Synthesis of Multi-Subunit Domain Gonadotropin Complexes: A Model for α/β Heterodimer Formation†

David Ben-Menahem, Ricia Hyde, Mary Pixley, Peter Berger,‡ and Irving Boime*

Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 Euclid Avenue, Campus Box 8103, St. Louis, Missouri 63110

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ABSTRACT: The human glycoprotein hormones chorionic gonadotropin (CG), thyrotropin (TSH), lutropin (LH), and follitropin (FSH) are heterodimers, composed of a common α subunit assembled to a hormone-specific β subunit. The subunits combine noncovalently early in the secretory pathway and exist as heterodimers, but not as multimers. Little information is available regarding the steps associated with the assembly reaction. It is unclear if the initial αβ engagment results either in the formation of only mature heterodimer or if the nascent complex is reversible and can undergo an exchange of subunits or combine transiently with an additional subunit. This is relevant for the case of LH and FSH, because both are synthesized in the same cell (i.e., pituitary gonadotrophs) and several of the α subunit sequences required for association with either the LHβ or FSHβ subunits are different. Such features could favor the generation of short-lived, multi-subunit forms prior to completion of assembly. Previously, we showed that the CGβ or FSHβ subunit genes can be genetically fused to the α gene to produce biologically active single chains, CGβα and Fβα, respectively. Studies using monoclonal antibodies sensitive to the conformation of the hCG subunits suggested that in contrast to the highly compact heterodimer, the interactions between the β and α domains in the single chain are in a more relaxed configuration. That the tethered domains do not interact tightly predicts that they could combine with an additional subunit to form triple domain complexes. We tested this point by cotransfecting CHO cells with the genes encoding Fβα and the CGβ subunit or the CGβα and FSHβ monomer. The CGβ subunit combined noncovalently with Fβα to form a Fβα /CGβ complex. Ternary complex formation was not restricted to a specific set of single chain/monomeric subunit, because a CGβα/FSHβ complex was also detected implying that triple domain intermediates could be transiently generated along the secretory pathway. Monoclonal antibodies specific for the CG heterodimer recognized the Fβα/CGβ complex, which suggests that the epitopes unique for dimeric CG were established. In addition, media containing Fβα/CGβ displayed high-affinity binding to both CG and FSH receptors. The presence of CG activity is presumptive for the existence of a functional Fβα /CGβ complex, because neither Fβα nor the uncombined CGβ subunit binds to CG receptor. These data show that the α subunit of the tether, although covalently linked to the FSHβ domain, can functionally interact with a different β subunit implying that the contacts in the nascent αβ dimer are reversible. The formation of a functional single chain/subunit complex was not restricted to the FSH single chain/CGβ subunit since CG single chain interacts with the monomeric FSHβ subunit and exhibits FSH activity. The presence of the triple domain configuration does not abolish bioactivity, suggesting that although the gonadotropins are heterodimers, the cognate receptor is capable of recognizing a larger ligand composed of three subunit domains.

A key feature of the glycoprotein hormone family, which consists of chorionic gonadotropin (CG)[1] follitropin (FSH), lutropin (LH), and thyrotropin (TSH), is their heterodimeric structure. The hormones are comprised of a common α subunit bounded noncovalently to a variable β subunit that confers the receptor binding specificity for each dimeric ligand (*1*). The subunits have been detected in vivo only as monomers or bioactive heterodimers, and although the α subunit is common to the four hormones, it appears that the contact sites in the subunit for FSHβ and CGβ subunits are not the same (*2*, *3*). The formation of an assembly competent subunit, at least in the case of the CGβ subunit, occurs through multiple intermediates (*4*, *5*). However, once this conformation is established, the steps involved in the combination of monomeric subunits to yield the heterodimer are not clear. For example, both LH and FSH are synthesized in the same cell, but it is not known what determines how a newly synthesized α subunit targets a particular β subunit that establishes the physiological ratio of LH/FSH in the reproductive cycle. There could be a sub-compartmentalization of the nascent ER pools in which there is spatial

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‡ Institute for Biomedical Aging Research, Austrian Academy of Sciences, A-6020 Innsbruck, Austria.

* Corresponding author. Telephone: 314-362-2556. Fax: 314-361-3560. E-mail: iboime@pcg.wustl.edu.

[1] Abbreviations: CG, human chorionic gonadotropin; LH, lutropin; FSH, follitropin; CGβα, single-chain hCG.; Fβα, unmodified tethered FSH.; FβCα, single chain FSH with CTP as a linker; CTP, carboxy terminal peptide of CGβ; CHO, Chinese hamster ovary; nrs, normal rabbit serum; mAb, monoclonal antibody.

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segregation of the LHβ and FSHβ subunits, each paired with an α subunit. Alternatively, the α subunit may co-mingle with a mixed population of β subunits, raising the question of whether the α subunit can interact intracellularly with more than one β subunit before reaching steady-state. Intermediates composed of subunits in a stoichiometric combination that differ from the mature glycoprotein were observed during the biosynthesis of the nicotinic acetylcholine receptor. While the mature pentamer is composed of $\alpha 2\beta\gamma\delta$, intermediates in various combinations (i.e., $\alpha\gamma\alpha\gamma$ or $\alpha 2\beta\delta 2$) were detected generated during biosynthesis of the ion channel (*6*, *7*).

