Double-CLIPS technology for the mimicry of structurally complex antibody binding sites on proteins
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MIMICRY OF A DISCONTINUOUS BINDING SITE ON HUMAN CHORIONIC GONADOTROPIN BETA (HCG-β)

Part of the work (§ 5.3 – § 5.4) described in this chapter is ready for publication: L. E. J. Smeenk, H. Hiemstra, J. H. van Maarseveen, P. Timmerman, manuscript in preparation

Part of the work (§ 5.3 – § 5.4) described in this chapter has been patented in: L. E. J. Smeenk, J. H. Van Maarseveen, N. S. Dailly, P. Timmerman, WO2012057624, 2012
5.1 GENERAL INTRODUCTION

In chapter 4, detailed investigations on the possibilities to use double-CLIPS technology for the mimicry of the discontinuous β1/β3-loop epitope on hFSH were described. Following the close structural and functional similarities between hFSH and the ‘sister’ protein hCG (Figure 5.1 A), and making use of the X-ray structures of both proteins (Figure 5.1 B1 and C3), we explored whether double-CLIPS technology could also be applied to design successful mimics of the protein hCG. Two new hCG mimics were designed and binding ELISA studies with five different monoclonal antibodies with clearly proven (mAb 3468) or potentially (8G5, 4F9, B2 and B4) discontinuous β1/β3-loop epitopes on hCG were used to primarily determine the potential benefits of the double-loop structure. These findings were then compared with a series of direct competition studies in ELISA, in order to further fine-tune and affinity-optimize the structure of these epitope mimics. Moreover, peptide arrays were applied to additionally study the relevance of each individual residue within the β1-loop of double-CLIPS hCG constructs. Finally, immunization studies in sheep were performed for the most optimal immunogen (in collaboration with the company Mologic Ltd.) with the emphasis to unravel if the strong hCG-mimicking properties can be used to raise antisera that show cross-reactivity with the native protein hCG in ELISA. Translation of knowledge obtained from double-CLIPS hFSH mimics to design potent hCG mimics, together with studying their immunological properties, forms the basis of this chapter and will be discussed in detail hereafter.

![Diagram](image)

**Figure 5.1:** Structural similarity between the sister proteins hCG and hFSH. (A) Schematic representation on the β-subunit of hCG. Amino acids in grey are identical to those found in hFSH. (B) X-ray structure of the β-subunit of hCG. (C) X-ray structure of the β-subunit of hFSH.
5.2 HUMAN CHORIONIC GONADOTROPIN (hCG)

5.2.1 human Chorionic Gonadotropin (hCG)
The heterodimeric protein hCG⁴ forms a particular interesting target for studying the mimicry of discontinuous epitopes, and, just like hFSH, is also a member of the cys-knot subfamily of glycoprotein hormones (GLHA/B). Apart from its biomarker function in pregnancy and potential target to control human fertility,⁵ it also plays a key role in cellular differentiation and proliferation and may activate apoptosis.⁷ The hormone hCG is produced by several cancerous tumors like ovarian, stomach, renal, bladder, pancreatic and colon adenocarcinomas and therefore its endogenous serum levels are additionally valuable for use in cancer diagnostics when the patient is not pregnant.⁸ Intelligent epitope selection is essential to achieve the required assay performance and to discriminate between hCG and for example its closely related sister hLH, which differs only by a single amino acid residue in the β1/β3 area.¹³ The hCG protein was found to be an ideal tumor marker in the diagnosis of aggressive forms of cancer, like the malignant trophoblastic disease (choriocarcinoma). The levels of hCG could be directly related to the number of choriocarcinoma cells and when this diagnosis was followed upon by appropriate therapies, increased overall survival for almost 100% of the patients was achieved.¹⁴ In addition, the clinical and immunological effects of a synthetic vaccine based on the 37-mer C-terminal peptide of β-hCG (CTP37), conjugated to diphtheria toxoid, was investigated in patients with metastatic colorectal cancer and turned out to induce anti-hCG antibodies that could also be associated with longer overall survival of the patients.¹⁶ Novel synthetic immunogens derived from hCG can therefore be of exceptional interest and very useful in developing improved cancer diagnostics and anti-cancer therapies.

5.2.2 Anti-hCG Monoclonal Antibodies (mAbs) 3468, 8G5, 4F9, B2 and B4
In order to monitor the structural resemblance of the discontinuous hCG mimics to the hCG protein itself, binding of the mimics to five different monoclonal antibodies (mAbs) was investigated. These include the mouse mAbs 3468, B2 and B4 and the sheep mAbs 8G5 and 4F9. These five IgG1 monoclonal antibodies were all raised against native hCG and shown to bind this protein with high affinity (for example Kₐ (3468)¹ = 1.0x10⁻¹⁰ M, Kₐ (8G5)¹⁷ = 6.9x10⁻¹² M and Kₐ (4F9)¹⁷ = 1.5x10⁻¹¹ M). The discontinuous interaction of mAb 3468 with hCG and the importance of the β3-loop residues P₁₃⁻S₃₁ for recognition were both confirmed by the published X-ray structure.¹ Therefore, investigations on the positive effect of the presence of an additional β1-loop within double-CLIPS mimics for binding with this antibody become highly interesting and are expected to reveal the clear enhancement of binding of double-CLIPS constructs as compared to their single-loop variants. The mouse mAbs B2 and B4 were expected to bind mainly to a hCG β3-loop epitope (R₄₈⁻N₇₇) and expected to only partially recognize the β1-loop, if at all. Thereby, it is completely unclear which of the amino acid residues of the β1-loop are involved.¹⁸ Finally, the sheep mAbs 8G5 and 4F9 were included in these studies mainly as positive controls, since they were known to recognize exclusively the β3-loop of hCG.¹⁷ Monoclonal antibody 8G5, for example, was known to bind to a linear peptide derived from the hCG β3-loop with

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selectivity over the corresponding hLH sequence, despite the fact that they only differ by a single amino acid residue in the β3-loop (Asparagine (Nγ) versus aspartate (D)).

