Antigen recognition by single-domain antibodies: structural latitudes and constraints

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ABSTRACT

Single-domain antibodies (sdAbs), the autonomous variable domains of heavy chain-only antibodies produced naturally by camelid ungulates and cartilaginous fishes, have evolved to bind antigen using only three complementarity-determining region (CDR) loops rather than the six present in conventional V\textsubscript{H}–V\textsubscript{L} antibodies. It has been suggested, based on limited evidence, that sdAbs may adopt paratope structures that predispose them to preferential recognition of recessed protein epitopes, but poor or non-recognition of protuberant epitopes and small molecules. Here, we comprehensively surveyed the evidence in support of this hypothesis. We found some support for a global structural difference in the paratope shapes of sdAbs compared with those of conventional antibodies: sdAb paratopes have smaller molecular surface areas and diameters, more commonly have non-canonical CDR1 and CDR2 structures, and have elongated CDR3 length distributions, but have similar amino acid compositions and are no more extended (interatomic distance measured from CDR base to tip) than conventional antibody paratopes. Comparison of X-ray crystal structures of sdAbs and conventional antibodies in complex with cognate antigens showed that sdAbs and conventional antibodies bury similar solvent-exposed surface areas on proteins and form similar types of non-covalent interactions, although these are more concentrated in the compact sdAb paratope. Thus, sdAbs likely have privileged access to distinct antigenic regions on proteins, but only owing to their small molecular size and not to general differences in molecular recognition mechanism. The evidence surrounding the purported inability of sdAbs to bind small molecules was less clear. The available data provide a structural framework for understanding the evolutionary emergence and function of autonomous heavy chain-only antibodies.

KEYWORDS

single-domain antibody; V\textsubscript{H}–V\textsubscript{L}; molecular recognition; antibody: antigen interaction; paratope; epitope

Introduction

Single-domain antibodies (sdAbs) are the monomeric binding domains of heavy chain-only antibodies that have arisen through convergent evolution at least three times (twice in Chondrichthyes and once in Camelidae, roughly 220 and 25 million year ago, respectively). The concept of autonomous, antigen binding-competent sdAbs was first described by Ward et al. in 1989, and several years later, naturally-occurring antibodies lacking light chains were discovered in dromedary camels and nurse sharks. The ~12–15 kDa variable domains of these antibodies (V\textsubscript{H} and V\textsubscript{NAR}, respectively; Figure 1) can be produced recombinantly and can recognize antigen in the absence of the remainder of the antibody heavy chain. The modular nature of V\textsubscript{H} and V\textsubscript{NAR} has been widely and productively exploited in the development of antibody-based drugs (reviewed in Ref. 5).

Structural studies of the first V\textsubscript{H} and V\textsubscript{NAR} isolated provided an early indication that these molecules might interact with antigens using mechanisms distinct from those of conventional antibodies. With hindsight, the notion that sdAbs might preferentially target particular types of antigenic structures may not seem totally unexpected, given their recombination from distinct repertoires of V, D and J genes (see Box 1), their potential ontogeny from separate B-cell precursors, and for camelid V\textsubscript{H}S, their specialized constant regions bearing very long hinge regions. However, the specific mechanisms of sdAb antigen recognition (e.g., the tertiary structures and physicochemical properties of sdAb:antigen interfaces, which may differ fundamentally from those of conventional antibody:antigen interfaces) remain unclear, although several studies have suggested protein cleft recognition as a general function for both V\textsubscript{H}S and V\textsubscript{NAR}. Over time, the idea that sdAbs can target ‘cryptic’ epitopes (so-called because they are inaccessible to conventional antibodies, either for steric reasons or due to their fundamental antigenic properties) has become entrenched, and although several case studies have supported it, its generality and implications are questionable. Several excellent recent reviews and opinion pieces have alluded to the nature of sdAb paratopes and their interactions with antigens, but have either not been rigorous in their approach or have incompletely addressed the topic, analyzing the properties of sdAb paratopes only, with no comparison to those of conventional antibodies. Thus, the aim of this review was to comprehensively investigate whether and how sdAb:antigen interactions differ from conventional antibody:antigen...
interactions, and to assess whether V_Hs and V_NARs share any similarities in this respect despite their evolutionary divergence. The answer to this question has direct relevance for the ‘druggable’ target space available to sdAbs vs. conventional antibodies.

