The major hormone-specific discontinuous epitopes on human chorionic gonadotrophin

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Abstract

The hormone human chorionic gonadotrophin (hCG) shows extensive sequence homology with LH. Thus, many of the antigenic epitopes on hCG are shared with LH, leading to immunological cross-reaction between these two hormones. Anti-fertility and anti-cancer vaccines based upon hCG should ideally target only the hCG-specific epitopes. The hCG-unique linear epitopes located in the C-terminal peptide of the hCG β -chain are well characterised. In contrast, the hCG-specific discontinuous epitopes, termed β 1, β 6 and β 7, have remained poorly defined. By generating hCG β -chain molecules containing single amino acid substitutions we have identified R10, N13, R60 and Q89 as being important in the formation of the β 1 epitope, with R60 providing a particularly dominant residue. We also show that the amino acid residue Q89 contributes to the β 7 epitope, whilst D61 plays a role in both the β 6 and β 7 epitopes.

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Introduction

The glycoprotein hormone family is composed of human chorionic gonadotrophin (hCG), luteinising hormone (LH), follicle-stimulating hormone and thyroid-stimulating hormone. These hormones are heterodimers comprising a common α -chain and a hormone-specific β -chain, both of which are glycosylated with N-linked carbohydrate (the β -chain of hCG also bearing O-linked carbohydrate). Both subunits share a common fold of two beta hairpins and a variable loop region and are rich in disulphide bonds, three of which form the central structural motif called a cysteine knot (Lapthorn *et al.* 1994, Wu *et al.* 1994). The two subunits are symmetrically interlocking, with the β -chain embracing its α -chain counterpart with a loop structure that is referred to as the 'seat-belt' (Lapthorn *et al.* 1994).

hCG is a pregnancy-related hormone produced by the pre-implantation blastocyst and subsequently by the trophoblast (Fox & Kharkongor 1970). Because hCG is implicated in implantation in the uterus and is essential for promoting the secretion of oestrogen and progesterone from the corpus luteum, and thus for maintaining the endometrium in a receptive state, blocking the action of this hormone with antibodies prevents pregnancy. Thus, vaccines based on this hormone have been developed as anti-fertility agents (Stevens 1999, Talwar 1999, Delves *et al.* 2002) and one such vaccine has successfully completed phase II efficacy trials (Talwar *et al.* 1994). Furthermore, hCG-based vaccines may have utility for the treatment of certain hormone-dependent cancers (Triozi & Stevens 1999, Moulton *et al.* 2002). However, there is an 85% sequence homology between the first 112 amino acids of the β -chains of

hCG and LH, leading to immunological cross-reaction between the two hormones due to a number of shared epitopes. An anti-hCG response which cross-reacts with LH may be undesirable as LH is not a pregnancy-specific hormone but a critical hormone in the process of ovulation and production of female sex hormones. Whilst adverse effects of antibody responses to LH have not been observed (Talwar 1999), it is possible that long-term exposure to a high level of LH-cross-reactive antibodies might lead to undesirable side-effects in hCG-vaccinated individuals (Dirnhofer *et al.* 1994).

A number of epitopes on hCG have been defined with monoclonal antibodies (mAbs) (Berger *et al.* 2002). Two linear epitopes, β 8 and β 9, have been mapped within the amino acid sequence 109–145, which constitutes the C-terminal peptide (CTP) of the molecule, a structure which is unique to hCG and is believed to adopt an extended, poorly ordered conformation. All the other B-cell epitopes on the hCG β -chain are discontinuous and therefore require the correct folding of the hormone. The β 1 epitope is of particular interest as, like the CTP, it is hCG-specific and thus also a desirable epitope to incorporate into an hCG-based vaccine. Structural assignment of the β 1 epitope region on the hCG β -chain has remained elusive. The majority of surface differences between hCG and LH on the body of the β -chain are located in a defined cluster close to the central cysteine knot (Fig. 1). We therefore hypothesised that this region must constitute the β 1 epitope, and residues within this area were selected for mutation. Residues R8, R10 and Q89 are unique to hCG and are present in a region protruding from the body of the molecule. In the three-dimensional structure, hCG-specific residues Q89 and R10 are in close proximity to R60 and D61. Thus, residues R8, R10, R60, D61 and Q89 were selected for mutation and the effects of these mutations on the establishment of contact with the antibody paratope analysed. LH and hCG differ in their carbohydrate content (Kessler *et al.* 1979), the hCG β -chain bearing two N-linked carbohydrate structures at N13 and N30, whereas the LH β -chain lacks the glycosylation on N13 due to the replacement of threonine at position 15 with a isoleucine in the LH sequence, abolishing the consensus sequence for N-linked glycosylation. Given that the β 1 epitope is present only on the

