Glycoconj J (2007) 24:97–106
DOI 10.1007/s10719-006-9016-x

Single chain human chorionic gonadotropin, hCGαβ: Effects of mutations in the α subunit on structure and bioactivity

Sunita R. Setlur · Rajan R. Dighe

Published online: 2 December 2006

Abstract The strategy of translationally fusing the subunits of heterodimeric proteins into single chain molecules is often used to overcome the mutagenesis-induced defects in subunit interactions. The approach of fusing the α and β subunits of human Chorionic Gonadotropin (hCG) to produce a single chain hormone (phCGαβ) was used to investigate roles of critical residues of the α subunit in hormone receptor interaction and biological activity. The α subunit was mutated using PCR-based site-directed mutagenesis, fused to the wild type β subunit and the fusion protein was expressed using *Pichia pastoris* expression system. Following partial purification, the mutant proteins were extensively characterized using immunological probes, receptor assays, and *in vitro* bioassays. The mutation hCGα P38A, which disrupts subunit interaction in the heterodimeric molecule, produced a fusion molecule exhibiting altered subunit interactions as judged by the immunological criteria, but could bind to the receptor with lower affinity and elicit biological response. Mutation of hCGα T54A disrupting the glycosylation at Asparagine 52, believed to be important for bioactivity, also yielded a biologically active molecule suggesting that the glycosylation at this site is not as critical for bioactivity as it is in the case of the heterodimer. The fusion protein approach was also used to generate a superagonist of hormone action. Introduction of four lysine residues in the Loop 1 of the α subunit led to the generation of a mutant having higher affinity for the receptor and enhanced bioactivity. Immunological characterization of single chain molecules revealed that the interactions between the subunits were not identical to those seen in the heterodimeric hormone, and the subunits appeared to retain their isolated conformations, and also retained the ability to bind to the receptors and elicit response. These data suggest the plasticity of the hormone-receptor interactions.

Keywords hCG · Gonadotropins · Single chain glycoprotein hormones · *Pichia pastoris* · Site directed mutagenesis

S. R. Setlur · R. R. Dighe (✉)
Department of Molecular Reproduction,
Development and Genetics,
Indian Institute of Science,
Bangalore 560012, India
e-mail: rdighe@mrdg.iisc.ernet.in

Introduction

The glycoprotein hormones, Lutropin (LH), Follitropin (FSH), Thyrotropin (TSH) and Chorionic Gonadotropin (hCG), are heterodimers composed of a common α subunit non-covalently associated with the hormone specific β subunits [1]. These hormones have proved to be interesting models for protein-protein interactions, for role of the carbohydrates in protein structure and function and for folding during biosynthesis. The crystal structures of hCG [2, 3] and FSH [4] revealed presence of cystine knots in both subunits, which is a hallmark of many growth factors (TGFβ, PDGF etc.). The overall conformation of the subunits was found to be similar, each possessing extended β-hairpin loops, two on one side of the cystine knot and one on the other.

The structure-function relationship studies of such multimeric proteins with non-covalently associated subunits have often been hindered due to mutagenesis induced defects in subunit association. Converting the heterodimeric molecules into their single chain derivatives by translationally fusing the two subunits is one of the methods to overcome this defect. Translational fusion of glycoprotein hormone subunits in βα orientation resulted in single chain hormones that were similar to their heterodimeric counterparts with respect

to receptor binding and biological activity [5–9]. In these studies, the C terminus of hCGβ served as a linker between the two subunits. However, previous studies from the laboratory have shown that fusion of the C terminus of the α subunit to the N terminus of the β subunit with a single glycine residue between the two subunits also results in a molecule with a conformation similar to that of the heterodimeric hormone as judged by immunological criteria. It was also able to bind to the receptor and elicit biological response. Further, using the fusion strategy it was also demonstrated that the C terminus of the α subunit plays an important role in the signal transduction, but not in hormone binding [10].

Generation of this fusion protein opened new avenues for further exploring finer aspects of structure and function of this class of proteins by carrying out site-directed mutagenesis. In the present study, effects of mutations in the α subunit on structure, receptor binding, and *in vitro* bioactivity of the single chain hCGαβ were investigated. The specific mutations investigated were Proline38 to Alanine that has been shown to abolish heterodimerization, disruption of glycosylation at Asparagine 52 known to be critical for bioactivity, and an increase in the positive charge in the L1 loop of the α subunit. Each of these mutants was expressed using the Pichia expression system, partially purified using hydrophobic interaction chromatography, characterized using monoclonal antibody-based radioimmunoassays (RIAs), receptor assays (RRAs), and *in vitro* bioassay.

Materials and methods

Chemicals and biologicals

Restriction enzymes used in this study were purchased from Boehringer Mannheim, Germany, Amersham Pharmacia Biotech, UK and MBI Fermentas, Germany. The DNA Purification kits were obtained from Promega Corporation, USA and Qiagen Inc., Germany. Yeast media were obtained from Invitrogen Corporation, USA and Difco Laboratories, USA.

hCG (CR127), hCGα (AFP1769A), and recombinant hCGβ (AFP8494) used as reference preparations RIAs, RRAs and *in vitro* bioassays, were obtained from National Hormone and Peptide Program, USA. ^{125}I NaI and [1,2,6,7,16,17-^{3}H (N)]—Testosterone were purchased from Perkin Elmer Life Science, USA. All other chemicals and reagents were purchased from Sigma Chemical Company, USA.

Antibodies

The polyclonal antisera raised in the rabbit and the monoclonal antibodies were characterized as described earlier [11, 12]. The monoclonal antibodies against hCGα subunit (52/16 and G10F7, C10) and hCGβ subunit (52/12, 52/20 and 13E10) used in this study recognize conformational epitopes since these antibodies do not bind reduced and carboxymethylated subunits of hCG (Dighe *et al.*, unpublished data). All these MAbs recognize distinct epitopes on both the subunits [11, 12] (unpublished data).

Construction of the expression vectors

Mutations in the α subunit were introduced using PCR-based site-directed mutagenesis using hCGα cDNA as the template. The PCR products were cloned into pBSK+ hCGβ fusing the α subunit cDNA to hCGβ subunit cDNA with an additional glycine residue between the two subunits [10].

