Functional homodimeric glycoprotein hormones: implications for hormone action and evolution

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Background: Human chorionic gonadotropin (hCG), lutropin, follitropin, and thyrotropin act as αβ heterodimers to control reproduction and thyroid function. The α and β subunits of these proteins are divided into three loops (α1,α2,α3; β1,β2,β3) by cysteine knots and the heterodimer is stabilized by 20 β-subunit residues wrapped around α2 like a seatbelt. Understanding how these hormones interact with their receptors, a matter of considerable dispute, would facilitate design of pro- and anti-fertility agents.

Results: By swapping α2 for β2 and vice versa and, in some cases, adding an amino-terminal coiled–coil dimerization domain, we prepared homodimeric analogs that have the conformation found in each 'half' of hCG. Homodimers containing loops β1,α2,β3 and none, part, or all of the seatbelt stimulated signal transduction to the same extent as hCG, albeit with lower potency. Those containing α1,β2,α3 were inactive.

Conclusions: The activities of homodimers containing the β1,α2,β3 groove exceed those of other minimized analogs more than 100–1000-fold, suggesting this portion of the hormone forms the major receptor contact. The discovery that glycoprotein hormone heterodimers can be converted to functional homodimers supports the proposal that this protein family evolved from an active homodimeric ancestor by gene duplication and acquisition of mutations to loop 2 that prevent homodimerization. This approach to protein minimization should be applicable to other proteins composed of architecturally related subunits, including those that might have arisen by gene duplication.

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Introduction

The glycoprotein hormones lutropin (LH), follitropin (FSH), and thyrotropin (TSH) control the functions of the ovaries, testes, and thyroid. Humans have two lutropins, hLH, an anterior pituitary gland hormone essential for gonadal steroid secretion and ovulation, and chorionic gonadotropin (hCG), a closely related placental protein required for maintenance of pregnancy. Both hCG and hFSH, a hormone that promotes gamete development, are used extensively to enhance fertility. A better understanding of how the structure of these hormones controls their biological activities would facilitate the development of new approaches to fertility regulation. Based on the idea that minimized hCG analogs could be used to identify residues critical for activity, we have begun to tease the hormone apart. Here we describe the preparation and characterization of the first glycoprotein hormone homodimers, minimized proteins that retain features topologically similar to either half of hCG. During these studies, we have also gained new insights into the parts of the glycoprotein hormone subunits that influence heterodimer formation.

A high-resolution structure of hCG serves as the model for this cysteine-knot protein family [1,2]. All four glycoprotein hormones are composed of an α subunit expressed from the same gene [3] and a unique β subunit that controls the biological specificity of the heterodimer [4]. Each subunit is divided into three elongated antiparallel loops (α1,α2,α3; β1,β2,β3) by a cysteine knot (Figure 1). Loops 1 and 3 of the same subunit are adjacent and located at the opposite end of the molecule from loop 2. In the heterodimer the subunits are aligned 'head-to-toe' such that α2 is adjacent to β1,β3 and β2 is adjacent to α1,α3 [1,2] (Figure 1). The β subunit contains 20 additional amino acids nicknamed the 'seatbelt' [1] that are wrapped around α2 to stabilize the heterodimer. Seatbelt residues also control the ability of hCG to distinguish between the LH, FSH, and TSH receptors [5–8], but it remains to be determined if this occurs through a direct receptor contact, an influence on hormone conformation, or both.

Two radically different models (Figure 2a,c) have been proposed to explain the interactions of the glycoprotein hormones with their receptors [9,10]. Model 1 (Figure 2a,b)

Figure 1

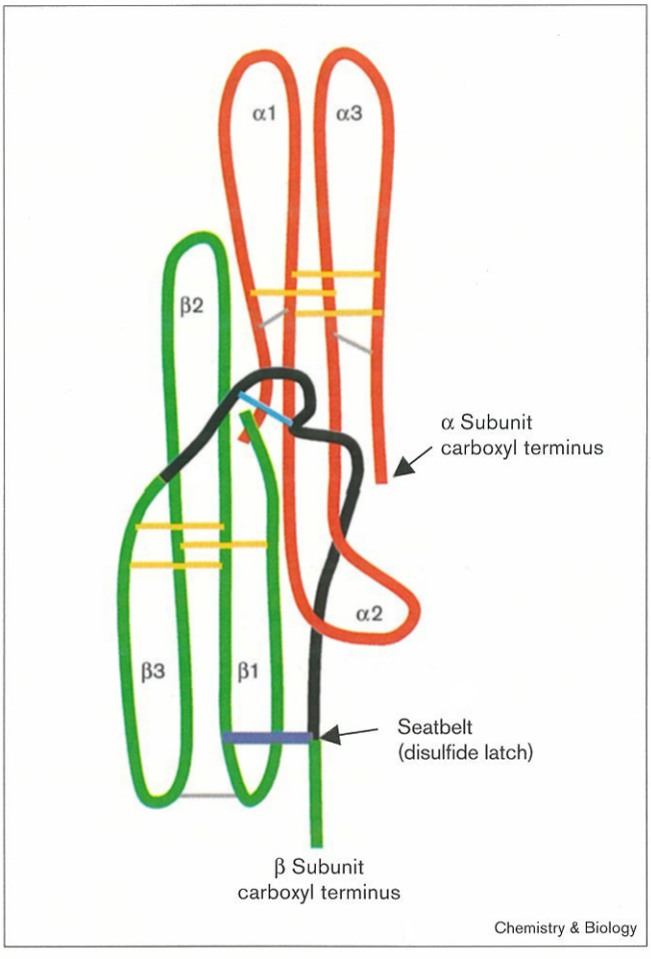


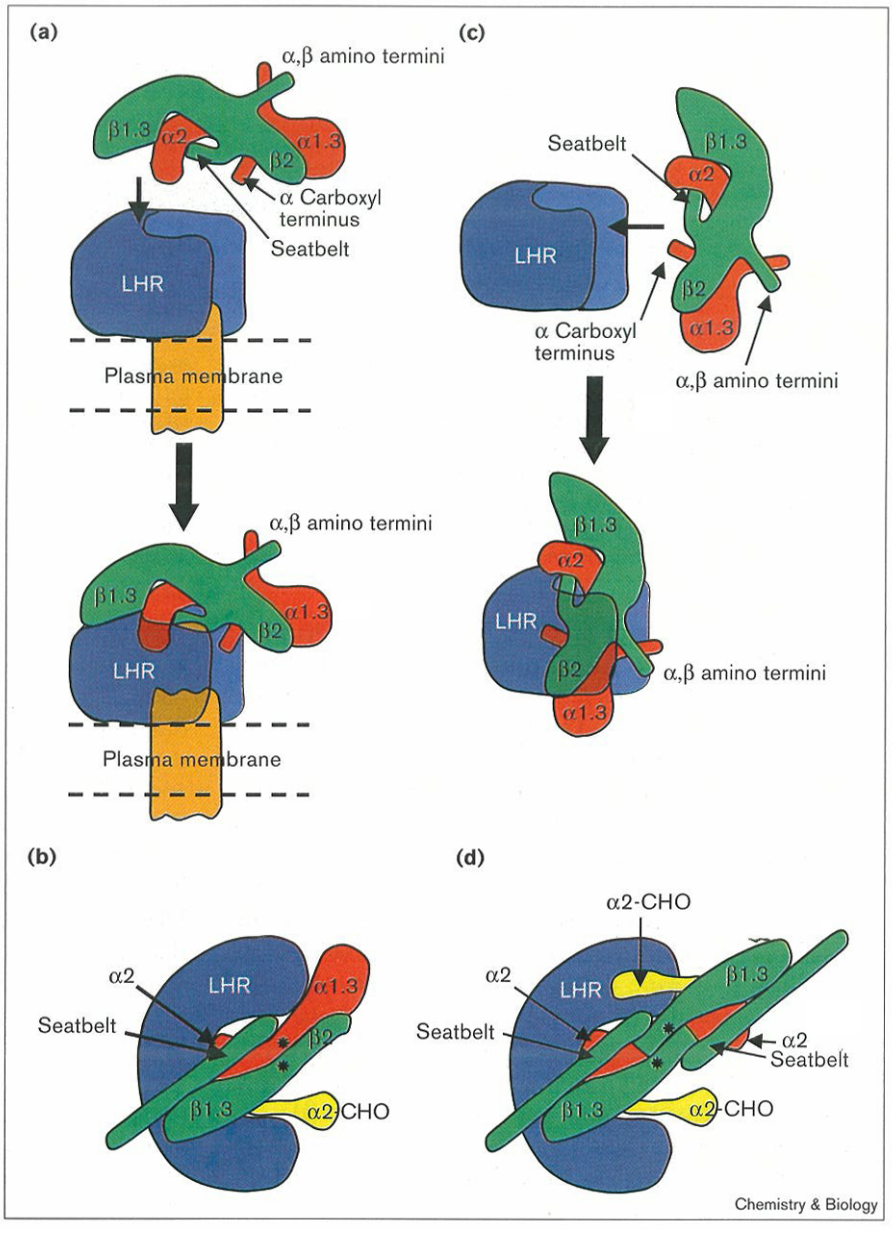
Diagram illustrating the relationship between the α and β subunits of all the glycoprotein hormones based on their relationship to hCG, the only family member for which a crystal structure has been obtained. The hCG heterodimer is formed by the combination of the α subunit (red) with the β subunit (green and black). Each subunit has a cysteine-knot motif (three yellow disulfide bonds) that divides them into three loops as shown. Loop 2 of the α subunit (α2) contains an oligosaccharide (not represented) required for the full ability of hCG to elicit a biological response. The β subunit also contains 20 additional residues (black) that surround α2. The seatbelt is 'latched' to β1 through a disulfide bond (blue) to stabilize the heterodimer. The seatbelt also contains a small loop created by a disulfide bond (light blue) in its amino-terminal half. Positively and negatively charged residues in the small seatbelt loop influence lutropin and thyrotropin activity, respectively. The remaining disulfide bonds (light grey) are not essential for protein folding or for lupotropin activity. In the heterodimer, the amino-terminal ends of both hCG subunits overlap (shown, but not labeled on the diagram). β subunit residues carboxy-terminal of the seatbelt disulfide bond are not required for biological activity. Those from hCG, however, contribute to its long half-life *in vivo*.

