Fine Tuning of the Specificity of an Anti-progesterone Antibody by First and Second Sphere Residue Engineering*\[S\]

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The specificity of anti-progesterone P15G12C12G11 antibody was improved by combination of in vitro scanning saturation mutagenesis and error-prone PCR. The most evolved mutant is able to discriminate against 5β- or 5α-dihydroprogesterone, 23 and 15 times better than the starting antibody, while maintaining the affinity for progesterone that remains in the picomolar range. The high level of homology with anti-progesterone monoclonal antibody DB3 allowed the construction of three-dimensional models of P15G12C12G11 based on the structures of DB3 in complex with various steroids. These models together with binding data, derived from site-directed mutagenesis, were used to build a phage library in which five first sphere positions in complementarity-determining regions 2H and 3L were varied. Variants selected by an initial screening in competition against a large excess of 5β- or 5α-dihydroprogesterone were characterized by a convergent amino acid signature different from that of the wild-type antibody and had lower cross-reactivity. Binding properties of this first set of mutants were further improved by the addition of second sphere mutations selected independently from an error-prone library. The three-dimensional models of the best variant show changes in the antigen binding site that explain well the increase in selectivity. The improvements are partly linked to a change in the canonical class of the light chain third hypervariable loop.

Progesterone is among the most important steroid hormones required for the maintenance of pregnancy. Its dosage is currently performed by immunomassay, and its quantification needs high quality antibodies (1). However, steroids are poorly immunogenic, and thus it is difficult to obtain monoclonal antibodies, particularly with high affinity and specificity. This is complicated by the fact that immunomassays must be carried out in complex media such as blood or urine, where the compound of interest must be quantified in the presence of other chemically related molecules. The contaminating progesterone-related derivatives are characterized by modifications at positions C-3, C-5, C-11, and C-17, which induce different conformations of the steroid skeleton (2). For example, C-5α and C-5β are ring A reduced formers. Although progesterone adopts the so-called conformation A, where ring A is at ~45° to the plane formed by rings B, C, and D, the addition of a proton at position C-5 results in conformations B or C (Fig. 1A). In conformation B, typical of C-5α, ring A is coplanar with the other rings, whereas in conformation C, adopted by C-5β, ring A is at 90° to the plane of the rest of the steroid skeleton (3).

Given these structural differences, it is surprising to find that after stimulation with progesterone, the immune system responds with antibodies like DB3 which strongly cross-react with C-5 dihydro-derivatives such as 5β-androstane-3–17-di-one, aetiocholanolone (C-5β), and 5α-pregnane-3-β-hemisuccinate (4, 5). The structural basis for its cross-reactivity in steroids in two different orientations using two alternative pockets P3 and P3'. The entire binding site of DB3 can be divided into four different pockets or compartments named P1, P2, P3, and P3' accommodating the different steroid rings (see Fig. 1C) (4). Rings D, C, and B of progesterone and C-5 dihydro-derivatives are fit in P1 and P2, whereas ring A of C-5α or C-5β derivatives uses P3 or P3', respectively. Progesterone and C-5α analogs that interact in a syn conformation (with their methyl groups facing TrpH100) place their ring A in pocket P3, whereas C-5β derivatives that adopt an anti-orientation (with their methyl groups facing TrpH100) position their ring A in P3'. The use of alternative binding pockets also comports small compensatory adjustments in the binding site residues to improve shape complementarity.

Monoclonal antibody P15G12C12G11 (referred to as C12G11 hereafter) has a high affinity for progesterone with a Kd of 20 pm. Its specificity, as evaluated in competitive immunomassays using biotinylated progesterone and various steroids analogs, includes a strong affinity for 5β- and 5α-dihydroprogesterone (DHP)3 with 30 and 20% of cross-reactivity, respectively. To develop a sensitive and highly specific in vitro immunomassay, we initiated a protein-engineering program with the aim of reducing the cross-reaction with these two analogs while maintaining its subnanomolar affinity for progesterone. The difficulties presented by such a project are associated with the lack of distinctive chemical groups that could be used to differentiate among these three compounds. With the only distinguishing features being the three-dimensional structures that these...
progestosterone derivatives adopt, the outcome of the search for variants possessing the right binding combination appeared difficult. The high homology with DB3 allowed the construction of models of our antibody, enabling us to plan a strategy of reengineering the combining site of C12G11 using combined semirational and random mutagenesis to select by phage display mutants with the desired characteristics. We present here the strategy we used and the fine characterization of the selected mutants. Models of the best variant were built and compared with that of wild-type C12G11 to account tentatively for the structural basis for the improved binding.

