General Strategy to Humanize a Cameld Single-domain Antibody and Identification of a Universal Humanized Nanobody Scaffold*

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Cécile Vincke15, Remy Loris5, Dirk Saerens15, Sergio Martinez-Rodriguez4*, Serge Muyldermans5†, and Katja Conrad†19

From the 4 Laboratory of Cellular and Molecular Immunology, 1 Laboratorium voor Ultrastructuur, Vrije Universiteit Brussel, Pleinlaan 2, Brussels B-1050 and the 5 Department of Molecular and Cellular Interactions, Vlaams Instituut voor Biotechnologie, Brussels, Belgium

Nanobodies, single-domain antigen-binding fragments of cameld-specific heavy-chain only antibodies offer special advantages in therapy over classic antibody fragments because of their smaller size, robustness, and preference to target unique epitopes. A Nanobody differs from a human heavy chain variable domain in about ten amino acids spread all over its surface, four hallmark Nanobody-specific amino acids in the framework-2 region (positions 42, 49, 50, and 52), and a longer third antigen-binding loop (H3) folding over this area. For therapeutic applications the cameld-specific amino acid sequences in the framework have to be mutated to their human heavy chain variable domain equivalent, i.e. humanized. We performed this humanization exercise with Nanobodies of the subfamily that represents close to 80% of all dromedary-derived Nanobodies and investigated the effects on antigen affinity, solubility, expression yield, and stability. It is demonstrated that the humanization of Nanobody-specific residues outside framework-2 are neutral to the Nanobody properties. Surprisingly, the Glu-49 → Gly and Arg-50 → Leu humanization of hallmark amino acids generates a single domain that is more stable though probably less soluble. The other framework-2 substitutions, Phe-42 → Val and Gly/Ala-52 → Trp, are detrimental for antigen affinity, due to a repositioning of the H3 loop as shown by their crystal structures. These insights were used to identify a soluble, stable, well expressed universal humanized Nanobody scaffold that allows grafts of antigen-binding loops from other Nanobodies with transfer of the antigen specificity and affinity.

Minimizing the size of antigen-binding entities from a multidomain protein such as a monoclonal antibody to a single-chain variable fragment or even a single domain has been one of the primary goals of antibody engineering. For drug therapy, these smaller formats can be beneficial in various aspects such as immunogenicity, biodistribution, renal clearance, serum half-life, tissue penetration, and target retention. However, the minimal sized antibody fragments need to retain sufficiently high antigen specificity and affinity, be expressed in high yields, and should have a low tendency to aggregate so as to maintain maximal potency and reduce possible risks of immunogenicity. Moreover functionality in adverse environments such as high concentrations of denaturant or elevated temperatures, and a concomitant increased shelf-life are additional assets.

A significant proportion of the functional antibodies within species of the Camelidae are devoid of light chains. These immunoglobulins are referred to as heavy-chain antibodies (1), and their antigen-binding fragment is reduced to a single domain (referred to as VHH or Nanobody), with a molecular size of only ~15 kDa, which is smaller in comparison to single-chain variable fragment fragments (30 kDa), Fab fragments (60 kDa), and whole antibodies (150 kDa). All Nanobodies belong to the same sequence family, closely related to that of the human VH 3 of family III, although different subfamilies can be distinguished in dromedary based on the CDR2 length and the position of an additional cysteine in the CDR1 or the framework-2 (2). Because extra cysteines are rare in llama VHH sequences, they cannot be used as a subfamily hallmark and alternative subfamily divisions had to be proposed for llama VHs (3, 4). Following immunization of a llama or dromedary, VHH genes can be easily cloned in a phagemid vector and antigen-specific VHHs can then be selected via phage display against virtually any antigen (5). Their small size, natural soluble behavior, and unique ability to target alternative epitopes make Nanobodies very attractive tools for tumor targeting, diagnostics, or even for in vivo therapy (6–10).

Analysis of the amino acid sequence of the Nanobodies obtained from immunized camelds revealed frequent substitutions in regions that are conserved in the VH domain of conventional antibodies. These Nanobody hallmark amino acids, located mainly in framework-2, are essential adaptations to avoid the association with the variable light chain domain (11). Most of these mutations are substitutions from a hydrophobic to a hydrophilic residue and are considered to increase the solubility of the isolated Nanobody (12, 13). In some cases, several of these residues at the “former VL-side” may also affect the

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The atomic coordinates and structure factors (codes 3DWT and 3EAK) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
1 To whom correspondence should be addressed. Tel.: 32-2-629-19-69; Fax: 32-2-629-19-81; E-mail: svmuyld@vub.ac.be.
2 present address: Galapagos NV, Generaal De Wittelaan L11 A3, B-2800 Mechelen, Belgium.
3 The abbreviations used are: VH, heavy chain variable domain; LDA, ligation during amplification protocol; GdmCl, guanidinium chloride; r.m.s.d., root mean square deviation; CDR, complementarity determining region.