suggests the groove between loops α2 and β1,β3 makes the primary receptor contact and the seatbelt controls hormone specificity through its influence on subunit interaction and the shape of the β1,α2,β3 groove [9]. In contrast, Model 2 (Figure 2c) postulates LH receptor contacts involve the α subunit carboxyl terminus, parts of β2, and parts of the seatbelt, but not the β1,α2,β3 groove [10]. In the second model the influence of the seatbelt on hormone specificity is mediated through its interaction with the receptor.

We assumed these models could be distinguished by monitoring the activities of 'minimized' hCG analogs lacking parts of the hormone considered essential in each. Molecular simulations based on the structure of hCG [1] suggested that α/β subunit chimeras prepared by substituting β subunit loop 2 with α2 should form symmetrical homodimers lacking loops α1,β2,α3 and the α subunit carboxyl terminus. These analogs, termed cBABS for their components (hCG, β1,α2,β3, seatbelt), would contain two copies of loops β1,α2,β3 and the seatbelt (Figures 3,4). These simulations also suggested that the conformations of β1,α2,β3 and seatbelt would be similar to those in hCG. Model 1 suggested that the cBABS homodimer could interact with LH receptors through either of its two β1,α2,β3 grooves. However, one of the two α2 oligosaccharides present in cBABS would make an inappropriate contact with the carboxy-terminal portion of the LH receptor extracellular domain to inhibit receptor interaction (Figures 2d, 4f, arrow). Thus, Model 1 also suggested that a related homodimer lacking the α2 oligosaccharide (cBABSδα2) would be substantially more active. Model 2 predicted that both cBABS homodimers would be inactive. This is because they lack the α subunit carboxyl terminus and loop β2, two portions of the hormone previously postulated to form essential receptor contacts, and they contain a second copy of the seatbelt within the hormone–receptor interface, a feature expected to disrupt binding.

Designing homodimers lacking the seatbelt required a strategy to mimic the roles this portion of the hormone has in subunit combination and dimer stability [11]. The apparent proximity of the amino-terminal ends of the subunits in hCG [1,2] and cBABS homodimers suggested the functions of the seatbelt could be replaced by an amino-terminal coiled–coil dimerization domain that contained a cysteine capable of forming an intersubunit disulfide bond tether. Based on the results of immunological studies showing that the amino-terminal ends of hCG do not contact the LH receptor [9], we assumed that the presence of additional residues in this region would not prevent the dimer from interacting with LH receptors. Models of cBABS homodimers suggested that the Cα and Cβ carbons of Arg6 in each subunit would be approximately 6 Å and 4 Å apart, distances similar to those between the cysteines in a typical disulfide bond. Therefore, we expected that replacing β subunit residues 1–5 with the coiled–coil domain of yeast transcription factor GCN4 [12] would promote dimerization, and that changing Arg6 to cysteine would form a stabilizing intersubunit disulfide bond. This would enable us to prepare homodimers having the α subunit carboxyl

Figure 2



Cartoon illustrating models of hCG–receptor interaction. **(a,b)** Model 1, in which the β1,α2,β3 groove is postulated to interact with the rim of a horseshoe-shaped extracellular domain of the receptor. Note that in this model the seatbelt and parts of α1,3 make only limited contacts with the receptor [9]. Model 1 also suggests a relationship between the extracellular and transmembrane domains of the receptor. A sideview is shown in (a), and (b) illustrates a view from outside the cell towards the cell surface. **(c)** Model 2, in which the α subunit carboxyl terminus, seatbelt, and loop β2 are postulated to interact with the concave surface of a horseshoe-shaped extracellular domain of the receptor. Note that in this model the β1,α2,β3 groove does not contact the receptor [10]. Model 2 makes no predictions about the location of the transmembrane domain or the orientation of hCG (i.e., it could be flipped top-to-bottom relative to how it is illustrated here). **(d)** The interaction of cBABS with the receptor as predicted by Model 1. The view shown is from the outside the cell towards the cell surface and illustrates how cBABS homodimers would bind to the extracellular domain of the LH receptor. Note that one of the α2 oligosaccharides (arrow) would be expected to make a steric contact with the carboxy-terminal portion of the receptor extracellular domain. Blue, LH receptor extracellular domain; orange, LH receptor transmembrane domain; red, α subunit or parts derived from α subunit; green, β subunit or parts derived from β subunit; asterisks in (b,d) show the location of amino termini. Oligosaccharides on the β subunit, α subunit loop 2 (a,c), α subunit loop 3, and the receptor are not depicted for purposes of clarity. Those on the β subunit and α3 would point away from the receptor in both models.

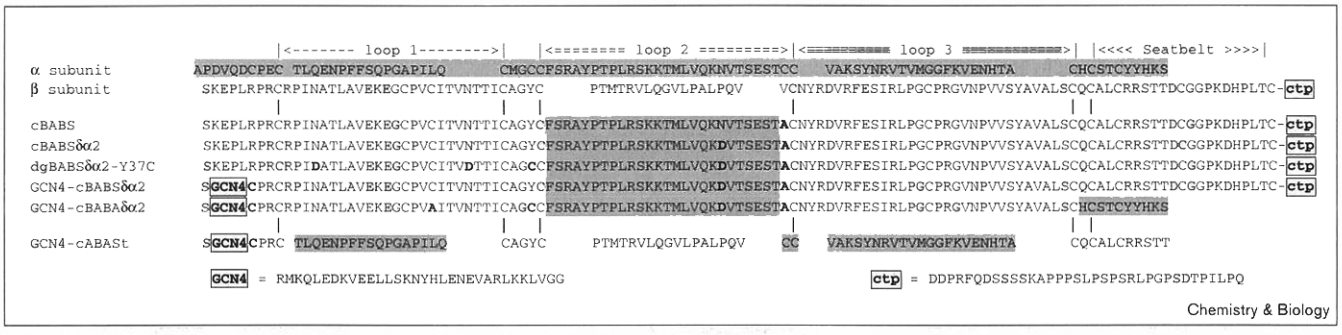
terminus in place of the seatbelt (GCN4–cBABAδα2, Figure 4c) or loops α1,β2,α3 and truncated seatbelt in place of loops β1,α2,β3 and full-length seatbelt (GCN4–cABASt, Figure 4d). By comparing the activities of these homodimers with those containing loops β1,α2,β3 and the entire seatbelt (cBABSδα2), we expected to distinguish between the functions of the β1,α2,β3 groove, the α subunit carboxyl terminus, and the seatbelt. Model 1 predicted that the seatbelt was not required for receptor interaction and that all homodimers containing loops β1,α2,β3 and no α2 oligosaccharide (i.e., cBABSδα2, GCN4–cBABSδα2, GCN4–cBABAδα2) would be active, albeit not necessarily with equal potency. According to Model 1, a homodimer lacking loops β1,α2,β3 (i.e., GCN4–cABASt) would not be active even though its design incorporated parts of the seatbelt thought to influence LH activity [6,13] and to recreate a heterodimer-specific epitope present in hCG [14]. Model 2 suggested that neither homodimer would interact with receptors because GCN4–cBABAδα2 is missing the entire seatbelt and GCN4–cABASt is missing the α subunit carboxyl terminus.

