Single Domain Antibodies Derived from Dromedary Lymph Node and Peripheral Blood Lymphocytes Sensing Conformational Variants of Prostate-specific Antigen*

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The importance of the lymphocyte source to generate hybridomas or to construct antibody gene libraries from which to identify potent monoclonal antibodies is understudied. However, the few comparative studies that exist seem to favor the lymph node tissue as a B-cell source. Here the peripheral blood and lymph node lymphocytes of a dromedary immunized with prostate-specific antigen (PSA) have been employed to clone two independent gene banks of the variable domains of heavy-chain antibodies (i.e. the VHHs). Several PSA-specific VHHs were retrieved after panning of these phage-displayed VHH libraries. Some of them were derived from the same B-cell lineage, possibly reflecting the restricted primary repertoire of heavy-chain antibodies. Other binders originated from different B-cell lineages and apparently converged toward a striking homologous amino acid sequence motif in their CDR3. This illustrates the strong somatic hypermutation and stringent antigen-driven selection ongoing in these animals. Although the various antigen binders exhibit a broad range of kinetic rate constants for their interaction with the PSA, leading to equilibrium constants from 70 pm to 100 nm, no significant difference existed between the binders from the two B-cell sources. The VHHs of both libraries were categorized in three groups based on non-overlapping epitopes. Some of these VHHs could inhibit and others could enhance the proteolytic activity of the antigen. Remarkably, VHHs seem to sense or induce conformational changes on different PSA isoforms, a feature that might be exploited to study the PSA conformational flexibility and to discriminate the stages of prostate cancer.

The hybridoma technology developed by Kohler and Milstein (1) is a cornerstone for molecular biology and medicine as it generates a massive amount of tools with extensive application opportunities. Despite the enormous success and efficiency to obtain monoclonal antibodies from hybridomas, novel technologies have been introduced over the past 15 years aimed at a faster identification of more antigen binders in a smaller format (2). The phage display and ribosome display, techniques whereby a physical linkage between the genotype and phenotype of the antibody fragment is maintained during the selection, are major breakthroughs to screen an immense number of candidate antigen binders. Ingenious adaptations during the retrieval procedure allow selecting for the more stable, better expressed binders and those of higher affinity (3, 4). In addition, new mutations, introduced naturally or on purpose, diversify the first set of binders and are subsequently subjected to the selection process to obtain optimized binders (5). To extend the application range or to tailor the antibodies to particular needs, quite diverse formats of antigen-binding entities (e.g. scFv, Fab, diabodies, etc.) that might substitute for the intact monoclonal antibodies have already been tested (2). The VHH,1 a single domain antigen-binding fragment derived from heavy-chain antibodies of camels, occupies a particular position within these possible formats. These monomeric antibody fragments offer special advantages in terms of expression yield (6), size and stability (7), and ease of generating bivalent, bispecific constructs or immunoconjugates (8, 9). Antigen-specific VHHs regularly target epitopes that are poorly immunogenic and less accessible for conventional antibodies (e.g. enzymatic clefts or phage receptors; see Refs. 10 and 11). This creates a number of opportunities for VHHs that might be more difficult to achieve with antigen-binding fragments from classical antibodies.

Antigen-specific VHHs can be retrieved after panning either a large naive VHH library displayed on phage or ribosomes (12, 13) or one that was cloned from B-cells of a llama or dromedary previously immunized with the antigen (11, 14). The latter procedure has the advantage that the VHHs have been affinity-matured in vivo for the antigen so that additional and time-consuming in vitro maturation steps can be avoided (15). Moreover, the B-cell proliferation within the animal during immunization makes certain that potent antigen binders are successfully retrieved even starting from relatively small VHH libraries of 106 individual clones, for example (10, 14, 16–18).

