Published in final edited form as:

Biochemistry. 2007 April 10; 46(14): 4417–4424. doi:10.1021/bi602449d.

A Novel Four Amino Acid Determinant Defines Conformational Freedom within Chorionic Gonadotropin β -Subunits[†]

Jason A Wilken^{‡,§} and Elliott Bedows^{‡,*}

[‡]Department of Biochemistry and Molecular Biology and the Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE 68198

Abstract

Based on apparent molecular mass heterogeneity following reducing versus non-reducing SDS-PAGE, we determined that the β -subunit of macaque (*Macaca fascicularis*) chorionic gonadotropin (mCG- β) is more conformationally constrained than is human chorionic gonadotropin (hCG- β). These two subunits share 81% amino acid identity. To determine the conformational variance source, which was not due to glycosylation differences, we generated a series of h-/mCG- β chimeras and identified domains that contributed to CG- β conformational freedom. We discovered that the CG- β 54-101 domain contained a small sub-domain, residues 74-77, that regulated the conformational freedom of the β -subunit, i.e., when residues 74-77 were of macaque origin (PGVD), mutated hCG- β subunit displayed macaque-like conformational rigidity; when residues 74-77 were of human origin (RGVN), mutated mCG- β subunit displayed human-like conformational freedom and microheterogeneity. Additionally, CG- β N-terminal domain residues (8, 18, 42, 46-48) were also found to influence CG- β conformational freedom when residues 74-77 were of human, but not macaque origin. The biological significance of the CG- β conformational variance was tested using a biological assay that showed that the h α /h β CG heterodimer facilitated human CG receptor-mediated cAMP-driven luciferase reporter gene activity in HEK cells nearly an order of magnitude more effectively than did the h α /mCG- β chimera. Together, these data demonstrate that two essential amino acid residues contained within a four amino acid sub-domain regulated CG- β conformational freedom and that a conformational difference between hCG- β and mCG- β was recapitulated in the context of receptor-mediated CG heterodimer signal transduction activation.

Chorionic gonadotropin (CG), the placental member of the glycoprotein hormone family, is a non-covalently linked heterodimer consisting of an α - and a β -subunit aligned head-to-tail. The α -subunit is common to all glycoprotein hormones that function in reproduction (i.e., the gonadotropins), while each β -subunit confers biological specificity to each respective family member (1). Both human (h)- and macaque (*Macaca fascicularis*) (m)CG- β subunits share a similar overall topology of three beta loops stabilized by a ladder of hydrogen bonds and a cystine knot motif. The cystine knot motif is comprised of a unique arrangement of six cysteine residues; counting from the N-terminus of the protein, a disulfide consisting of Cys2 and 5 and one consisting of Cys3 and 6 form a ring structure through which a disulfide consisting of Cys1 and 4 penetrates. CG- β loops 1 and 3 form antiparallel β -strands while the remaining hairpin-like structure, loop 2, appears as an extended loop connecting β -structural loops 1 and 3 (2, 3).

[†]This material is based upon work supported in part by NCI Cancer Center Support Grant P 30 CA36727, NCI Training Grant CA09746 to the Eppley Institute Cancer Center and a Grant from the Nebraska Dept. of Health (to E.B.). JAW is a recipient of an Emley Fellowship.

* To whom correspondence should be addressed: School of Allied Health Professions, College of Medicine, 984300 Nebraska Medical Center, Omaha, NE 68198-4300 Tel.: 402-559-6074 Fax: 402-559-8112 E-mail: ebedows@unmc.edu.

[§]Current address: Dept. of Obstetrics, Gynecology and Reproductive Sciences, Yale University, 300 George Street, Suite 8100, New Haven, CT 06511

Our laboratory has previously defined the intracellular kinetic folding pathway of both the hCG α -subunit (4) and hCG- β subunits (5,6) and, more recently, demonstrated that a disulfide exchange occurs as the cystine knot of the CG β -subunit folds (7). Identification of the disulfide exchange was accomplished by isolating key folding intermediates of mCG- β a naturally occurring homolog of hCG- β . mCG- β was chosen for these folding studies because both hCG- β and mCG- β are 145 amino acids in length and share 81% amino acid identity including all cysteine residues and consensus sequences for both sites of N-linked glycosylation and three of four sites of O-linked glycosylation (see Fig. 1) (8). During the course of the latter studies (7), however, we discovered that hCG- β exhibited more conformational freedom than mCG- β .

