Biological activity of single chain chorionic gonadotropin, hCG $\alpha\beta$, is decreased upon deletion of five carboxyl terminal amino acids of the α subunit without affecting its receptor binding

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ABSTRACT

The strategy of translationally fusing the two subunits of human chorionic gonadotropin (hCG) has been used to produce recombinant single chain hCG in which the C-terminus of the α subunit is fused to the N-terminus β without any linker using *Pichia pastoris* expression system. The *Pichia*-expressed hCG $\alpha\beta$ (phCG $\alpha\beta$) attained an overall conformation similar to that of hCG, and could bind to the receptor and elicit biological response, suggesting that receptor binding and signal transduction can take place even with a molecule having blocked the C-terminus of the α subunit. The carboxyl terminal of the α subunit has been shown to be involved in hormone binding and signal

transduction of all the heterodimeric glycoprotein hormones. However, deletion of five amino acids from the C-terminus of the α subunit in the single chain hCG did not alter the overall conformation of the fusion molecule and its receptor binding ability, but led to a significant reduction in its ability to elicit biological response. These data show that these five amino acids at the C-terminus of the α subunit in the single chain hCG are not absolutely essential for attaining a conformation required for receptor binding, but are essential for obtaining a full biological response.

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INTRODUCTION

The glycoprotein hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyrotropin (TSH) and chorionic gonadotropin (hCG) are heterodimers composed of the common α subunit noncovalently associated with the hormone-specific β subunits. Formation of the heterodimer from the independently synthesized subunits is accompanied by several conformational changes and is required for biological activity of these hormones (Pierce & Parsons 1981). The structure–function relationship studies of such multimeric proteins having noncovalently associated subunits have often been hindered due to mutagenesis-induced defects in subunit association. Recent studies show that all the single chain glycoprotein hormones, in which the α and the respective β subunits are genetically fused in tandem, are equivalent to their heterodimeric counterparts with respect to receptor

binding and biological activity (Narayan *et al.* 1995, Sugahara *et al.* 1995, 1996a,b, Gracia-Campayo *et al.* 1997, Grossmann *et al.* 1997a). Thus, this approach of fusing the two subunits into a single polypeptide chain enables one to overcome the limitation of heterodimerization, thus widening the scope of structure–function relationship studies. However, in all the single chain derivatives of glycoprotein hormones reported so far, the C-terminus of the specific β subunits were translationally fused to the N-terminus of the α subunit. The design of such a tethered form was based on the premise that the free C-terminus of the α subunit and the N-terminus of the β subunit are important for receptor binding (Merz 1979, Bielinski *et al.* 1990, Chen *et al.* 1992, Furuhashi *et al.* 1995). The question therefore arises whether single chain glycoprotein hormones in which the C-terminus of the α subunit is fused to the N-terminus of the β subunit would assume a

conformation that is similar to the heterodimeric hormone and whether such a molecule would be biologically active. The objective of the present study was to express such a molecule (hCG $\alpha\beta$) and study its conformation using immunological probes and also investigate its receptor binding and signal transduction abilities. Further, we were interested in studying the effect of deletion of five crucial C-terminal amino acid residues from the α subunit on the conformation and biological activity of the fusion protein. We have produced these derivatives of hCG using *Pichia pastoris* expression vectors.