5.2.3 Structural Design of Double-CLIPS hCG-Mimics

The very clear structural similarity between the proteins hCG-β and hFSH-β immediately becomes apparent by comparison of their X-ray structures, as shown in Figure 5.1 B and C, even though they display a sequence homology of only 37% for the first 114 amino acids of hCG (grey amino acids in Figure 5.1 A). The disulfide bridges connecting the β1- and β3-loop and the β1-loop and C-terminus are located at exactly the same positions for both proteins. These similarities emphasize the fact that both the β1-loop and the C-terminus of hCG are candidates to be involved in binding to the various mAbs, as was previously observed for the β1-loop of hFSH. Therefore, the design and synthesis of the discontinuous epitope mimics for hCG that were studied and described in this chapter, are comparable to a large extent to the hFSH-mimics described in chapter 4. For example, we decided not to reinvestigate the effects of linear peptides instead of looped versions, or alternative covalent linkages between the scaffolds, because it was expected to show a very similar outcome for hCG. However, it is also important to stress the potentially very different behavior observed for anti-FSH and anti-hCG antibodies. For example, the double-constrained β3-loop mimic FSH XII (see chapter 4) was the most potent mimic for mAb 5828 and 6602 on hFSH studied so far, while the hCG-equivalent of this mimic never showed any binding activity for mAb 3468. This indicates that, despite the strong structural resemblance between both proteins, a successful approach for mimicking discontinuous epitopes on hFSH is not by definition the optimal strategy for manufacturing proper hCG-mimics.

In contrast to hFSH and its antibodies mAb 5828 and 6602, an X-ray structure for the hCG protein in complex with mAb 3468 was published by Davis in 1999 (Figure 5.2 A), which was obviously used to support the design of our discontinuous hCG mimics.

![Figure 5.2: X-ray structure of the α-subunit and β-subunit of hCG in complex with its antibody mAb 3468 (purple). (A) Structure indicating the β1 (green), β2 (yellow), β3 (blue), C-terminus (in red) and the α-subunit (white) of hCG. (B) Space-filling model of the X-ray structure as presented under A. (C) Representation of the hydrogen bonds between residues of hCG and mAb 3468 (highlighted with yellow arrows).]
The space-filling model of this complex (Figure 5.2 B) clearly shows that several amino acids of the β3-loop (blue) and the β1-loop (green) are in close contact (<5Å) with the antibody (purple). Somewhat more distant, but still in close proximity, are the α2-loop (white) and the β-chain C-terminus (red). Hydrogen bonds between the antibody and the protein involve mostly the β3-loop directly (yellow arrows in Figure 5.2 C), but the β1-loop and/or the α-subunit of hCG could very well be involved in binding too, albeit only in an indirect manner, i.e. for example by structural stabilization of the β3-loop. Just like for hFSH, the potential influence of i) adding the β1-loop, ii) the presence of the native disulfide bond between the two loops, and iii) the peptide length of the β1-loop and the β3-loop on antibody binding was investigated in this chapter using the five monoclonal antibodies mentioned before. As a start, the two potential mimics CG I and II were designed and synthesized, together with several reference compounds (CG-β3 2g, CG 1f-2g, CG 2f-2g) as shown in Figure 5.3.

![Figure 5.3: Schematic representation of candidate hCG epitope mimics CG I-V and their reference compounds CG-β3 2g, CG 1f-2g, CG 2f-2g and CG 1Kf-2g.](image)

First of all, CG I represents the equivalent of FSH II (chapter 4) in the sense that it is built up from peptides with similar lengths (8-mer β1-loop and 18-mer β3-loop), equipped with a disulfide bond and the scaffolds positioned at the residues corresponding to those used in FSH II. In order to confirm whether the relevance of the β1-loop and the disulfide bond between the loops for five different antibody binding sites on hCG were similar as seen for hFSH, CG I was first compared in binding ELISA to its single β3-loop CG-β3 2g and the double-CLIPS compound CG 1f-2g, missing the disulfide bond. Similar studies were performed for the disulfide containing CG-mimics II and its non-constrained equivalent CG 2f-2g (Figure 5.3) to create a meaningful data set. For those antibodies where the influence
of the β1-loop and/or the disulfide bond between the loops indeed was found to be crucial, further investigations were performed, e.g. increasing the length of the β1-loops. Elongation of the β1-loop, however, was found to be impossible due to the difficult synthesis of this very hydrophobic peptide. Fortunately, application of the SIP-tail technology improved the synthesis and thereby allowed us to synthesize CG III, CG IV and CG V, together with the reference compound CG 1Kf-2g. Note that CG V not only contains the longest β1-loop, but also contains a longer β3-loop in order to position both scaffold in the same direction as optimized for hFSH. The compounds CG III and CG IV were included in this study in order to be able to determine the influence of the length of the β1-loop peptide, whilst excluding a negative or positive influence of the SIP-tail, that was indispensable for the synthesis of compound CG V, on antibody binding.
5.3 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

5.3.1 Evaluation of Discontinuous Binding Site Mimics for mAbs B4, B2 and 4F9
The study of hCG mimics I-V first describes the data on the mimicry of the binding sites of mAb B4, B2 and 4F9 on hCG. The potentially beneficial influence of the β1-loop was first examined using binding ELISA, by comparing the double-CLIPS construct CG 1f-2g with its single β3-loop CG-β3 2g for mAb B4. As can be seen in Figure 5.4, the single β3-loop is not recognized by this antibody (titer > 10.000 ng/ml, Table 5.1), while CG 1f-2g shows at least a 5-fold increase in binding (titer of 2227 ng/ml, Table 5.1). Nevertheless, an additional disulfide bond between the loops as presented in CG I does not further increase the binding to mAb B4 to a significant extent (Figure 5.4). Similar results were obtained when comparing CG II with CG 2f-2g (native SS-bond missing), that both show increased binding compared to the single β3-loop CG-β3 2g. Very similar titers were observed for these two constructs (i.e. titer for CG 2f-2g = 3935 ng/ml and CG II = 3108 ng/ml, Table 5.1) despite the additional SS-constraint in CG II. Moreover, the binding titer of CG I is almost three times better than that of CG II, indicating that the longer β1-loop in CG II disturbs its binding for mAb B4. Despite this, the binding titer of the best mimic CG I was still 12-fold lower than that of native hCG (106 ng/ml, Table 5.1).