Proline 38 in the α subunit was converted to Alanine by overlapping PCR based mutagenesis [13] using the sequence specific end primers, (a) 5′Full-Length Primer: 5′ CC**GAATTC**GCTCCTGATGTGCAGGATTGCCCA3′ (b) 3′ Full-Length Primer: 5′CC**GGATCC**AGATTTGTGA TAATAACA3′ and (c) two overlapping primers bearing the mutation, 5′ overlapping Primer: 5′ TCTAGAGCATAT***G***C CACTCCAC TA3′, 3′ overlapping Primer: 5′TAGTGGAGT GG***C***ATATGCTC TAGA3′.

Threonine 54 in the *N*-glycosylation consensus sequence Asn-X-Ser/Thr of the hCGα subunit was mutated to Alanine using overlapping PCR-based mutagenesis approach as described above. The sequences of the overlapping primers used for amplification were (a) 5′ overlapping Primer: 5′GTCCAAAAGAAC GTC***G***CCTCAGAGTCC3′ and (b) 3′ overlapping Primer: 5′GGACTCTGAGG***C***GAC GTTC TTTTGGA 3′.

A series of residues towards the N-terminal region of the α subunit were mutated to Lysine using asymmetric PCR-based mutagenesis [14] employing a single mutant primer, 3′ Primer: 5′CGGCT***T***GGAGAAGAA***CTT***GTT***CTT*** CT***T***TAGCGTGCATTC3′.

All the three hCGαβ cDNAs cloned in pBluescript SK+ were subsequently cloned into pPIC9K at the *Eco*RI and Not I sites. All the mutations were confirmed by DNA sequencing.

Transformation

The recombinant plasmids were linearized with *Bgl II* to release the expression cassettes with 5′AOX and 3′AOX sites necessary for integration into the yeast genome and used to transform *Pichia pastoris* by electroporation. The transformants were selected on histidine-deficient medium and then screened for secretion of hCG like activity using hCG RIA as described earlier [15].

Fermentation and partial purification of mutants

The best clones secreting hCG-like activity were used for large-scale production of mutants using the fermentation protocols, and the single chain mutants were partially purified using hydrophobic interaction chromatography (HIC) as described earlier [16].

Characterization of the recombinant protein

Immunological characterization

The partially purified recombinant mutants obtained from HIC chromatography were further characterized by RIAs using hCG, hCGα, and hCGβ antisera. The hCGα a/s [11] used for determination of hCGα-like activity had no cross reactivity with the β subunit at the dilution used in the assay, while the hCGβ a/s was processed through an affinity column of α subunit coupled to Sepharose to remove the hCGα-specific antibodies [17]. Solid-phase RIA was used to assay the immunoreactivity of the recombinant proteins with subunit specific monoclonal antibodies [12]. All the RIAs described above were carried with varying concentrations of recombinant hormones in duplicates and were carried out at least two times.

Radioreceptor assay

The ability of the recombinant proteins to bind to LH receptors was assayed by RRA using crude rat testicular membrane preparations as described earlier [15, 17]. All the RRAs were carried with varying concentrations of recombinant hormones in duplicates and were carried out at least two times.

Biological activity

Leydig cells were isolated from adult mouse testis as described earlier [17] and incubated with varying concentration of hCG or single chain mutants for 4 h at 34°C in a shaking water bath. Testosterone secreted into the medium was determined by specific RIA [17]. All the bioactivity determinations were carried out in quadruplets for each hormone concentration and each sample was analyzed for testosterone levels in duplicates. The *in vitro* bioassay for each preparation was carried out two times and the typical results obtained are shown in the 'Results Section'.

Analysis of data

All the RIA, RRA, and *in vitro* bioassay data were analyzed using Graphpad Prism 3 or Prism 4 software.

Results

Expression, fermentation and partial purification of single chain mutants

Three different mutations were introduced into the α subunit cDNA, fused to the β subunit cDNA, and expressed using Pichia expression system. The interesting clones were grown in a fermenter as described earlier [16]. The activities of recombinant fusion protein were determined by sensitive RIA employing a β subunit-specific MAb 52/12 since the epitope recognized by this antibody was not altered by fusion of the subunits or mutations in the α subunit as indicated by the parallelism of the displacement curves (Fig. 1). This antibody does not distinguish between the free hCGβ and the same subunit annealed with the α subunit in heterodimeric hCG. It was shown that hCG-like activity of phCGαβ, as determined by RIA with 52/12, was approximately 0.5 mg/l of fermentation medium. Approximately 10 l of culture supernatant obtained at the end of the fermentation was loaded onto a Phenyl Sepharose column (approximately 150 ml bed volume) in the presence of 1.6 M ammonium sulfate and 50 mM phosphate buffer, pH 7.5 and the column was sequentially eluted with 50 mM phosphate buffer and 50% acetonitrile. The fusion protein

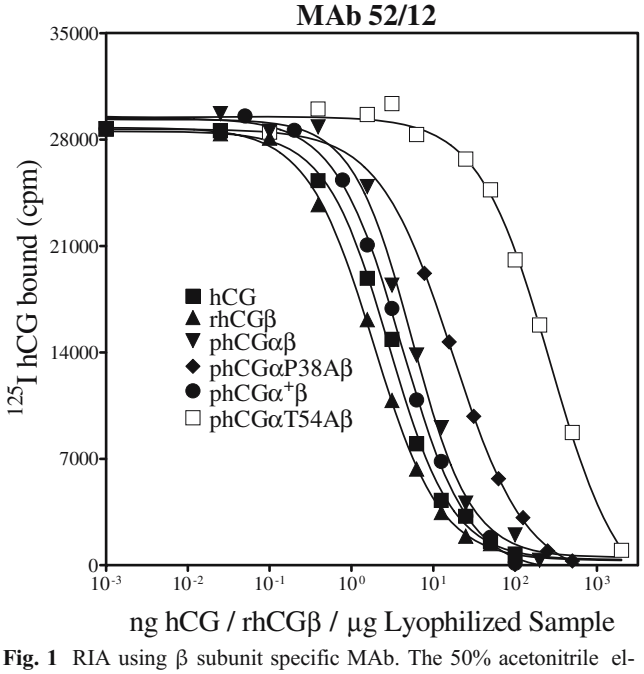

Fig. 1 RIA using β subunit specific MAb. The 50% acetonitrile eluates of the *Pichia*-expressed hormones were dialyzed and lyophilized prior to the assay. Varying concentrations of the lyophilized powder were tested for their ability to bind to the β subunit-specific monoclonal antibody 52/12. ^{125}I hCG was used as the tracer. hCG and recombinant hCGβ (AFP8494) were used as the reference preparations. Data were analyzed using Graphpad Prism 3 and represent duplicate values

activities were found in both buffer and acetonitrile eluates. However, the 50% acetonitrile eluates, which showed significant amount of receptor binding activity, were used in subsequent experiments [16]. The eluates were dialyzed, lyophilized, and used in the later studies without further purification. The contaminants likely to be present in the preparation did not affect receptor binding or response, as it was demonstrated that the Pichia fermentation medium did not interfere in the hormone specific RIAs or RRAs, as well as the response experiments [10, 15, 18].

