion supported by the finding that removal of this oligosaccharide accelerates assembly *in vitro* substantially (5), a process that occurs by a threading mechanism (6).

Pulse-chase analyses of hCG assembly in cells led to the suggestion that the seatbelt remains unlatched until after the subunits dock with one another (7). In this pathway, which we term "wraparound" (Fig. 1, *upper pathway*), formation of the seatbelt latch disulfide is the final step in heterodimer assembly and occurs after the seatbelt has been wrapped around loop α2. This pathway circumvents the need for the glycosylated end of loop α2 to pass through the hole in the β-subunit and explains the abilities of cells to make cross-linked hormone analogs in which the seatbelt is latched to cysteines added to the α-subunit (8). Assembly of glycoprotein hormone heterodimers can occur by a threading pathway *in vitro* at high subunit concentrations (6). Before heterodimer assembly begins in the threading pathway, the seatbelt is latched to a cysteine in loop β1 (Fig. 1, *lower pathway*).

Studies described here were initiated to learn if threading has a role in the intracellular assembly of the glycoprotein hormones using a strategy that circumvents the need for pulse-chase analysis, the most common approach to studying protein folding in cells. Pulse-chase methods require labeling, isolating, and characterizing partially folded intermediates. Important intermediates such as heterodimers that have not yet latched their seatbelts or in which loop α2 has not been threaded beneath the seatbelt are unstable and difficult, if not impossible to isolate. As shown here based on the abilities of β-subunit analogs to compete for formation of cross-linked and non-cross-linked heterodimers, cells can assemble hCG, hFSH, and hTSH by a threading mechanism. Indeed, it appears as if these human glycoproteins are assembled in the ER primarily by a threading route and that the wraparound pathway is used sparingly, if at all.

EXPERIMENTAL PROCEDURES

Pure recombinant hCG was obtained from Dr. Robert Campbell (Serono Reproductive Biology Institute, Rockland, MA). Constructs

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[1] The abbreviations used are: α1, α-subunit loop 1; α2, α-subunit loop 2; α3, α-subunit loop 3; β1, β-subunit loop 1; β2, β-subunit loop 2; β3, β-subunit loop 3; ER, endoplasmic reticulum; hCG, human choriogonadotropin; hFSH, human follitropin; hTSH, human thyrotropin; hCGβ, hCG β-subunit; abbreviations of the analogs used in these studies are described in Fig. 2.

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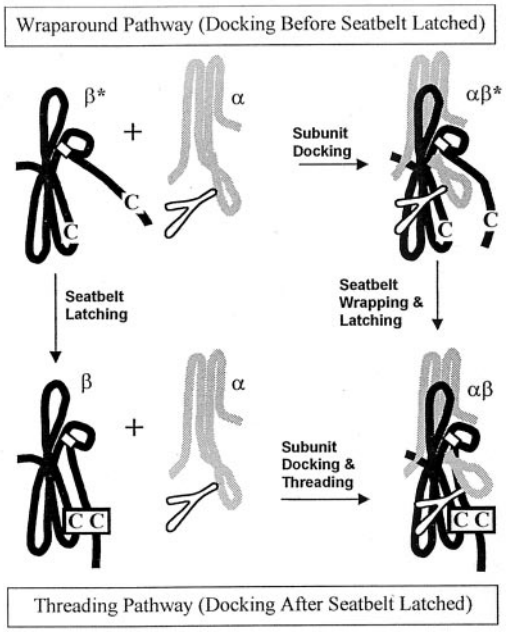

FIG. 1. **Pathways of glycoprotein hormone assembly.** During assembly that occurs by the wraparound pathway (7, 8), the subunits dock before the seatbelt is latched (*upper pathway*); formation of the heterodimer is completed when the seatbelt is wrapped around loop α2 and the latch disulfide is formed (*right vertical pathway*). In the threading pathway (6), the seatbelt latch disulfide forms before the subunits dock (*left vertical pathway*); assembly of the heterodimer is completed when loop α2 and its attached oligosaccharide traverse the hole in the β-subunit beneath the seatbelt (*lower pathway*). Key: α-subunit, *light gray*; β-subunit, *black*; *small rectangle*, disulfide that stabilizes the small loop within the seatbelt; *large rectangle*, seatbelt latch disulfide; *C*, β-subunit cysteines in loop 1 and at the end of the seatbelt; *open y-shaped* figure on α-subunit loop 2, loop α2 oligosaccharide. For simplicity, the remaining oligosaccharides are not depicted.

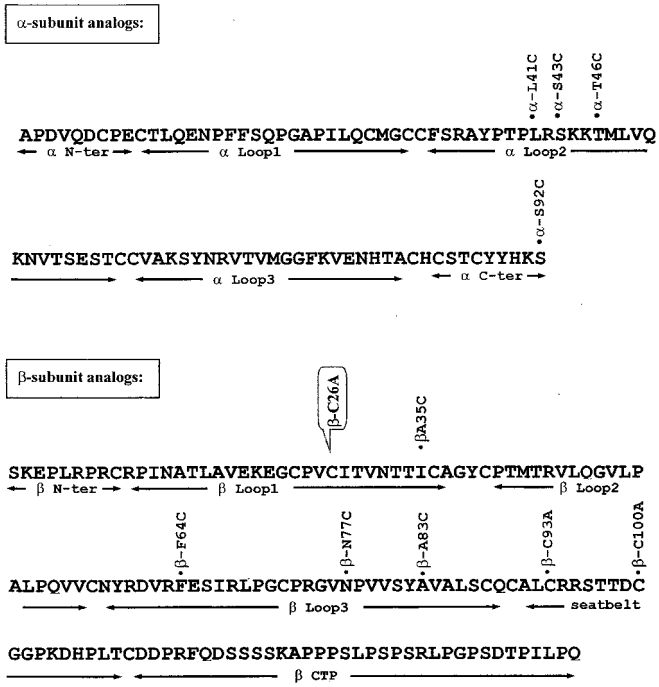

FIG. 2. **Sequences of hCG α- and β-subunit analogs used in this study.** The linear amino acid sequences of each hCG subunit are shown in single letter code. The locations of mutations used in these studies are shown in standard nomenclature. In some cases two mutations were present in a single protein and these are identified by the name of the subunit (*i.e.* hCGβ) and the mutations (*i.e.* -C26A,N77C). Several analogs contained four additional residues at the carboxyl-terminal end of the β-subunit shown as KDEL. The presence of these residues slowed secretion of the heterodimer, presumably because it caused it to be retained in the ER (18). The presence of this sequence is identified by the term -KDEL appended to the name of the analog. *Lines under* the amino acid sequence refer to its position in the protein.

used to express hCG in cultured cells were produced by polymerase chain reaction and cassette mutagenesis (9) and were sequenced prior to use. Analogs used in this study are listed in Fig. 2 and are readily identified by their names. Thus, α-L41C is the natural human α-subunit having a codon for cysteine in place of that for αLeu^{41}. Proteins were produced by transfecting the constructs into COS-7 cells obtained from the ATCC (Bethesda, MD) using a calcium phosphate procedure (10). The amounts of α- and β-subunit constructs used were 20 and 10 μg/10-cm culture plates, respectively. Secreted analogs were harvested 3 days after transfection and analyzed in monoclonal antibody sandwich immunoassays (11) employing antibodies A113, B111, B112, B404, B603, and B806, obtained from Dr. William Munroe (Hybritech Inc., San Diego, CA, a subsidiary of Beckman Coulter, Inc.), B101 was obtained from Dr. Robert Canfield (Columbia University, New York), B122 was obtained from Dr. Robert Campbell, and B110 was produced as described (12). The relative binding sites of these conformation-dependent antibodies have been determined and are indicated in Fig. 3. This figure also illustrates the locations of several residues discussed in this study. A113 recognizes a conformation-dependent α-subunit epitope in the heterodimer. B101 recognizes a conformation-dependent epitope in loop β2 of hCG and the uncombined β-subunit. B110 and B112 recognize conformation-dependent epitopes formed when loops β1 and β3 are adjacent in hCG and the uncombined β-subunit. B112 binds hCG and analogs in which βAsn^{77} is replaced by cysteine but not by aspartic acid or analogs in which a cysteine substitution participates in a disulfide bond. B111 recognizes a conformation-dependent epitope formed when βCys^{110} at the end of the hCG seatbelt is latched to βCys^{26} in the heterodimer and the uncombined β-subunit. B111 can also recognize analogs of hCG in which βCys^{26} and βCys^{110} are converted to alanine provided the heterodimer is stabilized in another fashion, such as fusing the NH_2-terminal end of the α-subunit to the COOH-terminal end of the β-subunit (8). This indicates that B111 does not recognize the βCys^{26}-βCys^{110} disulfide *per se*. B111 does not recognize hCG analogs in which the seatbelt is latched to any other residue other than βCys^{110}, however, or when residues near βCys^{110} are derived from hLH, hFSH, or hTSH. B603 and B806 recognize loops β1 and β3 in the β-subunits of hFSH and hTSH, respectively, but the binding sites of these antibodies have not been well characterized. None of the antibodies used in these studies recognize the β-subunit prior to formation of the cystine knot, a key step in formation of the β-subunit core.