MATERIALS AND METHODS

Construction of the expression plasmids

The cDNA portions encoding the mature secreted forms of the hCG α and hCG β subunits were amplified by PCR using the primers that introduced an EcoRI and a BamHI site at the 5' and the 3' ends of the hCG α cDNA and a BamHI and NotI site at the 5' and 3' ends of the hCG β cDNA respectively. The PCR amplifications using these primers also led to removal of the termination codon from the 3' end of hCG α cDNA and addition of a DNA segment encoding a hexahistidine tag at the 3' end of hCG β cDNA. The amplified hCG α cDNA was first cloned into pBluescriptSK⁺ at EcoRI and BamHI sites and then hCG β cDNA was cloned into the recombinant plasmid, thus translationally fusing the two subunits together with the introduction of a BamHI site at the junction of the α and β cDNAs, resulting in the addition of a single glycine residue between the two subunits. The hCG $\alpha\beta$ fused cDNA was next cloned into the EcoRI–NotI sites of the *P. pastoris* expression vector, pPIC9K, fusing hCG $\alpha\beta$ to the yeast α mating factor signal peptide to produce the pPIC9KhCG $\alpha\beta$ recombinant plasmid. To produce the deletion mutant in which the C-terminal five amino acid residues of the α subunit were eliminated, the portion of hCG α cDNA encoding the first 87 amino acid residues was amplified by PCR. The PCR amplified product was subsequently fused to the hCG β cDNA using the strategy described above to produce pPIC9K $\Delta\alpha\beta$.

Transformation and screening of *Pichia pastoris* recombinant clones

The recombinant plasmids (hCG $\alpha\beta$ and hCG $\Delta\alpha\beta$) linearized with BglII were used for the transformation of *P. pastoris* host strain GS115 (Samaddar *et al.* 1997). The transformants were selected on a histidine-deficient medium. The clones were screened for their ability to express and secrete

hCG-like immunoactivity (phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$) on induction with methanol (Samaddar *et al.* 1997, Sen Gupta & Dighe 1999) and the clones showing the highest secretion of hCG-like immunoactivity were chosen for further characterization.

Immunological characterization of *Pichia*-expressed hCG $\alpha\beta$ and hCG $\Delta\alpha\beta$

The induction media from the selected *P. pastoris* clones were dialyzed, concentrated by lyophilization and subjected to solid phase RIA (Dighe *et al.* 1990a,b) using conformation-specific hCG monoclonal antibodies (MAbs) that were specific for either the heterodimeric hCG (52/28), the α subunit (52/3, G10/F7) or the β subunit (13/E10 and 14/B7). The induction medium from host cells or vector-transformed *Pichia* cells did not show any displacement in any of the RIAs.

In vitro biological activity of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$

Radioreceptor assay

The ability of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ to bind to the LH receptor was demonstrated by incubating rat testicular LH receptor preparation with ¹²⁵I-hCG in presence of varying amounts of authentic urinary hCG or different aliquots of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ (Dighe & Moudgal 1983). The binding data obtained were converted into a Scatchard plot.

In vitro MA-10 Leydig cell bioassay

To determine the biological activity of these derivatives, approximately 30 000 MA-10 Leydig cells/well (Ascoli 1981) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 20% fetal calf serum (FCS) at 37 °C in a CO₂ incubator for 2 days and then stimulated with different doses of authentic and recombinant hCG derivatives in 1 ml IMDM containing 0.4% FCS for 3 h under identical conditions. Progesterone secreted into the medium was estimated by RIA. The experiments were repeated three times.

RESULTS

Expression and immunological characterization of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$

The *Pichia* cells transformed with pPIC9KhCG $\alpha\beta$ and pPIC9KhCG $\Delta\alpha\beta$ expression cassettes synthesized and secreted hCG-like activity into the medium on induction with methanol. The RIA displacement curves obtained with the secretory

FIGURE 1. RIA of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ using (a) hCG antiserum and (b) MAb 52/28. The induced culture supernatants from the highest secreting clones were assayed in an RIA using (a) hCG antiserum and (b) solid phase RIA using the heterodimer-specific MAb 52/28. ^{125}I -hCG as the tracer hCG (CR121) was used as reference preparation. ■ hCG; ▲ phCG $\alpha\beta$; ▼ phCG.

