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Expression and Characterization of Recombinant β-subunit hCG Homodimer

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We have linked two human chorionic gonadotropin (hCG) β-subunit cDNAs in tandem such that the expressed fusion protein consists of two mature β-subunits joined through the carboxy terminal peptide of the first β-subunit. A single glycine residue is inserted between the two subunits in the fusion protein. Chinese hamster ovary (CHO) cells transformed with a clone that contains the fused cDNAs express and secrete a protein that is consistent with it being a β-hCG homodimer protein. These β-homodimer molecules can recombine with two free α-subunits indicating that both β-subunits within the homodimer are likely folded in their native conformation. Our data also suggest that the two β-subunits fold upon each other as a globular protein and do not appear to exist as a simple fusion of two linear β-subunits. Furthermore, the two β-monomer subunits in the fusion protein form a stable homodimer that can bind and activate the hLH/CG receptor specifically. Recombination of the fusion protein with α-subunits appears to favor an arrangement where two α-subunits combine with a single molecule of the fusion protein. The recombined molecule consists of four subunits and is comparable to two tethered hCG moieties, which constitutes a hCG dimer. This hormone dimer can bind and activate the hLH/CG receptor with an activity approximating that of native hCG.

Key words: Gonadotropin, hCG, homodimer, β-subunit hCG, recombinant protein, hLH/CG receptor .

Introduction

Human chorionic gonadotropin (hCG) is a hormone secreted during pregnancy and consists of two distinct subunits. The β-subunit confers specificity to the hormone whereas the α-subunit is common to the other members of the glycoprotein hormone family (luteinizing hormone [LH], follicle-stimulating hormone [FSH], and thyroid-stimulating hormone [TSH]) within a species *(1)*. The hCG heterodimer has a crucial role in the reproductive pathway, to signal the corpus luteum to continue production of progesterone, which is required for the maintenance of pregnancy.

The structure of hCG was recently solved by X-ray crystallography *(2,3)*. Neither the α- or β-subunit are related in their protein sequence, however the crystal structure revealed striking similarities in their 3-dimensional structures *(2,3)*. In particular, both subunits exhibit a cysteine knot motif that is common among many of the growth factors such as nerve growth factor (NGF), transforming growth factor β (TGFβ), and platelet-derived growth factor β (PDGFβ) *(2,3)*. Interestingly, many of these growth factors can mediate signaling through the formation of homodimer molecules for which unique receptors exist *(4–6)*.

We have been studying potential alternate roles that the free subunits of hCG may play during pregnancy and development. To this end we have been investigating whether the free subunits of hCG exhibit a novel activity and act via unique receptor(s). Our studies have initially focused on the β-subunit of hCG. It is specific to hCG and secreted by a number of malignant cell types. Furthermore, some malignant cells that secrete the β-subunit of hCG are dependent upon its presence for their continued growth in culture *(7–9)*. This indicates that the β-subunit may serve as an autocrine growth factor for these cells and suggests an alternate role for this subunit that is not associated with pregnancy. Whether the β-subunit acts as a monomer or homodimer remains unknown. Because other growth factors exhibiting similar structures to the α- and β-subunits of hCG can act via formation of a homodimer, it is reasonable to postulate that the free β-subunit of hCG may act in a similar way.

The β-subunit of hCG is a protein of approx 22,000 daltons and can fold properly in the absence of the α-subunit. To test the hypothesis that it can form a stable homodimer we constructed an artificial homodimer molecule

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that consists of the fusion of two β-subunits. The carboxy terminus of β-subunit-1 was joined to the amino terminus of β-subunit-2 using the carboxy terminal peptide (CTP) of β-subunit-1 as the linker peptide. This fusion is similar in principle to the single chain hCG heterodimer constructed independently by Puett *(10,11)* and Boime *(12)*. The protein encoded by this fusion gene was designated "β-β."

Our studies of this fusion protein demonstrate that it is stably expressed and secreted by transfected CHO cells. The two tethered hCG β-subunits form a stable homodimer in which each β-subunit moiety is folded in a conformation that approximates that of native β hCG. In addition, in vitro recombination experiments between β-β and free α-subunit indicate that β-β molecules can recombine with two α-subunit monomers prepared from urinary hCG. Both recombined β-subunits in the dimeric molecule are in an orientation similar to the native hCG molecule as determined by monoclonal antibody recognition and receptor binding studies. Finally, a β-β molecule can both bind and activate the hLH/CG receptor, in vitro, specifically but with a lower apparent affinity than native hCG.

Results

Expression and Purification of the β-β Fusion Protein

To study the stability and properties of a homodimer of the β-subunit of hCG we constructed a fusion clone that covalently linked two hCG β-subunits through the "CTP" domain of the first subunit. A single glycine residue was inserted between the two subunits as a result of the placement of a unique restriction site located at the fusion of the subunit cDNAs. The construction of the fusion clone is illustrated in Fig. 1.

The fusion clone was cotransfected with a SV2neo marker clone into CHO cells and G418 resistant clones were isolated and assayed for the production of β-subunit protein using an anti-β-subunit monoclonal antibody (B105) that recognizes the free β-monomer (as well as the hCG heterodimer) *(13)*. We were able to readily identify a clone that was secreting approx 1 mg/L of the β fusion protein. This clone was then grown in spinner culture for the production of large quantities of material for analysis. The fusion protein was purified by affinity chromatography on a sepharose column containing bound B105 monoclonal antibody. The eluted material was analyzed by polyacrylamide gel electrophoresis on a nonreducing sodium dodecyl sulfate (SDS) gel. The silver stained gel (see Fig. 2) revealed that the fusion protein migrated at a size corresponding to twice that of monomeric β-subunit. In addition, the β-subunit fusion protein appeared to be largely intact and only showed moderate signs of nicking when reduced material was electrophoresed on the gel.

