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Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

III. THE SEATBELT AND ITS LATCH SITE DETERMINE THE ASSEMBLY PATHWAY*

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Vertebrate glycoprotein hormone heterodimers are stabilized by a strand of their β-subunits known as the "seatbelt" that is wrapped around loop 2 of their α-subunits (α2). The cysteine that terminates the seatbelt is "latched" by a disulfide to a cysteine in β-subunit loop 1 (β1) of all vertebrate hormones except some teleost follitropins (teFSH), wherein it is latched to a cysteine in the β-subunit NH_2 terminus. As reported here, teFSH analogs of human choriogonadotropin (hCG) are assembled by a pathway in which the subunits dock before the seatbelt is latched; assembly is completed by wrapping the seatbelt around loop α2 and latching it to the NH_2 terminus. This differs from hCG assembly, which occurs by threading the glycosylated end of loop α2 beneath the latched seatbelt through a hole in the β-subunit. The seatbelt is the part of the β-subunit that has the greatest influence on biological function. Changes in its sequence during the divergence of lutropins, follitropins, and thyrotropins and the speciation of teleost fish may have impeded heterodimer assembly by a threading mechanism, as observed when the hCG seatbelt was replaced with its salmon FSH counterpart. Whereas wrapping is less efficient than threading, it may have facilitated natural experimentation with the composition of the seatbelt during the co-evolution of glycoprotein hormones and their receptors. Migration of the seatbelt latch site to the NH_2-terminal end of the β-subunit would have facilitated teFSH assembly by a wraparound mechanism and may have contributed also to its ability to distinguish lutropin and follitropin receptors.

The glycoprotein hormones have key roles in reproduction and thyroid function (1). These heterodimers have an unusual topology in which a strand of their β-subunits surrounds a loop of their α-subunits (2–4). Because the carboxyl-terminal end of this strand is "latched" by a disulfide to the β-subunit, it has been likened to a "seatbelt" (2). In addition to its role in stabilizing the heterodimer, the seatbelt is responsible for much of the influence of the β-subunit on human glycoprotein hormone activity (5–8).

Most glycoprotein hormone β-subunits appear to have evolved from a single ancestor (9) and their folding pattern is highly conserved in all vertebrates except for that in FSH[1] of some teleost fish. Whereas in most species the seatbelt is latched to a cysteine in loop β1, in many teleosts it is latched to a cysteine in the NH_2-terminal end of the β-subunit corresponding to hCG residue βLeu^5 (10). This reduces the size of the hole in the β-subunit through which loop α2 is straddled (Fig. 1). The smaller size of this space in teFSH would be expected to impede threading of the glycosylated end of loop α2 through the β-subunit, a phenomenon that explains the greater acid stability of the teFSH heterodimer (10, 11) relative to that of glycoprotein hormones such as hCG. The latter dissociate completely within 15–20 min at pH 2, 37 °C (12).

The human glycoprotein hormones hCG, hFSH, and hTSH are assembled in mammalian cells primarily by a process in which loop α2 and its attached oligosaccharide are threaded through a hole in the β-subunit. This hole is formed after the seatbelt is latched to loop β1 (19, 20). The smaller space available for passage of loop α2 beneath the teFSH seatbelt suggested that teFSH might be assembled by a mechanism in which the subunits dock before the seatbelt is latched. This would enable the seatbelt to be wrapped around loop α2 before it is latched to a cysteine in the β-subunit NH_2 terminus. Experiments described here were designed to learn how teFSH analogs of hCG are assembled in the endoplasmic reticulum and to identify factors that contribute to the use of the threading and wraparound pathways for glycoprotein hormone assembly. Because the topology of teFSH differs substantially from that of hCG, we anticipated these studies would also enable us to test our procedures for analyzing hormone assembly in living cells (19, 20). By studying the assembly of a series of salmon FSH-hCG chimeras that are readily monitored using antibodies to hCG, we found that hCG analogs having the teFSH fold cannot be assembled by a threading mechanism and, as a consequence, are formed by a wraparound mechanism. Forcing the seatbelt to be latched to a site in the NH_2 terminus enhanced the assembly of teFSH analogs, most likely by reducing the inherent tendency of the seatbelt to be latched before the subunits dock.

EXPERIMENTAL PROCEDURES

The α- and β-subunit analogs used in these studies are illustrated in Fig. 2. Chimeras of salmon FSH and hCG β-subunits are identified using the root term s/hCGβ. For example, s/hCGβ-Nt,SB,C26A is an hCG β-subunit analog that contains the salmon FSH NH_2 terminus and seatbelt and an alanine in place of βCys^{26}. All other reagents and

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[1] The abbreviations used are: FSH, follitropin; hCG, human choriogonadotropin; hFSH, human follitropin; hTSH, human thyrotropin; α2, α-subunit loop 2; β1, β2, β3, β-subunit loops 1, 2, and 3, respectively; s/hCGβ, chimera of chum salmon FSH and hCG β-subunits; NH_2 terminus, amino-terminal end of the chum salmon β-subunit; SB, chum salmon seatbelt; TL, chum salmon seatbelt tensor loop; St, chum salmon seatbelt strap; tsFSH, teleost follitropin; LH, lutropin. The structures of the analogs used in this work can be determined by reference to Fig. 2.

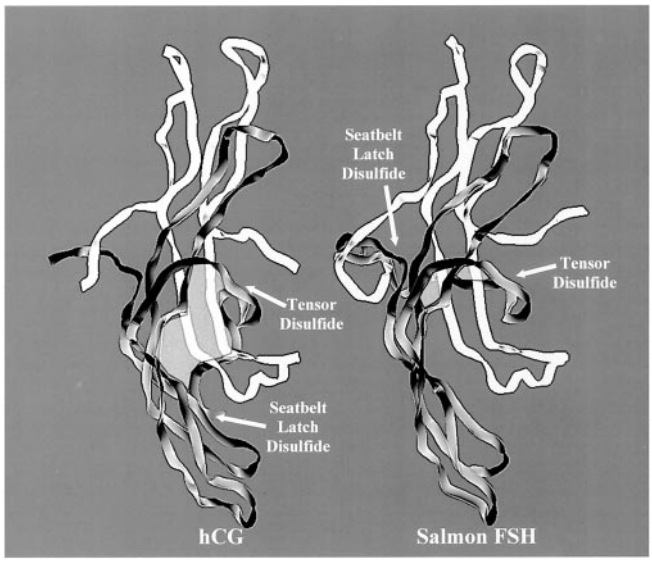

FIG. 1. **Comparison of the structures of hCG and salmon FSH showing the difference in the sizes of the holes in the β-subunits when the seatbelt is latched to β1 and the NH_2 terminus.** The Cα carbons of hCG (*left panel*) and a model of salmon FSH (*right panel*) are shown here. Atoms of β-subunit residues in the subunit core and seatbelt that surround α2 are highlighted. Note that the size of this hole appears to be considerably greater when the seatbelt is latched to a cysteine in β1 than when it is latched to a cysteine in the NH_2 terminus. The smaller size of the β-subunit hole in salmon FSH would make it more difficult to thread loop α2 beneath the seatbelt, a phenomenon that would account for its greater acid stability than hCG (10, 11). Therefore, one might expect that the assembly of salmon FSH differs from that of hCG. Color code: *white*, α-subunit; *dark gray*, β-subunit; *hatched patches*, β-subunit hole straddled by loop α2.