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antigen specificity of the Nanobody (13). These differences between VHH and VH conserved residues are Phe/Tyr-42 → Val, Glu-49 → Gly, Arg-50 → Leu, and Gly-52 → Trp (numbers refer to the International ImMunoGeneTics information system amino acid numbering (imgt.cines.fr)). Additionally, in ~10% of the Nanobodies, Trp-118 is substituted by an Arg (14). The Trp-118 → Arg substitution was proposed as an alternative option to further increase the solubility of single-domain antibody fragments (15).

Because mouse antibody fragments require a humanization step to be accepted as human therapeutics, it is likely that Nanobodies from camelid origin should also pass a humanization process. It is our objective in this work to assess the biochemical properties of several Nanobodies after such humanization effort. We investigated therefore the humanization of a couple of representative Nanobodies into more human-like antibody fragments and tested their retention of antigen-binding specificity. Two Nanobodies, members of subfamily-2, were chosen for this analysis: NbHuL6 (16) and NbBcII10 (17). Subfamily-2 is the most frequently occurring of the seven VHH families. It was previously demonstrated that the NbBcII10 framework-2 residues that were mutated to their human counterpart are shown in bold.

FIGURE 1. Amino acid sequences. A, NbHuL6 and NbHuL6gGLW with humanized residues in framework-2 (italic). B, human DP-47 reference framework sequence, NbBcII10 and h-NbBcII10gGLA, with humanized framework. Differences in sequence from the reference human sequence are underlined. Framework residues that were mutated to their human counterpart are shown in bold. Based on this knowledge, a general strategy is proposed to generate a humanized version of any Nanobody with maximal retention of stability and antigen-binding characteristics. Finally, a universal humanized Nanobody scaffold was identified that accommodates antigen-binding loops from Nanobodies, even those from other subfamilies or species.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutations were introduced by phosphorylated mutagenic oligonucleotides using the ligation during amplification protocol (LDA) (19). Multiple mutations were introduced simultaneously using multiple mutagenic primers, designed so as to add or eliminate a particular restriction enzyme site. The introduction of the different mutations was analyzed by restriction enzyme analysis and confirmed by DNA sequencing (ABI prism 3100 genetic analyzer, Applied Biosystems).

Expression and Purification of Nanobodies—The plasmid constructs were transformed into Escherichia coli WK6 cells. The expression in the periplasm and purification of recombinant His-tagged Nanobodies was performed as described previously (17). The purity of the proteins was evaluated by Coomassie-stained SDS-polyacrylamide gels. The protein concentration was determined spectrophotometrically at 280 nm using the computed extinction coefficient of each Nanobody, as calculated from their amino acid sequence (20).

Affinity Measurements—Different concentrations ranging from 500 nM to 7.8 μM of the NbHuL6, NbBcII10, and their respective variants were flown over a CM5 chip (Biacore) to which respectively human lysozyme (200 relative units) and BclII (800 relative units) had been coupled using the amine coupling chemistry (N-ethyl-N’-(dimethylaminopropyl)-carbodiimide/N-hydroxy succinimide) according to the manufacturer’s descriptions. All measurements were performed at a flow rate of 30 μl/min in HBS buffer (10 mM Heps, pH 7.5, 150 mM NaCl, 3.5 mM EDTA, and 0.005% Tween 20) and 10 mM glycine/HCl, pH 1.5, was used for regeneration. Data were fitted with the help of the BiAevaluation software version 4.1 (Biacore), on the basis of a 1:1 Langmuir binding model, with simultaneous fitting of the dissociation (k_d) and association (k_a) rate constants. The kinetic parameters k_on and k_off were subsequently used to calculate the K_D values.

Temperature-induced Unfolding—CD measurements were performed with a Jasco J715 spectropolarimeter in the far-UV (205–250 nm) region, using a protein concentration of 0.166
Generation of Chimeric Nanobody Constructs—The CDR-H loops from loop donor Nanobodies NbHuL6 (dromedary) and NbHSA (llama) were transferred to the scaffold of h-NbBcII10<sub>FGLA</sub> (humanized recipient Nanobody) by PCR-based mutagenesis. The sequence of each CDR-H loop from the loop donor Nanobody was encompassed by two primers, one back and one forward primer, containing at the 5’ and the 3’ ends the sequences corresponding to the framework residues of the recipient Nanobody. The chimeras were constructed as described previously with some minor modifications (18). The chimERIC Nanobody construct of NbHuL6 on h-NbBcII10<sub>FGLA</sub> was digested with Ncol and NotI, whereas the chimERIC construct with NbHSA as donor Nanobody was digested with HindIII and NotI. Both fragments were cloned in the expression vector pHE6<sub>G</sub> (17). The expression yield of both chimeras is comparable to the level of the humanized recipient Nanobody (2 mg/liter of culture).