For the gonadotropin case, we suspected that multi-subunit interactions are possible, because: (a) mutagenesis studies discussed above suggested that some of the contact sites between the common α and unique β subunits are not identical (*2*, *3*); (b) The crystal structure of hCG revealed that the molecule is elongated rather than globular, with minimal hydrophobic core and a large interface between the subunits (*8*, *9*). However, despite the extensive interface, it was concluded that the affinity between the subunits is relatively moderate (*9*). Taken together, these observations suggest that in the initial assembly phase, the contacts between the two subunits are reversible. This implies that an incoming β subunit can disrupt the $\alpha\beta$ interactions in the nascent dimer and supplant the original β monomer, or that more than two subunit domains can coexist in a single molecule before secretion of mature heterodimer. To test these predictions, we used a tethered subunit gonadotropin where only one β subunit is genetically fused to the α subunit (*10*−*14*). Because the integrity of the carboxy terminal of the α subunit is critical for maximal bioactivity, we and others linked the carboxy end of the β subunit to the amino terminus of the α subunit domain (*10*−*14*). These variants exhibit biological activity comparable to the corresponding heterodimers (*10*, *13*). Data obtained from analyses using a panel of monoclonal antibodies (mAbs) that are specific for either the heterodimer or for epitopes present only in the free subunit, suggest that single chain hCG is in a more open configuration than the corresponding wild-type dimer (*15*). Because the tethered gonadotropin displays characteristics of both a heterodimer and a monomeric subunit, we considered it as an incompletely assembled hormone. This implies it could complex with an additional monomeric subunit, because there would be less steric interference at the α/β contact sites compared to that seen in the heterodimer, which would exclude interactions between more than two mature subunits. To test this hypothesis, we coexpressed the gene encoding either CG or FSH single chain with a monomeric subunit. LHβ and CGβ share 85% identity in their first 110 amino acid residues (*1*), and both LH and CG bind to the identical receptor and elicit the same biological response. Because of the availability of crystal structure information for the CGβ subunit, and of numerous conformational sensitive mAbs against hCG, we used the CGβ subunit rather than LHβ subunit to study the assembly reaction with the α subunit domain in the single chain. The data show that the monomeric CGβ subunit combines noncovalently and intracellularly with the fused α domain in the tethered FSH resulting in a biologically active complex. Similarly, the FSHβ monomeric subunit combines with CG$\beta\alpha$ to form a bioactive CG$\beta\alpha$/FSHβ complex. That a large ligand composed of three subunit domains is bioactive in vitro supports the hypothesis that different conformers of a glycoprotein hormone can productively interact with the receptor (*16*, *17*). In addition, the data are consistent with the hypothesis that the initial $\alpha\beta$ interactions in the ER are transient.

Gonadotropin subunits and single chain variants

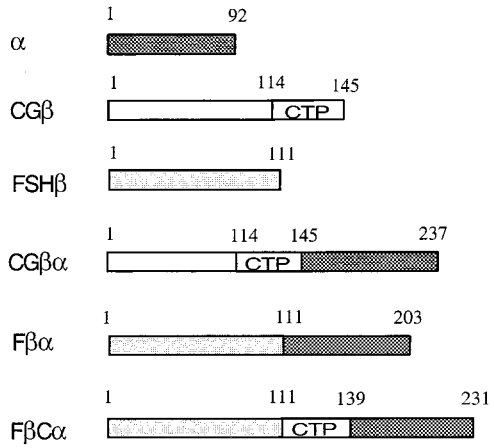

FIGURE 1: Protein structure of subunits and tethered gonadotropins. The α, CGβ, and FSHβ subunits are represented by stippled, white, and gray boxes, respectively. The number of the amino acid residues defines the amino and carboxyl termini and the CTP sequence. For the single chains, the carboxy terminus of the β subunit is genetically fused to the amino end of the common α subunit and the residues of the two subunits are numbered continuously. The carboxyl terminal peptide of CGβ (CTP; amino acid residues 115−145 of CGβ) is preserved in the single chain hCG (CG$\beta\alpha$). The two FSH single chain variants differ by the absence (F$\beta\alpha$) or presence (FβCα) of the CTP spacer between the tethered α and FSHβ subunit domains.

EXPERIMENTAL PROCEDURES

DNA Transfection and Cell Culture. Engineering of the genes encoding single chain hCG (CG$\beta\alpha$) and the tethered FSH without (F$\beta\alpha$) or with a 28 amino acid linker (FβCα) derived from the carboxy terminal peptide (CTP) of the CGβ subunit were described previously (*10*, *13*, *18*). Figure 1 illustrates the different subunits and single chain variants presented here. All variants were inserted into the mammalian expression vector pM[2] (*10*, *13*) and were transfected into CHO cells by calcium phosphate. Stable clones were selected approximately 11 days after transfection by using the neomycin analogue G418 (250 μg/mL). The clones were maintained in Ham's F-12 medium [supplemented with penicillin (100 units/mL), streptomycin (100μg/mL), and 2 mM glutamine] containing 5% fetal bovine serum and G418 (125 μg/mL) at 37 ^{o} C in a humidified atmosphere of 5% CO_2/95% air, as previously described (*18*).

Metabolic Labeling. Cells were labeled overnight in F-12 based medium, containing dialyzed calf serum containing 25 μCi/mL [^{35}S] cysteine or mixture of [^{35}S] cysteine and methionine (Pro-mix) as described (*16*−*18*). Aliquots of cell lysate and medium were immunoprecipitated with polyclonal antisera directed against either the common α or the CGβ subunit. The reduced proteins were resolved on 12.5% SDS-polyacrylamide gels.

Western Blot Analysis. Media samples were resolved on 12.5% SDS-polyacrylamide gels under nonreduced conditions and blotted onto nitrocellulose. The blots were probed with antisera or mAbs, as described in the figure legends. Purified hCG (CR 127) and recombinant (r) FSH were obtained from the NIH and Organon (Oss, The Netherlands), respectively. The antisera to the α or CGβ subunit are raised in the lab. The hCG conformational sensitive mAbs A407 and B109 that recognize primarily the heterodimer, but not the monomeric subunits, were kindly provided by Dr. Steven Birken (Columbia University Medical School, New York). The mAb 60H-2 (designated as mAb 60), which recognize all forms of FSHβ, was obtained from Organon (Oss, The Netherlands). The mAb INN-hFSH-117 (designated as 117) has a 100-fold greater affinity for dimeric FSH than to the monomeric FSHβ subunit (*19*, *20*). The blots were visualized with the Western Light detection system (Tropix) following the manufacturer's protocol.

Radio-Receptor Assay. Conditioned media were concentrated using either a Centricon concentrator (Amicon) or an ultra-free concentrator (Millipore). Subsequently, the samples were washed in PBS and quantitated using double polyclonal based RIA (Diagnostic Products Inc.), which includes antiserum that recognizes either the CGβ or FSHβ subunit. Heterodimeric-like epitopes were determined in a solid-phase immunoradiometric assay, using an IRMA Coat a Count kit (Diagnostic Products, Inc.), which includes monoclonal antibodies directed against epitopes in both the α and β subunits of the heterodimer. The cross reactivity of the Fβα single chain and or the CGβ monomeric subunit in the hCG IRMA was less than 3%. Likewise, no significant immunoreactivity was seen for the single chain hCG (CGβα) in either the FSH RIA or FSH IRMA.

