The Position of the α and β Subunits in a Single Chain Variant of Human Chorionic Gonadotropin Affects the Heterodimeric Interaction of the Subunits and Receptor-binding Epitopes*

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The glycoprotein hormone family represents a class of heterodimers, which include the placental hormone human chorionic gonadotropin (CG) and the anterior pituitary hormones follitropin, lutropin, and thyrotropin. They are composed of common α subunit and a hormone-specific β subunit. Based on the crystal structure, it was suggested that the quaternary subunit interactions are crucial for biological activity. However, recent observations using single chain glycoprotein hormone analogs, where the β and α subunits are linked (NH₂-CGβ-α; CGβα orientation), implied that the heterodimeric-like quaternary configuration is not a prerequisite for receptor binding/signal transduction. To study the heterodimeric alignment of the two subunit domains in a single chain and its role in the intracellular behavior and biological action of the hormone, a single chain CG variant was constructed in which the carboxyl terminus of α was fused to the CGβ amino terminus (NH₂-α-CGβ; αCGβ orientation). The secretion rate of αCGβ from transfected Chinese hamster ovary cells was less than that seen for CGβα. The αCGβ tether was not recognized by dimer-specific monoclonal antibodies and did not bind to lutropin/CG receptor. To define if one or both subunit domains were modified in αCGβ, it was co-transfected with a monomeric subunit. In each case, αCGβ/α and αCGβ/CGβ complexes were formed indicating that CG dimer-specific epitopes were established. The αCGβ/α complex bound to receptor indicating that the β domain in the αCGβ tether was still functional. In contrast, no significant receptor binding of αCGβ/CGβ was observed indicating a major perturbation in the α domain. These results suggest that although dimeric-like determinants are present in both αCGβ/α and αCGβ/CGβ complexes, the receptor binding determinants in the α domain of the tether are absent. These results show that generating heterodimeric determinants do not necessarily result in a bioactive molecule. Our data also indicate that the determinants for biological activity are distinct from those associated with intracellular behavior.

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The glycoprotein hormones lutropin (LH),1 follitropin (FSH), thyrotropin, and choriongonadotropin (CG) are heterodimers, which consist of a common α subunit and a unique β subunit that confer the receptor specificity of the ligand. The subunits combine non-covalently early in the secretory pathway, and formation of the heterodimer is crucial for binding to the gonadal and thyroid receptors. Although it is well established that both subunits contain residues critical for bioactivity, recent evidence indicates that the quaternary interactions between the two subunits are essential for intracellular behavior but not for in vitro bioactivity (1–3). This conclusion was based on the single chain gonadotropin model where the amino end of the common α subunit was genetically fused to the carboxyl end of CGβ subunit (designated CGβα; Fig. 1) (4). This analog was secreted efficiently and was active in vitro and in vivo bioassays. The CGβα orientation was chosen to keep the carboxyl end of the α subunit unmodified because this region is critical for high affinity receptor binding (5–11). However, it was unclear whether the orientation of the tethered subunit domains affects the heterodimeric alignment between the two subunits, resulting in functional determinants for secretion and bioactivity, or if the two domains can swivel with respect to each other producing heterodimer-like contacts regardless of their position. To examine this issue, we constructed a single chain hCG in which the carboxyl terminus of the α subunit was genetically fused to the amino end of CGβ subunit (αCGβ; Fig. 1). This design reverses the relative position of the linked subunit domains compared with the first generation of single chain analog described above.

Several parameters were examined for determining the function of the reverse-oriented constructs as follows: 1) the secretion rate and recovery from the media, 2) the ability of the tethered α/β domains to combine and form heterodimer-specific epitopes with co-transfected monomeric subunits, and 3) formation of receptor binding determinants. Here we show that the relative position of the α and CGβ tethered domains in the single chain is critical for bioactivity but not for secretion. In addition, αCGβ/α and αCGβ/CGβ complexes were observed when the single chain was co-expressed with individual subunits. Dimer-specific conformational epitopes were detected in both complexes, but only αCGβ/α was bioactive. This implies that formation of heterodimeric interactions does not ensure bioactivity. The results support the hypothesis that epitopes for gonadotropin assembly and bioactivity are distinct and can be uncoupled from each other.