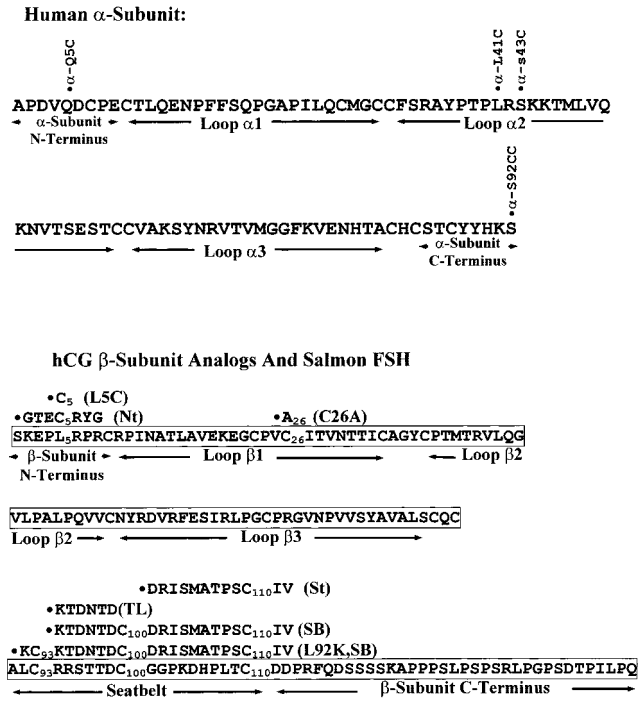

FIG. 2. **Sequences of the α- and β-subunit analogs used in these studies.** Cysteines were substituted for residues in the α-subunit to create analogs whose names are indicated at the sites of the mutations. Changes to the hCG β-subunit are indicated *above* the hCG β-subunit sequence, which is *boxed*. The name of each mutation described in the text is indicated in *parentheses* following its sequence. For example, C26A indicates that cysteine residue 26 in loop β1 had converted to alanine; *Nt* indicates that the hCG β-subunit NH_2-terminal sequence Lys-Glu-Pro-Leu-Arg-Pro-Arg had been replaced by the salmon NH_2-terminal sequence Gly-Thr-Glu-Cys^4-Arg-Tyr-Gly. Note that residues Cys^{26} and Cys^5 are seatbelt latch sites in β1 and NH_2 terminus, respectively. Some analogs are combinations of these mutations as suggested by their names. Thus, s/hCGβ-Nt,C26A,SB is a salmon FSH and hCG β-subunit chimera with the hCG β-subunit NH_2-terminal and seatbelt sequences replaced with those from salmon FSH and the seatbelt latch site in loop β1 Cys^{26} replaced with alanine.

methods used in these studies have been described in the preceding accompanying articles (19, 20).

RESULTS

Some Heterodimers Containing β-Subunits That Are Chimeras of Salmon FSH and hCG Are Assembled Differently from hCG—To study the assembly of heterodimers that have the teFSH folding pattern, we employed an hCG β-subunit analog that contained the salmon FSH β-subunit NH_2 terminus and seatbelt. This β-subunit chimera, s/hCGβ-Nt,SB,C26A (Fig. 2), retained binding sites for most hCG monoclonal antibodies that recognize epitopes formed by the subunit core, which includes its cystine knot and loops β1, β2, and β3. Because this analog contains an alanine in place of βCys^{26}, the normal hCG β-subunit seatbelt latch site, it was expected to latch its seatbelt to βCys^5, a cysteine within its NH_2-terminal salmon sequence. This β-subunit chimera was incorporated into heterodimers that contained the native human α-subunit, but much less efficiently than hCGβ (Table I, data rows 1 and 7). Unlike the hCG heterodimer, which dissociated completely at pH 2 within 30 min at 37 °C, only about 20% of the hCG/teFSH chimera heterodimer dissociated under these conditions. It was not as stable as heterodimers that contain an intersubunit disulfide, which do not dissociate under these conditions (13). These observations showed that we could distinguish heterodimers in which the seatbelt is latched to the α-subunit, heterodimers in which the seatbelt is latched to βCys^5, and heterodimers in which the seatbelt is latched to βCys^{26} by measuring differences in their stabilities at pH 2, 37 °C. These differences become much more apparent following an overnight treatment at pH 2, 37 °C (Fig. 3).

The hCGβ and s/hCGβ-Nt,SB,C26A subunits differ in their NH_2-terminal ends, their seatbelts, and in loop β1. Studies described next were designed to learn how each of these regions influence heterodimer assembly. Replacing hCG β-subunit residue βLeu^5 with cysteine created hCGβ-L5C, an hCG analog that has a seatbelt latch site in an NH_2-terminal location corresponding to that in salmon FSH. Heterodimers that contained the native human α-subunit and hCGβ-L5C dissociated rapidly at pH 2, 37 °C, and were recognized by B111 (Table I, row 2), indicating that their seatbelts were latched to βCys^{26} in loop β1, not to βCys^5. Thus, introduction of a potential latch site into the hCGβ NH_2 terminus was not sufficient to alter the manner in which the heterodimer is assembled. In contrast, elimination of the seatbelt latch site in loop β1 of hCGβ-L5C reduced its ability to form heterodimers with the native human α-subunit as can be seen by the lack of heterodimer formed when the α-subunit was co-expressed with hCGβ-L5C,C26A (Table I, row 3). This finding is consistent with the notion that the hCG seatbelt of hCGβ-L5C,C26A became latched to βCys^5 prior to subunit docking and/or that the seatbelt did not become latched to βCys^5 after the subunits had docked. The propensity of the hCG seatbelt to be latched prior to subunit docking (19) suggests that it may have become latched to this site prior to the start of assembly. This would indicate that it can interfere with threading when latched to a cysteine in the NH_2 terminus.

To learn how the salmon FSHβ NH_2 terminus affected assembly, we expressed s/hCGβ-Nt and s/hCGβ-Nt,C26A with the native human α-subunit (Table I, rows 4 and 5). The presence of the salmon FSHβ NH_2 terminus appeared not to affect most heterodimer assembly. Consequently, 90% of the het-

TABLE I

Relative influence of the β1 and N-terminal latch sites on heterodimer assembly

COS-7 cells were transfected with either the native human α-subunit and hCGβ or the indicated analog.

Data row	Analog transfected		Total dimer[a]	Dimer detected by B111[b]	Acid-stable dimer[c]	Probable seatbelt latch disulfide
	α-Subunit	β-Subunit				
				(ng/50μl ± SEM)[2]		
1	Native	hCGβ	6.72 ± 0.36	6.95 ± 0.26	<0.1	$\beta Cys^{110}/\beta Cys^{26}$
2	Native	hCGβ-L5C	7.32 ± 0.52	4.81 ± 0.18	<0.1	$\beta Cys^{110}/\beta Cys^{26}$
3	Native	hCGβ-L5C,C26A	<0.1	<0.1	<0.1	$\beta Cys^{110}/\beta 5$
4	Native	s/hCGβ-Nt	5.23 ± 0.20	6.37 ± 0.13	0.42 ± 0.08	$\beta Cys^{110}/\beta C26\beta Cys^{5}$
5	Native	s/hCGβ-Nt,C26A	<0.1	<0.1	<0.1	$\beta Cys^{110}/\beta 5$[d]
6	Native	s/hCGβ-Nt,SB	0.16 ± 0.01	<0.1	0.12 ± 0.03	$\beta Cys^{110}/\beta 5$[e]
7	Native	s/hCGβ-Nt,SB,C26A	0.91 ± 0.06	<0.1	0.74 ± 0.09	$\beta Cys^{110}/\beta 5$

[a] Heterodimer secreted into the culture medium was determined in A113/^{125}I-B110 sandwich assays.