Receptor binding and cAMP production were determined using transfected CHO or human fetal kidney 293 cell lines, expressing either the human LH or FSH receptor (*21*, *22*). In some experiments, cAMP accumulation was determined using the NEN Flashplate (Boston, MA) assay as per manufacturer's instructions. Briefly, 5×10^4 CHO cells, stably transfected, were incubated for 2 h at room temperature with ligands and ^{125}I cAMP added and incubated for 17 h at room temperature. The Flashplate was then read in Packard top counter. Total binding was 15% and nonspecific binding (in the presence of 5 μg hCG for LH/CG receptor binding assays or 5 IU of rFSH in FSH receptor binding experiments) was 1.5% of total counts (≈30 000 cpm or ≈100 000 cpm for LH/CG and FSH receptor binding assays, respectively).

RESULTS

Assembly of Single Chain Gonadotropin with Glycoprotein Hormone Subunit. Mutagenesis and structural analyses of hCG suggest the potential for transient interactions between the α and β subunits prior to secretion of the heterodimer. Because the subunit domains in the single chain gonadotropin display features of both assembled (heterodimer-like) and uncombined (free) subunits, we examined if the tethered α domain in the single chain FSH can combine with a monomeric CGβ subunit in transfected CHO cells. This combination was selected for two reasons: (a) site-directed mutagenesis of the α subunit revealed different contact sites for assembly depending on the gonadotropin β subunits (*2*, *3*) and (b) because free subunits do not bind to receptor (*1*), any observed CG biologic activity would be presumptive for the formation of a productive single chain/β-subunit complex.

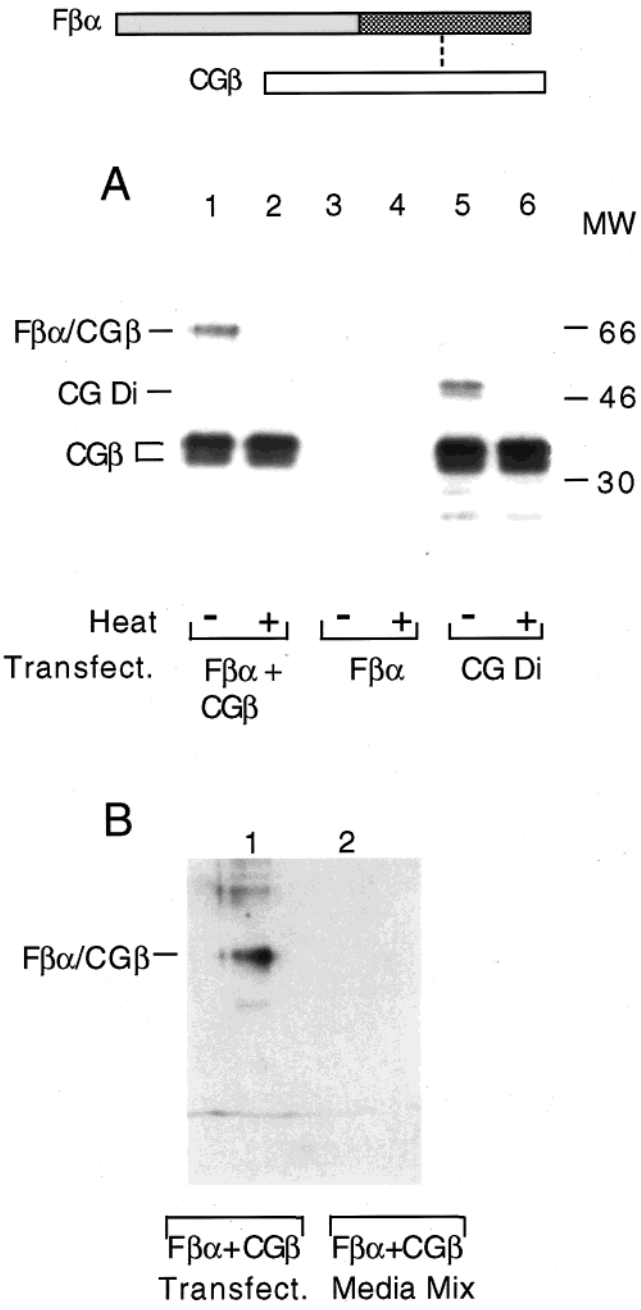

FIGURE 2: Panel A. Heat lability of the secreted Fβα/CGβ complex. Media derived from cells transfected with Fβα and CGβ (lanes 1 and 2), Fβα alone (lanes 3 and 4) or CG dimer (cotransfected α and CGβ subunits; lanes 5 and 6) were analyzed without (lanes 1, 3, and 5) or with heat (95 °C, 3 min) (lanes 2, 4, and 6) on nonreduced SDS-PAGE. The proteins were transferred to a nitrocellulose and probed with polyclonal CGβ antiserum (1:5000 dilution) and detected as described in Experimental Procedures. Molecular weight (MW) markers (Rainbow−Amersham) are shown. Panel B. Western blot analysis of Fβα/CGβ complex with hCG conformational sensitive mAb. Media from cells coexpressing Fβα and the CGβ subunit (lane 1), or Fβα or CGβ alone (lane 2) were electrophoresed and blotted. The membrane was probed with the hCG dimer−specific mAb B 109 (1:1000 dilition). In lane 2, media derived from cells expressing individually Fβα and CGβ were mixed (equal amounts as visualized with α or CGβ polyclonal antisera) and incubated for 16 h at 37 °C.