In this study, we used a series of molecular and biochemical analyses to compare the nature of the structural elements of h- and mCG- β responsible for the variance in conformational freedom between the respective CG- β subunits. We report that two specific amino acids residues were identified in a four amino acid sub-domain that, at least in large part, accounted for these differences.

EXPERIMENTAL PROCEDURES

Construction of h/mCG- β Chimeras

CG- β chimeras were constructed using “megaprimer” polymerase chain reaction (9) with Taq polymerase (Qiagen). The amino acid sequences of hCG- β (10), mCG- β (8) and all h/mCG- β chimeras are shown in Fig. 1. Primers complimentary to two regions of complete identity between hCG- β and mCG- β were used to generate megaprimers that were then used to generate CG- β chimeras, which were cloned into the pGS vector (11). Sequences were validated by the Eppley Cancer Center Molecular Biology Core Facility (University of Nebraska Medical Center, Omaha, NE). Plasmids were prepared using the Maxi Plasmid Kit (Qiagen) according to manufacturer’s protocol.

The amino acid sequence of CG- β was roughly divided into thirds, with the first, second, and third domains comprised of amino acids 1-53, 54-101 and 102-145, respectively. The species identity of each third is represented by an “h” or “m”; e.g., a chimera with mCG- β residues 1-53, hCG- β residues 54-101, and mCG- β residues 102-145 is denoted as m/h/m CG- β . Smaller substitutions generated by site-directed mutagenesis are indicated by species of origin followed by specific amino acid substitutions in parentheses; e.g., hCG- β containing macaque residues 74 and 77 is termed h(m74,77) CG- β . Single amino acid substitutions are similarly indicated with the identity of the species of origin followed by specific amino acid substitutions in parentheses; e.g., a hCG- β subunit containing macaque residue 18 is termed hCG- β V18A.

Cell Culture

293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with penicillin/streptomycin (100 units/ml and 100 μ g/ml, respectively; Invitrogen) and 10% fetal bovine serum (Atlanta Biologicals). CHO cells stably expressing human wild type hCG- α and mCG- β (7) were cultured in Ultraculture (Biowhittaker) supplemented with penicillin/streptomycin and 50 μ M methionine sulfoxamine (Sigma). Human embryonic kidney cells stably transfected with the human luteinizing hormone/CG receptor and a cAMP-responsive luciferase reporter (HEK LH/CG-R cells) (12) were cultured in low-glucose DMEM (Invitrogen) supplemented with penicillin/streptomycin, 2 mM L-glutamine, and 5% heat-inactivated fetal bovine serum (Invitrogen).

Transient Transfection and Metabolic Labeling of Cells

293T cells (1.9×10^6) were plated into 60-mm plastic dishes and grown overnight to 70-80% confluency. Plasmid DNA was precipitated as described previously (13). The resulting precipitate was added drop-wise to the dishes and agitated gently to mix. Cells were incubated for 2 days at 37 °C and used for metabolic labeling. Transfected 293T cells were pulse-labeled for 30 min with L-[^{35}S]cysteine (PerkinElmer Life Sciences; ~1100 Ci/mmol; at a concentration of 50-150 $\mu\text{Ci/ml}$) in serum-free medium lacking cysteine as previously described (11), followed by overnight incubation with complete media lacking radiolabel. Conditioned media was saved for analysis of secreted CG- β subunits.