![Figure 5.4: Binding ELISA for mAb B4. Left: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 1f-2g), and disulfide-constrained double-CLIPS mimic (CG I). Right: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 2f-2g), and disulfide-constrained double-CLIPS mimic (CG II).](image)

In contrast to mAb B4, the binding site of mAb B2 was found to be located almost entirely on the β3-loop, since highly comparable titers were observed for the single β3-loop 2g as compared to the double-CLIPS mimics CG 1f-2g and CG 2f-2g (Figure 5.5).

![Figure 5.5: Binding ELISA for mAb B2. Left: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 1f-2g), and disulfide-constrained double-CLIPS mimic (CG I). Right: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 2f-2g), and disulfide-constrained double-CLIPS mimic (CG II).](image)
Moreover, the additional disulfide bond introduced in CG I and CG II only slightly increased the binding, i.e. by a factor of 1.5, as compared to the constructs lacking this constraint (Figure 5.5). Still, the double-CLIPS mimic CG I closely approaches the titer of hCG itself (i.e. CG I = 543 ng/ml compared to hCG = 216 ng/ml). These results made us conclude that the correct mimicry of the mAb B2 binding site is mostly relying on the structural orientation of the β3-loop, which may be only slightly enhanced when the β1-loop is present, thereby supporting the correct topology of the β3-loop. Like for mAb B4, complex CG I shows slightly higher titers than CG II, again illustrating the negative effect of increasing the peptide β1-loop lengths. These titers approached that of native hCG (216 ng/ml, Table 5.1) more closely than was observed in the case of mAb B4.

Finally, it was found that the epitope of mAb 4F9 closely resembles that of mAb B2, because the single β3-loop mimic 2g is also recognized at similarly high titers (i.e. 396 ng/ml), and the discontinuous CG mimics 1f-2g and I bind better to this antibody than CG II (Figure 5.6).

![Figure 5.6: Binding ELISA for mAb 4F9. Left: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 1f-2g), and disulfide-constrained double-CLIPS mimic (CG I). Right: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 2f-2g), and disulfide-constrained double-CLIPS mimic (CG II).](image)

This latter observation again indicated a negative effect of a longer β1-loop in the constructs and also a decreased binding for the disulfide-constrained mimic CG II. It should also be noticed that the antibody titers of all the mimics are very close to that of the native hCG protein itself. Thus, the epitopes of the antibodies mAb B2 and 4F9 on hCG were found to have mostly a continuous nature, because addition of the β1-loop to the β3-loop only increased the antibody titers by a factor of 3-5 as compared to recognition of the single β3-loop. In view of the limited benefits of adding the β1-loop for these antibodies, their binding was not studied further in a competition ELISA-setup.

<table>
<thead>
<tr>
<th>Compound</th>
<th>mAb B2 (ng/ml)</th>
<th>mAb B4 (ng/ml)</th>
<th>mAb 4F9 (ng/ml)</th>
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<tr>
<td>hCG</td>
<td>216</td>
<td>106</td>
<td>53</td>
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<tr>
<td>CG-β3 2g</td>
<td>1818</td>
<td>&gt;10000</td>
<td>396</td>
</tr>
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<td>CG (1f-2g)</td>
<td>729</td>
<td>2227</td>
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<tr>
<td>CG I</td>
<td>543</td>
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</tr>
<tr>
<td>CG II</td>
<td>944</td>
<td>3108</td>
<td>155</td>
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</tbody>
</table>
5.3.2 Evaluation of Discontinuous Binding Site Mimics for mAb 8G5

The monoclonal antibody 8G5 also showed comparable results in ELISA as observed for mAb 4F9 in binding to the double-CLIPS mimics CG I and CG II and their non-constrained equivalents CG 1f-2g and CG 2f-2g (Figure 5.7).

**Figure 5.7:** Binding ELISA for mAb 8G5. Left: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 1f-2g), and disulfide-constrained double-CLIPS mimic (CG I). Right: binding of a single loop (CG-β3 2g), a double-CLIPS (CG 2f-2g), and disulfide-constrained double-CLIPS mimic (CG II).

Despite this, we envisioned that further investigation of this antibody by increasing the length of the β1-loop provides a good reference of a continuous epitope as compared to the discontinuous epitope of mAb 3468 discussed later. First of all, we should provide clear evidence that double-loop hCG-mimics with and without an additional SIP-tail (which was needed for successful synthesis of the longer β1-loops) showed similar activities, thus excluding that the simple addition of a SIP-tail would influence their binding behavior. Mimic CG I was therefore compared to CG III in both a binding (Figure 5.8 A) and a competition (Figure 5.8 B) ELISA experiment with hCG immobilized to the surface. As shown in Figure 5.8, no difference between CG I and CG III was observed in both studies. The binding ELISA thus revealed no significant enhancement of the mimic as a result of the SIP-tail as caused by e.g. improved surface-immobilization. Moreover, the competition ELISA allowed us to conclude that the SIP-tail also had no beneficial or detrimental influence on antibody binding in solution.