Results and discussion

Preparation and characterization of minimized homodimeric analogs

Vectors encoding the amino-acid sequences of cBABS, cBABS analogs, GCN4–cBABAδα2, and GCN4–cABASt (Figure 3) were built using standard molecular biology

Figure 3



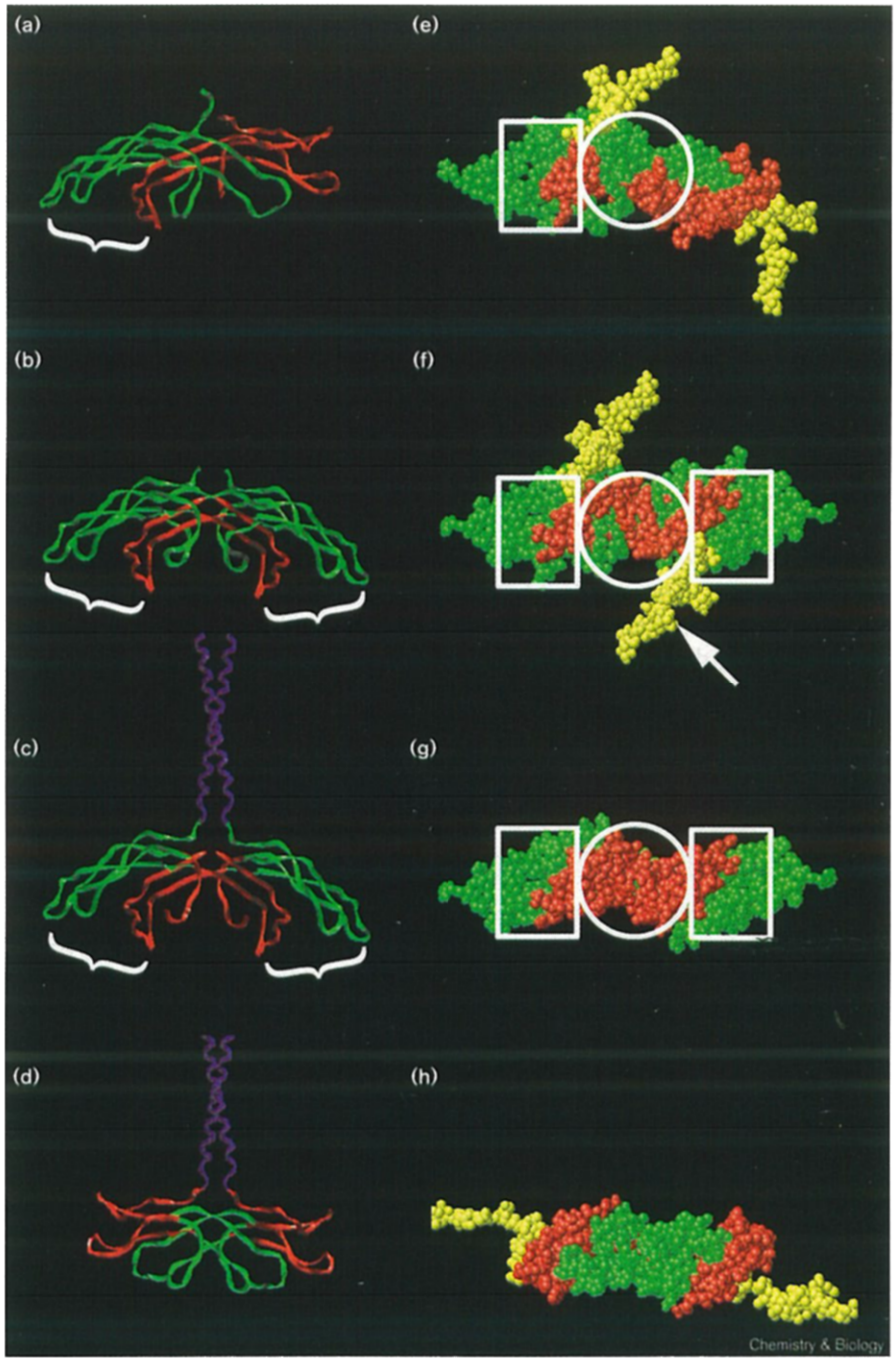
Sequences of hCG α and β subunits, cBABS, and several analogs (using single-letter amino-acid code) aligned to illustrate components thought to be structurally homologous. All analogs contain the hCG β subunit signal sequence (MEMFQGLLLLLLLSMGGTWA). Vertical lines refer to the locations of the cysteine residues in the cysteine knot. Other disulfide bonds: α subunit, C7–C31, C59–C87; β subunit, C23–C72, C26–C110 (seatbelt latch), C93–C100. Presumed disulfide bonds: cBABS and cBABSδα2, C23–C81, C26–C119, C102–C109; dgBABSδα2-Y37C, same as cBABS plus 37C–37C; GCN4–cBABSδα2, C34–C34, C51–C109, C54–C147, C130–C137; GCN4–cBABAδα2, C34–C34, C51–C109, C71–C71; GCN4–cABASt, C34–C34, C77–C105. Thus, GCN4–cBABAδα2 is expected to contain two intersubunit disulfide bonds (one at the end of the GCN4 domain and one between the cysteine knots).

methods and expressed in COS-7 and chinese hamster ovary (CHO) cell lines. All the proteins were secreted from the cells and readily detected using a sandwich immunoassay [15]. Analogs predicted to form homodimers based on molecular modeling did so, as revealed by their sizes in Western blots (Figure 5) and activities in immunoassays (Tables 1,2). Because hCG has only a single copy of each epitope, hCG cannot be measured in sandwich immunoassays employing the same antibody or antibodies to overlapping epitopes for capture and detection. In contrast, the homodimers contain two copies of every portion of hCG from which they were derived and were readily detected using two molecules of the same antibody, or using antibodies to overlapping epitopes (Tables 1,2). Thus, cBABS and cBABSδα2 reacted with monoclonal antibodies having conformation-sensitive epitopes exclusively on β1, β3, and/or the seatbelt (Table 1). GCN4–cBABA reacted with antibodies to epitopes on β1 and/or β3. GCN4–cABASt reacted with antibodies to α1, α3, or β2, including one (B109) that detects an hCG dimer-specific epitope created by the proximity of β2 and amino-terminal seatbelt residues [9,14,16] (Table 2). cBABS analogs were also recognized by antibody B111, an antibody to a conformation-specific epitope of hCG and the hCG β subunit that includes the terminal seatbelt disulfide bond and surrounding residues [16]. Recognition of cBABS analogs in B111/^{125}I–B111 demonstrated that both seatbelt disulfide bonds were latched (Table 1). As expected, compounds that lack a seatbelt (i.e., GCN4–cBABAδα2), or that have only a single seatbelt (i.e., hCG or the hCG β subunit) were inactive in B111/^{125}I–B111 sandwich immunoassays.

Detection of GCN4–cABASt by an antibody that recognized a heterodimer-dependent conformation in hCG showed that the subunits of this homodimer appear to be aligned similar to their positions in the α1,β2,α3 region of hCG. Attempts to study subunit organization in homodimers containing loops β1,α2,β3 were less straightforward due to the lack of heterodimer-specific antibodies to this region of hCG. Most antibodies to hCG β subunit loops β1 and β3 recognize a conformation that is present in the free β subunit as well as in the heterodimer. Recognition of cBABS and derivatives by antibodies to the β1 and β3 loops (e.g., B112) and to the seatbelt disulfide bond (e.g., B111) showed loops β1 and β3 folded as anticipated and that the seatbelt disulfide bond had formed. These measurements, however, did not prove that the seatbelts of these analogs were wrapped around α2. To test this possibility, we compared the stabilities of hCG and cBABS homodimers to urea-induced subunit dissociation. If both homodimer seatbelts were wrapped around α2, we expected that the homodimers would be more stable than hCG, a heterodimer stabilized by only a single seatbelt. cBABS analogs were more stable than hCG in 10 M urea (Figure 5a, lanes 7–9), a strong indication that each of their seatbelts was wrapped around an α2 loop. The urea stability of GCN4–cABASt (Figure 5b, lanes 3,4), a homodimer that lacks the seatbelt but has the potential to form a single intersubunit disulfide bond at the carboxyl terminus of the GCN4 domain [12], showed it to be stabilized by an intersubunit disulfide bond. Thus, it seemed likely that this intersubunit disulfide bond would form in all GCN4-containing homodimers. Because this disulfide bond would also prevent the subunits of the GCN4–cBABSδα2 homodimer from dissociating in urea, we were unable to determine if the seatbelts of this latter analog had become wrapped around α2.