1 The abbreviations used are: LH, lutropin; FSH, follitropin; CG, human chorionicgonadotropin; CTP, carboxyl-terminal peptide of the CGβ subunit; CHO, Chinese hamster ovary; mAbs, monoclonal antibodies.
Gonadotropin subunits and single chain variants

**FIG. 1.** The orientation of the α and CGβ subunit domains in single chains. The CGβ subunit (open box) is genetically fused to the α subunit (stippled box). CGβα indicates amino-terminal location of the β subunit, whereas αCGβ denotes that the carboxyl end position is occupied by the β subunit. In CGβ[delta]T the carboxyl-terminal peptide (CTP) subunit is absent. In the case of αCGβ[delta]T, the CTP was deleted from the carboxyl end of the CGβ subunit and inserted between the carboxyl end of the α and amino terminus of the CGβ subunits.

**EXPERIMENTAL PROCEDURES**

Construction of Single Chains—Engineering of the single chain CGβα was described previously (4). To construct αCGβ, the CGβ gene was inserted in frame at the carboxyl end of the α subunit gene using overlapping polymerase chain reaction. The following primers were used in the construction: 1) 5'-CTACAGGAAAACCCATT-3'; 2) 5'-GC-GGCTCTTTGGAAAGATTTGTGATAAT-3'; 3) 5'-ATTATCACAAATCT-TCCAAGGAGCCGCG-3'; 4) 5'-TGAGTCGACATGATAATTCAGTGATTT-GAT-3'. From the α minigene, product A was generated using primers 1 and 2. Primer 1 encodes residues 12–17 at the α subunit amino terminus. Primer 2 contains the first 4 codons of the CGβ subunit and the last 5 codons of the α subunit. In another reaction, primers 3 and 4 were used with CGβα as template. Primer 3 encodes the first 4 amino acids of the CGβ subunit (residues 1–4) and the last 5 amino acids of the α subunit (residues 88–92). Primer 4 contains some of the intron sequence between CGβ exons 2 and 3 and also includes a newly created SalI site. The fragment derived from this reaction is product B.

In a second reaction, fragments A and B were used as overlapping templates with primers 1 and 4 to generate product C. Product C was sequenced to ensure no errors were introduced during the polymerase chain reaction. Following NsiI and SalI digestion, the fragment was cloned into pBS containing the α minigene including its signal sequence (pBSαCG exon 2). The CGβ exon 2 flanked by SalI site was isolated from pM2HAαCGβ (4). CGβ exon 3 was ligated to SalI-digested pBSαCGβ in the correct orientation with αCGβ exon 2 resulting in pBSαCGβ. This was then cloned into BamHI-digested pM2HA. The final product contains NH₂-α (with signal peptide)-CGβ (without signal peptide) COOH.

To construct the αCGβ[delta]T single chain, a carboxyl-terminal peptide (CTP) was deleted from the CGβ subunit and inserted between the α and CGβ[delta]T domains. To facilitate construction of αCGβ[delta]T, the CTP-derived sequence linked to the carboxyl-terminal of the α subunit was truncated at amino acid residue 117 (instead of residue 114) and included 28 residues rather than 31 amino acids. This analog bearing the 28-amino acid linker exhibited the same intracellular and extracellular characteristics (data not shown) as reported previously for single chains containing a 31-amino acid linker (4).

DNA Transfection and Cell Culture—All variants were inserted into the mammalian expression vector pM2HA (4) and were transfected into CHO cells by the calcium phosphate method. Stable clones were selected—11 days after transfection by using the neomycin analog G418 (250 μg/ml). The clones were maintained in Ham's F-12 medium (supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mg glutamine) containing 5% fetal bovine serum and G418 (125 μg/ml) at 37 °C in a humidified atmosphere of 5% CO₂, 95% air, as described previously (12).

