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The Human Chorionic Gonadotropin- β Arginine⁶⁸ to Glutamic Acid Substitution Fixes the Conformation of the C-Terminal Peptide

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Wild-type human chorionic gonadotropin (hCG) has been used as a contraceptive vaccine. However, extensive sequence homology with LH elicits production of cross-reactive antibodies. Substitution of arginine⁶⁸ of the β -subunit (hCG β) with glutamic acid (R68E) profoundly reduces the cross-reactivity while refocusing the immune response to the hCG β -specific C-terminal peptide (CTP). To investigate the molecular basis for this change in epitope usage, we immunized mice with a plasmid encoding a truncated hCG β -R68E chain lacking the CTP. The animals produced LH-cross-reactive antibodies, suggesting that the refocused immunogenicity of R68E is a consequence of epitope masking by a novel disposition of the CTP in the mutant rather than a structural change in the cross-reactive epitope region. This explanation was strongly supported by surface plasmon reso-

nance analysis using a panel of anti-hCG β -specific and anti-hCG β /LH cross-reactive monoclonal antibodies (mAbs). Whereas the binding of the LH cross-reactive mAbs to hCG β -R68E was eliminated, mAbs reacting with hCG β -specific epitopes bound to hCG β and hCG β -R68E with identical affinities. In a separate series of experiments, we observed that LH cross-reactive epitopes were silent after immunization with a plasmid encoding a membrane form of hCG β -R68E, as previously observed with the soluble mutant protein itself. In contrast, the plasmid encoding the soluble secreted form of hCG β -R68E evoked LH cross-reactive antibodies, albeit of relatively low titer, suggesting that the handling and processing of the proteins produced by the two constructs differed. (*Molecular Endocrinology* 19: 1803–1811, 2005)

HUMAN CHORIONIC gonadotropin (hCG) is a member of the glycoprotein hormone family, which also includes LH, FSH, and TSH. The glycoprotein hormones are heterodimeric molecules consisting of a common α -chain noncovalently associated with a hormone-specific β -chain. In a healthy woman, biologically active hCG is only present in significant concentrations during pregnancy where it stimulates the corpus luteum to produce progesterone and estrogen required for sustaining the implanted embryo.

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Abbreviations: CBB, Carbonate-bicarbonate buffer; CTP, C-terminal peptide; HBS, HEPES-buffered saline; hCG, human chorionic gonadotropin; mAb, monoclonal antibody; PBS-T, PBS containing 0.05% Tween 20; RU, resonance unit.

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Incubation of marmoset embryos with hCG-specific antibodies prevents implantation (1), and hCG is considered an antifertility vaccine candidate (2–5). Phase II trials of a heterospecies vaccine consisting of an ovine α -chain and hCG β -chain coupled to tetanus toxoid or diphtheria toxoid resulted in only one pregnancy among 1224 cycles in those immunized women producing circulating anti-hCG antibodies above 50 ng per ml (2). However, due to the 85% sequence homology between the first 110 amino acid residues of hCG β and LH β , most of the anti-hCG antibodies produced cross-reacted with LH (3). Although no adverse effects of the LH-cross-reactive antibodies were observed in the cohort studied (3), the long-term presence of such antibodies may cause undesired side effects.

With the aim of producing a potentially safer hCG-based vaccine, we have constructed a series of hCG β mutants in which amino acid residues at presumed LH cross-reactive epitope regions have been substituted (6). One of these mutants, hCG β -R68E, with arginine⁶⁸

substituted with glutamic acid, failed to bind all tested LH-cross-reactive monoclonal antibodies (mAbs) without apparently affecting the natural folding of the molecule, as judged by the ability of the conformation-dependent hCG-specific mAbs to recognize the protein (6). Baculovirus-produced hCG β -R68E elicited antibodies with minimal LH cross-reactivity in both mice and rabbits (7, 8). The antibodies produced were predominantly directed against sequences present on the hCG β -specific C-terminal peptide region (hCG β CTP; amino acids 113–145), which is missing in LH β and has been generated during evolution by a read-through event. This peptide region adopts a free-floating entropy-rich conformation in hCG (9) and consequently is poorly immunogenic (7, 8). We hypothesized that glutamic acid⁶⁸ in the mutant forms a salt bridge with one or more of the basic amino acid residues present in the hCG β CTP, thus providing the hCG β CTP with a defined conformation that creates a new and more immunogenic loop.

We have used DNA immunization and analysis by surface plasmon resonance to further probe the structure of hCG β -R68E. We show here that truncation of the CTP element of hCG β -R68E (hCG β -R68E- Δ CTP) restores the immunodominance of the LH-cross-reactive epitope region so that mice immunized with a plasmid expressing hCG β -R68E- Δ CTP produce antibodies that recognize hCG, LH, and hCG β -R68E equally well. Biosensor analysis is often used to determine the thermodynamics of antibody-antigen interactions. Even subtle changes in the structure of the antigen can have profound consequences for the kinetics of formation or dissociation of the protein-protein complexes. Optical biosensor analysis of the conformational integrity of hCG β -R68E confirmed that whereas LH-cross-reactive epitopes were inaccessible in the mutant, hCG β -specific epitopes adjacent to the cross-reactive epitope region were unaffected by the substitution. This is consistent with our proposed model postulating formation of a salt bridge between glutamic acid⁶⁸ and a basic residue on the CTP, which masks the LH-cross-reacting epitopes. The identity of the charge on CTP remains to be determined, but both K¹²² and R¹³³ contribute to the formation of the loop structure.