**Single-domain antibodies directed against folded proteins**

As with conventional antibodies, the bulk of sdAbs studied have been directed against folded proteins. Certain regions and epitopes on folded proteins are inherently more immunogenic than others, a concept known as immunodominance. The immunological mechanisms underlying B-cell immunodominance are poorly understood, and patterns of immunodominance probably are not completely conserved across species.29

The first indication that sdAbs might preferentially target different sets of epitopes compared with conventional antibodies came from studies of anti-enzyme sdAbs (Table 1). Conventional antibodies can act as enzyme inhibitors, most commonly by inducing allosteric conformational changes or by sterically blocking substrate access to the active site.65 It was recognized from early structural studies of anti-lysozyme V_Hs6 and V_NARs7 that these molecules interacted with the enzyme in unusual fashion, probing deeply into its active site and epitopes on folded proteins. Certain regions of sdAbs,6,7,9,10 for example, protrude into the active site of lysozyme, as well as with active site-binding V_Hs against 13,14,15 carbonic anhydrase,16,17 and urokinase.18 Inhibition of a-amylase was achieved by one V_H through penetration of the active site cleft with its CDR2 loop,21

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**Box 1. Immunogenetics of sdAbs**

V_Hs, the variable domains of camelid heavy chain-only antibodies, are recomposed during B-cell development from a unique set of germline V genes and common D and J genes (shared with the V_L domains of conventional tetrameric antibodies) located within the igh locus on chromosome 4.5 Most camelid V_H and V_L genes are homologous to human IGHV3-family genes (~75–90% identity) and encode distinctive solubilizing residues in FR2 (Phe/Tyr42, Glu49, Arg50 and Gly 52 using IMGT numbering; these positions map to the V_H/V_L interface in conventional antibodies), although functional V_Hs lacking this consensus have been isolated.19 Some camelid V genes may ‘promiscuously’ recombine with both heavy chain-only and conventional antibody constant region genes.19 V_H domains bear unusually long CDR3 loops in comparison with human and murine conventional antibodies,20 probably reflecting increased non-template nucleotide addition, although this may be a feature of only a subset of V_Hs.21 In some V_Hs, the long CDR3 loop serves a dual purpose, folding over the former V_L interface as well as interacting with cognate antigen. The rearranged V_H exon is thought to undergo elevated rates of somatic hypermutation of both CDRs and FRs (e.g., FR1-encoding sequences immediately flanking CDR1,25–27 FR2-encoding sequences which may play a role in structuring the CDR3 loop,28,29 FR3-encoding sequences that form a β-turn which can make contact with antigen, sometimes called CDR430). V_Hs may also acquire somatic insertions and deletions at higher rates than conventional antibodies,24 and may under some circumstances undergo secondary rearrangement events using a cryptic recombination signal sequence in FR3.31 Some V_H genes encode non-canonical disulfide linkages formed between cysteine residue pairs (CDR1-CDR3, FR2-CDR3, CDR2-CDR3 or CDR3-CDR3; see Box 2). V_NARs, the variable domains of cartilaginous fish Ig new antigen receptors, share sequence homology with T-cell receptor and Ig light chain genes and may be descended from Ig-superfamily cell-surface receptors.26 Compared with Ig V genes, V_NARs lack two β strands (C and C) and consequently CDR2 is absent, although loops connecting the C-D and D-E strands (HV2 and HV4, respectively) can make contact with antigen. During B-cell development, V_NAR domains are rearranged from a small number of loci (perhaps only three) distinct from those encoding other types of Ig molecules detectable in serum (IgM, IgW). Each locus contains one V gene, two or three D genes and one J gene and thus primary repertoire diversity is almost entirely CDR3-based;8 since V_NAR CDR3 loops are formed through either three or four independent rearrangement events, these tend to be long.9 Unlike the V_H domains of IgMs and IgWs, V_NARs may also acquire somatic hypermutations upon encounter with antigen primarily in CDR1 and CDR3 but also in HV2 and HV4.27,28