Metabolic Labeling—Pulse-chase experiments were performed as described previously (4, 13). Aliquots of cell lysate and medium were immunoprecipitated with polyclonal antiserum directed against either the common α or the CGβ subunit, and the proteins were resolved on 12.5% SDS-polyacrylamide gels (4). The secretion half-time (t₁/₂) and recovery efficiency of the single chains were estimated by determining the time (min) when half of the maximal secreted variant was detected in the medium; the recovery of the secreted variants is expressed as a fraction (%) of the total (intracellular + secreted) (4, 13).

Western Blot Analysis—Media samples were resolved on 12.5% SDS-polyacrylamide gels without heating and under nonreduced conditions to prevent dissociation of the hCG heterodimer and be blotted onto nitrocellulose. The blots were probed with antisera or monoclonal antibodies (mAbs) as described in the figure legends. Purified hCG (CR 127) was kindly supplied by Dr. A. Parlow (National Hormone and Pituitary Program, Torrence, CA). Rabbit polyclonal antiserum against the human α or CGβ subunits were raised in our laboratory. The hCG conformational sensitive mAbs that recognize primarily the heterodimer, but not the monomeric CGβ subunit, were provided by Dr. Steven Birken (Columbia University Medical School, New York). The blots were visualized with the Western Light Detection System (Tropix, Inc., Bedford, MA) following the manufacturer's protocol. Western blot analysis was performed 3–5 times using 3 independent collection media.

Radioreceptor Assay—Conditioned media were concentrated using either a Centricon concentrator (Amicon Inc., Beverly, MA) or an ultrafree concentrator (Millipore Corp., Bedford, MA). Subsequently, the
**FIG. 2.** Secretion kinetics of CGβα and αCGβ. CHO cells expressing the chimeras were pulsed-labeled and chased for the indicated times (hr). Cell lysates and media were immunoprecipitated with α subunit antiserum, and the reduced and heated proteins were resolved on 12.5% SDS-polyacrylamide gel electrophoresis. The recovery (%) and secretion half-times ($t_{1/2}$) were calculated as described under “Experimental Procedures.”

![Diagram showing secretion kinetics](image)

**FIG. 3.** Western blot analyses of CGβα and αCGβ. The samples were electrophoresed under non-reduced conditions and probed with the following: α antiserum (panel A), mAb (designated 40) specific for the hCG heterodimer (Di mAb; panel B), and an mAb (designated 68) specific for the monomeric CGβ subunit (panel C). Di refers to the native heterodimer. The migration of the free α and β subunits and the non-aggregated single chain (tether) are indicated by the arrows. This experiment was repeated 5 times with 3 independent collection media.

![Western blot analyses](image)
samples were washed in phosphate-buffered saline and quantitated using a double polyclonal based radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA), containing antiserum that recognizes the CG/H9252 subunit. Receptor binding and cAMP production were determined using a transfected CHO cell line, expressing the human LH/CG receptor (14). Total binding was 15%, and nonspecific binding (in the presence of 5 g of hCG) was 1.5% of total counts. The cAMP accumulation was determined using the Adenyl Cyclase Activation Flash Plate kit (PerkinElmer Life Sciences) as per manufacturer’s instructions. Briefly, 5 × 10⁴ stably transfected CHO cells were incubated for 2 h at room temperature with ligands; 125I-cAMP was then added, and the cells were incubated for 17 h at room temperature. The flash plate was then read in a Packard Top Counter. Each experiment was performed at least 3 times, and the data are presented as the mean ± S.E. of 3 independent culture collections.