**Figure 5.8:** Binding (A) and competition (B) ELISA study with discontinuous hCG mimics I and III with mAb 8G5 to investigate the effect of SIP-tail containing CG mimics (III versus I).

The binding studies for mAb 8G5 were then continued by comparing the single β3-loop CG-β3 2g with the double-CLIPS mimic CG 1Kf-2g and its constrained equivalent CG III
in both a binding and a competition ELISA experiment (Figure 5.9 A). Clearly, the improved titers measured for CG 1Kf-2g (93 ng/ml, Table 5.2) and CG III (102 ng/ml) compared to the single β3-loop 2g (270 ng/ml) in the binding ELISA experiment was not observed in the competition ELISA studies (Figure 5.9 B), giving highly similar IC₅₀ values for both constructs (i.e. in the order of 3-8 μM, Table 5.2).

![Figure 5.9: Binding and competition ELISA studies for mAb 8G5. Binding (A) and competition (B) of a single loop (CG-β3 2g), a double-CLIPS (CG 1Kf-2g), and disulfide-constrained double-CLIPS mimic (CG III). Binding (C) and competition (D) of disulfide constrained double-CLIPS mimics with increasing β1-loop lengths (CG I-V).](image)

Interestingly, a comparative study with mimics CG III-V was applied in order to gain insight into the potentially positive influence of using longer β1-loop peptides in the double-loop hCG mimics on antibody binding (Figure 5.9 C). Binding ELISA studies only revealed comparable (CG III and CG IV) or even reduced (CG V) titers for the elongated mimics compared to the shorter reference hCG mimic CG I, but as observed earlier, this was not directly translated into the competition results. The competition data in Figure 5.9 D display a 30-fold increase in antibody binding when the β1-loop size was increased by 4 amino acids (i.e. IC₅₀ = 124 nM for CG II compared to 4 μM for CG I, Table 5.2) and even a 500-fold enhancement in binding when a 19-mer based β1-loop variant CG V was presented (IC₅₀ = 0.26 nM for CG V). In fact, the strongly enhanced binding of CG V must be related to a combination of both the longer β3-loop (22-mer) and the longer β1-loop presented in this complex as compared to CG I-IV (18-mer β3-loops). At the same time, this huge jump in IC₅₀ value for CG V may also be explained by a strongly enhanced interaction between the β1 and β3 loops when longer sequences are used. Such an interaction is expected to stimulate β-sheet and β-turn formation in the loops and thus the mimicry of a conformational binding site at the β3-loop for improved recognition by mAb 8G5.
Undoubtedly, CG V does indeed represent the most promising mimic for the 8G5 binding site on hCG, particularly in view of the fact that it competes with comparable strength as the native hCG protein itself (IC\textsubscript{50} values of 0.26 nM and 0.47 nM, respectively; see Table 5.2).

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<th>Compound</th>
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<th>mAb 8G5 IC\textsubscript{50} (nM)</th>
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<td>CG V</td>
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5.3.3 Evaluation of Discontinuous Binding Sites Mimics for mAb 3468

With these promising results in hand, the final step in our research was the mimicry of the most challenging discontinuous binding site of mAb 3468. The discontinuous nature of this antibody was most exquisitely illustrated by comparing binding of the double-CLIPS SS-constrained constructs CG I-III with their corresponding mimics CG 1f-2g, 2f-2g and 1Kf-2g having no disulfide bond (Figure 5.10 A).

![Figure 5.10: Binding ELISA for mAb 3468. (A) Binding of double-CLIPS mimics (CG 1f-2g, CG 2f-2g and CG 1Kf-2g), and disulfide constrained double-CLIPS mimics (CG I, CG II and CG III). (B) Binding of disulfide constrained double-CLIPS mimics with increasing β1/β3-loop lengths (CG I-V). (C) Binding effect of SIP-tail CG III compared to CG I.](image-url)
For mAb 3468, the titers of both the single β3-loop CG-β3 2g and the mimics lacking a disulfide bond were determined to be above 10,000 ng/ml, while in this example CG I has a titer of 1790 ng/ml and CG II showed binding to mAb 3468 with a titer of 897 ng/ml. This clearly shows the beneficial effect of the native disulfide bond in mimic CG II. In addition to this, a trend was observed that when the length of the β1-loop was increased, better recognition was observed in binding ELISA (i.e. CG II shows a better titer than CG I). However, such a trend was not observed in the binding ELISA data for increasing β1-loop lengths of CG III-V (Figure 5.10 B), because all compounds showed already similar binding titers in the range of native hCG (titer CG III/IV/V = 78/90/106 ng/ml compared to native hCG 51 ng/ml). Possibly, this was likely due to the presence of the SIP-tails, and this assumption was verified by comparing CG I and CG III on binding to mAb 3468, whereby CG III clearly showed a 20-fold increased binding when compared to CG I (Figure 5.10 C). This difference between CG I and CG III could be a non-specific recognition of the SIP-tail by the antibody, but this was unlikely because in that case it was expected that CG 1Kf-2g, the non-constrained equivalent of CG III that also contains a SIP-tail, should be recognized as well. Since CG 1Kf-2g was not recognized by mAb 3468 at significant levels (titer > 10,000, see Figure 5.10 A), non-specific recognition of the SIP-tail can therefore be excluded. Another explanation for the observed binding difference between CG I and CG III was a potential surface-immobilization effect. It is well known that peptides with multiple K-residues in the sequence generally can give a much more efficient immobilization at the ELISA-surface. This hypothesis was verified by comparing both complexes in a competition ELISA experiment. Unfortunately, no competition was observed for mimics CG I and CG III at concentrations below 10 μM. But since CG I and CG III showed no difference in binding to mAb 8G5 in both a binding and competition setup, we therefore assume that the SIP-tail on CG III may well enhance the folding and solubility of this complex when immobilized to the surface, but not when exposed to the antibody in solution.