Crystal Structure Determination—Data for the free form of NbBcII10 (pdb entry 3DWT) were collected at ESRF beamline ID14-2 under cryogenic conditions. The crystals diffract to 2.9-Å resolution. Data were reduced using the HKL suite of programs (29). Molecular replacement was done with the maximum-likelihood based program Phaser (30, 31), which allowed the unambiguous positioning of all eight VHH domains in the asymmetric unit. The framework region of crystal structure of NbBcII10 grafted with the CDR loops from NbLys3 (pdb entry 1ZMY) (18, 32) was used as the starting model. Rounds of simulated annealing refinement and B-factor refinement with CNS 1.0 (33) were alternated with manual rebuilding using TURBO (34). NCS restraints were used throughout the refinement, and the mll target function was used. Data collection and refinement statistics are given in Table 1. The final R and R<sub>free</sub> factors were 0.245 and 0.304, respectively, with the model fitting the electron density very well. Attempts to reduce the R factors by relaxing the NCS restraints or to build different CDR conformations in different monomers were unsuccessful.

X-ray data for the maximally humanized mutant of NbBcII10 (h-NbBcII10<sub>FGLA</sub> pdb entry 3EAQ) were collected at beamline BW7A of the DESY synchrotron, Hamburg, to a resolution of 1.85 Å and processed as for the wild-type data. The same starting model was used for molecular replacement and refinement as for the wild-type NbBcII10 structure to minimize bias. Refinement was carried out using the same protocol as for the wild-type NbBcII10, except that no NCS restraints were applied.

The same protocol was also applied to the partly humanized NbHuL6 complex (NbHuL6<sub>FGLA</sub> pdb entry 3EBA), for which the data were also collected on beamline BW7A. The wild-type NbHuL6:Hul<sub>III</sub> complex (pdb entry 1OP9) was used as the starting model for molecular replacement and refinement. All details of data collection and refinement are presented in Table 1.

RESULTS

Humanizing the Framework-2 Region of Nanobodies—Two Nanobodies, NbHuL6 and NbBcII10, with specificity for human lysozyme (Hul) and the β-lactamase BcII of Bacillus cereus, respectively, have been selected to determine the involvement of the VHH hallmark amino acid residues in framework-2 on expression yield, affinity, and stability. The
NbHuL6 was chosen for this study because of its potential therapeutic relevance, because it stabilizes an unstable human lysozyme mutant that forms fibrils and amyloids (35). The high stability (50.9 kJ mol\(^{-1}\)) of NbBcII10 (17) and the successful use of its framework to graft the antigen specificity loops from donor Nanobodies of the VHH subfamily-2 (18), makes it a logical candidate as universal Nanobody scaffold.

The two Nanobodies were mutated by LDA-PCR at the framework-2 hallmark amino acid positions 42, 49, 50, and 52 to the amino acids occurring at those positions in human VH domains, i.e., Val42, Gly49, Leu50 and Trp52, [ImMunoGeneTics numbering (imgt.cines.fr)]. These mutants, referred to as NbHuL6\(_{\text{VGLW}}\) and NbBcII10\(_{\text{VGLW}}\), respectively, were expressed in the periplasm of E. coli and purified by immobilized metal ion chromatography and gel filtration. (Nanobody mutants humanized in framework-2 are designated with a four-letter single letter code for the amino acid at positions 42, 49, 50, and 52; these letters are italicized if they correspond to the human sequence at that position). The amount of wild-type protein recovered after size-exclusion chromatography (data not shown). However, a delay in elution time from the column is noticed for both framework-2-humanized formats, suggesting nonspecific interaction of the humanized proteins with the gel matrix. This feature was also noted for isolated VH domains of conventional antibodies (13, 23, 36). In addition, the NbHuL6\(_{\text{VGLW}}\) has a tendency to precipitate upon prolonged storage at 4 °C.

The effect of humanizing the framework-2 region of NbHuL6 and NbBcII10 on the antigen-binding capacity was assessed by surface plasmon resonance. In both cases an increase in the equilibrium dissociation constants was observed (14.6 nM for NbHuL6\(_{\text{VGLW}}\) compared with 0.32 nM for wild-type NbHuL6 and 16 μM for NbBcII10\(_{\text{VGLW}}\) compared with 7.4 nM for wild-type NbBcII10 (Tables 2A and 3A)).

**Impact of Trp-118 \(\rightarrow\) Arg Substitution on Expression and Functionality of NbHuL6**—Another residue that participates in the interaction with the VL domain and that is highly conserved in conventional VH domain is Trp-118. In ~10% of the Nanobodies this residue, adjacent to the CDR3 loop, is substituted to a more hydrophilic Arg residue (14). This Trp-118 \(\rightarrow\) Arg mutation renders the domain more hydrophilic and was previously shown to have an effect on the expression and solubility of a cameldized rabbit VH domain (37). Therefore, the effect of the amino acid residue at position 118 on the expression and functionality of wild-type NbHuL6 and NbHuL6\(_{\text{VGLW}}\) was investigated.