**EXPERIMENTAL PROCEDURES**

**Cloning and Construction of scFvC12G11—**Hybridoma cells producing C12G11 were cultured in Iscove's modified Dulbecco's medium containing 10% fetal calf serum (Invitrogen) and antibiotics (100 μg/ml) at 37°C in a 5% CO₂ humidified chamber. Cloning and sequencing of mRNA encoding the light chain and the fl heavy chain of C12G11 were done as previously described (6). DNA encoding the scFvC12G11 (VL-linker-VH) was generated by PCR-based single overlap extension (7), using an 18-amino acid linker derived from the one initially described by Weissman et al. (8) according to Merocz et al. (9).

**Construction of scFv Libraries—**Wild-type scFvC12G11 DNA was cloned into the phagemid pAK100 (10) and used as template to build the libraries. A first library named 3L/2H was designed by overlap PCR via simultaneous saturation mutagenesis at positions L94, L95, L96 (CDR3L), H50, and H58 (CDR2H). PCR A was obtained using primers pAK100-3′-GTTCTCCGTTGTAGGTGTTTAT-5′ and pAK100-5′-GCTTGGTCCAGCACGCCGTTTMMNN-3′ and CDR3Lsyn493-5′-GCTTGGTCCAGCACGCCGTTTMMNN-3′ and CDR2Hsyn553-5′-GTTCTCCGTTGTAGGTGTTTAT-3′, respectively. PCR B and C were, respectively, performed using the sets of primers CDR3L-3′-TTCGGTGCCTGCCGAGGCACCTGAGCTGACCAGACG-5′/CDR3L-5′-ATACCAACGAGCACTGACGTTTMMNN-3′ and CDR2H-3′-ATACCAACGAGCACTGACGTTTMMNN-5′/CDR2H-5′-GTTCTCCGTTGTAGGTGTTTAT-3′. A second library was built independently by error-prone PCR (11) of the VH domain (epVH) via a Gene Morph PCR Mutagenesis kit (Stratagene). The GenBank accession number of the VL/CL nucleic and proteic sequences is AY854499 and of the VH/CH1 nucleic and proteic sequences is AY854500.

**Screening of the Libraries and Characterization of scFv Fragments—**The libraries were rescued using helper phage M13KO7, and screened and proteic sequences is AY854499 and of the VH/CH1 nucleic and proteic sequences is AY854500.

**RESULTS**

**Molecular Model of C12G11—**The isotype of the C12G11 is γ1, subgroup IX, D-sp2.9, εH-2 for the heavy chain and κ, subgroup I, V-Jκ5 for the light chain (18). A FASTA search on the structures deposited in the PDB identified DB3 (4, 5), another anti-progestosterone antibody, as being the antibody sharing the highest degree of homology with C12G11. The variable light and heavy domains of C12G11 display, respectively, 88.3 and 79.6% of sequence identity with those of DB3, and most changes are highly conservative. The six CDRs are identical in length, and CDR2L and CDR1H have identical sequences (Table I). CDR2H, with five substitutions, is the least conserved hypervariable loop. Overall, 12 of the 16 amino acid residues are conserved among the five DB3-like scFv probes of C12G11.

**Molecular Modeling—**Models of the Fv domain of C12G11 antibody were generated using the program MODELLER (16) with anti-progestosterone antibody DB3 (PDB code 1DBA) as template. The cross-reactive steroids 5β-DHP and 5α-DHP were built using the Cschem 3Dpro program. Models of the progesterone, 5β-DHP, and 5α-DHP complexes to C12G11 were constructed using the experimental structures of the DB3 complexes with progesterone, 5α-pregnane-20-one-3β-hemisuccinate (PDB code 1DBA), 5β-androstane-3β,17β-dione (PDB code 1DBR, 2α and 1DBK, respectively). The 5β-DHP and 5α-DHP analogs were fitted in the same orientation as the progesterone analogs bound to DB3. The backbone of the mutated third hypervariable loop in the combined variant Pro5β-His5α-Val5α-Val5α-Pro5α-Arg5α-Lys5α-Lys5α-His5α was built as a hybrid between the same loop in DB3 and antibody J539 (PDB code 2FBJ) (17).