Antibodies used for detection were radioiodinated to a specific activity of ~50 μCi/μg using IODO-GEN (Pierce) as described (13). The acid stability of the heterodimers was tested in 0.4-ml samples by reducing the pH to 2 by addition of microliter aliquots of 2 M HCl, while monitoring the pH, and incubating acidified samples 30 min at 37 °C, readjusting the pH to 7.5 by addition of sufficient microliter aliquots of a mixture of 10 N NaOH, 1 M Tris buffer (pH 7.5) (1:2), and then quantifying them by sandwich immunoassay (11). Material that contained the ER retention signal was isolated from the cells 1 or 2 days after transfection by scraping them from the culture dishes and solubilizing them in 10 mM sodium phosphate buffer (pH 7.5) containing 140 mM KCl, 20 mM EDTA, 1 μM leupeptin, 1.5 μM pepstatin, 500 μM pefablock, and 1% octyl glucoside (Sigma). Following sedimentation at 14,000 × *g* (10 min at 4 °C), the supernatants were diluted 6.7-fold with a phosphate-buffered saline solution (40 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 1.0 mM Na_2HPO_4, pH 7.2) and assayed by sandwich immunoassay (11) using the indicated antibodies and pure recombinant hCG as a standard. The hCG β-subunit used as a standard was purified from this hCG by high performance liquid chromatography on a C-18 resin using an acetonitrile gradient in water containing 0.1% trifluoroacetic acid as described (6). Standards were dissolved in octyl glucoside extracts of untransfected COS-7 cells for measurements of intracellular β-subunits and heterodimers. This minimized the possible influence of detergent and cell extract on the assay. Procedures to monitor assembly *in vitro* have been described (6). All sandwich assay estimates were determined statistically using Prism (GraphPad Software, San Diego, CA). Most analogs were studied three or more times. Differences in expression relative to that of hCG, which was always included as a standard, are typical despite the fact that some transfections were more efficient and led to the formation of larger amounts of heterodimers than others.

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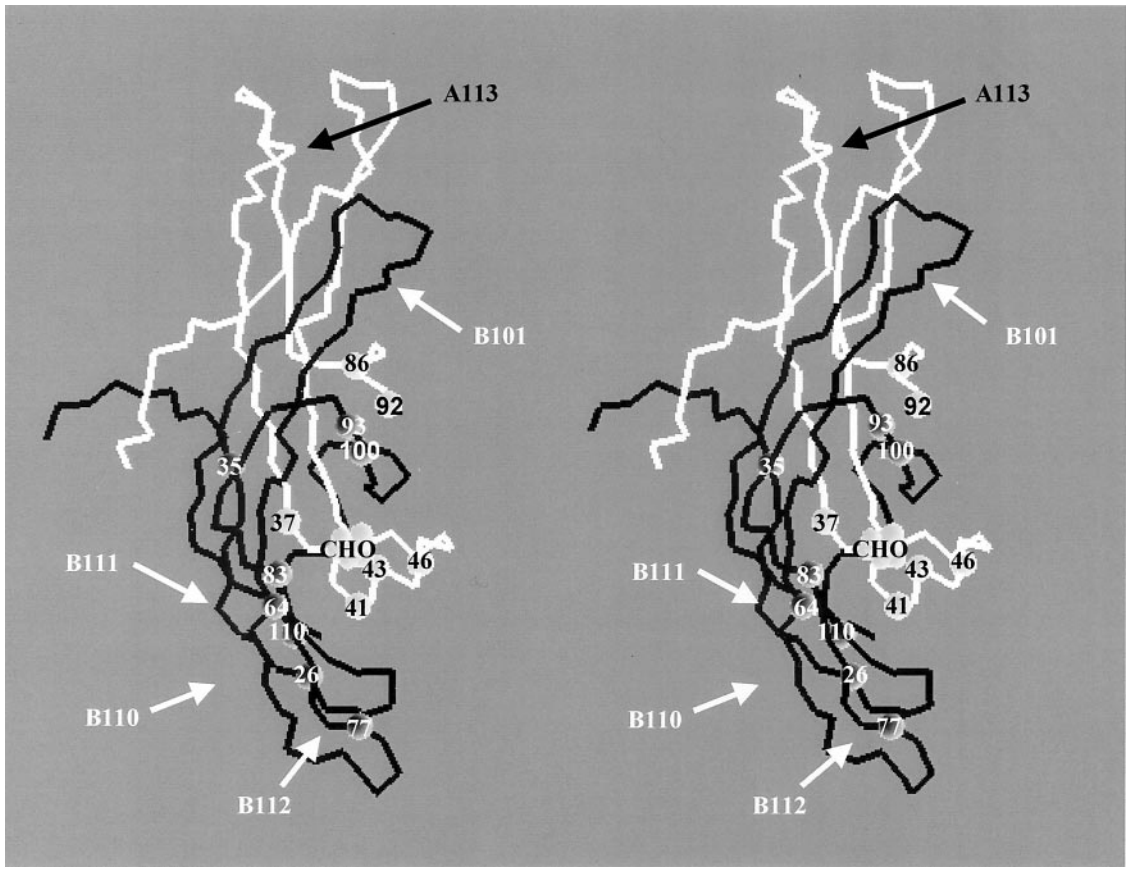

FIG. 3. **Relaxed stereo view depicting the Cα carbon atoms of hCG, the locations of mutations used in this study, and the relative antibody binding sites.** Key: α-subunit, *white*; α-subunit mutations, *light spheres with black text* denoting the amino acid residue number; *CHO*, location of residue αAsn^{52} that contains an *N*-linked oligosaccharide (not shown); β-subunit, *black*; β-subunit mutations, *dark spheres with white text* denoting the amino acid residue number; *A113* and *arrow*, approximate location of the binding site of the α-subunit antibody used for capture in sandwich immunoassays; *B101*, *B110*, *B111*, *B112*, and *arrows*, approximate locations of the binding sites of the β-subunit antibodies used for capture or detection in these studies. The seatbelt latch disulfide is normally formed between β-subunit residues 26 and 110. Note, the proximity of α-subunit residues 37, 41, and 43 to β-subunit residue 26, the normal seatbelt latch site. The small seatbelt loop is stabilized by a disulfide between β-subunit residues 93 and 100. B111, an antibody that was essential to distinguish the location of the seatbelt latch disulfide, recognizes a region of hCG near β-subunit cysteines 26 and 110, although it does not recognize the seatbelt latch disulfide *per se*. Although B111 can bind to single chain analogs of hCG in which β-subunit cysteines 26 and 110 are converted to alanine, it does not recognize any analog in which the seatbelt is latched to the α-subunit (8). B111 can also recognize heterodimers in which β-subunit cysteines 26 and 110 are converted to alanine if the heterodimer is stabilized by the presence of an NH_2-terminal Fos/Jun dimerization domain or if the heterodimer is stabilized by an N-terminal disulfide cross-link (27).

RESULTS

Rationale for Our Approach to Distinguish Heterodimer Assembly Pathways—The relative timing of subunit docking and seatbelt latch formation are reversed in the threading and wraparound pathways. Therefore, in principle, the threading and wraparound pathways could be distinguished by pulse-chase analysis (7, 14, 15), the most common method for studying protein folding in mammalian cells. This approach would not be useful for detecting trace quantities of transient unstable folding intermediates such as heterodimers that have not latched their seatbelts (16) or those in which loop α2 is only partially threaded through the β-subunit hole, however. Furthermore, pulse-chase methods can give undue weight to dead-end folding products that appear transient because they are degraded, not because they are folding intermediates. These considerations led us to monitor assembly using methods that depend on the abilities of folding intermediates to compete for the formation of cross-linked heterodimers.

We distinguished the threading and wraparound pathways by measuring the amounts of cross-linked heterodimer formed when α-subunit analogs containing an additional cysteine were co-transfected with the native β-subunit (Fig. 4, *middle* and *bottom lines*). We have found that the hCG seatbelt can be latched to a cysteine added to the α-subunit during the wraparound pathway of heterodimer assembly (8). Assembly by this route is efficient and many of the cross-linked heterodimers produced are as active as hCG in receptor binding and signal transduction assays (8). During assembly that occurs by a threading pathway (Fig. 4, *lower row*), the seatbelt is latched to the cysteine in loop β1 before assembly begins. As a result, the seatbelt would remain latched to loop β1, which would lead to the formation of a heterodimer that would be unstable at pH 2, 37 °C. During assembly that occurs by a wraparound mechanism, the seatbelt would have the opportunity to be latched to the cysteine in loop β1 or that had been added to the α-subunit. Consequently, the heterodimer would contain an intersubunit cross-link.