[b] Latching of the seatbelt to β1 was determined in A113/^{125}I-B111 sandwich assays. B111 does not recognize the salmon FSH seatbelt when it is latched to βCys^{26}, however.

[c] The acid stability of the heterodimer was determined in A113/^{125}I-B110 sandwich assays of media treated at pH 2 for 30 min at 37 °C.

[d] Because we were unable to detect any heterodimer, we assumed the seatbelts of these analogs were either unlatched or, more likely, became latched to the cysteine in the N_2-terminal end of the β-subunit prior to formation of the heterodimer.

[e] The seatbelt was assumed to be latched to the NH_2-terminal end of the β-subunit based on the finding that the small amounts of heterodimer formed are stable at low pH. We expected that the seatbelt was latched to βCys^{26} in most of the free β-subunit but this could not be determined using existing antibodies due to the fact that B111 does not recognize the salmon seatbelt, even when it is latched to βCys^{26}.

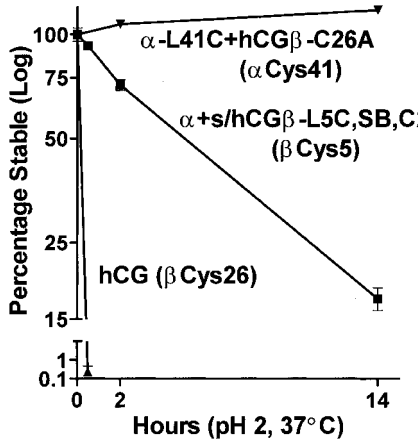

FIG. 3. **Variation in heterodimer stability as a function of the seatbelt latch site.** The acid stabilities of heterodimers in which the seatbelt is latched to βCys^{26} (*i.e.* hCG), βCys^{5} (*i.e.* α+s/hCGβ-L5C,SB,C26A), and α-subunit residue αCys41 (*i.e.* α-L41C+hCGβ-C26A) were determined at pH 2, 37 °C. The heterodimer containing the native hCG β-subunit dissociated completely within 30 min. That in which the seatbelt is latched to the α-subunit did not dissociate during an overnight incubation. The heterodimer in which the seatbelt was latched to βCys^{5} had a half-life of ~5–6 h.

erodimers containing s/hCGβ-Nt dissociated within 30 min at pH 2, 37 °C, and were readily recognized by antibody B111 (Table I, row 4). The latter observation showed that their seatbelts were latched to βCys^{26} in loop β1. The remaining 10% survived pH 2 treatment, 37 °C for 30 min, however, indicating that their seatbelts were latched to βCys^{5} in the salmon FSHβ NH_2 terminus. Preventing s/hCGβ-Nt from latching its seatbelt to loop β1 by converting βCys^{26} to alanine inhibited heterodimer assembly as seen by the lack of incorporation of s/hCGβ-Nt,C26A into heterodimers (Table I, row 5). Considered together, these observations suggested that the hCG seatbelt present in either s/hCGβ-Nt or s/hCGβ-Nt,C26A can become latched to βCys^{5} in the salmon FSH NH_2 terminus before or after the subunits dock; latching it to βCys^{5} prior to subunit docking appeared to inhibit assembly.

The teFSH seatbelt has a much greater influence than the NH_2-terminal latch site on heterodimer assembly. Much less heterodimer was formed when the native human α-subunit was co-expressed with an analog of s/hCGβ-Nt in which the hCG seatbelt was replaced with the salmon seatbelt (*i.e.* s/hCGβ-Nt,SB, Table I, row 6). The small amount of s/hCGβ-Nt,SB that was assembled into heterodimers containing the native α-subunit was roughly as stable as that containing s/hCGβ-Nt,SB,C26A, suggesting that its seatbelt was latched to βCys^{5} in the NH_2 terminus and not to βCys^{26} in loop β1. In fact, heterodimers containing s/hCGβ-Nt,SB,C26A, a β-subunit analog that has only a single seatbelt latch site, were produced more efficiently than those containing s/hCGβ-Nt,SB (Table I, data rows 6 and 7). This observation indicated that the salmon FSH seatbelt in s/hCGβ-Nt,SB became latched to each of these potential latch sites and that it may block threading when it is latched to βCys^{26}. In subsequent studies, we converted βCys^{5} of s/hCGβ-Nt,SB to alanine, thereby creating s/hCGβ-Nt,SB,C5A, which lacks an NH_2-terminal seatbelt latch site. This abolished heterodimer formation (not shown), confirming the notion that the seatbelt in heterodimers containing s/hCGβ-Nt,SB is latched to βCys^{5} and that latching the seatbelt to βCys^{26} in loop β1 suppressed assembly.

Assembly of Glycoprotein Hormones in Which the Seatbelt Is Latched to a Cysteine in the β-Subunit NH_2 Terminus Occurs by a Wraparound Route—Heterodimers in which the seatbelt was latched to the β-subunit NH_2 terminus dissociated at pH 2, 37 °C, albeit much slower than hCG (Fig. 3). This showed that during acid-induced dissociation of teFSH analogs the glycosylated end of loop α2 can pass through the space between the seatbelt and the β-subunit core. Theoretically, the reversal of this process would permit heterodimer assembly by a threading mechanism, albeit at a very slow rate. The small space between the seatbelt and the subunit core suggested that threading would be highly unlikely, even if the tensor disulfide were to be disrupted during threading as is the case during the assembly of hCG (20). The threading and wrapping pathways could be distinguished if we were able to determine whether the seatbelt had been latched before or after the subunits docked. As discussed next, this can be accomplished using disulfide cross-links to trap early assembly intermediates.

To trap early docking intermediates, we took advantage of the fact that the NH_2-terminal ends of the subunits become adjacent when the heterodimer is assembled (2, 3). We reasoned that converting αGln^{5} to cysteine to create α-Q5C would enable the formation of an α5-β5 intersubunit disulfide, but only if the subunits docked before the seatbelt was latched (Fig. 4). Furthermore, because αCys^{5} is located near βCys^{5}, the seatbelt latch site, we expected that during the wraparound pathway the seatbelt might also be latched to αCys^{5} instead of βCys^{5}, provided the α5-β5 disulfide had not already been formed. Either of these phenomena would have resulted in an