Western Blot Analysis of the β-β Fusion Protein

We also performed Western blot analysis of the β-β protein on SDS polyacrylamide gels under nonreducing conditions. These blots revealed that the β-β was recognized by a set of monoclonal antibodies that bound either free β-subunit only or the free β-subunit and the hCG heterodimer. These monoclonal antibodies included CTP 103, CTP 105 (data not shown), B105, and B201, which have all been previously described *(1,13–18)*. Monoclonal antibodies that are specific for hCG heterodimer, such as B107, do not bind the β-β fusion protein. The results of the Western blot analysis are displayed in Fig. 2.

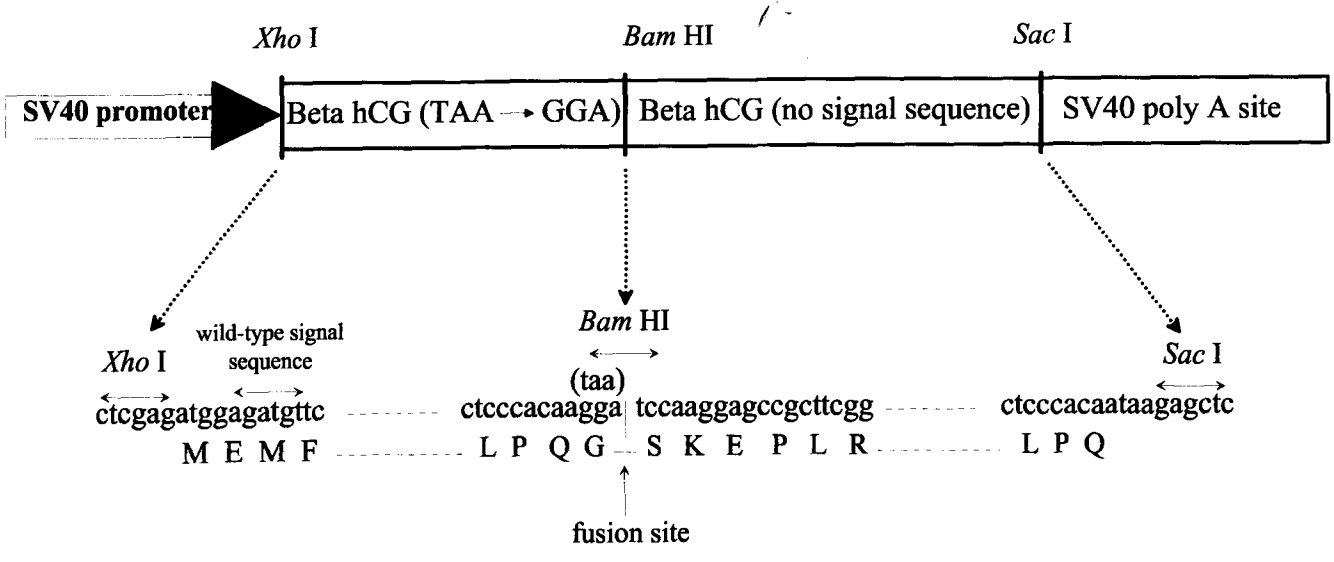

Fig. 1. The SV40 expression vector is depicted with the insert that encodes a fusion of two β hCG subunits. As illustrated, the first β-subunit has its native signal sequence but the TAA terminator codon was mutated to a GGA, which encodes glycine. This allows for read-through to the second β cDNA copy that lacks a signal sequence but terminates properly. The fusion protein encoded by this clone consists of two mature β-subunits fused through the carboxy terminal peptide of the first subunit. The addition of a single glycine residue between the subunits resulted from the engineered missense mutation of the terminator codon.