RESULTS

Immunization

BALB/c mice immunized with expression plasmids encoding either membrane-attached (pCDNA3-hCG β -TM) or soluble (pCDNA3-hCG β -His₆) forms of hCG β (Fig. 1) produced antibodies that bind equally well to holo-hCG, recombinant hCG β produced in baculovirus (Bac-hCG β -WT), and the mutant hCG β -R68E produced in baculovirus (Bac-hCG β -R68E) and cross-reacted with LH (Fig. 2). This contrasts with the results obtained after immunization with pCDNA3-hCG β -R68E. As expected, mice immunized with DNA encod-

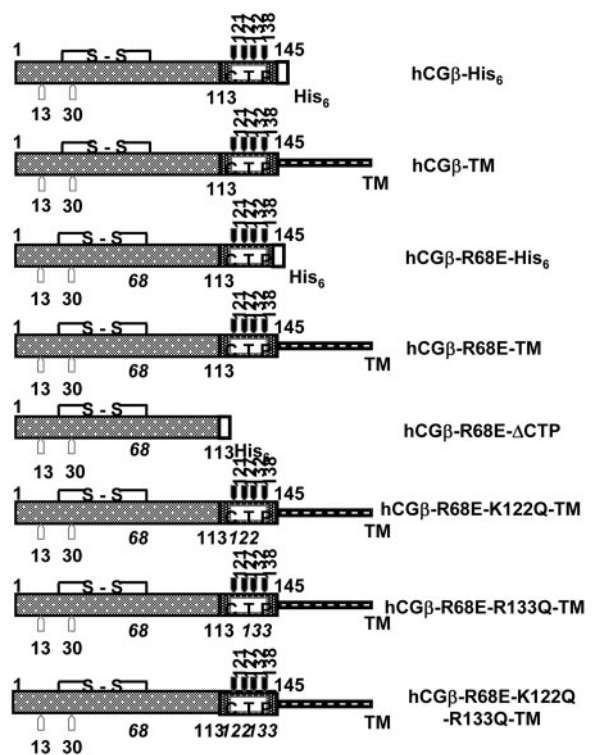


Fig. 1. Diagram of the hCG β -Related Immunogens Used

The N-linked and the O-linked glycosylation sites are indicated with an *open* and a *solid* arrow, respectively. The numbering of amino acid residues relates to hCG β , and the positions of the mutated residues are indicated in *italic*.

ing a membrane-attached form of hCG β -R68E (pCDNA3-hCG β -R68E-TM) produced antibodies that bind well to Bac-hCG β -R68E. The sera from these mice reacted poorly with holo-hCG and the free Bac-hCG β subunit and showed no cross-reactivity with LH (Fig. 2), consistent with the results obtained after intranasal immunization of mice with purified Bac-hCG β -R68E mixed with *Escherichia coli* heat-labile protein (7). This contrasts with the result obtained in mice immunized with pCDNA3-hCG β -R68E-His₆ encoding a secreted form of hCG β -R68E, which resulted in antibodies that bind to Bac-hCG β but less so to holo-hCG and LH although they reacted more strongly with Bac-hCG β -R68E.

This is consistent with our proposed structure of hCG β -R68E, which envisages the normally entropy-rich hCG β CTP becoming conformationally fixed by formation of a salt bridge between glutamic acid⁶⁸ and one or more of the basic residues present in the hCG β CTP (8, 10). This model predicts that mice immunized with a truncated hCG β -R68E molecule without CTP (hCG β -R68E- Δ CTP) would produce antibodies that bind to hCG, and notably LH, equally well as to hCG β -R68E itself. As shown in Fig. 2, mice immunized with pCDNA3-hCG β -R68E- Δ CTP did elicit antibodies that bind equally well to holo-hCG, LH, and Bac-hCG β . Although the antigen-specific IgGs react stron-

