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) 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 (Serenos Reproductive Biology Institute, Rockland, MA). Constructs

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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.
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-L14C is the natural human α-subunit having a codon for cysteine in place of that for αLeu111. 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 immunassays (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. B110 binds hCG and analogs in which βCys110 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 βCys110 at the end of the hCG seatbelt is latched to βCys26 in the heterodimer and the uncombined β-subunit. B111 can also recognize analogs of hCG in which βCys26 and βCys110 are converted to alanine provided the heterodimer is stabilized in another fashion, such as fusing the NH2-terminal end of the α-subunit to the COOH-terminal end of the β-subunit (6). This indicates that B111 does not recognize the βCys26 βCys110 disulfide per se. B111 does not recognize hCG analogs in which the seatbelt is latched to any other residue other than βCys110, however, or when residues near βCys110 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 n 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 NaCl, 20 mM EDTA, 1 μM leupeptin, 1.5 μM pepstatin, 500 μM phenylmethylsulfonyl fluoride, 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 KH2PO4, 140 mM NaCl, 1.0 mM Na2HPO4, 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.
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 1, 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 βCys26 (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...
observation that the seatbelts in hCG β-subunit analogs such as hCGβ-C26A, which cannot latch their seatbelts to loop β1 because they contain an alanine in place of βCys36, 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, hCGβ would be expected to have the same overall conformation as hCGβ-C26A (Fig. 4). Thus, hCGβ-C26A and the unlatched form of hCGβ (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 hCGβ-C26A. In contrast, because hCGβ-C26A can be incorporated into heterodimers only by the wraparound pathway, it would not be expected to compete with hCGβ 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 hCGβ-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 inter-subunit disulfide cross-link. These are distinguished readily from heterodimers in which the seatbelt is latched to βCys36 by their resistance to dissociation at pH 2, 37 °C, and by their inability to bind monoclonal antibody B111 (8). B111 binds a conformational hCG epitope formed when the seatbelt is latched normally, i.e. to βCys36. 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 hCGβ and each of the α-subunit analogs tested became latched primarily to βCys36°, 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 hCGβ was expressed with α-L41C (Table I, 1). Most heterodimers secreted by cells that were co-transfected with α-L41C and hCGβ lacked an inter-subunit 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 radiodinated antibody to the β-subunit for detection (i.e. 125I-B110) before, but not after low pH treatment. These heterodimers were also detected in similar immunoassays employing A113 for capture and 125I-B111 for detection before treatment at low pH. This indicated that their seatbelts were latched to βCys36°, not αCys41. 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 εCys26 in loop β1 after it had been latched to αCys41, we repeated the study in the presence of hCGβ-C26A, an analog that cannot latch its seatbelt to loop β1. When α-L41C was expressed with hCGβ-C26A, the heterodimer that formed was detected in A113/125I-B110 assays before and after acid treatment (Table I, 1, row 2). Furthermore, none of it was detected in A113/125I-B111 assays before or after low pH treatment. These findings showed that seatbelt residue εCys110 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 hCGβ and little or no hCGβ-C26A (Table I, 1, row 3). Because the seat-
hCG β-subunit analogs forced to latch their seatbelts to the α-subunit compete poorly with those that can latch their seatbelts to βCys<sup>92</sup>