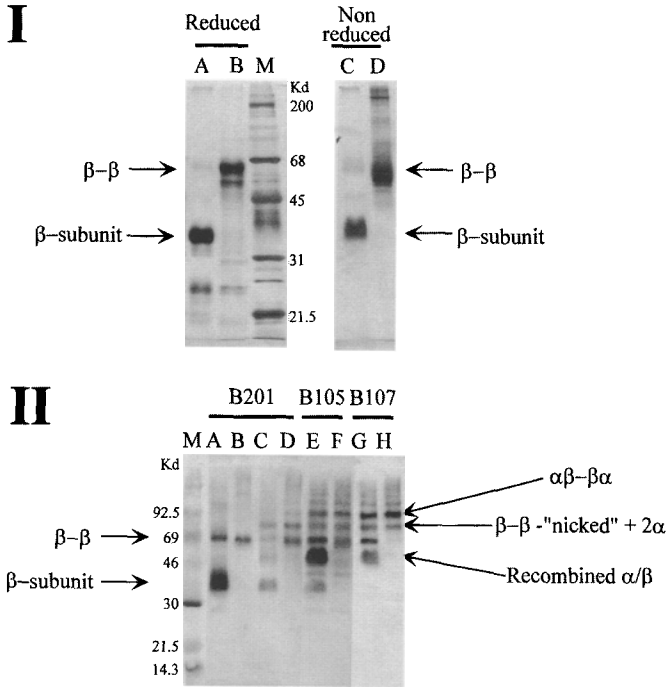

Fig. 2. Panel I—Two silver stained protein gels are shown. In lanes A and B the protein was reduced so that "nicked" material could be visualized. In lanes C and D the protein was not reduced. The lane marked *M* consists of molecular weight markers. In both gels it is evident that the β-β protein migrates at about twice the molecular weight of the monomeric β. In addition, as is evident in lane C, a small amount of the β monomer exists in the form of a homodimer that migrates at the same molecular weight as the β-β fusion protein. Under reducing conditions there is a loss of greater than 80% (determined by densitometry) of the naturally occurring β-subunit homodimer as is evident from a comparison of the electrophoretic pattterns of reduced (lane A) and nonreduced (lane C) β-subunit monomer. **Panel II**—Western blot analysis of nonreduced β-β protein using anti-hCG monoclonal antibodies that have different epitope specificities (see text for complete analysis of these blots). In lane A, free β-subunit was electrophoresed alongside of the β-β in lane B. Lanes C, E, and G contain recombined α- and β-subunit monomers whereas lanes D, F, and H contain β-β protein recombined with α-subunit. B201, B105, and B107 are three monolconal antibodies with different epitope specificities to hCG. B201 binds free β-subunit but not hCG heterodimer, B105 binds both hCG heterodimer and the free β-subunit and B107 binds nonnicked hCG heterodimer with a higher affinity than nicked hCG but does not bind the free subunits. Lane *M* consists of molecular weight markers.

Concurrently, a preparation of free β-subunit that was prepared from the dissociation of the hCG heterodimer, as previously described *(18)*, was electrophoresed. Interestingly, as is evident from the gel and Western blot in Fig. 2, the free β-subunit appears to form a homodimer molecule spontaneously in solution. This homodimer band migrates at approx the same molecular weight as the β-β fusion protein and is decreased by approx 80–90% when the material is reduced (see Fig. 2). It is also not recognized by B107 monoclonal antibody (data not shown). These results support our conclusion that the β-subunit monomer is able to spontaneously assemble to form a homodimer in solution.

Recombination of β-β with the α-Subunit

To determine if the hCG β-subunits in the fusion protein were folded as in the native protein we recombined the β-β protein with free α-subunit of hCG according to previously described methods *(18)* (*see* Methods). The recombined material was then electrophoresed on a nonreducing SDS polyacrylamide gel and transferred to Problott paper. Staining revealed two bands of higher-molecular-weight than the β-β protein band. Protein sequencing of the material in the higher molecular weight bands indicated that both bands contained β-β recombined with two α-subunits (data not shown). We designated this molecule αβ-βα. The lower band had a proteolytic cleavage between residue 43 and 44 of the β-subunit chain [similar to other "nicked" β-chains found in reference preparations of hCG *(19)*]. Whether the cleavage is present in the first or second of the linked β- monomers is unclear. We could not detect any β-β molecule that bound a single α-subunit. This suggests that the association of the β-subunit of hCG with its complementary α-subunit is energetically favored. Furthermore, it may account, in part, for the spontaneous formation of a "β-homodimer" molecule by the β-subunit monomer in the absence of the complementary α-subunit.

Western blot analysis (see Fig. 2 panel II lanes C-H) indicates that the tethered hCG molecules, αβ-βα (formed by recombination of the β-β with free α-subunit), are probably in a conformation that closely approximates that of the native hCG heterodimer (see Fig. 2). Both bands on the blot (of higher molecular weight than the β-β fusion protein) bind B105, which is specific for β-subunit and hCG. B107 (which binds nicked hCG poorly) *(13)* binds the upper nick-free band significantly stronger than the lower nicked band. B201, which binds β-subunit but not hCG heterodimer and can recognize nicked preparations of hCG heterodimer (Lustbader and Pollak, unpublished observations), binds the lower nicked band.

To test if the two hCG heterodimer moieties (within the αβ-βα molecule) are biologically active, we examined whether these molecules could bind the hLH/CG receptor and elicit a biological response. The results of this analysis are displayed in Fig. 3. The amount of recombined αβ-βα protein was determined by radioimmunoassay with monoclonal B107 (within one recombined αβ-βα molecule there exist two tethered molecules of hCG). Our data indicate that the recombined β-β fusion protein can bind the hLH/CG receptor with an apparent affinity slightly lower than that of the recombined subunit monomers (see Fig. 3 panel A). The lower apparent affinity of this material may result from the tethering of two native hCG molecules in this recombination product, which interferes with binding of one of the hCG moieties to the receptor binding domain.

The bioactivity assay tested the ability of the recombined material to elicit a cAMP response in cells that express

