Single-chain variable fragments selected on the 57–76 p21Ras neutralising epitope from phage antibody libraries recognise the parental protein

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Abstract Phage antibodies have been widely prospected as an alternative to the use of monoclonal antibodies prepared by traditional means. Many monoclonal antibodies prepared against peptides are able to recognise the native proteins from which they were derived. Here we show that the same is also true for phage antibodies. We have selected a number of single-chain variable fragments (scFv) from a large phage scFv library against a peptide from the switch region II of p21Ras. This peptide is known to reside in a mobile area of the native protein and is the epitope of a well characterised monoclonal antibody. Selected scFvs were able to recognise native p21Ras in both ELISA and Western blots, indicating that peptides are also likely to be very useful in selecting from phage antibody libraries.

Key words: Phage display; p21Ras; Ras neutralizing epitope; Single-chain variable fragment

1. Introduction

The immunisation of animals with peptides has long been used to isolate antibodies against the native proteins from which they were derived (reviewed in [1,2]). This technique is especially useful when the native protein is difficult to prepare or purify in sufficient quantities to allow immunisation, and is based on the initial observations of Anderer that antibodies raised against tobacco mosaic virus peptides could recognise the native protein [3,4]. Since then, a number of different authors have attempted to derive rules which allow the prediction of peptides which are more likely to elicit antibodies recognising the native protein. Individual parameters which have been shown to influence such peptide antigenicity include hydrophilicity [5], β-turn tendency [6,7], amphipathicity [8], surface exposure [9] and segmental mobility [10].

The rules governing peptide antigenicity take into account a complete immune system with functioning T and B cells. It has recently proved possible to derive antibodies in vitro against a large number of antigens from phage antibody libraries [11–16] in which antibodies are displayed on the surface of filamentous phage and selected on the basis of their binding activity to the antigen. As cells of the immune system are absent, the antibodies selected recognise the antigen as it is presented to the library and not after processing by the immune system.

The ability to select antibodies against peptides which recognise the native protein from these libraries has been tested with a 20 amino acid MUC1 peptide which corresponds to a repeated antigenic epitope in MUC1 mucin [17]. In this case a single antibody was isolated and shown to recognise the full-length protein. This is, however, a special case, since the epitope is repeated, and may not be applicable to all peptides.

The p21Ras protein is a small GTPase, which cycles between a GTP-bound active and GDP-bound inactive form, involved in many signal transduction pathways [18,19]. The active and inactive forms have different structures, caused by a large conformational change in the two switch regions (switch I: Asp-30–Asp-38 and switch II: Gly-60–Glu-76) which flank the guanine nucleotide binding pocket (reviewed in [20]). This conformational change allows the active form of p21Ras to interact with downstream effector proteins such as Raf-1, B-raf, PI-kinase and RalGDS [21] and so transmit signals into the cell from the surface.

The purpose of this work was to use phage display to derive new antibodies against p21Ras using the peptide approach; these to be used later for intracellular expression to interfere with p21Ras function (intracellular immunisation) [22]. Given the known mobility of switch region II and the fact that an antibody (Y13-259 [23]) which recognises this region [24] has been shown to be able to neutralise Ras function by antibody microinjection [25–27], and intracellular expression [28–31], we selected antibodies against the 57–76 peptide from a large phage display single-chain variable fragment (scFv) library [13]. This was presented to the antibody library in two different ways: as conjugated to bovine serum albumin (BSA) and displayed on the *Escherichia coli* cell surface within the outer membrane protein LamB (LamB-Ras) [32], a context in which it was previously shown to be capable of acting as a selector in a model system.

2. Materials and methods

2.1. Plasmids and library

4DJ [33] is a plasmid containing a p21Ras(57–76) epitope cloned (recognised by the mAb Y13-259) into the *Bam*HI site of pAJC264 [34], a prokaryote expression plasmid, which allows the expression of foreign epitopes within one of the exposed loops of the LamB protein [35]. The clone BW2029 is an *E. coli* N6105 strain harbouring a temperature-sensitive λ-repressor controlling expression of p21Ras from pJCL-E30 [36]; it was provided by B. Willumsen (University of Copenhagen) and was used to produce p21Ras protein according to [33].

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; MPBS, skimmed milk powder in PBS; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PCR, polymerase chain reaction; HRP, horseradish peroxidase; TMB, tetramethyl benzidine; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, nitroblue tetrazolium; GTP, guanosine triphosphate; GDP, guanosine diphosphate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; RU, response units

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The phage display library is characterised by 6.7×10^9 members of a naive human antibody repertoire, cloned in the pHEN1 vector [37] and displayed as scFvs [13]. pUC119 Sfi-NotmycHis [38] is a bacterial vector for the expression of scFvs with a hexa-histidine tag on their C-termini. The phage library and the pUC119 Sfi-NotmycHis vector were kind gifts of Jim Marks.