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**Figure 1.** Domain structures of camelid heavy chain-only IgG, shark immunoglobulin new antigen receptor (IgNAR) and conventional vertebrate tetrameric IgG. The variable domain(s) of each antibody molecule are shown in yellow and the antigen-combining site is indicated by a red box.
demonstrating that CDR3-centric binding is not the only mechanism of competitive enzyme inhibition by sdAbs. Competitive inhibition of these enzymes by conventional antibodies targeting their active sites has not been described despite intensive study, especially of murine antibodies against lysozyme. Naturally-occurring competitive inhibitors of protease enzymes are convex, and this appears to be a difficult geometry for the paratopes of conventional antibodies to achieve (see below): even in cases of near-truly competitive inhibition, conventional antibodies use a flat or concave V\_H/V\_L interface to bind protruding regions on enzymes and partially insert one or more CDRs into the active site cleft in a non-substrate-like manner.\textsuperscript{66,67} This hypothesis is supported by experiments using purified polyclonal immunoglobulin (Ig)Gs from enzyme-immunized dromedaries showing that competitive inhibition was a feature of heavy chain-only IgGs, but not of conventional IgGs.\textsuperscript{11,32} It remains unclear why immunization with some enzymes yields mostly sdAbs with planar paratopes and bind outside the active site, achieving allosteric or no inhibition, although tolerance mechanisms may play a role.

A second line of evidence clearly supporting distinct specificities of sdAbs vs. conventional antibodies can be found in studies of sdAbs against pathogenic microorganisms. Stijlemans et al.\textsuperscript{68} hypothesized that the ability of a dromedary V\_H1, C\_Ab-An33, to target a cryptic glycopeptid epitope conserved across all variant surface glycoprotein classes of Trypanosoma brucei was due to the V\_H1’s small size as well as, potentially, the nature of this epitope. This hypothesis was supported by the inability of rabbit and dromedary polyclonal conventional antibodies as well as a ~90-kDa lectin to access this site. Henderson et al.\textsuperscript{69} suggested that recognition of a conserved hydrophobic cleft on Plasmodium AMA1 by a V\_NAR (12Y-2 and its affinity-matured variants) reflected a novel binding mode; although the epitope of a murine conventional antibody (1F9) substantially overlapped that of V\_NAR 12Y-2, 1F9 binding depended to a greater degree on polymorphic loop residues surrounding the hydrophobic trough. Likewise, Ditev et al.\textsuperscript{70} attributed the binding of a panel of alpaca V\_Hs to multiple domains of the malarial VAR2CSA protein to an inherent ability of V\_Hs to recognize subdominant epitopes, although limited understanding of the human conventional antibody response against VAR2CSA as well as irreproducibility of these reactivity patterns by llama V\_Hs\textsuperscript{71} complicated this assessment.

Probably the clearest examples of epitopes that are more favorable for binding by sdAbs than conventional antibodies can be found in the envelope glycoprotein trimmer of HIV-1: heterologous cross-strain neutralization is extraordinarily difficult to achieve by conventional antibodies, requiring months of chronic infection and multiple rounds of somatic mutation and selection, yet cross-neutralizing camelid heavy chain-only antibodies directed against the CD4-binding site\textsuperscript{72-75} and CD4-induced sites\textsuperscript{76-78} can be easily elicited by routine immunizations with recombinant protein antigens. Similar examples can be found for other viral pathogens. Serotype cross-neutralizing antibodies targeting the CD155-binding ‘canyon’ of the poliovirus capsid are rarely produced by the murine or human humoral immune systems,\textsuperscript{79,80} but are apparently common in llama heavy-chain only responses.\textsuperscript{81} Likewise, V\_Hs targeting the HBGA-binding pocket of norovirus VP1 neutralized a broad range of genotypes,\textsuperscript{82} while larger conventional antibodies also made contact with antigenically variable residues surrounding the HBGA pocket and were thus strain-specific.\textsuperscript{83}