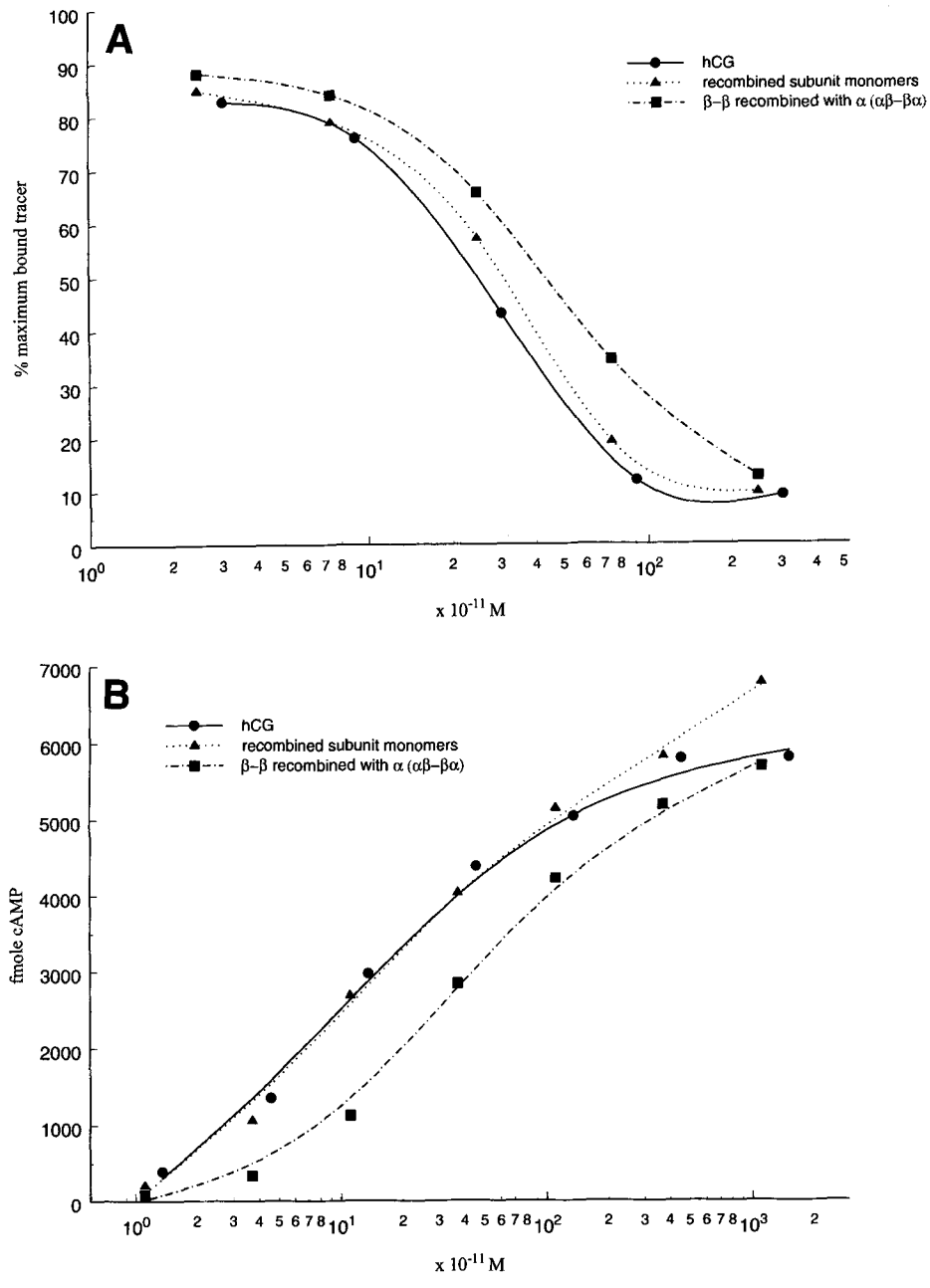

Fig. 3. Binding (panel A) and bioactivity (panel B) studies of recombined subunits and β-β recombined with α-subunit (see materials and methods). These studies were done in duplicate with cells harvested on the same day and the average was plotted as a single point. The standard error of the mean was small for all the means and as a result the error bars are left out for clarity. The data were graphed with the program AXUM 5 (Mathsoft, Cambridge, MA) and a best fit curve was drawn through the data points using the LOWESS algorithm.

the hLH/CG receptor. It demonstrated that the recombined fusion protein could activate the receptor (see Fig. 3, panel B). We conclude that the recombined αβ-βα molecule has two bound α-subunits that associate with the β-subunit moieties of the β-β protein in a conformation that closely approximates that of the native hCG heterodimer. These results also support the conclusion that the two β-subunits in β-β each fold in conformations that approximate that of the native β-subunit. In addition, it demonstrates the feasibility of tethering two native gonadotropin molecules together such that their native activity is retained.

Biological Activity of β-β on CHO Cells that Express the hLH/CG Receptor

Because the α- and β-subunits of hCG exhibit striking structural similarities, we reasoned that the β-β fusion protein may have some activity on the hLH/CG receptor. To test this hypothesis we measured both the apparent affinity and the ability of the β-β protein to elicit a cAMP response in cells that express the hLH/CG receptor. The results of these experiments are displayed in Fig. 4. Interestingly, the β-β fusion protein is able to specifically bind cells that express the hLH/CG receptor albeit with an apparent affin-