Introduction of an Arg at position 118 of NbHuL6\(_{\text{VGLW}}\) leads to a 3-fold improvement of the expression yield, whereas a 2-fold lower expression is observed for the same mutation in NbHuL6. Additionally, the wild-type NbHuL6 with an Arg at position 118 shows a 10-fold drop in affinity (Table 2A). The presence of Trp-118 of NbHuL6 had previously been demonstrated to be critical for proper positioning of the third antigen-binding loop (18). This effect on loop organization seems to be independent of the Nanobody hallmark regions in framework-2, because a similar drop (6-fold) in affinity is observed upon mutating the NbHuL6\(_{\text{VGLW}}\) to include Arg-118.

**Impact of Residues 42, 49, 50, and 52 on Biochemical Properties of NbHuL6**—Because the effect on affinity is most dramatic for the framework-2-humanized version of NbBcII10, we decided to dissect the contribution of each mutated residue on the biochemical properties of NbHuL6. The residues 49 and 50 are grouped and mutated simultaneously so that we constructed six different mutants between NbHuL6 and NbHuL6\(_{\text{VGLW}}\) covering all the possible residue combinations at positions 42, 49, 50, and 52. The partially humanized mutants of NbHuL6 were consistently recovered at levels comparable to the wild-type (3–4 mg/liter of culture) with one notable exception for the NbHuL6\(_{\text{VGLW}}\) mutant having an expression yield of 1.4 mg/liter of culture.
To get a better view on the involvement of each of the humanizing mutations on the kinetic parameters, the affinity of each partially humanized mutant of NbHuL6 was determined. The most obvious and consistent effect upon mutating Gly to Trp at position 52 is a 6- to 10-fold increase in the kinetic off-rate (Table 2A and Fig. 2). The concomitant increase in $K_D$ value is even more accentuated when a Val is located at position 42. This leads to the proposal that residue 52 is essential for high affinity antigen binding; most probably in conjunction with the residue at position 42. Only minor differences in affinity were observed when mutating the residues at positions 49 and 50.

The impact of the different mutations in framework-2 on the stability of NbHuL6 was assessed by determining the free energy of unfolding of all mutants from denaturant-induced unfolding and refolding experiments (Table 2A). The unfolding is completely reversible for all variants, because the fluorescence spectra measured after refolding are indistinguishable from those of the native states before unfolding (data not shown). The $m$ values around 15 kJ mol$^{-1}$ M$^{-1}$ are in the range expected for proteins of this size (14–15 kDa) (38). A single unfolding transition was observed, indicating a cooperative two-state unfolding of the Nanobodies (Fig. 3A). The mutations Glu-49→Gly and Arg-50→Leu result in a notable increase in protein stability, especially when another Gly occupies position 52 (Table 2A and Fig. 3, B and C). Conversely, a loss of stability was observed upon mutation of Phe to Val at position 42.

**TABLE 2**

Kinetic rate and equilibrium dissociation constants, changes in the free energies of unfolding by equilibrium denaturation (A) and overview of the heat-induced unfolding experiments (B) upon mutation of VH hallmark residues of NbHuL6

| A) | $k_{on}$ | $k_{off}$ | $K_D$ | $C_m$ | $m$-value | $\Delta G^\circ$ | $\Delta \Delta G^{VH}$ (relative to wild-type) |
|---|---|---|---|---|---|---|---|
| FERG | 2.39E+06 | 7.68E-04 | 0.32 | 3.02E+02 | 13.7 ± 1.0 | 41.4 ± 3.4 |
| FERW | 2.04E+06 | 6.85E-03 | 3.36 | 3.35E+01 | 15.5 ± 0.5 | 51.9 ± 1.9 |
| VERG | 2.70E+06 | 1.61E-03 | 0.60 | 2.58E+01 | 14.4 ± 0.6 | 37.1 ± 1.8 |
| VERW | 1.01E+06 | 1.59E-02 | 15.74 | 3.05E+01 | 16.7 ± 0.7 | 50.8 ± 2.4 |
| FGLG | 1.42E+06 | 5.78E-04 | 0.41 | 3.39E+02 | 19.0 ± 1.9 | 64.7 ± 6.6 |
| FGLW | 1.63E+06 | 3.85E-03 | 2.36 | 3.83E+02 | 14.3 ± 1.1 | 55.0 ± 4.4 |
| VGLG | 2.58E+06 | 1.24E-03 | 0.48 | 2.95E+01 | 19.7 ± 1.3 | 58.1 ± 3.9 |
| VGLW | 9.31E+05 | 1.36E-02 | 14.61 | 3.50E+01 | 15.4 ± 0.8 | 54.0 ± 3.1 |
| FERG W118R | 8.74E+05 | 2.98E-03 | 3.41 | 1.75E+02 | 9.0 ± 0.4 | 15.8 ± 0.9 |
| VGLG W118R | 2.12E+05 | 1.95E-02 | 91.98 | 3.11E+01 | 14.9 ± 0.5 | 64.6 ± 1.7 |