**Anti-progesterone Antibody Engineering**

**Experimental Procedures**

Cloning and Construction of scFvC12G11—Hybridoma cells producing C12G11 were cultured in Iscove's modified Dulbecco's medium containing 10% fetal calf serum (Invitrogen) and antibiotics (100 μg/ml) at 37°C in a 5% CO₂ humidified chamber. Cloning and sequencing of mRNA encoding the light chain and the fl heavy chain of C12G11 were done as previously described (6). DNA encoding the scFvC12G11 (VL-linker-VH) was generated by PCR-based single overlap extension (7), using an 18-amino acid linker derived from the one initially described by Weissman et al. (8) according to Merocz et al. (9).

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**Screening of the Libraries and Characterization of scFv Fragments—**The libraries were rescued using helper phage M13KO7, and screened following a procedure derived from that initially described by Hawkins et al. (12). A preselection round was carried out in the presence of 5 nM progesterone-11α-EMC-oligonucleotide-biotin. The resulting pre-screened library was then submitted to five rounds of competitive selection according to the strategy described by Saviranta et al. (9). A preselection round was carried out in the presence of 5 nM progesterone-11α-EMC-oligonucleotide-biotin together with 5 μM 5β-DHP or 5α-DHP. Screening of the error-prone library was performed in competition as described above against 5β-DHP only.

**Competition ELISA was used to characterize the selected scFv phages.** Phages were produced by infecting Escherichia coli strain DB1K cells grown in Iscove's modified Dulbecco's medium to an optical density of 0.6 to 0.8 at 600 nm, with DNA encoding the best variants were subcloned into pUMR vector and expressed as scFv-His, fragments in the periplasmic space of MC1061 E. coli DH5α bacteria. Resulting scFv-His, molecules were extracted and IMAC-purified as described previously (14). Protein concentrations were determined by amino acid analysis.

**Surface plasmon resonance analysis was performed on a BiAcore 2000 biosensor optical (BIAcore, Uppsala, Sweden).** Dissociation constants (Kₐ) for progesterone, 5β-DHP, and 5α-DHP were determined by competition BiAcore using a sensor chip saturated with biotinylated progesterone (= 690 resonance units) as described previously (15). A streptavidin surface without biotinylated progesterone was used as a control. 200-μl samples of 10 nM pure scFv variant in a buffer containing various amount of competitor, preincubated over 1 h at 37°C, were injected through the sample loop of the system. Data were evaluated with BIAevaluation software (Amersham Biosciences) and KaledisGraph (Synergy Software).

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Trp\(^{H47}\), Trp\(^{H50}\), Phe\(^{H97}\), Trp\(^{H100}\), and Phe\(^{H100k}\) lining the cavity that accommodates progesterone rings D, C, and B, and more specifically, rings B and C are sandwiched between Trp\(^{H50}\) and Trp\(^{H100}\) (Fig. 1B).

Antibody C12G11 displays strong cross-reactivity toward 5β-DHP and 5α-DHP, two hepatic metabolites of progesterone, in the same way as DB3 interacts with high affinity with analogs such as 5β-androstan-3–17-dione, aetiocholanolone (C-5β), and 5α-pregnane-3-β-hemisuccinate. The x-ray structures of these DB3-steroid complexes have been solved and detailed (4, 5). It was the similarities between the antigen binding sites of DB3 and C12G11 that allowed us to model the binding of 5β-DHP based on the 5β-androstan-3–17-dione and aetiocholanolone complexes, both C-5β, and 5α-DHP using 5α-pregnane-3-β-hemisuccinate. Thus we modeled 5α-DHP occupying the P1, P2, and P3 binding pockets in C12G11 in a syn configuration close to that of progesterone, whereas 5β-DHP would adopt an anti binding mode, with its ring A positioned into the alternative pocket P3’ delimited by Trp\(^{H50}\) and Val\(^{L94}\)user, the methyl groups facing TrpH47, TrpH50, PheH97, TrpH100, and PheH100k lining the cavity specifically, rings B and C, and more strongly from the 5α-DHP competitor with basic residues such as Arg and His becoming dominant at the 58% (11/19) level. Similarly, the wild-type residue Val\(^{L94}\) of the native C12G11 reappeared in only 21% (4/19) of the sequenced clones giving way again to basic amino acids (4%, 9/19), Lys and especially Arg, but also proline appeared in 31% (6/19) of the clones. A large majority (74%, 14/19) of basic amino acids, Arg and His, replaced the wild-type Thr\(^{L94}\). The study with 5α-DHP gave similar results, and the amino acid profiles for the two parallel studies were compared, showing that the diversity of the 5α-DHP-selected clones is more limited than that retained with 5β-DHP (Table II). The proline residue at position L95 disappeared, but proline at position L94 doubled to 74% (14/19). Finally, positions L96 and H50 completely reverted to the wild-type residues, and basic residues were found at position H58 at the same level in both cases.