Characterization of the single chain mutants

The 50% acetonitrile eluates of partially purified mutant fusion proteins were further characterized using hCG polyclonal antisera and a panel of α/β subunit-specific monoclonal antibodies. As discussed above, hCG-like activities were determined by RIA using MAb 52/12 (Fig. 1) and used for further analysis.

Immunological characterization of mutants with polyclonal antisera

RIA displacement analysis with highly specific polyclonal antisera against hCG, hCGα, and hCGβ was used to probe the conformation of the single chain mutants. The displacement curves obtained with hCG, hCGαβ, and the three mutants of the hCGαβ with ^{125}I hCG as the tracer are shown in the Fig. 2(a–c). With hCG a/s (Fig. 2a), hCGαβ and its proline and threonine mutants had higher EC_{50} compared to that of hCG indicating change in the conformation of these single chain mutants compared with that of the heterodimeric hormone. The lysine mutant, in contrast had lower EC_{50}, once again suggesting significant change in the conformation. The hCGα a/s used in this study was raised against hCGα, has no cross reactivity with the β subunit at the dilution used in the experiment, and can distinguish between the isolated α subunit and the α subunit in the heterodimeric hormone [11]. As shown in the Fig. 2b, all the displacement curves with single chain molecules exhibited a shift to the left of the hCG curve similar to that seen with hCGα. This suggested that the α subunit in the single chain molecules shows a structure similar to the isolated subunits further, indicating that the interaction between the subunits in the single chain molecule may not be identical to that seen in the case of heterodimeric molecule. With mutations in the α subunit, this lack of interaction is further accentuated as shifts in the EC_{50} are much more pronounced with proline and threonine mutants compared with that observed with hCGαβ. The hCGβ a/s, with no cross reactivity with the α subunit, can distinguish between the isolated β subunit and the β subunit in hCG. All single chain molecules show almost equal shifts to the left indicating that the conformations of the β subunits in these molecules are similar to that of the isolated β subunit. However, equal shifts seen here indicate that the conformations of the β subunit in different single chain molecules do not differ significantly from each other.

Immunological characterization with monoclonal antibodies:

β subunit specific monoclonal antibodies

RIAs were carried out using two monoclonal antibodies specific for the β subunit. These monoclonal antibodies were originally raised against hCG but also recognized the β subunits. They have different epitope specificities as demonstrated by using methodology established in the laboratory [11, 12] and recognized conformational epitopes as they did

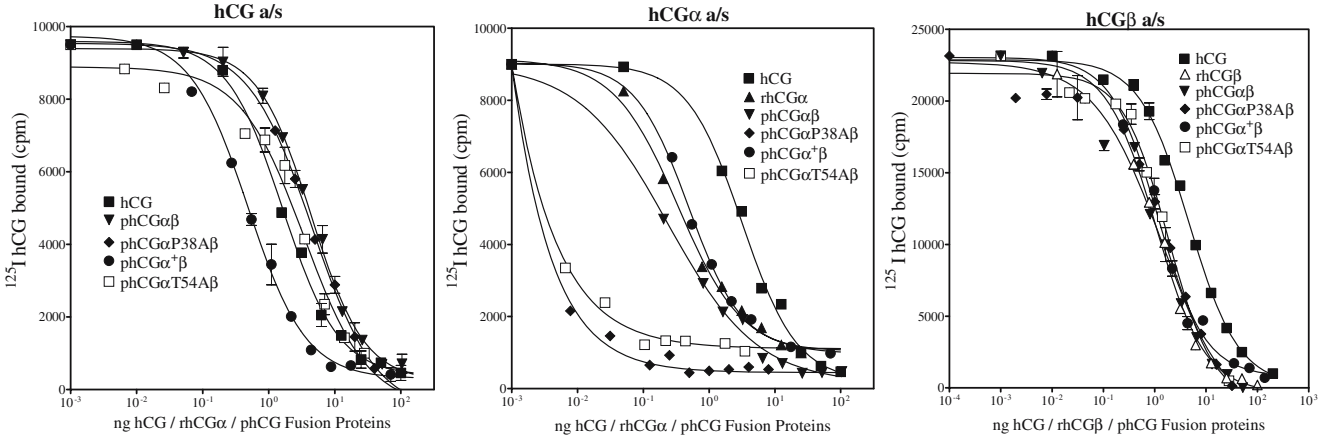

Fig. 2 RIA using polyclonal antisera. Various hormone preparations were analyzed using polyclonal hCG (a) hCGα, (b) and hCGβ, (c) antisera. The values on the *X*-axis indicate the amount of the hormones as quantified by RIA using MAb 52/12. ^{125}I hCG was used as the tracer and the statistical analysis was carried out using the Graphpad Prism software. The data represent duplicate samples

not bind reduced and carboxymethylated hCG subunits (unpublished data). As shown in Fig. 3a and b, all the three mutations in the α subunit did not significantly change the conformation of the epitopes recognized by the β subunit specific MAbs 52/20 (Fig. 3a) and 13/E10 (Fig. 3b).

α subunit specific antibodies

The conformation of the mutants was probed using an hCGα subunit specific monoclonal antibodies. All the three MAbs used here recognized different conformation—specific epitopes on hCG and also the isolated α subunit. As shown in Fig. 4a–c, the conformation of the epitopes recognized by these MAbs did not appear to be altered significantly following fusion of the two subunits as indicated by insignificant changes in EC_{50} of the displacement curves. However, mutations in the α subunit caused significant changes in the α conformation as indicated by changes in EC_{50} and slopes of the RIA curves. Interestingly, three MAbs exhibited different changes in the EC_{50} and slopes of the RIA curves suggesting that the mutations caused dissimilar changes in the conformation of the hormone subunits. More extensive changes in the conformation were also evident in the cases of hCGαβP38A and hCGαβT54A.

Receptor binding

The ability of each mutant to inhibit binding of ^{125}I hCG to rat LH receptor was determined by incubating the labeled hormone with LH receptor in the presence of different concentrations of each mutant (as determined by RIA using MAb 52/12. As shown in the Fig. 5, mutants bound to the receptor with different affinities). While the wild type hCGαβ had nearly the same affinity for the receptor, there was an increase in the affinity of mutants for the receptor.