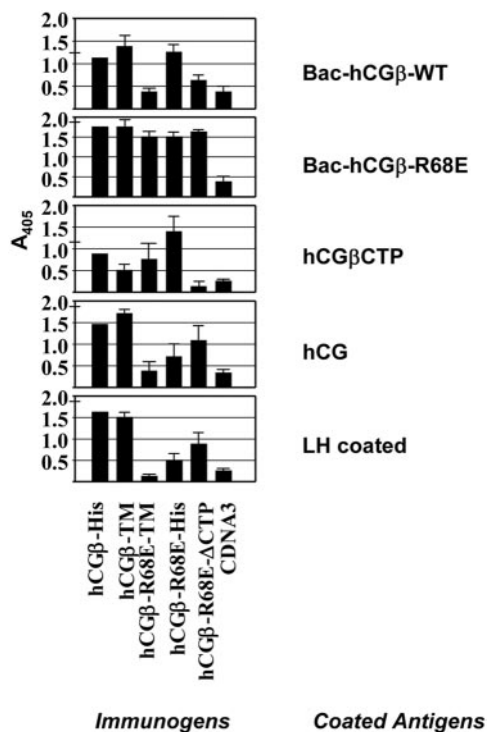


Fig. 2. The Level of hCG-Specific Antibodies after Immunization with Plasmid DNA

Female BALB/c mice ($n = 5$) were immunized with 50 μ g plasmid DNA expressing membrane-attached (TM) or secreted (His) hCG β or hCG β -R68E or truncated hCG β -R68E (hCG β -R68E- Δ CTP) devoid of the CTP. Sera (dilution 1:50) were analyzed in ELISA using plates coated with proteins as indicated, and the results are expressed as optical density \pm SD.

ger with Bac-hCG β -R68E, there was no statistical difference [$P = 0.11$, paired Student's t test (LH coating vs. Bac-hCG β coating); $P = 0.09$, paired Student's t test (LH coating vs. recombinant hCG coating)] between the results obtained with Bac-hCG β , holo-hCG, or LH. This supports the hypothesis that the CTP attains a conformation in hCG β -R68E, which blocks the accessibility of cognate B cells to the cross-reactive epitope region on the body of the hCG β molecule.

Thermodynamic Analysis

To probe further into the conformation of hCG β -R68E, we used surface plasmon resonance to determine the association and dissociation rates and affinity constants of selected hCG-reactive mAbs, because even modest structural changes in the overall conformation of the core domain of hCG β as a result of the glutamic acid⁶⁸ substitution would affect the thermodynamic properties of the antibodies' binding. For this analysis, a panel of mAbs directed to LH-cross-reactive and hCG β -specific epitopes (11, 12) were selected (Table 1). INN-22 and INN-58 have specificity for the immunodominant LH-cross-reactive epitopes, β 2 and β 5

Table 1. Specificities of the mAbs Used in the Present Study

mAb	Epitope	Epitope Properties
INN-2	β 1	hCG specific
INN-22	β 2	hCG/LH cross-reactive
INN-58	β 5	hCG/LH cross-reactive
INN-64	β 6	hCG specific ($\alpha\beta$ -interface)
INN-68	β 7	hCG specific ($\alpha\beta$ -interface)

(12), respectively. As representative for the hCG β -specific mAbs, INN-2, INN-64, and INN-68, which bind to β 1, β 6, and β 7 (12), respectively, were selected. Epitope β 1 is accessible on holo-hCG and includes among its contact residues arginine¹⁰ and arginine⁶⁰ close to the cystine knot (13), whereas β 6 and β 7 are both located in the interface between the α - and β -subunit and are inaccessible on holo-hCG (12). Epitope β 6 is spatially close to lysine²⁰, glutamic acid²¹, glycine²², and glycine⁷⁵ (6), whereas epitope β 7 is affected by mutations of the residues in position 61 and 89 (13).

To reduce cross-linking by the bivalent IgGs, Fab fragments of each mAb were generated by papain digestion. The Fab fragments obtained from the individual Igs contained limited but varying amounts of undigested Igs in the samples. Although the calculated equilibrium constant therefore cannot be interpreted as an absolute value, the data obtained for wild-type mutant hCG β can nevertheless be compared, because the same Fab/antibody ratio was used for both proteins.

Figure 3 shows the sensorgrams for the binding of INN-2, INN-22, INN-58, INN-64, and INN-68 to Bac-hCG β -WT and Bac-hCG β -R68E, and the resulting association rate constant (k_a), dissociation rate constant (k_d), and apparent dissociation equilibrium constant K_D are summarized in Table 2. The five Fabs recognized Bac-hCG β -WT with a dissociation equilibrium constant varying between 8.2×10^{-8} M for INN-2 and 0.7×10^{-8} M for INN-68 Fab. This compares with the affinities of the mAbs determined by saturation or competitive RIA (14, 15). This demonstrates that the recognition of the five spatially distinct antigenic sites on Bac-hCG β -WT by these antibodies has not been affected dramatically by production of the hCG β subunit in insect cells, indicating that differences in post-translational modifications, such as glycosylation, in insect cells do not substantially modify the overall three-dimensional structure of the protein.

The glutamic acid⁶⁸ substitution in Bac-hCG β -R68E abrogates the binding of the LH-cross-reactive Fabs INN-22 and INN-58, whereas binding of the Fabs to the hCG-specific epitopes, β 1, β 6, and β 7, was unaffected by the mutation (Fig. 3 and Table 2) in agreement with the results obtained by flow cytometric analysis of transiently transfected COS-7 cells (6). At the same concentration at which the cross-reactive Fabs (INN-22 and INN-58) reacted with Bac-hCG β -WT, minimal binding to Bac-hCG β -R68E was observed.