These observations demonstrated that we had accomplished our first goal, namely to prepare minimized

Figure 4



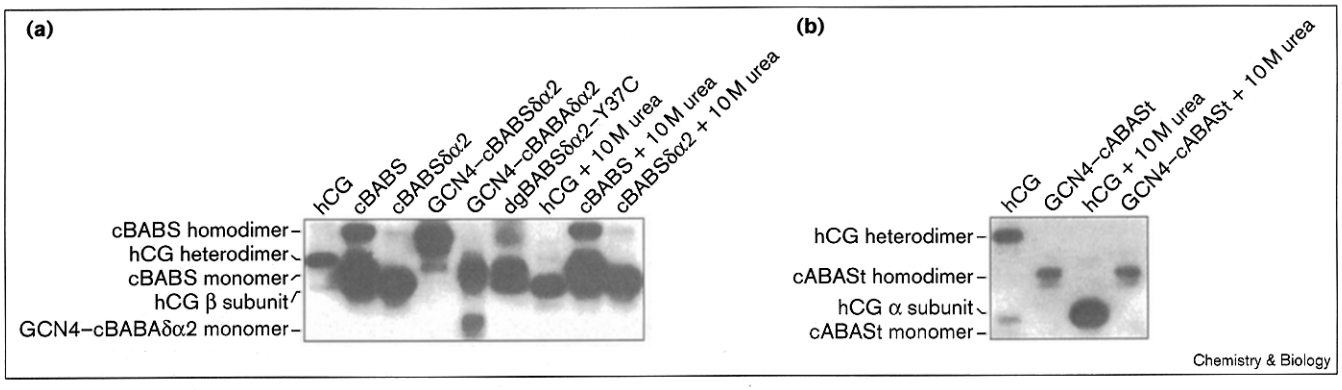
Ribbon diagrams of **(a)** crystal coordinates of hCG heterodimer, **(b)** model of cBABS homodimer, **(c)** model of GCN4–cBABAδα2 homodimer, **(d)** model of GCN4–cABASt homodimer (red, α-subunit regions; green, β-subunit regions; GCN4 residues, blue). Brackets illustrate the locations of the β1,α2,β3 grooves of hCG and homodimeric analogs. In this view, the receptor would be located beneath the hormone. **(e–h)** Space-filled views of the surface believed to face the LH receptor [9,10] shown for (e) hCG, (f) cBABS, (g) GCN4–cBABAδα2, and (h) GCN4–cABASt. The views shown in these panels were obtained by rotating those of panels a–d 90° about the x axis. The color code is similar to that in (a–d). The oligosaccharides of the α subunit have been added and colored yellow. (To minimize the complexity of the figure, the β-subunit oligosaccharides are not shown. In this orientation, they would be located behind the hormone and extend below the surface of the paper.) Rectangular areas outline the β1,α2,β3 grooves of hCG and analogs, the region of these proteins that we suggest interacts with the receptors [9]. Note that areas within rectangular areas are similar in hCG (e) and in the active homodimers cBABS (f) and cBABA (g). A circle outlines the region of hCG (e) estimated from the report of Jiang *et al.* [10] to interact with the receptor. Note that the composition of this region differs substantially in active homodimers such as analogs of cBABS (f) and cBABA (g). The arrow in (f) indicates the location of the α2 oligosaccharide of cBABS expected to hinder binding to the LH receptor.

proteins that enabled us to study each 'half' of hCG separately, with or without the seatbelt (Figure 4). The relationship between the structures of cBABS (Figure 4b) and GCN4–cBABA (Figure 4c) and hCG can be seen by comparing the analogs with the left half of hCG shown in Figure 4a. The relationship between the structure of GCN4–cABASt (Figure 4d) and hCG can be seen by comparing the analog with the right half of hCG shown in Figure 4a.

Implications of these observations for subunit combination

None of the natural glycoprotein hormone subunits form stable homodimers under physiological conditions. In contrast, when β2 was replaced by α2, only those hCG β subunit analogs lacking both a seatbelt and an amino-terminal dimerization domain failed to form homodimers. Furthermore, cBABS analogs containing hCG loops β1,α2,β3 and a seatbelt derived from hFSH also formed homodimers (data not shown), indicating that

Figure 5



Western blots illustrating the formation of homodimers by cBABS, GCN4–cBABA, GCN4–cABASt, and related analogs. **(a)** cBABS and related proteins contain β1,α2,β3 and were detected by radioiodinated antibody B112 in Western blots. **(b)** GCN4–cABASt contains β1,α2,β3 and was detected in Western blots by radioiodinated antibody A113.

dimerization was not limited to the presence of the hCG seatbelt. The propensity of cBABS analogs to dimerize, albeit some better than others, implies that β2 inhibits hCG β subunit dimerization and, most likely, dimerization of all glycoprotein hormone β subunits. Failure of the β subunits to dimerize could occur because they lack contacts between α2 and β1,β3 observed in the heterodimer [1,2] and/or because they are prevented from dimerizing by inappropriate contacts between β2 and other parts of the β subunit.

The α subunit might dimerize poorly because it lacks a strong dimerization domain, because it lacks contacts that occur in the heterodimer between β2 and α1,α3, and/or because it lacks a seatbelt. Cells transfected with cDNAs of both cBABS and the human α subunit secreted cBABS homodimers and cBABS/α subunit heterodimers (Table 1). In the hCG α subunit/cBABS heterodimer, one copy of α2 becomes located adjacent to α1 and α3. Thus, there appear to be no bad contacts between these loops, making it unlikely that contacts between α2 and α1,α3 prevent the α subunit from forming homodimers.

The α2 oligosaccharide is important for hCG subunit combination [17]. Cells engineered to express hCG α subunit lacking this oligosaccharide secrete much less heterodimer than those expressing the native protein. Similarly, the α2 oligosaccharide appears to be important for cBABS dimerization. Analogs lacking this oligosaccharide were secreted primarily as monomers (Figure 5a). This included cBABSδα2 and dgBABSδα2–Y37C, an analog in which all *N*-linked oligosaccharides have been removed and a cysteine has been added to permit formation of an intersubunit disulfide bond. The finding that

Table 1

Antibody recognition of cBABS analogs containing β1,α2,β3 components (B112 capture).

	Antibody pair				
Analyte	B112/^{125}I–A113	B112/^{125}I–B109	B112/^{125}I–B111	B112/^{125}I–B112	B111/^{125}I–B111
hCG β subunit	90 ± 171	−126 ± 285	17,596 ± 501	−77 ± 262	−502 ± 84
hCG	43,218 ± 2071	14,439 ± 569	30,162 ± 127	97 ± 148	−239 ± 184
cBABS	157 ± 192	−297 ± 104	27,573 ± 1025	14,271 ± 139	25697 ± 1148
cBABS + α subunit	6,437 ± 295	−356 ± 110	26,007 ± 773	1,008 ± 174	1018 ± 139
GCN4–cBABSδα2	−839 ± 289	27 ± 293	35,482 ± 1465	23,419 ± 371	33,551 ± 2412
GCN4–cBABAδα2	375 ± 388	264 ± 233	268 ± 505	24,357 ± 949	−122 ± 1487

Sandwich immunoassays employed B112 for capture and radiolabeled antibodies (specific activity, 50 μCi/μg) to known hCG epitopes [5,6,9,16] for detection. Antibody binding sites: A109/A113/A202, α1 and/or α3; B101, β2; B109, hCG dimer-specific epitope containing parts of β2 and seatbelt; B111, seatbelt and carboxy-terminal residues near Cys110; B112, β3. A113, B101, and B109 bind overlapping hCG epitopes. Thus, only homodimers are detected by B111/^{125}I–B111, B112/^{125}I–B112, A113/^{125}I–A113, A113/^{125}I–B101, and A113/^{125}I–B109 sandwich immunoassays. Values are means (counts per minute) of triplicates minus the blank. Those greater than 1000 are significant ($p < 0.05$). It is clear from these data that cBABS can combine with the α subunit to form heterodimers. cBABS analogs and GCN4–cBABAδα2 did not bind B101 (data not shown).

Table 2

Antibody recognition of GCN4–cABASt containing α1,β2,α3 components (A113 capture).

	Antibody pair			
Analyte	A113/^{125}I–A113	A113/^{125}I–B101	A113/^{125}I–B109	A113/^{125}I–B112
hCG	−370 ± 130	263 ± 612	704 ± 280	13418 ± 1643
GCN4–cABASt	4358 ± 1064	14307 ± 441	2224 ± 305	202 ± 699

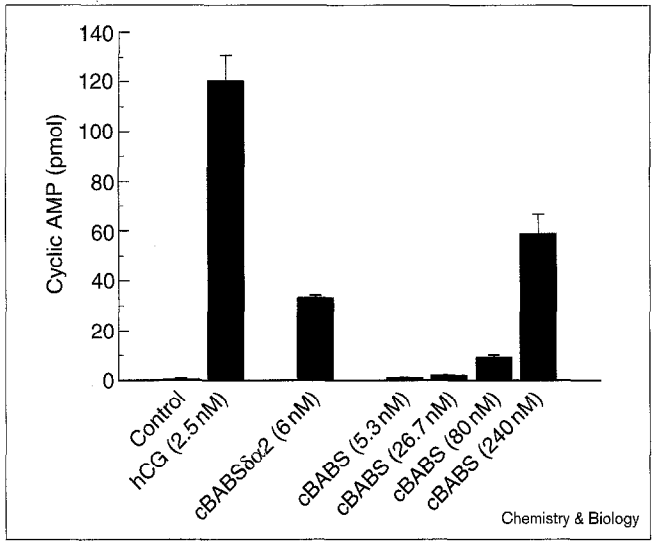
These sandwich assays employed A113; conditions are the same as those reported in Table 1. GCN4–cABASt did not bind B111 (data not shown).

only a small amount of dgBABSδα2–Y37C was secreted as a homodimer showed that the potential to form an intersubunit disulfide bond was not sufficient to drive efficient dimerization (Figure 5a). Amino-terminal addition of a strong dimerization domain substantially increased the formation of dimers lacking the α2 oligosaccharide and/or seatbelt (compare GCN4–cBABSδα2, GCN4–cBABAδα2, and GCN4–cABASt, Figure 5a,b). As discussed later, however, it is possible that some of the GCN4–cBABSδα2 homodimers are the equivalent of tethered monomers lacking the arrangement of the β1,α2,β3 loops expected in cBABS or cBABSδα2 homodimers.