Biological response

The *in vitro* biological activity of each mutant was determined by incubating varying concentrations of each mutant with the mouse Leydig cells for 4 h at 32°C and determining testosterone secreted into the medium. As shown in the Fig. 6, response to hCGαβ was nearly identical to that observed with hCG. The mutant phCGαP38Aβ was capable of eliciting a biological response comparable to that of the parent phCGαβ fusion.

In the case of phCGαT54Aβ, its ability to stimulate biological response was substantially decreased (Fig. 6). The maximal response stimulated was far lower than that seen in the case of both phCGαβ and hCG. However, an apparent change in the EC_{50} value was observed when the specific activity was quantified based on the 52/12 RIA. Hence, the mutant was a partial agonist unlike the deglycosylated native heterodimer which is devoid of any biological activity.

The mutant, phCGα^{+}β was found to be a superagonist of the hormone action as shown by the maximal stimulation and the shift in the EC_{50} values compared to both phCGαβ fusion protein and the native heterodimer (Fig. 6).

Discussion

It is known that mutations introduced into subunits of heterodimeric molecules often result in lowering of affinity between the subunits rendering the results of such studies difficult to interpret. The strategy of bringing the two subunits in the physical proximity by translationally fusing them has been employed to investigate the role of some of the critical residues in the α subunit in its interaction with the β subunit, as well as receptor binding and bioactivity of the resultant molecules. Translational fusion of the C terminus of the α subunit to the N terminus of the β subunit with an additional

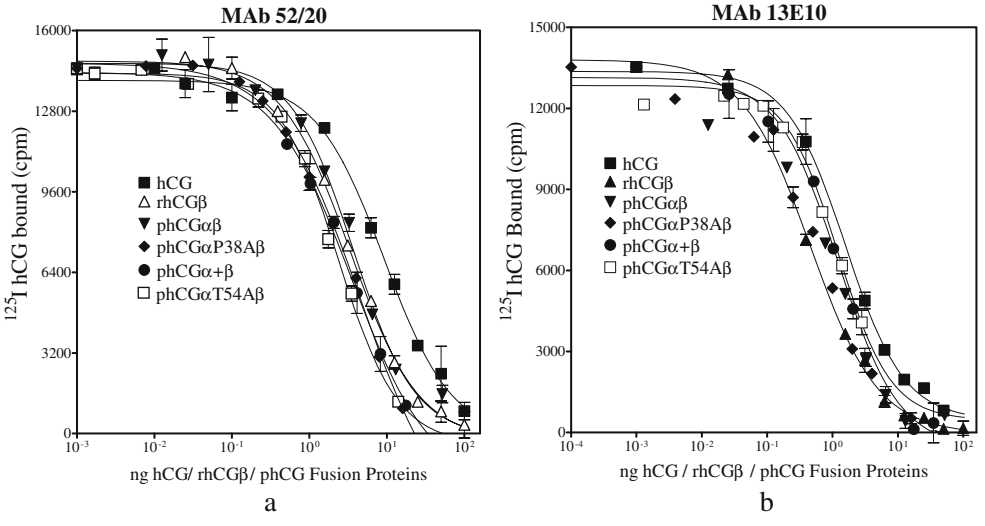

Fig. 3 RIA using β subunit specific MAbs. Various hormone preparations were analyzed using β subunit specific MAbs using ^{125}I hCG as the tracer; MAb 52/20 (a) and MAb 13E10 (b) The values on the *X*-axis indicate the amount of the hormones as quantified by RIA using MAb 52/12

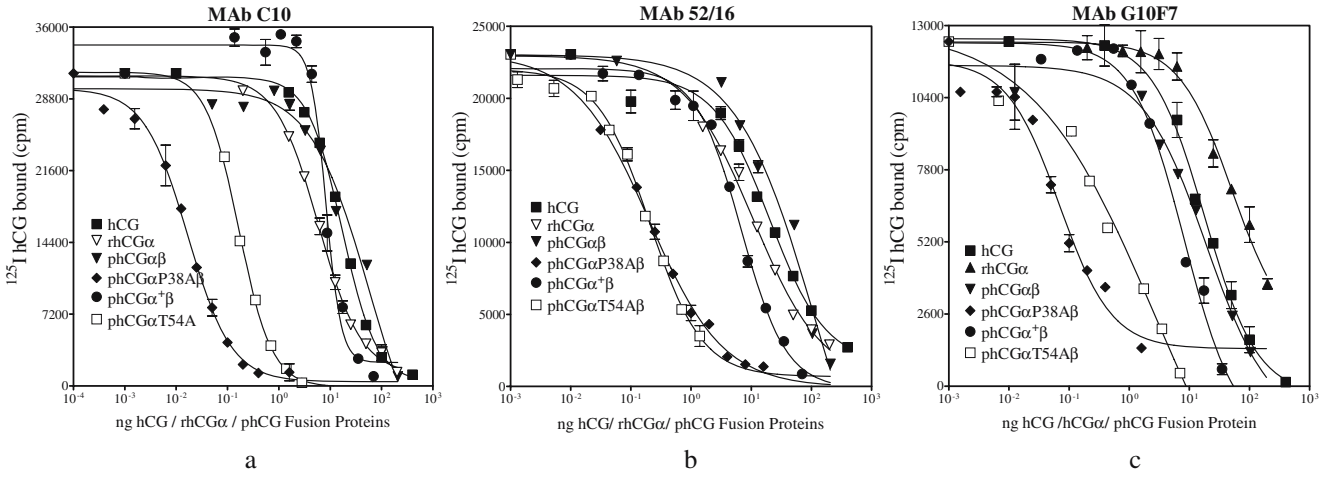

Fig. 4 RIA using α subunit specific MAbs. Various hormone preparations were analyzed using α subunit-specific MAbs using ^{125}I hCG as the tracer; MAb C10 (a), MAb 52/16 (b), and MAb G10F7 (c). The values on the *X*-axis indicate the amount of the hormones as quantified by RIA using MAb 52/12

glycine residue between the two subunits of hCG resulted in a biologically active molecule [10]. Using this hCGαβ as the parent molecule, three single chain mutants with mutations in the α subunits were produced and characterized for their immunological and biological properties.