Immunoprecipitation and Purification of CG- β Subunits

CG- β subunits were precipitated with polyclonal antisera that recognizes all known conformations of hCG- β and mCG- β (7,14). Immunoprecipitations were carried out at 4 °C overnight with rotation in the dark. Immune complexes were precipitated with protein A-Sepharose (Sigma) and prepared for reversed-phase high performance liquid chromatography as previously described (15). Briefly, protein A-Sepharose beads/antibody-antigen immunocomplexes were washed three times with phosphate-buffered saline containing detergents (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) followed by four washes with phosphate-buffered saline lacking detergents. Immunocomplexes were pelleted between washes by centrifugation for 1 min at $2000 \times g$. To dissociate the Sepharose/antibody/antigen interactions, immunocomplexes were treated with 6 M guanidine HCl, pH 3.0 (sequanal grade; Pierce), for 16-20 h while rotating at room temperature. 100 μg of myoglobin (Sigma) was added as a carrier. The guanidine eluates were injected onto a Vydac 300- $\text{\AA}$ C₄ reversed-phase column equilibrated with 0.1% trifluoroacetic acid and eluted using an acetonitrile gradient as described previously (16). Fractions were collected in 1-min intervals and quantified by scintillation counting. Fractions containing the major peak were pooled and stored at -20 °C for further characterization.

PAGE Analysis of Conformational Freedom

Radiolabeled CG- β subunits, purified to homogeneity as described above, were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as follows. Resolution by non-reducing or reducing SDS-PAGE involved dilution of aliquots into non-reducing sample buffer (125 mM Tris-HCl, pH 6.8, containing 2% SDS, 20% glycerol, and 40 $\mu\text{g/ml}$ bromophenol blue) or reducing buffer (non-reducing gel buffer plus 2% β -mercaptoethanol), boiling for 5 minutes and loading onto a 5-20% gradient polyacrylamide slab gel run by the method described by Laemmli (17). Gels were dried *in vacuo* on filter paper and exposed to a phosphorscreen (Molecular Dynamics) and scanned on a Molecular Dynamics Storm 860 phosphorimager.

CG Bioassay

The ability of CG to induce cyclic adenosine monophosphate (cAMP) production was assayed by a CG-responsive luminescence bioassay (12,18). Briefly, HEK LH/CG-R cells were seeded into 96-well plates containing growth media with experimental samples (hCG- α /mCG- β heterodimer, purified as previously described for the mCG- β subunit, at pH 7.4 (7) or recombinant hCG standard (Sigma)) for 18 hours. Concentration of CG heterodimers was determined by densitometry of Coomassie-stained gel compared to hCG standard. Cells were lysed with 100 μl of lysis buffer as per manufacturer's instructions (Promega) and incubated with gentle shaking at room temperature for 30 minutes. 25 μl lysate aliquots were mixed with 100 μl luciferase assay reagent (Promega) and chemiluminescence measured using a TopCount luminometer (Packard).

RESULTS

h- and mCG- β display differential migration rates and microheterogeneity by non-reducing SDS-PAGE

Methods of resolving proteins based on Stokes radii can distinguish between conformationally free and restrained species (19-23). Because flexible domains contribute to an abnormally large Stokes radius, a disordered protein can appear to have a larger than expected apparent molecular mass by gel filtration or gel electrophoresis. Therefore, the respective conformational freedoms of secreted, radiolabeled, purified h- and mCG- β were resolved by SDS-PAGE under reducing and non-reducing conditions.

A striking difference in the apparent molecular mass of hCG- β (Fig. 2A, lane 1) and mCG- β (Fig. 2A, lane 8) was observed under non-reducing conditions ($M_r \sim 35,000$ vs. $\sim 30,000$, respectively). Further, hCG- β migrated as a more diffuse, microheterogeneous band than did mCG- β . Under reducing conditions, h- and mCG- β subunits nearly co-migrated, with hCG- β (Fig. 2B, lane 1) having a ~ 1 kDa greater apparent molecular mass than mCG- β (Fig. 2B, lane 8) because hCG- β contains one additional O-linked glycosylation site (hCG- β Ser138 vs. mCG- β Ala138) (8). In previous studies defining the kinetic folding pathway of CG- β , we demonstrated that conformational differences of hCG- β folding intermediates observed under non-reducing SDS-PAGE conditions were not apparent under reducing SDS-PAGE conditions (11,15,16), consistent with results seen in Figs. 2A and 2B.

