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A naturally occurring NAR variable domain binds the Kgp protease from *Porphyromonas gingivalis*

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Abstract The new antigen receptor (NAR) from sharks consists of a single immunoglobulin variable domain attached to five constant domains, and is hypothesised to function as an antibody. Two closely related NARs with affinity for the Kgp (lysine-specific) gingipain protease from *Porphyromonas gingivalis* were selected by panning an NAR variable domain library. When produced in *Escherichia coli*, these recombinant NARs were stable, correctly folded, and specifically bound Kgp ($K_d = 1.31 \pm 0.26 \times 10^{-7}$ M). Binding localised to the Kgp adhesin domains, however without inhibiting adhesin activity. These naturally occurring proteins indicate an immune response to pathogenic bacteria and suggest that the NAR is a true antibody-like molecule.

Key words: Scaffold; Peptide display; V_H; Variable domain; New antigen receptor; Lysine-specific gingipain protease from *Porphyromonas gingivalis*

1. Introduction

The immune systems of cartilaginous fish employ a diverse range of antibody and antibody-like proteins, including monomeric and pentameric IgM, IgX, and IgW [1,2]. These proteins are all of conventional antibody architecture, relying on the interaction of heavy (V_H) and light (V_L) domains to form an antigen-binding site comprising four to six variable CDR loops. In contrast, the new antigen receptors (NARs) from *Ginglymostoma cirratum* (nurse sharks, *n*NAR) and *Orectolobus maculatus* (wobbegong sharks, *w*NAR) encapsulate variability within two CDR loops of a single V_H domain. It is clear from immune electron microscopy that there is no associated light chain and that the variable domains do not associate together across a V_H/V_L-like interface [3]. Structurally, the intact NAR molecule consists of a disulphide-bonded dimer of two protein chains of five constant and one variable immunoglobulin domains. This arrangement, and particularly the single variable domain, is very similar to the V_HH antibodies found in camelid species in a clear case of convergent evolution at the molecular level [3,4]. V_HHs are capable of binding a wide range of protein, hapten and peptide targets and represent a significant proportion of the camelid immune response [5,6]. While a similar function for the NAR in the shark immune response awaits formal proof, there is strong evidence that NARs are functional antibodies. For example, NARs show (i) protein variability that is almost exclusively encapsulated into the two major CDR loop regions, with maintenance of a conserved underlying immunoglobulin structural framework; and (ii) a pattern of hypermutation in the CDRs between secretory and transmembrane forms, analogous to the process of affinity maturation in mammalian IgG class molecules [7,8].

In a previous study, we showed that the individual *w*NAR variable domains could be expressed as soluble monomers in the *Escherichia coli* periplasm. An in vitro type II *w*NAR library was then designed with synthetic CDR3 loops, and successfully displayed on the surface of fd bacteriophages. Library panning using standard techniques against target proteins resulted in the isolation of NAR domains specific for proteins from *Porphyromonas gingivalis* [9]. *P. gingivalis* is an anaerobic bacterium strongly associated with human periodontal disease where virulence is mediated through a range of extracellular factors including the related gingipain cysteine proteases Kgp (specific for lysine residues) and HRgp (specific for arginine residues) [10]. Both proteases are high molecular weight complexes of a gingipain catalytic domain and have up to four haemagglutinin/adhesion subunits.

Here we report the results of further screening of NAR single variable domain libraries against Kgp, using an expanded library and screening techniques different to those previously reported. Surprisingly, we observed strong selection for two naturally occurring NAR proteins not derived from our synthetic CDR3 library. In this first description of antigen specificity in natural NARs, we analyse the binding characteristics of these antibody-like domains.

2. Material and methods

2.1. Equipment and reagents

Vent DNA polymerase and all restriction enzymes were purchased from New England Biolabs (Beverley, MA, USA) and used according to the manufacturer's instructions. T4 DNA ligase was from Biotech (Australia). DNA fragment recovery and purification was by QIA-

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Abbreviations: *w*NAR, new antigen receptor from wobbegong sharks; *n*NAR, new antigen receptor from nurse sharks; Kgp, lysine-specific gingipain protease from *Porphyromonas gingivalis*; HRgpA, high molecular weight arginine-specific gingipain protease from *Porphyromonas gingivalis*

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quick Gel Extraction Kit, Qiagen (Germany). Small-scale preparations of DNA from *E. coli* were by QIAprep Spin Miniprep kit, Qiagen (Germany). Monoclonal anti-FLAG antibody was purified from hybridoma cell line KM5-1C7-8-5 (provided by Dr. N. Nicola, CRC for Cellular Growth Factors, WEHI, Australia) using rProtein A Sepharose fast flow resin from Amersham Pharmacia Biotech (Australia) according to the manufacturer's instructions. Purified anti-FLAG antibody was immobilised onto Mini-Leak™ Low resin from Kem-En-Tec (Denmark) following the manufacturer's instructions, to generate anti-FLAG affinity resin. Goat anti-mouse IgG (Fc)-horseradish peroxidase (HRP) was from Pierce. BenchMark™ Prestained Protein Ladder Cat. # 10748-010 was from Gibco BRL Life Technologies (Gaithersburg, MA, USA). Standard molecular biological techniques were performed as described [11]. HRgpA, RgpB and Kgp were purified from the H66 strain culture fluid as described previously [12].