The dependence of the B-cell source to produce the “immune” VHH library and the performance of the VHH retrieved from

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1 The abbreviations used are: VHH, variable domain of the heavy chain of a heavy-chain antibody; LNL, lymph node lymphocyte; PBL, peripheral blood lymphocyte; PSA, prostate-specific antigen; ACT, α1-antichymotrypsin; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; V-D-J, variable-diversity-joining.
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such libraries have not yet been addressed. This might be an overlooked aspect because a recent study (19) revealed that more antigen-specific antibodies producing hybridoma lines were obtained from lymph node tissue compared with those from spleen or bone marrow. Although their results might be biased by the preferential fusion of lymph node B-cells to the myeloma cells, a similar preference for lymph node tissue over other lymphatic tissues to construct high quality human immunoglobulin gene libraries was also observed (20).

In this work, we generated two immune VHH libraries from the same dromedary. The VHH genes amplified from cDNA derived from peripheral blood lymphocytes (PBL) constituted the first library; those from palpable lymph node lymphocytes (LNL) formed the other library. Multiple prostate-specific-antigen (PSA) binding VHHS were retrieved from both libraries, some of which were originating from the same B-cell lineage. The number, sequence variation, and performance of these various VHHS derived from the two lymphatic tissues were compared in terms of antigen affinity, epitope recognition, and effect on the enzymatic activity of the antigen. The PSA was chosen as antigen because it is the most widely used target for early prostate cancer screening (21). However, the PSA circulating in blood occurs in different forms, i.e. as free protein or complexed with ACT (22). It seems that the diagnosis of prostate cancer is far more reliable if several of these forms can be quantified individually, as the presence of some of those isoforms are better correlated with prostate disorders than others (23, 24). Therefore, it is essential to possess more than one set of antibodies that can discriminate among the different PSA isoforms; therefore, we compared the binding characteristics of the isolated VHHS (21).

EXPERIMENTAL PROCEDURES

Immunization of Dromedary—One female dromedary (Camelus dromedarius) kept at the Central Veterinary Research Laboratory (Dubai, United Arab Emirates) was injected seven times subcutaneously at weekly intervals with 500 μg of purified prostate-specific antigen (DiaMed) mixed in Gerbu adjuvant (GERBU Biochemicals). Forty five days after the start of the immunization, 50 ml of anticogulant blood was collected, from which plasma and peripheral blood lymphocytes were isolated (WAK-Chemie). At the same time, parts of the cervicals superficiales ventrales lymph node were retrieved via biopsy. Prior to the collection of lymph node, sedation was performed by the intravenous administration (jugular vein) of 0.05 mg/kg xylazine (Bayer AG). Sternal recumbency occurred 5–10 min following xylazine administration on Vivaspin concentrators (Vivaspin) with a molecular mass cutoff of 5 kDa. The purity of the protein was checked by SDS-PAGE. The final yield was determined from the UV absorption at 280 nm, and the theoretical extinction coefficient of the VHH was calculated for its amino acid content.

Solid-phase ELISA—Maxisorb 96-well plates (Nunc) were coated overnight at 4 °C with Neutravidin or a PSA-specific monoclonal anti-PSA dromedary IgG antiserum, a horseradish peroxidase anti-M13 conjugate (Amersham Biosciences), or a mouse anti-hemagglutinin decapetide tag (Nabco) or a mouse anti-histidine tag (Serotec), respectively. Subsequent detection of the rabbit antiserum or the mouse anti-tag antibodies was done with an alkaline phosphatase anti-rabbit or anti-mouse conjugate (Sigma), respectively. The absorption at 405 nm was measured 15 min after addition of the substrate p-nitrophenyl phosphate or 2,2′-azino-bis(3-ethylbenz thiazoline-6-sulfonic acid) for phosphatase or peroxidase conjugates, respectively. Enzyme Mapping ELISA—Either Neutravidin (5 μg/ml) or a PSA-specific monoclonal antibody 3E6 (5 μg/ml) was coated on the wells of a Maxisorb 96-well plate, followed by a 2-h incubation at room temperature with 1% casein in phosphate-buffered saline. Residual protein-binding sites in the wells were blocked for 2 h with 200 μg/ml bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO). After washing, Neutravidin (5 μg/ml) was added serial dilutions of purified IgG subclasses, virions from different sources were added to the wells. After washing, rabbit anti-fragment C of Neutravidin, a mouse anti-histidine tag (Serotec), or a rabbit anti-dromedary IgG antiserum, a horseradish peroxidase anti-M13 conjugate (Amersham Biosciences), or a mouse anti-hemagglutinin decapetide tag (Nabco) or a mouse anti-histidine tag (Serotec), respectively. The absorbance at 405 nm was measured 15 min after addition of the substrate p-nitrophenyl phosphate or 2,2′-azino-bis(3-ethylbenz thiazoline-6-sulfonic acid) for phosphatase or peroxidase conjugates, respectively.