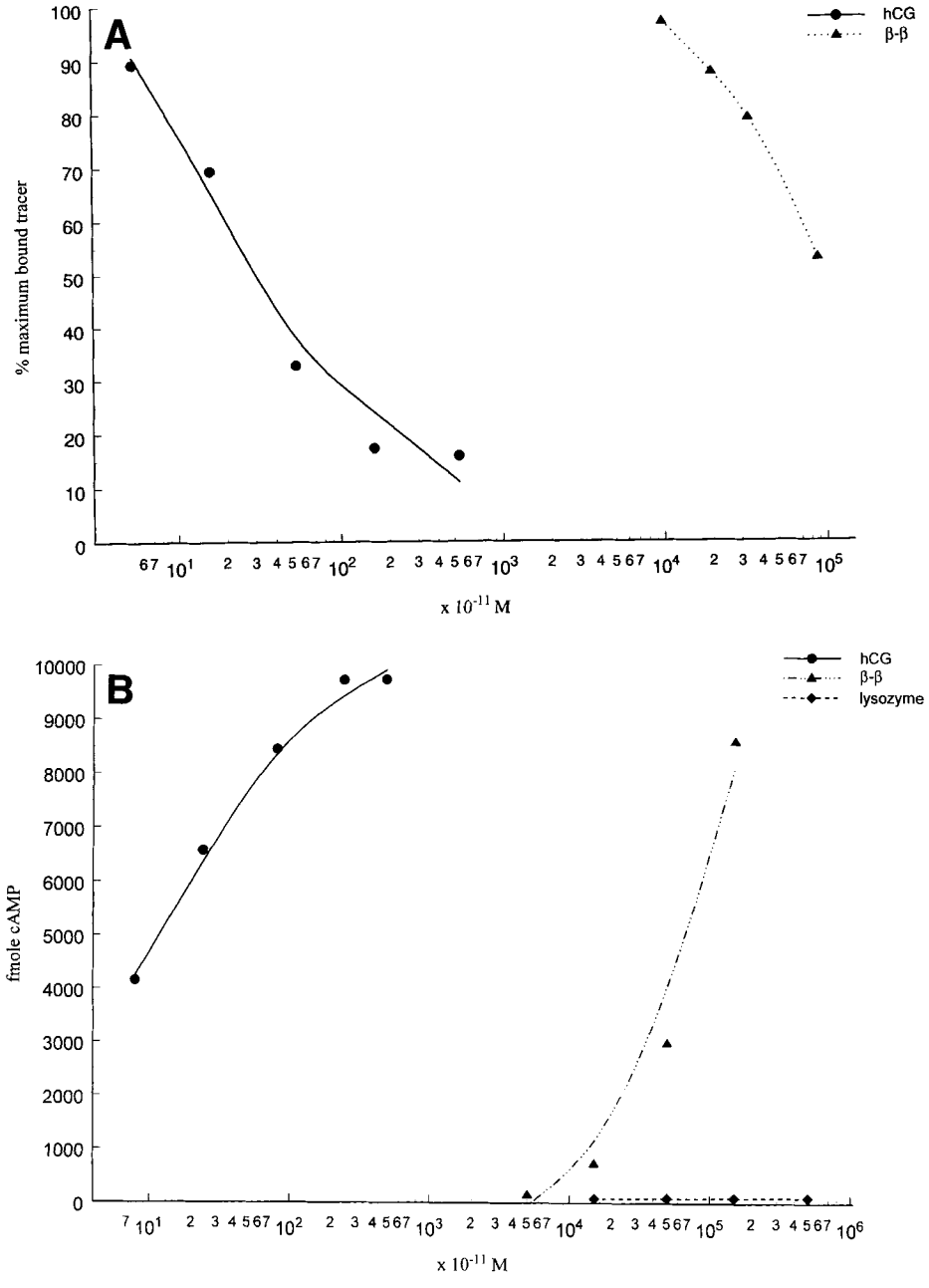

Fig. 4. Binding (panel A) and bioactivity (panel B) of β-β on cells that express the hLH/CG receptor are compared to hCG (*see* Materials and Methods). These studies were done in duplicate with cells harvested on the same day and the average was plotted as a single point. The standard error of the mean was small for all the means and as a result the error bars are left out for clarity. The results of these experiments were graphed with the program AXUM 5 (Mathsoft, Cambridge, MA) and a best fit curve was drawn through the data points using the LOWESS algorithm. In the bioactivity plot lysozyme was included to demonstrate the specificity of the cAMP assay.

ity approximately three orders of magnitude lower than that of hCG for the receptor (Fig. 4 panel A). Similarly, the fusion protein can also elicit a maximal cAMP response in these cells (in a concentration range that corresponds to its apparent affinity, i.e., three orders of magnitude higher concentration than hCG) that is similar to that induced by hCG (Fig. 4 panel B).

The affinity of the β-β for the hLH/CG receptor and its ability to induce a biological response at an apparent affinity that is on the order of 10^{-6} M prompted us to examine the specificity of this apparent affinity and activity. We therefore tested NGF and lysozyme for their abilities to both bind and activate the hLH/CG receptor. Neither protein had any detectable affinity for the hLH/CG receptor nor could either induce a cAMP response in cells that expressed the receptor (data only shown for bioactivity assay of lysozyme).

Molecular Modeling of the β-β Protein and the αβ-βα Complex

A molecular model for the β-β protein, depicted in Fig. 5, was constructed using the hCG heterodimer structure *(2)* as

a template. The two monomeric chains in hCG were observed to be somewhat similar in conformation and share a common network of cystine disulfide bridges (10–60, 28–82, and 34–84 in the α-subunit and 9–57, 34–88, and 38–90 in the β-subunit), located in what is commonly referred to as the disulfide "cradle" region. The β-subunit was separated and superimposed by least-squares refinement onto the α-subunit, using the the cystine "cradle" region as a coordinate target. The result showed both encouraging steric and electronic complementarity, except for the C-terminal regions. The αβ heterodimeric structure is stabilized by the β-subunit, which wraps itself around its α-partner in a "seatbelt" by forming a disulfide bridge (26–110) around loop 2 of the α-subunit. This structure is absent in the β-β homodimer creating a slight disparity due to the structural differences that exist in this loop region between the α- and β- subunits, but this was resolved by readjusting and energetically minimizing these regions so that they are interlocked in a conformationally similar manner to that observed for the αβ heterodimer *(2)*.

The next step was to determine the possible binding sites for the α-subunits on the β-β model so that one could be constructed for β-β recombined with two α monomer subunits. This represented a more challenging problem, since we had little experimental data to direct the initial placement. The proposed α-subunits were positioned based mainly on shape, steric and electrostatic complementarity at their binding interface. The resulting model, as shown in Fig. 5 has not been refined to eliminate all steric conflicts; however, it does indicate a possible plausible recognition site for the α-subunits which can be tested experimentally.

Discussion

We have demonstrated the feasibility of constructing a synthetic homodimer of the β-subunit of hCG by fusing two subunits directly using the carboxy terminal peptide of the first subunit as a linker peptide. The fusion protein is expressed and secreted well by CHO cells. In addition, our results suggest that the β-monomeric subunit of hCG forms homodimers spontaneously in vitro. Recombination of the β-β fusion protein with hCG α-subunits yields a functionally active dimeric hormone consisting of two tethered β-subunits that are associated with two monomeric α-subunits (αβ-βα).