2.2. E. coli strains

The cell line used for library propagation and selection and protein expression was *E. coli* TG1 (K12 *sup*E Δ(*lac-pro*AB) *thi hsd*Δ5 F′{*tra*D36 *pro*AB$^+$ *lac*I^q *lac*ZΔM15}. *E. coli* transformants were maintained and grown in 2×YT broth supplemented with 100 μg/ml (w/v) ampicillin +/− 2% (w/v) glucose. Solid media contained 2% (w/v) Bacto-agar. Transformation of *E. coli* was by standard procedures [11] performed using electro-competent cells.

2.3. Library construction and panning

DNA library cassettes encoding the *w*NAR were constructed from cDNA as described [9]. The total library size was $\sim 4.0\times10^8$ independent clones, consisting of $>3\times10^8$ clones with synthetic CDR3 sequences, and $\sim 7.0\times10^6$ clones derived from natural cDNAs. Library contributions were normalised in proportion to their sizes prior to panning, and phagemid particles carrying the NAR-gene 3 protein were propagated and isolated by standard procedures [13]. For biopanning of the phagemid library, Kgp (2 μg/ml in phosphate-buffered saline (PBS)) was coated onto Maxisorb Immunotubes and incubated at 4°C overnight. Immunotubes were rinsed (PBS), blocked with PBS/2% Blotto for 1 h at room temperature (RT), and incubated with freshly prepared phagemid particles (in PBS/2% Blotto) for 30 min at RT with gentle agitation, followed by 90 min without agitation. After incubation, immunotubes were washed (PBS/0.1% Tween 20; 7, 8, 10 washes for panning rounds 1–3), followed by an identical set of washes with PBS. Phagemid particles were eluted using 0.1 M HCl, pH 2.2/1 mg/ml bovine serum albumin, neutralised by the addition of 2 M Tris base, and either immediately reinfected into *E. coli* TG1 or stored at 4°C.

2.4. Nucleic acid isolation and cloning

Following final selection, phagemid particles were infected into *E. coli* TG1 and propagated as plasmids, followed by DNA extraction. The NAR cassette was extracted as a *Not*1/*Sfi*1 fragment and subcloned into the similarly restricted cloning/expression vector pGC [14]. DNA clones were sequenced on both strands using a BigDye terminator cycle sequencing kit (Applied Biosystems, USA) and a Perkin Elmer Sequenator. The nucleotide sequence of clones 12A-9 and 12A-14 associated with this study are deposited in the GenBank database under accession numbers AF466395 and AF466396.

2.5. Soluble expression of wNAR constructs from expression vector pGC

Recombinant proteins were expressed in the bacterial periplasm as described [9]. Briefly, *E. coli* TG1 starter cultures were grown overnight in 2×YT medium/ampicillin (100 μg/ml)/ glucose (2.0% w/v), diluted 1/100 into fresh 2×YT/100 μg/ml ampicillin/glucose (0.1% w/v) and then grown at 37°C/200 rpm until $OD_{550\,nm} = 0.2–0.4$. Cultures were then induced with IPTG (1 mM final), grown for a further 16 h at 28°C and harvested by centrifugation (Beckman JA-14/6K/10 min/4°C). Periplasmic fractions were isolated by the method of Minsky [15] and either used as crude fractions or recombinant protein purified by affinity chromatography using an anti-FLAG antibody–Sepharose column (10×1 cm). The affinity column was equilibrated in PBS, pH 7.4, and bound protein eluted with ImmunoPure™ gentle elution buffer (Pierce). Eluted proteins were dialysed against two changes of PBS/0.02% sodium azide, concentrated by ultrafiltration over a 3000 Da cutoff membrane (YM3, Diaflo), and analysed by FPLC on a precalibrated Superdex75 column (Pharmacia) in PBS pH 7.4. Recombinant proteins were analysed by SDS–polyacrylamide gel electrophoresis through 15% Tris/glycine gels.