2.2. Preparation of selectors

BSA-peptide conjugation was performed by the glutaraldehyde coupling procedure [1] which employs NH_2 and SH groups for coupling. Since there are no lysines or cysteines present in the amino acid sequence of the peptide, the peptide should be coupled to BSA via its amino terminus, so preserving its secondary structure and antigenic activity. The Ras peptide 57–76 (DTAGQEEYSAMRDQYMRTGE, synthesised in ICGEB, Trieste) was added to a 1 mg/ml solution of BSA in phosphate buffered saline (PBS) pH 7.4, at molar ratios of protein to peptide varying from 1 to 20. When the mixture was cooled by keeping on ice, a 2% glutaraldehyde solution was added in an equal volume to the protein mixture with constant stirring. After 1 h the reaction was stopped by addition of sodium borohydride ($NaBH_4$) to a final concentration of 10 mg/ml. The reaction mixture was dialysed against three changes of PBS and stored in aliquots at −20°C.

The clone 4DJ was used for preparation of LamB-Ras(57–76) bacteria [32].

2.3. Selection

Immunotubes were coated overnight at 4°C with 10 μg/ml of BSA-Ras(57–76) peptide in 100 mM carbonate buffer (pH 9.4), blocked for 1 h at room temperature with 2% skimmed milk powder in PBS (2% MPBS). The first cycle of selection was performed using 10^{13} phage in 1 ml of MPBS left in contact with the antigen in the immunotube for 1 h at room temperature. Phage were washed 20 times in PBS+Tween 20 (0.05%) and 20 times in PBS. Phage were eluted in 100 mM triethylamine for 10 min and neutralised by the addition of Tris 1 M pH 8 [12].

The selection of the library on the LamB-Ras(57–76) selector was performed in two ways: directly and with a depletion step for 4 h on non-recombinant bacteria before each cycle of selection.

The selection was performed on 300 μl of bacterial suspension (equivalent to 30 μl of bacterial pellet and approximately 3×10^9 bacteria). The selection procedure was the same as described above, except that the fixed bacteria were centrifuged for 5 min at 5000 rpm at room temperature between washing cycles.

When depletion was applied before each cycle of phage binding to the fixed bacteria displaying the epitope, the phage were incubated on 1 ml of fixed DH5α bacterial suspension for 2 h on the rotating wheel at room temperature. After centrifugation (for 5 min at 5000 rpm) the supernatant containing unbound phage was employed for binding to the bacteria displaying the epitope.

2.4. Screening for unique binders

BSA-Ras(57–76) binding was determined by ELISA using soluble scFvs [12]. The coating and saturation of microtitre plates were done in the same way as for selection in immunotubes. After incubation with the soluble scFvs, wells were incubated with the anti-myc mouse monoclonal antibody, 9E10 [39] and with a 1:2000 dilution of an anti-mouse-HRP (horseradish peroxidase) conjugate (Dako). Binding was revealed with TMB (tetramethyl benzidine) and stopped with 1 M sulphuric acid.

The number of unique scFvs was estimated by PCR fingerprinting using *Bst*NI as described in [12].

2.5. scFv purification and affinity measurements

The selected scFv genes were subcloned into the bacterial expression vector pUC119Sfi-NotmycHis, expressed, harvested from the periplasm [40] and purified by immobilised metal affinity chromatography [41]. To separate monomeric scFvs from dimeric and aggregated scFvs, samples were fractionated on a Superdex 75 column using PBS as running buffer in a Biologic (Bio-Rad). scFv concentrations were quantified with the micro-BCA kit (Pierce).

scFv dissociation equilibrium constants (K_d) were calculated from the association (k_{on}) and dissociation (k_{off}) rate constants determined using surface plasmon resonance in a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). Using standard amine coupling as described in the Biacore manuals, approximately 1200 RU of BSA-Ras(57–76) was coupled to a CM5 sensor chip (Pharmacia). These amounts of coupled antigen yielded scFv RU_{max} of approximately 400 RU. Association rate constants were measured using scFv concentrations in the appropriate range (around K_d values). Calculation of K_d values was performed by fitting the data according to a single-site model, using the BIAevaluation 2.1 software. Where possible, association rate constants were determined from secondary plots (k_s versus concentration).

2.6. Western blot

The clone BW2029 was grown and induced according to [33]. Induced bacteria were treated with sample buffer, boiled and run on SDS-PAGE, and then blotted onto nitrocellulose sheets. These were cut into 5 mm strips. Soluble scFv proteins were expressed, harvested from the bacterial periplasm [42] and incubated with the nitrocellulose strips, overnight at +4°C. Washings were performed with PBS+0.05% Tween 20 and saturation with 2% MPBS. The nitrocellulose strips were incubated at room temperature for 1 h with the 9E10 monoclonal antibody, washed and incubated for 1 h with a 1:1000 dilution of an anti-mouse-AP (alkaline phosphatase) conjugate (Dako). Binding was revealed with BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitroblue tetrazolium) chromogenic substrate.