After the two panning procedures, most of the selected clones were produced individually fused to the minor coat protein gIII on the surface of the phage to establish their overall binding properties (see “Experimental Procedures”). Table III gives the change in the IC\(_{50}\) values for progesterone, 5β-DHP, and 5α-DHP for each mutant compared with wild-type (see “Experimental Procedures” and Table III footnotes). The cross-reaction profiles of the mutants for the two analogs were calculated as the ratios of IC\(_{50}\) Prog/IC\(_{50}\) 5β-DHP, or IC\(_{50}\) Prog/IC\(_{50}\) 5α-DHP, respectively. The first group of variants is characterized by basic amino acid (Lys or Arg) at position L94 and the absence of proline residue at position L95; clones B16, A23, B4, A31, B7, and B19. All but clone A31 display a decrease of their cross-reactivity for 5β-DHP (15.9 to 22.8% versus 26% for the wild-type antibody fragment), and 5α-DHP (4.7 to 5.9% versus 19% for the wild-type). This result suggests that in our case, functional C12G11 variants can be generated despite the absence of proline residue in the CDR3L hypervariable loop, including the canonical one at position L95. Most interestingly, four variants in particular, A3 (Pro\(^{L94}\)-Tyr\(^{L95}\)-Val\(^{L96}\)/Trp\(^{H50}\)-His\(^{H58}\)\), B5 (Pro\(^{L94}\)-Arg\(^{L95}\)-Val\(^{L96}\)/Trp\(^{H50}\)-Arg\(^{H58}\)\), B21 (Pro\(^{L94}\)-His\(^{L95}\)-

### Table 1

| Antibody | CDR1L | CDR2L | CDR3L |
|----------|-------|-------|-------|
| DB3      | 27abcde 30 50 | 90 95 | SQQSHVPP |
| C12G11   | ------- | V-N- | ------ |

| Antibody | CDR1H | CDR2H | CDR3H |
|----------|-------|-------|-------|
| DB3      | NYGN | WINLYTGEPTYVDFK | GYDNMYFEDV |
| C12G11   | ------- | T-N- | L-R- | VFDG- | Y- |

**Screening of 3L2H scFv-phage Library**—Prior to starting the selection by competition, the naïve library was voided of low affinity and nonfunctional scFv-phages by carrying out one round of preselection with just biotinylated progesterone. The prepanned library was then subjected to five rounds of selection in the presence of a large excess of either 5β-DHP or 5α-DHP. After five rounds of competitive panning selection using 5β-DHP or 5α-DHP as competitor, we noticed that the nature of the side chains found at the degenerated positions had evolved during the panning, leading to a marked reduction of the initial diversity (Table II). Thus, position H50 totally recovered the wild-type tryptophan, and position L96 was essentially populated by hydrophobic residues with a strong preference for the wild-type valine. Despite the deliberate bias toward the wild-type proline at position L95, we observed a steady drop, from the 70% level forced into the library during construction, to just 21% (4/19) after five rounds of selection with the 5β-DHP competitor with basic residues such as Arg and His becoming dominant at the 58% (11/19) level. Similarly, the wild-type residue Val\(^{L94}\) of the native C12G11 reappeared in only 21% (4/19) of the sequenced clones giving way again to basic amino acids (4%, 9/19), Lys and especially Arg, but also proline appeared in 31% (6/19) of the clones. A large majority (74%, 14/19) of basic amino acids, Arg and His, replaced the wild-type Thr\(^{L94}\). The study with 5α-DHP gave similar results, and the amino acid profiles for the two parallel studies were compared, showing that the diversity of the 5α-DHP-selected clones is more limited than that retained with 5β-DHP (Table II). The proline residue at position L95 disappeared, but proline at position L94 doubled to 74% (14/19). Finally, positions L96 and H50 completely reverted to the wild-type residues, and basic residues were found at position H58 at the same level in both cases.