2.6. Enzyme-linked immunosorbent assays

Protein antigens (0.5 μg/well) in PBS were coated onto Maxisorb Immuno-plates (Nunc, Germany) and incubated at 4°C overnight. Plates were rinsed, blocked with PBS/5% Blotto for 1 h at RT, and incubated with periplasmic fractions or recombinant protein for 1 h at RT. Plates were rinsed with PBS, washed three times with PBS/0.05% Tween 20, and anti-FLAG antibody (1/1000 in PBS/5% Blotto) added. Plates were incubated and washed as above, and the HRP-conjugated secondary anti-mouse Fc antibody added (1/1000 in PBS/5% Blotto). Plates were washed again and developed using 2,2-azino-di-(ethyl) benzothiazoline sulphonic acid (Boehringer Mannheim, Germany) and read at OD_{405} nm.

For localisation of Kgp binding, fibrinogen (10 nM) in PBS was coated as above, and the plates then rinsed, blocked with PBS/1% Blotto for 1 h at 37°C, and washed three times with PBS/0.1% Tween 20. Plates were then incubated with either 30 nM of gingipain alone or gingipain+3 μM 12A-9 for 1 h at 37°C, washed as above, and incubated with 10 μg/ml chicken anti-gingipain antibody for 1 h at 37°C before addition of a HRP-conjugated anti-chicken antibody (1/10000 in PBS/1%Blotto). Plates were washed again and developed using 3,3′,5,5′-tetramethylbenzidine (Sigma, USA) and read at 450 nm.

2.7. Biosensor binding analysis

A BIAcore™ 1000 biosensor (BIAcore AB, Uppsala, Sweden) was used to measure the interaction between *w*NAR proteins 12A-9 and 12A-14, and Kgp. Kgp at a concentration of 50 μg/ml in 10 mM sodium acetate buffer, pH 4.5, was immobilised onto a CM5 sensor chip via amine groups using the Amine Coupling kit (BIAcore AB) [16]. The immobilisation was performed at 25°C and 5 μl/min flow rate. Injection of 50 μl of 50 μg/ml Kgp coupled 2000RU to the surface. Binding experiments were performed in HBS buffer (10 mM HEPES, 0.15 M NaCl, 1 mM $CaCl_2$, 0.005% surfactant P20, pH 8.15) at 25°C and a constant flow rate of 5 μl/min with a series of analyte concentrations (825–52.5 nM). Regeneration of the Kgp surface was achieved by running the dissociation reaction to completion before the next injection of analyte. The binding data was evaluated with BIAevaluation 3.0.2 [17].

3. Results

3.1. Panning of an expanded wNAR variable domain library on Kgp

Previously we described the design and construction of a *w*NAR variable domain library with synthetic CDR3 loops. This library, although small ($\sim 3\times10^7$), was successfully displayed on the surface of fd bacteriophage and panned against Kgp displayed in the context of ELISA plate wells [9]. In order to isolate further antigen-specific NAR domains, the library was expanded to $\sim 4\times10^8$ independent clones by incorporation of both synthetic and naturally occurring (derived from cDNA) CDR3 sequences, followed by further transformations into *E. coli* TG1. Phagemid particles were then rescued and panned against the Kgp antigen immobilised on immunotubes. After three rounds of biopanning an ~100-fold increase in bacteriophage titre was observed, with 100% of phagemid-transfected colonies positive for *w*NAR sequences suggesting that positive selection was occurring. Thus, *w*NAR variable domain cassettes were rescued from phagemids, cloned into the periplasmic expression vector pGC, and transformed into *E. coli* TG1.

Periplasmic fractions from recombinant clones were tested for binding to Kgp and to negative control antigens by ELISA (not shown). Over 50% of the clones tested showed significant binding above background. When sequenced, only two different sequences were present, represented by the clones designated 12A-9 and 12A-14. The primary and de-

duced amino acid sequences of 12A-9 and 12A-14 are presented in Fig. 1A. Both proteins represent 108 residue *w*NAR variable domains and are obviously closely related (Fig. 1B), with differences distributed evenly between framework and loop regions (particularly CDR3). Surprisingly, the CDR3 loops are both only 13 residues in length, compared to 15–18 incorporated in the in vitro CDR3 library. Similarly, framework residue 84, which was conserved as either a glutamine or alanine in the synthetic library, encoded a lysine residue in both proteins (Fig. 1B). This indicates that proteins 12A-9 and 12A-14 are naturally selected domains, as it is highly unlikely that two antigen-specific clones could have been independently mutated in the artificial library to the same size CDR3, both containing Lys[84] and with other differ-