RESULTS

**Biosynthesis of hCG Tether Variants**—To compare the secretion of CG/H9252 and CG/H9251 subunits from stably transfected CHO cells, pulse-chase experiments were performed. Cells were labeled with 35S-Promix, chased with unlabeled amino acids, and aliquots of lysate and medium immunoprecipitated with polyclonal α antiserum (Fig. 2). Although recovery of both variants from the media was similar, αCGβ was secreted slower (t₁/₂ = 155 min) than CG/H9252 (t₁/₂ = 90 min).

To examine if the two subunit domains in αCGβ could form intrachain heterodimeric-like interactions, media samples were electrophoresed under nonreduced conditions without heating, and the blots were probed with polyclonal α antiserum and a panel of CG dimer-specific mAbs (Fig. 3). The α antiserum recognizes both native CG heterodimer and the noncombined α subunit (Fig. 3, panel A, lane 1) and the monomeric α subunit (mAb 80; lanes 3 and 4). This experiment was repeated 3–5 times with 3 independent collection media.

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**FIG. 4. Intracellular behavior of αCGβAT.** The chimera αCGβAT was subjected to pulse-chase kinetics (panel A) as described in Fig. 2. Panels B and C are Western blots containing non-reduced proteins. Blot in panel B was probed with α antiserum (lanes 1 and 2), and mAb 40 against the hCG heterodimer (Di mAb; lanes 3 and 4). Blot in panel C was probed with mAbs specific for either the monomeric CGβ (mAb 68; lanes 1 and 2) or the monomeric α subunit (mAb 80; lanes 3 and 4). This experiment was repeated 3–5 times with 3 independent collection media.
nomeric form of the CGβ subunit rather than the heterodimer (panel C). As expected, the non-combined CGβ subunit control secreted from transfected cells was immunoreactive (lane 4), and the heterodimer was not detected (lane 1). Whereas CGβα was poorly recognized by the CGβ-specific mAb (lane 2), αCGβ was much more immunoreactive (lane 3). These data imply that the conformation of one or both subunit domains in αCGβ is altered, which inhibits an intrachain heterodimeric-like interaction.

A major difference between the two single chain variants is the presence of the native CTP in the CGβ subunit (17) between the subunit domains in CGβα. As discussed previously, the CTP sequence lacks extensive secondary structure and serves as a natural linker (18). The αCGβ analog was constructed without a linker to maintain consistency with the subunit structure of CGβα. To assess if the intracellular effects of αCGβ are related to the absence of a linker sequence, a different chimera was constructed where the CTP was deleted from the carboxyl end of the CGβ subunit and inserted between the α and CGβ subunits (αCGβΔT) (Fig. 1). When examined by pulse-chase kinetics (Fig. 4, panel A), the secretion rate was 3-fold greater (t½ = 50 min), and the recovery was higher (80%) than αCGβ (t½ = 155 min; recovery = 70%). To examine the ability of αCGβΔT to generate heterodimer-like determinants, blots containing this variant were screened with the mAbs described above (Fig. 4, panels B and C). In contrast to the results seen with αCGβ, αCGβΔT is recognized by dimer-specific mAb (panel B, lanes 3 and 4), although its immunoreactivity to CGβ or α monomer-specific mAbs is reduced considerably (panel C, lanes 1–4). Thus, the presence of the linker sequence in αCGβΔT increases the heterodimeric alignment of the α and β domains.

Biological Activity of the Variants—The receptor binding affinity of the variants was determined using CHO cells stably expressing the human LH/CG receptor. CGβα displayed high affinity binding, displacing the iodinated tracer similar to that of native hCG (Fig. 5A; Table I). In contrast, no binding was observed with αCGβ. However, αCGβΔT does bind to receptor, although its affinity is reduced about 100- and 70-fold when compared with CGβα and the heterodimer, respectively. Signal transduction of the chimeras was assessed by quantitating adenylyl cyclase activation (Fig. 5B; Table I). Native hCG, CGβα, and αCGβΔT caused a concentration-dependent increase in cAMP accumulation. Although, the stimulation of αCGβΔT was 25–30-fold less than the controls, the induced levels of cAMP were greater than expected based on the receptor binding data (see “Discussion”). The results show that for the variant in the αβ configuration (αCGβΔT), the linker can rescue a significant portion of the receptor binding activity, but the lack of a free carboxyl end in the α subunit nevertheless reduces the binding affinity. These observations are similar to data seen with the heterodimer containing an α subunit with a CTP unit at its carboxyl end (10). In that study it was shown that the receptor binding affinity of this analog was reduced over 100-fold.

