A Single-chain Bifunctional Gonadotropin Analog Is Secreted from Chinese Hamster Ovary Cells as Two Distinct Bioactive Species

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The major developments in exploring structure activity relationships of the glycoprotein hormone family was the genetic engineering of single chains comprised of the common α subunit and one or more of the hormone-specific β subunits tandemly arranged. These studies indicate that there is a structural permissiveness in the quaternary relationships between the subunits and biological activity. However, the conformational relationships between the ligand and the receptor are unclear. Bifunctional triple-domain analogs represent an ideal model to address this issue. Does a single molecule possess the ability to simultaneously interact with both specific receptors or are there two functionally distinct species in the chimeric population? Here we show, using a preadsorption protocol comprised of Chinese hamster ovary cells expressing either the luteinizing hormone (LH)/chorionic gonadotropin (CG) or follicle-stimulating hormone (FSH) receptor, that at least two distinct bioactive populations of the dually active triple-domain chimera FSHβ-CGα-β are synthesized, each corresponding to a single activity (CG or FSH). Furthermore, we show that these bioactive populations form distinct stable heterodimer-like contacts. That there is not a single biologically active species formed during synthesis of the chimera implies that in vivo the heterodimer exists in multiple conformations and is not a static rigid molecule.

The gonadotropin hormones, CG, LH, and FSH are glycoprotein heterodimers comprised of a common α subunit non-covalently associated with a hormone-specific β subunit. Structure-function studies of multimeric proteins having non-covalently associated subunits have often been hindered because of mutagenesis-induced defects in subunit association. This is especially an issue for the glycoprotein hormones, since only the heterodimers exert biological activity. Genetically linking the α and β subunits to form single-chain derivatives, in which the assembly step is bypassed, is an excellent approach to expand structure-function studies on these proteins (1–3). One variation of this model, used to examine the conformational relationships between the ligand and the receptor, was the design of a triple-domain gene chimera encoding the sequence FSHβ-CGβα (4). This analog, composed of a single α subunit covalently linked with two tandemly arranged β subunits, displayed high-affinity binding to their respective human receptors (FSH-R and LH/CG-R) and activated adenylyl cyclase comparable with the heterodimers. Although this chimera exhibited dual gonadotropin activity, it was unclear, however, if a single molecule possessed both FSH and CG activities or if two distinct stable species in the trimer population were generated, each corresponding to a single activity. To distinguish between these possibilities, we used a preadsorption receptor binding protocol in which conditioned medium containing the triple-domain analog was first preincubated with transfected CHO cells expressing either the LH/CG or FSH receptors followed by binding to cells expressing either receptor (Fig. 1). We predicted that if a single molecule exhibits bifunctional activity, regardless of which receptor cell line is used in the initial preincubation, both of the activities in the secondary binding assays would be reduced in parallel (Fig. 1A). Alternatively, if there are two distinct biologically active species, there would be a preferential loss of a single activity following the first incubation (Fig. 1B). Our results are consistent with the hypothesis that at least two distinct bioactive populations of chimera are secreted, each corresponding to a single activity, and that the single-chain ligand forms heterodimer-like FSH and CG conformations.

EXPERIMENTAL PROCEDURES

Receptor-expressing Cell Lines—CHO cells expressing the rat LH receptor (rLH-R) or the human FSH receptor (hFSH-R) were grown in Ham’s F-12 medium supplemented with 5% serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂/95% air as described previously (4). CHO cells expressing the rat LH receptor rather than homologous human LH/CG receptor were used here due to their higher affinity for CG (5). This would increase the capture efficiency of the analog during preincubation steps.

Preparation of Concentrated Analog—The FSHβ-CTP-CGβα single chain was previously constructed and expressed in CHO cells (4). Conditioned media (Ham’s F-12 medium supplemented with 2 mM glutamine 100 units/ml penicillin, 100 μg/ml streptomycin) from this mutant was concentrated by Ultrafree centrifugal filter devices (Millipore Corp., Bedford, MA). Estimates of the concentrations of the two components of the single-chain analog were performed by FSH and CG dimer-specific RIAs according to the manufacturer’s instructions (Diagnostics Products Corp.). Each of the two RIAs used is hormone-specific and shows low cross-reactivity (less than 0.1%) to the other glycoprotein. The analog exhibited 2.4 IU of CG for each IU of FSH and CG immunoreactivity detected.