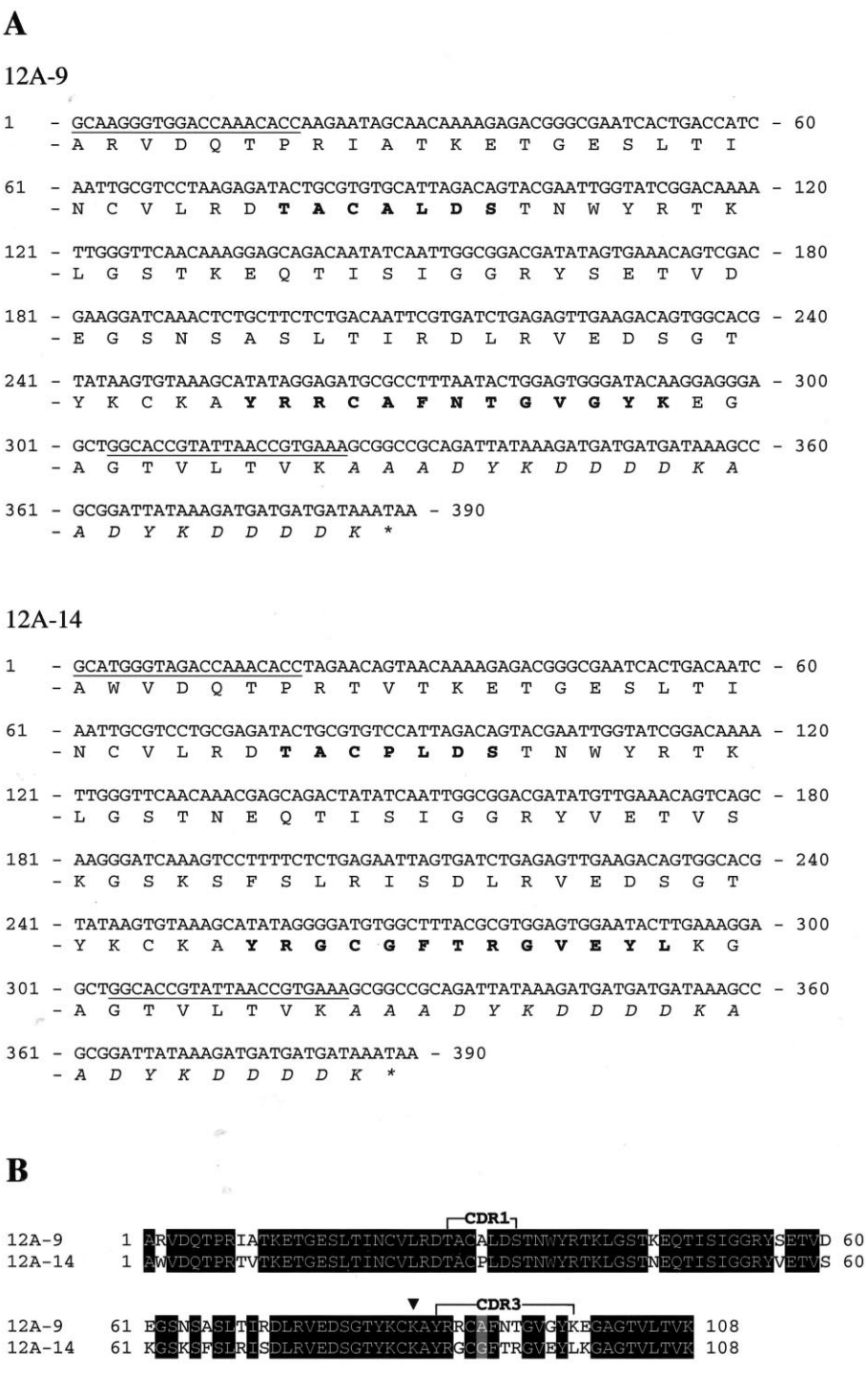

Fig. 1. Nucleotide and deduced amino acid sequences of the *w*NAR 12A-9 and 12A-14 variable domains. A: Nucleotide and deduced amino acid sequences of clones 12A-9 and 12A-14. The conserved termini dictated by the oligonucleotide primer sequences used in library construction are underlined, and the alanine linker and dual octapeptide FLAG tags are italicised. The positions of the CDR1 and -3 regions are indicated in bold type. B: Alignment of proteins 12A-9 and 12A-14. Amino acids are designated with the single-letter code, and identical residues (dark shading) and conservative replacements (light shading; I/V/L/M, D/E, K/R, A/G, T/S, Q/N, F/Y) are indicated. The framework residue Lys[84], which indicates that these are naturally occurring NARs, is arrowed, and the CDR1 and -3 regions are indicated.

