Communication

The Biologic Action of Single-chain Choriogonadotropin Is Not Dependent on the Individual Disulfide Bonds of the β Subunit*

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David Ben-Menahem‡, Masataka Kudo§,
Mary R. Pixley, Asomi Sato,
Nobuhiko Suganuma¶, Emerald Perlas‖, Aaron J. W. Hsueh§, and Irving Boime¶

From the Department of Molecular Biology & Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110 and the Division of Reproductive Biology, Department of Gynecology/Obstetrics, Stanford University Medical Center, Stanford, California 94305-5317

Disrupting disulfide loops in the human chorionic gonadotropin β subunit (CGβ) inhibits combination with the α subunit. Because the bioactivity requires a heterodimer, studies on the role of disulfide bonds on receptor binding/signal transduction have previously been precluded. To address this problem, we bypassed the assembly step and genetically fused CGβ subunits bearing paired cysteine mutations to a wild-type α (WTα) subunit. The changes altered secretion of the single-chain mutants which parallel that seen for the CGβ monomeric subunit. Despite conformational changes in CG disulfide bond mutants (assayed by gel electrophoresis and conformationally sensitive monoclonal antibodies), the variants bind to the lutropin/CG receptor and activated adenylate cyclase in vitro. The data show that the structural requirements for secretion and bioactivity are not the same. The results also suggest that the extensive native subunit interactions determined by the cystine bonds are not required for signal transduction. Moreover, these studies demonstrate that the single-chain model is an effective approach to structure-activity relationships of residues and structural domains associated with assembly of multisubunit ligands.

Mutational and structural studies have revealed that small clusters of amino acids rather than large structural motifs often contribute to most of the energy involved in protein-protein interactions such as hormone binding to its receptor (for review, see Refs. 1–3). However, it is not clear how the conformation of a peptide ligand contributes to the coupling of binding to signal transduction. This is especially an issue for the function of hormone-receptor complexes involving multi-subunit ligands. A convenient model for studying the tertiary and quaternary determinants in signaling is the glycoprotein hormone family which include human chorionic gonadotropin (hCG), lutropin (LH), follitropin (FSH), and thyrotropin. Each is a non-covalent heterodimer composed of a common α and unique β subunit which allows recognition of the corresponding G-protein-coupled receptor (for review, see Ref. 4).

Recent crystallographic studies of hCG revealed a significant structural similarity to several growth factor families, e.g. transforming growth factor β, which contain a cystine knot motif composed of three pairs of bridged cysteine residues (5–8). Each hCG subunit contains a cystine knot configuring three disulfide-bonded loops which are the major structural motifs (7, 8). Based on the crystallographic studies of hCG (7, 8), the disulfide bonds in the CGβ subunit are at positions 9–57, 34–88, 38–90, 23–72, 93–100, and 26–110 (Fig. 1A). The folding intermediates associated with the ordered formation of these bridges to acquire an assembly-competent form is well documented (9, 10). Current models of hCG action presume that the conformation of the dimer and the highly interactive contacts between the two subunits are critical for function (11–14). One method to examine the functional role of the structural motifs in the glycoprotein hormones is to assess the biologic activity of variants containing mutated cysteine residues. However, breaking single disulfide bonds of the β subunit inhibits secretion and assembly with the α subunit, and as a result dimer recovery is dramatically reduced (Ref. 15 and Table I). Since only the heterodimer binds to the receptor, it is virtually impossible to examine the bioactivity of these variants. Recently, a single gene encoding a protein containing CGβ and a subunit was constructed (16, 17). The tethered hormone exhibited secretion kinetics and bioactivity similar to that of the non-covalent heterodimer. Here we compile into a single protein subunits that cannot combine efficiently, namely the α subunit and CGβ with cysteine to alanine mutations. Because this tethered construction by-passes the assembly step, we can examine the role of the disulfide bonds on the biological activity of hCG. The data show that they are primarily required for assembly with the α subunit and secretion of the heterodimer. The extensive native subunit interaction in the dimer which is altered by these mutations is not critical for receptor binding and signal transduction.