Preadsorption to CHO Receptor Cells—The experiments presented here were designed to test whether there is a single form that exerts bifunctional activity or whether there are multiple species each exhibiting a specificity for only a single receptor. The principle of the ap-
proach is the ability of receptor-expressing CHO cells to selectively sequester specific populations of gonadotropin activities after incubating a known amount of the chimera with CHO cells expressing either the FSH-R or the rLH-R. As a control, the chimera was also incubated with non-transfected CHO wild type cells. The supernatant recovered after this preadsorption step was used for receptor binding assays in parallel with the other samples.

We first determined the optimal conditions in the preadsorption assay to achieve maximal binding and retention of the triple-domain FSHβ-CGβ-α chimera. The criterion used to optimize the system was the difference between the amount of FSH ligand remaining in the recovered sample following the preadsorption step and the amount of immunoreactive FSH added. The optimal protocol included two preadsorption steps performed at 21 °C, one for 18 h, and then a subsequent 5-h incubation. Based on our preliminary analyses, all the experiments described here were performed as follows. Cells expressing the hFSH-R or rLH-R or non-transfected wild type CHO cells were plated on 24-well plates at 10^5 cells per well and grown at 37 °C for 24 h to form monolayer. The medium was removed, and the cells were washed twice with 0.5 ml each of cold modified Krebs-Ringer buffer and incubated at 21 °C with media containing different concentrations of purified hCG, recombinant FSH standards, or conditioned medium containing the FSHβ-CTP-CGβ-α analog. After 18 h, the media were recovered and centrifuged to eliminate dead cells, and the supernatant was placed into new plates containing monolayers of hFSH-R, rLH-R, or wild type CHO cells. This second adsorption period was performed for 5 h at 21 °C. The media were centrifuged again, and this supernatant was used for FSH and CG receptor binding assays and Western blot analyses. For each cell type, two to four wells of cells were used per concentration of analog during preadsorption experiments.

Receptor Binding Assays—Receptor binding assays were modified from previously described conditions (4). Briefly, 200 μl of the analog-containing medium recovered after the two adsorption steps described above were incubated for 18 h at 21 °C with 4 × 10^5 of either rLH or hFSH receptor cells in 300 μl of cold modified Krebs-Ringer buffer containing 0.1% bovine serum albumin and 100,000 cpm ^125I-hCG or ^125I-rFSH, respectively. To terminate the binding reaction and to remove unbound isotope, the cells were washed twice with cold modified Krebs-Ringer buffer containing 0.1% bovine serum albumin and centrifuged and the supernatant discarded. A γ-counter was used to determine the radioactivity bound to the cell pellet, and for each sample, two to four duplicates were assayed and at least three repetitions of the binding assays were performed. Each supernatant collected after the preadsorption sequence was used for both LH and FSH receptor binding assays. We refer to this second receptor binding step as a secondary assay.

Western Blot Analysis—Single-chain gonadotropin mutants in concentrated conditioned media were quantitated by both FSH and LH dimer-specific RIAs according to the manufacturer’s instructions. Equal amounts of analogs were resolved on 12.5% SDS-PAGE in the absence of heat and reducing agent and transferred onto nitrocellulose. Proteins were probed with monoclonal antibodies (mAb) FSH dimer-specific (F554), FSHβ-specific, and CG dimer-specific (B109) and with CGβ polyclonal antiserum. Immunodetection was performed with Tropix chemiluminescent system (Tropix, Bedford, MA). The FSH mAbs were obtained from Organon Bv (Oss, The Netherlands) and B109 mAb from Dr. Steven Birken at Columbia University, College of Physicians and Surgeons. The CG polyclonal antiserum was prepared in this laboratory.