Our studies also examined the activity of the β-β molecule on CHO cells that express the hLH/CG receptor. This homodimer molecule binds the hLH/CG receptor with an affinity approximately three orders of magnitude lower than urinary or recombinant hCG. In addition, cAMP response studies indicate that the β-β fusion protein can elicit as high a cAMP response as hCG at concentrations in which it can bind the receptor. Although the affinity of the β-β molecule for the hLH/CG receptor is relatively low, it can, in principle, be significant when free β-subunit is produced ectopically *(9,20)* since we have observed the spontaneous

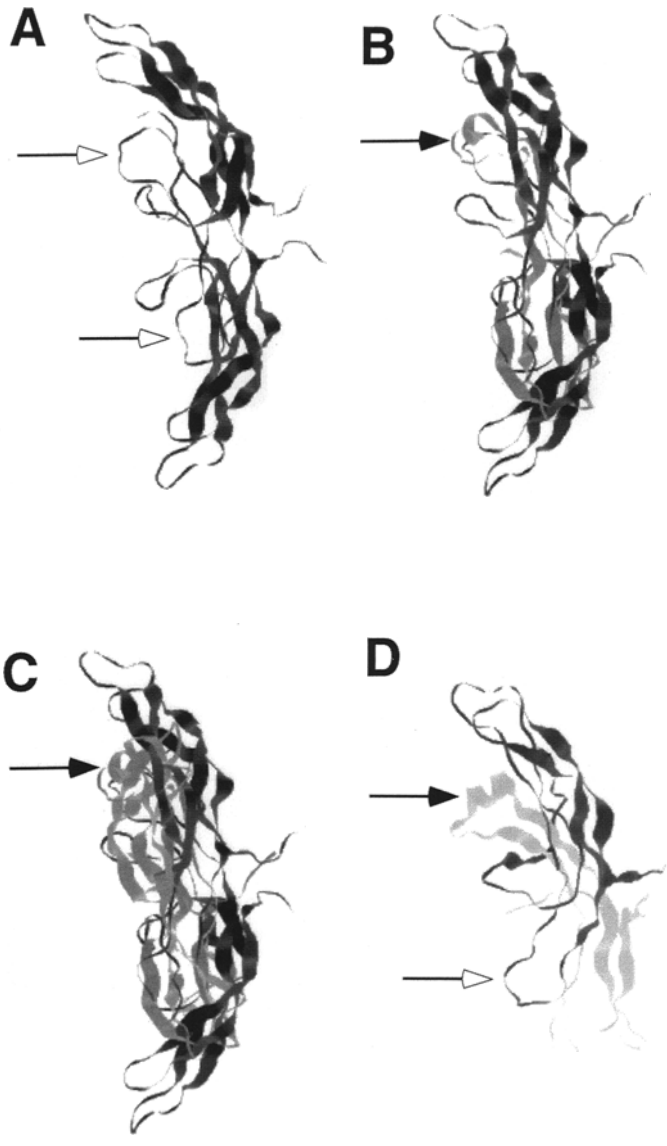

Fig. 5. In panel A a model for the β-β fusion protein is displayed (see results section for the methods used for the construction of this model). Panel B is a model of the β-β protein associated with one α-subunit and panel C is a model of β-β associated with two α-subunits. Panel D contains the structure of hCG as determined from crystallography. The arrows with the white arrowhead point to loop 2 of the β-subunit as a point of reference in the β-β model and the structure of hCG. The arrows with the black arrowheads point to the α helical region of the α-subunit associated with β-β in panels B and C as well as in the structure of hCG in panel D. Note that the model predicts a symmetrical structure for the β-β protein that has a similar symmetry to the crystallographic structure of hCG. The upper loop 2 of β in the homodimer is in a corresponding position to the helical region of the α-subunit in the hCG structure. As evident in panel B, the modeling studies suggest that the position of loops 1 and 3 of the β-subunit relative to the α helical region of the α-subunit in the recombined β-β material is similar although not identical to that derived from the structure of hCG.

formation of β homodimer molecules in vitro from free β-subunit. This is an intriguing possibility in light of the "cross-over" activity of TSH on gonadotropin receptors in

instances of juvenile hypothyroidism *(21–24)*, and hCG on human TSH receptors in thymocytes *(25–32)*. Furthermore, studies suggest that human follicle-stimulating hormone (hFSH) has some "cross-over" activity on the hLH/CG receptor *(33)*. We have been studying this activity in vitro and have preliminary data suggesting that the β-β fusion protein has a similar apparent affinity for the hLH/CG receptor as hFSH. This is evidenced by its equivalent potency to hFSH in inducing a cAMP response from the hLH/CG receptor (L. Lobel, S. Pollak and J. Lustbader, unpublished observations).

The activity and affinity of the β-β fusion protein for the hLH/CG receptor is interesting in light of the studies of Moyle et al. *(34)* with chimeras of the α and β subunits of hCG. Their work indicates that a chimera of the β-subunit in which β loop 2 is replaced with α loop 2 (containing the α helical domain) can bind and activate the hLH/CG receptor albeit with a low affinity. Other chimeras that instead replace loops 1 and 3 of the β-subunit with those of the α-subunit are completely inactive. These results suggest that loops 1 and 3 of the β subunit and loop 2 of the α subunit are critical for receptor binding and activation. Nonetheless, the results of our experiments and modeling studies suggest that loop 2 of the β-subunit can substitute for loop 2 of the α-subunit in the structure of hCG and still maintain an active structure (with lower affinity). As depicted in the model in Fig. 5 panel A, the β-β molecule likely adopts a conformation in which loops 1 and 3 of the first β-subunit are in close proximity with loop 2 of the second β-subunit of the fusion protein. In this model, loop 2 of the β-subunit substitutes for loop 2 of the α-subunit. Accordingly, this suggests that the symmetry of these loops may be just as important for receptor binding and activation as the primary and secondary structure.