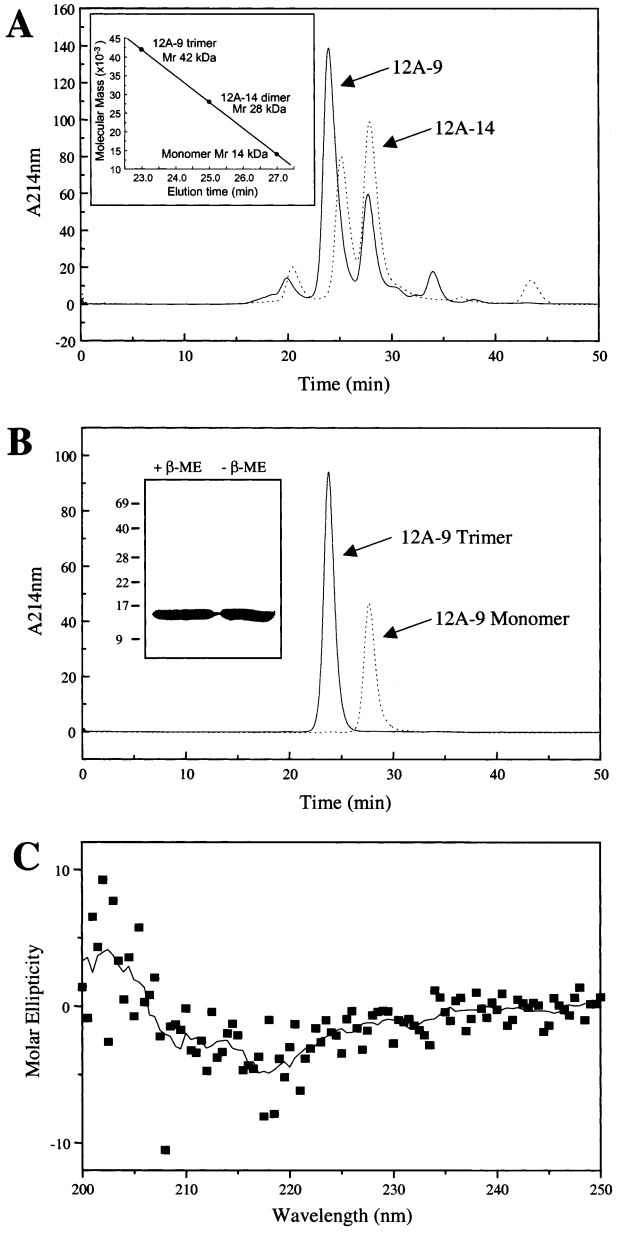

Fig. 2. Size exclusion chromatography and CD analysis of proteins 12A-9 and 12A-14. A: Elution profiles of affinity-purified 12A-9 and 12A-14 proteins on a calibrated Superdex75 HR10/30 column equilibrated in PBS, pH 7.4, and run at a flow rate of 0.5 ml/min. The 12A-14 and 12A-9 peaks eluting at 27 min are consistent with a monomeric domain (calculated M_r of 14 225 for 12A-14 and 14 122 for 12A-9). The 12A-14 peak eluting at 25 min is consistent with a dimer ($M_r = 28$ kDa) and the 12A-9 peak eluting at 23 min is consistent with a trimer ($M_r = 42$ kDa). The inset shows the relationship between molecular mass and elution time for this family of *w*NAR domains. The optical density at 214 nm is given in arbitrary units. B: Re-chromatography of isolated 12A-9 monomer and trimer peaks on Superdex75 column under the same conditions as for (A). The inset shows the *w*NAR 12A-9 trimer treated in the presence (+) or absence (−) of β-mercaptoethanol and analysed by SDS–polyacrylamide gel electrophoresis through a 15% (w/v) polyacrylamide Tris/glycine gel and stained with Coomassie brilliant blue. C: Circular dichroic spectrum of affinity-purified *w*NAR 12A-9 in 0.05 M sodium phosphate buffer, pH 7.4. The scatter plot shows data collected and the unbroken line represents an average of these data points.

ences scattered throughout both CDR and framework regions (Fig. 1B). It is notable that conserved cysteine residues are present in both CDR1 and -3 loops, and probably form stabilising inter-loop disulphide linkages [3,6,9].

3.2. Characterisation of recombinant 12A-9 and 12A-14 variable domains

To compare proteins 12A-9 and 12A-14 and to define their binding characteristics, recombinant proteins were isolated from the *E. coli* periplasm by affinity chromatography using an anti-FLAG antibody affinity resin and their oligomeric status analysed by size exclusion chromatography on a calibrated Superdex75 HR10/30 column. The elution profiles showed that both proteins contained two major oligomeric forms (Fig. 2A). The elution times indicated that 12A-14 consisted of a monomer ($M_r \sim 14$ kDa) and a dimer ($M_r \sim 28$ kDa), while 12A-9 consisted of a monomer and a trimer (~ 42 kDa) (Fig. 2A, inset). The presence of a dimer and trimer in affinity-purified fractions of 12A-14 and 12A-9, respectively, was confirmed by dynamic light scattering analysis (data not shown). As clone 12A-9 showed higher expression levels than clone 12A-14 (1 mg/l compared to 0.2 mg/l purified protein) and showed apparently higher binding activity (see next section), protein 12A-9 was chosen for further analysis.