Substitution of Proline38 with Alanine in the α subunit of the heterodimer resulted in complete loss of interaction with the β subunit [19, 20]. The residues flanking Proline 38 play important roles in receptor binding and subunit interaction in a hormone-specific manner [21]. Proline 38 is situated at the end of the β sheet and forms a part of the three- residue segment Pro38-Thr39-Pro40 which breaks the β sheet bending the peptide backbone at an angle of 100°, resulting in the formation of the succeeding α-helix, αL2, wrapped by the seat-belt region of the β subunit. Orientation of this loop presumably plays a role in attaining a conformation of the heterodimer recognized by the receptor [22] as it shows different orientations in FSH [4]

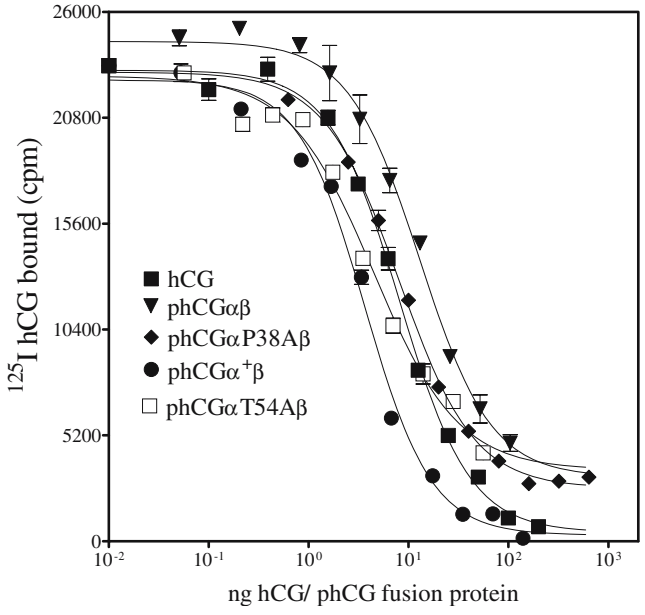

Fig. 5 Receptor assay of mutants. Ability of each mutant to bind to the receptor was determined by RRA using ^{125}I hCG as the tracer and rat testicular membrane as the source of receptor. The values on the *X*-axis indicate the amount of hormones as quantified by the RIA using MAb 52/12

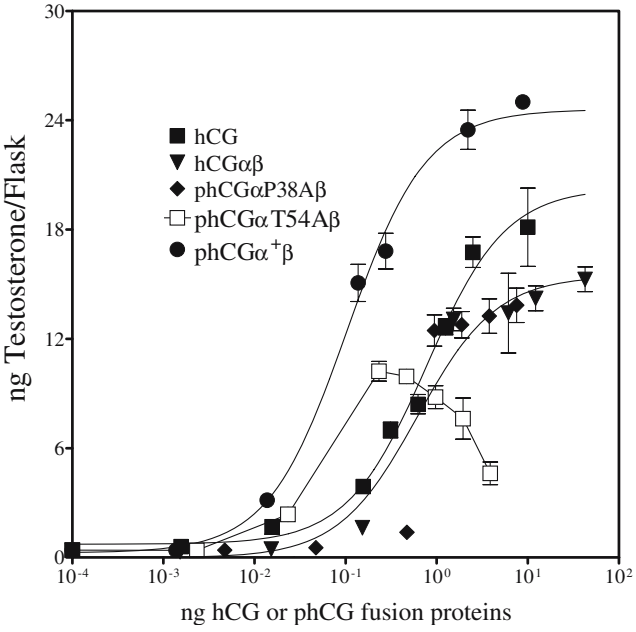

Fig. 6 Bioactivity of mutants. Varying concentrations of the hormones were incubated with the mouse Leydig cells, and testosterone secreted into the medium was estimated by RIA. The values indicated on the *X*-axis are based on quantification using MAb 52/12. The values presented are mean ± S.D of triplicates [phCGαP38Aβ (a); phCGα T54Aβ(b); phCGα^{+}β(c)]

and hCG [2, 3] indicating its possible role in conferring receptor specificity. Substitution of Proline 38 by Alanine leads to relaxation of the kink introduced in the protein backbone by the presence of the Proline residue which probably results in lack of interaction between two isolated subunits. We tested whether fusion of two subunits would overcome this lack of interaction caused by the mutation.

RIA analysis of the mutant with different subunit specific antibodies revealed that the interaction between two subunits in the fusion protein was quite different from that seen in heterodimeric hormone or the wild type hCGαβ. The polyclonal hCGα a/s used can distinguish between the free α subunit and the α subunit in all heterodimeric Glycoprotein hormones, and at the dilution used in the assay, has no cross reactivity with the β subunit [11]. As shown in the Fig. 2b, EC_{50} obtained with the Proline mutant was much lower than that obtained with hCGαβ. Similarly, the EC_{50} of the mutant was also lower in case of hCGβ a/s which can distinguish between the free hCGβ subunit and the same subunit in hCG. Data obtained with the α subunit-specific MAbs were also in agreement with the data obtained with hCGα polyclonal a/s suggesting that the conformation of the proline mutant was different from that of hCGαβ and that the interaction between the two subunits in the mutant single chain derivative is not exactly identical to that seen in the heterodimeric hormone. Intriguingly, despite these changes in subunit interaction, the single chain proline mutant was able to bind to the receptor and elicit biological response.

Similar variations in the conformation were also observed in a mutant with disruption of glycosylation at Asparagine 52 of the α subunit which converts the molecule into an antagonist in case of hCG [23], but not in case of TSH [24]. It was of interest to examine if the carbohydrate residues at this position played an equally important role in the bioactivity of the fusion protein. The overall conformation of phCGαβT54αβ was similar to that of hCG heterodimer as judged by immunological criteria. A mutation M29V was inadvertently introduced during PCR mutagenesis. Although this residue lies in the C-X-G-X-C (CMGCC) motif implicated to play a role in subunit interaction, mutation of this residue has been shown to have no effect on the heterodimer function [25].