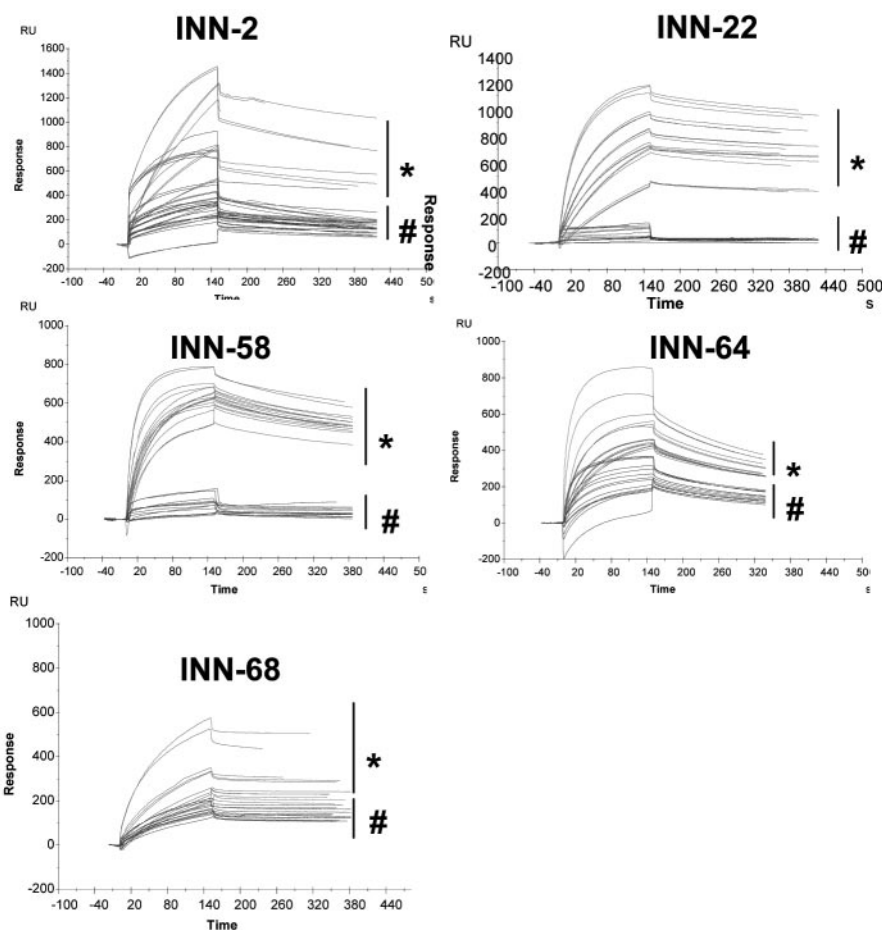


Fig. 3. Sensorgram Showing the Binding of Fabs to Bac-hCG β or Bac-hCG β -R68E

Recombinant Bac-hCG β or Bac-hCG β -R68E was bound to Ni²⁺-charged nitrilotriacetic acid sensor chips at a concentration equivalent to 500 RU. A solution (50 μ l) containing a predetermined dilution of the appropriate Fab (INN-2; INN-22; INN-58; INN-64; and INN-68) was passed onto the chip at a rate of 20 μ l/min. The sensorgrams were corrected for the constant release of His-tagged recombinant protein, normalized, and overlaid. *, Sensorgrams obtained with Bac-hCG β ; #, sensorgrams obtained with Bac-hCG β -R68E.

Indeed, the association rates of the two cross-reactive Fabs were very similar to that obtained for the isotype-matched control antibody (data not shown). In contrast, the β 6 (INN-64) and β 7 (INN-68) epitopes located at the interface between the α - and β -subunit remained unchanged by the mutation. The association and dissociation rate constants for the binding of each of these antibodies to Bac-hCG β -WT and Bac-hCG β -R68E were not statistically different (Table 2). Both β 6 and β 7 are spatially relatively adjacent to the cross-reactive epitope region recognized by INN-22 and INN-58. Thus the fact that the binding affinities of INN-64 and INN-68 are unaffected by the glutamic acid⁶⁸ substitution implies that the overall structure of hCG β is maintained.