CG- β residues 74 through 77 represent a determinant that modulates CG- β conformation

Our results have indicated that mCG- β is more conformationally restrained than hCG. Because hCG- β and mCG- β share 81% amino acid identity (8), we predicted that non-conserved residues in h- and mCG- β influence CG- β conformational freedom. To test this, we constructed a series of chimeras comprised of h- and mCG- β domains to elucidate the sequence-structure relationship(s) of CG- β . CG- β subunits were divided roughly into thirds (residues 1-53, 54-101, and 102-145) and domains were substituted by standard molecular biology techniques to generate the human/maque CG- β chimeras h/h/m, h/m/h, h/m/m, m/h/h, m/h/m and m/m/h, shown in Fig. 1.

Plasmids encoding CG- β chimeras were transiently transfected into 293T cells, metabolically labeled as described in Methods and the resulting radiolabeled, purified CG- β subunits were resolved by reducing and non-reducing SDS-PAGE since differences in conformational freedom are revealed under these conditions. As shown in Fig. 2A, under non-reducing conditions we observed that all CG- β subunit chimeras whose middle domain (residues 54-101) contained macaque residues, (i.e., h/m/h-, h/m/m-, m/m/h- and m/m/m CG- β ; lanes 3, 4, 7, and 8, respectively) had a reduced apparent molecular mass and microheterogeneity, whereas CG- β subunits whose middle domain contained human residues (i.e., h/h/h-, h/h/m-, m/h/h- and m/h/m CG- β ; lanes 1, 2, 5, and 6, respectively) exhibited increased and highly variable diffusion profiles and apparent molecular masses. That these differences were conformational in nature is demonstrated in Fig. 2B, where all subunits migrated similarly by SDS-PAGE under reducing conditions.

Since h- and mCG- β amino acid sequences 54-101 differ by only 8 amino acids, we further divided this domain into three sub-domains. As shown in Fig. 1, residues 74 and 77, 82 and 83, and 89 and 91 differ between h- and mCG- β and, therefore, were exchanged generating constructs h(m74,77)-, h(m82,83)-, h(m89,91)-, m(h74,77)-, m(h82,83)-, and m(89,91) CG- β , where h- or m- represents the CG- β species origin and the residues in parentheses represent the substitutions made in these subunits. (Residues 68 and 91 were not tested because the cross-species differences in amino acid identity were conservative [Asp68 vs. Glu68 and Thr91 vs.

Ser91]). h(74,77) CG- β (Fig. 3A, lane 3) appeared to be less heterogeneous and of lower apparent molecular mass than was hCG- β (lane 1), and m(h74,77) CG- β (lane 6) was more heterogeneous and of higher apparent molecular mass than was mCG- β (lane 2). Substituting residues 82 and 83 or 89 and 91 in either CG- β species had no noticeable effect on the migration of these CG- β constructs.

To determine how specific residues within the 74-77 domain affected CG- β conformational freedom, the mutant subunits hCG- β R74P, R74A, and N77D (Fig. 3A, lanes 9, 10, and 11, respectively), as well as mCG- β P74R, P74A, and D77N (Fig. 3A, lanes 12, 13, and 14, respectively) were constructed, purified and assayed by SDS-PAGE as described above. (Substitution of mCG- β and hCG- β residue 74 with Ala was also tested because of the change in side-chain charge and backbone flexibility imparted by R74P and P74R substitutions.) In all cases, the resulting CG- β mutant subunits were similar to mCG- β in terms of microheterogeneity and apparent molecular mass by non-reducing SDS-PAGE, implying that both hCG- β residues Arg74 and Asn77 are necessary and sufficient to impart hCG- β -like conformational freedom to the CG- β subunit.