Selection of Specific Antibody Fragments—The VHH library was expressed on phage after infection with M13K07 helper phages. Specific VHHS were obtained afterPSA were eluted with 100 μl triethylamine (pH 10.0). The eluted particles were immediately neutralized with 1 M Tris-HCl (pH 7.4) and used to infect exponentially growing E. coli TG1 cells. The enrichment of phage particles carrying the antigen-specific VHHS were assessed by comparing the number of phages eluted from wells with captured versus noncaptured antigen.

After the second and third round of panning, individual colonies were picked, and expression of soluble periplasmic PSA was induced with 1 μM isopropyl-β-D-thiogalactopyranoside. The recombinant VHH was eluted from the periplasm for antigen recognition in an ELISA.

Expression and Purification of Antibody Fragments—The VHH genes of the clones that scored positive in ELISA were recloned into the expression vector pHEN6 (16), using the restriction enzymes PstI and BstEI. The plasmid constructs were transformed into E. coli WK6 cells. Production of recombinant VHH was performed in shaker flasks by growing the bacteria in Terrific Broth (25) supplemented with 0.1% glucose and ampicillin until an absorbance at 600 nm between 0.6 and 0.9 was reached. VHH expression was then induced with 1 μM isopropyl-β-D-thiogalactopyranoside for 16 h at 28 °C. After pelleting the cells, the periplasmic proteins were extracted by osmotic shock (26). This periplasmic extract was loaded on a nickel-nitritocetic acid superflow Sepharose column (Qiagen), and after washing, the bound proteins were eluted with an acetate buffer (pH 4.7). The eluted fraction was neutralized with 1 M Tris-Cl and concentrated in Vivaspin concentrators (Vivaspin) with a molecular mass cutoff of 5 kDa. The purity of the protein was checked by SDS-PAGE. The final yield was determined from the UV absorption at 280 nm, and the theoretical extinction coefficient of the VHH was calculated for its amino acid content.

Enzyme Kinetic Measurements—The PSA enzymatic activity was determined by hydrolysis of 0.1 mM substrate MeO-Suc-Arg-Pro-Asp-1-fluoromethyl ketone (chloromethylketone). According to the manufacturer's descriptions, the kinetic and equilibrium parameters (kcat, K0.5, and Km) were determined with the BiAevaluation software version 3.0 (BiAcore). The same procedure was followed to characterize the binding to the PSA isoforms from SCIPAC: PSAheavy, PSA-non-ACT binding, and PSA-ACT complex.

BIACore Measurements—Affinity measurements were assessed by injection of different concentrations of PSA (DiaMed) to purified His-tagged VHH attached to a nickel-nitrotetrazole (Ni-NTA) matrix (BIAcore) according to the manufacturer's descriptions. The kinetic and equilibrium parameters (kcat, K0.5, and Km) were determined with the BiAevaluation software version 3.0 (BiAcore). The same procedure was followed to characterize the binding to the PSA isoforms from SCIPAC: PSAheavy, PSA-non-ACT binding, and PSA-ACT complex.
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Identification of PSA Specific VHVs

Isolation of PSA Specific VHVs—From both libraries, 36 and 24 individual colonies were randomly picked after the 2nd and 3rd round of panning, respectively, and cultured to produce soluble VH. The VH clones derived from the PBL or LNL library were named Cx or Nx, respectively (with x indicating a serial number from 1 to 60). The periplasmic extracts were tested individually in an ELISA for the presence of PSA-specific VHVs (Table I). The success rate of identifying positive scoring clones for this antigen was higher for the library derived from PBL (90%) than that from LNL (65%). However, the number of different PSA-specific clones retrieved from the LNL library exceeded those from the PBL libraries. The nucleotide sequence analysis of the VHVs revealed 11 and 14 distinct PSA binders from the PBL and from the LNL libraries, respectively. Twelve clones were unique to the LNL panning, and nine were retrieved only from the PBL library. Two sets of clones sharing the same amino acid sequences were retrieved from both libraries. Only one representative of N8 and N37 is given in Fig. 2, although these binders also occurred in the PBL library (C12 and C11, respectively).