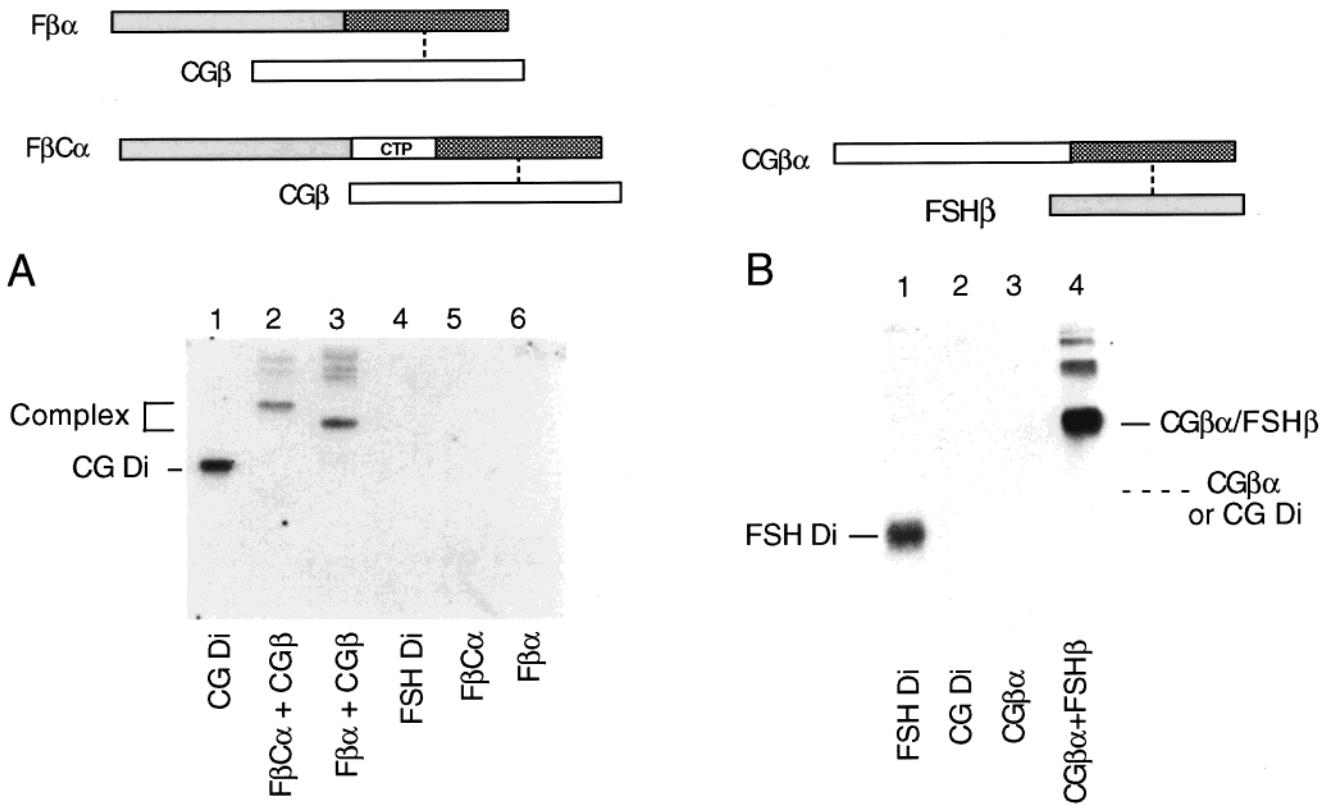

FIGURE 3: Panel A. Interaction of CGβ subunit with single chain FSH variants with (FβCα) or without linker (F$\beta\alpha$) between the tethered domains. The following samples were electrophoresed under nonreduced conditions: media from cells expressing the CGβ subunit cotransfected with either FβCα (lane 2) or F$\beta\alpha$ (lane 3); media containing only FβCα (lane 5) or Fα (lane 6); purified FSH dimer (Di; lane 4) and CG dimer (Di; CR127; lane 1). The blot was probed with B 109 (1:1000 dilution). Panel B. Analysis of CG$\beta\alpha$/FSHβ complex with a conformational sensitive FSH mAB. Western blot analyses of media samples of all, expressing FSH Di (lane 1), CG Di (lane 2), CG$\beta\alpha$ alone (lane 3), or coexpressed with FSHβ subunit (lane 4). The membrane was probed with mAb 117 (1:20 000 dilution) that recognizes FSH dimeric epitope. The dashed line indicates the approximate position of CG Di and CG$\beta\alpha$ on the membrane.

Media derived from cells coexpressing F$\beta\alpha$ and excess a CGβ subunit were Western blotted under nonreduced conditions and probed with CGβ antiserum. A 62K band distinct from F$\beta\alpha$ and CGβ was observed (Figure 2, panel A, lane 1; F$\beta\alpha$/CGβ); CGβ migrates as a doublet, reflecting the addition of one or two carbohydrate chains (*23*). No signal was seen when medium isolated from cells expressing only F$\beta\alpha$ was analyzed (lane 3). That a noncovalent association between the CGβ subunit and the α domain occurs in the single chain is shown by the disappearance of the complex (lane 1) and the heterodimer (lane 5) when the media are boiled without 2-mercaptoethanol for three minutes (lanes 2 and 6). To estimate the efficiency of F$\beta\alpha$/CGβ complex formation, we compared the amount of CGβ monomer complexed with the F$\beta\alpha$ single chain to the total CGβ pool (i.e., an uncombined as well as assembled subunit as determined by RIA) in the sample. Of the total CGβ (92.8 $\pm$ 17.1 IU/mL), 63% (58.6 $\pm$ 18.6 IU/mL) was configured in heterodimeric association with the F$\beta\alpha$/CGβ complex as determined by the IRMA (see Experimental Procedures). Taken together, these data imply that the noncovalent interaction between the CGβ subunit and the α domain in F$\beta\alpha$ resembles the α/β combination of the hCG heterodimer.

Further evidence for a heterodimer-like interaction between the CGβ subunit and F$\beta\alpha$ is the immunoreactivity of the complex to hCG dimer-specific monoclonal antibodies B109 and A407 (*24*−*27*). Both antibodies reacted with the F$\beta\alpha$/CGβ complex (Figure 2, panel B, lane 1) (only the data with B109 is shown). Unlike that seen with the polyclonal antiserum, B109 detects immuno reactive high molecular weight forms. Aggregates of the single chains were observed and while their composition is unclear such aggregation has been observed previously for native hCG (*28*, *29*) and a variety of single chain mutants (*16*, *17*). In addition, previous studies demonstrated no correlation between the presence of aggregates and biological activity in a series of tethered gonadotropin mutants (*16*, *17*). The formation of the F$\beta\alpha$/CGβ complexes occurred intracellularly rather than fortuitous interaction between free subunit and single chain in the medium, because no detectable complex was observed when equal amounts of conditioned media from cells expressing only the CGβ subunit and F$\beta\alpha$ were mixed (panel B; compare lane 2 with lane 1).