To examine whether dimer-like populations detected in conditioned medium containing the chimera are stable at room temperature, a time course experiment was performed, followed by Western blot analysis. To obtain optimal signal on blots, the conditioned medium was diluted in modified Krebs-Ringer buffer. One ml of this medium was incubated at room temperature under conditions similar to those used for the prebinding receptor incubations. Duplicate aliquots were taken at 0, 0.5, 1, 3, and 5 h and after 18 h of incubation. Samples were frozen until Western blot analysis using CG and FSH dimer-specific mAbs. This experiment was performed three times.

RESULTS

Receptor Binding Experiments with Purified Recombinant FSH and hCG Standards—We tested the validity of the preadsorption protocol to fractionate a mixture of distinct gonadotropin activities using human recombinant FSH (rFSH) and urinary CG (CG) heterodimers. Accordingly, 300 mIU of human rFSH, CG, or a combination of both were preincubated with FSH receptor cells, rLH receptor cells, or CHO wild type cells under the conditions described above. The rFSH and CG inputs were previously determined to give, respectively, 60 and 40% displacement of bound ^125I tracer from their cognate receptor cells in standard binding assays. Both FSH and CG binding assays were performed on samples obtained after the two-step preadsorption incubation (Fig. 2). As discussed under

FIG. 1. Models of distinct populations of trimer single chains secreted by CHO cells and their predicted bioactivities. A, single bifunctional molecules; preadsorption to either specific receptor results in a parallel reduction of both CG and FSH activities. B, multiple bioactive species; one of both activities is selectively reduced following preadsorption to the specific receptor for that activity.
with wild type and LH-R cells. This difference reflects that essentially all of the CG was sequestered by LH-R cells, while the untransfected cells did not bind CG, reflected by the observed $^{125}\text{I}$ displacement. When FSH and CG standards were mixed, a comparable difference was obtained, indicating that the presence of hFSH did not interfere with the binding of the hCG to its receptor during the preadsorption step.

A reciprocal FSH receptor binding experiment was performed in which the above samples were incubated with FSH receptor (open bars) or wild type (closed bars) cells (Fig. 2B). The FSH standard recovered after binding with wild type cells displaced 60% of $^{125}\text{I}$-FSH in the test assay, but there was only a 20% displacement in bound $^{125}\text{I}$ following preincubation with FSH-R cells. This shows that the amount of available FSH after the initial incubation was reduced by 40% due to the sequestration of hormone by the FSH-R cells during preadsorption. Significantly, similar differences between receptor cells and control wild type cells were obtained when the FSH standard was mixed with the hCG standard. Finally, there was no detectable loss of CG activity, i.e., there was no binding of CG standard to the FSH receptors following the preadsorption step. Using both hCG and hFSH standards, these data indicate that the two-step preadsorption protocol represents a stable assay and a feasible approach to fractionate a potential mixture of FSH and CG bioactive species generated during synthesis of the triple-domain chimera.

**Preadesorption of the Chimera with Receptor-expressing Cells**—Having demonstrated that preadsorbing either purified hFSH or hCG to the FSH-R or rLH-R cells effectively alters the extent of binding of the cognate ligand recovered after preincubation, we examined the preadsorption of the triple-domain analog in the two-step assay. Previously we demonstrated that the FSH$\beta$-CG$\beta$-α chimera bound to the FSH and LH receptors with affinities comparable with the corresponding heterodimers (4). As discussed above, if the analog is a single bifunctional entity, loss of one activity should be paralleled by loss of the other. Alternatively, if two mutually exclusive biologically active populations are present, each hormonal activity should be independent from the other. Several concentrations of the chimera were preadsorbed in parallel to three sets of cells: LH-R, FSH-R, and CHO-wt (Fig. 3). Both CG and FSH binding affinities remaining in the supernatants were determined in the secondary binding assay using LH-R (A) and FSH-R cells (B). As expected, the secondary CG receptor binding assay showed much lower binding activity in the supernatant following preadsorption of the chimera to the LH-R cells compared with the supernatant following incubation with control CHO-wt cells (initial binding activity) (Fig. 3A). The supernatant obtained from the samples containing the two lower concentrations of analog tested (42 and 125 mIU/ml of FSH) lacked binding activity, i.e., no noticeable displacement of $^{125}\text{I}$ was observed. These data suggest that at these concentrations, nearly all the active CG activity initially present in the conditioned media was sequestered by the LH receptors during the preadsorption steps. Moreover, when identical concentrations of analog were preadsorbed to FSH-R cells, there was no difference in the CG binding activity compared with the supernatant following incubation with control CHO-wt cells (initial binding activity) (Fig. 3A). The supernatant obtained from the samples containing the two lower concentrations of analog tested (42 and 125 mIU/ml of FSH) lacked binding activity, i.e., no noticeable displacement of $^{125}\text{I}$ was observed. These data suggest that at these concentrations, nearly all the active CG activity initially present in the conditioned media was sequestered by the LH receptors during the preadsorption steps. Moreover, when identical concentrations of analog were preadsorbed to FSH-R cells, there was no difference in the CG binding activity compared with the supernatant following incubation with control CHO-wt cells. These data indicate quantitative binding of CG by the rLH-R during the preadsorption steps. The data also show that there was no coordinate loss of both CG and FSH activities, which implies the presence of at least two independent entities.