hCG molecule, it is possible that the presence of the carbohydrate structure might induce minor conformational changes in the structure of hCG and thereby indirectly contribute to the generation of this epitope. Thus, residue N13 was also targeted for mutation. We additionally investigated amino acids contributing to the β 6 and β 7 epitopes associated with two discrete antigenic regions accessible only on the free β -chain, and therefore presumed to involve residues located at the α/β interface (Berger *et al.* 2002).

Material and methods

mAbs

All mAbs have been described previously (Berger *et al.* 1990, Jackson *et al.* 1996) and recognise various epitopes as indicated; INN-2 mAb (β 1), INN-32 mAb (β 1), INN-111 mAb (β 3), INN-24 mAb (β 4), INN-58 mAb (β 5), INN-51 mAb (β 3/5), INN-20 mAb (β 3/5), INN-64 mAb (β 6), INN-68 mAb (β 7) and 3E2 mAb (β 3/5). The OT3A2 mAb recognises the linear β 8 CTP epitope 133–139. Isotype control mAbs for flow cytometry were QB-END/10 anti-CD34 (Quantum Biosystems, Cambridge, UK) and TT04 anti-tetanus toxoid (National Institute for Biological Standards and Control, Potters Bar, Herts, UK) (Sheppard *et al.* 1984).

Construction of the mutants

The generation of the expression vector construct has been previously described (Jackson *et al.* 1996). The mutants were produced by an overlap PCR mutagenesis method (Horton & Pease 1991) using hCG β -chain cDNA as a template. The primer sequences are listed in Table 1. The mutated genes were initially cloned into pBluescript (Stratgene, Ceder Creek, TX, USA) and the position of the desired mutations, and the absence of any undesired mutations, confirmed by sequencing. The wild-type and mutants were then subcloned into a pCDM8 construct (Seed 1987), in which a DNA fragment coding for the first 17 membrane-proximal residues, the transmembrane region and the cytoplasmic tail of the H2-D^b protein had been previously cloned in frame with the hCG β -chain gene (Jackson *et al.* 1996). The fragments containing the wild-type or the mutants R10E,

Figure 1 Surface exposed differences between hCG and LH. The hCG heterodimer structure (PDB 1HRP, Laphorn *et al.* 1994) in surface representation with the position of individual amino acid residues which are changed in LH highlighted. The α -chain surface is shaded in dark grey and the β -chain surface in light grey. The N-acetylglucosamine sugars visible in the hCG crystal structure which are attached at N13 and N30 of the β -chain and N52 and N78 of the α -chain are shown in stick representation. These provide an indication of where the biantennary N-linked oligosaccharides cover the structure. The sugar at N13 is highlighted as this is absent in LH due to the change T15I. The β -chain CTP is not resolved in the X-ray structure and is therefore not shown.

Table 1 Primer sequences used for construction of the hCG β-chain mutants

Primer sequences	
Mutant	
R8L	Sense: 5' GGCCACTGTGCCGCCCCATCAATGCCACC 3' Antisense: 5' GGGCGGCACAGTGGCCGAAGCGGCTCC 3'
R10E	Sense: 5' GCGAGCCCATCAATGCCACCCTGGCTGTGG 3' Antisense: 5' GGCATTGATGGGCTCGCACCGTGGCCGAAGC 3'
N13E	Sense: 5' GCCACCCTGGCTGTGGAG 3' Antisense: 5' CTCCACAGCCAGGGTGGCCTCGATGGGGCGCACCGTGG 3'
R60H+R63S+E65A	An additional mutation (R60H) generated by a PCR error during an initial attempt at constructing R63S+E65A (see R63S+E65A below for primer sequences)
D61R	Sense: 5' GTGCGCTTCGAGTCCATCCGG 3' Antisense: 5' GATGGACTCGAAGCGCACCKSGCGGTAGTT 3'
R63S+E65A	Sense: 5' GTGTCCTTCGCCTCCATCCGGCTCCCTGGC 3' Antisense: 5' GGAGGCGAAGGACACATCGCGGTAGTTG 3'
Q89R	Sense: 5' CAGCTGTCGCTGTGCACTCTGCCGCCGC 3' Antisense: 5' GCACAGCGACAGCTGAGAGCCACGGC 3'
A91N	Sense: 5' GTCAATGTAACCTCTGCCGCCGCAGCACC 3' Antisense: 5' GCAGAGGTTACATTGACAGCTGAGAGCC 3'
L92P	Sense: 5' AATGTGCACCCTGCCGCCGCAGCACCACA 3' Antisense: 5' CGGCAGGGTGCACATTGACAGCTGAGAGC 3'