Multiple CG- β 1-53 domain residues may also affect CG- β conformational freedom

Shown in Fig. 2A and 3A, the m/h/h CG- β , m/h/m CG- β , and m(h74,77) CG- β chimeras appeared more heterogeneous than wild type hCG- β , suggesting that when CG- β residues 74 and 77 were of human origin, additional amino acids within the CG- β 1-53 domain influenced CG- β conformational freedom. To test this, we generated a series of CG- β constructs in which mCG- β chosen residues between 1-53 were swapped into hCG- β , generating hCG- β K2R, R8L, V18A, G22A, and T42M single amino acid substitutions (where each substitution represented a change from a wild type hCG- β residue to the mCG- β residue located at the same position), as well as h(m46-48) CG- β and h(m51-52) CG- β mutants. Purified, radiolabeled CG- β subunits were resolved by reducing and non-reducing SDS-PAGE.

Fig. 4A demonstrates that although the mobilities of hCG- β K2R, hCG- β G22A and h(m51,22) CG- β (lanes 3, 6 and 9, respectively) were indistinguishable from that of wild-type hCG- β (lane 2), hCG- β R8L, V18A, and T42M and h(m46-48) CG- β (lanes 4, 5, 7, and 8, respectively) migrated with a greater apparent molecular mass and microheterogeneity than wild type hCG- β (lane 2), and intermediate to that of m/h/h CG- β (lane 1) by non-reducing SDS-PAGE. That all observed differences were conformational in nature is shown in Fig. 4B, which reveals that all CG- β subunits migrated similarly under reducing conditions.

Since hCG- β R8L, V18A, T42M and m46-48 mutants displayed intermediate apparent molecular mass and microheterogeneity compared to the m/h/h CG- β chimera, it indicates that each of these individual substitutions made an independent contribution to the overall conformational freedom of the hCG- β subunit. Further, when we substituted the hV18 residue, which is known to make a major hydrophobic contribution to the earliest folding events of the hCG- β loop 1 domain (24), for the mA18 residue in chimera m/h/h CG- β (thus creating m/h/h CG- β A18V; lane 10), which is less hydrophobic in nature, this construct also displayed an intermediate level of microheterogeneity compared to the m/h/h CG- β chimera and comparable to those of hCG- β R8L, V18A, T42M and h(m46-48) CG- β subunits. Taken together, our data demonstrate that several individual hCG- β loop 1 and loop 2 amino acids also make a contribution to the conformational freedom of hCG- β , but that these contributions are nullified if CG- β residues 74 and 77 are of macaque origin (see Figs. 2 and 3).

Conformational freedom of the CG- β subunit may influence the biological activity of the CG heterodimer

Our results have demonstrated that hCG- β and mCG- β differ in their conformational freedom, but raises the question of whether these differences in β -subunit conformation are recapitulated in the CG heterodimer. To address this question, we compared the biological activity of the two CG heterodimers each containing the same, human α subunit. Recombinant hCG (Sigma) and high performance liquid chromatography-purified CG heterodimer consisting of hCG- α and mCG- β (here termed h- α /m- β CG) were used. Using HEK LH/CG-R cells expressing the human receptor and a cAMP-regulated luciferase reporter gene (12), the bioactivities of hCG and h- α /m- β CG were compared as described in Methods, generating the dose response curves shown in Fig. 5. Both hCG and h- α /m- β CG exhibited classic receptor-ligand response curves with overlapping minimal and maximal responses and with most of the difference between minimal and maximal responses spanning a two-log difference in CG concentration. hCG and h- α /m- β differed in their EC₅₀ values by roughly one-log concentration (~40 ng/ml vs. ~300 ng/ml, respectively). These data indicate that conformational differences like those seen in the hCG- β and mCG- β subunits were also seen in the respective CG heterodimers.

Discussion

The dimeric glycoprotein hormones hCG and mCG, which share >80% amino acid identity (8), both appear to have parallel physiological functions in pregnancy maintenance (25,26) and elicit PKA-mediated signal transduction events (18). Nonetheless, in the experiments reported here we demonstrate that the β -subunits of these two CG molecules were conformationally distinct. The differences in conformational freedom between h- and mCG- β suggest that mCG- β , which had a lower apparent molecular mass under non-reducing conditions, was more rod-shaped while hCG- β was more globular in structure. The nearly identical apparent molecular mass of these two CG- β species seen by reducing SDS-PAGE and our previous demonstration that mCG- β disulfide bond formation parallels that of hCG- β (7), suggest that the observed migrational differences between the two subunits under non-reducing conditions were not only conformational in nature, but also were independent of CG- β domains requiring native disulfide bonds.