Intrachain Subunit Domains and Assembly Competence—It is evident that compared with CGβα, αCGβ is biologically inactive, indicating that reversing the positions of the subunits dramatically alters the overall structure of the single chain. A key question is whether or not the reduction in receptor binding is related to modifications of one or both of the α/β domains. To address this point, we co-transfected αCGβ with either the α or CGβ subunit gene. We reasoned that if the intrachain domains were substantially misfolded, this would be reflected in their inability to form heterodimeric-like contacts with either of the co-transfected monomeric subunits. For example,

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**TABLE I**

| Ligand | IC₅₀ (ng/ml) | EC₅₀ (ng/ml) |
|--------|-------------|-------------|
| hCG dimer | 9.8 ± 0.3 | 1.6 ± 0.1 |
| CGβα | 6.7 ± 0.7 | 1.2 ± 0.3 |
| αCGβ | ND | ND |
| αCGβ/CGβ | ND | ND |
| αCGβ/CGβΔT | 135.7 ± 3.0 | 16.0 ± 1.4 |
| αCGβ/α | 104.4 ± 9.1 | 9.7 ± 0.9 |
| αCGβΔT | 712.0 ± 31.5 | 36.2 ± 2.8 |
| αCGβΔT/CGβ | 612.5 ± 34.9 | 18.7 ± 0.7 |

**FIG. 5. Bioactivity of hCG single chain chimeras.** Varying concentrations of single chains were incubated for 16–18 h at room temperature with stably transfected CHO cells expressing the LH/CG receptor (panel A). Signal transduction of the ligands was determined under the same conditions by measuring cAMP (panel B). Data are mean ± S.E. of 4–6 experiments.
would be presumptive for the presence of a αCGβ/monomeric subunit functional complex.

We first examined the integrity of the tethered CGβ domain by co-expressing the monomeric α subunit with αCGβ (Fig. 6). The secreted proteins were analyzed in Western blots using polyclonal α antiserum (panel A) and a CG dimer-specific mAb 40 (panel B). The α antiserum recognized heterodimer and uncombined α subunit (lane 1) and αCGβ (lane 2 and 3). In addition, a 70-kDa band corresponding to αCGβ/α was detected in cells co-expressing both the single chain and the monomeric subunit (lane 3). The interaction between the incoming α subunit and the tethered CGβ subunit domain formed heterodimer-like epitopes because the complex was recognized by a dimer-specific mAb (panel B, lane 3).

The integrity of the intrachain α domain was examined with cells co-expressing αCGβ and the monomeric CGβ subunit (Fig. 7). The dimer-specific mAb 53 recognizes CGβα (lane 1) but not αCGβ (lane 2). However, the αCGβ/CGβ complex is immunoreactive (lane 3, asterisk). Six other conformational sensitive mAbs also detected both the αCGβ/CGβ and the CGβ/α complexes (data not shown). Thus, the intrachain α subunit can form a heterodimer-like interaction with a co-expressed monomeric CGβ subunit. Both αCGβ/α and αCGβ/CGβ complexes and the heterodimer dissociate after heating (3 min, 95 °C) in the absence of β-mercaptoethanol (data not shown). This result indicates that the association of the monomeric subunit with the tethered domain is non-covalent, comparable to subunit interactions seen in the native heterodimer. These experiments show that both the α and the CGβ subunit domains in αCGβ retain the ability to assemble with a monomeric subunit despite their inability to exhibit an intrachain heterodimeric configuration.