The monomeric and trimeric peaks of 12A-9 were isolated by size exclusion chromatography and found to be stable with no evidence of re-equilibration (Fig. 2B). Furthermore, treatment of 12A-9 trimer in 8 M urea followed by size exclusion chromatography into PBS yielded back only trimer. The stability of the trimer was not due to disulfide bond linkages, as treatment of 12A-9 trimer with SDS in the absence of reducing agent produced a single protein band of ~ 14 kDa, expected for the monomer (Fig. 2B, inset). N-terminal amino acid sequencing of affinity-purified 12A-9 monomer/trimer mixture showed that only one protein species was present with the expected N-terminus ([1]ARVDQTP–; Fig. 1A) indicating that the signal peptide had been correctly cleaved on secretion into the *E. coli* periplasm. Far ultraviolet CD spectra of aqueous solutions of protein 12A-9 trimer showed a profile with a negative band with λ_{max} at 217–219 nm (Fig. 2C). This spectrum is characteristic of β protein and not a disordered structure [18], confirming that the 12A-9 variable domain folds into a compact, β-sheet immunoglobulin fold in the *E. coli* periplasmic space.

3.3. Specificity and binding activity of recombinant protein 12A-9

The specificity of affinity-purified 12A-9 and 12A-14 proteins for Kgp was demonstrated by ELISA. Both proteins reacted specifically with Kgp but not the other antigens tested (Fig. 3A). Protein 12A-9 showed clearly superior binding characteristics with at least five-fold higher activity than protein 12A-14 (Fig. 3B). The binding kinetics of the monomeric 12A-9 and 12A-14 were also measured by BIAcore biosensor analysis with Kgp protein immobilised via amine coupling to the sensor surface. A comparison of the binding interactions of 12A-9 and 12A-14 binding to immobilised Kgp showed that ~ 10 times more 12A-14 was required to elicit a response similar to that obtained with 12A-9 (Fig. 4A), consistent with the result observed in the ELISA reaction. The apparent lower binding activity of 12A-14 can be attributed to either weaker binding (slow association rate and fast dissociation rate con-

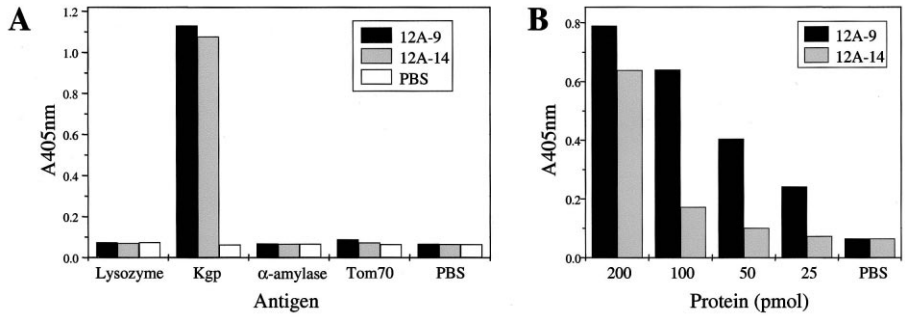

Fig. 3. Analysis of proteins 12A-9 and 12A-14 by ELISA. A: Proteins were purified from the periplasmic fraction of *E. coli* TG1 by affinity chromatography through an anti-FLAG M2 antibody column and tested for binding to lysozyme, Kgp, Tom70, and α-amylase. Results represent the average of triplicate wells. B: As for (A) except serial two-fold dilutions of equal amounts of 12A-9 and 12A-14 proteins were tested for binding to Kgp. Results represent the average of duplicate wells.

stants) or that only a small fraction (~5%) of the purified 12A-14 is active in binding immobilised Kgp. Interestingly, a comparison of the binding kinetics of affinity-purified 12A-9, 12A-9 monomer, and 12A-9 trimer, showed no difference in the dissociation rates between monomer and trimer (data not shown) suggesting that the 12A-9 trimer does not exhibit multivalent binding to immobilised Kgp. Whether the apparent inability of *w*NAR 12A-9 trimer to exhibit multivalent binding is due to the orientation and accessibility of Kgp epitope on the sensor surface or to the steric orientation of the CDRs in the 12A-9 trimer remains to be resolved. Protein 12A-9 showed no binding to a blank surface (activated and then blocked with ethanolamine) in either its monomeric or trimeric form, indicating that there is no non-specific interaction with the sensor surface (Fig. 4A, inset; and not shown).