The Hillslopes of the displacement are given below:

	hCG	phCG $\alpha\beta$	phCG $\Delta\alpha\beta$
hCG antisera	-1.292 ± 0.0694	-0.5269 ± 0.0585	-0.4145 ± 0.0427
MAb 52/28	-1.159 ± 0.1685	-0.7107 ± 0.1099	-1.141 ± 0.1600

medium from the selected clones (phCG $\alpha\beta$, phCG $\Delta\alpha\beta$) using hCG antiserum and ^{125}I -hCG as the tracer are shown in Fig. 1a. Although phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ completely inhibited binding of ^{125}I hCG to the antiserum, the differences in the slopes of the displacement curves obtained with the single chain derivatives and that obtained with authentic hCG suggest that the fusion proteins are capable of attaining an overall conformation similar, if not identical, to that of hCG. This observation was further confirmed by RIA using a heterodimer-specific MAb 52/28 that did not recognize isolated hCG subunits (C Sen Gupta & R R Dighe, unpublished observation). The displacement curves obtained using this MAb (Fig. 1b) indicate that this heterodimer-specific epitope is formed in both phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$, although subtle differences may exist in the conformation of this epitope when the two subunits are translationally fused.

Further characterization of fusion proteins was carried out using subunit-specific MAbs that were raised against hCG, but recognized different

conformation-specific epitopes in either the α (MAbs 52/3 and G10/F7) or β (13/E10 and 14/B7) subunit of hCG. It is evident from the displacement curves shown in Fig. 2 that specific epitopes recognized by these MAbs are indeed formed in both the phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ fusion proteins. It also appears that the slopes of the displacement curves obtained with phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ using hCG β -specific MAbs appear to be more similar to that obtained with hCG than that observed with hCG α -specific MAbs.

In vitro biological activity of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$

(a) Receptor binding

As shown in Fig. 3a, both the phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ fusion proteins could completely inhibit ^{125}I -labeled hCG binding to the receptor. Scatchard plot analysis shows that phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ can bind to the LH receptor with affinities comparable

FIGURE 2. RIA of phCGαβ and phCGΔαβ using hCG MAbs. The secretory media from the clones described above were dialyzed, concentrated by lyophilization and assayed in solid phase RIA using hCGα MAbs 52/3, G10/F7 (α subunit specific) and hCGβ MAbs 13/E10, 14/B7 (β subunit specific). In all the assays, hCG (CR121) was used as the reference preparation and ¹²⁵I-hCG as the tracer. ■ hCG; ▲ phCGαβ; ▼ phCGΔαβ. The Hillslopes of the displacement are given below:

	hCG	phCGαβ	phCGΔαβ
MAb 52/3	- 0.5345 ± 0.0865	- 0.0651 ± 0.0825	- 0.3038 ± 0.0749
MAb G10/F7	- 0.5606 ± 0.0713	- 0.6313 ± 0.0834	- 0.4330 ± 0.0543
MAb 13/E10	- 0.9564 ± 0.0746	- 1.154 ± 0.0624	- 0.9630 ± 0.0547
MAb 14/B7	- 0.9447 ± 0.0319	- 1.094 ± 0.0322	- 0.8980 ± 0.0405

to heterodimeric hCG (K_d for phCGαβ = 6.2×10^{-10} M, K_d for phCGΔαβ = 3.3×10^{-10} M, K_d for hCG = 7.7×10^{-11} M).

(b) *In vitro biological response*

The *in vitro* bioassay using MA-10 Leydig cells (Fig. 3b) shows that both phCGαβ and phCGΔαβ

could stimulate progesterone production over the basal progesterone production by the cells. Whereas the maximal stimulation obtained with phCGαβ was comparable to that obtained with hCG, deletion of the five crucial amino acid residues from the C-terminus of the α subunit led to reduction of the maximal stimulation obtained.

DISCUSSION

In the present study we demonstrate that a single-chain derivative of hCG with the C-terminus of the α subunit translationally fused to the N-terminus of the β subunit attains an overall conformation similar to that of heterodimeric hCG, binds to the LH receptor and elicits a biological response. Surprisingly, a deletion mutant of single chain hCG $\alpha\beta$ with five C-terminal amino acids of the α subunit eliminated

was similar to hCG with respect to overall conformation and receptor binding. This mutant also stimulated a biological response, although maximal stimulation obtained was significantly less compared with that obtained with hCG. The *P. pastoris* yeast expression system, which has been shown in the laboratory to be capable of hyperexpressing and secreting immunologically similar and biologically active hCG (Sen Gupta & Dighe 1999), was used for expression of the single chain phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ derivatives.