**Determinants for Bioactivity in αCGβ**—The αCGβ/α complex binds to the human LH/CG receptor although with reduced affinity compared with the heterodimer (Fig. 8A; Table I). The observed dose-dependent binding could only result from synthesis of a αCGβ/α complex since neither αCGβ nor the monomeric α subunit alone exhibits significant receptor binding. In contrast, αCGβ/CGβ did not displace the tracer (Fig. 8A) despite the formation of immunoreactive dimer-specific epitopes (see Fig. 7, lane 3). Because both αCGβ and the monomeric β subunit contain the CTP at their carboxyl termini, which are glycosylated and sialylated, the absence of αCGβ/CGβ receptor binding could be related to charge interference of two CTPs. To examine this point αCGβ was co-transfected with a CGβ monomeric variant lacking the CTP (CGβΔT). Although αCGβ/CGβΔT complex exhibited heterodimer-like interactions (see Fig. 7, lane 4), it nevertheless did not bind to the receptor (Fig. 8A; Table I). The data imply that lack of bioactivity in the αCGβ/CGβ complex is not due to the additional CTP. The cAMP levels induced by the complexes paralleled receptor binding (Fig. 8B; Table I). Thus, although the monomeric subunits in both αCGβ/α and αCGβ/CGβ form heterodimeric contacts, only the αCGβ/α complex is bioactive. These results indicate that determinants for receptor binding/signal transduction are preserved in the intrachain β subunit of αCGβ but not in the α domain.

The data described above demonstrated that the presence of the CTP as a linker in ααCGβΔT partially restores activity of the αCGβ. We examined if this linker would reconstitute bind-
Fig. 7. Complex formation between αCGβ and co-transfected monomeric CGβ or CGβAT subunits. Media derived from cells co-expressing the αCGβ single chain and monomeric CGβ subunit (αCGβ+CGβ, lane 3) or monomeric CGβ devoid of the CTP (αCGβ+CGβAT; lane 4) were blotted under non-reduced conditions and probed with mAb 53, which is specific for the hCG heterodimer. The asterisks in lanes 3 and 4 denote the αCGβ/CGβ and αCGβ/CGβAT complexes, respectively. This experiment was repeated 3–5 times with 3 independent cell cultures.

Fig. 8. Bioactivity of αCGβ single chain complexed with either the monomeric α (αCGβ/α), CGβ (αCGβ/CGβ), or CGβAT (αCGβ/CGβAT) subunits. The experiment was performed as described in the legend to Fig. 5. Data are mean ± S.E. of 4–6 experiments.

To examine if the relative position of the two subunit domains is critical for generating a functional single chain gonadotropin, we engineered a tethered hCG in which the position of the two domains was reversed (αCGβ) relative to the CGβα orientation. In both αβ and βα configurations, the variants were secreted efficiently which shows that the relative position of the linked subunits was not critical for secretion. However, in contrast to CGβα, the intra-chain subunits in αCGβ exhibited no detectable reactivity to hCG dimer-specific mAbs, although each domain was capable of forming a heterodimeric-like complex with its complementary monomeric subunit. This suggests that the structural constraints generated at the α-β linkage do not favor a heterodimeric alignment for the two domains. We also observed that αCGβ did not bind to the receptor compared with CGβα, which implies that accessibility of receptor binding determinants is dependent on the position of the αβ subunits in the single chain. A major structural difference between CGβα and αCGβ is the presence of a CTP sequence between the two tethered domains in CGβα. As reported previously (18), we considered this sequence a natural linker since it is serine/proline-rich and thus lacks significant secondary structure. In the case of αCGβ, the CTP is at the carboxyl end of the single chain, and thus it does not serve as a linker. When the CTP was deleted from the carboxyl end of the CGβ subunit and inserted between the α and β subunits, the resulting αCGβAT was rapidly and efficiently secreted and...