An alternative method of distinguishing the threading and wraparound mechanisms depends on the competition between two β-subunits (Fig. 4, *top* and *bottom lines*). We employed this method to eliminate the possibility that the seatbelt latch site might be rearranged during assembly in the cell. For example, it might be possible for the seatbelt of the native hCG β-subunit to be latched to a cysteine in the α-subunit during assembly that occurs by a wraparound mechanism. Subsequently, the seatbelt latch site might "migrate" to its native latch site by a disulfide exchange with βCys^{26} (Fig. 4, *dashed arrow*), making it appear that the heterodimer had been formed by a threading pathway. To avoid this possibility, we took advantage of the

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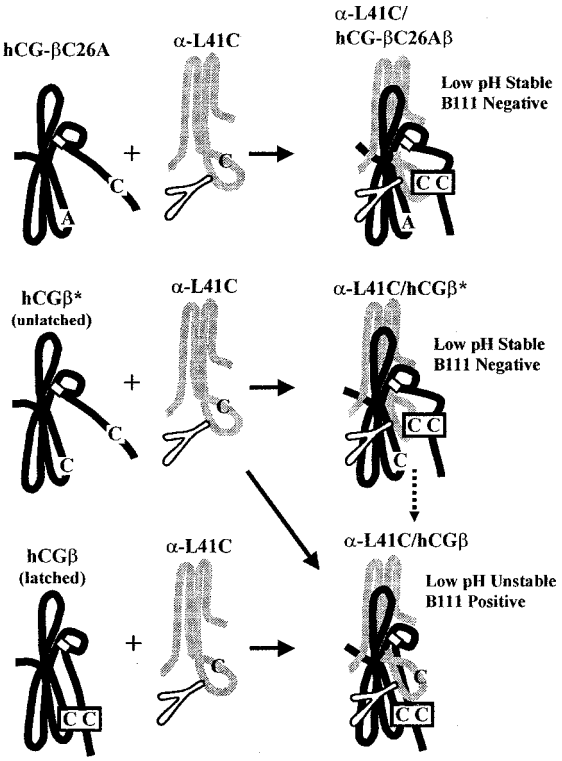

FIG. 4. **Rationale for the experiments involving a competition between hCG*β* and hCG*β*-C26A for *α*-subunit analogs that contain an additional cysteine residue.** We presume that hCG*β* can exist in two states, one in which the seatbelt is latched (hCG*β*) and another in which the seatbelt is unlatched (hCG*β**). Because hCG*β*-C26A cannot latch its seatbelt to *β*1, it can exist only in a state that is comparable with hCG*β**. As outlined in the top reaction, hCG*β*-C26A can form an intersubunit disulfide cross-linked heterodimer by the wraparound pathway with several *α*-subunit analogs that contain an additional cysteine (8). This heterodimer is stable at low pH and is readily recognized by conformation-dependent monoclonal antibodies A113, B101, B110, B112, and B122. The heterodimer is not recognized by B111, however. This shows that both subunits have folded properly but that the seatbelt is not latched as it is in hCG. hCG*β** would also be expected to form a heterodimer by a wraparound pathway. Heterodimers in which the seatbelt is latched to βCys^{26} and a cysteine added to the *α*-subunit would be readily distinguished by differences in their acid stabilities and their recognition by B111 as indicated. We expect that hCG*β*-C26A and hCG*β** would compete for docking with the *α*-subunit analogs used in these studies. Because the seatbelt of hCG*β*-C26A can be latched efficiently to several sites on the *α*-subunit (8), some seatbelts of hCG*β** should also become latched to the *α*-subunit. Theoretically, heterodimers in which hCG*β** is latched to a cysteine in the *α*-subunit could also undergo an internal disulfide rearrangement such that the seatbelt migrated from the cysteine in the *α*-subunit to βCys^{26}. This is indicated by a *dashed arrow*. A rearrangement such as this cannot occur for heterodimers that contain hCG*β*-C26A. In the fully folded form of hCG*β*, the seatbelt is latched to loop *β*1 and we anticipate that it would be assembled into a heterodimer only by the threading route. As shown in the text and Table I, we observed that the hCG*β*-C26A seatbelt becomes latched to the *α*-subunit. We did not detect latching of the hCG*β* seatbelt to the *α*-subunit or more than marginal competition of hCG*β*-C26A with hCG*β* for any *α*-subunit analog. These findings support the notions that most hCG is assembled by a threading pathway and little, if any, is formed by the wraparound pathway.

observation that the seatbelts in hCG *β*-subunit analogs such as hCG*β*-C26A, which cannot latch their seatbelts to loop *β*1 because they contain an alanine in place of βCys^{26}, can become latched to cysteines added to the *α*-subunit in place of residues 35, 37, 41–50, 64, 86, 88, and 90–92, among others (8). Because the seatbelts of these analogs cannot be latched until after the subunits contact one another, heterodimers containing these *β*-subunits can only be assembled by the wraparound pathway. Furthermore, once latched, the seatbelt in these heterodimers cannot migrate to the *β*-subunit unless the heterodimer dissociates. The observation that these heterodimers are secreted efficiently indicates that they do not dissociate.

Before its seatbelt is latched, hCG*β* would be expected to have the same overall conformation as hCG*β*-C26A (Fig. 4). Thus, hCG*β*-C26A and the unlatched form of hCG*β* (*i.e.* *hCGβ**) would be expected to compete with one another for docking with the *α*-subunit in the wraparound pathway. If most heterodimers became assembled by the wraparound pathway, one would expect to find a significant fraction of the total heterodimer that contained hCG*β*-C26A. In contrast, because hCG*β*-C26A can be incorporated into heterodimers only by the wraparound pathway, it would not be expected to compete with hCG*β* for heterodimers that are assembled after the seatbelt is latched, *i.e.* by threading (Fig. 4). Therefore, if most assembly occurs by threading, very little of the heterodimer formed would contain hCG*β*-C26A.

The position of the seatbelt latch site in hCG can be determined by the acid stability of the heterodimer and by its ability to be recognized by monoclonal antibody B111. Heterodimers in which the seatbelt is latched to the *α*-subunit contain an intersubunit disulfide cross-link. These are distinguished readily from heterodimers in which the seatbelt is latched to βCys^{26} by their resistance to dissociation at pH 2, 37 °C, and by their inabilities to bind monoclonal antibody B111 (8). B111 binds a conformational hCG epitope formed when the seatbelt is latched normally, *i.e.* to βCys^{26}. It does not bind heterodimers in which the seatbelt is latched to other cysteines such as those that have been added to either subunit (8, 17).

Human Choriogonadotropin Can Be Assembled in the ER by Two Different Routes, but Most Is Made by the Threading Pathway—The seatbelts of heterodimers produced by co-expressing hCG*β* and each of the *α*-subunit analogs tested became latched primarily to βCys^{26}, not to the cysteine added to the *α*-subunit. This was the first indication that hCG is formed in the ER by a threading pathway. An example of this is seen by comparing the properties of heterodimers produced when hCG*β*, was expressed with *α*-L41C (Table I, 1). Most heterodimers secreted by cells that were co-transfected with *α*-L41C and hCG*β* lacked an intersubunit disulfide and were unstable following 30 min at pH 2, 37 °C (Table I, 1, row 1). As a result they were detected readily in heterodimer-specific sandwich immunoassays employing an antibody to the *α*-subunit for capture (*i.e.* A113) and a radioiodinated antibody to the *β*-subunit for detection (*i.e.* ^{125}I-B110) before, but not after low pH treatment. These heterodimers were also detected in similar immunoassays employing A113 for capture and ^{125}I-B111 for detection before treatment at low pH. This indicated that their seatbelts were latched to βCys^{26}, not αCys^{41}. Both findings are consistent with the conclusion that the heterodimer was formed by a threading mechanism.

To exclude the possibility that the location of the seatbelt in the heterodimer had undergone a disulfide exchange and become latched to βCys^{26} in loop *β*1 after it had been latched to αCys^{41}, we repeated the study in the presence of hCG*β*-C26A, an analog that cannot latch its seatbelt to loop *β*1. When *α*-L41C was expressed with hCG*β*-C26A, the heterodimer that formed was detected in A113/^{125}I-B110 assays before and after acid treatment (Table I, 1, row 2). Furthermore, none of it was detected in A113/^{125}I-B111 assays before or after low pH treatment. These findings showed that seatbelt residue βCys^{110} of this heterodimer was cross-linked to the *α*-subunit, a consequence of its formation by a wraparound mechanism. When both *β*-subunits were expressed simultaneously with *α*-L41C, heterodimers secreted into the medium contained hCG*β* and little or no hCG*β*-C26A (Table I, 1, row 3). Because the seat-

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TABLE I

hCG β-subunit analogs forced to latch their seatbelts to the α-subunit compete poorly with those that can latch their seatbelts to βCys[26]