Interactions of the homodimers with receptors

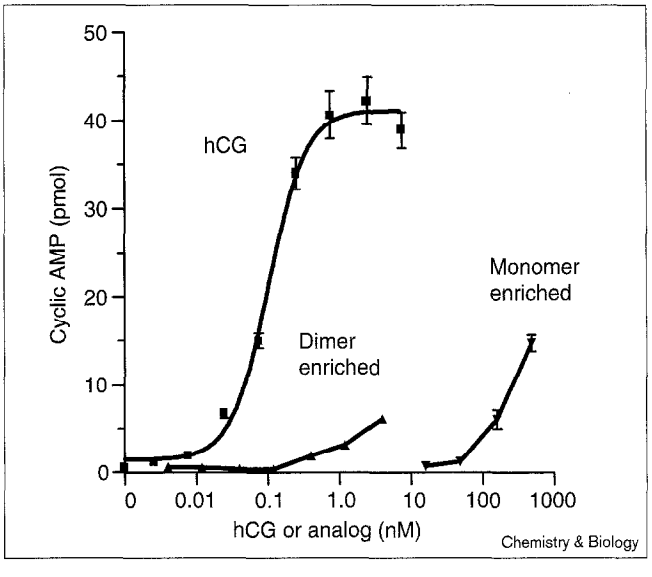
As predicted by Model 1, cBABS and cBABSδα2 stimulated LH-receptor-mediated cyclic AMP accumulation (Figure 6). The most active analog we prepared, cBABSδα2, had 1–3% of the potency of hCG. cBABS was 40-fold less active than cBABSδα2 even though it contains two copies of the α2 oligosaccharide, a structure known to potentiate the signal-transduction activity of hCG [18]. This suggested that one or both copies of this oligosaccharide reduced LH receptor interactions and confirmed the prediction made by Model 1 that removing the α2 oligosaccharide would enhance the activity of the homodimer (see Figure 2d). This observation is also consistent with the idea that parts of the hormone become located in the cavity created by the horseshoe shape of the receptor's extracellular domain [9]. The relative activities of homodimer- and monomer-enriched fractions of cBABSδα2 (Figure 7) suggested the receptor interacted with the homodimer, not the free subunit. Unfortunately, because cBABSδα2 was produced primarily as a monomer and because the monomer was not readily separated from the homodimer by size-exclusion chromatography, we were unable to prepare sufficient purified material to obtain full dose-response curves.

Figure 6



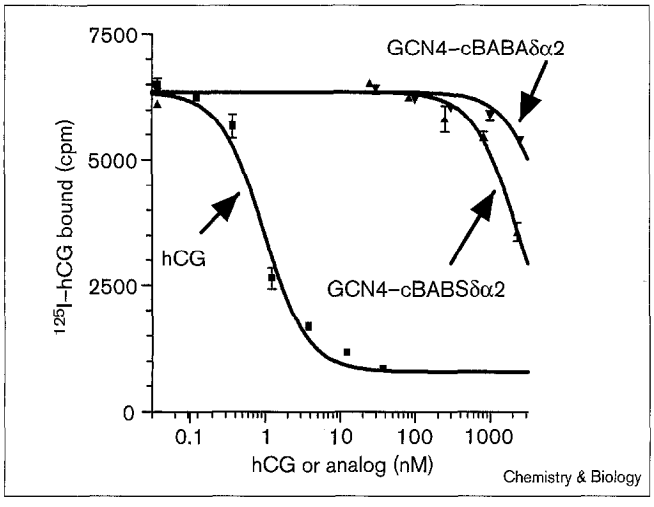
Biological activities of cBABS and cBABSδα2 in rat LH receptor signal transduction assays, showing the effect of hCG and these analogs on LH receptor signal transducton. Note that the amounts of cBABS, cBABSδα2, GCN4–cBABSδα2, and GCN4–cBABAδα2 homodimers used in the assays shown in Figures 6–11 were determined by sandwich immunoassay using purified cBABS as a standard, and confirmed by Western blot analysis.

Figure 7



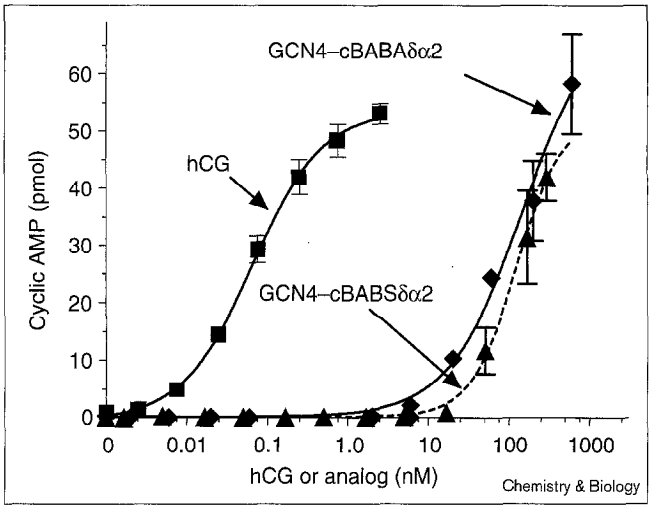
LH signal transduction assays of hCG and fractions for cBABSδα2 enriched in homodimer (quantified as dimer) and monomer (quantified as monomer).

Figure 8



Abilities of GCN4–cBABSδα2 and GCN4–cBABAδα2 to inhibit binding of ^{125}I–hCG in CHO cells expressing rat LH receptors.

Figure 9

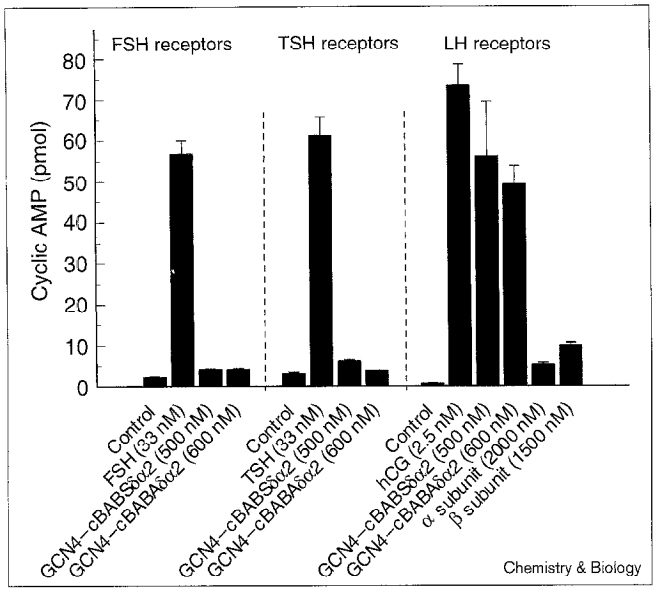


Abilities of GCN4–cBABSδα2 and GCN4–cBABAδα2 to stimulate cyclic AMP accumulation in CHO cells expressing rat LH receptors.

The abilities of cBABS and cBABSδα2 to elicit signal transduction contradict the hypothesis that the α subunit carboxyl terminus is essential for receptor binding and signaling, an important tenet of Model 2 [10]. Both cBABS and cBABSδα2 lack the α subunit carboxyl terminus, a five-residue sequence required for hCG function that had previously been implicated as a major receptor contact [4,19,20]. Chen *et al.* [19] were unable to detect receptor binding or signal transduction in response to an hCG analog lacking the α subunit carboxyl terminus. Indeed, relative to hCG, it appears from the data [19] that their truncated hCG analog was less active than cBABSδα2. We postulate that the low activity of hCG analogs lacking the α subunit carboxyl terminus is due primarily to the influence of these five residues on hormone conformation, not elimination of a key receptor contact. This idea is supported by the observation that removal of the α subunit carboxyl terminus improved the ability of the heterodimer to be recognized by antibodies that bind the free α subunit much better than hCG [9]. Nonetheless, because the activities of cBABS and cBABSδα2 are lower than that of hCG, we cannot exclude the possibility that the α subunit carboxyl terminus makes some contribution to the receptor contact site. Model 1 suggests the α subunit carboxyl terminus is located near both the extracellular and transmembrane domains where it could contact the receptor [9].