Encouraged by the positive effects observed for β1-loop elongation for mAb 8G5, mimics CG IV and CG V were also studied in competition with mAb 3468. The loops as present in CG-mimics I-IV were either to short or, alternatively, were not sufficiently structured or organized in order to properly resemble the binding site for this antibody, because no competition was observed at all (IC_{50} > 10 μM). In contrast, very strong competition was finally observed for the CG-mimic V (Figure 5.11) that showed an IC_{50} value of 149 nM (Table 5.3), only a factor 100 away from native hCG (IC_{50} = 1.26 nM, Table 5.3). CG-mimic V served as the first successful epitope mimic of mAb 3468 on hCG. Obviously, the good competition of CG V against mAb 3468 could be influenced by increased solubility or enhanced folding due to the presented SIP-tail in this complex, but
this entity on itself could never be the only explanation for the observed results, because CG III and CG IV did not compete with mAb 3468 at all even though they contain the same SIP-tail as CG V.

It could be debated whether further fine-tuning of mimic CG V by extending the peptide lengths in combination with the introduction of additional constraints between the loops would enhance its antigenic properties. In view of our time schedule and curiosity, it was concluded that CG V already functions as an impressive mimic of a discontinuous epitope of both mAbs 8G5 and 3468 and that the ultimate test to prove this assumption was to vaccinate this mimic into sheep. Investigation of the immunological properties of CG V in generating antibodies that could cross-react with hCG is therefore described in § 5.5.

Table 5.3: titers and IC$_{50}$ values of several hCG mimics in binding or competition ELISA-studies with mAb 3468

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<th>mAb 3468 IC$_{50}$ (nM)</th>
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<tr>
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</table>
5.4 SYNTHESIS AND SCREENING OF DOUBLE-CLIPS hCG-MIMICS IN PEPTIDE ARRAYS

First, we performed an amino acid replacement study for the β1-loop of double-CLIPS hCG mimics in peptide arrays, in order to gain more insight into the residue relevance of the amino acids within the β1-loop. Like for hFSH, peptide arrays allowed the simultaneous screening with the five anti-hCG mAbs (i.e. B2, B4, 3468, 4F9 and 8G5) for a library of 500 different double-CLIPS mimics. The library was based on residue replacements of double-CLIPS mimics with an 8-mer, 10-mer or 12-mer β1-loop located between A₁₇ and T₂₈. The optimized protocol for hFSH resulted in the synthesis of the β1-loop peptides at the surface that were thereafter cyclized via a CLIPS reaction using the oS₂-ONH₂ scaffold. This was followed by oxime reaction with the aldehyde functionalized β3-loop peptide CG-β3 2g with sequence Ac-CSIRLPGJPVRGVPVSC-NH₂ (= S₆₆-S₈₁). Subsequent Acm removal followed by oxidation of the liberated thiol groups led to disulfide formation between the β1- and β3-loops in the cases were both loops were provided with an Acm-protected cysteine residue. The peptide arrays were consequently incubated with different dilutions of mAbs ranging from 1 ng/ml to 10 μg/ml in the incubation buffer.

Interesting results were obtained for binding with the sheep mAbs 4F9 and 8G5, causing a uniform coloring of all the wells already at antibody concentrations as low as 1 ng/ml. This indicated that these two antibodies already bind strongly to the CLIPS-constrained β3-loop alone that is presented in each well, thereby expressing no additional benefit from the second β1-loop in any of the expressed mimics. In contrast to this, mAbs B2 and B4 were very well able to discriminate between the double-CLIPS mimics, but only at mAb concentrations of 1-10 μg/ml (Figure 5.12). In those examples, the residues V₁₈ and K₂₀ for both antibodies were found to be most critical, although replacement by the 19 other natural amino acids resulted in only small effects (i.e. maximum decrease in OD₄₅₀nm-value of 500). In addition to this, replacement of the residues P₂₄ and V₂₅ results in slightly better binders for mAb B2, indicating a structural effect that is not seen for mAb B4.

![Figure 5.12: Replacement analysis of hCG β1-loop sequence Ac-CVEKEGJPSVSC-NH₂ indicating the overall residue relevance (average deviation) for mAb B2 (1 μg/ml) on the left and mAb B4 (1 μg/ml) on the right, whereby the red line represents the binding of the natural sequence.](image)

Finally, screening the library with mAb 3468 clearly revealed the explicit recognition of double-CLIPS constructs. Single β1/β3-loops were not included in this study, because they were known not to show any binding with mAb 3468, based on earlier hCG loop-scan
results obtained at PepScan.\textsuperscript{20} In contrast to the binding studies with mAb 5828 and 6602 on hFSH-mimics, much higher antibody concentrations were needed for mAb 3468 (i.e. 1-10 µg/ml instead of 10-100 ng/ml) in order to observe significant binding (\(\text{OD}_{405\text{nm}} > 200\)). This confirmed our previous observations in binding and competition ELISA studies, showing that mAb 3468 was highly sensitive to loop-length and loop-orientation. Very similar binding values were observed for double-CLIPS mimics with and without disulfide constraint (Figure 5.13), having a difference in \(\text{OD}_{405\text{nm}} < 100\). A similar observation was made for hFSH, stressing again the fact that correct formation of the intramolecular disulfide bond within the double-CLIPS constructs could not be directly confirmed.