| B) | $T_m$ | Reversibility | Restored activity |
|---|---|---|---|
| FERG | 79.7 ± 0.2 | 93.3 | 86.8 |
| FERW | 84.2 ± 0.5 | 93.4 | ND |
| VERG | 76.2 ± 0.2 | 93.0 | 58.8 |
| VERW | 79.4 ± 0.2 | 92.6 | 100 |
| FGLG | 82.5 ± 0.3 | 96.3 | 64.9 |
| FGLW | ND | 103.9 | 100 |
| VGLG | 78.4 ± 0.2 | 81.6 | 65.4 |
| VGLW | 79.5 ± 0.3 | 66.7 | 79.9 |
| FERG W118R | 70.0 ± 0.2 | 69.9 | 58.4 |
| VGLG W118R | 79.9 ± 0.2 | 96.3 | 55.2 |

* Partially humanized mutants in framework-2 are designated with a four-letter label referring to the residues at, respectively, positions 49, 50, 52 and 52. The human hallmark residues at these positions are in italic font.

$\Delta \Delta G^{VH} = \Delta G^\circ$ (wild type) − $\Delta G^\circ$ (mutant) (27, 28).

$ND$, not determined.

To get a better view on the involvement of each of the humanizing mutations on the kinetic parameters, the affinity of each partially humanized mutant of NbHuL6 was determined. The most obvious and consistent effect upon mutating Gly to Trp at position 52 is a 6- to 10-fold increase in the kinetic off-rate (Table 2A and Fig. 2). The concomitant increase in $K_D$ value is even more accentuated when a Val is located at position 42. This leads to the proposal that residue 52 is essential for high affinity antigen binding; most probably in conjunction with the residue at position 42. Only minor differences in affinity were observed when mutating the residues at positions 49 and 50.

The impact of the different mutations in framework-2 on the stability of NbHuL6 was assessed by determining the free energy of unfolding of all mutants from denaturant-induced unfolding and refolding experiments (Table 2A). The unfolding is completely reversible for all variants, because the fluorescence spectra measured after refolding are indistinguishable from those of the native states before unfolding (data not shown). The $m$ values around 15 kJ mol$^{-1}$ M$^{-1}$ are in the range expected for proteins of this size (14–15 kDa) (38). A single unfolding transition was observed, indicating a cooperative two-state unfolding of the Nanobodies (Fig. 3A). The mutations Glu-49→Gly and Arg-50→Leu result in a notable increase in protein stability, especially when another Gly occupies position 52 (Table 2A and Fig. 3, B and C). Conversely, a loss of stability was observed upon mutation of Phe to Val at position 42.
Strategy to Humanize Nanobodies

Temperature-induced unfolding followed by CD spectroscopy largely confirms these results (Table 2B). For all the different variants of NbHuL6 a single transition was observed, consistent with two-state folded-unfolded behavior (Fig. 4A). As expected, a large proportion (>90%) of the native ellipticity was recovered upon cooling the samples from 95 °C to 35 °C, with exception for the NbHuL6 with only very few main-chain atoms involved. This contrasts to earlier observations that suggested that the high affinity of heavy-chain antibodies might in part be due to a high tendency to recruit the polypeptide backbone as this would reduce the entropy penalty of the association.

Humanization by Veneering of the Complete Framework of NbBcII10—Apart from the Nanobody hallmark residues in framework-2, other amino acids differences occur between the VHH framework and the VH framework of a conventional antibody at various positions. To obtain a single-domain antibody fragment with the highest possible similarity to human germ line sequences, we also humanized these residues using the human germ line DP-47 as a reference (41) (Fig. 1B). DP-47 belongs to the VH family III, which also includes the camelid VHH domains and is well expressed/displayed in bacterial/phage systems (42). The VH family III occurs in antibodies against a wide variety of different antigens.

NbBcII10 was preferred for this analysis because of its high conformational stability (50.9 kJ/mol ± 2.1) and the successful use of its framework in several loop-grafting experiments (18). At first, eleven camelid VH framework residues outside framework-2 of NbBcII10 were humanized to mimic the DP-47 sequence (Ser-12 → Leu, Ala-15 → Pro, Thr-24 → Ala, Ala-83 → Ser, Val-87 → Leu, Thr-88 → Tyr, Asn-93 → Ser, Lys-95 → Arg, Pro-96 → Ala, Ile-101 → Val, and Glu-123 → Leu) (h-NbBcII10). These solvent-exposed residues are expected to have only minor influence on the CDR conformation (43, 44), which is confirmed by the wild-type-like binding behavior of h-NbBcII10 (Table 3 and Fig. 2). However, this resurfacing affects the stability of NbBcII10, because its melting point decreases from 77.5 °C to 72.0 °C for h-NbBcII10.