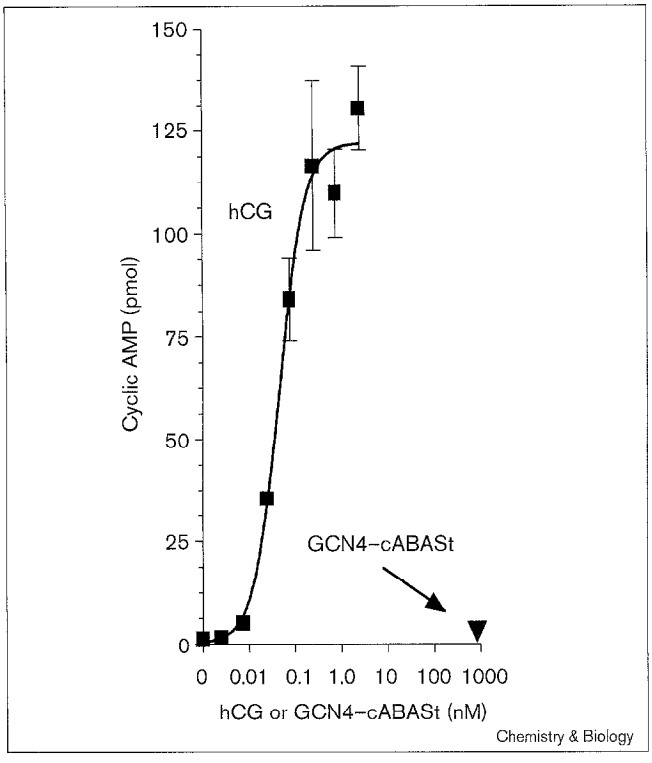
The GCN4-dimerization domain [12] was required for preparing homodimers lacking all or part of the seatbelt (i.e., cBABA and cABASt). As a 'control', we also added the GCN4 domain to cBABSδα2. Although GCN4–cBABSδ2 dimerized much better than cBABSδα2 (Figure 5a), it was less active. GCN4–cBABSδα2 blocked binding of ^{125}I–hCG to CHO cells expressing LH receptors with only 0.01% the potency of hCG (Figure 8). The potency of GCN4–cBABSδα2 in signal transduction assays was 10-fold lower than that of cBABSδα2 and approximately 0.1% that of hCG (Figure 9). This result is probably not related to an inappropriate contact between the GCN4 coiled–coil domain and the receptor because an analog of hCG containing similarly located coiled–coil domains of Fos and Jun had identical receptor binding and signal transduction activities as hCG (W.L., M.P.B., and W.R.M., unpublished observations). A more likely explanation for the lower activity of GCN4–cBABSδα2 is that it contained substantial amounts of inactive dimers consisting of two GCN4–cBABSδα2 monomers joined at their amino-terminal ends. This type of homodimer would be produced by formation of the seatbelt disulfide bond prior to subunit combination. If the seatbelt became latched prior to dimerization, it would not be wrapped around α2 to stabilize the β1,α2,β3 groove. Instead, due to its location, the seatbelt would be expected to prevent loop α2 of one subunit from associating with loops β1,β3 of the other subunit, thereby precluding formation of the β1,α2,β3 groove. Premature formation of the seatbelt disulfide bond during expression of cBABSδα2 would explain the secretion of high amounts of monomer and low amounts of dimer into the culture media. However, premature closure of the seatbelt in GCN4-cBABSδα2 would not prevent the subunits from being drawn together by a dimerization domain and held together in an inactive form by an intersubunit disulfide bond. Unfortunately, we would not be able to distinguish tethered monomers from homodimers that contained two copies of α2 surrounded by a seatbelt because both would be recognized by the same antibodies in sandwich immunoassays, both would be the same size, and both would be stable in 10 M urea.

Figure 10



Abilities of GCN4–cBABSδα2, GCN4–cBABAδα2, and hCG α and β subunits to stimulate cyclic AMP accumulation in CHO cells expressing human FSH and TSH receptors and in cells expressing rat LH receptors.

Figure 11



Activity of GCN4–cABASt in rat LH receptor signal transduction assays.

An analog with both seatbelts replaced by the α subunit carboxyl terminus, GCN4–cBABAδα2, had similar activities to GCN4–cBABSδα2 in receptor-binding and signal-transduction assays (Figures 8,9). The finding that GCN4–cBABAδα2 retains substantial signal transduction activity makes it highly unlikely that the seatbelt is responsible for the major interaction between the hormone and the receptor. Because it has lower activity than hCG, however, we cannot exclude the possibility that seatbelt residues participate in a receptor contact. The low activity of GCN4–cBABAδα2 relative to that of cBABSδα2 is most likely to be related to the fact that the seatbelt appears to be required to stabilize the β1,α2,β3 groove. Unlike the structure of α2 in hCG [1,2], the conformation of α2 in the free subunit is unconstrained [21]. In hCG, the conformation of α2 is constrained by contacts it makes with residues in the seatbelt and parts of β1,β3. The shape of α2 is much less likely to be constrained in analogs that lack the seatbelt than it is in hCG, resulting in some loss of activity.

All analogs containing loops β1,α2,β3 were more active than the individual α or β subunits, that is, analogs containing single copies of α1,α2,α3 and α subunit carboxyl terminus or β1,β2,β3, and the seatbelt (Figure 10). They were also more active than the analog containing two copies of α1,β2,α3, and the part of the seatbelt postulated to interact with LH receptors (GCN4–cABASt, Figure 11). Concentrations of hCG-based homodimers that elicited maximal LH receptor stimulation were only marginally active in signal-transduction assays employing CHO cell lines expressing FSH or TSH receptors (GCN4–cBABSδα2; GCN4–cBABAδα2, Figure 10), which showed that the activities of the homodimers depended on an interaction with the LH receptor, not an influence of the homodimers on CHO cells *per se*. A tenfold higher concentration of these analogs elicited a partial FSH response (data not shown; these amounts not tested in TSH assays). As observed previously [5,7], analogs containing hCG loops β1,α2,β3 can interact with all three receptors, indicating that this part of the molecule has only a small influence on receptor-binding specificity, as discussed later.

Implications of these data for glycoprotein hormone receptor interaction

Crystal structures are available for several hormone–receptor complexes [22–24]. Alanine scanning mutagenesis of residues within the contact site has shown that relatively few ligand and receptor residues participate in essential contacts. Replacing the sidechain of only one of these 'hot spots' with alanine usually results in 100-fold or more loss in potency [25,26]. Construction of homodimers from the hCG heterodimer involved exchanging entire loops of the hormone subunits, a procedure expected to have a much more dramatic effect on hormone structure than replacement of single amino-acid residue sidechains by alanine. Thus, the homodimers would retain substantial hormonal activity in LH-receptor-mediated signal-transduction assays

only if they retained much of the receptor contact site and if the contact site was similar in conformation to that in hCG.

The findings that the α subunit carboxyl terminus, loop β2, and seatbelt are not absolutely required for hormone activity argue strongly against the view of receptor binding outlined in Model 2 (Figure 2c). The observations that all analogs that have loops β1,α2,β3 were active and those that lack an oligosaccharide on the α2 loop were the most active support the view that residues in loops β1,α2,β3 participate in essential receptor contacts, a prediction of Model 1 (Figure 2a,b). The entire surface of β subunit loops 1 and 3 furthest from the α2 loop can be recognized by monoclonal antibodies that bind the hCG–receptor complex [14,27]. Thus, this region of hCG does not participate in receptor contacts. The only remaining surface common to hCG and all active homodimers available for participating in receptor contacts includes the end of the α2 loop and nearby residues in loops β1 and β3. Surfaces of hCG expected to contact the LH receptor in Models 1 and 2 are shown as rectangles and ovals, respectively, in Figure 4. Those parts shown within the rectangles (i.e., residues of α2 and β1,β3 postulated in Model 1) are conserved in all the active molecules (Figure 4e–g). Those shown within the ovals are not conserved and, therefore, not expected to form a key receptor contact.

At only 1–3% the activity of hCG, the potency of cBABSδα2 might appear low. However, it is equal to or greater than that of many lutropins in 'cross species' assays [28], more than 100–1000-fold greater than that of the most active synthetic peptide fragment [29], and substantially greater than that of either free hormone subunit. The homodimers described here are 'first generation' minimized proteins. As has been the case with other minimized proteins [30], it should be possible to improve their activities using mutagenesis, a process that will provide further insights into the relationship between hormone structure and function.