N13E and D61R hCG β-chain–MHC fusion genes were further subcloned into pcDNA3 as NotI–SalI fragments.

Expression of wild-type and mutated hCG β-chains

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Hank's balanced salt solution (HBSS), trypsin–versene solution, chloro-quinine, gentamycin and G-418 were purchased from Invitrogen-Life Technologies Ltd (Paisley, Renfrewshire, UK). DMEM was supplemented with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1 mM sodium pyruvate. Chinese hamster ovary (CHO) cells were cultured in DMEM containing 10% FCS and 1% penicillin–streptomycin at 37 °C in a 5% CO₂ incubator in a humidified atmosphere. Liposome-mediated transfection of the CHO cells was undertaken using cationic liposomes, formulated from 3-beta [N-(N',N'-dimethylaminoethane) carbamoyl]-cholesterol and dioleoyl L-alpha-phosphatidylethanolamine, kindly provided by Dr Karl Murray (Department of Psychiatry and Behavioural Sciences, University College London, London, UK) (McQuillin *et al.* 1997). The cells were transfected at three different (w/w) ratios of plasmid DNA to liposomes (1/1, 1/3, 1/6). The

appropriate amount of DNA and liposomes were added to 400 µl serum-free DMEM, and the mix vortexed and then incubated for 30 min at room temperature. This mix was deposited onto 50% confluent cells present in a well of a Costar six-well plate (Corning BV, Schiphol-Rijk, The Netherlands). After 2 h of incubation, growth medium was added to each well and the cells harvested 48 h later. To generate stable cell lines expressing wild-type or mutants R10E, N13E and D61R of the hCG β-chain, or expressing either green fluorescent protein (GFP) or only neomycin resistance, the cells were allowed to grow for 5 days post-transfection under non-selective conditions prior to selection of antibiotic-resistant clones in growth medium supplemented with 800 µg/ml G-418 sulphate. After 14 days (2 days after 100% of the mock transfected cells had died under the G-418 selection) single colonies were aspirated and transferred to individual wells of a 24-well plate containing growth medium–G-418. Individual clones were expanded and analysed.

Flow cytometry

Cells (2 × 10⁵) were stained with 1–10 µg/ml primary antibody in 2% (w/v) BSA, 0.02% (v/v) sodium azide in HBSS and incubated on ice for 45 min. After three washes in HBSS, the cells

were resuspended in fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ rabbit anti-mouse IgG (Dako, High Wycombe, Bucks, UK) in the above buffer and incubated on ice for 30 min. After three washes, the cells were resuspended in HBSS and analysed by fluorescence-activated cell sorting using a Becton-Dickinson Facsan (Becton Dickinson, Mountain View, CA, USA). The gate was set on the negative control, an irrelevant isotype-matched antibody. All mAbs were titrated to give roughly the same staining intensity on the wild-type surface-expressed hCG β -chain. Each mutant/mAb combination was tested at least three times. The relative expression of each epitope normalised to that of CTP β 8 was calculated using the following ratio for each antibody: relative expression = (percent positive cells obtained with the antibody) / (percent positive cells obtained with OT3A2). Any reduction in binding < 20% was disregarded.

The ratios were statistically analysed. An F-test showed that the variance between each population was the same and therefore a Student's *t*-test was utilised to assess statistical significance.