Recent studies with all the glycoprotein hormones (Narayan *et al.* 1995, Sugahara *et al.* 1995, 1996a,b, Gracia-Campayo *et al.* 1997, Grossmann *et al.* 1997a) have demonstrated that the single chain derivatives in which the α and respective β subunits are fused into a single polypeptide chain are biologically active. However, in all the single chain glycoprotein hormones reported to date, the C-termini of the respective β subunits were fused to the N-terminus of the α subunit. This strategy was based on the observations that the C-terminus of the α subunit and the N-terminus of the β subunit are important for receptor binding and signal transduction (Merz 1979, Bielinski *et al.* 1990, Chen *et al.* 1992, Furuhashi *et al.* 1995). In the present study, the positions of the two subunits in the fusion molecule have been reversed and still the protein produced is biologically active. These data suggest that the C-terminus of the α and the N-terminus of β need not be free to obtain biological response.

FIGURE 3. Bioactivity of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$. (a) Radioreceptor assay (RRA). The secretory medium as described in Fig. 2 was assayed in a RRA using adult rat testicular membrane as the source of LH receptors, ^{125}I -hCG was used as the tracer and hCG (CR121) as the reference preparation. The amounts of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ plotted along the x-axis were quantified in an assay with the MAb 52/28 of the medium described above. ■ hCG; ▲ phCG $\alpha\beta$; ▼ phCG $\Delta\alpha\beta$. The dissociation constants calculated from the RRA were: K_d for phCG $\alpha\beta$ = 6.2×10^{-10} M, K_d for phCG $\Delta\alpha\beta$ = 3.3×10^{-10} M, K_d for hCG = 7.7×10^{-11} M. (b) *In vitro* MA-10 cell bioassay. The MA-10 Leydig cell assay was employed to assess the bioactivity of phCG and its $\alpha\beta$ single chain derivatives. A typical response curve obtained is shown. The MA-10 cells were incubated with increasing concentrations of hCG and phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ (as quantified by RRA) (0.2–200 ng/ml per well) for 3 h at 37 °C. Progesterone secreted into the medium was assayed using progesterone antiserum and [^3H]progesterone as the tracer. Each point represents the mean $\pm$ s.d. of triplicate wells. ■ hCG; ▲ phCG $\alpha\beta$; ▼ phCG $\Delta\alpha\beta$. (EC_{50} for hCG: 6.35 ± 1.34 , EC_{50} for phCG $\alpha\beta$: 2.418 ± 1.36 , EC_{50} for phCG $\Delta\alpha\beta$: 1.038 ± 1.43 .)

This contention has been supported by recent reports in which a similar strategy of fusing the subunits has been employed (Puett *et al.* 1998, Narayan *et al.* 1999, Ben-Mannhein *et al.* 1999). Further, in the hCG $\beta\alpha$ fusion protein, the C-terminal peptide (CTP) of the hCG β subunit having a high content of serine and proline residues is highly flexible and acts as an efficient linker between the two subunits. Further studies also indicated that this linker facilitates secretion of the fusion protein without having any influence on the bioactivity and was also observed in the case of FSH and TSH (Sugahara *et al.* 1996a,b, Gracia-Campayo *et al.* 1997, Grossmann *et al.* 1997a).