Several factors may contribute to the differences in the activity of hCG and the most potent homodimer, cBABSδα2. First, cBABSδα2 is larger than hCG (Figure 4), a consequence of the fact that it contains two seatbelts and two copies of β1,α2,β3, the largest loops in hCG. Support for the idea that the difference in size might hinder receptor binding comes from the observation that reducing the size of the protein by removing the oligosaccharide from loop 2 resulted in a 40-fold gain in potency (Figure 6). Second, it is possible that the conformation of the groove between loops β1,α2,β3 in hCG and cBABSδα2 may not be identical. Subtle differences in the conformations of lutropins are known to disrupt their abilities to interact with receptors [27]. Third, unlike most cysteine-knot proteins, glycoprotein hormone dimers are stabilized by a seatbelt, not one or more intersubunit disulfide bond. Therefore, they may not be as rigid as some cysteine-knot proteins, for example, vascular endothelial growth factor (VEGF) [25]. Instead, glycoprotein hormone dimers may acquire two or more stable conformations similar to those of glial cell-derived neurotrophic factor (GDNF) [31]. Based on the abilities of some anti-hCG monoclonal antibodies to bind hormone–receptor complexes with greater affinity than the free hormone, we have suggested that the conformation of lutropins is altered during receptor binding [16,32]. The presence of two seatbelts in cBABSδα2 might have hindered its ability to undergo the conformational change, thereby reducing its ability to interact with LH receptors. Finally, it is conceivable that other parts of hCG missing in cBABSδα2 might also participate in receptor contacts that augment binding now known to be associated with loops β1,α2,β3. Because the contributions of multiple binding sites to the overall affinity of a ligand–receptor pair are usually cooperative, in combination with the binding energy due to loops β1,α2,β3, the additional contribution of even a weak contact might be sufficient to account for the full potency of hCG.

Potential utility of the homodimers

In addition to their interest for studies of hormone structure and function, the homodimers are expected to have practical uses for eliciting immunological responses to particular regions of hCG, a postulate that we have recently confirmed (P.H.E., G.J. Macdonald, and W.R.M., unpublished observations). Because these homodimer analogs retain selected epitopes, they might have potential therapeutic uses in the development of site-specific responses such as those needed for a contraceptive vaccine.

Implications of these data for the evolution of glycoprotein hormones

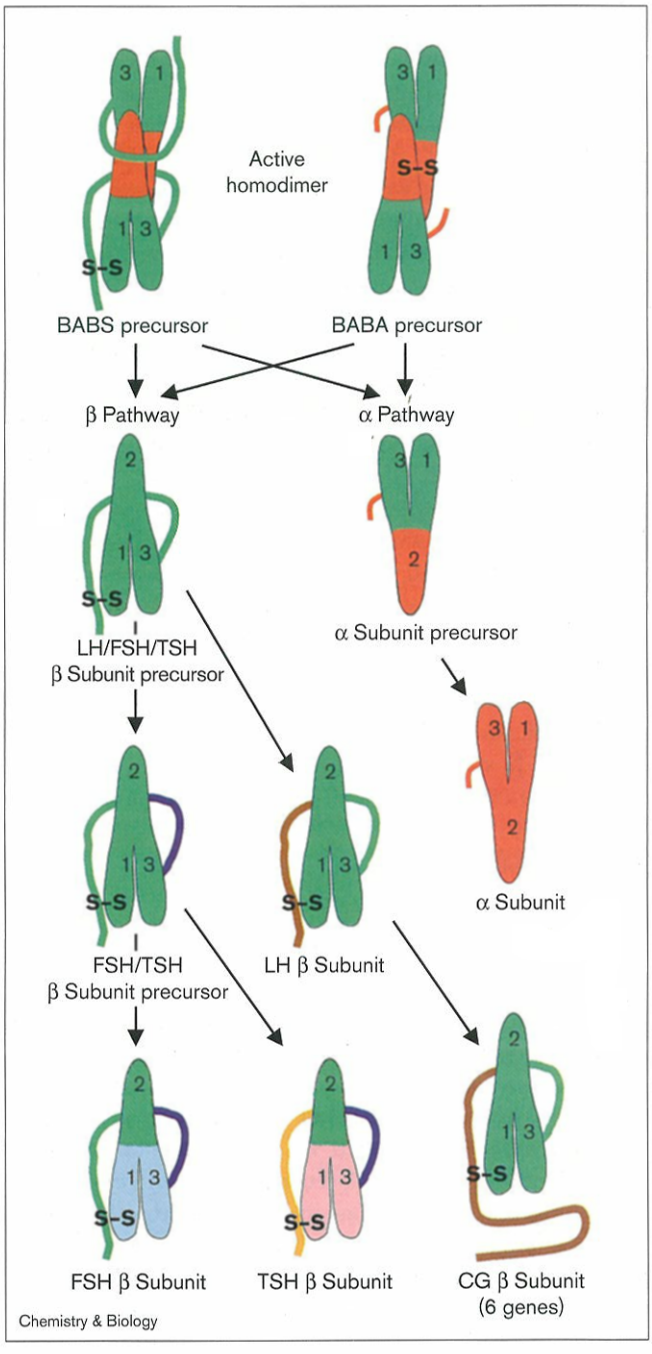
The evolutionary origin of proteins is usually traced by sequence analysis [33]. This may not always be possible, however, particularly when sequences diverge quickly or when key intermediates are lost or unknown. The discovery that intersubunit chimeras form functional homodimers provides the first biochemical support for the idea that both glycoprotein hormone subunits could have evolved from a common ancestor [34]. We envision that following gene duplication, mutations that prevented homodimer formation accumulated in both subunits (Figure 12). The finding that co-expression of cBABS and the α subunit led to formation of cBABS homodimers and cBABS/α heterodimers (Table 1) shows this might have occurred in a stepwise fashion wherein one gene product lost the ability to homodimerize before the other. Such a process might have created families typified by activins and inhibins [35] in which some (i.e., inhibin α subunit) but not all (i.e., activin β subunits) members have lost their abilities to form homodimers. As in the case of the inhibin/activin family, this process would have the potential to create agonist and antagonist analogs.

We propose a model for pituitary glycoprotein hormone evolution (Figure 12) in which the simplest ancestral glycoprotein hormone was a cysteine-knot protein homodimer similar to BABA, a protein likely to be stabilized by a disulfide bond, or BABS, a protein stabilized by two seatbelts. Homodimers lacking a seatbelt or the α2 oligosaccharide would probably not have been formed without an amino-terminal dimerization domain (not diagrammed). Following gene duplication, gain (BABA) or loss (BABS) of the seatbelt, modification of loop 2 and reduction in the dimerization domain yielded subunits that formed heterodimers, not homodimers. Reduced affinity of a dimerization domain (both pathways), changes to loop 2 (β-pathway), or loss of the seatbelt (α-pathway) would have prevented homodimer formation. Intersubunit disulfide bonds between the α subunit and β subunit cysteine knots cross-link and stabilize hCG (M. Einstein and W.R.M., unpublished observations). Cys31, a component in the α subunit Cys7–Cys31 disulfide bond not required for subunit combination or hormone activity [36], might be a vestige of a primordial intersubunit disulfide bond. Although this disulfide bond would increase dimer stability, it did not promote efficient dimerization of deglycosylated cBABS analogs such as cBABSδα2,β1Y37C (Figure 5a), and therefore would be unlikely to drive dimerization of progenitors. Efficient dimerization required specific subunit interactions, a function fulfilled here by GCN4 residues located in the region corresponding to the prohormone sequence found in most other cysteine-knot proteins. Introduction of negatively charged residues into the seatbelt would have eliminated the need for portions of the dimerization domain found at the β subunit amino terminus of lutropins. Thus, amino-terminal β subunit residues are required for efficient formation of lutropins [37,38], but not follitropins, thyrotropins, or lutropin analogs containing negatively charged seatbelts [38]. The findings that GCN4–cBABSδα2 and GCN4–cBABAδα2 had similar activities (Figure 8,9) and that cBABS/α heterodimers were active (not shown) suggest many of these changes could have accumulated without disrupting essential hormone functions.

In the α-subunit pathway elimination of the ability of the α-subunit precursor to form stable homodimers might have involved reduction in the strength of the amino-terminal dimerization domain and/or loss of an intersubunit disulfide. The highly conserved cysteine corresponding to human α subunit Cys31 [4], a residue capable of participating in an intersubunit disulfide, might be a vestige of an early BABA-like homodimer that was stabilized by a disulfide.

In the β-subunit pathway the seatbelt might have been introduced into a BABA-type subunit by 'read-through' of the termination codon, a process similar to that proposed to extend the hLH β subunit carboxyl terminus during the

Figure 12



A model for glycoprotein hormone evolution. Following gene duplication, inhibitory determinants were introduced into the subunits that prevented them from forming homodimers. The resulting β-subunit gene appeared to be duplicated several times. Based on the abilities of β-subunit analogs to interact with all three receptors, we postulate that loops 1 and 3 of the ancestral hormone were the most chemically similar to those of lutropins. However, this should not be taken to imply that the earliest hormones had the physiological activities of lutropins, a characteristic dependent on tissues that express the hormone receptors. Numbers identify loops. Red, portions of homodimers derived from hCG α subunit; green, portions of homodimers derived from hCG β subunit; dark blue, amino-terminal half of seatbelt; brown or orange, carboxy-terminal half of seatbelt; light blue, FSH loops β1,β3; pink, TSH loops β1,β3.

evolution of the hCG β subunit [39]. The first and second seatbelt cysteines (Cys93, Cys100, hCG β subunit numbering) form a disulfide bond that creates the small seatbelt loop in all β subunits. Participation of Cys93 in the Cys93–Cys100 disulfide bond would have prevented it from forming a disulfide bond with the cysteine in loop 2, a process 'reversed' during design of GCN4–cABASt. β subunit specificity would have been achieved through subsequent gene duplication and divergence led to heterodimers with LH, FSH, and TSH activity, a process initially dominated by mutations in the seatbelt [5,6] and later involved changes in β1 and/or β3 [7]. β2 does not appear to have a significant influence on receptor-binding specificity [5,7]. Therefore, it would be expected that changes to β1,β3, and the seatbelt would have the greatest influence on β subunit evolution. It is not yet possible to determine which β subunit evolved first. However, chimeras containing β1 and β3 from hCG, but not FSH or TSH, interact well with all three receptors [5–8,40], an indication that β1 and β3 of the primordial ancestor were more similar to those found in existing mammalian lutropins than those in mammalian follitropins or thyrotropins. hCG-based analogs containing an LH/FSH seatbelt chimera have full LH activity [6], high FSH activity [6], and substantial TSH activity [7]. Thus, a progenitor β subunit containing this chimeric seatbelt could have interacted with all three receptors, eliminating the need for simultaneous duplication of the β subunit and receptor genes.