The β 1-specific INN-2 antibody was previously thought to recognize the wild-type and mutant protein equally well (6). The biosensor analysis showed, however, a slightly higher association rate ($k_a = 2.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) for INN-2 binding to the mutant protein

than that observed for the binding to Bac-hCG β -WT ($k_a = 1.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$). Nonetheless, the complex formed with the mutant protein appeared to be less stable with a $k_d = 2.7 \times 10^{-3} \text{ sec}^{-1}$ for Bac-hCG β -R68E compared with $k_d = 1.8 \times 10^{-3} \text{ sec}^{-1}$ for the wild-type form of hCG β (Table 2). Overall, the affinity of binding was significantly greater for the wild-type protein ($K_D = 8.2 \times 10^{-8} \text{ M}$) compared with that of the mutant ($K_D = 11.1 \times 10^{-8} \text{ M}$; $P < 0.001$). Nevertheless, the difference in free energy changes between INN-2 binding to the mutant and the wild-type protein was minimal ($\Delta\Delta G = 0.163 \text{ kcal/mol}$). The binding of INN-2 to hCG involves residues arginine¹⁰ and arginine⁶⁰ (13). It seems unlikely, however, that an amino acid substitution localized more than 30 Å away from the β 1 epitope, which does not modify the conformation of more proximal epitopes, could affect the recognition pattern of this epitope. However, the formation of a salt bridge between the glutamic acid⁶⁸ and the basic residues of the hCG β CTP could affect the spatial ori-

Table 2. Kinetic Parameters for Reaction of Fab Fragments of the INN-2, INN-22, INN-58, INN-64, and INN-68 Antibodies with Bac-hCG β -WT and Bac-hCG β -R68E

Antibody	Antigen	Apparent K_D (10^{-8} M)	k_a (10^4 M $^{-1}$ sec $^{-1}$)	k_d (10^{-3} sec $^{-1}$)	WT/R68E ^a	ΔG^b (kcal/mol)	$\Delta\Delta G^c$ (kcal/mol)
INN-2 Fab	WT	8.2 \pm 1.1 ^d	1.5 \pm 0.2	1.8 \pm 0.37	$P = 0.0003^a$	-9.6	0.163
	R68E	11.1 \pm 2.1	2.5 \pm 0.6	2.7 \pm 0.5		-9.4	
INN-22 Fab	WT	1.8 \pm 0.4	3.6 \pm 0.7	0.6 \pm 0.05		-10.5	
	R68E	N.A.	N.A.	N.A.			
INN-58 Fab	WT	1.1 \pm 0.2	12.1 \pm 1.9	1.3 \pm 0.1		-10.8	
	R68E	N.A.	N.A.	N.A.			
INN-64 Fab	WT	4.4 \pm 1.1	6.6 \pm 1.4	2.8 \pm 0.2	$P = 3.7$	-10.0	0.002
	R68E	4.2 \pm 1.6	5.4 \pm 1.1	2.3 \pm 0.1		-10.0	
INN-68 Fab	WT	0.7 \pm 0.2	2.8 \pm 0.6	0.2 \pm 0.04	$P = 0.12$	-11.0	0.162
	R68E	1.1 \pm 0.3	2.4 \pm 0.6	0.2 \pm 0.1		-10.9	

N.A., Not ascertainable because binding is comparable to isotype control Fab and therefore too low to permit calculation of binding constants; WT, wild type.

^a Student's *t* test was used to calculate the *P* value for K_D .

^b The variation in free energy upon binding was determined using: $\Delta G = -RT \ln(k_a/k_d)$.

^c The difference in free energy changes between the mutant and the wild-type protein were calculated as: $\Delta\Delta G = \Delta G_{R68E} - \Delta G_{WT}$.

^d \pm SD.

entation of the oligosaccharide antennae, resulting in an increase in the association rate of INN-2. The increased instability of the complex formed with the mutant protein could be related to some steric hindrance of the antibody by the fixed CTP of hCG β -R68E.

CTP Mutations

Our model predicts that the presence of an acidic residue (glutamic acid) at position 68 of hCG β changes the chemical characteristic of this region of the protein structure. Normally this region of the structure is flat with two protruding amino acid residues, R68 and R74, approximately 10Å apart, both with a net positive charge. The substitution R68E changes both the shape and charge of this area of hCG β , which is part of the cross-reactive epitope, although it is unlikely to greatly modify the folded structure of the protein that is tethered by the disulfide C²³-C⁷². A working hypothesis for the masking of the cross-reactive epitopes is that R68E forms a salt bridge with one or more of the basic amino acid residues in the CTP. There are three possible candidate residues, R¹¹⁴, K¹²², and R¹³³ (Fig. 4). Both R¹¹⁴ and R¹³³ have negatively charged residues in relative close proximity within the peptide, which could form additional charged interactions with R⁷⁴ on the same surface. R¹³³ is an unlikely candidate as it forms part of the epitope for mAb OT3A (amino acids 133–139) the binding of which has been shown to be unaffected in the R68E mutant (6). R¹¹⁴ is also in doubt if the β -subunit disulfide C²⁶-C¹¹⁰ is formed as this would tether the CTP on the opposite side of the β -subunit and R¹¹⁴ would be too far away from R68E to form a salt bridge. However if this disulfide were not formed (due to the absence of the α -subunit), then the flexibility of the "seat belt" region of the β -subunit would probably

permit R¹¹⁴ to form a salt bridge. In an attempt to identify the putative donor residue for the postulated salt bridge with R68E, we selectively substituted K¹²² and R¹³³ on hCG β -R68E with glutamine individually or together (Fig. 1) and immunized groups of BALB/c mice with plasmids precipitated onto gold particles. As shown in Fig. 5, the strongly CTP-focused and reduced hLH cross-reactive antibody response obtained with pCDNA3-hCG β -R68E-TM was not obtained after immunization with any of the three CTP mutants: pCDNA3-hCG β -R68E-K122Q-TM, pCDNA3-hCG β -R68E-R133Q-TM, or pCDNA3-hCG β -R68E-K122Q-R133Q-TM. The immune response with each of the CTP mutants was similar to that obtained after immunization with pCDNA3-hCG β -TM. This confirmed that the conformation of CTP is fixed in hCG β -R68E but that both K¹²² and R¹³³ contribute to establish this conformational change.