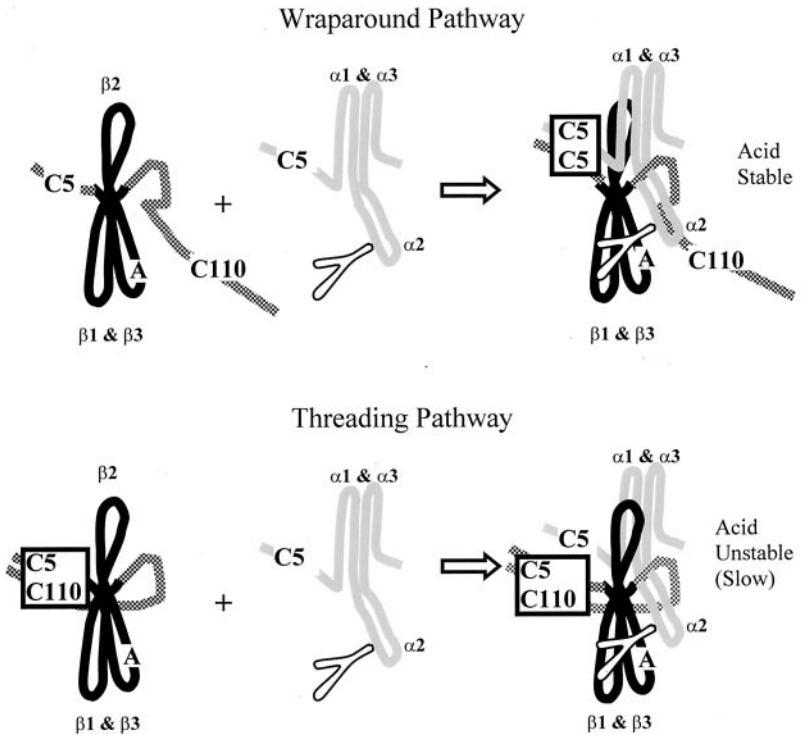

FIG. 4. **The mechanism of teFSH assembly can be distinguished by monitoring the assembly of heterodimers that contain α-Q5C.** *Top row*, during assembly of teFSH analogs by a wraparound mechanism, the β-subunit docks with the α-subunit before the seatbelt is latched. This will position αCys^5 adjacent to βCys^5, with which it is likely to form an intersubunit disulfide. This will make the resulting heterodimer completely resistant to dissociation at pH 2, 37 °C. *Bottom row*, during assembly of these analogs by a threading mechanism, the seatbelt becomes latched to βCys^5 before the subunits dock. This would prevent the formation of an intersubunit αCys^5-βCys^5 disulfide and the heterodimer will have a stability similar to that of teFSH. Consequently, the heterodimer will dissociate with a half-life of ~5–6 h at pH 2, 37 °C.

intersubunit disulfide cross-link that would prevent the heterodimer from dissociating upon treatment overnight at pH 2, 37 °C. In contrast, if assembly occurs by a threading mechanism instead of a wraparound mechanism, seatbelt residue βCys^{110} would have been cross-linked to βCys^5 prior to docking. This would have prevented the formation of both the α5-β5 and α5-β110 disulfides and led to the formation of a heterodimer that dissociated with a half-life of 5–6 h during treatment at pH 2, 37 °C. Of course, it was also possible that during wrapping, seatbelt residue βCys^{110} would become latched to βCys^5 to create a heterodimer that would also dissociate with a half-life of 5–6 h. Therefore, a finding that all the heterodimer dissociated with a half-life of 5–6 h would indicate but not prove that assembly occurs by a threading mechanism. However, the finding that a substantial fraction of the heterodimer was acid stable would show that most, if not all, heterodimer assembly had occurred by a wraparound mechanism.

There was one other caveat to this approach. Co-expression of α-Q5C and hCGβ-R8C, analogs in which cysteines are substituted for hCG residues αGln^5 and βArg^8 has been shown to form a disulfide cross-linked heterodimer (14). Formation of this disulfide is explained by the distances between the Cα and Cβ atoms of these residues (*i.e.* roughly 5.2 and 3.9 Å), which are similar to those in typical glycoprotein hormone disulfides (*i.e.* roughly 5–6.5 and 4 Å, respectively). The location of the salmon FSH β-subunit latch site corresponds to hCG β-subunit residue βLeu^5, not βArg^8, however. The distances between the Cα and Cβ atoms of αGln^5 and βLeu^5 are ~9.1 and 11.1 Å, suggesting that when these residues are replaced with cysteines, their positions in the heterodimer might prevent them from forming a disulfide.

To learn if an α5-β5 disulfide bridge can form during heterodimer assembly, we co-expressed hCGβ-L5C with the native α-subunit and with α-Q5C. Heterodimers formed with the native α-subunit were unstable at acid pH and dissociated within 30 min (Table I, data row 2); those formed when hCGβ-L5C was co-expressed with α-Q5C remained intact following an overnight incubation at pH 2, 37 °C (Table II, data row 1). Both heterodimers were recognized by antibody B111 (not shown), demonstrating that their seatbelts were latched to residue βCys^{26}. This showed that heterodimers containing α-Q5C and hCGβ-L5C are stabilized by an α5-β5 disulfide bridge.

We reasoned that if seatbelt residue βCys^{110} is latched to βCys^5 before the teFSH β-subunit docks with α-Q5C, neither of these β-subunit cysteines can be cross-linked to αCys^5. We tested this possibility by co-expressing α-Q5C with hCGβ-L5C,C26A, an analog that has a teFSH folding pattern and in which the seatbelt appears to be latched efficiently to βCys^5 before the subunits dock. This analog did not form detectable amounts of heterodimer with the native α-subunit (Table I, data row 3) and only small amounts were incorporated into heterodimers containing α-Q5C (Table II, row 2). Apparently, the seatbelt of hCGβ-L5C,C26A became latched to βCys^5 before the subunits could dock, which precluded the formation of an αCys^5-βCys^5 disulfide bridge. The finding that small amounts of hCGβ-L5C,C26A were incorporated into heterodimers containing α-Q5C (Table II, row 2) showed that α-Q5C can be used to trap β-subunits that contain unlatched seatbelts, even when these represent a small fraction of the total β-subunit population. Based on the finding that less than 10% of these heterodimers dissociated after an overnight incubation at pH 2, 37 °C (Table II, data row 2), we anticipate that 9 of 10 heterodimer molecules contained an intersubunit disulfide between αCys^5 and βCys^5 or βCys^{110}, which indicates that the subunits docked before the seatbelts were latched and that they were formed by a wraparound mechanism. The remaining heterodimer molecules appeared to contain an intrasubunit disulfide between βCys^5 and βCys^{110}. Whereas these could have been formed by a threading mechanism, the finding that the vast majority of heterodimers containing hCGβ-L5C,C26A were assembled by a wraparound mechanism suggested that the β5-β110 disulfide had also been formed by a wraparound mechanism after the subunits dock. As expected from the fact that hCGβ-L5C,C26A lacks a loop β1 latch site, heterodimers containing α-Q5C and hCGβ-L5C,C26A were not detected by B111 (not shown).

These findings showed that the pathway used to assemble heterodimers containing teFSH analogs can be deduced from

TABLE II

The ability of αCys[5] to compete for seatbelt for the β-subunit N-terminal latch site

COS-7 cells were transfected with α-Q5C and hCGβ analogs with their NH_2-terminal and/or seatbelt sequences replaced by the counterparts of their salmon FSH counterparts as indicated. The loop β1 seatbelt latch site was also removed in some analogs by replacing βCys[26] with alanine. Heterodimer secreted into the culture medium was quantified in A113/^{125}I-B110 sandwich assays. The stabilities of the heterodimers were determined in A113/^{125}I-B110 sandwich assays of media treated at pH 2 for 30 min or overnight at 37 °C. All values are mean ± S.E. of triplicate transfections.