Experiments conducted following construction of hCG-/mCG- β chimeras identified residues of CG- β that were responsible for the conformational variance between the hCG- β and mCG- β subunits. Human residues 54-101 most influenced overall hCG- β -like conformation; minimization of the 54-101 domain led to the discovery that a four amino acid stretch, residues 74-77, regulated CG- β conformation and of this small stretch, only two residues at CG- β codons 74 and 77, differed between h- and mCG- β . Both residues must be of the human type (Arg74 and Asn77) to allow the CG- β subunit to display the flexible hCG- β -like conformation; substitution of macaque-type residues (Pro74 and Asp77) resulted in a subunit with less flexible mCG- β -like characteristics.

The conformational switch attributed to residues 74 and 77 was unexpected. Residues 74-77 of CG- β are found near the apex of loop 3 and in the region of the CG- β 23-72 disulfide bond (see Fig. 6A) (27,28). Substitution of either human residues 74 or 77 with the respective macaque residues results in an overall negative charge for the region (R74P, and N77D). We can envision two explanations for how residues 74 and 77 may act to alter CG- β conformation. According to the hCG crystal structure (27,28), Lys20 resides within 5 Å of Asn77. Substitution of the human Asn77 with the negatively charged macaque Asp77 appears capable of creating a stabilizing salt bridge with Lys20 (see Fig. 6). Substitution of the charged human Arg74 with the conformationally-constraining macaque Pro74 also appears to decrease overall flexibility of loop 3, affecting side-chain packing between β loops 1 and 3, thereby affecting overall

conformational stability (see Fig. 6). Because conserved residue $\beta 73$ is also a proline, mCG- β loop 3 contains adjacent proline residues, which would be expected to constrain loop 3 backbone rotational freedom. Thus, it appears that the observed decreased inherent flexibility of mCG- β could arise from either of these amino acid differences, likely being reinforced by the presence of both.

Alignment of the hCG- β crystal structure with the data presented here revealed more unexpected findings. As shown in Fig. 6B, most of the residues that influence hCG- β conformational freedom were found distal to the CG- β core residing, instead, at the periphery of the subunit. Residues 46-48 and 74 are located at the apexes of loops 2 and 3, respectively, while 42 and 77, while somewhat closer to the CG- β core, protrude outwards into solution. Residue 18 has been previously identified as a component of a CG- β hydrophobic core with Leu16, Ile27, Val29, Ile67, Leu69, Val80, and Tyr82 (28) and a substitution from 18Val to 18Ala could potentially destabilize this core, increasing the conformational freedom of CG- β . This helps explain why m/h/h CG- β exhibited such extensive microheterogeneity compared to either h- or mCG- β , while m/h/h CG- β A18V exhibited less extensive microheterogeneity than m/h/h CG- β (Fig. 4A, lane 1, 2, and 10).

While the crystal structure (27,28) and the in vivo folding pathway of CG- β (7) provide insight into the structural nature of CG, both assume a static final structure and do not take into account the potential flexibility of the molecule. Erbel et al. (29) have demonstrated that the solution structure of the heterodimeric partner of CG- β , the α -subunit, possesses extraordinary structural flexibility, or inherent disorder, between residues 33 and 57, with the remainder of the protein exhibiting structurally defined domains. This flexible domain of the α -subunit adopts a more rigid conformation when bound to a glycoprotein hormone β -subunit and this conformation varies according to the respective α -subunit binding partner (27,28,30,31). This suggests that CG subunits are not static but rather flexible, owing, at least in part, to inherent disorder.