A series of sensorgrams for the binding of 12A-9 peak-purified monomer are shown in Fig. 4B. The binding data were fitted at each concentration to the 1:1 Langmuir binding model and the kinetic constants evaluated. The data showed a reasonably good fit to the 1:1 binding model, consistent with *w*NAR 12A-9 monomer binding to a single epitope on Kgp, although some deviation from the binding model is apparent towards the end of the dissociation phase. The binding data gave a value for the k_a of $4.29 \pm 0.68 \times 10^4$ M^{-1} s^{-1} and k_d of $7.81 \pm 1.30 \times 10^{-3}$ s^{-1} to yield a dissociation constant (K_d) of $1.31 \pm 0.26 \times 10^{-7}$ M.

3.4. Mapping of the Kgp epitope

To determine the Kgp epitope targeted by protein 12A-9, different forms of gingipain were tested for binding. Protein 12A-9 bound both Kgp and the related arginine-specific gingipain, HRgpA, but not the lower molecular weight RgpB form that lacks most adhesin subunits (results not shown). This suggested that the adhesin domains formed at least part of the 12A-9 epitope. However, in a series of competition ELISA experiment, high concentrations of 12A-9 (3 μM, more than 10-fold above K_d) failed to inhibit binding of Kgp or HRgpA to immobilised fibrinogen and other proteins such a fibronectin (Fig. 5; and results not shown). Thus, either the 12A-9 epitope is removed from the adhesin regions involved in agglutination, or single NAR variable domains are of insufficient size to block the adhesin binding. Similarly, protein 12A-9 did not affect the enzyme activity of the gingipains, suggesting that it does not target the catalytic site of the enzymes.

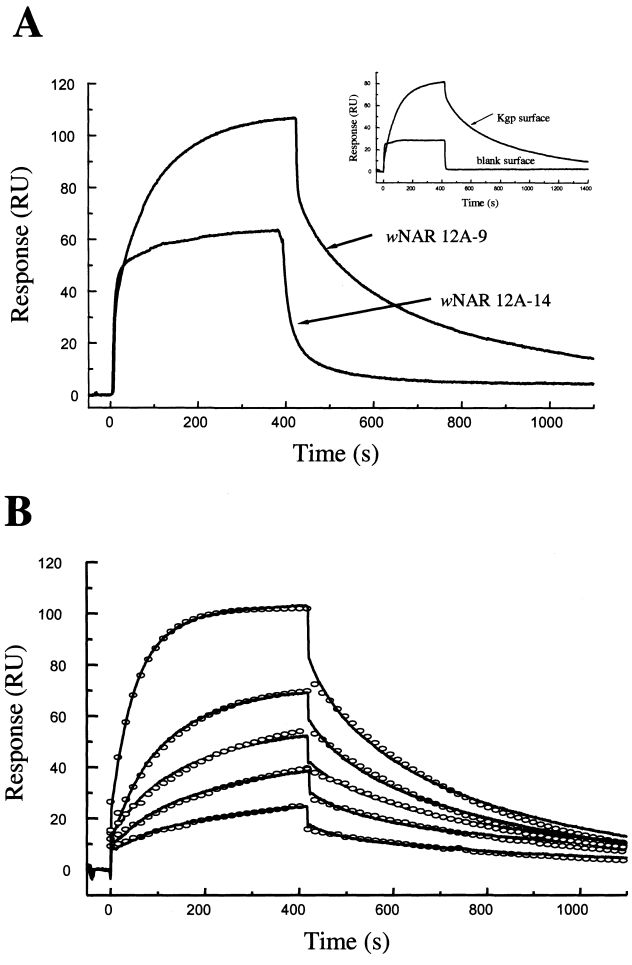

Fig. 4. Analysis of proteins 12A-9 and 12A-14 by BIAcore. Binding of *w*NAR monomeric proteins to immobilised Kgp (2000RU) was measured at a constant flow rate of 5 μl/min with an injection volume of 35 μl. Dissociation was continued with HBS buffer until the response returned to the initial value before injecting the next sample. A: Sensorgrams showing the binding of *w*NAR monomeric proteins 12A-9 (6 μg/ml) and 12A-14 (115 μg/ml). The inset shows the binding profile of monomeric *w*NAR protein 12A-9 (6 μg/ml) to immobilised Kgp and a blank surface (NHS/EDC activated and blocked with ethanolamine). B: Sensorgrams showing the binding of a series of concentrations of *w*NAR 12A-9 protein (825, 413, 210, 105, 52.5 nM; conditions as in A). The circles show the fit to the data obtained on analysis with the 1:1 Langmuir binding model for the evaluation of the kinetic rate constants.