Finally, compared with conventional antibodies, sdAbs have been implied to have privileged access to recessed sites on membrane proteins,\textsuperscript{84} such as ion channels and G protein-coupled receptors (GPCRs). While this is an intriguing hypothesis, it has yet to be substantiated by any data. Camelid V\_Hs generated against the Kv1.3 ion channel targeted extracellular loops, not the channel cavity,\textsuperscript{85} and the epitopes of V\_Hs against the P2X7 ion channel were not defined.\textsuperscript{86} Similarly, camelid V\_Hs developed as potential therapeutics against the chemokine receptors CXCR4\textsuperscript{84} and CXCR5\textsuperscript{87} and ChemR23,\textsuperscript{88} as well as V\_Hs used as crystalization chaperones for several GPCRs, channels and

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**Table 1. Single-domain antibodies as enzyme inhibitors.**

| Enzyme          | Type of sdAb(s) | Inhibition | Mechanism(s) of Inhibition | Reference(s) |
|----------------|----------------|------------|---------------------------|--------------|
| Aldolase       | V\_H\_s        | ND         | NA\textsuperscript{3}     | 30           |
| α-amylase      | V\_H\_s +/-   |            | Competitive\textsuperscript{1}, allosteric\textsuperscript{1}, NA | 31,32        |
| ART2.2         | V\_H\_s +/-   |            | Competitive\textsuperscript{1}, ND, NA | 33           |
| Aurora-A kinase | V\_NAR +/-   |            | Allosteric\textsuperscript{1}, NA | 34           |
| β-lactamase    | V\_H\_s +/-   |            | Allosteric\textsuperscript{2,3}, NA | 35           |
| Botulinum toxin | V\_H\_s +/- |            | Steric exclusion of substrate\textsuperscript{1} | 36           |
| Carbonic anhydrase | V\_H\_s +/- |            | Steric exclusion of substrate\textsuperscript{1} | 37           |
| CD38           | V\_H\_s ND    |            | Allosteric\textsuperscript{2}, NA | 38           |
| CDT binary toxin | V\_H\_s     |            | ND                        | 40           |
| DHFR           | V\_H\_s +/-   |            | Allosteric\textsuperscript{1,3} | 41           |
| Furin          | V\_H\_s +/-   |            | Steric exclusion of substrate\textsuperscript{1,3} | 42           |
| Lysozyme       | V\_H\_s       |            | Competitive\textsuperscript{1,2}, NA | 43           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,2}, NA | 44           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 45           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 46           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 47           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 48           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 49           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 50           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 51           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 52           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 53           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 54           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 55           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 56           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 57           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 58           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 59           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 60           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 61           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 62           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 63           |
|                | V\_H\_s       |            | Competitive\textsuperscript{1,3}, NA | 64           |

\textsuperscript{1}Mechanism inferred from antibody:enzyme X-ray co-crystal structures.

\textsuperscript{2}Specificity for active site or non-active site regions demonstrated through epitope mapping experiments.

\textsuperscript{3}Mechanism inferred from studies of enzyme kinetics.

\textsuperscript{4}Inhibition was not assessed, but structural studies showed that the antibody did not target the active site.

Abbreviations used: ART2.2, ecto-ADP-ribosyltransferase 2.2; CD, Clostridium difficile transferase; DHFR, dihydrofolate reductase; HCV NS3, hepatitis C virus non-structural protein 3; NA, not applicable; ND, not determined; NOR, nitric oxide reductase; SBE-A, starch branching enzyme A.
transporters, all appear to bind solvent-exposed extracellular or intracellular loops of these receptors in a manner similar to conventional antibodies and their fragments. By contrast, a synthetic CXCR4-binding “i-body” engineered from an Ig-like NCAM domain was found to penetrate deep into the receptor’s ligand-binding pocket to occupy a truly cryptic, partially transmembrane epitope. Thus, there is at least some reason to believe that the small size of sdAbs may grant them access to recessed regions on pores and channels, although experimental evidence is still lacking.