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<sup>125</sup>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<sup>125</sup>I-B110 detection sandwich assays. Those in the third and fourth data columns are the results of A113 capture<sup>125</sup>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) and expression of α113 (row 1) and 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, and in one 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, 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, 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. 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appearance 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 βCys26. 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 A113/225I-B111 assays. Whereas its acid stability was not due solely to the formation of a disulfide cross-link, its ability to be recognized by B111 showed that its seatbelt was latched normally and that it may have been formed by a wraparound mechanism. The finding that the seatbelt was latched to βCys26 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 βCys26 out competed αCys41 as a seatbelt latch site. Whereas this is consistent with the view that the seatbelt had become latched to βCys26 before the subunits docked, it does not preclude the possibility that the seatbelt had become latched to βCys26 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 A113/225I-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 A113/225I-B111 assays (Table I, 2, row 3). These findings are consistent with the conclusions that the seatbelts of most heterodimer molecules were latched to βCys26, not αCys41 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 αCys41 and either βCys26 or βCys100. 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 αCys41 and βCys100. 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,NS52D, 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...
detected readily in A113/L251-B111 assays, showing that its seatbelt was latched to βCys26 (Table I, 3, row 1). Heterodimer formed with hCGβ-C26A-KDEL was acid stable and not recognized in A113/L251-B111 assays, indicating that seatbelt residue βCys110 became bridged to αCys41, a consequence of the wraparound pathway (Table I, 3, row 2). Most heterodimers formed when both subunits were co-expressed with α-L41C,N52D contained hCGβ-CDEL 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/L251-B111 assays showed that its seatbelt was latched to βCys26, not αCys41. Less than 8% of the heterodimer made in the presence of both α-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 αCys41 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 βCys26 in the heterodimer. Thus, we expected that seatbelt residue βCys110 would be latched to αCys41 readily, a phenomenon observed only when the seatbelt is prevented from being latched to its natural β-subunit site (i.e. βCys26).

We tested the abilities of hCGKDEL 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 hCGKDEL, 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 βCys83 or, more likely, βCys100. 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 βCys100 (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 βCys100, 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 βCys83 or βCys100. 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 αCys83 and αCys100 are readily bridged to βCys110. Thus, these cysteines should have competed effectively with βCys26 during assembly of heterodimers that formed by the wraparound pathway. The finding that little, if any, intersubunit disulfide between βCys110 and either αCys83 or αCys100 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 co-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 αCys83βCys110 or αCys100βCys110 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
route. To test the notion that cysteines in other regions of the \(\alpha\)-subunit would permit us to detect hCG assembly by the wraparound pathway, we tested the influence of a cysteine in place of \(\alpha\)Ser\(^{92}\). 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 \(\beta\)Cys\(^{92}\) or \(\beta\)Cys\(^{100}\) 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 \(\alpha\)-S92C and hCG\(\beta\)-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/L41C-B111 assays as it was in A113/125I-B111 assays. Based on the abilities of \(\alpha\)-S92C to form heterodimers with either hCG\(\beta\)-C93A-KDEL and hCG\(\beta\)-C100A-KDEL (Table II), we anticipate that this small amount of cross-linked heterodimer contains an intersubunit disulfide between Cys\(^{92}\) and either Cys\(^{92}\) or Cys\(^{100}\). Both of these are recognized well by B111. Expression of \(\alpha\)-S92C with hCG\(\beta\)-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 Cys\(^{92}\)-Cys\(^{110}\) disulfide and had been formed by a wraparound pathway. Co-expression of \(\alpha\)-S92C, hCG\(\beta\)-KDEL, and hCG\(\beta\)-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 a Cys\(^{92}\)-Cys\(^{110}\) disulfide.

Again, this supported the notion that hCG is assembled by a threading pathway.

Human Folliculin and Thyrotropin Also Appear to be Assembled by a Threading Pathway—hFSH and hTSH are structurally similar to hCG and contain the same \(\alpha\)-subunit. These \(\beta\)-subunits formed heterodimers with \(\alpha\)-subunit analogs containing cysteines added to the \(\alpha\)-subunit in place of residues 35, 37, 41–50, 64, 86, 88, and 90–92 (not shown). Unlike the hCG \(\beta\)-subunit, however, analogs of hFSH and hTSH \(\beta\)-subunits lacking the abilities to latch their seatbelts to \(\beta\)-subunit loop 1 (i.e. hFSH\(\beta\)-C20A, hFSH\(\beta\)-C20A-KDEL, and hTSH\(\beta\)-C19A) did not form heterodimers with any of these \(\alpha\)-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 \(\beta\)-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 \(\beta\)-subunit before the subunits dock, even if it were forced to be latched to sites other than \(\beta\)Cys\(^{26}\). 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 hCG\(\beta\) and hCG\(\beta\)-C26A that contained an additional cysteine as described (Fig. 6).