Sequence Alignment of the PSA-specific VHVs—The deduced amino acid sequences of all PSA-specific antibody fragments are aligned against the human VH consensus sequence (Fig. 2). For the VHVs retrieved from the LNL library, it is clear that the clones N4, N7, N9, N14, N20, N23, N24, N25, and N50 are derived from heavy-chain antibody-specific VH germ line genes, as they contain the hallmark amino acid substitutions in framework 2 (Val-37 → Tyr/Phe, Gly-44 → Glu, Leu-45 → Arg, Trp-47 → Xaa; see Ref. 15). Fewer fragments containing these hallmark substitutions were isolated from the PBL library; however, they are clearly present in VH VH clones C2, C7, C8, C17, and C23. Although several other clones from both libraries do not harbor the VH VH hallmark amino acid substitutions, there is substantial evidence that these are also derived from heavy-chain antibodies since the gene for the variable fragment attached to conventional H-chain was eliminated by agarse gels (see “Experimental Procedures”). Moreover the Trp-103 → Arg or Trp-103 → Gln mutation present in these clones will undoubtedly disrupt the interaction with a variable domain of a light chain (29).

Extra cysteines residing in the CDR1 and CDR3 and forming an interloop disulfide bond are frequently observed in dromedary VHVs (30). An extra pair of cysteines in the antigen binding loops is also present in nearly half of the PSA-specific VH fragments irrespective of the starting library. The CDR3 length varies from 7 to 18 amino acids with an average 11.7 amino acids (28). The enrichment of antigen-specific clones during the consecutive rounds of panning was assessed by a “phage ELISA” on biotinylated PSA coated on the microtiter plates. This approach was preferred because the passive coating of PSA on microtiter plates has been reported to provoke protein denaturation resulting in loss of conformational epitopes (28). The enrichment of antigen-specific clones during the consecutive rounds of panning was assessed by a “phage ELISA” on biotinylated PSA captured by a Neutravidin coating upon addition of a fixed amount of virions (10^9) per well (Fig. 1). The highest anti-PSA titer was obtained for virions after the second round of panning, independent of the VHH library that was used.

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from at least three different B-cell lineages as there are three binders with a GAL sequence in the beginning of a CDR3 of 7 amino acids (clones N3, C1, C11, = N37), one binder has the GAL motif at the start of an 8 amino acids long CDR3 (N8 = C12) that probably resulted from a different D-J recombination, and one binder where the GAL occurs halfway the CDR3 of 8 amino acids as well (C23). The comparison of the GAL codons and their nucleotide surroundings suggest that different D genes have been employed during the V-D-J recombination followed by a convergent evolution toward the same amino acids upon antigen maturation. From the binders with a 7-amino acid-long CDR3, the N3 and N37 (= C11) are definitely clonally related and thus derived from the same V-D-J recombination event (one nucleotide difference in a 15-nucleotide stretch containing the GAL codons). The third member of this CDR3 length might originate from another B-cell lineage as it contains 6 or 7 nucleotide differences with the former clones in the same stretch. The nucleotide differences among the other GAL motif carrying binders of different V-D-J origin were also 6–8 for the corresponding region of 15 nucleotides.

Clones C9 and C24 share an identical amino acid sequence from the beginning of the framework 2 until the end of their framework 4, i.e. including CDR2 and CDR3, but have a different CDR1 sequence amino. These clones are most likely derived from the same B-cell lineage, although a PCR crossover artifact might have caused the sequence variation (31).