The goal of our studies on a fusion protein consisting of two hCG β-subunits is threefold. We were at first interested in determining the feasibility of augmenting the size of the hCG molecule such that it retained hCG activity. A hormone dimer that was active was a simple approach to this goal and our results demonstrate that this is feasible. Dimeric hormones may be useful as long-acting analogs of the hormone monomer and as such may be useful in in vitro fertilization protocols or for prophylaxis against certain breast cancers *(35,36)*. In addition, recent studies from other laboratories suggest that both the invasive phenotype and invasion of *Neisseria Gonorrhoeae* through endometrial tissue is mediated by contact of gonococci with hLH/CG receptors that are expressed in the endometrium *(37)*. A molecule that could block the receptor locally might be effective for inhibition of the invasive phenotype and invasion by this pathogenic organism. Because small molecule inhibitors of the hLH/CG receptor would likely be rapidly absorbed, a larger analog of the hCG molecule may be potent locally as an inhibitor of invasion yet not be absorbed rapidly and thus have limited systemic affects. Studies are currently underway to determine the clearance of the hCG dimer hormone in animal models, as well as its absorption through epithelial tissue.

The second goal of these experiments was to determine whether a hCG β-β fusion protein could form a stable molecule in the absence of the α-subunit. Both our experimental results and our modeling studies indicate that the β-β protein folds in a structurally stable conformation. This demonstration appeared important in light of previously published studies that have suggested a role for the free α- and β-subunits of hCG in the maintenance and progression of certain neoplasias. Furthermore, the striking structural homology between the subunits of hCG and the family of cysteine knot growth factors suggests that the free subunits of hCG may act via the formation of homodimers. This hypothesis is based upon the action of some growth factors through formation of homodimers *(4)*. NGF *(5,6)* is just one notable example.

Finally, we are also interested in establishing that homodimers of the free subunits of the gonadotropins may have biologically relevant activities. This current report suggests that a homodimer of β-hCG may have an alternate activity, on the hLH/CG receptor that could be physiologically relevant under special circumstances. Whereas this is not a novel activity it does suggest a potential new role for the free β-subunit of hCG.

Our previous crystallographic studies of the structure of hCG revealed a striking similarity between the β- and α-subunits of hCG and the family of cysteine knot growth factors *(2)*. Many of these growth factors function through the formation of homodimer molecules. The work detailed in this study indicates that a β-β fusion protein can form a stable homodimer molecule that can bind to the hLH/CG receptor. Therefore, an alternate role for the β-subunit of hCG (and perhaps the α-subunit) in the form of a homodimer molecule appears to be a feasible hypothesis since we demonstrated that free β-subunit spontaneously dimerizes in vitro. Our current and previous studies suggest that one potential alternative role for the β-subunit of hCG may be as a growth factor during human reproduction. We are presently investigating this hypothesis as well as attempting to identify a novel receptor for a β-β like homodimer molecule in human reproductive tissue.

Materials and Methods

Molecular Biology

All techniques relating to recombinant DNA were performed according to standard methods. PCR reactions were performed with Vent DNA polymerase (New England Biolabs, Beverly, MA) and clones were sequenced to ensure that no mutations were introduced by the PCR reactions. Clones were grown in the *E. coli* strain DH5α. Transfection of DNA into CHO cells was performed by the calcium phosphate coprecipitation technique *(38)* with a SV2neo clone as the selectable marker.

HCG Reference Preparations

The reference preparations of urinary hCG (CR127), the β-subunit (βCR129 and βCR119-2), and the α-subunit (αCR123 and αCR127) used in this study have been previously described *(19,39)*. The CR 127 preparation of hCG is the current widely distributed reference preparation *(39)*.

Construction of a β-β Fusion Clone

To fuse two β-subunits of hCG, we constructed two variations of the coding domain of the β-subunit. This was accomplished using PCR with oligonucleotides that contained either specific mutations or that amplified truncated domains of the β-cDNA. The first consists of the entire coding domain from the ATG to the terminator codon. In this case, the terminator codon was mutated to GGA so that a glycine residue was specified in place of termination. The second β-subunit was constructed such that the signal sequence was absent. Otherwise the entire coding domain was present including the proper termination codon. This second β-subunit begins with a serine residue, which is the first amino acid of the mature β-subunit. This residue is specified by a TCC codon which facilitates the fusion of the two β-subunits at a unique synthetic *Bam*HI site (GGATCC). The fusion construct, therefore, encodes an amino terminal β-subunit consisting of the entire coding domain of the β-subunit cDNA with a mutated terminator codon. The carboxy terminal β-subunit cDNA in this fusion begins with the codon for the first residue of the mature β-subunit protein, contains the entire coding domain of the β-subunit and then terminates properly. This arrangement specifies a mature protein that should be composed of two complete mature β-subunits that are tethered at the end of the CTP domain of the first β-subunit through the addition of a single glycine residue. The fusion construct is illustrated in Fig. 1.

Construction of β-β Producing Cell Lines

To produce the fusion protein specified by the cDNA fusion construct described above, we cotransfected the construct into CHO cells with a SV2neo clone according to the calcium phosphate co-precipitation technique *(38)*. In addition we also performed a second transfection with the addition of a cDNA clone that encodes that α-subunit. Cells transfected with the β-hCG homodimer construct (without the α subunit) and selected for resistance to G418 were harvested and subcloned to isolate clonal cell lines producing the β-β protein. Clones were readily identified that produced approx 1 mg/L of recombinant β-β. Cells transfected with both the β-β fusion construct and α-subunit cDNA produced primarily β-β fusion protein.