After the two panning procedures, most of the selected clones were produced individually fused to the minor coat protein gIII on the surface of the phage to establish their overall binding properties (see “Experimental Procedures”). Table III gives the change in the IC\(_{50}\) values for progesterone, 5β-DHP, and 5α-DHP for each mutant compared with wild-type (see “Experimental Procedures” and Table III footnotes). The cross-reaction profiles of the mutants for the two analogs were calculated as the ratios of IC\(_{50}\) Prog/IC\(_{50}\) 5β-DHP, or IC\(_{50}\) Prog/IC\(_{50}\) 5α-DHP, respectively. The first group of variants is characterized by basic amino acid (Lys or Arg) at position L94 and the absence of proline residue at position L95; clones B16, A23, B4, A31, B7, and B19. All but clone A31 display a decrease of their cross-reactivity for 5β-DHP (15.9 to 22.8% versus 26% for the wild-type antibody fragment), and 5α-DHP (4.7 to 5.9% versus 19% for the wild-type). This result suggests that in our case, functional C12G11 variants can be generated despite the absence of proline residue in the CDR3L hypervariable loop, including the canonical one at position L95. Most interestingly, four variants in particular, A3 (Pro\(^{L94}\)-Tyr\(^{L95}\)-Val\(^{L96}\)/Trp\(^{H50}\)-His\(^{H58}\)\), B5 (Pro\(^{L94}\)-Arg\(^{L95}\)-Val\(^{L96}\)/Trp\(^{H50}\)-Arg\(^{H58}\)\), B21 (Pro\(^{L94}\)-His\(^{L95}\)-

**Comparison of amino acid sequences of VL-CDRs and VH-CDRs of the anti-progesterone antibodies DB3 versus C12G11**

| Antibody | CDR1L | CDR2L | CDR3L |
|----------|-------|-------|-------|
| DB3      | 27abcde 30 50 | 90 95 | SQQSHVPP |
| C12G11   | ------- | V-N- | ------ |

| Antibody | CDR1H | CDR2H | CDR3H |
|----------|-------|-------|-------|
| DB3      | NYGN | WINLYTGEPTYVDFK | GYDNMYFEDV |
| C12G11   | ------- | T-N- | L-R- | VFDG- | Y- |
Val^L96/Trp^H50-Asp^H58), and B14 (Pro^L94-His^L95-Val^L96/Trp^H50-Arg^H58), are characterized by a more important reduction of cross-reactivity toward both 5α/H9252-DHP and 5α/H9251-DHP, than the clones of the first group. Indeed, the percentages of cross-reactivity of the latter are an average of 7.0% versus 26% and 3.0% versus 19%, for 5α/H9252-DHP and 5α/H9251-DHP, respectively. Furthermore, the deduced IC₅₀ values for progesterone of all selected and tested mutants remain close to that of the wild-type scFv, with a variation comprised between 1.3 and 3.3 only.

Identification of Second Sphere Residues—Undoubtedly, the VH domain of C12G11 plays an important role both in the formation of the binding cavities and in its lack of specificity.