![Figure 5.13: Optical density (OD) at \(\lambda=405\) nm wavelength for binding to mAb 3468 (1 µg/ml and 10 µg/ml) for constrained double-CLIPS constructs (Disulfide), double-CLIPS constructs having a scrambled \(\beta1\)-loop (*Double-CLIPS) and double-CLIPS constructs immobilized onto the surface for both 10-mer and 12-mer \(\beta1\)-loop peptides.](image)

Furthermore, it was found that double-CLIPS mimics with ‘scrambled’ versions of the \(\beta1\)-loop (*Double-CLIPS in Figure 5.13) still showed remarkably good binding to mAb 3468 (only factor 2-3 lower than the native sequences), which indicates that the native amino acid sequence within this \(\beta1\)-loop was less relevant to binding as observed for corresponding hFSH mimics. In contrast, screening the peptide arrays with mAb 3468 revealed the importance of amino acid sequence V\(_{18}\)-E\(_{19}\)-K\(_{20}\)-E\(_{21}\)-G\(_{22}\), being most essential for 8-mer and 12-mer \(\beta1\)-loop peptides and illustrating the close resemblance of these amino acids with that for hFSH (i.e. I\(_{12}\)-E\(_{13}\)-K\(_{14}\)-E\(_{15}\)-E\(_{16}\)). This effect becomes apparent from the 12-mer peptide in the upper graph of Figure 5.14, where the average \(\text{OD}_{405\text{nm}}\)-values for these particular amino acids are located below the red line, thus indicating reduced binding when these amino acids are replaced by others. The bottom graphs in Figure 5.14 show the same results, but here the effect per amino acid is presented for each individual replacement. The green color illustrates a replacement with consequently decreased binding towards mAb 3468, whereas the red color represents enhanced binding. From both graphs, amino acid K\(_{20}\) was found to be essential for binding of the double-CLIPS construct with a 12-mer \(\beta1\)-loop, because replacement by any other amino acid reduced binding (i.e. lies below the red line in Figure 5.14 (top) or gives a green color in Figure 5.14 (bottom)). This feature on residue K\(_{20}\), however, was less clearly observed for the constructs with an 8-mer or 10-mer \(\beta1\)-loop peptide, and likely indicates that K\(_{20}\) may play a more structural role that is key to the overall structure of the longer double-CLIPS construct, rather than being an essential amino
acid that is in direct contact with mAb 3468. On the other hand, replacement of the P_{24}
residue in both the 8-mer and 10-mer β1-loop peptides showed an explicit binding
enhancement for almost all amino acid replacements (i.e. lies above the red line in Figure
5.14 (top) or gives a red color in Figure 5.14 (bottom)), while this was not observed for the
12-mer β1-loop peptide. This probably shows that for smaller peptides the turn-inducing
proline disrupts the structure induced by the CLIPS-scaffolds.

![Figure 5.14](image)

**Figure 5.14:** Replacement analysis of hCG β1-loop sequence Ac-CVEKEGJPVSIC-NH_{2} (12-mer) and
Ac-CEKEGJPVSC-NH_{2} (10-mer) for binding to mAb 3468 (1 μg/ml). Top: the residue relevance
(average deviation), whereby the red line represents the binding of the natural sequence. Bottom:
replacement effect per amino acid whereby the green color indicates a negative effect on the binding,
while the red color illustrates enhanced binding.

In summary, the peptide array data for mAbs 4F9 and 8G5 showed no beneficial influence
of the β1-loop to the β3-loop, with equally strong binding observed for all double-loop
constructs. On the other hand, mAb B2 and B4 turned out to recognize a more
discontinuous epitope, with selected residues in the β1-loop (i.e. V_{18} and K_{20}) found to be
essential. Finally, mAb 3468 clearly revealed that the additional loop was needed for
binding, but crucial amino acid could not be assigned, apart from K_{20} and P_{24} that seemed to
support the structural orientation of the loops rather than being directly involved in binding
to mAb 3468.
5.5 SHEEP IMMUNIZATION STUDIES

In order to investigate the antigenic and immunological properties of the most promising double-CLIPS hCG mimic i.e. CG V (see § 5.3), sheep immunization studies were carried out in collaboration with the company Mologic Ltd in Bedfordshire, England. Two different double-CLIPS mimics, CG V and CG VI, differing only in their mode of presentation to the immune system, were used as immunogen for raising antibodies, and a total of four animals were used for immunization. Mimic CG V was administered without further conjugation to a carrier protein, whereas CG VI carried an additional GGG-biotin linker at the N-terminal end of the β3-loop. The latter provides a means to conjugate the peptide to streptavidin prior to immunization, thus creating a significantly larger complex that could promote a much stronger immune response. Sheep 1584 and 1585 were immunized with CG-mimic V, and sheep 1586 and 1587 with CG VI. Immunizations were performed at Ig-Innovations using a 14-weeks immunization protocol (see Experimental Section for a detailed schedule). Primary immunization was carried out with Complete Freunds Adjuvant (CFA), and subsequent booster immunization with Incomplete Freunds Adjuvant (IFA). The bleed samples collected after 14 weeks (i.e. the second bleed) were included in the presented study, where pre-immune sera were taken as a reference. Figure 5.15 shows the ELISA titers of the sera from all four sheep on CG VI-immobilized plates, indicating that the second bleed resulted in enhanced responses as compared to the pre-bleeds, as desired. Moreover, a stronger response in sheep 1584 and 1586 was observed when comparing the second bleeds with the first bleeds (after 10 weeks, not shown in Figure 5.15), indicating the generation of additional anti-peptide antibodies in between the two periods. In addition to this, it was noticed that the measured titers of the second bleed were relatively low. This was most likely due to the small size of the immunogens, although the same effect was observed when CG VI was premixed with Streptavidin in order to increase the overall size of the immunogenic complex.

![Diagram of CG V and CG VI](image)

**Figure 5.15:** Binding ELISA of CG VI-immobilized plates with diluted sera from four sheep (1584-1587), to determine the generation of anti-peptide antibodies after 14 weeks compared to the pre-bleed samples (sheep 1584*-1587*).
This alternative strategy did not appear to have a significant positive effect on the response to the peptide at this stage.

Subsequently, the potential cross-reactivity of the polyclonal sera with hCG was investigated by performing an ELISA assay with surface-immobilized hCG. Diluted sera (starting at 1/500) were examined and compared to the pre-bleed samples (Figure 5.16).