We demonstrated earlier the severe impact of the humanization of framework-2 on the antigen-binding capacity of NbBcII10. Our analysis on NbHuL6 demonstrates that this effect was mainly attributed to the residues at positions 42 and 52. Therefore only the residues at positions 49 and 50 in framework-2 were changed to their human counterpart in NbBcII10 and h-NbBcII10. We then compared the biochemical properties of NbBcII10 and h-NbBcII10, respectively, and observed a considerable gain in thermodynamic as well as thermal stability, whereas the antigen-binding capacity and solubility are preserved (Table 3, A and B, and Fig. 2). The increase in stability even compensates for the loss of stability observed for the veneered h-NbBcII10. As for NbHuL6, the mutations at positions 49 and 50 cause a more sticky behavior of the mutated proteins, resulting in a longer retention time on the size-exclusion chromatography column. Strikingly, the maximal humanization of the NbBcII10 resulted in a complete loss of the reversible refolding and binding activity after heat treatment (Table 3B). This seems to be communally attributed to the mutations spread throughout the entire scaffold region of NbBcII10 and is not only confined to those of framework-2.
Structural Effects of Humanizing NbBcII10—The crystal structure of wild-type and maximally humanized NbBcII10 (h-NbBcII10GLA) were determined to 2.9 Å and 2.1 Å resolution, respectively (Fig. 6, A and B). The maximally humanized mutant of NbBcII10 crystallizes with two molecules in the asymmetric unit. Both molecules are very similar (r.m.s.d. 0.37 Å for 128 Cα atoms). All mutated residues have their side-chain solvent exposed on the surface of the molecule and provoke a minimal structural adaptation of the protein. The mutant structures are nevertheless distinct from the wild-type structures with r.m.s.d. values between 0.77 and 0.88 Å for 125 Cα atoms (three residues have missing coordinates in the wild-type structure), similar to the situation in NbHuL6. Pairwise r.m.s.d. plots as a function of residue number show that the corresponding structural adaptations are spread over the entire structure rather than being located in specific regions, in agreement with the thirteen mutations being distributed over the entire sequence. Nevertheless, somewhat larger deviations are observed for the N terminus, CDR2, and residues 80–88. These differences are, however, difficult to link to specific mutations and may, at least in part, be a consequence of different crystallization conditions and crystal lattice interactions.

The crystal structure of the h-NbBcII10GLA was superimposed on the single-domain human VH structures 1OHQ and 3BV9 from Jespers, et al. (45) and Barthelemy, et al. (36), respectively, to assess the structural effect of the humanized sequences in our Nanobody. With the exception of the residues Ser-83 and Val-101, where a 120° turned rotamer of the side chain was noticed without further effect on surrounding residues, all other amino acids had an identical positioning.

Grafting Experiments on the Humanized h-NbBcII10GLA—The identification of a highly stable, humanized Nanobody scaffold that allows grafting of the antigen-binding loops of single-domain antibodies is an ultimate goal. For h-NbBcII10GLA to be a good universal scaffold we need to demonstrate that its maximally humanized framework serves as a universal acceptor for loop grafting and concomitantly for transfer of antigen specificity. We therefore constructed two Nanobody chimeras by CDR grafting and analyzed their biophysical properties. As mentioned before, NbHuL6 is a member of the dromedary subfamily-2, whereas the llama NbHSA belongs to the VH subfamily-2 according to the classification of Achoor et al. (3). The latter Nanobody was chosen to demonstrate the tolerance to transfer the antigen specificity and affinity of a non-dromedary VHH to our humanized dromedary VHH scaffold. Grafting the CDRs of NbHSA and NbHuL6 on the framework of h-NbBcII10GLA to generate h-NbBcII10GLA-S-S-S and h-NbBcII10GLA-L-L-L, respectively, had no significant effect on the expression and the affinity of the recombinant proteins (Table 3A and Fig. 2). The thermodynamic stability of the
h-NbBcII10FGLA S-S-S was slightly decreased compared with that of the original clone, whereas in the case of h-NbBcII10FGLA L-L-L, the stability was higher than for NbHuL6. This result is confirmed by the heat-induced unfolding experiments showing a small increase in $T_m$ value of h-NbBcII10FGLA L-L-L (Table 3B). In addition, the reversible refolding after heat-induced unfolding of h-NbBcII10FGLA was partially restored after grafting the CDR loops of NbHuL6. It corroborates that, apart from the scaffold, the CDRs also contribute to this particular property of camelid single-variable domains.

**DISCUSSION**

Camelid single-domain antibody fragments or Nanobodies have a high potential for biotechnological and medical applications because of their small size, good stability, high antigen affinity and specificity, and improved solubility. Due to their small size and the high degree of identity of their framework to the human VH framework of family III, Nanobodies are expected to exhibit a low immunogenicity (14). Nevertheless, for tumor therapy the perception is that the Nanobody needs to be humanized to a maximal degree, evidently without compromising on their expression level, affinity, solubility, and stability.