Overall, the evidence is compelling that camelid V<sub>H</sub>Hs, at least, can interact with recessed epitopes on proteins that are poorly available for binding by conventional antibodies. Additional examples of binding to recessed epitopes on proteins (clefts, cavities, crevices or grooves) can be found for sdAbs against lactococcal siphophage, <sup>97</sup> Plasmodium falciparum MTIP, <sup>98</sup> epidermal growth factor receptor, <sup>99</sup> and respiratory syncytial virus fusion protein, <sup>100</sup> although in these cases it is less clear that these sites are inaccessible to conventional antibodies. While it is possible that V<sub>NAR</sub><sub>H</sub> may share similar cleft-binding proclivities, such claims are based on very limited published data (three structures, <sup>7,12,69</sup>). Moreover, it should be noted that there are many examples (not covered in this review) of partial or complete overlap between the epitopes of sdAbs and conventional antibodies, and thus the degree to which sdAbs bind cryptic epitopes vs. conventional antibody-accessible epitopes, as well as whether the magnitude of this difference exceeds more general species-to-species reactivity differences of conventional antibodies, remain unknown.

### Single-domain antibodies direct against linear protein epitopes

It is generally recognized that the majority of conventional antibodies raised against folded proteins are directed against conformational epitopes (≥90%), <sup>101</sup> although this may depend to some extent on the nature of the antigen. Several authors have suggested that V<sub>H</sub>Hs, at least, are even less likely than conventional antibodies to bind linear peptides with high affinity. <sup>102,103</sup> Although this is a plausible hypothesis based on the typical structures of sdAb paratopes (see below), it has not yet been substantiated by any data. Moreover, the relatively large number of studies reporting sdAb reactivity by western blotting suggests that sdAbs directed against continuous epitopes are probably not vanishingly rare.

### Single-domain antibody paratope structures

The paratopes of conventional antibodies directed against folded proteins tend to be flat or concave; <sup>104</sup> convex binding sites are difficult to achieve, at least by murine and human conventional antibodies, although synthetic conventional antibodies can be engineered to adopt such geometries. <sup>105</sup> By contrast, sdAb paratopes can clearly adopt both flat <sup>106,107</sup> and convex <sup>11</sup> topologies, although possibly only inefficiently adopt concave ones. The CDR1 and CDR2 loops of V<sub>H</sub>Hs depart from the typical canonical structures of conventional antibodies (Figure 2A), potentially through somatic mutation since germline human V<sub>H</sub> and camelid V<sub>H</sub>H repertoires appear to have similar canonical structures. <sup>18</sup> Only a handful of V<sub>NAR</sub><sub>H</sub> have been crystallized, and several showed a structural class of CDR1 (H1–13–9) that is more common in V<sub>H</sub>Hs than in conventional antibodies, although others had CDR1 canonical structures closer to those of V<sub>L</sub> domains. The CDR3 length distributions of both V<sub>H</sub>Hs and V<sub>NAR</sub><sub>H</sub> (Figure 2B) are broader than those of conventional antibodies and biased towards longer lengths; the long CDR loops of sdAbs may be structurally constrained by non-canonical disulfide linkages (see Box 2). Despite potentially elevated somatic mutation rates (at least of V<sub>H</sub>Hs), the paratopes of V<sub>H</sub>Hs, V<sub>NAR</sub><sub>H</sub> and conventional antibodies have similar amino acid contents, all being enriched for Gly, Ser and Tyr, and their CDR sequences bear no obvious patterns of sequence homology (Figure 2C, D). Both V<sub>H</sub>H and V<sub>NAR</sub><sub>H</sub> paratopes have smaller molecular surface areas and smaller diameters than conventional antibodies (Figure 2E, F). However, sdAb paratopes as a group are not more globally extended than those of conventional antibodies, as reflected by the maximum interatomic distance between the tips and the bases of any CDR loop (Figure 2G).