EXPERIMENTAL PROCEDURES

Vector Construction—A KpnI-XhoI fragment (2.7 kilobases; Fig. 1B) containing single-chain hCG (CGβα) in vector pM3HA (16) was subcloned into pBluescript II KS (Stratagene). Construction of cysteine (Cys) to alanine (Ala) mutants in the CGβ subunit gene (CGβΔCys) was described (15). The ApoI fragment containing the first 135 amino acids of CGβ sequence in CGβα was exchanged for the ApoI fragment in CGβΔCys. The new KpnI-XhoI fragment was inserted into pM3HA and rechecked by restriction enzyme analysis. The mutations were confirmed using Taq DyeDeoxy Terminator Cycle Sequencing Kit and an ABI prism DNA Sequencer (Perkin Elmer).

Cell Culture and Metabolic Labeling—Clones of transfected Chinese hamster ovary (CHO) cells were maintained as described (15, 16). We used polyclonal α antisera for precipitation and Western blots since WTα subunit is tethered to the mutated CGβ sequence, and the immunochemical mapping of hormone-receptor complexes involving multi-subunit ligands. A convenient model for studying the tertiary and quaternary determinants in signaling is the glycoprotein hormone family which include human chorionic gonadotropin (hCG), lutropin (LH), follitropin (FSH), and thyrotropin. Each is a non-covalent heterodimer composed of a common α and unique β subunit which allows recognition of the corresponding G-protein-coupled receptor (for review, see Ref. 4).

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RESULTS

Secretion of hCG Single-chain Mutants—To assess the intracellular effect of the disulfide loops in the CGβ domain, we examined the secretion of tethered hCG variants in which both cysteines of a proposed pair were mutated. Transfected CHO cells stably expressing the Cys mutants were metabolically labeled and subjected to SDS-polyacrylamide gel electrophoresis analysis under reduced conditions. The electrophoretic migration of the secreted proteins is slower than the corresponding intracellular species (Fig. 2A) which reflects both the intermolecular disulfide bonds and addition of the N-linked oligosaccharides to the CGβ subunit just prior to secretion (15). The experiment reveals that the secretion kinetics and extent of recovery of the mutants from the media are variable. For example, the 34–88 mutant is not detected in the media after 4 h of labeling (lane 7). Pulse-chase analysis shows that the unmodified single-chain hCG (CGβα) is secreted efficiently (t1/2 = 85 min; recovery = 75%; Table I) which is comparable to that of the heterodimer (15). As previously observed for the monomeric CGβ subunit (15), release of the 26–110 mutant is accelerated (t1/2 = 60 min, recovery 90%). In contrast, mutations in the cystine knot (i.e. 9–57, 34–88, and 38–90) resulted in variants that were secreted slower and less efficiently. This is especially evident for the 34–88 mutant where the t1/2 is 350 min and 25% of the protein is recovered. The reduced recovery suggests that the mutants were altered resulting in degradation of a significant fraction of the pool. This is substantiated by pulse-chase analyses that show in contrast to CGβα, where most of the variant is secreted, 75% of the 34–88 mutant that accumulates in the lysate does not exit the cell and is no longer detected after 24 h (Fig. 2B). Thus, the disulfide bonds which comprise the core of the subunit, namely 9–57, 34–88, and to a lesser extent 38–90, are essential for efficient secretion of the CG single chain. The secretion kinetics generally parallel those seen for CGβ monomer (Table I), which implies that these mutation-induced alterations in intracellular behavior are not the result of the single-chain construction.
Conformational Changes of the Cys Analogs—If the conformation is altered by breaking the disulfide bonds, we should detect differences in electrophoretic mobility and immunoreactivity on SDS gels. To test this prediction, Western blots were performed under non-reduced conditions using a monoclonal antibody directed against a subunit epitope that are exposed primarily in the heterodimer (designated A407; Ref. 19). The antibody recognized the CG\(\beta\)a, suggesting that dimer-like structure is preserved despite the tethering (Fig. 3A, lanes 1 versus 6). The mutants lacking the bonds that form late in the folding sequence of CG\(\beta\) monomer, i.e. 23–72, 26–110, and 93–100 (9, 10), were immunoreactive (Fig. 3A, lanes 2, 7, and 8). The recognition of the 23–72 and 26–110 Cys variants by the dimer-specific antibodies presumably reflects the limited assembly of such mutated CG\(\beta\) monomers with the \(\alpha\) subunit (Table I and Ref. 15). It is unclear why the 93–100 Cys mutant is detected because no discernible assembly is seen with monomer CG\(\beta\) subunit bearing this mutation (Table I and Ref. 15), but this single-chain mutant may be partially configured to a heterodimeric form. Of significance, analogs lacking bonds of the cystine knot motif are the scaffold for the structure, and the cysteine mutations induce conformational changes in the single chains. In contrast to the inability of such CG\(\beta\) subunits to efficiently combine and thus interact with the \(\alpha\) subunit, the mutated \(\beta\) domains in the single chain are nevertheless tethered to an \(\alpha\) domain and secreted. The improved recovery (Table I) now permits analysis of the effects of disrupting the disulfide-bonded loops on biological activity.