The most remarkable differences between Nanobodies and human VH domains are the adaptations in the framework-2 region. Most of these mutations involve a substitution from a hydrophobic to a hydrophilic residue and are considered to be important for the solubility of the isolated Nanobody (12, 13). Indeed, human VH domains occur in nature only in complex with a VL domain, and the removal of this VL domain would expose a large hydrophobic surface to the solvent. Therefore, most isolated VH domains have a pronounced tendency to aggregate (46). Consequently, it is crucial to understand the influence of each of these framework-2 substitutions on the biophysical properties of Nanobodies to produce the most optimal single-domain antibody format. To date, most studies attempting to unravel the function of these VHH hallmark residues have focused on the partial camelization of conventional VH single domains to exploit the favorable properties of Nanobodies (12, 37, 47, 48). This approach achieved only limited success as camelizing mutations on human or mouse VH was shown to be thermodynamically destabilizing due to framework deformations and did not completely eliminate the tendency to dimerize and aggregate (49–51). In another approach, Barthelemy et al. (36) introduced a guided molecular evolution technique to mutategenize a human VH into a stabilized, reversibly refolding, better expressing, and autonomous single-domain antibody fragment. Here we start from a properly behaving dromedary-derived Nanobody and propose a strategy to humanize this single-domain antibody. The advantage of humanized Nanobodies over camelized human VHs is that VHH domains evolved naturally, matured in the absence of a VL partner and will therefore behave as soluble, strictly monomeric, specific single domains. Other adaptations outside the framework-2, spread over the entire VHH

**FIGURE 4.** Temperature-induced unfolding transitions of (A) NbHuL6 and (B) NbBcII10 variants monitored at 205 nm. Curves are normalized to the fraction of unfolded protein. Partially humanized mutants in framework-2 are designated with a four-letter label referring to the residues at, respectively, positions 42, 49, 50, and 52. The human hallmark residues at these positions are in italics.
sequence, probably assist in obtaining the best possible single-domain format.

In a previous study, we assessed the effect of mutating the Nanobody hallmark amino acid in framework-2 on the stability, solubility, and loop conformation of NbAn33 (13). However, this NbAn33 (with Tyr-42 and Trp-52) belongs to the rare VHH subfamily-5, whereas the majority of the dromedary-derived Nanobodies belong to the subfamily-2. In addition, the short CDR3 loop of NbAn33 does not fold over the “former VL side” as observed for Nanobodies with longer CDR3 loops (15, 52, 53). Consequently, NbAn33 was more straightforward to humanize in contrast to the abundant subfamily-2 Nanobodies with longer CDR3.

In this study we analyzed the effect of the substitutions of residues Phe-42, Glu-49, Arg-50, and Gly-52 in framework-2 to their human counterpart on expression, affinity, stability, and biophysical properties of NbHuL6 and NbBcII10, representative members of VHH subfamily-2. Nanobodies of this VHH subfamily encompass 75% of all dromedary antigen-specific Nanobodies (2). These results allow us to determine which residues in framework-2 can be humanized without hampering the functionality of these single-domain antibodies. Thereafter, we humanized the remaining Nanobody scaffold to generate a more human-like generic VHH-derivative with maximal retention of its original Nanobody beneficial properties.

The important increase in stability of NbHuL6 and NbBcII10 upon humanization of residues 49 and 50, especially when the residue at position 52 is not mutated to the VH hallmark residue (i.e. Trp) is a striking result. A similar effect on stability had already been noticed for NbAn33, although in this case the increase in stability upon mutation of residues 49 and 50 is less pronounced possibly, because the natural residue at position 52 is already occupied by a VH-like Trp (13). These results are consistent with studies on camelized VHs, where mutations in framework-2 usually lead to a decrease in domain stability (12). The residues at positions 49 and 50 are always charged residues in the VHH format, which reduces the exposed hydrophobic area of the former VL interface and renders the domain more hydrophilic. A reasonable explanation is that the VH domain
Strategy to Humanize Nanobodies

FIGURE 7. Schematic overview of the Nanobody humanization strategy. Left panel: resurfacing the framework of a Nanobody with an antigen specificity of interest. Residues 42 and 52 are maintained due to their impact on antigen affinity and/or stability. Right panel: grafting antigen specificity loops from donor Nanobody on the universal humanized Nanobody scaffold. Solvent-exposed residues in framework (blue lines), VH-H hallmark residues in framework-2 (black lines), non-mutated VH hallmark residues (red lines).

concedes in stability by replacing non-polar with polar residues at positions 49 and 50 to enhance its solubility. Therefore, camelizing the Gly-49 and Leu-50 of a VH sequence will provoke a domain destabilization, whereas humanization of the Glu-49 and Arg-50 of a VHH will generate a stabilized variable domain. Furthermore, the monomeric state of the Nanobodies is not compromised by the Glu-49 → Gly and Arg-50 → Leu mutations as observed from the single symmetrical elution peak on size-exclusion chromatography, despite a more sticky behavior on the gel matrix. Several studies already demonstrated that other inherent features in the sequences of single-domain antibodies of Camelidae also contribute to this soluble and monomeric behavior (54, 55). Moreover, the mutations at positions 49 and 50 seem to be completely neutral for the antigen-binding capacity of the Nanobodies we studied.