The strategy used for producing the fusion protein in this study introduced a single additional glycine residue at the junction of α and β subunits without any additional linker. Using immunological probes, it was observed that the fusion protein was capable of attaining an overall conformation resembling that of hCG. phCG $\alpha\beta$ was able to completely inhibit binding of the ^{125}I -hCG to hCG antiserum. However, differences in the slopes of the RIA displacement curves indicate changes in the conformation of the fusion protein (Fig. 1a). Studies with conformation-specific MAbs showed that all the epitopes recognized by these antibodies are formed in the fusion protein. The slopes of the displacement curves also indicate that similarity in the conformation between hCG and phCG $\alpha\beta$ is more remarkable with respect to the β subunit, whereas subtle differences in the conformation of the α subunit were evident.

Interestingly, a heterodimer-specific epitope in hCG recognized by MAb 52/28 was also properly formed in the fusion molecule (Fig. 1b). This MAb recognizes an epitope in hCG that is formed only when the two subunits anneal to give rise to heterodimeric hormone. Further, this MAb does not interact with isolated subunits (C Sen Gupta & R R Dighe, unpublished observations). Formation of this epitope in phCG $\alpha\beta$ suggested that possibly the interactions between the translationally fused subunits in this molecule are similar to those in hCG. The receptor binding assay showed that the fusion protein was capable of binding to LH receptors with an affinity similar to that of native heterodimeric hCG.

The fusion protein was also biologically active, as indicated by the *in vitro* response assay. The maximal stimulation observed with phCG $\alpha\beta$ was equivalent to that obtained with hCG. In our earlier studies, using the *Pichia* expression system, we have co-expressed both α and β subunits of hCG in the same cell giving rise to heterodimeric hCG (phCG), which was immunologically similar to hCG and

could bind to the LH receptor with comparable affinity. However, it was found that the maximal *in vitro* response obtained with phCG was twofold higher than that observed with hCG (Sen Gupta & Dighe 1999). This observation could possibly be attributed to the differences in glycosylation pattern associated with *Pichia*-expressed recombinant protein. If phCG is considered the appropriate control, phCG $\alpha\beta$ is certainly less active than the heterodimeric protein. Whether the differences between the biological activities of phCG and phCG $\alpha\beta$ could be attributed to certain differences in the conformations of the two proteins, thereby leading to the reduced accessibility of the determinants necessary for signal transduction, remains to be answered.

Several chemical modification and mutagenesis studies have demonstrated that the C-terminal region of the α subunit plays a very important role in receptor binding and signal transduction for all the heterodimeric glycoprotein hormones (Merz 1979, Bielinski *et al.* 1990, Chen *et al.* 1992, Yoo *et al.* 1993, Grossmann *et al.* 1995). In each of these mutants, annealing of the mutant α subunit with the respective β subunits appears to be unaffected, leading to the conclusion that the C-terminus of the α subunit is required for the receptor binding and subsequent signal transduction. Therefore, we next investigated the importance of the five C-terminal residues of the α subunit by deleting these residues from the fusion protein. As shown with immunological probes (Figs 1 and 2), the overall conformation of phCG $\Delta\alpha\beta$ appeared to be similar to those of phCG $\alpha\beta$ and hCG, although differences in the conformation of the α subunits could be detected. The data obtained with MAb 52/28 clearly indicated that this heterodimer-specific epitope, shown to be critical for receptor binding, was also formed in this derivative. The receptor-binding assay showed that phCG $\Delta\alpha\beta$ could bind to the receptor with the same affinity as that of phCG $\alpha\beta$. As shown in Fig. 3b, phCG $\Delta\alpha\beta$ could also stimulate the response over the basal response, although the maximal stimulation was threefold lower than that obtained with phCG $\alpha\beta$. It appears from these data that the five C-terminal amino acid residues of the α subunit may not be essential for receptor binding of the fusion protein, but are certainly necessary to ensure full signal transduction activity.