Results

Expression of wild-type and mutant hCG β -chain on CHO cells

The cell-surface expression of the chimeric β -hCG proteins was quantified using the OT3A2 mAb, which recognises a CTP linear epitope on the hCG β -chain of each mutant molecule. Similarly to our previous studies using COS7 cells (Jackson *et al.* 1996), both wild-type and mutant hCG β -chains were capable of being expressed on the surface of CHO cells (Fig. 2). Furthermore, as assessed using ten different conformation-dependent mAbs, surface expression of recombinant wild-type hCG β -chain on the CHO cells also did not dramatically affect the folding of the molecule (Fig. 3a). The amount of antibody per cell required to obtain a similar level of staining for each epitope was therefore established using CHO cells expressing wild-type hCG β -chain.

Mutations affecting the β 6 and β 7 epitopes

The β 3, β 4, and β 5 epitopes were recognised equally well on the mutants R8L, A91N, L92P,

R63S+E65A (data not shown), R10E, N13E, D61R, Q89R and R60H+R63S+E65A (Fig. 3b–f) indicating that these mutations did not substantially modify the overall structure of the protein. However, the Q89R mutation led to an 80% decrease in the binding of the INN-68 mAb recognising the β 7 epitope ($P < 0.0005$, Fig. 3e). The glutamine at position 89 is most likely located within the immediate environment of the β 7 epitope. Modification of the aspartic acid at position 61 (D61R) decreased the binding of the β 6- and β 7-specific antibodies by approximately 30% ($P < 0.005$ for INN-64 and < 0.05 for INN-68) (Fig. 3d).

Mutations affecting the β 1 epitope

Mutating the hCG β chain-specific residues R8 to a glutamic acid, A91 to an asparagine or L92 to proline as single point mutants did not affect the binding of any of the mAbs used including the hCG-specific β 1 epitope antibodies INN-2 and INN-32 (data not shown). However, recognition of the mutant R10E by the mAbs INN-2 and INN-32 was reduced by approximately 30% ($P < 0.005$) compared with the wild-type clone (Fig. 3b), but none of the other epitopes were affected by this mutation. Substituting Q89 for arginine led to a decrease in binding on the β 1-specific antibodies of about 30% ($P < 0.005$, Fig. 3e). The R60H+R63S+E65A triple mutation totally abolished the recognition of the β 1 epitope (Fig. 3f) ($P < 0.0005$), but had no effect on any of the other epitopes. In contrast, the R63S+E65A double mutation bound all antibodies equally as well as the wild-type β -hCG (data not shown), thereby implying that the R60H mutation is responsible for the abolition of the β 1 epitope. The likely approximate location of the β 1 epitope, indicating the position of the amino acids R10, R60 and Q89, whose mutation led to a reduction in binding of the β 1-specific mAbs, is shown in Fig. 4.

Role of carbohydrate in antibody binding

hCG is the most highly glycosylated member of the glycoprotein hormone family of proteins, with two N-linked biantennary complex carbohydrate chains per subunit and four O-linked carbohydrates in the unique β -subunit CTP. LH in contrast has only one β chain carbohydrate due to the replacement

Figure 2 Stable expression of wild-type and mutated hCG β -chain on the cell surface of CHO cells. Events (5000) are shown for CHO cells optimally expressing GFP, wild-type hCG β -chain or, as representative examples, mutants R10E, N13E and D61R stained with OT3A2 mAb (black), which recognises the linear β 8 epitope (amino acids 133–139) of the hCG β -chain CTP. Expression levels often varied when the same plasmid was used on different occasions. The negative isotype-matched controls are shown in red. The indicated percentage of cells expressing the chimeric construct was determined by the cells with a higher fluorescence intensity than the negative isotype-matched control.

of threonine at position 15 with an isoleucine in the consensus sequence for N-linked glycosylation. These differences in glycosylation could significantly affect the regions of the β -subunit surface accessible to an antigenic response in the two hormones. As expected, the mutation N13E did not substantially affect the β 3, β 4 and β 5 epitopes as these are distant from the glycosylation site. However, the mutation led to an approximately 20% increased recognition by the β 1-specific antibody INN-32 compared with binding to the wild-type protein ($P < 0.05$) (Fig. 3c). This suggests that the carbohydrate associated with residue 13 partly interferes with the binding of the antibody INN-32 and is therefore at the periphery of the β 1

epitope site. The N13E mutation was the least well expressed of the mutants (Fig. 2), perhaps reflecting the known influence of the N-linked glycosylation sites on the interaction of the hCG β -chain with molecular chaperones such as BiP, Erp72 and Erp94 (Feng *et al.* 1996).