This table describes the properties of heterodimers produced by co-expressing the α-subunit analog indicated at the top of each block with β-subunits capable of participating in wrapping or threading pathways (*i.e.* hCGβ and hCGβ-KDEL), β-subunits that are limited to wrapping pathways (*i.e.* hCGβ-C26A and hCGβ-C26A-KDEL), or with both types of β-subunits simultaneously. The total amount of heterodimer in 50-μl aliquots of unconcentrated culture media (secreted) or 7 μl of cell lysate (intracellular) was quantified in an A113 capture/^{125}I-B110 detection sandwich assay using purified recombinant hCG as standard. The fraction of the total heterodimer that was acid stable was determined after treatment at pH 2 for 30 min at 37 °C. Data in the first and second columns were determined in A113 capture/^{125}I-B110 detection sandwich assays. Those in the third and fourth data columns are the results of A113 capture/^{125}I-B111 detection sandwich assays. Values in the first to fourth data columns are mean ± S.E. for 3 independent transfection plates. Similar results were observed in at least three other independent studies for each analog. The values in the fifth column, row 1 of blocks 4 and 5 were derived from these means by comparing differences in the B111 assays before and after treatment at acid pH (row 1) (Co-expression of α -subunit analogs having a free cysteine and the native hCG β -subunit can give rise to four populations of heterodimers, *i.e.* one that does not have a cross-link, one in which tensor cysteine 93 is cross-linked to the cysteine added to the α -subunit, one in which tensor cysteine 100 is cross-linked to the cysteine added to the α -subunit, and one in which the seatbelt is latched to the cysteine added to the α -subunit. We calculated the maximum amount of heterodimer in which the seatbelt is latched to the α -subunit in Table I, rows 1, 4 and 5, as follows. For simplicity, we assign variable x to be the amount of uncross-linked heterodimer, variable y to be the amount of heterodimer in which either tensor cysteine is cross-linked to the α -subunit, and variable z to be the amount of heterodimer in which the seatbelt is latched to the α -subunit. The latter could be formed only by the wraparound pathway. Constants a, b, and c represent the abilities of B111 to bind heterodimers with these configurations relative to B110, respectively. Because B111 does not bind heterodimers in which the seatbelt is latched to the α -subunit, $c = 0$. Data in the first column (C_1) is the total amount of heterodimer detected by antibody B110, namely $C_1 = x + y + z$. Data in the second column (C_2) is the fraction of heterodimer detected by antibody B110 that contains a cross-link. Thus, $C_1C_2 = y + z$. Data in the third (C_3) and and fourth (C_4) columns represent the abilities of B111 to bind the total and cross-linked heterodimers relative to those measured by B110. Note that for all calculations the values shown in C_2, C_3, and C_4 were converted to their fractional equivalents by dividing them by 100. The values of C_3 and C_4 can be expressed as: $C_3 = (ax + by + cz)/(x + y + z) = (ax + by)/(x + y + z) = (ax + by)/C_1$ and $C_4 = (by + cz)/(y + z) = by/(y + z) = by/(C_1C_2)$. To estimate maximal amount of z, we first divided C_3 by C_4 and rearranged the terms to give: $C_3/C_4 = C_2(ax + by)/(by)$. Because the ability of B111 to recognize the cross-linked heterodimer in 4 and 5 in Table I appears to be less than its ability to recognize the total heterodimer, $b \leq a$ and this equation can be written as the inequality: $C_3/C_4 \geq C_2b(x + y)/(by) = C_2(x + y)/y$, and solved for z by replacing (x + y) with $(C_1 - z)$ and by replacing y with $(C_1C_2 - z)$. These relationships are known from the definitions of C_1 and C_2 noted above. Thus, $C_3/C_4 \geq C_2(C_1 - z)/(C_1C_2 - z)$, which can be rearranged to solve for z as: $z/C_1 \leq C_2(C_3 - C_4)/(C_3 - C_2C_4)$. The value of z/C_1 represents the maximal fraction of the total heterodimer that has its seatbelt latched to the α -subunit. The values of z/C_1 shown in the fifth column, row 1, of 4 and 5, were converted to percentages by multiplying them by 100). Values in the fifth column, row 2 of each data block were determined from the stability of the heterodimer. Values in the fifth column, row 3 of each data block were determined by comparing the amount of acid stable heterodimer in the absence and presence of hCGβ -C26A or hCGβ -C26A-KDEL. For example, in the case of block 2, row 3, the fraction of the acid stable heterodimer likely to contain hCGβ -C26A-KDEL was calculated as (55.78–35.50)/55.78 or 0.364. This was then used to calculate the amount of this material in the total heterodimer by multiplying it by the fraction of the total that is acid stable (*i.e.* 0.364 × 18.60%) to give the value of 6.8% shown in the table. The binding site of antibody B111 is shown in Fig. 3 and described in the corresponding legend.

β-Subunit analog	Heterodimer total/50 μl	Low pH stable heterodimer	B111/B110 Ratio total dimer	B111/B110 ratio stable dimer	Wraparound pathway, αCys to βCys[110]
	ng ± S.E.	*% Total ± S.E.*		*% Stable ± S.E.*	
1) Heterodimers containing α-L41C measured in media					
hCGβ	2.42 ± 0.23	Undetectable	65.5 ± 3.4	Not done	Undetectable
hCGβ-C26A	2.73 ± 0.23	96.5 ± 0.8	0 ± 0.3	Not done	≥96%
Both	2.13 ± 0.12	Undetectable	67.0 ± 4.3	Not done	Undetectable
2) Heterodimers containing α-L41C measured in cell lysates					
hCGβ-KDEL	6.16 ± 0.40	9.69 ± 0.39	54.66 ± 8.40	55.78 ± 3.66	Undetectable
hCGβ-C26A-KDEL	9.13 ± 0.63	103.01 ± 1.83	0.64 ± 0.18	0.50 ± 0.10	100%
Both	6.68 ± 0.57	18.60 ± 0.42	56.65 ± 8.99	35.50 ± 1.63	6.8%
3) Heterodimers containing α-L41C,N52D measured in cell lysates					
hCGβ-KDEL	3.16 ± 0.19	6.08 ± 0.45	80.64 ± 3.80	79.64 ± 4.68	Undetectable
hCGβ-C26A-KDEL	2.25 ± 0.10	110.76 ± 2.69	1.68 ± 0.57	1.78 ± 0.31	100%
Both	2.05 ± 0.08	17.30 ± 1.72	68.90 ± 10.45	43.78 ± 4.25	7.8%
4) Heterodimers containing α-S43C measured in cell lysates					
hCGβ-KDEL	5.27 ± 0.35	18.1 ± 1.0	64.10 ± 4.03	59.6 ± 1.2	≤1.5%
hCGβ-C26A-KDEL	10.92 ± 1.73	99.8 ± 18.8	0.37 ± 0.07	0.4 ± 0.1	100%
Both	6.35 ± 0.41	23.9 ± 2.0	42.47 ± 5.96	40.6 ± 0.6	7.6%
5) Heterodimers containing α-T46C measured in cell lysates					
hCGβ-KDEL	7.71 ± 0.44	14.4 ± 1.1	63.33 ± 7.04	49 ± 3	≤3.7%
hCGβ-C26A-KDEL	14.1 ± 0.65	92.8 ± 4	0.67 ± 0.03	0.8 ± 0	≥93%
Both	7.1 ± 0.55	37.5 ± 2.5	50.57 ± 3.68	21.2 ± 2.2	21.3%
6) Heterodimers containing α-S92C measured in cell lysates					
hCGβ-KDEL	9.45 ± 0.89	6.03 ± 0.4	47.77 ± 7.19	85.03 ± 4.98	Undetectable
hCGβ-C26A-KDEL	11.66 ± 1.05	128.47 ± 16.8	0.97 ± 0.12	0.37 ± 0.07	100%
Both	7.4 ± 0.31	6.60 ± 0.7	49.00 ± 1.42	65.00 ± 5.68	1.6%

belts in these heterodimers were latched to βCys[26], not αCys[41], they were detected readily in A113/^{125}I-B110 and A113/^{125}I-B111 assays before, but not after low pH treatment. The finding that hCGβ-C26A did not compete with hCGβ for formation of heterodimers containing α-L41C indicated that hCG assembly occurs primarily by threading.

One could argue that heterodimers having their seatbelts latched to αCys[41] were disrupted or degraded during secretion and that this prevented us from detecting them in preparations containing α-L41C and hCGβ. This appeared highly unlikely because heterodimers containing hCGβ-C26A were found in the medium when it was the only β-subunit used in the transfection. Nonetheless, we tested this possibility using analogs of hCGβ and hCGβ-C26A that contained four COOH-terminal residues (*i.e.* KDEL) known to delay the secretion of other proteins from the ER (18). To determine how this affected the secretion of hCG, we transfected COS-7 cells with the native α-subunit and hCGβ or hCGβ-KDEL and measured the ap-

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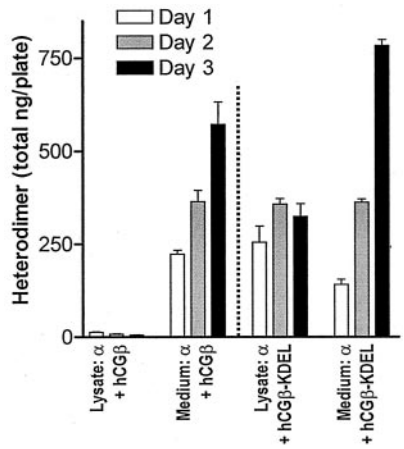

FIG. 5. **Influence of KDEL sequence on heterodimer secretion.** Cells transfected with hCG analogs containing KDEL sequences and materials that were retained within the cell (lysates) and secreted into the media during 3 days of incubation were measured as described in the text.

pearance of heterodimer in media and cell lysates. The KDEL tag delayed heterodimer secretion from the cell and caused it to be accumulated in the cells (Fig. 5). Prolonged incubation resulted in release of the KDEL-tagged material from the cells, probably because the ER retention mechanism had been saturated. These studies suggested that assays of KDEL-tagged heterodimers in cell lysates would reflect material located primarily in the ER.