The same concentrations of the triple-domain analog used in Fig. 3A were preadsorbed in parallel to the three sets of cells as described above, but now the binding activities were determined in the secondary assay with FSH-R cells (Fig. 3B). Only

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**Fig. 2. Receptor binding experiments with purified recombinant hFSH and urinary hCG standards.** A, comparative CG receptor binding assay (CG-RBA) of the standards following preadsorption to control wild type CHO cells (open bars) and rLH-R cells (closed bars); B, comparative FSH receptor binding assay (FSH-RBA) of the standards following preadsorption to control wild type CHO cells (open bars) and FSH-R cells (closed bars). Results are shown as the mean ± S.E. of at least three experiments each performed with four duplicates.
the sample exposed to the FSH-R cells during preadsorption displayed lower binding compared with wild type cells. In this case, values of nearly 100% of the standard $^{125}$I-FSH bound in the secondary assay were obtained after preadsorbing to FSH-R cells for concentrations up to 175 mIU/ml of FSH, indicating no competing FSH activity in the post-adsorption supernatant. Moreover, the FSH-R binding activity in the supernatant determined after preadsorption to rLH-R cells is comparable with the supernatant obtained after preadsorption to CHO-wt cells. These results show that FSH binding activity is quantitatively retained by the FSH-R during the adsorption steps, and there was no apparent coupled decrease in CG activity.

Taken together, our data show that when one of the two hormonal binding activities of the chimera is eliminated by preadsorption to its receptors, the binding activity of the other component remains relatively unaffected. These results suggest that at least two distinct bioactive populations of the analogs are secreted in the conditioned medium, which exhibit either CG or FSH binding activity.

**Western Blot Analysis of Chimera**—Although the above receptor binding data demonstrate the presence of at least two distinct biologically active species, their configuration and structural stability are not clear. Do these two bioactive populations form heterodimer-like interactions or do they exist in an exclusively relaxed non-dimeric conformation? To address this question, we examined the immunoreactivity of the chimera on Western blots using dimer-specific monoclonal antibodies. That these mAbs were specific for heterodimers is shown in Fig. 4. The FSH-specific mAb detects only recombinant FSH dimers (A, lanes 1 and 2) but not the non-combined secreted FSHβ subunit (lane 3), whereas in the case of the CG dimer-specific mAb, only the CG heterodimer (B, lanes 1 and 2) and not the free subunit (lane 3) is immunoreactive. These antibodies also do not recognize the common α subunit (6)\(^2\). By contrast, an mAb that recognizes epitopes in the FSHβ subunit reacts with both the heterodimer (A, lanes 4 and 5) and free subunit (lane 6). CG polyclonal antiserum (B) detects both the heterodimer (lanes 4 and 5) and free subunit (lane 6). Thus, these mAbs should distinguish chimeric forms bearing FSHβ/CGβ heterodimeric contacts from those in which the β subunit is in a non-assembled configuration. If the chimera lacks α-β heterodimeric contacts, no signals should be observed with dimer-specific mAbs. It is clear that both FSH and CG dimer-specific mAbs detect the chimera (Fig. 5, A and B, lane 1) and their respective native heterodimers (Fig. 5, A and B, lane 2). As expected, no signals were detected when the corresponding uncombined FSHβ and CGβ subunits were probed with the dimer-specific mAbs (lane 3) even after prolonged exposure of the blot (data not shown). These results corroborate the above