**FIG. 1.** Structures of progesterone, 5β-DHP, and 5α-DHP derivatives (A) and molecular models of native (B) and (C) and engineered (D) C12G11 binding site. A, representation of progesterone (upper diagram) showing the numbering scheme for the skeleton and the nomenclature of the steroid rings. Superimposing the different rings (top diagram onto bottom) shows the conformation variability of the steroid skeleton: progesterone in yellow (conformation A), 5α-DHP in magenta (conformation B), and 5β-DHP in green (conformation C). B, molecular model of the C12G11 wild-type antigen binding site with the three structurally distinct steroids: progesterone (yellow), 5α-DHP (magenta), and 5β-DHP (green). The side chains of residues important for the binding are shown. Progesterone and 5α-DHP adopt a syn configuration with the methyl groups facing Trp^H50; ring A is positioned in pocket P3 surrounded by residues Trp^H100, His^L27d, Val^L94, Pro^L95, and Val^L96. 5β-DHP adopts an anti conformation with its methyl groups facing Trp^H50 and uses the alternative pocket P3' composed by Val^L94, Pro^L95, Val^L96, Trp^H47, Trp^H50, and Thr^H58 for the ring A binding. Residues submitted to saturation mutagenesis are labeled in blue. C, location of the different binding subpockets P1, P2, P3, and P3', which form the steroid binding site of the C12G11 wild-type antigen binding site with progesterone (yellow) and 5β-DHP (green). D, close-up view of the C12G11 mutant B14 + Lys^H31-His^H32 binding site model. The CDR3L loops are shown in the backbone trace. The original canonical class 1 CDR3L loop is indicated in orange, and the predicted new CDR3L loop with L93-L95 in canonical class 2 is in yellow. The surface representation corresponds to that of the engineered scFv variant B14 + Lys^H31-His^H32. The color scheme is as in A: progesterone, yellow; 5α-DHP, magenta; and 5β-DHP, green. The five mutated residues that led to the mutant B14 + Lys^H31-His^H32 are labeled in blue.
Consequently, an error-prone PCR library of variants focused on the VH domain only was built in parallel to the 3L2H library, and this was to complete tentatively with the structure-based engineering of C12G11. The resulting library was only screened against 5β-DHP because its binding takes in particular advantage of the alternative binding pocket P3* that is mainly defined by the VH domain (Fig. 1A). After six round of panning, selected clones displayed multiple mutations, a reduced recognition of 5β-DHP with 10 versus 26% of cross-reactivity, but also a significant loss of affinity for progesterone (data not shown). It is noteworthy that a majority of these multiple random mutants contained repeatedly one of following substitutions: asparagine into lysine at position 52 (CDR2H) or glutamic acid into lysine at position 56 (CDR2H) (Table II). Among the bests combined mutants and compared with the wild-type fragment, whereas the affinity for progesterone remains in the picomolar range (Fig. 2). Moreover, comparison of the $K_d$ values obtained for mutant B14 and B14 + Lys$^{51\text{H11001}}$, His$^{32\text{H11001}}$ confirms the beneficial effect upon addition of the double mutation Asn$^{31\text{H11001}}$ → Lys/Tyr$^{52\text{H11001}}$ → His. The improvement in specificity for our best mutant B14 + Lys$^{51\text{H11001}}$His$^{32\text{H11001}}$ compared with the wild-type scFv$_{c12g11}$ was calculated from measurement obtained under identical experimental conditions (Fig. 2). It shows an increase in discrimination against 5β-DHP and 5α-DHP, 23-fold and 15-fold, respectively, compared with the starting antibody. These improvements were calculated as cross-reaction percentages from experimentally measured $K_d$ values (Fig. 2). Furthermore, the lower cross-reactivity of mutant B14 + Lys$^{51\text{H11001}}$His$^{32\text{H11001}}$ is associated with a moderate 3.8-fold increase in the dissociation constant for progesterone (75 pM versus 20 pM).

**DISCUSSION**

**Strategies of Engineering of Anti-progesterone C12G11 Antibody**—In the last decade, the ability of protein engineering to modulate the affinity and specificity of antibodies has been steadily improving, especially in the case of haptens such as steroids, sulfa-antibiotics, cardiac and cancer markers (12, 20–31). Here we describe the first successful engineering work on an anti-progesterone antibody aimed at reducing its cross-reactivity toward 5β-DHP and 5α-DHP, two hepatic analogs, while preserving its high affinity for progesterone. The best mutant we generated is not only more specific than the starting antibody with a 23-fold and 15-fold lower cross-reactivity for 5β-DHP and 5α-DHP, respectively, but it has also maintained its affinity for progesterone within the picomolar range. One should note that this was achieved progressively using a precisely targeted combination of site-directed saturation and random mutagenesis.

The binding site of DB3 was instrumental for building models of our antibody to assess first prior to this work the possibility of improving the binding specificity of C12G11 by site-directed substitutions of the main residues that define the alternative binding pockets P3 and P3'. However, none of the constructed single site mutants displayed the expected specificity while still retaining the ability to bind progesterone (data not shown). This emphasized the structural plasticity and the functional adaptability of the C12G11 antigen binding site and confirmed that it is difficult to carry out successful engineering by site-directed mutagenesis alone, as was found since the first antibody engineering of an anti-digoxin binding site (32). Aware of these problems, we thus opted for a strategy of combinational site-directed-saturation mutagenesis aimed at modifying simultaneously positions L94, L95, L96, H50, and H58, which our models have identified as involved in the geometry of the binding pockets P3 and P3' responsible for lack of specificity of C12G11.