We wanted to determine if differences in conformation between hCG- β and mCG- β were recapitulated in the assembled, bioactive CG heterodimer, but we could not address this directly by SDS-PAGE because the non-covalently bound subunits of the CG heterodimer dissociate under SDS-PAGE conditions. Instead, a biological assay was used to investigate the significance of these differences in conformation in the context of an intact heterodimer. As shown in Fig. 5, a CG heterodimer containing the mCG- β subunit, while biologically active, was less efficacious than hCG at eliciting a PKA-mediated signal transduction event via the human LH/CG-R. Because hCG and h- α /m- β CG both contain the same α -subunit, the observed difference in biological activity between these heterodimers was attributed to conformational differences in the β -subunits. These results suggest that conformational freedom within the ligand subunits also influenced the signal transduction capabilities of the assembled heterodimer.

Recent advances in the study of protein intrinsic disorder predict that many, if not most, proteins encoded by eukaryotic genomes contain intrinsically disordered regions, if not entire domains (32). This estimation was generated by the Predictor of Natural Disorder (PONDR [Molecular Kinetics]; www.pondr.com), a commonly cited algorithm for the prediction of regions of intrinsic disorder (33-35). While PONDR is not capable of predicting interactions spanning long distances in terms of primary amino acid sequence (i.e., interdomain or interprotein disulfide bonds or ionic interactions), it is useful for identifying domains that are potentially disordered. As shown in Fig. 7A, PONDR analysis of the amino acid sequence of the α -subunit successfully predicts the existence of an inherently disordered domain that perfectly matches the flexible domain detected by Erbel et al. (29). PONDR analysis of hCG- β and mCG- β also provided clues to the conformational freedom of the CG- β subunits. Shown in Fig. 7B, the CG- β subunit was predicted to have extensive conformational freedom at its C-terminus, which is

in agreement with numerous studies documenting the inherent flexibility of the C-terminal peptide of CG- β (1,28). PONDR analysis also predicted a disordered domain between residues 1-20 and our results demonstrated that residues 8 and 18 regulated CG- β conformational freedom. However, while PONDR predicted that the macaque domain 70-80 would be more disordered than a human domain 70-80, our results show that the opposite was true. The mechanism by which residues 74 and 77 regulated conformational freedom may involve global interactions in mCG- β not present in hCG- β , which was not predicted by either PONDR (Fig. 7) or SYBYL modeling software (data not shown).

In summary, we have identified the 74-77 sub-domain of CG- β loop 3 as a mediator of CG- β conformational freedom, which in mCG- β may result from interactions with residues of β loop 1 (i.e., a potential salt bridge between residues Lys20 and Asp77 in mCG- β) and/or conformational constraints centered about mCG- β residues Pro73 and Pro74. As alluded to above, the ultimate conformation of all dimeric glycoprotein hormones is reliant on the conformation of their β -subunits, which suggests that CG biological activity could be affected by its inherently disordered domains, which was consistent with the results of our biological activity assay. Perhaps during the course of human evolution, the overall inherent disorder of CG- β increased and in doing so, provided a selective signaling advantage for hCG following binding of the more flexible ligand to its receptor. This is consistent with the observation that hCG- β residues 74-77 are conserved in non-human primates (8) and raises further questions about the functional significance of this evolutionary divergence.

Acknowledgements

The authors thank Dr. John S. Davis for helpful discussions and Claudia MacDonald for her technical assistance.

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ABBREVIATIONS

cAMP

cyclic adenosine monophosphate

hCG

human chorionic gonadotropin

HEK LH/CG-R

human embryonic kidney cells expressing the luteinizing hormone/chorionic gonadotropin receptor

mCG

macaque chorionic gonadotropin

PONDR

predictor of natural disorder

SDS-PAGE

sodium dodecyl sulfate polyacrylamide gel electrophoresis

Figure 1. Amino acid sequences of CG- β constructs

Shown are the complete primary amino acid sequences of hCG- β (10), mCG- β (8), and chimeric CG- β constructs. Bolded amino acids represent sequences of non-identity between human and macaque CG- β . The arrows delineate the boundaries between the 1-53, 54-101, and 102-145 domains.