DISCUSSION

A single arginine⁶⁸ to glutamic acid substitution in hCG β dramatically alters the antigenicity and immunogenicity of the mutant molecule. The immunodominant LH cross-reactive epitope cluster on hCG has been defined by several mAbs and comprises several epitopes (β 2, β 3, β 4, and β 5) (12) that may share one or more contact residues as we have previously suggested (16). Representative mAbs defining each of these epitopes fail to bind to recombinant hCG β -R68E expressed in either COS7 cells (6) or insect cells (Table 2). We have identified, however, one CTP-reactive mAb (2F4/3) that reacts more avidly with hCG β -R68E than with hCG or hCG β (7), suggesting that the glutamic acid⁶⁸ substitution affects the conformation of the normally entropy-rich, free-floating CTP. Indeed, immunogenicity studies with baculovirus-derived

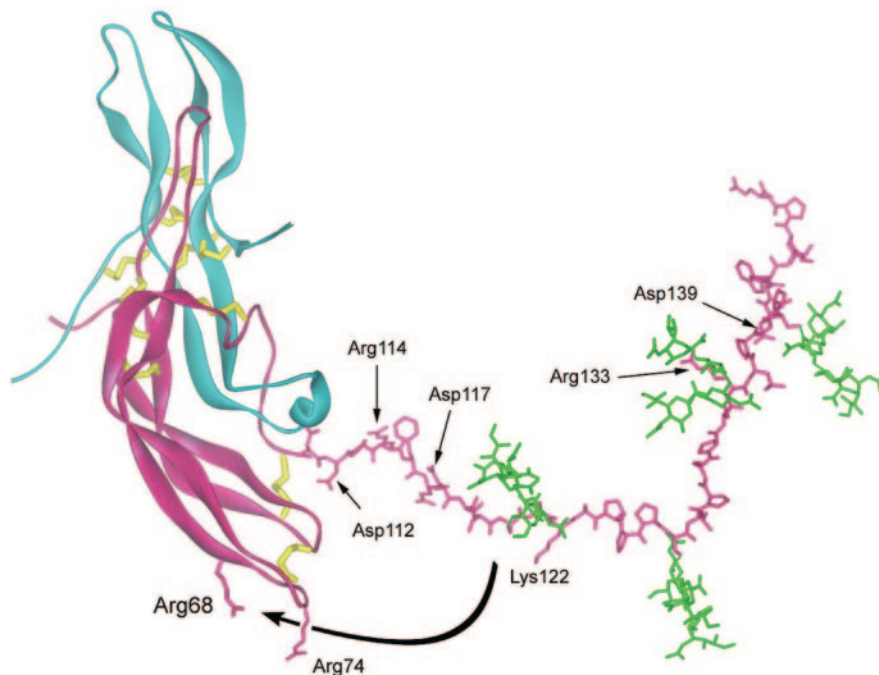


Fig. 4. A Ribbon Representation of a Molecular Model of hCG (Based on Protein Data Base Entry 1 HRP) with the α -Subunit (Cyan), β -Subunit (Magenta), and Disulfide Bridges (Shown in Yellow)

A chemically sensible conformation of the CTP (not seen in the x-ray structure) is modeled with O-linked carbohydrates shown in green. The CTP is able to wrap around the β -subunit in the R68E mutant forming an interaction that blocks the LH cross-reactive epitope. Charged amino acid residues of the CTP are labeled.

hCG β -R68E in both mice (7) and rabbits (8) show that the immune response is refocused toward epitope(s) present on the CTP at the expense of the major LH cross-reactive epitope normally used by hCG/hCG β as also demonstrated in the present study after DNA immunization of mice with pCDNA3-hCG β -R68E-TM or pCDNA3-hCG β -R68E-His₆ (Figs. 2 and 5).