Data row	Analog	Total dimer	pH 2, 0.5 hours	pH 2, 16 hours
		ng/50μl ± S.E.	*% total ± S.E.*	
1	α-Q5C + hCGβ-L5C	20.02 ± 1.94	102.1 ± 6.1	100.3 ± 3.6
2	α-Q5C + hCGβ-L5C,C26A	1.12 ± 0.07	92.7 ± 0.7	91.9 ± 1.8
3	α-Q5C + s/hCGβ-Nt	7.25 ± 0.09	97.1 ± 1.5	102.9 ± 2.5
4	α-Q5C + s/hCGβ-Nt,C26A	0.73 ± 0.10	91.9 ± 3.7	86.0 ± 3.5
5	α-Q5C + s/hCGβ-Nt,SB,C26A	1.98 ± 0.05	89.9 ± 1.0	89.0 ± 0.9
6	α-Q5C + s/hCGβ-L5C,SB,C26A	8.22 ± 0.12	104.1 ± 2.8	102.7 ± 0.9

the abilities of their NH_2-terminal β-subunit cysteines to become cross-linked to the NH_2-terminal cysteine of α-Q5C. To learn how the salmon NH_2 terminus affected subunit docking and seatbelt latching, we monitored the acid stabilities of heterodimers containing α-Q5C and s/hCGβ-Nt or s/hCGβ-Nt,C26A. These studies revealed that the pathway of assembly was essentially the same when the NH_2-terminal latch site was surrounded by hCG or salmon FSH β-subunit residues. Coexpression of α-Q5C with s/hCGβ,Nt led to a substantial amount of acid-stable heterodimer (Table II, row 3) that was detected readily by B111 (not shown). This revealed that the hCG seatbelt of s/hCGβ-Nt had been latched to βCys[26] in loop β1 prior to subunit docking, thereby enabling βCys[5] in the β-subunit NH_2 terminus to be cross-linked to αCys[5] in the α-subunit NH_2 terminus. In contrast, much smaller amounts of heterodimer were formed when βCys[26] was replaced with alanine to create s/hCGβ-Nt,C26A (Table II, row 4). The reduction in heterodimer assembly caused by this change showed that the hCG seatbelt in s/hCGβ-Nt,C26A had been latched to βCys[5] prior to assembly. In this position the seatbelt inhibited threading and, by being in a disulfide with βCys[5], prevented the formation of the α5-β8 disulfide. As had been observed for heterodimers containing α-Q5C and hCGβ-L5C,C26A, a small fraction of the heterodimers containing α-Q5C and hCGβ-Nt,C26A were acid labile. This indicated that some of the seatbelt had been latched to βCys[5], most likely after the subunits had docked.

To learn how the composition of the seatbelt affected subunit docking and seatbelt latching, we monitored the acid stabilities of heterodimers containing α-Q5C and β-subunits s/hCGβ-Nt,SB,C26A and s/hCGβ-L5C,SB,C26A. These β-subunits are analogs of s/hCGβ-Nt,C26A and hCGβ-L5C,C26A in which the hCG seatbelt has been replaced with its salmon FSH counterpart. The presence of the salmon seatbelt increased the amount of heterodimer formed significantly. For example, 3-fold more heterodimer was obtained with s/hCGβ-Nt,SB,C26A than with s/hCGβ-Nt,C26A (Table II, rows 5 and 4) and 7–8-fold more heterodimer was obtained with s/hCGβ-L5C,SB,C26A than with hCGβ-L5C,C26A (Table II, rows 6 and 2). Most of the heterodimers formed were acid stable, which is consistent with the notion that they had been formed by a wraparound mechanism. These findings indicated that the salmon FSH seatbelt is not latched to βCys[5] as rapidly as the hCG seatbelt and, as a result, more βCys[5] was available to form a disulfide with αCys[5]. As had been observed for heterodimers containing the hCGβ-L5C,C26A and s/hCGβ-Nt,C26A subunits, about 10% of the heterodimer containing the s/hCGβ-Nt,SB,C26A subunit dissociated at low pH. This suggested that the seatbelt had also become latched to βCys[5] in a small fraction of the heterodimer, most likely by a wraparound mechanism.

The teFSH Seatbelt Can Be Latched to the α-Subunit, an Observation That Confirms Assembly Occurs by a Wraparound Mechanism—The observation that αCys[5] became cross-linked to the β-subunit during the assembly of heterodimers containing α-Q5C and β-subunits having the teFSHβ folding pattern strongly supports the notion that these teFSH analogs are assembled by a wraparound mechanism. To test this result in an alternative fashion, we repeated these studies using α-subunit analogs having unpaired cysteines located at other sites (Table III). We have found that hCG seatbelt residue βCys[110] can be latched to cysteines added to the α-subunit when it is prevented from being latched to βCys[26] in loop β1 (13). To learn if the salmon FSH seatbelt behaved similarly to the hCG seatbelt in either regard, we compared the stabilities of heterodimers containing α-L41C or α-S43C and either s/hCGβ-Nt,SB,C26A or s/hCGβ-L5C,SB,C26A (Table III). Heterodimers containing the native α-subunit (Table III, data rows 3 and 6) were more stable than hCG (Table III, data row 1), but not nearly as stable as the cross-linked heterodimers that contained α-L41C and hCGβ-C26A in which the seatbelt is latched to αCys[41] (Table III, row 2) or heterodimers that contained cysteines added to parts of loop α2 (Table III, rows 4, 5, 7, and 8). This observation shows that cysteines added to the α-subunit compete with βCys[5] as a seatbelt latch site, confirming the notion that hCG analogs in which the seatbelt is latched to a cysteine in the NH_2-terminal end of the β-subunit are formed by a wraparound pathway. This suggests that all glycoprotein hormone analogs having the teFSH architecture are likely to be formed by a wraparound mechanism, not by threading.

These studies revealed that teFSHβ analogs containing the salmon FSH seatbelt and an hCGβ NH_2-terminal latch site were incorporated into heterodimers better than those in which both regions were derived from salmon FSHβ (Table III, data rows 3–5 and 6–8). This observation contradicted our expectations that the salmon NH_2 terminus would serve as the more efficient latch site. One explanation for this observation is that the salmon FSH seatbelt has a greater tendency to be latched to the salmon FSHβ NH_2 terminus before the subunits dock, a phenomenon that would reduce assembly. Another explanation is that residues at the NH_2-terminal end of the β-subunit have a role in subunit docking (15, 16). Those in hCGβ may be more effective in promoting subunit docking than those in salmon FSHβ.

The Composition of the Seatbelt Can Influence Threading—Differences in the assembly of heterodimers containing s/hCGβ-Nt and s/hCGβ-Nt,SB indicated that the composition of the seatbelt might have a substantial influence on the mechanism of heterodimer assembly. Although each of these β-subunits has two potential seatbelt latch sites, the hCG seatbelt of s/hCGβ-Nt became latched to βCys[26] and permitted assembly by a threading mechanism. As had been seen earlier (Table I, data row 6), assembly of heterodimers containing the native

TABLE III

Cysteines added to the α-subunit compete with that at βCys[5] for latching the seatbelt

Heterodimers secreted into the culture medium by COS-7 cells co-transfected in triplicate with the indicated α- and β-subunits were concentrated 10-fold and quantified in A113/^{125}I-B110 assays. The samples were then treated at pH 2, at 37 °C for 30 min, 120 min, and overnight before being assayed a second time. Values shown (mean ± S.E. for triplicates) represent the amount of material in the concentrated medium and the percentage that remained as heterodimer following acid treatment. The least acid-stable heterodimer is hCG, in which the seatbelt is latched to βCys[26] in loop β1. The stability of α-L41C/hCGβ-C26A is typical of heterodimers in which the seatbelt is latched to a cysteine in the α-subunit. The stabilities of heterodimers having the seatbelt latched to βCys[5] (*i.e.* those containing the native α-subunit and s/hCGβ-Nt,SB,C26A, s/hCGβ-L5C,SB,C26A, s/hCGβ-Nt,SB, or hCGβ-L5C,C26A-KDEL) is between that of hCG and α-L41C/hCGβ-C26A. The increased stability of chimeras containing α-subunit analogs having an additional cysteine is due to the presence of an intersubunit disulfide. Formation of this cross-link can occur only if the seatbelt is latched by a wraparound mechanism. (Note that in two other experiments, we observed that 15.2 and 19.2% of the s/hCGβ-L5C,SB,C26A containing heterodimer was stable after overnight incubation, values that indicate that the heterodimers containing the native human α-subunit and s/hCGβ-L5C,SB,C26A or s/hCGβ-Nt,SB,C26A have approximately the same stability.)