The results from this study showed weak binding of the sera to hCG, requiring at least overnight development of the substrate. The data reveal a slightly improved titer for sheep 1584 and 1586 as compared to the pre-bleed samples, whereas the sera of sheep 1585 and 1587 hardly showed detectable binding to hCG. Overall, the hCG-titer development is relatively slow and not equally observed in both animals, which tends us to conclude that the sera do not contain significant numbers of anti-hCG antibodies after two immunizations.

The lack of hCG cross-reactivity for anti-CG V and CG-VI sera suggests that the 3D-structure of CG V in solution does not yet mimic the native protein sufficiently well in order to produce the desired cross-reactive antibodies. An induced-fit mechanism, in which mimic CG-V only adopts the correct structure when presented to the antibody, should then account for the strong binding in solution as observed with mAbs 8G5 and 3468 (see above). This hypothesis should obviously be tested and verified by further studying the solution structure of CG V, e.g. with CD spectroscopy or 2D ¹H-NMR spectroscopy.

Furthermore, it is expected that the achievements of CG V can still be enhanced further by structural optimization and redesign, for example by an even further elongation of the β3-loop and β1-loop. In addition to this, the flexible loops can be additionally constrained at the bottom with a second non-native disulfide bond (within or between the loops), following a similar approach as described for hFSH (see chapter 4). The latter strategy could result in improved secondary structures, possibly bringing the resemblance of the discontinuous epitope of hCG to an even higher level.

Finally, the hCG-mimics may be also improved further by attachment of additional hCG substructures that reside within the area of the epitope of mAb 3468, i.e. the α2-loop or C-terminus, and so enhance recognition for these mimics even further.
5.6 SUMMARY

This chapter evaluates the design and binding properties of double-CLIPS hCG mimics of the (discontinuous) binding sites for five different anti-hCG-β antibodies: 4F9, B2, B4, 8G5 and 3468. The relevance of the β1-loop was investigated in detail for all five antibodies, as well as increasing the peptide lengths within the mimics, though the latter was only investigated for those antibodies showing binding to hCG in a discontinuous matter. Based on previous results from our study on hFSH, the oxime linkage between the scaffolds was considered an optimal covalent connection and its orientation was not re-investigated. Moreover, the presented study on hCG mimics only includes CLIPS-peptides, following earlier observations with hFSH showing that linear peptides did not bind to a significant level.

Monoclonal antibody 4F9 clearly binds a continuous epitope on hCG. Recognition of the single β3-loop was almost equally good as for the double-CLIPS alternatives. Peptide array screening studies confirmed this showing equally strong binding of all double-CLIPS constructs and thus no additional benefit of the β1-loop. For mAbs B2 and B4, the binding site has a discontinuous nature, even though binding in ELISA was only slightly enhanced with discontinuous β1/β3-loop constructs. Nevertheless, peptide array screening studies clearly showed recognition of the top of the β1-loop (V18-E19-K20), which seems to indicate that the top of the β1-loop is indeed involved in binding.

For mAb 8G5, the equally strong binding of all double-CLIPS constructs in peptide array screening initially suggested a strictly continuous binding site on hCG. In sharp contrast to this, clear binding enhancements in ELISA for discontinuous β1/β3-loop constructs were clearly observed, while in competition studies an enormous improvement was observed for elongated β1-loop (and β3-loop) sequences. The best binding site mimic for this antibody (CG V) showed equally strong competition as compared to the native protein itself, which unequivocally confirms the importance of the β1-loop present in CG V. Finally, the discontinuous nature of the binding site of mAb 3468 as observed from X-ray data, was clearly confirmed in our binding studies, where none of the single loops showed any binding. This antibody benefits from longer β1-loop sequences in binding ELISA experiments, where the presence of SIP-tails positively influences the binding. The strong competition (only ~100-fold less than hCG itself) observed for CG-mimic V, the only mimic that showed activity below 10,000 nM, clearly indicates the vital importance of peptide length for binding activity to this antibody. As yet, the peptide sequences might well be sub-optimal and need a final step of structural optimization for complete mimicry of the mAb 3468 binding site.

Finally, mimic CG V was studied for its ability to generate anti-hCG antibodies via sheep immunization studies. Despite the enhanced peptide titers observed for two of the four sheep after 14 weeks, no significant cross-reactivity with hCG was observed. It is
expected that this is a result of an incorrect overall topology of the loops within CG V, that could possibly be enhanced by application of longer peptide loops constrained with an extra disulfide bond, or by the presentation of additional/alternative hCG fragments within mimic V.
5.7 EXPERIMENTAL SECTION

**Peptide Array Synthesis and Binding Studies with Monoclonal Antibodies.**
Preparation of peptide-peptide arrays on polypropylene support was performed using standard Fmoc chemistry. After side chain deprotection using TFA and scavengers, the peptide arrays were washed with excess of milliQ-water (5-10 min) and treated with a 0.5 mM solution of scaffold **S2-ONH$_2$** in a 1:3 mixture of MeCN-buffer NH$_4$HCO$_3$ (20 mM, pH 7.8) to afford the corresponding CLIPS-peptides. Oxime ligation was performed in 30 min by addition of a 1 mM solution of CG-β3 2g in 100 mM aniline/citric acid buffer. The cards were washed again with excess of milliQ-water (5-10 min) and treated with a solution of iodine in methanol (3.4 mg/ml) for 30 min, followed by washing with a 1M solution of DTT. Finally, the peptide arrays were washed with excess of MeCN/H$_2$O 1:1 (3-10 min) and sonicated in disrupt-buffer (1% SDS/0.1% BME in phosphate-buffered saline (PBS, pH 7.2) at 70 °C for 30 min followed by sonication in milliQ-water for another 45 min. Oxidation was performed prior to antibody binding studies by storing the cards in 0.1% NH$_4$HCO$_3$ buffer pH 8 in open air for 1-2 days.