To explain these findings we have proposed that the presence of an acidic residue (glutamic acid) at position 68 of hCG β changes the chemical characteristic of this region of the protein structure. The mutation R68E could potentially permit the formation of an internal salt bridge between the glutamic acid and a positive charge associated with the CTP, thereby fixing the structure of the CTP by forming a new loop structure. Elimination of the CTP on the hCG β -R68E immunogen (hCG β -R68E- Δ CTP) elicited antibodies that reacted equally well with hCG, LH, and Bac-hCG β -R68E in contrast to antibodies produced after immunization with pCDNA3-hCG β -R68E-TM or pCDNA3-hCG β -R68E-His₆. The presence of a new loop structure is further supported by real-time binding kinetics of mAbs to wild-type and mutant Bac-hCG β using surface plasmon resonance. Whereas the β 2- and β 5-specific LH cross-reactive mAbs fail to bind Bac-hCG β -R68E, the mAbs INN-64 and INN-68, specific for epitopes β 6 and β 7, which include residues located both on loop 1 and loop 3 of the β -subunit (6), bind to the wild-type and the mutant subunit with nearly identical association and dissociation rates (Table 2). This demonstrates that glu-

tamic acid⁶⁸ does not introduce major structural changes that affect the relative spatial arrangement of loops 1 and 3, which present these two epitopes. Rather, it seems more probable that a salt bridge might be formed between glutamic acid⁶⁸ and one or more of the basic residues on CTP (K¹²² and R¹³³), thereby masking the entire cross-reactive epitope cluster and eliminating their accessibility for both isolated LH-cross-reactive mAbs and potential B cell receptors with specificity for this region of hCG. However, both K¹²² and R¹³³ appear important for establishing this additional loop structure, because selectively substituting either of these residues with a noncharged glutamine abolished the CTP-loop structure of hCG β -R68E. It is surprising that both these mutants have an effect, ruling out a simple salt bridge between E⁶⁸ and one of the residues. Both K¹²² and R¹³³ occupy rather similar positions in sequence (*i.e.* both follow a serine that is O-linked glycosylated in the CTP), so substituting either of these residues with the neutral glutamine may alter the O-linked glycans on the CTP. Equally, these residues may be important in fixing the CTP structure in hCG β -R68E but not directly via an interaction with residue 68. Clearly, a more detailed structural analysis of hCG β -R68E is required to determine the structure of the CTP in this mutant protein and the role of the individual amino acids in interactions with the main body of hCG β .

Regarding antibody recognition of the other, non-CTP but hCG β -specific, epitopes we noted less binding (at a molar basis) of the panel of mAbs to Bac-

Mathiesen (18). Briefly, silver rod electrodes were placed on the skin at the site of DNA injection, and the muscles were subjected to eight trains of 1000 pulses delivered at a frequency of 1000 Hz using a Hear 6-bp stimulator (Frederick Hear, Bowdoinham, ME). Each pulse lasted 400 μ sec. The electric field strength was approximately 50 V over 3–4 mm, and each train was delivered at 2-sec intervals with each train lasting 1 sec.

For particle-mediated DNA delivery the plasmids (2 μ g/mg gold) were precipitated onto 2- μ m diameter gold particles (DeGussa Metals Group, South Plainfield, NJ) in the presence of 0.05 M spermidine (Sigma) and 1 M calcium chloride. The gold particles were washed three times in absolute alcohol containing 0.15 M polyvinylpyrrolidone (Sigma), and then adsorbed to the inner surface of Tefzel tubing (TFX Medical, Inc., Jaffrey, NH) by centrifugal force. The Tefzel tubes were subsequently cut into 1.27-cm long cartridges and stored desiccated at 4 C. A cartridge contains 0.5–0.75 μ g plasmid DNA/0.5 mg gold particles. The DNA-coated gold particles were delivered from cassettes at 500 pounds/square inch of pressure at two sites of shaved abdomen (1.0–1.5 μ g total DNA) by particle-mediated DNA delivery using the Helios Gene Gun (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Mice were immunized at wk 0 and received a boost 4 wk later.

At the end of the experiment, the animals were killed by exsanguination. All animal experiments were carried out according to United Kingdom Home Office guidelines.

Immunoassays

MaxisorpC plates were coated at 4 C overnight with recombinant hCG (Sigma), hLH purified from pituitary (a kind gift from Dr. A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA), and Bac-hCG β and Bac-hCG β -R68E purified as described (7), at 1.0 μ g/ml with 50 μ l/well in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (CBB). The plates were washed three times in PBS containing 0.05% Tween 20 (PBS-T), followed by blocking with 2% dried skimmed milk powder in CBB overnight at 4 C. After washing three times with PBS-T, 50 μ l serum (from the immunized or nonimmunized mice) diluted 100 times in PBS-T was added and incubated for 2 h at 37 C. The plates were washed three times with PBS-T before goat antimouse IgG alkaline phosphatase-conjugated polyclonal antibodies (Sigma) were added for 2 h at 37 C. The substrate *p*-nitrophenylphosphate in CBB containing 2 mM MgCl₂ was added, for 15 min, and the plates were read at A₄₀₅ using an MR5000 ELISA plate reader (Dynatech Laboratories Ltd., Billingham, Sussex, UK).