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

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**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 hCG\(\beta\) or hCG\(\beta\)-C26A would create a new seatbelt latch site within the \(\beta\)-subunit. As shown in the top two rows, the presence of this cysteine in hCG\(\beta\)-C26A creates an analog (hCG\(\beta\)-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 \(\beta\)-subunit before the subunits dock. In contrast, formation of an intrasubunit disulfide prior to docking would prevent the seatbelt from being latched to the \(\alpha\)-subunit. Consequently, no heterodimer would form unless it were possible for the \(\alpha\)-subunit to be threaded through the altered \(\beta\)-subunit. In the case of hCG\(\beta\)-C26A,N77C, latching the seatbelt to \(\beta\)Cys\(^{77}\) would also disrupt the ability of the free subunit to be recognized by B112 as well as by B111. Addition of a cysteine to hCG\(\beta\) to create hCG\(\beta\)-N77C would provide the seatbelt with a second latch site. If the seatbelt became latched to its native site, this \(\beta\)-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 wraparound pathway. As described in the text, the presence of additional cysteines in hCG\(\beta\) did not prevent heterodimer formation, suggesting that the seatbelt was more likely to form a stable disulfide with \(\beta\)Cys\(^{26}\), its natural site. In contrast, the presence of additional cysteines in hCG\(\beta\)-C26A prevented this \(\beta\)-subunit analog from being incorporated into a heterodimer.
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 βCys23 and βCys77 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 βCys77.

In contrast, if the seatbelt has a strong tendency to be latched to the α-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 βAsn77 to create hCGβ-C26A,N77C creates a potential latch site in loop β3. Because latching the seatbelt to βCys77 before hCGβ-C26A,N77C docks with α-L41C would prevent the seatbelt from being latched to αCys41, 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 βAsn77 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 βCys77 did not block subunit folding or prevent the seatbelt from being latched to βCys77. The total amount of heterodimer formed was usually decreased, however, indicating that βCys77 may have competed with βCys23 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 βCys41, not βCys77.

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 βCys77. This strongly suggested that its seatbelt was latched to βCys77 before the subunits dock, a phenomenon that would be expected to interfere with threading. Furthermore, because βCys77 was present in a disulfide with βCys77, the seatbelt was unable to be wrapped around loop α2 and form an intersubunit disulfide cross-link with αCys41.

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 βAla35, βPhe64, and βAla63 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 βAla35, βPhe64, or βAla63. 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 LH 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 βCys23 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 βCys20 and βCys19 were replaced by αCys41.

| Analog transfected | α-Subunit | β-Subunit | Total dimer secreted | B111 binding | Acid stable dimer | Probable location of latch disulfide | ng/50 μl ± S.E. |
|--------------------|-----------|-----------|---------------------|---------------|------------------|-------------------------------------|----------------|
| Study 1: Cysteine in place of Asn77 in β3 | α-L41C | hCGβ | 6.52 ± 0.42 | 2.72 ± 0.38 | <0.1 | βCys110/βCys23 | 0.57 ± 0.15 |
| | α-L41C | hCGβ-C26A | 5.60 ± 0.10 | <0.1 | 4.67 ± 0.45 | βCys110/βCys41 | 0.38 ± 0.03 |
| | α-L41C | hCGβ-N77C | 2.37 ± 0.09 | 1.27 ± 0.09 | <0.1 | βCys110/βCys23 | 0.03 Not tested |
| | α-L41C | hCGβ-C26A,N77C | <0.1 | Not tested | Not tested | βCys110/βCys77 | 0.10 |
| Study 2: Cysteines in locations nearer the seatbelt origin | α-L41C | hCGβ-C26A | 1.50 ± 0.03 | Not tested | 1.41 ± 0.06 | βCys110/βCys41 | 1.50 |
| | α-L41C | hCGβ-A35C,C26A | 0.18 ± 0.01 | Not tested | 0.17 ± 0.01 | βCys110/βCys41 | 0.03 |
| | α-L41C | hCGβ-Phe64,C26A | <0.1 | Not tested | <0.1 | βCys110/βCys41 | 0.03 |
| | α-L41C | hCGβ-A35C,C26A | <0.1 | Not tested | <0.1 | βCys110/βCys83 | 0.03 |
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 βCys26 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 locked 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 βCys26 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 βCys77 (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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