Binding Characteristics of Antigen-specific VHHs

VHH Expression—All the binders were recloned into an expression vector (pHEN6) (16). The induced recombinant is transported to the periplasm of E. coli and is carrying a histidine tag that facilitates purification by immobilized metal-affinity chromatography. After an additional gel filtration chromatography, >95% pure protein was obtained as deduced from Coomassie staining of the SDS-PAGE. The overall yield of purified VHH varied between 0.1 and 6 mg per liter of culture and was clone-dependent. However, no significant difference in the range of expression and purification yield was noticed between the PBL- and LNL-derived clones and also not between the VHHs carrying the framework 2 hallmark substitutions and those having a nonconventional amino acid at position 103.

VHH Epitope Mapping—Pure antibody fragments were prepared and tested in ELISA to define their epitope on PSA. Binding to PSA was assessed in ELISA by using different coating methods of PSA or different PSA forms, e.g. by capturing PSA Free or PSA-ACT on the PSA-specific monoclonal antibody 3E6 or by capturing biotinylated PSA onto Neutravidin. The binders could be categorized into three different epitope groups, depending on their recognition pattern of the PSA presented in various formats (Table II). However, it is clear that each category is populated with VHHs from the PBL and the LNL library.

The first category of antibody fragments contains VHHs binding on PSA that is already associated with ACT or 3E6. These VHHs recognize epitopes located outside the ACT and 3E6 interaction sites. This group of antibody fragments encompasses VHHs C1, C2, C8, C23, C59, N3, N4, N7, N8, N9, N13, N20, N24, and N37. All of the VHHs having the GAL pattern in their CDR3 belong to this group, corroborating the idea that this subgroup of VHHs binds a common epitope. Likewise, the clones N24 and C59 have a sequence stretch of Gly-Tyr-Gly-Tyr-Cys/Tyr-Tyr in their CDR3 in common, although at a slightly different localization within the loop. Their nucleotide sequence indicates that they are originating from the same D gene. In any case the different size of the CDR3 indicates that these binders are from different V-D-J rearrangements and hence of different lineages.

The second group of VHHs binds biotinylated PSA when coupled on Neutravidin but is unable to recognize PSA or PSA-ACT when captured by 3E6. The epitopes of these VHHs (C17, N15, N23, and N25) probably overlap with that of 3E6 as only biotinylated PSA is recognized. All four VHHs show very low amino acid sequence identity in their CDRs, although one clone, N23, harbors a region (Ser-Cys-Ser-Leu-Xaa-Xaa-Tyr) that was also found in one of the binders (C7) of the third epitope group. There was little nucleotide sequence homology in the codons for these CDR3 sequence regions, supporting a different D origin.

The binders from the third group encompassing C7, C9, C24, N14, and N50 recognize biotinylated PSA captured by Neutravidin or mAb 3E6 and fail to associate with the PSA-ACT complex when captured by 3E6. This pool of clones contain the clones C9 and C24 that share an identical CDR2 and CDR3 amino acid sequence but differ in their CDR1 sequence. Three possibilities can be proposed to explain this recognition pattern. First and the most obvious possibility would be that the ACT- and VHH-binding sites overlap with each other. Second, the epitope for these VHHs might be adjacent to the ACT interaction spot so that the detecting antibody (i.e. the anti-His antibody) is unable to target the VHH tag. Third, the binding site of the VHHs of this group is at a PSA site that undergoes a conformational change upon mAb 3E6 and ACT interaction. These possibilities can be discriminated by testing the enzymatic inhibition of PSA by these VHHs and a detailed kinetic binding analysis.

VHH Modulating the Proteolytic PSA Activity—It is well established that camel single domain antibody fragments against enzymes have a preference to interact with the cata-

Fig. 2. Amino acid sequence of PSA-specific VHHs aligned against a human VH consensus sequence (top). Clones C11 and C12 from the PBL library were omitted as they shared the sequence of clones N37 and N8, respectively, from the LNL library.
lytic site of their antigen (10, 14). Hence, an enzymatic PSA assay with a standard fluorogenic substrate (S-2586) was performed to assess the possible interaction of the VHHs from group 3 with the active site of PSA, as these VHHs are likely candidates to act as inhibitors. The initial velocity of substrate hydrolysis by the enzyme was measured in the presence or absence of a molar excess of purified VHH (Fig. 3). Addition of a non-PSA binding VHH (cAbAn33; see Ref. 18) to PSA did not change the initial hydrolysis velocity, suggesting that the mere presence of VHH protein on itself is ineffective for this enzymatic activity of PSA. Compared with the reaction without VHH or a non-PSA binder, the presence of C9, C24, N14, or N50 caused a significant drop (ranging from 75% for C9 to 99% for N50) in PSA activity. The reduction of PSA activity in presence of these VHHs confirms their possible binding into or nearby the active site and supports the previous epitope mapping experiments based on the ACT binding competition.