The mutant phCGαT54Aβ could bind to the receptor with enhanced affinity, a situation analogous to the glycosylation mutant of the native heterodimer. However, its ability to elicit biological response was not affected. This result is quite different from that seen in the case of the native heterodimer and shows that glycosylation at this site is not as important for bioactivity in the fusion protein as it is in case of the heterodimer. It has been speculated that the carbohydrates played a role in receptor binding by direct interaction with a lectin-like moiety in the receptor as suggested by detection of sequence similarities between regions of the hCG/LH receptor with the soybean agglutinin and the *Dolichos biflorus* seed lectin [26, 27]. However, the crystal structure indicates that the carbohydrates at Asn52 are involved in subunit interaction and are in contact with β subunit residues, β59Tyr, β62Val, β64Phe, β83Ala, and β97Thr, thus maintaining the subunit-subunit interaction. Importance of carbohydrates in maintaining the conformation of the heterodimer was supported by the observation that bioactivity of deglycosylated hCG could be restored by binding of certain antibodies [28]. Apparent loss in bioactivity occurs because of weakening of interaction between the two subunits causing them to fall apart [29]. If the interaction is stabilized by means of an inter-subunit disulfide bridge, the bioactivity can be salvaged. Hence, it appears that the carbohydrates are involved in subunit interaction and in maintaining a structure optimal for signal transduction. An alternate hypothesis suggested that the carbohydrates are required to stabilize a conformational change occurring as a consequence of receptor binding essential for signal transduction [30]. Our data indicate that the carbohydrates in the heterodimer may indeed be acting to stabilize the conformation of the heterodimer, be it a post-receptor binding acquired conformation or a conformation acquired due to subunit interaction. Hence, fusing the two subunits has indeed precluded the need for carbohydrates to be present at this position for generation of an optimal conformation necessary for bioactivity. However, since the maximal stimulation of response has been affected, it does appear that carbohydrates at this position do play some role in the bioactivity of the fusion protein. An alternate explanation for the lowering of response can also be the mutation of Threonine54 residue *per se*, since this residue has been implicated to be important in subunit interaction and has been found to form non-bonded interactions with βAsp99. Hence, the signaling ability might have been affected due to disruption of subunit interaction as a result of this mutation. The fact that the subunit interaction was indeed disrupted is evident from the conformational changes observed with this mutant using immunological criteria.

The conformational changes in the subunits this mutant were seen to be surprisingly similar to those seen in the case of phCGαP38Aβ. The similarities in the conformation of the α subunit can be explained on the basis of the crystal structure where it is seen that both the mutations map in the area of the α subunit where there is extensive interaction between the subunits. The residues α33 to 38 and α53 to 57 form a part of β barrel, which is composed of β sheets from both subunits bringing together segments from near the cystine knot, thus forming a major site of subunit interaction. Any alteration in this area seems to disrupt subunit interactions. Hence, the α subunit in the mutant fusion protein

assumes a conformation resembling the uncombined α subunit. However, this indeed makes it difficult to understand the increase in receptor binding and suggests compensatory changes elsewhere in the fusion protein might lead to optimization of receptor binding.

The fusion protein approach, in addition to being useful in carrying out structure–function analyses, also provides a platform for logically synthesizing analogs of hormone action with therapeutic implications. It is known that the fusion proteins have extended plasma half-lives [9]. Hence, a mutant α subunit having superactivity when annealed with wild type β subunit, upon translational fusion with the β subunit should result in a superactive analog with extended *in vivo* stability. Therefore, residues 13, 14, 16, and 20 of the α subunit were replaced by Lysine. Such substitutions in the L1 loop of the α subunit of the heterodimeric hCG resulted in an eight-fold increase in receptor binding with a concomitant four-fold increase in cAMP stimulation with the maximum stimulation being increased by two-fold [31]. In case of TSH, the receptor binding and signal transduction were observed to be increased to as much as 39-fold. This region is believed to be a part of the receptor-binding domain [32]. This was shown by examining antibody binding to the hormone–receptor complex. Jiang *et al.* [33] suggested that the region 16 to 23 might participate in receptor binding. A support for this came from the studies of Couture *et al.* [34] who showed that a peptide spanning exo-loop2 of the receptor transmembrane domain could prevent the binding of an α subunit monoclonal antibody which recognizes an epitope formed by the residues α15 to 17 and α73 to 75. The peptide was also seen to reduce cAMP response, thus establishing the presence of interaction between exo-loop2 of the receptor and the αL1 loop of the hormone. An electrostatic interaction is envisioned between the negatively charged residues of the receptor and the positively charged residues of the hormone. Further, Sohn *et al.* [35] used alanine scanning mutagenesis and photoaffinity labeling and showed that the exoloop 3 of FSH receptor is in contact with the αL1 loop. Co-operativity of receptor binding between αL1, αL3, and βL3 loops was then unraveled by mutating residues in all the three loop regions. This led to a tremendous increase in both receptor binding and bioactivity of the mutants [36, 37].

The conformation of the mutant fusion protein phCGα$^+$β, was similar to that of the parent phCGαβ fusion protein and the native heterodimer except for subtle variations as judged by its ability to interact with a spectrum of antibodies. The mutant phCGα$^+$β could bind to the receptor with a pronounced high affinity and acted as a superagonist of hormone action with increased maximal stimulation and potency. This result also shows that the orientation of this loop with respect to the receptor or the countersubunit is similar in the case of both the fusion protein and the native heterodimer. Hence, it does appear that the local conformation remains unaltered.

The data presented here show that translational fusion of two subunits of the hCG overcomes the effect of mutations on subunit interactions. However, the subunit interactions in fusion proteins are not exactly identical to that seen in the heterodimeric molecules, and the subunits seem to have conformations similar to those in their isolated status. Our preliminary studies with purified recombinant hCGαβ suggest that acid denaturation of the analog results in loss of receptor binding which is not regenerated at the physiological pH, while the recombinant heterodimeric hormone regains full biological activity confirming that the interaction between the subunits is identical to the one seen in the heterodimeric molecule (manuscript in preparation). Despite this, the mutant fusion proteins can bind to the receptor and elicit biological response suggesting that the approach of tethering the subunits had overcome the constraint of heterodimerization imposed by the mutations. The physical proximity of the subunits apparently aids in the folding and realization of a basic conformation necessary for receptor binding and signal transduction. This indicates a tremendous degree of flexibility in the hormone receptor interactions and shows that the receptor has the ability to accommodate analogs with altered conformation as seen in the cases of phCGαP38Aβ and phCGαT54Aβ. A similar degree of plasticity of glycoprotein-hormone receptors has been reported in several studies involving analogs with compromised subunit interaction [38] and multi-subunit derivatives [39–42]. All the analogs synthesized in these studies were shown to be biologically active, demonstrating that a heterodimer-like conformation is not an absolute necessity for receptor interaction and that as long as the key hormone–receptor contacts are maintained, the analogs can be accommodated by the receptor. Ability of the large ECD of the receptor in accommodating large ligands may be understood in the light of evolution of these hormones from single chain derivatives to specialized heterodimeric hormones. Hence, this makes it very interesting to locate the exact key contacts in both subunit interaction and receptor binding. Purification and physicochemical characterization of the single chain mutants in terms of NMR and X-ray crystallography will provide new insights into the domains, and residues involved in subunit interaction, receptor binding and signal transduction.