The receptor binding ability of phCG $\Delta\alpha\beta$ is certainly surprising in view of earlier work (Bielinski *et al.* 1990, Chen *et al.* 1992, Yoo *et al.* 1993, Grossmann *et al.* 1995). It is also contrary to the model proposed based on the crystal structure of hCG (Lapthorn *et al.* 1994) and the recent studies carried out with FSH, in which this region of the α subunit has been shown to constitute an important

part of the receptor binding domain of the hormone heterodimer (Arnold *et al.* 1998). However, as has been suggested previously (Ben-Mannhein *et al.* 1997), it is possible that hCG may contain multiple receptor binding and signaling determinants and the effect of elimination of a particular determinant may not result in complete elimination of the receptor recognition domain and may be compensated by the others. As seen in case of both phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$, the β subunits' conformations bear considerable resemblance to that of the β subunit in the native heterodimeric hCG. Our earlier studies have shown that the initial interaction between the glycoprotein hormones and the respective receptors takes place mainly through the β subunits, while the α subunit plays an important role in the signal transduction process (Dighe & Moudgal 1983, Moudgal *et al.* 1989). The recent model for TSH-receptor interaction is in agreement with this suggestion (Grossmann *et al.* 1997b). Therefore these derivatives, in which the conformation of the β subunit is essentially unaltered and that of the α subunit is partially modified, are able to interact with the receptor and also elicit a biological response. The ability of phCG $\Delta\alpha\beta$ to stimulate only partial response can be attributed to the absence of the five C-terminus residues. Absence of lysine 91 of hCG α , which has been shown to interact with aspartic acid 397 of the receptor for signal transduction (Ji *et al.* 1993), may be one of the reasons for lower biological activity of phCG $\Delta\alpha\beta$. However, the partial stimulation also suggests that the C-terminus of the α subunit is not the only determinant required for full agonistic activity and there are other determinants that contribute to the signal transduction process. With this strategy it will be possible to design single chain derivatives with intact β subunits of different glycoprotein hormones fused to different portions of the α subunits resulting in derivatives with intact receptor binding and varying signal transduction abilities.

The single chain derivatives, both $\alpha\beta$ or $\beta\alpha$ without linker, of glycoprotein hormones, are produced by fusing the C-terminus of one subunit to the N-terminus of the other subunit. The crystal structure of hCG suggests that the C-termini of both subunits are in close proximity to each other, as are the N-termini. Therefore it is difficult to explain the attainment of the overall conformation resembling heterodimeric hCG by the fusion proteins as well as the biological activities of these derivatives on the basis of the crystal structure. Molecular modeling of hCG fusion protein with a linker sequence carried out by Narayan *et al.* (1995) and Sugahara *et al.* (1996b) suggests that the linker

allows appropriate alignment of the α and β subunits as shown by the crystal structure, maintaining the native disulfide bond pattern of each subunit. Therefore, the determinants essential for receptor binding and signal transduction are still maintained in proper mutual orientation on the surface of the protein. However, it has also been suggested that, in absence of the linker, the fusion hormone cannot adopt the wild-type structure observed in the X-ray structure of hCG and still maintain a native disulfide bonded pattern (Sugahara *et al.* 1996b).

Further structural studies are necessary to obtain a clearer understanding of the structure-function relationship of such fusion protein analogs. The *Pichia pastoris* yeast expression system, which is capable of producing large quantities of recombinant proteins (Sudbery 1996), has the potential of producing adequate amounts of the fusion proteins for such structural analysis to be carried out. The levels of expression of phCG $\alpha\beta$ and phCG $\Delta\alpha\beta$ were in the range 50–100 $\mu\text{g/l}$ of the medium at the shake culture level. Preliminary studies with fermentation of phCG $\alpha\beta$ have indicated several-fold increases in the expression. This mutant is now being purified. Preliminary Western blot analysis of phCG $\alpha\beta$ showed a single band of approximately 42–43 kDa interacting with both hCG α - and hCG β -specific MAbs (R Gadkari, C Sen Gupta & R R Dighe, unpublished observations). Using the strategy of keeping the β subunits of glycoprotein hormones intact and fusing them to the mutants of the α subunit, it should be possible to produce derivatives of these hormones with binding properties similar to those of the native counterparts, but with different bioactivities. The *Pichia* expression system is the appropriate expression system for hyperexpression of such derivatives.

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