### Single-domain antibody:antigen interactions

The footprints of sdAbs on antigens are smaller than those of conventional antibodies, given that the paratopes of the former molecules are roughly half the size of the latter ones. Using only three CDR loops (two CDR loops and potentially two HV loops for V<sub>NAR</sub><sub>H</sub>s), sdAbs can bury similar solvent-accessible surface areas on proteins compared with conventional antibodies (Figure 3A). This is made possible by a number of molecular contacts (hydrogen bonds, salt bridges) that is slightly lower for sdAbs than in conventional antibodies, but higher on a per-chain basis (Figure 3B, 3C). Moreover, the surface complementarity of sdAb:protein interfaces is on the high end for antibody:antigen interactions (Figure 3D). Thus, sdAbs and conventional antibodies bind protein antigens through similar types of non-covalent interactions, but these are more concentrated in the smaller paratopes of sdAbs.

### Single-domain antibodies directed against small molecules

The dominant mechanism by which conventional antibodies interact with haptns, small-molecule lipids and oligosaccharides is by forming a binding pocket at the interface between the V<sub>H</sub> and V<sub>L</sub> domains, typically involving the bases of the CDR-H3 and CDR-L3 loops. <sup>113–115</sup> Similarly, conventional antibodies tend to accommodate short linear peptides and nucleic acid polymers within grooves formed from both heavy- and light-chain CDRs. <sup>104</sup> Four studies have reported structures of camelid V<sub>H</sub>Hs in complex with haptns and peptides (Table 2); the recognition mechanism of all but one (a methotrexate-specific V<sub>H</sub>H with a non-canonical binding site involving framework region (FR)3 residues located below CDR1) <sup>113</sup> was basically similar to that of conventional antibodies, with the hapten-binding pocket formed from two or more CDRs and extending in some instances into the former V<sub>L</sub> interface. Notably, three of these V<sub>H</sub>Hs have non-
Fig 2. Properties of sdAb vs. conventional antibody paratopes. (A) Structural classification of CDR1 and CDR2 according to PyIgClassify.\textsuperscript{108} (B) CDR3 length distributions. (C) Amino acid compositions of conventional antibody (V\textsubscript{H} domain) and sdAb paratopes. For V\textsubscript{H}\textsubscript{H}s, sequences of CDR1 and CDR3 only were used. (D) Relatedness of conventional antibody (V\textsubscript{H} domain) and sdAb CDR3 sequences. The phylogenetic tree was produced using neighbor-joining methods in ClustalW2 and the cladogram was visualized using iTOL\textsuperscript{109} with CDR3s colored according to species origin as in part B. (E) Molecular surface areas of conventional antibody (V\textsubscript{H}:V\textsubscript{L}) and sdAb paratopes. Areas were calculated for merged CDR sequences (Honegger-Plückthun numbering) using PyMol. (F) Diameters of conventional antibody (V\textsubscript{H}:V\textsubscript{L}) and sdAb paratopes. Diameters were calculated as the maximum interatomic distance between any two FR-CDR boundary residues (Honegger-Plückthun numbering). (G) Extension of conventional antibody (V\textsubscript{H}:V\textsubscript{L}) and sdAb paratopes. Extension was calculated as the maximum interatomic distance between the CDR base (first or last residue according to Honegger-Plückthun numbering) and the CDR tip. The CDR(H)3 loop is shown in blue. In parts (E) – (G), boxplot lines represent medians, the box boundaries represent quartiles and the box whiskers represent ranges. Red dots indicate sdAbs targeting cryptic epitopes discussed in the main text. Data are representative of all complete antibody structures available in the Protein Data Bank and indexed in PyIgClassify as of January 2018.
canonical structures of either CDR1 or CDR2 that have not been observed in structures of other V H HS and may not be germline-encoded.

Multiple studies have reported the isolation of hapten-specific V H HS without investigating their structures, although several also reported weaker and inconsistent serum heavy chain-only IgG titers compared with conventional IgG titers against the hapten. No studies have reported hapten-specific V NAR HS, and only one study has described a carbohydrate-specific V H H directed against Neisseria meningitidis lipopolysaccharide; at least two camelid V H HS have been described that bind to glycopeptide epitopes. No sdAbs of any type have been described that convincingly bind lipids or nucleic acids. Together, the consensus of the data is that it is probably difficult, but not impossible, for sdAb paratopes to accommodate haptons and that three CDRs are sufficient to form the binding pockets and grooves required for such interactions, although potential involvement of solubility-enhancing FR2 residues at the former V L interface in pocket formation may impose restrictions on hapten-binding specificities.