The resulting structure-based library was expressed and screened by phage display, using a competition panning procedure, a strategy that appears particularly well adapted to engineer the specificity of anti-hapten antibodies (13, 20, 21, 24, 33). After first screening assays using variable concentrations of ligand, we kept the concentrations of biotinylated progesterone, two hepatic analogs, at 1 nM. The second screening then allowed us to select a competitive population.

**Table II**

| Panning selection with competitor | 5β-DHP | 5α-DHP |
|----------------------------------|-------|-------|
| Wild type                        |       |       |
| Library                          |       |       |
| P-V-T                            |       |       |
| K-I-H                            |       |       |
| R-R-R                            |       |       |
| R-H-A                            |       |       |
| R-R-H                            |       |       |
| R-R-R                            |       |       |
| P-L-A                            |       |       |
| P-R-Y                            |       |       |
| P-H-Y                            |       |       |
| P-H-R                            |       |       |
| P-H-R                            |       |       |
| P-H-R                            |       |       |

*“–“ indicates the same amino acids as those in wild-type sequence. Residue numbering is according to the Kabat scheme.*
combination of mutations capable of reducing the affinity of both progesterone metabolites simultaneously. We feared that a competitive procedure with all three natural ligands of C12G11 at once would have overselected wild-type-like fragments, to the detriment of more specific variants. This was borne out by the sequence profiles that emerged from both screening procedures because no wild-type fragments were selected (Table II), all common and distinct solutions were retained, and little would have been gained from the simultaneous approach to justify the risks.

The first set of selected mutants that display the desired discriminating binding properties are characterized by the consensus sequence: ProL94-(Leu,Tyr,Arg,His)L95-ValL96/TrpH50-(His,Arg,Asp,Ala)H58. Very early during the selection procedures, positions L96 of CDR3L and H50 within CDR2H returned to the natural residues found in the initial C12G11 antibody, indicating that they are likely both structurally and functionally essential for high affinity recognition of progesterone. It appeared not really surprising to find a tryptophan residue at position H50 because its side chain stacks against rings B and C of the steroids, but the important role of ValL96 was a surprise because single site mutants Val L96Ile/Leu/Met or Asn had been found to have conserved progesterone recognition abilities (data not shown). Among the residues found at position H58, we identified a predominance (80%, 15/19) of basic side chains such as Arg and His and a minority of Tyr and Ala residues, in agreement with the unchanged binding capacities toward progesterone observed for the single site mutant ThrH58Arg (data not shown). The changes in the residues at the tip of CDR3L, L94-L95, were surprising. Sequence analysis of the most specific C12G11 variants (Table II) showed a selection against the native canonical ProL95, which we had deliberately biased at 70% in the initial library, in favor of unrelated basic or aromatic side chains, whereas a proline residue appears one position before in the sequence, at L94. However, such “proline exchange” is not neutral as far as 5β-H9251- and 5α-H9252-DHP recognition is concerned because both are now discriminated against, whereas the subnanomolar affinity for progesterone is maintained.

Interestingly, the specificity of recognition of this first set of variants was further improved by the addition of the double mutation AsnH31Lys/TyrH32His. The latter was selected independently by screening of the error-prone VL-linker-epVH scFv library built in parallel to the structure-based one and was found interesting because it is located behind CDR3H, which contributes to the formation of the P1 pocket. However, that positive effect was not obvious because the double mutation AsnH31Lys/TyrH32His alone was neutral upon recognition of progesterone and 5β-H9251- and 5α-H9252-DHP analogs. Thus, our results show that combining mutations issued from distinct libraries and approaches may have added benefits. Similar strategies have recently been used to modulate the binding properties of two anti-hapten antibodies (23, 25). Furthermore, it is noteworthy that our best mutant associates first sphere (31, 32) and second sphere residues (29, 34, 35), confirming the importance of noncontact amino acid residues upon antigen recognition (26, 29).

In the case of the wild-type scFv C12G11 for 5α-H9252-DHP, we note a 2-fold discrepancy between the cross-reaction percentage deduced from the competitive phage-ELISA (Table III) and the dissociation constant value determined from competitive BIAcore experiments (Fig. 2). Such discrepancies were also re-