Thus, the fusion protein strategy has proved to be an interesting approach for carrying out such studies and in providing insights into hormone-receptor interaction and also in logically engineering analogs of hormone action with altered functionalities for clinical application.

Acknowledgments The work presented here was supported by grants from the Department of Biotechnology, Government of India,

the Indian Council of Medical Research, the University Grant Commission, New Delhi, and the Council for Scientific and Industrial Research, India (fellowship for SRS). We also wish to thank National Hormone Pituitary program, NIH, USA for providing the reference preparations of hormones.

References

1. Pierce, J.G., Parsons, T.F.: Glycoprotein hormones: structure and function. Annu. Rev. Biochem. **50**, 465–495 (1981)
2. Wu, H., Lustbader, J.W., Liu, Y., Canfield, R.E., Hendrickson, W.A.: Structure of human chorionic gonadotropin at 2.6 A resolution from MAD analysis of the selenomethionyl protein. Structure **2**, 545–558 (1994)
3. Lapthorn, A.J., Harris, D.C., Littlejohn, A., Lustbader, J.W., Canfield, R.E., Machin, K.J., Morgan, F.J., Isaacs, N.W.: Crystal structure of human chorionic gonadotropin. Nature **369**, 455–461 (1994)
4. Fox, K.M., Dias, J.A., Van Roey, P.: Three-dimensional structure of human follicle-stimulating hormone. Mol. Endocrinol. **15**, 378–389 (2001)
5. Sugahara, T., Grootenhuis, P.D., Sato, A., Kudo, M., Ben-Menahem, D., Pixley, M.R., Hsueh, A.J., Boime, I.: Expression of biologically active fusion genes encoding the common alpha subunit and either the CG beta or FSH beta subunits: role of a linker sequence. Mol. Cell. Endocrinol. **125**, 71–77 (1996)
6. Narayan, P., Gray, J., Puett, D.: A biologically active single chain human chorionic gonadotropin analog with altered receptor binding properties. Endocrinology **141**, 67–71 (2000)
7. Garcia-Campayo, V., Sato, A., Hirsch, B., Sugahara, T., Muyan, M., Hsueh, A.J., Boime, I.: Design of stable biologically active recombinant lutropin analogs. Nat. Biotechnol. **15**, 663–667 (1997)
8. Sugahara, T., Pixley, M.R., Minami, S., Perlas, E., Ben-Menahem, D., Hsueh, A.J., Boime, I.: Biosynthesis of a biologically active single peptide chain containing the human common alpha and chorionic gonadotropin beta subunits in tandem. Proc. Natl. Acad. Sci. U. S. A. **92**, 2041–2045 (1995)
9. Grossmann, M., Wong, R., Szkudlinski, M.W., Weintraub, B.D.: Human thyroid-stimulating hormone (hTSH) subunit gene fusion produces hTSH with increased stability and serum half-life and compensates for mutagenesis-induced defects in subunit association. J. Biol. Chem. **272**, 21312–21316 (1997)
10. Sen Gupta, C., Dighe, R.R.: Biological activity of single chain chorionic gonadotropin, hCGalphabeta, is decreased upon deletion of five carboxyl terminal amino acids of the alpha subunit without affecting its receptor binding. J. Mol. Endocrinol. **24**, 157–164 (2000)
11. Dighe, R.R., Murthy, G.S., Kurkalli, B.S., Moudgal, N.R.: Conformation of the alpha-subunit of glycoprotein hormones: a study using polyclonal and monoclonal antibodies. Mol. Cell. Endocrinol. **72**, 63–70 (1990)
12. Dighe, R.R., Murthy, G.S., Moudgal, N.R.: Two simple and rapid methods to detect monoclonal antibodies with identical epitope specificities in a large population of monoclonal antibodies. J. Immunol. Methods **131**, 229–236 (1990)
13. Higuchi, R., Krummel, B., Saiki, R.K.: A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16**, 7351–7367 (1988)
14. Perrin, S., Gilliland, G.: Site-specific mutagenesis using asymmetric polymerase chain reaction and a single mutant primer. Nucleic Acids Res. **18**, 7433–7438 (1990)
15. Sen Gupta, C., Dighe, R.R.: Hyperexpression of biologically active human chorionic gonadotropin using the methylotropic yeast, *Pichia pastoris*. J. Mol. Endocrinol. **22**, 273–283 (1999)
16. Gadkari, R., Deshpande, R., Dighe, R.R.: Hyperexpression and purification of biologically active human luteinizing hormone and human chorionic gonadotropin using the methylotropic yeast, *Pichia pastoris*. Protein Expr. Purif. **32**, 175–184 (2003)
17. Dighe, R.R., Moudgal, N.R.: Use of alpha- and beta-subunit specific antibodies in studying interaction of hCG with Leydig cell receptors. Arch. Biochem. Biophys. **225**, 490–499 (1983)
18. Samaddar, M., Catterall, J.F., Dighe, R.R.: Expression of biologically active beta subunit of bovine follicle-stimulating hormone in the methylotrophic yeast *Pichia pastoris*. Protein Expr. Purif. **10**, 345–355 (1997)
19. Xia, H., Chen, F., Puett, D.: A region in the human glycoprotein hormone alpha-subunit important in holoprotein formation and receptor binding. Endocrinology **134**, 1768–1770 (1994)
20. Grossmann, M., Szkudlinski, M.W., Dias, J.A., Xia, H., Wong, R., Puett, D., Weintraub, B.D.: Site-directed mutagenesis of amino acids 33–44 of the common alpha-subunit reveals different structural requirements for heterodimer expression among the glycoprotein hormones and suggests that cyclic adenosine 3′,5′-monophosphate production and growth promotion are potentially dissociable functions of human thyrotropin. Mol. Endocrinol. **10**, 769–779 (1996)
21. Grossmann, M., Weintraub, B.D., Szkudlinski, M.W.: Novel insights into the molecular mechanisms of human thyrotropin action: structural, physiological, and therapeutic implications for the glycoprotein hormone family. Endocr. Rev. **18**, 476–501 (1997)
22. De Beer, T., Van Zuylen, C.W., Leeflang, B.R., Hard, K., Boelens, R., Kaptein, R., Kamerling, J.P., Vliegenthart, J.F.: NMR studies of the free alpha subunit of human chorionic gonadotropin. Structural influences of N-glycosylation and the beta subunit on the conformation of the alpha subunit. Eur. J. Biochem. **241**, 229–242 (1996)
23. Matzuk, M.M., Boime, I.: The role of the asparagine-linked oligosaccharides of the alpha subunit in the secretion and assembly of human chorionic gonadotrophin. J. Cell. Biol. **106**, 1049–1059 (1988)
24. Grossmann, M., Szkudlinski, M.W., Tropea, J.E., Bishop, L.A., Thotakura, N.R., Schofield, P.R., Weintraub, B.D.: Expression of human thyrotropin in cell lines with different glycosylation patterns combined with mutagenesis of specific glycosylation sites. Characterization of a novel role for the oligosaccharides in the *in vitro* and *in vivo* bioactivity. J. Biol. Chem. **270**, 29378–29385 (1995)
25. Kikuchi, T., Koyama, M., Miyai, K., Kimura, T., Nishikiori, N., Azuma, C., Kusunoki, M., Saji, F., Tanizawa, O.: Loss of biological activity of human chorionic gonadotropin (hCG) by the amino acid substitution on the "CMGCC" region of the alpha-subunit. Mol. Cell. Endocrinol. **102**, 1–7 (1994)
26. McFarland, K.C., Sprengel, R., Phillips, H.S., Kohler, M., Rosemblit, N., Nikolics, K., Segaloff, D.L., Seeburg, P.H.: Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science **245**, 494–499 (1989)
27. Calvo, F.O., Ryan, R.J.: Inhibition of adenylyl cyclase activity in rat corpora luteal tissue by glycopeptides of human chorionic gonadotropin and the alpha-subunit of human chorionic gonadotropin. Biochemistry **24**, 1953–1959 (1985)
28. Rebois, R.V., Liss, M.T.: Antibody binding to the beta-subunit of deglycosylated chorionic gonadotropin converts the antagonist to an agonist. J. Biol. Chem. **262**, 3891–3896 (1987)
29. Heikoop, J.C., van den Boogaart, P., de Leeuw, R., Rose, U.M., Mulders, J.W., Grootenhuis, P.D.: Partially deglycosylated human choriogonadotropin, stabilized by intersubunit disulfide bonds, shows full bioactivity. Eur. J. Biochem. **253**, 354–356 (1998)