In another control we tested the effect on the proteolytic activity of PSA in the presence of mAb 3E6 or one arbitrarily selected VHH from group 1, the VHH N7. Remarkably, a 2- or 7-fold higher relative activity to that of uncomplexed PSA was noticed for these binders, respectively. Possibly, the attachment of an antibody on either one of these epitopes (3E6 and N7 have nonoverlapping epitopes) (Table II) induces a conformational change that facilitates the subsequent hydrolysis of the small substrate by PSA.

Affinity Measurements—The affinity of all VHHs for PSA was determined by surface plasmon resonance on a BIAcore 3000. All measurements were performed on a nickel-nitrilotriacetic acid biochip that allows complexing the His tail present at the C terminus of the VHHs. The PSA (DiaMed) at concentrations between 0 and 500 nM was then injected at a flow rate of 30 μl/min, and its association to the VHH was recorded. The binding kinetics between PSA and VHH yielded \( k_{on} \) values in the range of \((0.16–19) \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) and \( k_{off} \) values of \((533 \text{ to } 1.5) \times 10^{-5} \text{ s}^{-1}\) (Fig 4). Corresponding \( K_D \) values varying from 100 nM to 79 pM could be obtained from these kinetic rate constants. The comparison of the \( k_{on}, k_{off} \) or \( K_D \) values obtained for the VHHs isolated from the LNL or PBL library revealed very similar values as illustrated in the RaPID plot (Fig. 4).

**Differential Recognition of PSA Isoforms by VHHs**—The five VHHs that were used to measure the modulation of the enzymatic PSA activity were now used to evaluate their binding parameters to three PSA isoforms, i.e. PSAfree, PSA-ACT binding, and PSA-ACT complex as obtained from SCIPAC (Table III). Minor differences in kinetic rate constants were noticed when measuring the VHH binding to PSA purified by DiaMed or by SCIPAC, possibly reflecting some residual heterogeneity among the PSA samples. In addition, the equilibrium binding constants of any of these VHHs for PSAfree and PSA-non-ACT binding resulted in comparable \( K_D \) values. The
slight deviations in $k_{\text{on}}$ and $k_{\text{off}}$ values that were occasionally measured between PSAfree and PSA non-ACT-binding for some of these VHHs remained within a factor of 2.

For the VHHs under investigation, it was anticipated that only N7 could bind to the PSA-ACT complex because of previous epitope mapping experiments based on the ELISA tests (Table II and Fig. 3). Most surprisingly, the PSA inhibiting VHHs, C9 and C24, originating from the same B-cell lineage could be demonstrated to interact with the PSA-ACT complex. However, for both VHHs the $k_{\text{on}}$ value for the PSA-ACT complex was ~5-fold lower compared with that of the free PSA, indicating the involvement of an induced conformational switch of the epitope or of a steric hindrance by the ACT. In any case, it is clear from the biosensor experiment that the ACT footprint and the PSA isoforms. The kinetic data, the ELISA epitope mapping, and the protease-stimulating effect of N7 on PSA point out that ACT binding, and substrate binding.

**DISCUSSION**

This study proves that lymphocytes from lymph nodes are a good alternative to those from peripheral blood for cloning and isolating high affinity antibodies, at least with our immunization protocol, with PSA as antigen and in this dromedary. At first sight, taking lymph node biopsies seems to involve more sophisticated handlings; however, these should not pose any difficulty in a veterinarian environment.