### Table III

**Characterization of mutants by competition phase-ELISA**

| Clone | Mutations | Progesterone IC50 change | 5β-DHP Cross-reaction (%) | 5α-DHP Cross-reaction (%) |
|-------|-----------|--------------------------|--------------------------|--------------------------|
| Wild type | L94 L95 H50 V Y P V N Y W R E T | | | |
| L3H12 library: | | | | |
| B16 | K I - - - - - - R | 2.0 | 2.9 | 6.9 | 20.0 ± 3.6 | 5.3 ± 1.6 |
| A23 | K I - - - - - - H | 2.0 | 3.7 | 8.6 | 15.9 ± 0.3 | 4.7 ± 0.9 |
| B4 | K R - - - - - - R | 2.5 | 4.1 | 5.6 | 17.5 ± 3.3 | 5.9 ± 1.5 |
| A31 | R R - - - - - - D | 2.5 | 1.3 | 4.7 | 31.5 ± 6.3 | 7.4 ± 0.2 |
| B7 | R R - - - - - - H | 2.2 | 3.1 | 6.1 | 20.5 ± 4.6 | 5.7 ± 1.3 |
| B19 | R R - - - - - - R | 3.3 | 6.3 | 4.9 | 22.8 ± 3.6 | 5.4 ± 0.7 |
| B23 | P L - - - - - - A | 1.4 | 4.3 | 8.4 | 11.4 ± 3.2 | 3.3 ± 0.15 |
| A3 | P Y - - - - - - H | 3.0 | 9.6 | 14.8 | 8.2 ± 1.6 | 2.4 ± 1.2 |
| B5 | P R - - - - - - R | 2.0 | 9.1 | 12.7 | 7.0 ± 1.1 | 2.8 ± 0.4 |
| B21 | P H - - - - - - D | 1.3 | 4.0 | 8.3 | 7.0 ± 1.8 | 2.9 ± 0.1 |
| B14 | P H - - - - - - A | 2.8 | 13.5 | 20.8 | 5.6 ± 1.9 | 2.2 ± 0.2 |

**a** The IC50 values of the wild-type are average 3.7 × 10−10 M for progesterone, 1.4 × 10−9 M for 5β-DHP, and 1.9 × 10−9 M for 5α-DHP. The relative affinity of each mutated scFv-phage was evaluated by determining the IC50 change that was calculated by dividing the IC50 value of the variant looked for a defined competitor by the IC50 value of the wild-type for the same competitor. An IC50 change greater than 1 reflects a decrease of relative affinity of the variant for the competitor compared with the wild-type.

**b** The percentage of cross-reaction was calculated as follows: (IC50 progesterone/IC50 5β-DHP or 5α-DHP) × 100.
Fig. 2. Competition BIAcore experiments. Purified scFv-His$_6$ wild-type, B14 and B14 + Lys$_{H31}$-His$_{H32}$ mutants were incubated with different concentrations of progesterone, 5α-DHP, or 5β-DHP for 1 h and injected over a progesterone-coated sensor chip. From the linear sensorgrams, the slopes (resonance units vs. time in seconds) were plotted against the corresponding total competitive steroid concentration. The slopes correlating the free scFv-His$_6$ in the injected mixture and the dissociation constants ($K_d$) were calculated according to Nieba et al. (15). Cross-reaction percentages were determined as follows: $(K_d$ progesterone$/K_d$ 5α-DHP or 5β-DHP $\times 100$).

| Clone               | Dissociation constant $K_d$ (x10$^9$M$^{-1}$) | % cross-reaction |
|---------------------|---------------------------------------------|------------------|
| Wild type           | 0.020 ± 0.009                               | 71%              |
| B14                 | 0.068 ± 0.020                               | 10%              |
| B14 + K$_{H31}$H$_{H32}$ | 0.075 ± 0.018                               | 3.1%             |

In conclusion, we have been able to select variants of the C12G11 anti-progesterone antibody that displays improved specificity toward two structurally different competitive steroids, and this without significant loss of affinity for progesterone. More precisely, the engineering of C12G11 has resulted in the case of the variant Pro$_{154}$-His$_{L95}$-Val$_{L96}$-Trp$_{H95}$Arg$_{H58}$ + Lys$_{H31}$-His$_{H32}$ (clone B14 + Lys$_{H31}$-His$_{H32}$) in a simultaneous 54- and 85-fold decrease in affinity for 5α-DHP and 5β-DHP, respectively, together with a more limited 3.8-fold reduction in affinity for progesterone. To our knowledge this constitutes the first description of such a discriminating engineering of an anti-progesterone antibody versus three structurally different ligands that establish contacts via two alternative binding sites. These experimental results together with the predicted model are now exploited to design a new library of variants to assess the possibility to improve further the specificity of recognition of C12G11.

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