DISCUSSION

Previously, we and others (4, 21–24) observed that single chain variants of the glycoprotein hormone family exhibit biological activity comparable to the corresponding heterodimers. In such chimeras, e.g. CGβα, the β subunit occupies the aminoterminal end of the molecule linked to the α subunit. This orientation was chosen to preserve the free carboxyl end in the α subunit due to its importance for maximal receptor binding efficiency (5–11).
suggest that in the absence of linker the alignment of the α/β domains is perturbed especially at the carboxyl end of the subunit, the data imply that the conformational changes in this region expose determinants that are more efficient in stimulating downstream intracellular signaling reactions compared with the wild type heterodimer.

That these α-carboxyl-terminal mutants exhibited signal transduction is not in agreement with a previous study (26) that mutating or deleting carboxyl-terminal amino acids 88–92 of the α subunit abolished adenylate cyclase activation for the hCG heterodimer. One explanation to account for these variances is the use of a different bioassay for assessing signal transduction in that work (26). Recently, Gupta and Dighe (27) constructed a chimera composed of the α subunit attached to the amino terminus of the β subunit through a single glycine residue linker. The receptor binding of this analog was reduced only 10-fold. Thus, it is apparent from the above studies that the binding affinity of αCGβ is reduced, but there are quantitative differences in the reported binding affinities. The disparity between our αCGβ data and that of Gupta and Sighe (27) is due to the presence of the glycine linker in their construct, which may have increased the binding affinity. This is analogous to the results seen with αCGβAT.

Previously, we compared the secretion and biological activity of the CGβα lacking the CTP sequence and FSHβα single chains constructed devoid of a linker (28). We observed that for these variants the secretion rate was substantially reduced, but receptor binding/signal transduction was unaffected. Although absence of the linker sequence in the αCGβ tether lowered the secretion rate, no biological activity was detected. One explanation that could account for these differences is the interference between the adjacent disulfide bonds at the junction between the subunits since the 26–110 disulfide bond in the CGβ subunit (conserved position 20–104 in the FSHβ subunit) is critical for secretion (13). Based on chemical modification and mutagenesis studies, it appears that the CGβ carboxyl and α subunit amino-terminal regions do not contain key receptor contact sites (10, 29). Presumably, this accounts for high affinity receptor binding of CGβα devoid of CTP and FSHβα without linker despite their altered secretion kinetics. In the αCGβ single chain, however, there could be a significant perturbation of the last disulfide bridge, i.e. Cys-59–87 in the α subunit and the adjacent amino-terminally located disulfide bond in the β subunit. It is well documented that the 87–92-amino acid sequence in the α subunit is crucial for high affinity receptor binding by the heterodimers (5–11). That the Cys-59–87 bond does not impair secretion or assembly of the wild type heterodimer (12) might explain why the monomeric CGβ subunit can form a heterodimer-like complex with αCGβα and αCGβα is biologically inactive. In the case of the αCGβ/α complex, the carboxyl terminus of the bound monomeric α subunit is free, and thus αCGβα binds to the LH/CG receptor and activates adenylyl cyclase.

Despite the absence of the linker sequence in αCGβ, it is evident that much of the native α subunit structure is intact since it has the capacity to form heterodimeric contacts with

exhibited strong immunoreactivity to dimer- but not monomer-specific mAbs. The αCGβAT variant was bioactive, but the binding affinity was reduced compared with CGβα. These data suggest that in the absence of linker the alignment of the α/β domains is perturbed especially at the carboxyl end of the α subunit.