**Synthetic single-domain antibodies and non-antibody scaffolds**

Fully synthetic sdAbs, derived from V H Hs, V NAR8 or from rare human and murine V H H and V L domain APPs that remain stable and soluble outside the context of the natural V H H/V L pairing, can be engineered to bind antigens using *in vitro* methods (e.g., phage display). More recently, technologies have been developed for generating semi-synthetic sdAbs using engineered cell lines capable of inducible V(D)J recombination and transgenic mice bearing either hybrid llama-human or fully human *igh* loci; in both cases, a limited set of V H H, D, and J H genes (some of which are in non-germline configurations to promote autonomous folding) are rearranged in a foreign cellular or *in vivo* system. Limited numbers of synthetic sdAbs have been described and fewer still have been studied structurally in complex with antigens. Nevertheless, the available data suggests that some synthetic sdAbs have cleft-binding properties akin to those of V H Hs and V NAR51 while others employ unusual mechanisms to interact with planar protein epitopes (e.g., dramatic CDR3 restructuring of a MDM4-specific V H H domain to accommodate packing against a hydrophobic helix),

significant involvement of FRs in binding of V H Hs to vascular endothelial growth factor, and CD40 using distinct mechanisms. Even less is known regarding the paratope structures and binding modes of non-Ig-based antibodies such as variable lymphocyte receptors and non-antibody scaffolds (based on monomeric non-Ig domains such as fibronectin type III and SRC homology 3 domains), and their synthetic origin may imply that they follow no general patterns. If so, restrictions on the binding specificities of naturally-occurring sdAbs may not equally affect synthetic sdAbs and non-antibody scaffolds, although fundamental structural constraints on the amino acid sequences that can be tolerated by stable Ig folds would still apply.

**Conclusions and perspectives**

Recent work on unusual antibodies produced by unorthodox model organisms (e.g., cows, chickens) has spurred renewed interest in the comparative immunology of antibody responses. Some ‘cryptic’ regions on proteins (e.g., enzyme active sites, recessed regions of viral glycoproteins) are clearly more accessible to sdAbs than to conventional antibodies. More generally, we surmise that the major advantage of sdAb recognition is the ability to target conserved cleft and pocket regions (typically binding sites) on hypervariable pathogens without making ancillary contact with the easily mutable perimeters of these sites. Why and how pathogen selection produced two evolutionarily-unrelated sdAb systems in sharks and camelds, but not in other organisms, remains to be clarified. In the case of sdAbs, privileged access is conferred by their compact paratope diameters (in the absence of a paired V L domain) rather than any global difference in paratope shape or structure. Similar non-covalent interactions mediate the binding of conventional antibodies and sdAbs, although these are more efficiently concentrated in the compact paratopes of sdAbs to produce high-affinity interactions. Although it is likely that sdAb paratopes have difficulty adopting concave geometries and recognizing small molecules, it remains unclear whether such paratope restrictions disfavor interaction with certain types of protein epitopes as well.

Future studies will need to rigorously assess the degree of separation and overlap in the protein epitope space...
targeted by sdAbs vs. conventional antibodies, and to explore whether sdAb-accessible (and inaccessible) epitopes can be predicted in silico. Basic studies of the immunological functions of conventional vs. heavy chain-only antibodies in host defense (e.g., neutralization; opsonization; antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity) would also be highly valuable. Given the apparent sufficiency of sdAb paratopes to mediate high-affinity interactions with proteins, both the evolutionary forces responsible for shaping the more complex paired \( V_H:V_L \) antibody system in vertebrates, as well as the overall functions of light chains, are open questions.

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**Abbreviations**

- CDR: complementarity-determining region
- FR: framework region
- GPCR: G protein-coupled receptor
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