Conversely, the nature of residue 52 appears to affect strongly the antigen-binding capacity of NbHuL6 and NbBcII10. The most probable explanation for this effect is that the replacement of a Gly by the bulky side chain of Trp-52 provokes a conformational shift of the CDR3 loop as seen in the crystal structure with NbHuL6GLW (Fig. 5C). This confirms the importance of the Gly-52 in Nanobodies for proper positioning of the third antigen-binding loop (13). Indeed, it seems that Gly-52 was evolutionarily introduced into the many VH germ line genes to allow positioning of the long CDR3 over the former VL side. However, the occurrence of Gly-52 might be at the expense of the domain stability, as demonstrated by the higher Cm and Tm values when a Trp substitutes Gly at this position (Tm and Cm values of 84.2 °C and 3.35 M, respectively, for the NbHuL6FERW format compared with 79.7 °C and 3.02 M for wild type).

The exact role of Phe-42 is less straightforward. In this study, substitution by Val-42 changes the expression yield, binding structure of the camelized human VH fragment, VH-P8, where Val maintained at position 42 causes a distortion of the former VL-side (50). In conclusion, Phe-42 seems to be essential to maintain the structural integrity and the correct global fold of the Nanobody scaffold. This is confirmed by the better expression levels and the higher stability of the domains with a Phe instead of a Val located at position 42. As a result the crystal structure could be determined for the NbHuL6GLW format, which was not possible for the NbHuL6FERW mutant, because of low expression yield and aggregation at lower concentrations.

The Trp-118 → Arg mutation increases the expression level of NbHuL6GLW. A similar significant yield improvement was previously observed for both the wild-type and the framework-2-humanized format of NbAn33. This emphasizes the critical role of Arg-118 for the expression of Nanobodies. However, the presence of a Trp at this position in most Nanobodies cannot be substituted by Arg because of its detrimental influence on the antigen affinity of these Nanobodies, probably by hampering the proper positioning of the third antigen-binding loop.

In summary, in this part of our study we demonstrated that the conserved Nanobody residues at positions 42 and 52 have a major impact on the integrity of the antigen interaction. Surprisingly, humanizing the residues at positions 49 and 50 stabilizes the Nanobodies, without affecting dramatically their solubility and antigen affinity.

We further focused on humanizing the remaining framework to mimic the human VH sequence as much as possible. The sequence DP-47, derived from human subgroup III, was chosen as template. This choice corresponds to the most commonly used VH subgroup in the natural repertoire of human antibodies (56, 57). In addition there is an interesting correla-
ternation between stability and framework-1 classification (58). The most(5,17),(996,989)
most stable human VH3 domain belongs to structural subtype II, as do DP-47 and the Nanobodies. Eleven additional residues outside framework-2 were mutated to their human counterpart in the framework of NbBcl110 (h-NbBcl110). These mutations do not significantly affect the antigen-binding capacity of the Nanobody but cause a decrease in expression yield and stability of the single domain. This was anticipated, because some of the substituted residues (e.g. Ala-83 → Ser, Lys-95 → Arg, and Gln-123 → Leu) were previously identified as foldability/stability determinants of water-soluble llama VH fragments (55). However, the beneficial effects of humanizing the residues at positions 49 and 50 were sufficient to compensate for this loss in stability. As for NbHu6, these mutations render the scaffold more sticky on the gel matrix, however, without affecting its stability. As for NbHul6, these mutations render the scaffold determinants of water-soluble llama VH fragments (55).

Finally, we anticipate that our universal humanized Nanobody scaffold may disturb outside framework-1, 3, and 4 will have a minimal effect, whereas the mutations of amino acids in framework-2 at positions 49 and 50 to Gly and Leu, respectively, will even stabilize the autonomous domain (at the expense of solubility and reversibly unfolding). The humanization of amino acids at positions 42 and 52 in framework-2 is discouraged due to their proven involvement in antigen affinity and stability of Nanobodies.

Finally, we anticipate that our universal humanized Nano-body h-NbBcl110_FGLA is an excellent candidate to construct a single-framework, synthetic library by introducing variability into its CDRs.

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Strategy to Humanize Nanobodies

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General Strategy to Humanize a Camelid Single-domain Antibody and Identification of a Universal Humanized Nanobody Scaffold
Cécile Vincke, Remy Loris, Dirk Saerens, Sergio Martinez-Rodriguez, Serge Muyldermans and Katja Conrath

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