Production of β-β Protein in Cell Culture

Cultures were maintained in tissue culture plates in Ham's F-12 (Mediatech), 10% fetal calf serum, and 400 µg/mL G418 (Life Technologies, Grand Island, NY) in a humidified 37°C incubator with 5% CO_2. For optimal recombinant β-β protein production, the cells were grown in suspension in biological stirrers (Techne, Princeton, NJ) in serum-free medium. The serum-free medium used was the original formula of CHO-S-SFM (Life Technologies) with the addition of 400 µg/mL G418 (Life Technologies). No adaptation period was necessary; the cells were treated with trypsin to detach them from the tissue culture flasks and seeded at 10^5 cells/mL in stirrer bottles. Cells were grown for 3–4 d, reaching a density of approx 10^6 cells/mL. The cells were collected by centrifugation and resuspended in fresh CHO-S-SFM containing G418. The bottles were incubated for an additional 3–4 d and the β-β containing medium was harvested by centrifugation and filtration through a 0.45 micron membrane.

Affinity Purification of β-β Protein

Affinity columns were prepared by coupling the monoclonal antibody B105 (specific for β-subunit of hCG or intact hCG) to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) at a concentration of 5 mg B105/mL of Sepharose. The β-β protein containing cell supernatants were passed through the 7 mL bed-volume column and the column was washed with 15–20 bed volumes of phosphate-buffered saline (PBS) plus a final wash with 1–2 bed volumes deionized water. Bound β-β was eluted with 1 *M* acetic acid, and the eluant was immediately frozen and then dried in a speed-vac concentrator.

Receptor Binding and Biological Activities

CHO cells engineered to express the rat LH receptor on their surface were developed by Dr. William Moyle (Rutgers University, Piscataway, NJ) and generously donated for these studies. These cells were designated CHO-LR-HHH by Dr. Moyle. A competitive receptor assay was constructed as follows: The cells were removed from the flask surface by adding versene. 100 µL of competing hormone (or buffer) in 0.1% BSA/PBS was added to 100 µL CHO-LR-HHH (10^6 cells in Hank's balanced salt solution + 1 m*M* PMSF) and the mixtures were incubated for 15 min at 37°C. 100 µL of ^{125}I-hCG (approx 100,000 cpm in 0.1% bovine serum albumin/phosphate-buffered saline [BSA/PBS]) was added and the mixtures were incubated another 1–4 h at 37°C and then overnight at 4°C. The tubes were centrifuged at 750*g* for 10 min, the supernatant was aspirated and the cell pellets were counted.

Biological activity was determined on the basis of cAMP production. 2 to 3 × 10^4 CHO-LR-HHH cells were suspended in 100 µL of 20 m*M* Hepes, 0.1% BSA, 4.75 m*M* KCl, 1 m*M* EDTA, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, 150 m*M* NaCl pH 7.4 and stimulated by the addition of varying concentrations of hormone in 100 µL of PBS, 0.1% BSA for a total reaction volume of 200 µL. Cells were incubated for 15 min at 37°C and then for 3 min in a 75–80°C water bath. Tubes were spun at 750*g* for 10 min and then 100 µL of the supernatant was assayed for cAMP content using a com-

mercial kit (DuPont/New England Nuclear, Boston, MA) according to manufacturer's instructions.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the procedure described by Laemmli *(40,41)*. The sample buffer contained 125 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue (reduced samples contained 5% 2-mercaptoethanol). The gel was silver stained according to established techniques *(42)*.

Subunit Recombination

For the biological activity and binding studies, 2.7 nmol of β-β (or 5 nmol urinary hCG β-subunit CR119-2) were mixed with 1 nmole of urinary hCG α-subunit CR127 in a total volume of 10 µL 0.2 *M* ammonium bicarbonate and 1 mg/mL sodium azide. All samples were then incubated overnight at 37°C. A similar procedure was followed for the electrophoresis and Western blot studies, except that a molar excess of α-subunit was used.

Western Blot Analysis:

After electrophoresis, the proteins in the gel were transferred to nitrocellulose paper using a variation of the methods of Towbin *(43)* and Burnette *(44)*. After blocking in 5% BSA, 0.01 *M* Tris-HCl, 0.15 *M* NaCl pH 7.6 (BSA-TBS), the paper was incubated overnight with monoclonal antibody diluted in BSA-TBS (at concentrations of approximately 10–20 µg antibody/mL). All monoclonal antibodies used in these studies have been previously described *(1,13)*. B105 binds both intact hCG and free β-subunit, B107 binds hCG only and B201 binds only the free β-subunit. The binding of the primary antibodies was visualized as previously described *(13)*.

Sequencing of Individual Bands from SDS-Gels

Following electrophoresis, proteins were transferred to a Problott membrane (PE Biosystems, Foster City, CA) using 10 m*M* CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) pH 11, 10% methanol under conditions recommended by the manufacturer. The membrane was stained with Coomassie blue G-250 and the stained bands were cut out and sequenced on a PE Biosystems model 494 protein sequencer.

Molecular Modeling of β-β *and the* αβ-βα *Recombined Complex*

The molecular modeling software QUANTA, version 4.1 (Molecular Simulations, Inc., San Diego, CA) was used for molecular construction and display. All force field calculations were performed with CHARMm 23.0 *(45,46)* using the supplied parameter set PARM.RTF.

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