\(^2\) V. Garcia-Campayo, A. Jablonka-Shariff, and I. Boime, unpublished observations.
findings that at least two bioactive species are generated during the synthesis of the triple-domain chimera, and they are configured with intramolecular heterodimer-like interactions.

That these mAbs react with the heterodimer-like forms of the triple-domain chimera provides an independent approach to verify our preadsorption model. Using the same preincubation protocol described for Fig. 3, 300 mIU/ml of chimera were incubated with CHO-wt cells, FSH-R cells, and LH-R cells. The quantity of chimera used here is based on the amount that gave sufficient displacement in the binding studies but at an adequate concentration to yield a signal that is quantifiable on the Western blots. Following the preadsorption to the cells, the supernatants were electrophoresed and the blots probed with the two mAbs. Each cell type was run in duplicate on the blot (Fig. 6). Immunodetection with FSH dimer-specific mAb (A) shows that the FSH dimer-like population of the trimer following preadsorption to rLH-R cells (lanes 3 and 4) remains constant relative to the detection obtained after preadsorption to control CHO-wt cells (lanes 1 and 2). However, the FSH dimer-like population is specifically reduced 3–4-fold when the chimera is preadsorbed to FSH-R cells (lanes 5 and 6). The reciprocal data were seen when the chimera was immunodetected with CG dimer specific mAb (B). The CG dimer-like population of the

![Fig. 4. Immunoreactivity of FSH (A) and CG (B) heterodimer-specific monoclonal antibodies with their recombinant and native hormones. For both A and B, lanes 2, 3, 5, and 6 contain proteins expressed from transfected CHO cells. Similarly, lanes 1 and 4 show purified recombinant FSH (Org) and purified urinary hCG (CR121). The immuno-probe designated as FSH in A corresponds to the FSH dimer-specific mAb. FSHβ probe recognizes epitopes in both the non-combined and heterodimeric forms of the FSHβ subunit. In B, immuno-probe CG corresponds to the CG dimer-specific mAb; probe CGβ polyclonal antiserum detects all forms of the CGβ subunit. Note in A, lane 6, that the FSHβ subunit resolves into two bands, which presumably reflects the presence of one and two asparagine-linked oligosaccharides. Some non-assembled CGβ subunits can be seen in the purified CG dimer preparation (presumably due to degradation) and recombinant CG preparations. The latter is the result of an excess synthesis of CGβ subunit compared with α subunit in the transfected clones.](https://www.jbc.org/Downloaded from)

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trimer decreases 3-fold following preadsorption to rLH-R cells (lanes 3 and 4) relative to the detection after preadsorption to either control CHO-wt cells (lanes 1 and 2) or FSH-R cells (lanes 5 and 6). The data show the presence of two distinct populations, each sequestered by a specific receptor, and thus support the receptor binding experiments that the chimera is comprised of at least two distinct biologically active populations.

One question that arises from these results is whether or not these dimer-like populations detected in the media are stable or if there are transient changes in the proportions of the secreted dimer-like FSH/CG conformers. To examine this issue, aliquots of conditioned medium containing the chimera were incubated with CHO cells at room temperature under conditions identical to those described above for the prebinding incubations (see “Experimental Procedures”). The samples were taken at various time points and probed with CG/FSH dimer-specific mAbs (Fig. 7). It is evident that throughout the time course, there were no marked changes in the signals up to 18 h of incubation. Moreover, there was no preferential shift to one species at the expense of the other. These data suggest that at least for the secreted forms, there is no marked interchange between CG and FSH complexes and the secreted forms are in a stable configuration.