Production and Purification of Baculovirus-Produced hCG β

The construction of pBac2-hCG β -WT and pBac2-hCG β -R68E encoding wild-type hCG β (Bac-hCG β -WT) and mutant hCG β -R68E (Bac-hCG β -R68E) and their viruses has been described previously (7). Briefly, Bac-hCG β -WT and Bac-hCG β -R68E were both extended at the carboxy terminus with a His₆-tag that can be used to capture the proteins on the sensor chip by chelating to nickel ions. The recombinant proteins were purified as previously reported (7). The media from virus-infected High Five cultures infected at a multiplicity of infection of 10 were harvested by centrifugation at 24, 48, and 72 h post infection and analyzed by PAGE and Western blotting. The concentrations of the recombinant proteins in the supernatants harvested 72 h post infection were determined using an ELISA and rabbit polyclonal serum directed against the hCG β CTP (kindly provided by Dr. Vernon C. Stevens, Ohio State University, Columbus, OH). The concentrations of Bac-hCG β -WT and Bac-hCG β -R68E supernatants were 1–2 μ g/ml and 2–3 μ g/ml, respectively, based on a comparison with hCG β (Zymed Laboratories, Inc., South

San Francisco, CA). To determine whether the β -subunits were aggregated in the supernatant, the nondenatured proteins were analyzed by size exclusion chromatography using the SMART system (Amersham Pharmacia Biotechnology Ltd., Little Chalfont, Buckinghamshire, UK). Baculovirus supernatants (50 μ l) were separated on a Sepharose 12 PC 3.2/30 column (bed volume of 2.3 ml) (Amersham Pharmacia Biotechnology Ltd.) at a speed of 50 μ l/min. Twenty fractions of 50 μ l were collected, and 10% SDS-PAGE and Western blotting using an hCG β CTP-specific rabbit polyclonal serum were used to determine the presence of recombinant protein in the fractions. The molecular weight of the hCG β subunits was assessed using a High and a Low Molecular Weight Calibration kit (Amersham Pharmacia Biotechnology Ltd.) containing catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa).

Antibodies

The INN-2 mAb (β 1), INN-22 mAb (β 2), INN-58 mAb (β 5), INN-64 mAb (β 6), and INN-68 mAb (β 7) (12) were HPLC-purified IgG1. Fab fragments of the mAbs were generated using the Pierce Immunopure Fab kit following the manufacturer's instructions (Pierce Chemical Co., Cheshire, UK). The mouse IgGs were digested by immobilized papain for 5 h before the Fc fragment, together with undigested Igs, was removed by passage over a Protein A column. The composition of the Fab fractions was examined using SMART size exclusion chromatography as described above, PAGE, and Western blotting. The concentration of each Fab/antibody solution was determined using the Bradford protein assay following the manufacturer's instructions (Bradford assay protein quantification kit; Pierce Chemical Co.).

Surface Plasmon Resonance

Antigen/antibody complex formation was recorded using a Biacore X biosensor (Biacore, Uppsala, Sweden) and analyzed using the BIAevaluation 2.1 software. All solutions were prepared in HEPES-buffered saline (HBS) at pH 7.5 and passed through the flow cell at a speed of 20 μ l/min. The His₆-tagged recombinant hCG β proteins, as well as all the Fab fragments, were extensively dialyzed against HBS, pH 7.5, before use. Each binding cycle was composed of four events. First, the nitrilotriacetic acid chip was cleaned using 20 μ l of 10% sodium dodecyl sulfate followed by 20 μ l of 0.1 M NaOH. Second, 20 μ l of 25 mM NiCl₂ was passed through the flow cell, resulting in an increase of 80 resonance units (RU) due to the noncovalent binding of the nickel ions. Third, 50 μ l of the dialyzed baculovirus-derived protein was injected at a concentration selected to give an increase of 500 RU. Finally, 50 μ l of a solution containing the appropriately diluted Fab fragments was injected into the flow cell. Once the antibody had passed the surface, the formed complex was washed with HBS at pH 7.5 for an additional 1000 sec to dissociate bound antibody. A minimum of 15 sensorgrams was used for the determination of the association and dissociation constants of each antigen/Fab pair. To obtain an accurate calculation of the kinetic constants, triplicate sensorgrams of five different concentrations for each Fab fragment were obtained. INN-2 was used at concentrations varying between 8.76×10^{-7} M and 8.76×10^{-8} M; INN-22 was used at range between 11.67×10^{-7} M and 1.167×10^{-7} M; the concentrations of INN-58 Fab varied between 19×10^{-7} M and 1.58×10^{-7} M; only five concentrations of INN-64 Fab (8.84×10^{-7} M to 1.77×10^{-7} M) were necessary to establish the association and dissociation rates for the binding of this antibody to Bac-hCG β -WT; and INN-68 was used at concentrations ranging between 11.2×10^{-7} M and 2.24×10^{-7} M. All experiments were performed at 25 C. Both the association and dissociation curves were recorded. Before

each analysis, the slow release of the His₆-tagged protein was recorded and subtracted from the sensorgrams showing the binding characteristics of the antibodies. The sensorgrams were finally normalized and overlaid before analysis in a 1:1 Langmuir model using the BIAevaluation 2.1 program. This program uses nonlinear regression analysis for the determination of rate binding constants for macromolecular interactions.

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