Data row	α-Subunit analog	β-Subunit analog	Total dimer	Remaining pH 2, 30 min	Remaining pH 2, 120 min	Remaining pH 2, 16 h	Probable seatbelt latch site
			ng/50 μl		*% total ± S.E.*		
1	Native	hCGβ	28.81 ± 0.05	1.4 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	β110:β26
2	α-L41C	hCGβ-C26A	14.81 ± 0.18	93.0 ± 7.1	107.1 ± 0.6	117.8 ± 4.9	β110:α41
3	Native	s/hCGβ-Nt,SB,C26A	5.11 ± 0.06	53.7 ± 1.0	35.1 ± 1.2	22.3 ± 1.7	β110:β5
4	α-L41C	s/hCGβ-Nt,SB,C26A	2.66 ± 0.01	61.7 ± 0.6	58.9 ± 1.7	62.8 ± 4.3	β110:α41>β5
5	α-S43C	s/hCGβ-Nt,SB,C26A	3.94 ± 0.15	77.1 ± 0.6	68.9 ± 3.3	70.8 ± 1.7	β110:α43>β5
6	Native	s/hCGβ-L5C,SB,C26A	16.60 ± 0.60	109.9 ± 1.3	84.5 ± 0.6	34.2 ± 1.1	β110:β5
7	α-L41C	s/hCGβ-L5C,SB,C26A	18.01 ± 2.17	86.7 ± 0.6	72.3 ± 2.3	76.0 ± 0.3	β110:α41>β5
8	α-S43C	s/hCGβ-L5C,SB,C26A	16.16 ± 0.17	88.9 ± 0.3	70.3 ± 2.7	113.3 ± 1.3	β110:α43>β5

α-subunit and s/hCGβ-Nt,SB was very inefficient (Table IV, row 3). Both s/hCGβ-Nt and s/hCGβ-Nt,SB were incorporated efficiently into heterodimers that contain α-Q5C (Table IV, row 1 and 2), showing that their seatbelts were latched primarily to loop β1 residue βCys[26] prior to subunit docking. The difference in the amount of heterodimer formed when s/hCGβ-Nt,SB was expressed with the native α-subunit and α-Q5C suggested that the salmon FSH seatbelt inhibited threading when it was latched to βCys[26]. Studies described next were initiated to test this notion and to identify the portion of the seatbelt that was most likely to be responsible for its ability to interfere with threading.

We monitored the formation of heterodimers containing the native α-subunit and s/hCGβ-SB or s/hCGβ-SB,L92K; the former seatbelt has two hCG residues at its NH_2 terminus, namely βAla[91] and βLeu[92]. In the chum salmon FSH seatbelt, these residues are isoleucine and lysine and, to learn how a positively charged residue adjacent to the tensor loop would affect assembly, we replaced βLeu[92] with lysine. The amount of heterodimer made when either of these β-subunit chimeras was co-expressed with the native α-subunit was much lower than that of hCG (Table V, data rows 1–3). This showed that the presence of the salmon FSH seatbelt reduced assembly, most likely by impeding threading.

The NH_2- and COOH-terminal halves of the hCG and salmon FSH seatbelts differ significantly and we studied each of these regions separately. The NH_2-terminal half of the seatbelt contains a small loop that is stabilized by a disulfide that we term the "tensor" because of its ability to change the length of the seatbelt during heterodimer assembly. The tensor disulfide is disrupted during threading, which elongates the seatbelt and facilitates passage of the glycosylated end of loop α2 through the β-subunit (20). Reformation of the tensor disulfide following threading stabilizes the heterodimer. To test the notion that differences in the stabilities of the hCG and salmon FSH tensor loops were responsible for the reduced ability of the salmon FSH seatbelt to permit threading, we replaced the hCG tensor loop with its salmon FSH counterpart and monitored heterodimer assembly. The salmon FSH tensor loop had little or no inhibitory influence on heterodimer assembly. Consequently, s/hCGβ-TL was incorporated into heterodimers as well as or better than hCGβ and much better than s/hCGβ-SB or s/hCGβ-SB,L92K, analogs that have nearly the entire salmon FSH seatbelt (Table V, data rows 1–4).

The COOH-terminal half of the seatbelt constrains the position of loop α2 but makes few specific contacts with this portion of the α-subunit needed to stabilize the heterodimer. Furthermore, the position of this part of the seatbelt depends primarily on the location of the seatbelt latch site, which is found in loop β1 of most vertebrate hormones and in the β-subunit NH_2 terminus in salmon FSH (1). This can be varied experimentally in hCG analogs by moving the seatbelt latch site to different parts of the α-subunit (13). Because this portion of the hCG seatbelt appears to contribute little to lutropin activity other than to keep the heterodimer intact, we refer to it as the "strap." The strap region has been shown to have a dramatic influence on FSH and TSH activity, however, even though the mechanism of this phenomenon remains unknown (6, 8, 17). Substitution of the hCG strap with its salmon FSH counterpart reduced heterodimer assembly dramatically (Table V, data row 5). This showed that the strap region of the salmon FSH seatbelt is responsible for its ability to suppress threading when it is latched to βCys[26]. Heterodimers that contain this portion of the seatbelt dissociate rapidly at pH 2, 37 °C, indicating that when the strap is latched to βCys[26] in loop β1 it does not block threading during acid-induced heterodimer dissociation.

To learn if heterodimers in which the salmon FSH seatbelt strap regions were latched to βCys[26] had been formed by threading or wraparound mechanisms, we expressed s/hCGβ-St and s/hCGβ-St,C26A with α-S43C and α-S92C. Previously, we had found that the hCG seatbelt in hCGβ-C26A is latched readily by a wraparound mechanism to either of these α-subunit analogs when it is prevented from latching with βCys[26] (13). By analogy, we reasoned that if heterodimers containing the salmon seatbelt strap were latched by a wraparound mechanism, s/hCGβ-St,C26A would be latched readily to both α-S43C and α-S92C. Co-expression of s/hCGβ-St,C26A with either α-subunit analog resulted in the formation of only trace amounts of heterodimer (not shown). This suggested that chimeras containing the native α-subunit and the salmon FSH seatbelt strap are more likely to form by a threading mechanism than a wraparound mechanism. The finding that only small quantities of heterodimer are formed suggested that threading is very inefficient, most likely because the strap region interfered with passage of loop α2 beneath the seatbelt.

We reasoned that if the salmon strap reduced the rate of threading relative to that seen during hCG assembly, it would facilitate the formation of a disulfide cross-link between a cys-

TABLE IV

Formation of the α5-β5 intersubunit cross-link rescued the formation of heterodimers containing s/hCGβ-Nt,SB

COS-7 cells were incubated with α-Q5C and either s/hCGβ-Nt or s/hCGβ-Nt,SB to determine the influence of the hCG and salmon seatbelt on latching the seatbelt to loop β1. The stabilities of the heterodimers were measured following an incubation at pH 2, 37 °C, for 30 min or overnight. Values shown are means of triplicate transfections ± S.E. Values for heterodimers containing the native human α-subunit and s/hCGβ-Nt,SB were estimated following concentration of the media 10-fold and are expressed here in units that reflect the original concentration of the medium. The reduction in the amount of this heterodimer caused by pH 2, 37 °C treatment show that its seatbelt is latched to βCys^{5}. The seatbelt in the heterodimer containing α-Q5C and s/hCGβ-Nt was recognized by antibody B111 (not shown) indicating that it was latched to βCys^{26}. A similar quantity of acid-stable heterodimer was produced when s/hCGβ-Nt,SB was co-transfected with α-Q5C. Since B111 does not recognize heterodimers containing the salmon seatbelt, we were unable to determine the location of the seatbelt latch site in this heterodimer directly. We expect that it is latched to βCys^{26}, however, due to the fact that elimination of this cysteine enhances the assembly of heterodimers containing the native human α-subunit and s/hCGβ-Nt,SB,C26A (Table I). Thus, we anticipate that most heterodimers containing α-Q5C and s/hCGβ-Nt,SB are stabilized by an α5-β5 cross-link and that much less, if any, is stabilized by the formation of a cross-link between βCys^{110} at the end of the seatbelt and αCys^{5}.