30. Erbel, P.J., Haseley, S.R., Kamerling, J.P., Vliegenthart, J.F.: Studies on the relevance of the glycan at Asn-52 of the alpha-subunit of human chorionic gonadotropin in the alphabeta dimer. Biochem. J. **364**, 485–495 (2002)
31. Szkudlinski, M.W., Teh, N.G., Grossmann, M., Tropea, J.E., Weintraub, B.D.: Engineering human glycoprotein hormone superactive analogues. Nat. Biotechnol. **14**, 1257–1263 (1996)
32. Remy, J.J., Couture, L., Pantel, J., Haertle, T., Rabesona, H., Bozon, V., Pajot-Augy, E., Robert, P., Troalen, F., Salesse, R., Bidart, J.M.: Mapping of HCG-receptor complexes. Mol. Cell. Endocrinol. **125**, 79–91 (1996)
33. Jiang, X., Dreano, M., Buckler, D.R., Cheng, S., Ythier, A., Wu, H., Hendrickson, W.A., el Tayar, N.: Structural predictions for the ligand-binding region of glycoprotein hormone receptors and the nature of hormone–receptor interactions. Structure **3**, 1341–1353 (1995)
34. Couture, L., Remy, J.J., Rabesona, H., Troalen, F., Pajot-Augy, E., Bozon, V., Haertle, T., Bidart, J.M., Salesse, R.: A defined epitope on the human choriogonadotropin alpha-subunit interacts with the second extracellular loop of the transmembrane domain of the lutropin/choriogonadotropin receptor. Eur. J. Biochem. **241**, 627–632 (1996)
35. Sohn, J., Youn, H., Jeoung, M., Koo, Y., Yi, C., Ji, I., Ji, T.H.: Orientation of follicle-stimulating hormone (FSH) subunits complexed with the FSH receptor. Beta subunit toward the N terminus of exodomain and alpha subunit to exoloop 3. J. Biol. Chem. **278**, 47868–47876 (2003)
36. Grossmann, M., Leitolf, H., Weintraub, B.D., Szkudlinski, M.W.: A rational design strategy for protein hormone superagonists. Nat. Biotechnol. **16**, 871–875 (1998)
37. Leitolf, H., Tong, K.P., Grossmann, M., Weintraub, B.D., Szkudlinski, M.W.: Bioengineering of human thyrotropin superactive analogs by site-directed "lysine-scanning" mutagenesis. Cooperative effects between peripheral loops. J. Biol. Chem. **275**, 27457–27465 (2000)
38. Jackson, A.M., Berger, P., Pixley, M., Klein, C., Hsueh, A.J., Boime, I.: The biological action of choriogonadotropin is not dependent on the complete native quaternary interactions between the subunits. Mol. Endocrinol. **13**, 2175–2188 (1999)
39. Lobel, L., Pollak, S, Lustbader, B., Klein, J., Lustbader, J.W.: Bacterial expression of a natively folded extracellular domain fusion protein of the hFSH receptor in the cytoplasm of *Escherichia coli*. Protein Expr. Purif. **25**, 124–133 (2002)
40. Ben-Menahem, D., Hyde, R., Pixley, M., Berger, P., Boime, I.: Synthesis of multi-subunit domain gonadotropin complexes: a model for alpha/beta heterodimer formation. Biochemistry **38**, 15070–15077 (1999)
41. Kanda, M., Jablonka-Shariff, A., Sato, A., Pixley, M.R., Bos, E., Hiro'oka, T., Ben-Menahem, D., Boime, I.: Genetic fusion of an alpha-subunit gene to the follicle-stimulating hormone and chorionic gonadotropin-beta subunit genes: production of a bifunctional protein. Mol. Endocrinol. **13**, 1873–1881 (1999)
42. Garcia-Campayo, V., Kumar, T.R., Boime, I.: Thyrotropin, follitropin, and chorionic gonadotropin expressed as a single multifunctional unit reveal remarkable permissiveness in receptor–ligand interactions. Endocrinology **143**, 3773–3778 (2002)