Figure 2. A determinant in the 54-101 domain of CG-β modulates CG-β conformational freedom
Panel A. Purified hCG-β, mCG-β and selected chimeras assayed by non-reducing SDS-PAGE. Lane 1: hCG-β; lane 2: h/h/m CG-β; lane 3: h/m/h CG-β; lane 4: h/m/m CG-β; lane 5: m/h/h CG-β; lane 6: m/h/m CG-β; lane 7: m/m/h CG-β; lane 8: mCG-β. Panel B. The same CG-β subunits described in Panel A were assayed by reducing SDS-PAGE.

Figure 3. The CG-β 74-77 domain modulates CG-β conformational freedom

Panel A. Purified hCG-β 54-101 domain mutants assayed by non-reducing SDS-PAGE. Lane 1: hCG-β; lane 2: mCG-β; lane 3: h(m74,77) CG-β; lane 4: h(m82,83) CG-β; lane 5: h(m89,91) CG-β; lane 6: m(h74,77) CG-β; lane 7: m(h82,83) CG-β; lane 8: m(h89,91) CG-β; lane 9: hCG-β R74P; lane 10: hCG-β R74A; lane 11: hCG-β N77D; lane 12: mCG-β P74R; lane 13: mCG-β P74A; lane 14: mCG-β D77N. Panel B. The same CG-β subunits described in Panel A were assayed by non-reducing SDS-PAGE.

Figure 4. Multiple residues of the hCG- β 1-53 domain can influence conformational freedom

Panel A. Purified hCG- β 1-53 domain mutants assayed by non-reducing SDS-PAGE. Lane 1: m/h/h CG- β ; lane 2: h CG- β ; lane 3: hCG- β K2P; lane 4: hCG- β R2L; lane 5: hCG- β V18A; lane 6: hCG- β G22A; lane 7: hCG- β T42M; lane 8: h(m46-48) CG- β ; lane 9: h(m51-52) CG- β ; lane 10: m/h/h CG- β A18V. Panel B. The same CG- β subunits described in Panel A were assayed by non-reducing SDS-PAGE.

Figure 5. Dose response of hCG vs. h- α /m- β CG on HEK LH/CG-R cells

HEK LH/CG-R cells were treated with hCG (open squares) or h- α /m- β CG (open circles) in half-log concentration increments. Following overnight treatment, cells were lysed and assayed for cAMP-regulated luciferase reporter activity by luminescence assay. All samples were tested in triplicate, with standard deviation bars shown.

Figure 6. Molecular modeling of CG-β

Panel A. The crystal structure of hCG- β loops 1 and 3, based on the coordinates defined by Wu et al. (27), is depicted using MBT Protein Workshop 1.35 software, with the human and macaque (h/m) residues 74 and 77 highlighted. Lys20 is also highlighted to demonstrate the potential for electrostatic interaction between Lys20 and Asp77 in mCG- β . Cys23 and Cys72 are highlighted to demonstrate the proximity of the 23-72 disulfide bond. Panel B. Shown are two views of hCG- β (27), rotated 180 degrees, using MBT Protein Workshop 1.35 software. Residues influencing CG- β conformational freedom when residues β 74 and β 77 were of human origin (i.e., β -8, 18, 42, 46-48, 74, and 77) are shaded dark.

Figure 7. PONDR analysis of CG subunit conformational freedom

The computer algorithm PONDR (Molecular Kinetics; www.pondr.com) (33-35) VL-XT Predictor was used to predict regions of conformational freedom (or disorder) within CG subunits. Panel A. PONDR analysis of hCG- α predicted a disordered domain between amino acid residues 35-55, in agreement with the findings of Erbel et al. (29). Panel B. PONDR analysis of hCG- β predicted disordered regions spanning residues 40-50, 70-80, and 110-145. No structural data for residues beyond Asp111 are defined by Lapthorn et. al. (28), in agreement with PONDR analysis. Panel C. PONDR analysis of mCG- β predicted domains that were similarly disordered to those of hCG- β .