Data row	Analog	Total heterodimer	pH 2 stable, 30 min	pH 2 stable, 16 h
		ng/50 μl ± S.E.	*% total ± S.E.*	
1	α-Q5C+s/hCGβ-Nt	5.48 ± 0.44	99.4 ± 3.7	103.7 ± 4.7
2	α-Q5C + s/hCGβ-Nt,SB	4.73 ± 0.09	112.9 ± 8.3	99.2 ± 4.6
3	α + s/hCGβ-Nt,SB	0.12 ± 0.01	67.5 ± 3.1	34.9 ± 3.7

TABLE V

Influence of the salmon seatbelt tensor loop and strap on heterodimer assembly

The indicated β-subunit analogs were co-transfected into COS-7 cells with the native α-subunit. Production of heterodimer was monitored in the medium three days later. The stability of the heterodimer was measured following an incubation at pH 2, 37 °C for 30 min. Values shown are mean ± S.E. for triplicate transfections.

Row	β-Subunit	Total	% Stable
		ng/50μl	*% total ± S.E.*
1	hCGβ	2.34 ± 0.28	0.4 ± 0.3
2	s/hCGβ-SB	0.39 ± 0.12	0.2 ± 0.1
3	s/hCGβ-SB,L92K	0.67 ± 0.12	0.7 ± 0.3
4	s/hCGβ-TL	2.27 ± 0.26	0.3 ± 0.2
5	s/hCGβ-St	0.28 ± 0.07	5.2 ± 0.5

TABLE VI

Stability of heterodimers containing the salmon seatbelt strap region following co-expression with α-S43C and α-S92C

The indicated β-subunit analogs were co-transfected into COS-7 cells with the α-subunit analog αS43C and αS92C, respectively. Production of heterodimer was monitored in the medium three days later in A113/^{125}I-B111 sandwich immunoassays. The stability of the heterodimer was measured following an incubation at pH 2, 37 °C for 30 mins. Values shown are mean ± S.E. for triplicate incubations.

Data row		ng/50 μl ± S.E.	% Stable ± S.E.
α-S43C			
1	+hCGβ	7.27 ± 0.49	10.3 ± 0.6
2	+s/hCGβ-St	1.58 ± 0.04	44.4 ± 1.4
3	+s/hCGβ-St,C93A	0.44 ± 0.03	102.9 ± 1.2
4	+s/hCGβ-St,C93A,C100A	Not detected	Not done
α-S92C			
5	+hCGβ	7.67 ± 0.44	3.8 ± 0.1
6	+s/hCG-St	2.09 ± 0.10	10.1 ± 0.9
7	+s/hCGβ-St,C93A	0.36 ± 0.03	113.4 ± 5.3
8	+s/hCGβ-St,C93A,C100A	Not detected	Not done

teine added to loop 2 of the α-subunit and one of the tensor cysteines. This type of cross-link had been observed during the assembly of hCG (20). As expected for assembly that occurs by a threading mechanism, a substantial fraction of the heterodimer formed when α-S43C was co-expressed with s/hCGβ-St was stable at pH 2, 37 °C (Table VI, data row 2). This suggested that a cross-link had formed between one of the tensor cysteines in s/hCGβ-St and αCys^{43}. The finding that a much larger fraction of the heterodimer containing s/hCGβ-St was cross-linked than that containing hCGβ supported the hypothesis that threading had been delayed. This notion was also supported by the finding that expression of s/hCGβ-St with α-S92C, a region of the α-subunit that is not threaded beneath the seatbelt, led to lesser amounts of cross-link (Table VI, data row 6). The hypothesis that these disulfides involved a tensor cysteine is consistent with the finding that co-expression of either α-S43C or α-S92C with s/hCGβ-St,C93A, an analog that has an unpaired tensor cysteine at βCys^{100} led to the formation of heterodimers that were completely acid stable (Table VI, data rows 3 and 7). In contrast, co-expression of α-S43C and α-S92C with s/hCGβ-St,C93A,C100A, an analog that lacks both tensor cysteines resulted in the formation of only trace amounts of heterodimers (Table VI, data rows 4 and 8).

DISCUSSION

Mechanisms of Glycoprotein Hormone Assembly in Vertebrates—Because of the tendency of the seatbelt to be latched to a cysteine in loop β1 before the subunits dock, we anticipate that most vertebrate glycoprotein hormones are assembled by a threading mechanism. Indeed, as shown here, even the salmon FSH seatbelt tends to be latched to a cysteine in loop β1 when it has a choice of seatbelt latch sites. When the seatbelt can only be latched to an NH_2-terminal β-subunit site, however, as it is in the case for salmon FSH and other related teleost species (Table VII), heterodimer assembly can occur by a wraparound mechanism, but not by threading. This appears due to the combination of the low ability of the salmon seatbelt to be latched to the NH_2 terminus and the difficulty of threading loop α2 beneath these seatbelts once they are latched. The finding that the salmon seatbelt can impede threading when it is latched to loop β1 suggests that some glycoprotein hormones may be assembled by a wraparound pathway even when their seatbelts are latched to loop β1. We would expect this process to be inefficient, however.

Implications of these Observations for the Evolution of teFSH and the Interaction of teFSH with Piscine FSH Receptors—The finding that hCG analogs which have the teFSH folding pattern are assembled by a wraparound mechanism suggest that hormones such as salmon FSH are also assembled in this fashion. Why would the wraparound pathway, an assembly mechanism that appears to be relatively inefficient (19, 20), be used to produce hormones that are usually thought to be critical for the reproduction of vertebrates? The wraparound pathway permits formation of heterodimers that cannot be assembled by threading. As such, it would have facilitated natural experimentation with the seatbelt during co-evolution of these heterodimeric ligands and their receptors. The seatbelt is responsible for much of the influence of the hormone-specific β-subunit on biological activity (5–8) and, as shown here, the efficiency of threading. When changes in the β-subunit create seatbelts that block threading, the use of the wraparound mechanism offers the organism a mechanism of producing het-