F$\beta\alpha$ lacks a linker between the β and α domains, but its biologic activity is comparable to the spacer-containing tethered FSH (FβCα; ref *13*) despite the different configuration [C denotes the linker that is a stretch of 28 amino acids derived from the carboxyl terminus of CGβ (*1*, *30*)]. To assess if the presence of the linker significantly affects the formation of the triple domain complex, FβCα was cotransfected with CGβ. A complex (FβCα/CGβ), distinguishable from the heterodimeric hCG, was observed on western blots probed with mAb B109 (Figure 3, panel A; lanes 1 and 2). The FβCα/CGβ (lane 2) migrates slower than F$\beta\alpha$/CGβ (lane 3) due to the presence of the linker in the single chain component of the former. As expected, B109 did not detect FSH heterodimer (lane 4) nor the single chain

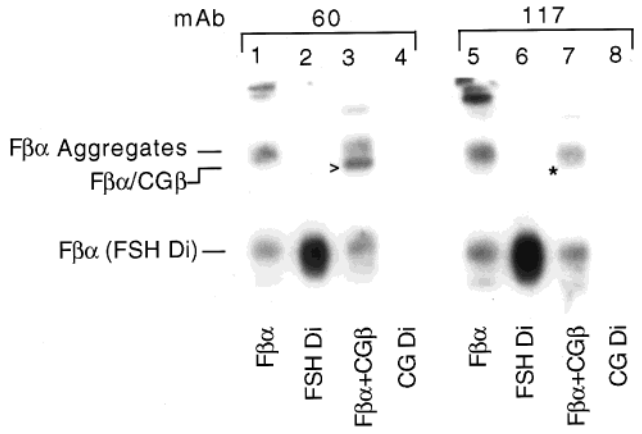

FIGURE 4: Analysis of F$\beta\alpha$/CGβ complex with a dimer specific FSH mAb. Media samples of cells expressing F$\beta\alpha$ alone (lanes 1 and 5) or cotransfected CGβ subunit, (lanes 3 and 7), and purified FSH dimer (Di; lanes 2 and 6) and CG dimer (Di; CR127) (lanes 4 and 8) were blotted and probed with mAb 60 (1:1000 dilution) (lanes 1−4) that recognize all forms of FSHβ or with FSH dimer specific mAb 117 (1:20 000 dilution) (lanes 5−8). The arrowhead in lane 3 identifies the F$\beta\alpha$/CGβ complex not seen in lane 1. The asterisk in lane 7 shows the position where the complex would migrate if present.

variants FβCα and F$\beta\alpha$ (lanes 5 and 6, respectively). All FSH variants scored positive when probed with mAb 60 which recognizes all forms of FSHβ (not shown), which indicates the presence of sufficient protein in each lane. The data show that heterodimeric-like complexes between a monomeric CGβ and tethered FSH with or without a linker are formed.

That the tethered α domain/monomeric β subunit complex is not restricted to a particular β subunit was examined by the ability of the CG single chain (CG$\beta\alpha$) to interact with the monomeric FSHβ subunit. Using a heterodimeric FSH IRMA assay, 52% of the total monomeric FSHβ subunit was detected in association with CG$\beta\alpha$ to yield a CG$\beta\alpha$/FSHβ complex (10.8 ± 3.6 IU/mL vs 20.6 ± 3.4 IU/mL measured by IRMA and RIA ($n = 3$), respectively). The CG$\beta\alpha$/FSHβ complex was observed by Western blot analysis (Figure 3, panel B). FSH dimer specific mAb (designated 117) recognizes both heterodimeric FSH (lane 1) and the CG$\beta\alpha$/FSHβ complex (lane 4). Neither the CG heterodimer nor tethered CG were detected by the FSH mAb (lanes 2 and 3). The data show that the formation of a subunit/single chain complex is not restricted to a unique β subunit in the tether or the monomeric component.

Conformational Epitopes of F$\beta\alpha$/CGβ Complex. A question that arises form these observations is whether the α subunit in the F$\beta\alpha$/CGβ complexes with two β subunits simultaneously or if interaction with one β monomer precludes a heterodimeric-like configuration with an additional β domain. To address this issue, we examined if heterodimeric-like FSH epitopes coexisted with those generated by the tether-CGβ interaction (Figure 3, panel A) in the F$\beta\alpha$/CGβ complex. Media derived from cells expressing both F$\beta\alpha$ and CGβ was probed with a mAb (#60) that reacts with all forms of the FSHβ subunit or with heterodimeric-specific FSH mAb (#117) (Figure 4). The mAb 60 reacts with F$\beta\alpha$ (lane 1), FSH dimer (lane 2), and the complex (lane 3, arrowhead), implying that the FSHβ domain in both F$\beta\alpha$ and F$\beta\alpha$/CGβ is not denatured. High molecular bands (apparent MW > 67K), as discussed above, were also observed when F$\beta\alpha$ was expressed alone or together with CGβ (lanes 1 and 3, respectively). As expected, no signal was seen with CG dimer (lane 4). When a blot derived from the same gel was probed with dimer specific mAb 117 (lanes 5−8), signals corresponding to F$\beta\alpha$ (lane 5) and FSH heterodimer (lane 6) were observed. However, mAb 117 did not recognize the F$\beta\alpha$/CGβ complex (compare lane 7—asterik and lane 3—arrowhead), suggesting that while in the F$\beta\alpha$/CGβ complex heterodimeric CG epitopes are established (Figure 3, panel A), the corresponding FSH dimer epitope is not seen. The potential to establish a follitropic epitope is present and uncombined F$\beta\alpha$ coexpressed in the cells is recognized by mAb 117. In a complementary set of experiments, a complex was detected when CG$\beta\alpha$ single chain and a FSHβ subunit were coexpressed. A dimeric FSH epitope was formed in the complex (Figure 3, panel B lane 4) but a panel of conformational sensitive mAbs, which recognize dimeric hCG, were unable to recognize this structure (not shown). The results further demonstrate that a complex is not recognized by both CG and FSH dimer specific mAbs, suggesting that at least for the epitopes tested it does not exhibit, simultaneously, FSH and CG heterodimeric-like conformations. The results imply that an incoming β subunit competes with the β subunit domain in the single chain to eventually establish a single, rather than dual, heterodimeric interface with the tethered α subunit domain. Taken together, the data support the hypothesis that prior the formation of mature hormone the interactions between the two subunits in the nascent heterodimer are reversible.