Discussion

We have identified key amino acids involved in the β 1, β 6 and β 7 epitopes on the hCG β -chain. The glutamine at position 89 and the aspartic acid at position 61 contribute to the β 7 epitope, with mutation of D61 in addition to the residues K20,

Figure 3 Epitope mapping of mutated hCG β -chains. The expression of each epitope on a mutant was assessed by the percentage of the cell population having a fluorescence intensity superior to the fluorescence intensity of the population stained with the negative isotype-matched control for each cell line stained with a panel of mAbs (INN-2 (β 1), INN-32 (β 1), INN-111 (β 3), INN-24 (β 4), INN-58 (β 5), INN-51 (β 3/5), INN-20 (β 3/5), 3E2 (β 3/5), INN-64 (β 6), INN-68 (β 7) and OT3A2 (CTP)). Each flow cytometry experiment was carried out at least four times on cells optimally expressing the transfectant. To allow for variable surface expression of each transfected cell line, the relative expression of each epitope was calculated for each antibody by reference to staining of the CTP β 8 epitope, as described in Materials and methods. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, comparing antibody binding to the mutant with that to the wild-type hCG β -chain.

Figure 4 Location of site-directed mutations leading to decreased antibody binding. A detail of the middle of the hCG heterodimer structure represented in space-filling with the position of individual mutations highlighted. The α -chain is shaded in dark grey and the β -chain in light grey. The N-acetylglucosamine sugars visible in the hCG crystal structure, which are attached at N13 and N30 of the β -chain and N52 of the α -chain, are shown in stick representation.

E21, G22 and G75 (Jackson *et al.* 1996) also affecting the β 6 epitope. These two epitopes are located at the α/β interface and therefore accessible only on the free β -chain.

The β 1 region represents the major hormone-specific conformation-dependent epitope on hCG (Berger *et al.* 2002). However, the precise location of this epitope region on the hCG β -chain has been unclear. Easily accessible areas of a protein surface, as identified by a high protrusion index and solvent accessibility, are often the site of antibody recognition (Novotny *et al.* 1986, Thornton *et al.* 1986). Analysis of the crystal structure of hCG (Lapthorn *et al.* 1994, Wu *et al.* 1994) revealed a protruding area not previously identified as an

antigenic area (Berger *et al.* 1996). Hypothesising that this might constitute the β 1 epitope, we mutated amino acid residues at this location.

The R60H+R63S+E65A triple mutation spans approximately 15 Å of the length of the β -subunit and therefore disrupts a significant portion of the β -subunit surface that has not been investigated in previous studies (Fig. 4) (Jackson *et al.* 1996). This mutant totally abolishes binding of both the anti- β 1 antibodies but is in fact further down the molecule than the hormone-specific changes between hCG and LH. The R63S+E65A double mutation bound all antibodies equally as well as the wild-type hCG β -chain, clearly suggesting that the R60H is the key mutation that abolishes binding of both the anti- β 1

antibodies. This substitution, which modifies the size but maintains the hydrophilicity and the charge, did not alter the binding of antibodies directed to any other epitopes on hCG. This indicates that there was no substantial effect on the overall conformation of the protein but that R60 establishes contact with one or more residues in the paratope of the anti- β 1 antibodies. When either the arginine residue at position 10 was changed to a glutamic acid or the glutamine at position 89 changed to an arginine, the binding of both β 1-specific antibodies was reduced to approximately 70% of the binding to the wild-type protein. Residues R60 and Q89 are adjacent in the three-dimensional structure of the hCG β -subunit and therefore would be expected to contribute to the β 1 epitope. The side-chain of R10 adopts a conformation which places it further away from the other residues implicated in the β 1 epitope. When this residue is mutated to glutamate, the side-chain is likely to adopt an orientation facing towards R60, possibly forming an electrostatic interaction. In the native heterodimer, accessibility of the residue at position 10 might be restricted by the position of the N-terminal region of the α -chain and by the presence of the N-linked carbohydrate at position 13 on the β -chain (Fig. 4). Therefore, it is possible that the residue R10 does not directly bind the hypervariable regions of the INN-2 and INN-32 mAbs but rather is located on the periphery of the β 1 epitope and establishes positive interactions with framework residues, thereby stabilising antibody binding. Alternatively, its substitution could modify the conformation of the epitope resulting in decreased recognition. However, changing the adjacent arginine at residue 8 to a leucine did not affect the binding of the β 1-specific mAbs.