The seatbelts of heterodimers measured in lysates of cells co-transfected with α-L41C and hCGβ-KDEL were latched to βCys^{26}. As a result, most were unstable at low pH and readily detected by B111 (Table I, 2, row 1). A small fraction (9.7%) of the heterodimer was stable at low pH even though it was detected in A113/^{125}I-B111 assays. Whereas its acid stability suggested that this fraction contained an intersubunit disulfide cross-link, its ability to be recognized by B111 showed that its acid stability was not due solely to the formation of a disulfide between seatbelt residue βCys^{110} and loop α2 residue αCys^{41}. This indicated that αCys^{41} can participate in an intersubunit disulfide with β-subunit cysteines other than that at the end of the seatbelt (*i.e.* βCys^{110}). The amounts of the acid stable α-L41C/hCGβ-C26A-KDEL heterodimer in cell lysates were too small for us to attempt identifying this intersubunit disulfide using traditional biochemical methods. Therefore, we modeled each of the 12 intersubunit disulfides that could be formed between αCys^{41} and cysteines in the β-subunit and excluded those that were likely to disrupt the conformation of the heterodimer to an extent that it would no longer be recognized by antibodies used in these studies. Only two intersubunit disulfides, namely αCys^{41}-βCys^{93} and, more likely, αCys^{41}-βCys^{100} appeared to fit these criteria. These β-subunit cysteines stabilize a small loop within the seatbelt and are near several residues in loop α2, including αCys^{41}.

To test the notion that βCys^{100} or βCys^{93} might participate in the formation of an intersubunit disulfide with αCys^{41} without disrupting the B111 binding site, we expressed α-L41C with hCGβ-C93A-KDEL and hCGβ-C100A-KDEL. These analogs cannot form the small seatbelt loop and have free cysteines at βCys^{100} and βCys^{93}, respectively. We also expressed α-L41C with hCGβ-C93A,C100A-KDEL, an analog lacking both cysteines. Because the small seatbelt loop has been shown to be essential for the formation of heterodimers containing the native α-subunit (16), we expected that these β-subunits would form heterodimers with α-L41C only if they could form an intersubunit disulfide. This prediction was satisfied by the observation that hCGβ-C93A-KDEL, the analog having a free cysteine at βCys^{100}, formed a heterodimer with α-L41C (Table II). The findings that α-L41C did not combine stably with hCGβ-C93A,C100A-KDEL and that the native α-subunit did not combine stably with hCGβ-C93A-KDEL supported the notion that the intersubunit disulfide involved residues αCys^{41} and βCys^{100}. As had been predicted from modeling studies, the amount of heterodimer formed when α-L41C was expressed with hCGβ-C93A-KDEL was much greater than that formed when it was expressed with hCGβ-C100A-KDEL (Table II).

Based on this observation and the finding that both the α-L41C/hCGβ-KDEL and α-L41C/hCGβ-C93A-KDEL heterodimers were detected readily by B111 (Tables I and II, respectively), we expect that the acid-stable fraction of α-L41C/hCGβ-KDEL contains an intersubunit disulfide cross-link between αCys^{41} and βCys^{100}. Nonetheless, we cannot exclude the possibility that a portion of this material contains a disulfide between αCys^{41} and βCys^{110}, indicating that it had been formed by a wraparound mechanism. The finding that the seatbelt was latched to βCys^{26} in the vast majority of the acid unstable α-L41C/hCGβ-KDEL heterodimer and a substantial fraction of the acid-stable α-L41C/hCGβ-KDEL heterodimer showed that βCys^{26} out competed αCys^{41} as a seatbelt latch site. Whereas this is consistent with the view that the seatbelt had become latched to βCys^{26} before the subunits docked, it does not preclude the possibility that the seatbelt had become latched to βCys^{26} by a process of disulfide exchange. We tested this possibility by comparing the abilities of hCGβ-KDEL and hCGβ-C26A-KDEL to compete for heterodimer formation.

The heterodimer made by co-expressing α-L41C and hCGβ-C26A-KDEL was acid stable and could not be detected in A113/^{125}I-B111 assays before or after low pH treatment (Table I, 2, row 2). This showed that it contained an intersubunit disulfide, a consequence of its assembly by a wraparound mechanism. hCGβ-C26A-KDEL barely competed with hCGβ-KDEL for heterodimer formation, however. Most heterodimers made in the presence of both β-subunit analogs were unstable at pH 2, 37 °C, and were readily detected in A113/^{125}I-B111 assays (Table I, 2, row 3). These findings are consistent with the conclusions that the seatbelts of most heterodimer molecules were latched to βCys^{26}, not αCys^{41} and that they had been formed by the threading pathway. Much of the acid-stable fraction was recognized by B111, indicating that its seatbelt was latched normally and that it may have been stabilized by an intersubunit disulfide between αCys^{41} and either βCys^{93} or βCys^{100}. Differences in the ability of B111 to recognize the acid-stable heterodimer made in transfections lacking and containing hCGβ-C26A-KDEL (*i.e.* Table I, 2, rows 1 and 3) indicated that a small amount of the acid-stable heterodimer made in the presence of hCGβ-C26A-KDEL contained an intersubunit disulfide cross-link between αCys^{41} and βCys^{110}. Based on calculations described in the legend to Table I, it appears as if 6.8% of the heterodimer in cell lysates may have been formed by the wraparound pathway when both β-subunits were present. This small amount is consistent with the notion that threading is much more efficient than wrapping, particularly because much more hCGβ-C26A-KDEL was incorporated into heterodimers containing α-L41C in the absence of hCGβ-KDEL.

We have found that removal of the loop α2 oligosaccharide facilitates threading *in vitro* (5) and considered the possibility that it would also enhance assembly in cells. To test this, we studied assembly of heterodimers that contain α-L41C,N52D, an analog of α-L41C that lacks the loop α2 glycosylation signal. We co-expressed this α-subunit analog with hCGβ-KDEL, hCGβ-C26A-KDEL, or both β-subunit analogs and measured the formation of heterodimer in cell lysates (Table I, 3). The heterodimer formed with hCGβ-KDEL was acid labile and was

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TABLE II

Cross-linking of βCys^{93} and βCys^{100} to α-subunit analogs having an extra cysteine

COS-7 cells were co-transfected with constructs encoding the indicated α- and β-subunit analogs. The β-subunits employed are unable to form the tensor disulfide and in most instances did not combine stably with the α-subunit unless it contained a cysteine at an appropriate location. The free cysteine in hCGβ-C93A is βCys^{100} and that in hCGβ-C100A is βCys^{93}. The values under the heading B111 (% total) refer to the binding of these heterodimers by B111 relative to that of B110. These data show that βCys^{100} can be cross-linked to many α-subunits containing an additional cysteine and that the resulting heterodimers can be recognized by B110 and B111. A few can also be cross-linked to βCys^{93}. Not detectable refers to heterodimer that is less than 0.02 ng/50 μl. In many cases we detected the presence of material that is just above this blank value but not enough to quantify accurately. This has been identified by the symbol "<0.10."

α-Subunit	β-Subunit analog					
	hCGβ-C93A-KDEL (βCys^{100})		hCGβ-C100A-KDEL (βCys^{93})		hCGβ-C93A,C100A-KDEL	
	Total ng/50 μl ± S.E.	B111	Total ng/50 μl ± S.E.	B111	Total ng/50 μl ± S.E.	B111
		% total		*% total*		*% total*
Native α	Not detectable	Not detectable	Not detectable	Not detectable	Not tested	Not tested
α-L41C	0.18 ± 0.02	81.5 ± 15.3	<0.10	Not estimated	Not detectable	Not detectable
α-S43C	0.85 ± 0.03	91.2 ± 5.7	<0.10	Not estimated	<0.1	Not estimated
α-T46C	0.68 ± 0.03	62.0 ± 2.0	0.11 ± 0.01	42.9 ± 4.0	0.11 ± 0.01	98.7 ± 4.9
α-S92C	1.69 ± 0.04	107.8 ± 5.7	0.52 ± 0.07	116.7 ± 3.8	Not detectable	Not detectable

detected readily in A113/^{125}I-B111 assays, showing that its seatbelt was latched to βCys^{26} (Table I, 3, row 1). Heterodimer formed with hCGβ-C26A-KDEL was acid stable and not recognized in A113/^{125}I-B111 assays, indicating that seatbelt residue βCys^{110} became bridged to αCys^{41}, a consequence of the wraparound pathway (Table I, 3, row 2). Most heterodimers formed when both β-subunits were co-expressed with α-L41C,N52D contained hCGβ-KDEL and 80% dissociated during the pH 2 treatment (Table I, 3, row 3). The finding that most of the acid-stable heterodimer was also recognized in A113/^{125}I-B111 assays showed that its seatbelt was latched to βCys^{26}, not αCys^{41}. Less than 8% of the heterodimer made in the presence of both β-subunit analogs appeared to contain hCGβ-C26A-KDEL. Thus, preventing the glycosylation of loop α2 did not affect the route of assembly in cells, most likely because nearly all hCG is assembled by a threading mechanism, even when the loop α2 oligosaccharide is present.