TABLE VII
Migration of the seatbelt latch site in fish

We anticipate that all glycoprotein hormones originated from a precursor in which the seatbelt was latched to a cysteine in β1. This would permit assembly by either a threading or wraparound mechanism. Selection pressure to optimize reproduction and thyroid function led to the duplication and reduplication of the β-subunit to create β-subunits found in lutropins, follitropins, and thyrotropins. Subsequent divergence of the resulting β-subunits occurred in response to selection pressures on reproduction and development. The seatbelt is the portion of the β-subunit that has the greatest influence on hormone activity (5–8), making it more sensitive to selection pressure than other parts of the protein. Its roles in heterodimer assembly and stability would also have influenced β-subunit evolution. Mutations of the seatbelt that prevented assembly would have been lost regardless of their abilities to regulate receptor function. The existence of the wraparound assembly mechanism would have permitted mutations to the seatbelt that prevented threading, even though wrapping appears to be much less efficient than threading. In part, this is due to the tendency of the seatbelt to become latched to the β-subunit before it docks with the α-subunit. Factors that drove the evolution of teFSH remain unknown. In teleosts, these are likely to have involved seatbelt mutations that affected the interaction of teleost FSH with FSH, LH, and TSH receptors. Relocation of the seatbelt to the NH_2 terminus of the β-subunit would be expected to disrupt high affinity interactions between the COOH terminal end of the seatbelt and any of these receptors. Based on the studies of Yan *et al.* (18), which show that salmon FSH does not bind the salmon LH receptor, we propose that the evolution of the teFSH structure was driven by the need to reduce cross-reactions with the LH receptor and/or to increase the stability of the heterodimer. Shown here (single letter code) are parts of the β-subunit corresponding to the NH_2 terminus (Nt) and seatbelt along with the residue corresponding to hCG βCys^{26}, the β1 seatbelt latch site. The signal sequence cleavage site has not been determined experimentally in all cases and the NH_2-terminal residues shown here were determined by analogy to known sequences. The cleavage sequence for s/hCGβ-Nt,SB,C26A was assumed to be identical to that of hCGβ, which would give it an additional serine residue that is not found in the salmon FSH β-subunit. It should be noted that the signal peptides of many vertebrate FSH β-subunits contain additional cysteines. Therefore, the introduction of an NH_2-terminal cysteine in the β-subunit may have arisen from a modification of the signal peptide cleavage site. The assembly pathway is known only for hCG, hFSH, hTSH, and salmon FSH. That for the remaining β-subunits is inferred from these. The term "unknown-T" is used to indicate that two pathways are possible but that threading appears to be most likely.

Species and β-subunit	Acquisition number	Assembly pathway	Amino terminus	Residue in β1	Seatbelt region	
					Tensor	Strap
Human hCGβ	NP_000728	Threading	SKEPLRPRC	C	AL CRRSTTDC	GGPKDHPLTC
Human FSHβ	1FL7B	Threading	NSC	C	GK CDSDSTDC	TVRGLGPSYC
Human TSHβ	AAB30828	Threading	FC	C	GK CNTDYSDC	IHEAIKTNYC
Shark FSHβ	CAC43235	Threading	NRC	C	GM CNTETTDC	TVSAMEPTHC
Japanese eel	Q9YGK3	Threading	RA**S**TSC	C	SK CNTDSTDC	GPLNTEVSGC
Conger eel	CAB93518	Wrapping	RA**C**SSC	**W**	SR CNTNSTDC	GQLNTEASGC
Black carp FSHβ	AAK07415	Threading	GSE**F**RSSC	C	SK CNSDIADC	GVLSQQTSSC
Common carp FSHβ	O13050	Unknown-T	GSE**C**RSSC	C	SK CNSDITDC	GALSQQTLSC
Goldfish FSHβ	Q98848	Unknown-T	GSE**C**RSSC	C	SK CNSDITDC	GVLSQQTLGC
Chum salmon FSHβ	P10257	Wrapping	GTE**C**RYGC	**S**	IK CKTDNTDC	DRISMATPSC

erodimers that may enable it to reproduce.[2]

The evolutionary pressures which drove the change in glycoprotein hormone topology that created teFSH are enigmatic, particularly because hormones having this structure appear to be more difficult to assemble. One factor that may have contributed to the development of teFSH is its increased stability, which may offset the additional difficulty of assembling the heterodimer. More likely, the changes in the teFSH β-subunit occurred during the continual co-evolution of the gonadotropins and their receptors (6). Both salmon LH and salmon FSH interact with the salmon FSH receptor (18), indicating that some of the actions of salmon FSH can be replaced by salmon LH. In contrast, salmon FSH does not interact with the salmon LH receptor, a property that may reflect its altered seatbelt latch site. The observation that the FSH strap region of the seatbelt occupies two very different positions in the Japanese eel and the conger eel (Table VII) suggests that the strap may not participate in high affinity contacts with the FSH receptor in either of these related species. If the strap region contributed to the ability of FSH to bind to LH receptors when it was latched to loop β1, changing its position to the NH_2 terminus would reduce binding to the LH receptor without affecting binding to the FSH receptor.

The most ancient species for which a sequence of the FSH β-subunit is known is the shark. The seatbelt of this follitropin appears to be latched to a site in loop β1. We have found that hCG-shark FSH chimeras containing the shark FSH seatbelt are assembled into heterodimers readily and that this is not affected by adding the salmon NH_2-terminal seatbelt latch site.[3] The finding that the shark FSH seatbelt is readily latched to loop β1 and that it does not inhibit threading suggests strongly that shark FSH is assembled by a threading mechanism similar to β-subunit analog s/hCGβ-Nt (Table II, row 3). The FSH β-subunit in goldfish and the common carp appears to have two seatbelt latch sites, one in the NH_2 terminus and one in loop β1, but it is not known which of these latch sites are used (Table VII). Indeed, whereas it is conceivable that these fish produce two forms of FSH, we expect that similar to s/hCGβ-Nt most of their FSH will be assembled by a threading mechanism and its seatbelt will be latched to loop β1. This is supported by the finding that black carp FSH, which has a seatbelt that is similar to that of the common carp and goldfish, appears to have only the seatbelt latch site in loop β1 (Table VII). Thus, it would be expected that this heterodimer forms by threading. The presence of two potential latch sites within the goldfish and common carp β-subunits suggests that the precursor of teFSHβ may have also had two potential latch sites. Based on our finding that s/hCGβ-Nt,SB is assembled into heterodimers poorly, we expect that the site in loop β1 was eliminated to enhance heterodimer assembly. Loss of the loop β1 latch site would have reduced premature latching of the seatbelt, thereby facilitating assembly by the wraparound pathway.

The Strategies Devised to Study the Assembly of hCG and Analogs Having the Folding Pattern of teFSH Can be Used to Study the Assembly of Other Vertebrate Glycoprotein Hormones—The amino acid sequences of several vertebrate hormones have been reported, but it remains to be determined how

[2] Fish gonadotropins often differ from their mammalian counterparts. In many cases, the piscine lutropin, which is also known as GTH-II is capable of interacting with LH and FSH receptors. This may have also had a role during the evolution of teFSH.

[3] M. P. Bernard, R. V. Myers, D. Cao, and W. R. Moyle, unpublished data.

these are assembled. Considerable variations occur in the sizes of the α- and β-subunit cores and the seatbelt regions of many glycoprotein hormones, particularly in fish. These have the potential to provide new insights into the mechanisms of protein folding within the endoplasmic reticulum. We anticipate that the approaches described here for studying the assembly of teFSH will be applicable to studies of the assembly of glycoprotein hormones from most vertebrates including fish. The most important aspect of our approach is the use of α- and β-subunit analogs that contain unpaired cysteines. Although we took advantage of a well characterized panel of monoclonal antibodies to hCG, we anticipate that any procedure capable of measuring the amounts of heterodimers produced following transfection of cells with subunit analogs containing appropriate unpaired cysteines would suffice. This includes the use of epitope tagged α- and β-subunit analogs. Many of the studies described in this and in the accompanying articles (19, 20) were performed to check the internal consistency of our findings. We anticipate that only a few of the analogs that we produced and characterized would be required to distinguish most assembly pathways. These would include analogs corresponding to α-Q5C, α-S43C, α-S92C, and hCGβ-C26A.

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