In Vitro Bioactivity of the Subunit/Tether Complexes. As an additional independent marker for the presence of heterodimeric-like epitopes in the F$\beta\alpha$/CGβ complex, we examined if it acquired receptor binding determinants. Condition media from CHO cells, expressing both FSH single chain (F$\beta\alpha$) and the CGβ subunit, were examined using transfected cells expressing either the LH/CG or FSH receptor. LH/CG receptor binding activity could only result from the formation of CGβ subunit/tether molecule, because neither uncombined CGβ monomer nor F$\beta\alpha$ single chain alone exhibits LH/CG receptor binding. As shown in panel A of Figure 5, the binding affinities of the F$\beta\alpha$/CGβ triple domain complex and heterodimeric hCG were similar. Because the medium also contains free CGβ subunit and uncombined FSH single chain, the RIA determination captured the F$\beta\alpha$/CGβ and free CGβ. Neither the monomeric CGβ subunit nor the FSH single chain bind significantly to the LH receptor (data not shown and Figure 4 in ref *16*). Thus, the activity observed is presumptive for the formation of a productive interaction between the monomeric CGβ subunit and the α domain in the single chain to establish receptor binding determinants unique to the complex. Adenylate cyclase was activated and cAMP production paralleled the binding (Figure 5, panel B). Although FSH bioactivity was also observed with cells expressing the FSH receptor (data not shown), it is difficult to assess if the complex has FSH activity, because it is virtually impossible to distinguish its follitropic activity from activity manifested by the uncomplexed single chain Fα in the sample. However, the LH/CG activity could only have been generated from the complex F$\beta\alpha$/CGβ. Thus, the appearance of high-affinity-

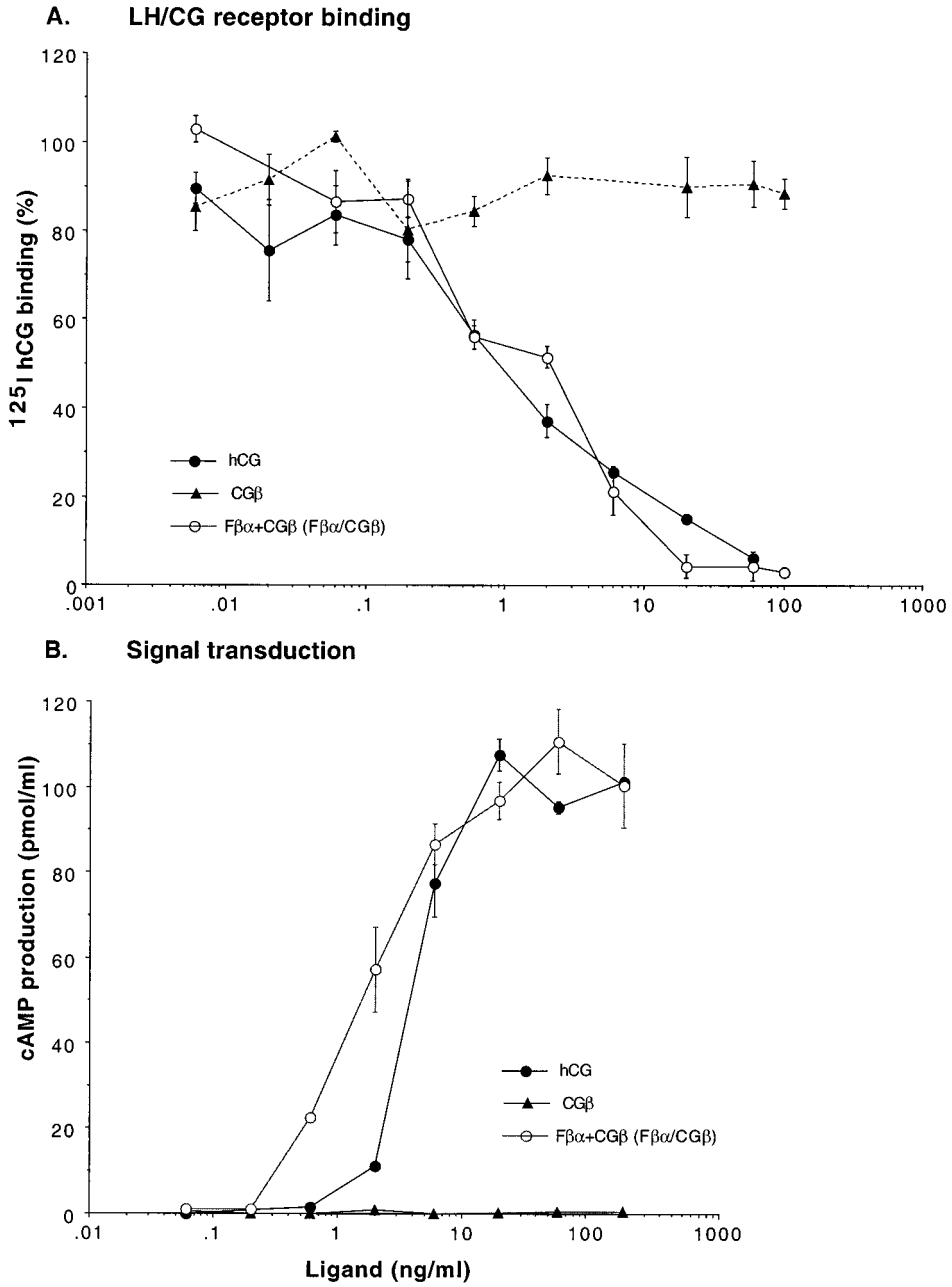

FIGURE 5: LH activity of media containing F$\beta\alpha$/CGβ complex. Binding to human LH/CG receptor (panel A) was measured in human embryonic kidney 293 cells expressing the recombinant receptor. Displacement curves of ^{125}I hCG at each volume of unlabeled ligand is expressed as the percentage of maximal binding of the tracer (see Methods). Heterodimeric hCG was used as standard. Signal trnasduction of the ligands was assayed in 293 cells expressing the receptor and cAMP production was determined by RIA (panel B). Heterodimeric hCG was used as standard. The data are presented as the mean ± S.E. of a representative experiment from three experiments with similar results.

CG/LH bioactivity is presumptive evidence that the α subunit in the F$\beta\alpha$ single chain forms a biologically active complex with the CGβ subunit.

Gain of function in the ternary complex based on the incoming subunit was also detected when the pituitary derived FSHβ subunit combined with tethered CG. Condition media from CHO cells coexpressing the CG$\beta\alpha$ single chain and FSHβ monomer was examined for receptor binding/signal transduction using transfected CHO cells expressing FSH or LH/CG human receptor. High-affinity binding to the FSH receptor (Figure 6, panel A) and cAMP accumulation (Figure 6, panel B) similar to the activity of recombinant FSH were observed. The follitropic activity is generated by CG$\beta\alpha$/FSHβ complex, because both the tethered hCG and the FSHβ monomeric subunit do not significantly bind to the FSH receptor. The sample also displayed lutropic acitvity (data not shown). As discussed above, it is difficult to link the LH/CG activity to the CG$\beta\alpha$/FSHβ complex, because of the presence of uncombined bioactive tethered hCG in the sample. The results suggest that various gonadotropin complexes can bind to and activate the receptor, implying that despite the evolutionary pairing of heterodimeric ligand/receptor (*31*), a triple domain gonadotropin is functional.