Mutation of the asparagine at position 13, one of the two N-linked glycosylation sites of the β -chain, removes this large oligosaccharide. The loss of this carbohydrate structure was associated with increased recognition of one of the antibodies specific for the β 1 epitope, INN-32. This is most likely due to the removal of some steric hindrance of the antibody by the glycosylation.

Substitution of the aspartic acid residue at position 61 in the hCG β -chain by an arginine dramatically changes both the size and charge of the amino acid residue. However, despite its proximity to residue 60 (Fig. 4) and the fact that the R60H mutation abolished binding of the anti- β 1

antibodies, the substitution D61R had no significant effect on the binding by the β 1-specific mAbs. This suggests that the carboxylate of the aspartic acid is not involved in the establishment of side-chain electrostatic interactions with the antibodies. Moreover, if the residue D61 is indeed present at the interface with the anti- β 1 antibody, the finding that both antibodies accommodated the variation of volume introduced by the D61R replacement suggests that the interface between the antibodies and hCG β -chain are not highly complementary in shape at this position. Surprisingly the substitution D61R did decrease recognition of the β 6 and β 7 epitopes. Previous work by our groups has established that residues at positions 20, 21, 22 and 75 and 77 contribute to the β 6 epitope (Jackson *et al.* 1996, Berger *et al.* 2002). These residues are located in an area over 25 Å from position 61 in the tertiary structure of hCG. Thus, it is unclear why the mutation of this residue modifies the recognition of the β 6 epitope. This mutation could induce an alteration of the conformation to which the β 6 epitope is sensitive or produce functional variation by indirect or long-range effects (Lavoie *et al.* 1992), for example by influencing the side-chain positions on loops 1 and 3 in relation to each other. However, given the absence of any effect on the other β epitopes that are close to the β 6 epitope argues against this as an explanation. Whether the conformation of the C-terminus of the β -chain (residues 109–145) is affected by this mutation and blocks the β 6 epitope is also a possible explanation for this effect.

Insight into the position of the β 7 epitope is provided by the Q89R mutation, which dramatically reduces the binding of the INN-68 mAb. This clearly shows that Q89 is involved in antibody binding to the β 7 epitope. However this should not be over interpreted, as the introduction of arginine at residue 89, due to its size, would be expected to have an effect over a larger region than a smaller substitution. The fact that the triple mutant including R60H does not affect INN-68 binding suggests that Q89 is not the centre of the β 7 epitope. However, our finding that residue 89 plays a role in the binding of both β 1 and β 7 antibodies would accord with the previously observed close relationship between these two epitopes (Berger *et al.* 1990, 2002).

In discontinuous epitopes, usually 15–20 residues establish contact with the residues of the paratope

(Jones & Thornton 1996). A majority of these residues may be energetically significant in binding the ligand (Dall'Acqua *et al.* 1996, Goldman *et al.* 1997), or sometimes only two or three of the contact residues provide most of the binding energy (Novotny *et al.* 1989, Cunningham & Wells 1993, Kelley & O'Connell 1993). The fact that the substitution R60H abolished the binding of the anti- β 1 antibody suggests that this epitope is energetically dependent on only a few residues. Moreover, this would explain the lack of effect of the substitutions of residues in close proximity on the crystal structure that might be in contact, such as the aspartic acid at position 61. Mutation of a contacting residue which does not play a significant energetic role may be accompanied by reorganisation of water molecules, which restores the original interaction (Dall'Acqua *et al.* 1998).

In conclusion, our results suggest that the recognition of the major hormone-specific discontinuous epitope, β 1, on hCG is based on only a very few critical interactions. We have identified these energetically dominant residues as including the arginines at positions 10 and 60, and the glutamine at position 89. Moreover, we have showed that this epitope is dramatically tolerant to substitutions of other residues probably located at the binding site, such as the aspartic acid residue at position 61. Thus, to supplement the inherently weak immunogenicity of the hormone-unique linear CTP structures, this epitope region should be maintained in mutated or subunit vaccines aimed at optimising a hormone-specific response against hCG for control of fertility or cancer. The same considerations hold true for residues contributing to the β 6 and β 7 epitopes for vaccines which target cancers where isolated β -chains function as growth factors (Butler *et al.* 2000).

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