Together, these observations suggested that most heterodimers containing α-L41C are assembled by a threading route. Whereas it might be argued that the apparent inefficiency of wrapping is because of the possibility that αCys^{41} is a poor seatbelt latch site, this seemed unlikely given the finding that α-L41C assembled readily with hCGβ-C26A and hCGβ-C26A-KDEL. Furthermore, we had chosen this α-subunit residue because of its proximity to βCys^{26} in the heterodimer. Thus, we expected that seatbelt residue βCys^{110} would be latched to αCys^{41} readily, a phenomenon observed only when the seatbelt is prevented from being latched to its natural β-subunit site (*i.e.* βCys^{26}).

We tested the abilities of hCGβ-KDEL and hCGβ-C26A-KDEL to compete for two other α-subunit analogs containing a cysteine in loop α2. When α-S43C and α-T46C were co-expressed with hCGβ-KDEL, we observed that 18.1 and 14.4%, of the heterodimer that remained in the cells did not dissociate at acid pH even though it was readily detected with B111 (Table I, 4 and 5, row 1). The acid stability of these heterodimers appeared because of the formation of a disulfide between the cysteine that had been added to loop α2 and βCys^{93} or, more likely, βCys^{100}. Both of these α-subunit analogs formed significant amounts of heterodimer when expressed with hCGβ-C93A-KDEL, indicating they are likely to contain an intersubunit disulfide cross-link with βCys^{100} (Table II). Because the recognition of the acid-stable forms of these heterodimers by B111 is similar to that of heterodimers in which these α-subunit cysteines were cross-linked to βCys^{100}, it appeared likely that most, if not all of the cross-linked material contains a disulfide between the cysteine added to the α-subunit and either βCys^{93} or βCys^{100}. Consequently, the threading pathway was expected to be responsible for more than 95% of the heterodimers formed when hCGβ-KDEL was expressed with either α-S43C or α-T46C (Table I, 6 and 7, row 1).

To test the possibility that we were being mislead by our choice of an α-subunit latch site, we repeated these studies with α-S43C and α-T46C, analogs that also formed heterodimers efficiently with hCGβ-C26A (8). When these α-subunit analogs were co-expressed with hCGβ-C26A-KDEL, nearly all the heterodimer retained within the cell was acid stable (Table I, 4 and 5, row 2). Neither heterodimer was detected by B111, indicating that its seatbelt had become latched to the α-subunit. The ample amount of heterodimer formed with either α-subunit analog suggested that αCys^{43} and αCys^{46} are readily bridged to βCys^{110}. Thus, these cysteines should have competed effectively with βCys^{26} during assembly of heterodimers that formed by the wraparound pathway. The finding that little, if any, intersubunit disulfide between βCys^{110} and either αCys^{43} or αCys^{46} was formed when α-S43C and α-T46C were expressed with hCGβ-KDEL is consistent with the notion that most heterodimers are assembled by threading (Table I, 4 and 5, row 1).

To learn if hCGβ-C26A-KDEL would compete with hCGβ-KDEL during assembly of heterodimers containing α-S43C and α-T46C, we co-expressed each α-subunit analog with both β-subunit analogs. The amounts of heterodimer formed were always similar to those seen when each α-subunit analog was co-expressed with hCGβ-KDEL alone (Table I, 4 and 5, row 3). Furthermore, we often observed that a higher percentage of heterodimers containing α-S43C and α-T46C were acid stable than when α-L41C was used as the assembly partner. For example, 23.9 and 37.5% of the heterodimer appeared to be acid stable when α-S43C and α-T46C were expressed with both β-subunit analogs (Table I, 4 and 5). B111 recognized more of the acid-stable heterodimer containing α-S43C than that containing α-T46C, an indication that the latter contained a significant fraction of hCGβ-C26A-KDEL. This difference is correlated with the amounts of heterodimer observed following transfection of the cells with hCGβ-C26A-KDEL in the absence of hCGβ-KDEL, showing that hCGβ-C26A-KDEL can compete with hCGβ-KDEL when it is present in sufficient excess. The fraction of acid-stable heterodimer in these preparations likely to contain an αCys^{43}-βCys^{110} or αCys^{46}-βCys^{110} disulfide was 7.6 and 21.3%, respectively. Note that in both cases, the presence of hCGβ-KDEL inhibited heterodimer production, indicating that heterodimer assembly occurred preferentially by a threading mechanism.

Observations made using α-subunit analogs containing a cysteine in loop α2 suggested that most hCG assembly occurred after the seatbelt had been latched. Consequently, the majority of these hCG analogs appeared to be assembled by a threading

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route. To test the notion that cysteines in other regions of the α-subunit would permit us to detect hCG assembly by the wrap-around pathway, we tested the influence of a cysteine in place of αSer^{92}. We have reported that this residue can be readily latched to the seatbelt (8). This cysteine would not be expected to pass beneath the seatbelt during assembly by a threading mechanism and we reasoned that it would be less likely to form a disulfide with either βCys^{93} or βCys^{100} during assembly even though it can be cross-linked efficiently to either of these cysteines when the other is absent (Table II). Co-expression of α-S92C and hCGβ-KDEL led to the formation of considerable amounts of heterodimer, nearly all of which dissociated at acid pH (Table I, 6, row 1). The acid-stable fraction of this heterodimer was recognized almost as well in A113/^{125}I-B111 assays as it was in A113/^{125}I-B110 assays. Based on the abilities of α-S92C to form heterodimers with either hCGβ-C93A-KDEL and hCGβ-C100A-KDEL (Table II), we anticipate that this small amount of cross-linked heterodimer contains an intersubunit disulfide between αCys^{92} and either βCys^{93} or βCys^{100}. Both of these are recognized well by B111. Expression of α-S92C with hCGβ-C26A-KDEL also led to the formation of a substantial amount of acid-stable heterodimer (Table I, 6, row 2), but this was not recognized by B111, indicating that it was stabilized by the αCys^{92}-βCys^{110} disulfide and had been formed by a wraparound pathway. Co-expression of α-S92C, hCGβ-KDEL, and hCGβ-C26A-KDEL resulted in a heterodimer that was acid unstable and recognized well by B111, indicating that it had been formed by a threading mechanism. These data suggested that only 1.6% of the heterodimer formed might contain an αCys^{92}-βCys^{110} disulfide. Again, this supported the notion that hCG is assembled by a threading pathway.

Human Follitropin and Thyrotropin Also Appear to be Assembled by a Threading Pathway—hFSH and hTSH are structurally similar to hCG and contain the same α-subunit. These β-subunits formed heterodimers with α-subunit analogs containing cysteines added to the α-subunit in place of residues 35, 37, 41–50, 64, 86, 88, and 90–92 (not shown). Unlike the hCG β-subunit, however, analogs of hFSH and hTSH β-subunits lacking the abilities to latch their seatbelts to β-subunit loop 1 (*i.e.* hFSHβ-C20A, hFSH-C20A-KDEL, and hTSHβ-C19A) did not form heterodimers with any of these α-subunit analogs (not shown). This suggested that the wraparound pathway makes few contributions, if any, to the assembly of hFSH or hTSH.

Latching the Seatbelt to a Cysteine in hCG β-Subunit Loop 1 Occurs Before the Subunits Dock—We began these studies to learn if threading might be capable of making small contributions to glycoprotein hormone assembly, but did not anticipate finding that it was the major route of human glycoprotein hormone assembly in the ER. Indeed, the notion that hCG is assembled by a threading mechanism contradicted earlier suggestions that it was assembled by a wraparound mechanism (7). Efforts to confirm the use of the threading pathway for hCG assembly led us to test this possibility by an alternative strategy. We reasoned that if threading is the main route of hCG assembly, the seatbelt would have a strong tendency to be latched to the β-subunit before the subunits dock, even if it were forced to be latched to sites other than βCys^{26}. Furthermore, we anticipated that when the seatbelt is latched to an inappropriate site, it would interfere with heterodimer assembly. We tested these possibilities using analogs of hCGβ and hCGβ-C26A that contained an additional cysteine as described (Fig. 6).