DISCUSSION

Previously we demonstrated that single-chain chimeras of the glycoprotein hormone family bearing either two or three tandemly arranged β subunits genetically linked to a single α subunit displayed multiple bioactivities (4, 7). While it was evident that a single protein manifested these activities (two in the case of FSHβ-CGβ-α chimera or three for the TSH-FSHβ-CGβ-α analog), it was not clear if one form exhibited the multiple functions, or if each activity could be attributed to different populations of molecules, each possessing a single hormonal activity.

To address this question, the biological activities encoded in the FSHβ-CGβ-α chimera were fractionated by preincubating conditioned media containing the analog with CHO cells expressing either the FSH or LH receptor. Using two independent approaches, we show here that at least two functional
entities are generated during the synthesis of the chimera, each corresponding to either a FSH or LH/CG binding activity. These data represent the first indication that the single-chain glycoprotein hormone analogs are secreted as functionally distinct populations. Previously, we demonstrated that significant changes in the quaternary structure of the hormone dimers or single chains comprised of mutated α and β subunits did not impair the in vitro biological activity of such analogs (8–10). These observations were compatible with the idea that a population of only a single form would manifest dual activity, since presumably it would not configure into a complete heterodimeric structure. In such a model, a specific receptor orients the appropriate α/β domains into the respective biologically active species (11). However, based on this study, we would have expected a coordinate loss of both FSH and CG activities, since preincubation with either the FSH-R or LH-R protein would have sequestered the unique dually active single form. Our results show that the FSH and CG activities of the single-chain triple-domain chimera are independent and thus argue against synthesis of only a single bioactive class of molecules. Moreover, because these bulky single-chain variants, relative to the native heterodimer, are bioactive, this implies flexibility in the ligand-receptor interaction and that this conformational plasticity may account for their binding to their cognate receptors via the common α subunit.

The ability for two intrachain β subunits to interact with one α subunit and display two different biologically active conformations has important physiological implications. It is well established that both FSH and LH are made in the same cell, i.e. in the gonadotroph, and yet exhibit differences in the rates of assembly of their corresponding subunits in the endoplasmic reticulum (ER). It is, however, unclear how in the ER the β subunits sort out from each other and assemble with a common α subunit. Our data provide an explanation for this specificity. Once the α-β contacts are formed, the resultant species are stable, since the forms analyzed here are secretory products that accumulate in the media. This implies that there is little interchange between the native heterodimers during synthesis or between the heterodimer-like forms of the triple-domain chimera. This conclusion is supported by our data that there is no significant shift between the extracellular chimeric heterodimer-like forms of CG/FSH during prolonged incubation. Moreover, in previous studies (12) it was shown that when a single-chain double-domain construct (e.g. CGβ-α) is co-transfected with the gene encoding the FSHβ subunit, or when the gene encoding the FSHβ-α single chain is co-transfected with the CGβ subunit, analyses of the products by dimer-specific mAbs revealed no single complex contained both FSH and CG dimer-specific epitopes. This suggested that those intracellular
complexes did not exhibit simultaneous FSH and CG heterodimer-like conformations.

There have been several reports that the ER is comprised of a mosaic of specialized subdomains (13–16). These subcompartments have distinct functions and have a different distribution of resident proteins. Thus, one explanation for the formation of FSH/LH heterodimers in the gonadotrope is that each hormone is assembled at a distinct sub-ER compartment. Our in vitro experiments indicate that the α subunit of the chimera primarily interacts with only a single β subunit. This would explain why in vivo a three-subunit complex comprised of LHβ, FSHβ, and a single α subunit is not observed in the gonadotrope. The absence of stable triple-domain, dually active gonadotropins in vivo ensures the coordinate events of endocrine homeostasis by the circulating monofunctional heterodimers required for distinct physiological responses.

The model system described here may have wider applicability. Given the availability of cell lines expressing the numerous high-affinity receptors and their corresponding solubilized forms, design of experimental systems comparable with those described here can be performed for identifying other proteins that exhibit multiple activities via activation of different receptors.

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