Two independent VHH libraries were cloned from either the peripheral blood lymphocytes or the cervicales superficiales ventrales lymph node tissue extracted from one immunized dromedary. Higher PCR amplification levels were observed for LNL cDNA, a finding that confirms previous results (20) on the amplification of human LNL cDNA. Although the in vitro selection and identification of antigen-binding clones from the PBL derived library seemed to be more productive (90 versus 65% positive), the actual number of different clones retrieved from either library matches each other closely or was perhaps slightly in favor of the sample from the LNL.

Two binders (N8 and N37) had the same amino acid sequence than binders (C12 and C11, respectively) that were retrieved from the other library. In addition, the amino acid sequences, especially those of the CDR3 sequence, of other binders revealed that some of them originated from the same B-cell lineage (N3 and N37 = C11). Conversely, some of our binders (N24 and C59) might have used the same D gene but in a different V-D-J rearrangement event, where part of their D gene was subsequently heavily modified. Finally, other binders that have emerged from different V-D-J rearrangements, even using different D genes, seem to have undergone a striking
convergent evolution, e.g. N23 and C7. It is of note that only a small biopsy sample of one lymph node was taken to generate the LNL library, and only 50 ml of blood out of the ~30 liters of blood circulating in the adult animal for the PBL library. Such small aliquots yielding already B-cell-related VHIs in the libraries suggest that our dromedary had only limited diversity in its primary antigen-binding receptors in its B-cells at the time of the immunization. In view of the poorly elaborated immature repertoire, to a large extend caused by the lack of a VH-VL combinatorial diversification process, it is surprising to find multiple binders that underwent a convergent evolution toward similar sequence motifs in their CDRs. This argues for an extraordinarily well developed affinity maturation process and B-cell proliferation ongoing in these animals once a particular primary B-cell receptor has been selected. Such an efficient in vivo selection and maturation against any given epitope might be difficult to surpass by in vitro selection and maturation techniques with the possible exception of ribosome display based strategies (5, 13).

The differences in $k_{\text{on}}$ and $k_{\text{off}}$ rate constants for PSA interaction with the various VHIs could be as large as a factor 100 and 500, respectively. The equilibrium binding reaction between the individual VHH to PSA revealed a 1000-fold difference between the most potent and weakest binder. However, the equilibrium and kinetic rate constants for PSA interaction were not significantly different for the binders from either library. It is essential to possess more than one set of antibodies that can discriminate among the different PSA isoforms, and so we compared the binding characteristics of the isolated VHIs.

A simple ELISA experiment whereby the PSA was either accessible or covered by ACT or a mAb (3E6) allowed us to define three distinct epitopes for our VHIs. Members of each library were equally distributed over these three epitope classes, demonstrating that multiple surfaces of PSA are antigenic and targeted by VHIs from the libraries.

In particular, the binders that were competing for PSA binding with ACT attracted our attention as it is well established that VHIs can inhibit the enzymatic activity of their antigen because of a tight association with the catalytic site (10, 16, 32). Measurement of the proteolytic activity of PSA with a standard substrate in the presence or absence of VHIs (C9, C24, N14, and N50) resulted in an important reduction of the enzymatic activity, ranging from 75 to 99%. This finding is in line with the inability of these VHIs of the epitope group “3” to bind PSA-ACT complex as observed in the epitope mapping from ELISA. Also mouse monoclonal antibodies have been described to inhibit the PSA-ACT complex formation. In this case, the PSA inhibition might have been caused by the mAb interacting with the kallikrein loop, leading to direct steric hindrance for ACT, or by a possible conformational change in the ACT-binding site upon mAb binding elsewhere on the PSA surface (33). Hence, multiple schemes can be proposed for the inhibition, and the PSA association should be followed by several techniques to discriminate between the various possibilities. This is exemplified by the C9 and C24 binders at one end and the N14 and N50 at the other end. All these VHIs scored in ELISA as having an affinity of the PSA-ACT complex as observed in the epitope mapping from ELISA.

Finally, the availability of small, single domain antibody fragments recognizing PSA with high affinity could lead to the design of new PSA detection tools. In a collaborative effort, the VHH with the highest $k_{\text{on}}$ has been used successfully to detect PSA at ng/ml quantities when fused to S-layer protein and crystallized on the sensing surface of a biosensor (27), or by cross-linking to a self-assembling monolayer.2

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