Only a few changes would have been required to convert the activity of the progenitor β subunit intermediate into that associated with current mammalian β subunits. Following the first β-subunit gene duplication, introduction of negative charges into the amino-terminal portion of the seatbelt region would have decreased LH activity [5,13], increased TSH activity [7], left FSH activity unchanged [13], and reduced the need for the amino terminus in subunit combination [38]. A second β-subunit gene duplication followed by mutations in β1 and β3 that separated FSH and TSH activities would account for the failure of chimeras containing TSH and FSH β1,β3 to interact with all three receptors. Subsequent mutations appear to have occurred in a region of the TSH seatbelt not important for TSH activity. This would account for the observations that TSH/FSH chimeras containing the FSH seatbelt interact well with TSH receptors but FSH/TSH chimeras containing the TSH seatbelt do not interact with FSH receptors [5]. The hCG β subunits appear to have evolved by duplication of the lutropin β-subunit precursor and read-through of the termination codon [39].

Significance

In humans, repoductive and thyroid function are controlled in part by four glycoprotein hormones, human chorionic gonadotropin (hCG), lutropin, follitropin and thyrotropin, which act as αβ heterodimers. We are interested in understanding how these hormones interact with their receptors. Here we describe the construction and analysis of the first homodimeric glycoprotein hormone analogs, symmetrical proteins that have substantial biological and immunological activity. These studies have yielded new insights into glycoprotein hormone subunit combination, receptor interaction, and potential evolutionary mechanisms.

Although many of the homodimers are larger than hCG itself, they can be considered minimized proteins because they are derived only from parts of the hormone. Previous efforts to minimize these hormones have relied almost exclusively on the preparation of synthetic fragments, peptides active only at very high concentrations (i.e., 10 μM–10 mM), if at all. The very low activity of these peptides has confounded identification of parts of the hormones that interact with their receptors. In contrast, the homodimers constructed in this study retain many key aspects of hormone conformation, including some aspects known to depend on interactions between the two hCG subunits. The finding that all active homodimers contain the groove between α subunit loop 2 and β subunit loops 1 and 3 provides the first direct evidence that this portion of hCG (and presumably other glycoprotein hormones) participates in the primary receptor contact. This region of the protein should now become the major focus of efforts to further dissect ligand–receptor interactions. The homodimer approach to protein minimization should be applicable to studies of other heterodimeric proteins that have similar architectures.

Finally, hCG is known to be essential for human pregnancy, an observation that has been the basis for widespread efforts to devise a contraceptive vaccine. A major difficulty in vaccine development is related to the similarity of hCG and lutropin (hLH). Most immunogens designed to raise neutralizing antibodies against hCG also elicit antibodies to hLH. The finding that the homodimers retain the structures from the parts of hCG from which they are derived should facilitate design of immunogens that elicit antisera capable of immunoneutralizing hCG but not cross-reacting with hLH.

Materials and methods

Modeling

Homodimers were designed using coordinates from the crystal structure of hCG [1] and the modeling package Sybyl (Tripos, St. Louis, MO). The cysteine knots in the subunits of cBABS, cBABA, and cABASt analogs were aligned with those of each hCG subunit. Both halves of each homodimer are nearly identical to one another and to the components of hCG from which they were derived (Figure 4a,b,e,f). The Cα and Cβ carbons of Arg6 in each subunit of these model homodimers are approximately 6 Å and 4 Å apart, distances similar to those of cysteines in a disulfide bond. During design of GCN4-containing homodimers, each Arg6 was converted to cysteine, the homodimers

joined by a Cys6–Cys6 disulfide bond, and coordinates of amino-terminal residues deleted. The cysteine coordinates of a GCN4 homodimer that contains a carboxy-terminal disulfide bond [12] were aligned with the Cys6–Cys6 disulfide bond of the homodimers. Merging the GCN4 and homodimer coordinates led to structures typified by that shown in Figure 4c. During design of cABASt, we attempted to maintain the relative positions of β2 and the amino-terminal portion of the seatbelt required to form the binding site for B109, an hCG heterodimer-specific antibody [14]. Simulations suggested this could be accomplished by replacing Val56 of β2 with cysteine and truncating the seatbelt at residue 98 to create two free adjacent cysteines (i.e., Cys56 of β2 and hCG seatbelt residue Cys93), which have the same potential to form a disulfide bond as α subunit residues Cys59 and Cys87. Analysis by PROCHECK [41] showed that the phi–psi bond angles of all models were in allowed regions.

Homodimer expression

Constructs driving the expression of cBABS and cBABA analogs (Figure 3) were prepared using standard cassette and PCR mutagenesis methods, taking advantage of natural or previously introduced restriction-enzyme sites in the hCG β subunit cDNA [5,14]. The codons for GCN4 and cABASt were entirely synthetic and prepared by Vent polymerase (New England Biolabs, Beverly, MA) fill-in of long overlapping oligonucleotides. All sequences were verified by dideoxy sequencing methods. Proteins were produced by expression in COS-7 and CHO cells using commercially available expression vectors (pSVL, Pharmacia, Piscataway, NJ; pCI, Promega, Madison, WI) and methods described previously [5]. BABS and ABASt analogs were immunoaffinity purified using anti-subunit monoclonal antibodies B110 (β1,β3-specific) or B101 (β2-specific) [15] coupled to Affi-Gel Hz Hydrazide resin (BioRad, Hercules, CA) according to the manufacturer's directions. To prevent cross-contamination, each analog was purified on a new resin. Final purification involved high-performance liquid chromatography (HPLC) gel filtration on 0.75×30 cm TSK G3000SW (TosoHaas, Philadelphia, PA) or 1×30 cm Superose6-HR (Pharmacia, Piscataway, NJ) columns. These resins only partially separated the cBABSδα2 homodimer from the monomer. cBABSδα2 homodimer-enriched fractions used in Figure 7 contained material from the leading edge of the overlapping dimer/monomer peak and cBABSδα2-monomer-enriched fractions contained material from the peak and trailing edge.

Homodimer purification and assay

cBABS analogs were quantified by sandwich immunoassays employing B112 for capture and radioiodinated B112 for detection using a purified cBABS standard whose concentration had been determined by amino-acid analysis at LSUMC Auxiliary Enterprises (Louisiana State University, New Orleans, LA). GCN4–cABASt was quantified by sandwich immunoassays employing A113 for capture and radioiodinated A202 for detection using an hCG standard. Receptor binding and signal transduction assays were performed using 80,000–200,000 CHO cells stably expressing LH, FSH, or TSH receptors [6,7]. hCG α and β subunit assays were performed in the presence of 3 μg of anti-hCG β or α subunit antibodies, respectively, to neutralize the hCG contamination of the subunit preparations.

Assay reproducibility

The ability of homodimers containing hCG loops β1,α2,β3 to stimulate signal transduction was seen in every assay in which it was tested (26 assays) performed over a period of two years by three different individuals using several different preparations (R.V.M., Y.H., and Y.W.). It should be noted, however, that we observed maximal stimulation equal to that of hCG only in those assays employing material that had been purified from cell culture media.

Note added in proof

We call attention to the recent article by Li and Ford (Li, M.D. & Ford, J.J. (1998). A comprehensive evolutionary analysis based on nucleotide and amino acid sequences of the α and β subunits of the glycoprotein hormone gene family. *J Endocrinol.* **156**, 529-542) who suggest glycoprotein hormones evolved from a primordial ancestor more than 900 million years ago, based on comparisons of nucleotide and amino-acid sequences of 58 α and β subunits from piscine, amphibian, avian and mammalian species. Some of these organisms predate the appearance of vertebrates, the only animals in which function of these hormones has been clearly demonstrated. Sequence comparisons also suggest that lutropin-like β subunits predated those of follitropins and thyrotropins, a conclusion consistent with the model in Figure 12 reached on the basis of protein engineering.

Acknowledgements

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