Adding a cysteine to hCGβ creates the potential for new disulfide bond arrangements. The structure of the β-subunit suggests relatively few of these will be formed, however, without distorting the protein to the extent that it is no longer

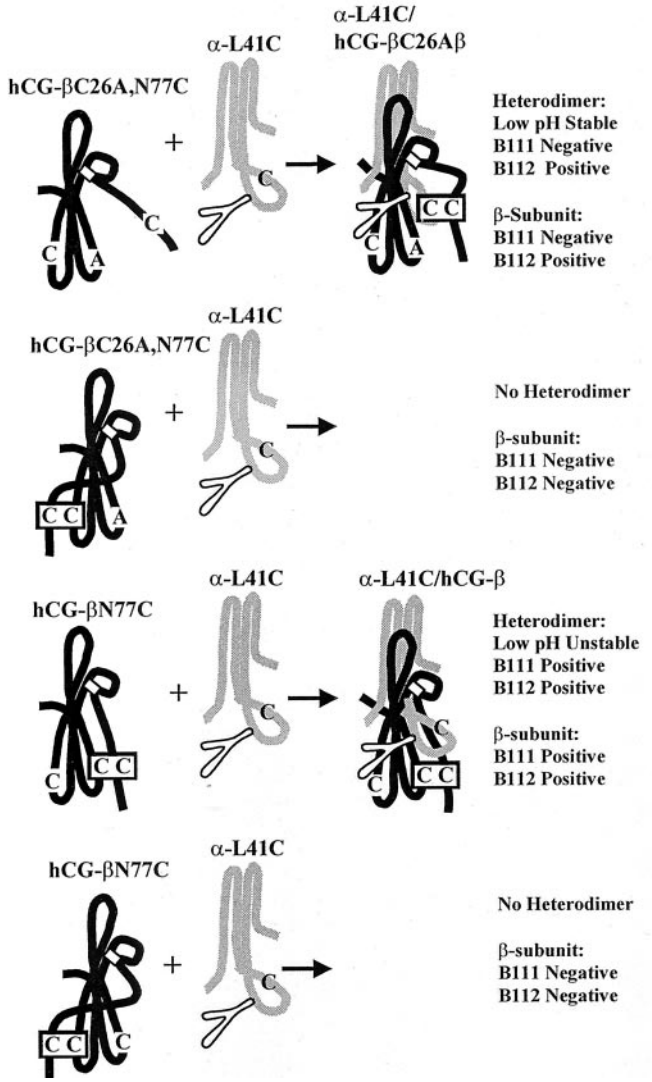

FIG. 6. **Rationale for studies involving a competition between intrasubunit cysteines and intersubunit cysteines for formation of the seatbelt latch disulfide.** The presence of an additional cysteine in hCGβ or hCGβ-C26A would create a new seatbelt latch site within the β-subunit. As shown in the *top two rows*, the presence of this cysteine in hCGβ-C26A creates an analog (hCGβ-C26A,N77C) that can exist in two states. In one of these, the seatbelt remains unlatched because of the difficulty of forming the non-native intrasubunit disulfide. In the other state, the seatbelt becomes latched to the cysteine added to the β-subunit before the subunit has time to dock with an α-subunit analog such as α-L41C. Formation of an acid-stable heterodimer would imply that the seatbelt had been latched to the cysteine added to the α-subunit. This would show that it had not become latched to the β-subunit before the subunits docked. In contrast, formation of an intrasubunit disulfide prior to docking would prevent the seatbelt from being latched to the α-subunit. Consequently, no heterodimer would form unless it were possible for the α-subunit to be threaded through the altered β-subunit. In the case of hCGβ-C26A,N77C, latching the seatbelt to βCys^{77} would also disrupt the ability of the free subunit to be recognized by B112 as well as by B111. Addition of a cysteine to hCGβ to create hCGβ-N77C would provide the seatbelt with a second latch site. If the seatbelt became latched to its native site, this β-subunit would be able to form an acid unstable heterodimer by a threading mechanism and both the free subunit and the heterodimer should be detected by B111 and B112. In contrast, latching the seatbelt to the added cysteine would be expected to disrupt assembly by either the threading or the wrap-around pathway. As described in the text, the presence of additional cysteines in hCGβ did not prevent heterodimer formation, suggesting that the seatbelt was more likely to form a stable disulfide with βCys^{26}, its natural site. In contrast, the presence of additional cysteines in hCGβ-C26A prevented this β-subunit analog from being incorporated into a heterodimer.

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TABLE III

Competition between β-subunit cysteines and a cysteine added to α2

This table describes the production of heterodimers following co-transfection of COS-7 cells in triplicate with α-L41C and the indicated β-subunit analog. The total heterodimer in the medium was determined in A113/^{125}I-B110 sandwich assays. Latching of the seatbelt to loop β1 was determined in A113/^{125}I-B111 sandwich assays. The presence of a cysteine in α-L41C reduced the binding of B111 to the α-L41C/hCGβ heterodimer to 58.9 ± 7.3% that of hCG (average of 5 independent studies). This ratio was not changed by the presence of the cysteine in the α-L41C/hCG-βN77C heterodimer. Free hCGβ-C26A,N77C was readily detected in the medium using antibody B101 for capture and ^{125}I-B122 for detection (0.67 ± 0.01 ng/50 μl medium, Study 1). hCGβ-N77C is recognized by both ^{125}I-B111 and ^{125}I-B112, antibodies that bind the hCG β-subunit near residues βCys^{110} and βAsn^{77}, respectively. This showed that the seatbelt of hCGβ-N77C is latched to βCys^{26}. In contrast, hCGβ-C26A,N77C was not recognized by either ^{125}I-B111 or ^{125}I-B112, showing that its seatbelt is latched to the cysteine introduced in place of βCys^{77}.

Analog transfected		Total dimer secreted	B111 binding	Acid stable dimer	Probable location of latch disulfide
α-Subunit	β-Subunit				
			ng/50 μl ± S.E.		
Study 1: Cysteine in place of Asn^{77} in β3					
α-L41C	hCGβ	6.52 ± 0.42	2.72 ± 0.38	<0.1	βCys^{110}/βCys^{26}
α-L41C	hCGβ-C26A	5.60 ± 0.10	<0.1	4.67 ± 0.45	βCys^{110}/αCys^{41}
α-L41C	hCGβ-N77C	2.37 ± 0.09	1.27 ± 0.09	<0.1	βCys^{110}/βCys^{26}
α-L41C	hCGβ-C26A,N77C	<0.1	Not tested	Not tested	βCys^{110}/βCys^{77}
Study 2: Cysteines in locations nearer the seatbelt origin					
α-L41C	hCGβ-C26A	1.50 ± 0.03	Not tested	1.41 ± 0.06	βCys^{110}/αCys^{41}
α-L41C	hCGβ-A35C,C26A	0.18 ± 0.01	Not tested	0.17 ± 0.01	βCys^{110}/βCys^{35}>αCys^{41}
α-L41C	hCGβ-F64C,C26A	<0.1	Not tested	<0.1	βCys^{110}/βCys^{64}
α-L41C	hCGβ-A83C,C26A	<0.1	Not tested	<0.1	βCys^{110}/βCys^{83}

measurable by conformation-sensitive antibodies such as those employed in these studies. For example, a disulfide that disrupted the cystine knot would be likely to prevent the protein from folding and being assembled into a heterodimer with the α-subunit (16). A cysteine that disrupted the disulfide between βCys^{23} and βCys^{72} would prevent it from being recognized by B110. As noted earlier, a cysteine that interfered with seatbelt latching would prevent B111 binding. Several cysteines have been added to the hCG β-subunit without preventing its assembly into heterodimers or being recognized by monoclonal antibodies (20, 21). This shows that adding a cysteine to the native hCG β-subunit does not prevent its seatbelt from being latched to βCys^{26}.

In contrast, if the seatbelt has a strong tendency to be latched to the β-subunit before the subunits dock with one another, addition of a cysteine to hCGβ-C26A would prevent it from being assembled with α-subunit analogs that contain an additional cysteine unless assembly can occur by a threading mechanism. As noted earlier, heterodimers that contain hCGβ-C26A can form only by a wraparound mechanism. The addition of a cysteine to hCGβ-C26A in place of βAsn^{77} to create hCGβ-C26A,N77C creates a potential latch site in loop β3. Because latching the seatbelt to βCys^{77} before hCGβ-C26A,N77C docks with α-L41C would prevent the seatbelt from being latched to αCys^{41}, this substitution would block heterodimer formation by a wraparound mechanism. If the latching of the seatbelt to this location also blocked threading, this substitution would also prevent any heterodimer formation (Fig. 6).