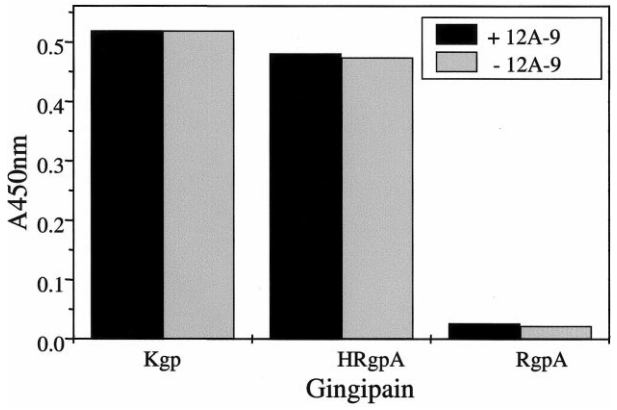

Fig. 5. *w*NAR protein 12A-9 does not inhibit the adhesin activity of Kgp and Rgp gingipains. Fibrinogen (10 nM) was coated onto ELISA wells and binding to this surface of Kgp and HRgpA gingipains tested in the presence or absence of 3 μM 12A-9 protein. The lower molecular weight RgpA form, lacking adhesin domains, is shown for comparison.

4. Discussion

The isolation of two naturally occurring and antigen-specific NAR variable domains confirms the NAR from sharks as a functional antibody-like molecule. With an affinity for the target antigen of ~130 nM, these single domains have antigen specificity comparable to recombinant forms of the camelid V_HH single domain antibodies, where the affinity varies between 2 and 300 nM [5,6]. However, NARs encompass this affinity in two, rather than three CDR loops, as the CDR2 region is severely truncated [3,9]. The cysteine residues seen within many NAR (and camelid) CDR loops are conserved in both proteins 12A-9 and 12A-14, and probably contribute to the antigen-binding affinity by disulphide bond formation and structural stability.

Both the NAR variable domains reported here can be readily produced as recombinant proteins in *E. coli*. However, while a significant proportion of the 12A-9 and 12A-14 proteins are produced in monomeric form, multimers (dimer and trimer) also occur. Surprisingly, the isolated monomeric and multimeric species are remarkably stable, with no evidence of inter-conversion of peak purified proteins. The relationship between the 12A-9 monomer and trimer forms requires further investigation and may reflect non-covalent framework residue interactions peculiar to these proteins, as other NAR variable domains we have analysed do not show similar multimer formation and stability and are predominantly monomeric proteins. As a trimeric architecture is not expected to occur in native NAR molecules, where the variable domains are tethered to constant regions, it is possible that their existence here as trimeric variable domains is an unusual side-effect of recombinant expression. Ultimately, resolution of these questions will probably require X-ray crystallographic data to define the precise nature of the NAR solvent-exposed residues and surfaces.

The 12A-9 and 12A-14 NAR domains are clearly derived from functional shark cDNAs and show no similarity, beyond invariant regions in the underlying framework, to the anti-Kgp NARs isolated previously [9]. These earlier proteins have synthetic rather than naturally selected CDR3 loops and were not affinity matured. Thus, it is likely that clones 12A-9 and 12A-14, introduced in the expanded library, efficiently out-competed any other Kgp-specific proteins. Alternatively, it is well documented that different selection matrices (immunotubes, this study; versus ELISA plates [9]) can produce radically different antibody selection solutions, even when screening is performed exactly in parallel [19]. The isolation of natural Kgp gingipain-specific NARs in sharks is perhaps surprising. However, while little is known about the oral microflora of sharks, one study reported isolation of *Vibrio*, *Staphylococcus*, and *Pseudomonas* species from the teeth of a great white shark [20]. Therefore, it is possible that wobbegong sharks carry *Porphyromonas* species and have mounted an immune response against the protease virulence determinants. The isolation of the two different but obviously closely related NAR proteins may also indicate the action of affinity maturation processes on an initial low-affinity NAR molecule. Indeed, clone 12A-14 could represent such a low-affinity progenitor, with subsequent 'maturation' to the 12A-9 form. If so, then both CDR loops and framework regions are being targeted, in what may be the shark equivalent of mammalian antibody somatic hypermutation.

With specific NAR binding molecules now isolated from both synthetic libraries generated using artificial loop sequences, and from the natural immune repertoire of sharks, it is clear that recombinant NAR variable domain libraries represent a valuable source of high-affinity single domain binding reagents. Future experimentation is clearly required to further compare their stability and structure with that of other antibody fragments, and such work is currently in progress.

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