Our results are consistent with a recent report that a single chain hCG constructed in the αβ configuration and a CTP between the subunits is biologically active (25). In that work the binding affinity of the chimera was reduced 25–30-fold, whereas signal transduction was decreased only 10-fold. Similarly, we find that whereas the binding affinity of αCGβAT is reduced 70–100-fold, adenylate cyclase activation is reduced about 20-fold. Thus, there is an apparent uncoupling of binding/signal transduction when the subunit domains in the single chain are in the α/β orientation. This hypothesis is supported by data that show if the hCG heterodimer contains an α subunit with a deleted 59–87 disulfide bond, this analog stimulated CAMP synthesis to a greater extent than expected based on its low binding affinity (12). Because cysteine residue 87 is at the carboxyl end of the subunit, the data imply that the conformational changes in this region expose determinants that are more efficient in stimulating downstream intracellular signaling reactions compared with the wild type heterodimer.

**TABLE II**

*Summary of the Western blot analyses and bioactivity of hCG single chain chimeras*

| Ligand   | Dimeric epitopes | Monomeric epitopes | Bioactivity |
|----------|-----------------|-------------------|-------------|
| hCG dimer | ++              | –                 | +++         |
| CGβα     | ++              | –                 | ++         |
| αCGβ     | –               | –                 | –          |
| αCGβ/CGβ | +               | NT                | –          |
| αCGβ/CGβAT| ++             | NT                | –          |
| αCGβ/α   | ++              | NT                | ++         |
| αCGβAT   | ++              | –                 | +          |
| αCGβAT/CGβ| ++             | –                 | +          |
| αCGβAT/α | ++              | –                 | ++         |

The immunoreactivity of chimeras in Western blots are indicated as follows: +++, or ++, very strong; +, strong; +–, weak; –, negative; NT, not tested.
the co-transfected CGβ subunit (Table II). The absence of heterodimer-like interactions in αCGβ per se implies that the two domains cannot swivel with respect to each other. The data support the hypothesis that flexibility at the carboxyl end of the α subunit is a critical component for receptor recognition by the single chain variants and by native heterodimers but not for the intracellular behavior of the subunit.

The data presented in Fig. 9 suggest that altering the amino end of the CGβ subunit affects the binding affinity. This is consistent with a previous study of Xia et al. (30), which examined the role of the lysine residue at position 2 in the CGβ subunit. They suggested that the conformation of the amino end might be associated with receptor binding. In another investigation (31), a synthetic peptide corresponding to residues 1–16 of the CGβ subunit was shown to inhibit binding of 125I-hCG to porcine Leydig cells.

Our results also show that the presence of heterodimeric determinants does not necessarily result in a biologically active molecule (Table II), which is in agreement with the report of a naturally occurring mutation in the LHβ gene (32). A single amino acid substitution in the LHβ subunit (glutamine to arginine at residue 54) was associated with hypogonadism. It was shown that the mutated subunit formed a heterodimer that did not bind to the receptor in vitro. The uncoupling of a quaternary event and target binding are also supported by recent mutation studies of the Escherichia coli lactose repressor protein (LacI), where it was demonstrated that although assembly and folding of the two functional domains were not significantly affected, their affinity for the DNA target sequence was reduced dramatically (33). These results are consistent with our earlier studies that gonadotropin variants composed of more than 2 subunit domains bind and activate the receptor (21). That such variants with bulky constituents relative to the native heterodimer are bioactive implies flexibility in the ligand-receptor interaction. The data show that the relative position of the α- and CGβ-tethered domains in single chain CG is critical for bioactivity but not for secretion. Moreover, since no amino acid mutations were created in the αCGβ molecule, the data imply that receptor binding determinants in this variant are not accessible to the receptor due to an altered conformation or by interference created from the other intrachain β subunit. Thus, quaternary interactions are essential for the intracellular trafficking of the heterodimers but not for receptor recognition and signal activation. In the latter case, the role of the heterodimeric structure is to ensure that the appropriate epitopes in each subunit are brought in contact with the receptor triggering the biological response.

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The Position of the α and β Subunits in a Single Chain Variant of Human Chorionic Gonadotropin Affects the Heterodimeric Interaction of the Subunits and Receptor-binding Epitopes

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