DISCUSSION

That the complexes generated between a monomeric β subunit and the single chain resembles the subunit association

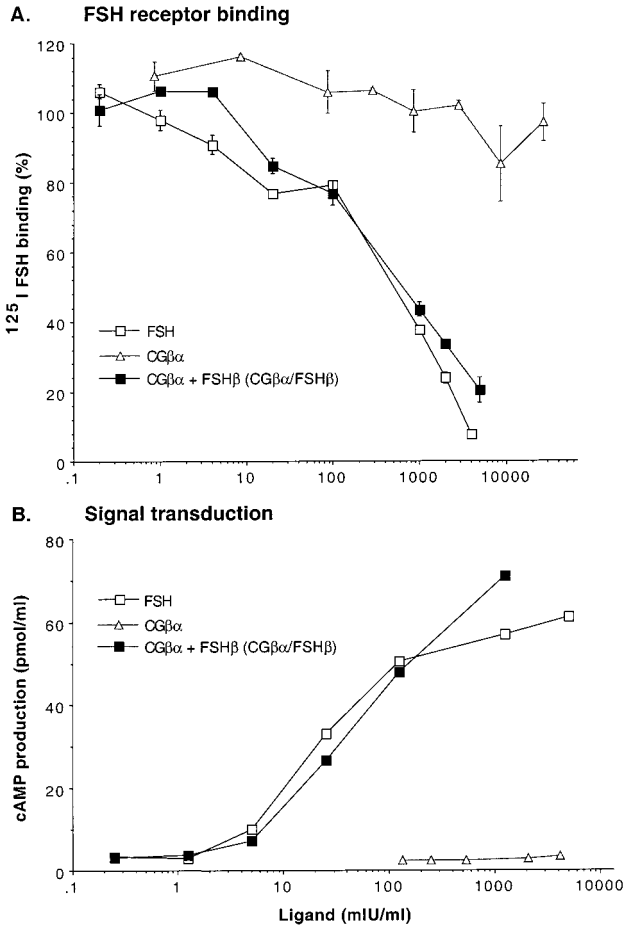

FIGURE 6: FSH activity of media containing CG$\beta\alpha$/FSHβ complex. Binding to human FSH receptor (panel A) was measured in CHO cells stably expressing the recombinant receptor (see Experimental Procedures). Signal transduction of the ligands was assayed by measuring cAMP production (panel B). Heterodimeric FSH was used as standard. The data are presented as the mean ± S. E. of a representative experiment from two experiments with similar results.

seen in the native heterodimer is suggested by the following: (a) the interaction is noncovalent; (b) it occurs intracellularly rather than generated by nonspecific interactions after secretion of the individual components; (c) it can be detected by dimer-specific monoclonal antibodies; (d) the complex binds to the receptor corresponding to the β subunit that combines with the single chain. Given the increased size of the triple domain structure, it was surprising that the complexes were biologically active. Although determinants from both α and β subunits are critical for bioactivity, our previous studies suggested that ligands with apparently different conformations can bind to the receptor (*13*, *16*, *17*). The high-affinity binding of the triple domain complex supports this hypothesis. The extracellular domain of the gonadotropin receptors is very large as compared to other serpentine receptors, but the significance of this unique feature is unknown. Several models were developed for the gonadotropin/receptor interaction, and they predict precise alignment of the heterodimeric ligand and receptor. Because the receptor productively interacts with a three subunit domain gonadotropin, this suggests it can tolerate a much larger ligand. It is not clear if the size of the receptor "pocket" is sufficient to accommodate the triple domain gonadotropin or the ligand for the most part is outside the binding site. Together with our previous results describing the bioactivity of various disulfide bond hCG mutants (*16*, *17*), we suggest that flexibility exists in both the ligand and receptor to establish a functional unit.

The assembly of the glycoprotein hormone subunits occurs in the endoplasmic reticulum (*32*, *33*). In the pituitary, LH and FSH are synthesized in the same cell and are packaged in secretory granules after exiting the ER. CHO cells lack a regulated secretory pathway, i.e., they secrete proteins constitutively. Although we cannot exclude that gonadotrope-specific sorting and packaging may stablize the native α/β interactions, the efficiency of assembly of the common α subunit with LHβ, CGβ, or FSHβ subunits is the same in CHO cells and cell lines containing a regulated pathway (*34*, *35*). Moreover, the ability to form a monomeric tether complexes is not restricted to CGβ subunit and the LHβ subunit combines with F$\beta\alpha$ to form the F$\beta\alpha$/LHβ complex (data not shown).

At least two models can explain the interaction of the three domains in the complex (e.g., F$\beta\alpha$/CGβ, CG$\beta\alpha$/FSHβ, or F$\beta\alpha$/LHβ. (A) The α subunit in the single chain can form a native heterodimeric interface with a single β monomer. In this model, an additional β subunit could displace or prevent the interaction between the tethered α and β domain. (B) The α subunit can share itself simultaneously with both β subunits through different contact sites. This predicts that such individual molecules will display dual activity rather than only single phenotype as expected from model A. The absence of both CG and FSH dimer specific epitopes (see Figure 4) in either the F$\beta\alpha$/ CGβ or CG$\beta\alpha$/ FSHβ complexes supports the suggestion that the interactions between each β subunit and the α domain in the single chain are reversible and are mutually exclusive (model A). This implies that although the tethered α domain is covalently linked to the FSHβ domain, F$\beta\alpha$ is not committed to establish only a follitropic phenotype (i.e., FSH dimer specific epitopes and bioactivity), but that it can form a heterodimeric-like interface with the incoming CGβ subunit, resulting in lutropic activity. However, we cannot exclude that binding of the CGβ subunit masks, but does not abolish FSH conformational sensitive epitopes. In such a case, the α subunit could still maintain a functional dual relationship with the two β domains.