Substitution of a cysteine for βAsn^{77} in hCGβ did not prevent it from forming heterodimers with α-L41C that were recognized by B111 (Table III, Study 1). This showed that the presence of βCys^{77} did not block subunit folding or prevent the seatbelt from being latched to βCys^{26}. The total amount of heterodimer formed was usually decreased, however, indicating that βCys^{77} may have competed with βCys^{26} as a seatbelt latch site. Based on the ability of α-L41C/hCGβ-N77C to be recognized by B111, we expect that its seatbelt is latched to βCys^{26}, not βCys^{77}.

As shown earlier (Table I, 1), α-L41C can be assembled with hCGβ or hCGβ-C26A to form acid unstable and cross-linked heterodimers, respectively. We observed that addition of a cysteine to hCGβ to create hCGβ-N77C did not prevent it from being assembled into heterodimers with α-L41C (Table III, Study 1, rows 1 and 3). In marked contrast, addition of a cysteine to hCGβ-C26A, which created hCGβ-C26A,N77C, prevented heterodimer formation (Table III, Study 1, rows 2 and 4). This was not because of the inability of the cell to make this β-subunit analog. As noted in the legend to Table III, we detected a significant amount of the free β-subunit in the culture medium (0.67 ng/50 μl) that was not recognized by monoclonal antibodies B111 or B112, indicating that its seatbelt was latched to βCys^{77}. This strongly suggested that its seatbelt was latched to βCys^{77} before the subunits dock, a phenomenon that would be expected to interfere with threading. Furthermore, because βCys^{110} was present in a disulfide with βCys^{77}, the seatbelt was unable to be wrapped around loop α2 and form an intersubunit disulfide cross-link with αCys^{41}.

To minimize the possibility that there was something unique about the presence of a cysteine at β-subunit residue 77, we repeated these studies using three different analogs of hCGβ-C26A. The presence of a cysteine in place of βAla^{35}, βPhe^{64}, and βAla^{83} disrupted its ability to form cross-linked heterodimers with α-L41C (Table III, Study 2). Thus, we found 1.5 ng of heterodimer containing hCGβ-C26A in 50 μl of culture medium, but almost no heterodimer containing hCGβ-C26A analogs having cysteines in place of βAla^{35}, βPhe^{64}, or βAla^{83}. These observations supported the notion that most seatbelt latching occurred before the subunits docked. Formation of trace amounts of acid-stable heterodimer containing hCGβ-A35C,C26A (Table III, Study 2) indicated that a small fraction of the seatbelt might remain unlatched until after assembly of this heterodimer.

DISCUSSION

Threading Appears to Be the Major Route of Assembly for the Human Glycoprotein Hormones—The crystal structures of hCG (1, 2) and hFSH (3) show that their seatbelts are latched to a conserved cysteine in loop β1. Because of similarities in the locations of the cysteines of the hLH and hTSH β-subunits, the seatbelts of these hormones are also likely to be latched to this site. The efficiency with which the seatbelt can be latched to βCys^{26} of hCG suggests that the seatbelts of the other human β-subunits are also likely to be latched before their subunits dock. This is supported by our inability to detect heterodimer assembly when any of several α-subunit analogs containing an additional cysteine were expressed with hFSH and hTSH β-subunits in which βCys^{20} and βCys^{19} were replaced by ala-

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nine. Thus, unless the seatbelt becomes unlatched transiently during the assembly process, hFSH and hTSH are also likely to be produced by a threading mechanism. Furthermore, the failure of hCGβ-C26A,N77C to be assembled into heterodimers with α-L41C suggests that the seatbelt latch disulfide is not readily disrupted once it has formed. This argues against the likelihood that the seatbelt becomes unlatched transiently during assembly.

Throughout these studies we were concerned by the possibility that we were being misled by our methodology, particularly because we had expected to find that most heterodimer assembly would occur by a wraparound pathway. We were especially cognizant of the possibility that adding a cysteine to the α-subunit might alter the route of assembly, which is why we studied several α-subunit analogs. We were also concerned that replacing hCG βCys^{26} with alanine would disrupt the structure of the β-subunit. This is also unlikely based on the finding that hCGβ-C26A containing heterodimers were readily recognized by all conformation-sensitive antibodies tested except B111, an antibody that recognizes the position of the latched hCG seatbelt. Furthermore, when hCGβ-C26A or hCGβ-C26A-KDEL were expressed with cysteine containing α-subunit analogs in the absence of hCGβ or hCGβ-KDEL, they formed comparable or greater amounts of heterodimers. This indicated that hCGβ-C26A and hCGβ-C26A-KDEL were capable of being incorporated into heterodimers at rates that should have enabled them to out compete hCGβ and hCGβ-KDEL, if assembly occurs primarily by a wraparound pathway. The finding that only small amounts of hCGβ-C26A and hCGβ-C26A-KDEL were incorporated into heterodimers in the presence of hCGβ or hCGβ-KDEL suggests that the wraparound pathway is a relatively minor route for formation of the human glycoprotein hormones.

As a final effort to test the notion that threading is the more favored route of assembly, we measured the abilities of cysteines within the β-subunit to compete for formation of the seatbelt latch disulfide. The threading pathway predicts that the seatbelt would be latched prior to heterodimer assembly. The finding that addition of a latch site to hCGβ-C26A disrupted its ability to form heterodimers with α-L41C satisfied this prediction. The observation that independent methods led us to the same result, namely that threading is the dominant assembly pathway, strengthens our confidence in this conclusion significantly.

Cysteines Added to the α-Subunit Can Become Cross-linked to the β-Subunit during Assembly—A fraction of several α-subunit analogs became cross-linked to the native hCG β-subunit during assembly. This suggests that one or more β-subunit disulfides are disrupted during assembly and/or that the proximity of a β-subunit disulfide to the cysteine added to the α-subunit makes it subject to a disulfide exchange. The presence of the ER retention signal KDEL facilitated cross-linking of analogs α-L41C, α-S43C, and α-T46C (Table I, 2, 4, and 5; data for α-S43C and α-T46C expressed with β-subunits lacking KDEL retention signal not shown), indicating that this phenomenon occurred in the ER. The ability of α-S92C to be cross-linked to the native β-subunit was as low or lower than that of any other cysteine tested, even though it became cross-linked to hCGβ-C93A-KDEL and hCGβ-C100A-KDEL better than any other cysteine that was added to the α-subunit. This suggested that cross-linking depended on the proximity of the α-subunit cysteine to the seatbelt during threading. As will be described elsewhere (26), the disulfide that stabilizes a small loop within the seatbelt is disrupted during threading, which permits it to form this cross-link. This disulfide is also the only α- or β-subunit disulfide that we found to be disrupted significantly during β-mercaptoethanol catalyzed threading *in vitro* (6).

We considered the possibility that the cross-linking we observed during assembly of heterodimers that contained hCGβ and hCGβ-KDEL might be because of latching of the seatbelt to the cysteine that had been added to the α-subunit. Whereas we cannot exclude this possibility completely, the fact that the hCGβ analogs lacking the abilities to latch their seatbelts to the β-subunit competed poorly with those that can latch their seatbelts to βCys^{26} in every case is inconsistent with this possibility. This includes more than 30 independent experiments done by different individuals over a period of three years, some of which were performed with 6-fold more hCGβ-C26A than hCGβ. Furthermore, the notion that the wraparound pathway is a significant mode of hCG assembly is inconsistent with the finding that the hCG seatbelt has a marked tendency to be latched to the β-subunit before the subunits dock productively, even when the seatbelt can be latched only to a site such as βCys^{77} (Table III).

The Wraparound Pathway May Be a Salvage Pathway That Is Particularly Useful for Assembling Molecules with Lutropin Activity—The finding that hCGβ-C26A can be incorporated into heterodimers shows that the wraparound pathway can be used for heterodimer assembly. The observation that it is not as efficient as the threading pathway indicates that it is available as a potential salvage mechanism that would permit natural experimentation with the structure of the seatbelt. The seatbelt is the portion of the β-subunit that has the greatest influence on hormone activity (9, 22–24) and is among the most divergent parts of lutropins, follitropins, and thyrotropins (25). We were not able to detect synthesis of any hFSH or hTSH by the wraparound pathway, a phenomenon that may indicate the seatbelts of these human β-subunits are not wrapped around loop α2 efficiently. Whereas this precluded us from performing competition studies with these β-subunits, as will be discussed in greater detail elsewhere (26), cross-linked heterodimers containing either the hFSH and hTSH β-subunits were found in cells that co-express these β-subunits with α-subunits containing an additional cysteine.

Methods Used Here May Be Suited to Studying the Folding and Assembly of Other Proteins in the ER—Earlier analyses of hCG assembly *in vivo* employed pulse-chase methods that are less suited to detecting transient intermediates than the competition approach outlined here (7). This may explain why the threading pathway was not seen previously. We suggest that the competition strategies outlined here, which can be tailored to permit the detection of transient intermediates, will be useful adjuncts to pulse-chase methods for studying protein folding in cells.

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Glycoprotein Hormone Assembly in the Endoplasmic Reticulum: I. THE GLYCOSYLATED END OF HUMAN α-SUBUNIT LOOP 2 IS THREADED THROUGH A β-SUBUNIT HOLE

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