Subsequent binding studies with several anti-hCG mAbs were performed by pre-treatment with PBS for 30 min followed by pre-coating with incubation buffer (PBS containing 5% ovalbumin, 5% horse serum and 1% Tween-80) for 1 h. Then, the peptide arrays were incubated with mAbs (B2, B4, 3468, 8G5 and 4F9) diluted in incubation buffer to 1 ng/ml, 10 ng/ml, 100 ng/ml or 1 µg/ml solutions) for 1 h at 37 °C. After washing (3 × 10 min) with PBS/Tween-80 (0.05%), the cards incubated with mAb B2, B4 or 3468 were additionally incubated with peroxidase labeled rabbit anti-mouse antibody for 1 h at 25 °C (1/1000; Dako, Glostrup, Denmark). The cards incubated with mAb 8G5 and 4F9 were incubated with peroxidase labeled rabbit anti-sheep antibody for 1 h at 25 °C (1/1000). Subsequently, after washing the cards with PBS/Tween-80 (0.05%), 3 × 10 min), they were incubated with the peroxidase substrate 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS; 50 mg in 100 ml 0.1 M citric acid-sodium phosphate buffer (pH 4.0) containing 20 µl 30% H$_2$O$_2$). After 1 h the absorbance (at 405 nm) was measured using a CCD-camera (XC-77RR, Sony, Japan). Bound mAb was removed by sonication in disrupt-buffer as described above. Oxidation was repeated to enable re-using the peptide arrays.

**Binding ELISA Studies**
Polystyrene 96-well plates (Greiner, Germany) were treated with 100 µl/well of 0.2% glutaric dialdehyde in phosphate-buffer (0.1 M, pH 5) for 4 h at room temperature while shaking, followed by washing (3x 10 min) with phosphate-buffer (0.1 M, pH 8). Then, the wells were coated with 100 µl/well of a 1 µg/ml solution of hCG or a 10 µg/ml solution of hCG-mimic in phosphate-buffer (0.1 M, pH 8) overnight at 37 °C. After washing with 1% Tween-80 (3x) and stabilizing in horse serum (4% in PBS/1% Tween-80/3% NaCl) for 30 min, the plates were incubated with antibody (B2, B4, 3468, 8G5 or 4F9) at various dilutions, starting with a standard antibody dilution of 3 µg/ml in the first well and threefold dilution steps in subsequent wells. Incubation was performed for 1 h at 37 °C, followed by washing with 1% Tween-80 (3x). Then, the plates treated with mAb B2, B4 or 3468 were incubated with 100 µl/well of peroxidase-labeled rabbit-anti-mouse serum (1/1000 dilution in 4% horse serum, see above) for 1 h at 25 °C, followed by washing with 1% Tween-80 (4x). The plates treated with mAb 8G5 and 4F9 were incubated with 100 µl/well of peroxidase-labeled rabbit-anti-sheep serum (1/1000 dilution in 4% horse serum, see above) for 1 h at 25 °C, followed by washing with 1% Tween-80 (4x). Finally, the plates were incubated with a 0.5 µg/ml solution of ABTS (2,2′-azinodi(ethylbenzthiazoline sulfonate)) containing 0.006% H$_2$O$_2$ in citric acid/phosphate-buffer (0.1 M each, pH 4).
OD_{405nm}-values (AU) were measured after 45 min standing at room temperature in the dark. The anti-hFSH monoclonal antibodies were included in the analysis as positive controls.

**Competition ELISA Studies**

The procedure is identical as described for protein- and peptide-binding in ELISA (see above), with the difference that after coating of a 1 μg/ml solution of hCG, pre-incubation of antibody (mAb 3468 or 8G5) in the presence of the competing hCG or hCG-mimic at different concentrations is carried out on a separate ELISA plate (using 1/3 dilution per step) for 30 min at 37 °C, followed by transfer of the pre-incubated solutions to the sample ELISA-plate and subsequent incubation for 1 h at 37 °C. The required antibody/mimic starting dilutions giving an OD_{405nm}-value between 1.0 and 1.5 in binding ELISA (60 ng/ml for mAb 3468 and 8G5) were found to give optimal results as starting dilutions in the competition experiments.

**Sheep Immunization Studies**

Both peptides CG V and CG VI have been used as immunogens for raising antibodies in Female Adult sheep, with CG V being administered without further conjugation and CG VI (1.5 mg) being premixed with streptavidin (3 mg, prepared in 2 ml PBS and left 20 min at r.t.) prior to immunization. Sheep were immunized on day 0. Immunizations were carried out at Ig-Innovations using a standard 14-week protocol with PBS/CFA 1:1 (v/v) (CFV, complete Freund’s adjuvant) in the primary immunization (2.6 CFA and 250 μg peptide immunogen) and with IFA (incomplete Freund’s adjuvant) in subsequent boosts (2.6 ml IFA and 100 μg peptide immunogen) at 4 and 8 weeks. Pre-immune samples were taken and these have been received along with the first test bleeds taken at week 6. The sheep were first bled after 10 weeks and the antisera collected and analyzed. This was repeated after 14 weeks. The immunization schedule applied for immunogens CG V and CG VI in sheep 1584-1585 and 1586-1587 respectively is shown below:

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<td>1584 and 1585</td>
<td>P/S</td>
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<td>CG VI</td>
<td>1586 and 1587</td>
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<td>CG VI</td>
<td>1586 and 1587</td>
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</tbody>
</table>

P = Primary Immunization; R = Booster immunization; S = Sample; B = Bleed;
5.8 REFERENCES

[17] Data kindly provided by Sandra Hemmington, Mologics Ltd.
[20] Data kindly provided by Wouter Puijk and Peter Timmerman, PepScan Therapeutics