That an incoming subunit can combine with a single chain gonadotropin is consistent with the modest affinity between the subunits of the heterodimer as suggested by Wu et al. (*9*). We propose that the nascent heterodimer can dissociate and the released monomeric subunits undergo another subsequent assembly cycle. Alternatively, an incoming β subunit may engage the α subunit in the partially assembled heterodimer, resulting in a tri-subunit intermediate (e.g., α/CGβ/α, CGβ/α/CGβ, or LHβ/α/FSHβ) prior to the formation of mature hormone. This proposed model is especially relevant to FSH/LH, because both are synthesized in the pituitary gonadotropes and several sequences in the common α subunit required for association with the different β subunits are not the same (*2*, *3*). The absence of stable trimeric, dually active gonadotropins ensure the coordinate events of endocrine homeostasis by the circulating monofunctional heterodimers. This implies that based on the physiological demand complexed subunits could dissociate rapidly in the ER to bias gonadotropin secretion either toward LH or FSH during the reproductive cycle.

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REFERENCES

1. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem. 50*, 465−495.
2. Furuhashi, M., Suzuki, S., and Suganuma, N. (1996) *Endocrinology 137*, 4196−4200.
3. Grossmann, M., Szkudlinski, M. W., Dias, J. A., Xia, H., Wong, R., Puett, D., and Weintraub, B. D. (1996) *Mol. Endocrinol. 10*, 769−779.
4. Huth, J. R., Mountjoy, K., Perini, F., and Ruddon, R. W. (1992) *J. Biol. Chem. 267*, 8870−8879.
5. Bedows, E., Huth, J. R., Suganuma, N., Bartels, C. F., Boime, I., and Ruddon, R. W. (1993) *J. Biol. Chem. 268*, 11655−11662.
6. Sine, S. M., and Claudio, T. (1991) *J. Biol. Chem. 266*, 19369−19377.
7. Kreienkamp, H.-J., Maeda, R. K., Sine, S. M., and Taylor, P. (1995) *Neuron 14*, 635−644.
8. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature 369*, 455−461.
9. Wu, H., Lustbader, A. J., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) *Structure 2*, 545−558.
10. Sugahara, T., Pixley, M. R., Minami, S., Perlas, E., Ben-Menahem, D., Hsueh, A. J. W., and Boime, I. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2041−2045.
11. Narayan, P., Wu C., and Puett, D. (1995) *Mol. Endocrinol. 9*, 1720−1726.
12. Grossmann, M., Wong, R., Szkudlinski, M. W., and Weintraub, B. D. (1997) *J. Biol. Chem. 272*, 21312−21316.
13. Sugahara, T., Sato, A., Kudo, M., Ben-Menahem, D., Pixley, M. R., Hsueh, A. J. W., and Boime, I. (1996) *J. Biol. Chem. 271*, 10445−10448.
14. Sugahara, T., Grootenhuis, P. D. J., Sato, A., Kudo, M., Ben-Menahem, D., Pixley, M. R., Hsueh, A. J. W., and Boime, I. (1996) *Mol. Cell. Endocrinol. 125*, 71−77.
15. Jackson, A. M., Berger, P., Pixley, M. R., Hsueh, A. J. W., and Boime, I. (1998) Abstract for the 80th Annual Endocrine Society Meeting, pg. 88.
16. Ben-Menahem, D., Kudo, M., Pixley, M. R., Sato, A., Suganuma, N., Perlas, E., Hsueh, A. J. W., and Boime, I. (1997) *J. Biol. Chem. 272*, 6827−6830.
17. Sato, A., Perlas, E., Ben-Menahem, D., Kudo, M., Pixley, M. R., Furuhashi, M., Hsueh, A. J. W., and Boime, I. (1997) *J. Biol. Chem. 272*, 18098−18103.
18. Suganuma, N., Matzuk, M. M., and Boime, I. (1989) *J. Biol. Chem. 264*, 19302−19307.
19. Berger, P., Panmoung, W., Khaschabi, D., Mayregger, B., and Wick, G. (1988) *Endocrinology 123*, 2351−2359.
20. Madersbacher, S., Shu-Chen, T., Schwarz, S., Dirnhofer, S., Wick, G., and Berger, P. (1993) *Clin. Chem. 39*, 1435−1439.
21. Jia, X.-C., Oikawa, M., Bo, M., Tanaka, T., Ny, T., Boime, I., and Hsueh, A. J. W. (1991) *Mol. Endocrinol 5*, 759−768.
22. Kudo, M., Osuga, Y., Kobilka, B. K., and Hsueh, A. J. W. (1996) *J. Biol. Chem. 266*, 22470−22478.
23. Matzuk, M. M., and Boime, I. (1988) *J. Biol. Chem. 263*, 17106−17111.
24. Krichevsky, A., Armstrong, E. G., Schlatterer, J., Birken, S., O'Connor, J., Bikel, K., Silverberg, S., Lustbader, J. W., and Canfield, R. E. (1988) *Endocrinology 123*, 584−593.
25. Moyle, W. R., Matzuk, M. M., Campbell, R. K., Cogliani, E., Dean-Emig, D. M. Krichevsky, A., Barnett, R. W., and Boime, I. (1990) *J. Biol. Chem. 265*, 8511−8518.
26. Krichevsky, A., Birken, S., O′Connor, J. F., Bikel, K., Schlatterer, J. P., and Canfield, R. E. (1994) *Endocrine 2*, 511−520.
27. Cosowsky, L., Rao, S. N. V., Macdonald, G. J., Papkoff, H., Campbell, R. K., and Moyle, W. R. (1995) *J. Biol. Chem. 270*, 20011−20019.
28. Lustbader, J., Birken, S., Pollak, S., Leveinson, L., Bernstine, E., Hsiung, N., and Canfield, R. (1987) *J. Biol. Chem. 262*, 14204−14212.
29. Birken, S., Maydelman, Y., Gawinowicz, M. A., Pound, A., Liu, Y., and Hartree, A. S. (1996) *Endocrinology 137*, 1402−1411.
30. Talmadge, K., Vamvakopoulos, N. C., and Fiddes, J. C. (1984) *Nature 307*, 37−40.
31. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) *Nature 368*, 251−255.
32. Hoshina, H. and Boime, I. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7649−7653.
33. Magner, J., and Weintraub, B. (1982) *J. Biol. Chem. 257*, 6709−6716.
34. Corless, C., Matzuk, M., Ramabhadran, T., Krichevsky, A., and Boime, I. (1987) *J. Cell. Biol. 104*, 1173−1181.
35. Muyan, M., Rzymkiewicz, D., and Boime, I. (1994) *Mol. Endocrinol. 8*, 1789−1797.

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