Receptor Binding and Signal Transduction—Binding of single-chain variants to the human LH/CG receptor stably expressed in 293 cells was examined. Unexpectedly found that all CG variants bind to the receptor including those mutants that as subunits assemble poorly (i.e. 34–88, 9–57, 93–100, and 38–90). The single-chain mutants, except analogs 38–90 and 34–88, displayed similar dose-response curves to the pu-
Bioactivity of hCG Single-chain Disulfide Bond Mutants

| Heterodimer | Binding IC<sub>50</sub> | cAMP EC<sub>50</sub> | Coupling factor (IC<sub>50/EC<sub>50</sub></sub>) |
|-------------|-------------------|-----------------|----------------------------------------|
| 9.5 ± 2.4   | 4.5 ± 0.6         | 2.1             |                                        |
| 34–88       | 39 ± 23           | 32 ± 5          | 2.9                                    |
| 38–90       | 67 ± 11           | 51 ± 21         | 1.3                                    |
| 9–57        | 20 ± 4            | 13 ± 1          | 1.5                                    |
| 23–72       | 8.0 ± 2           | 5.0 ± 2         | 1.6                                    |
| 93–100      | 15 ± 4            | 13 ± 2.4        | 1.2                                    |
| 26–110      | 23 ± 6            | 9.1 ± 4.5       | 2.5                                    |

 DISCUSSION

Structural analyses of hCG revealed that the overall shape of the glycoprotein hormones is elongated rather than globular with the disulphide-bonded loops primarily surface-exposed (7, 8). However, despite their relatively large structures, conformational changes induced by deleting each of the disulfide bonds and altering any of the loops in the CGβ domain does not abolish binding/signal transduction nor does it significantly affect coupling. These results were not anticipated since it is well accepted that the intracellular/extracellular behavior of the glycoprotein hormones is conformationally sensitive (11–14).

Mutations inhibiting heterodimer formation and eliminating dimer-specific epitopes in the tethered analogs did not significantly affect the bioactivity of the single-chain mutants. The glycoprotein hormone-specific quaternary structure, i.e. the heterodimer, signals key functional intracellular events that result in efficient secretion and hormone-specific processing of the subunit and includes domains for bioactivity such as the “determinant loop” (i.e. residues 93–100; Ref. 29). Because the single-chain 26–110 or 93–100 mutants were as active as CGβα, the major role for this sequence is apparently to stabilize the noncovalent heterodimer (7, 8). Thus, the primary and/or secondary structure of loci within each of these individual loops rather than their tertiary structure are likely determinants for functional receptor recognition.

By-passing the assembly step with the single-chain approach enabled us to expand the spectrum of analogs for structure-function analysis. Similarly, mutations in other multisubunit hormones and growth factors of the cystine knot superfamily which result in inefficient assembly, secretion, and loss of bioactivity (6 and references therein) can be examined.

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* National Hormone and Pituitary Agency.