2.7. Native Ras ELISA

p21Ras was purified according to the method described in [43]. The GTPase activity of the recombinant p21Ras was checked using the method described in [44]. Purified p21Ras (100 ng) was coated in carbonate buffer overnight at 4°C, washed 3 times with TBS (Tris 20 mM pH 7.4, NaCl 150 mM) and blocked with 3% BSA in TBS for 1 h at 37°C. Subsequently wells were washed once with TBS and undiluted periplasmic fractions were incubated for 1 h at 37°C. Wells were washed with TBST (TBS containing 0.01% Tween 20) and incubated with biotinylated mAb 9E10 for 1 h at 37°C. Wells were washed with TBST and incubated with HRP/streptavidin (1:5000 Dako) for 15 min at room temperature. After washing, wells were stained with TMB and stopped with 1 M sulphuric acid. Absorbances were measured at 450 nm.

Table 1
(A) Elution profiles from the phage antibody library selected on the indicated selectors[a] and (B) screening of anti-Ras(57–76) clones derived from the third cycle of selection on the indicated selectors[b]

(A) Selector	Phage recovery (%)		
	1st cycle	2nd cycle	3rd cycle
BSA-Ras(57–76)	1.6×10^{-8}	1.3×10^{-6}	4×10^{-5}
LamB-Ras(57–76) bacteria (without depletion)	2.5×10^{-4}	8.3×10^{-4}	0.23
LamB-Ras(57–76) bacteria (with depletion)	5.8×10^{-5}	2.5×10^{-3}	1
(B) Selector	**Positives (%)**	**Unique clones**	
BSA-Ras(57–76)	25.0	7	
LamB-Ras(57–76) bacteria (without depletion)	6.5	1	
LamB-Ras(57–76) bacteria (with depletion)	4.3	3	

[a]Phage recovery in every cycle is indicated as percentage of phage eluted compared to those loaded.
[b]Peptide Ras(57–76) binders, as detected by BSA-Ras(57–76) ELISA, are indicated as percentages of positives on total number of 100 analysed clones; unique clones among binders are determined by *Bst*NI fingerprinting.

3. Results

The Ras peptide chosen (57–76) for selection is α-helical within the context of the p21Ras protein [20], and changes its orientation upon activation (see [45] for discussion and references). The secondary structure of the free peptide is also likely to be α-helical on the basis of structural predictions using Predict-Protein (Heidelberg) [46,47] and molecular mechanics [47,48] and dynamics [49] calculations. This structural maintenance and the known conformational mobility of this segment represent a valuable prerequisite for its use as potential selector.

The phage library [13] was selected on two selectors: BSA-Ras(57–76) and LamB-Ras(57–76) bacteria. LamB-Ras(57–76) bacteria were used as it has been demonstrated that the antibody Y13-259, the derived scFv, and a phage displaying Y13-259 scFv are specifically able to recognise the Ras neutralising epitope when displayed within the context of the outer membrane protein, LamB [32,33]. Moreover, the LamB-Ras(57–76) bacteria column was shown to be effective in selecting specific phage-antibody binders from a non-binder background [32]. One problem in the use of bacteria displaying epitopes on their surface is the possibility of selecting antibodies against natural epitopes found on the bacterial cell surface. Therefore in addition to direct selection on LamB-Ras(57–76) bacteria, a depletion procedure, which involved preincubating the library on wild type bacteria before each cycle of selection on LamB-Ras(57–76) bacteria, was also used. The BSA-Ras(57–76) selector was made by coupling the Ras peptide to BSA at an epitope density of 1:20. The selections on BSA-Ras(57–76), LamB-Ras(57–76) bacteria and LamB-Ras(57–76) bacteria with depletion were performed with three cycles of binding and elution. The elution profiles are presented in Table 1A. In all three cases, a steady enrichment of phage from each of the selectors with increasing cycles is seen.

Phage clones derived from the third cycle of all three selections were analysed for their ability to bind BSA-Ras(57–76) in an ELISA. In all three cases anti-Ras peptide binders were identified. The percentages of unique clones among binders, as determined by *Bst*NI fingerprint analysis [50] of the PCR amplified scFv gene, are presented in Table 1B. The use of bacteria resulted in a much lower number of clones able to recognise BSA-Ras(57–76) when compared to the use of BSA-Ras(57–76) as a selector. This is likely to be due to the simultaneous selection of antibodies against bacterial epitopes, as described above. Selection on bacteria did not appear to be greatly influenced by prior depletion, in that in both cases anti-peptide Ras antibodies with similar affinities (see later) were selected.

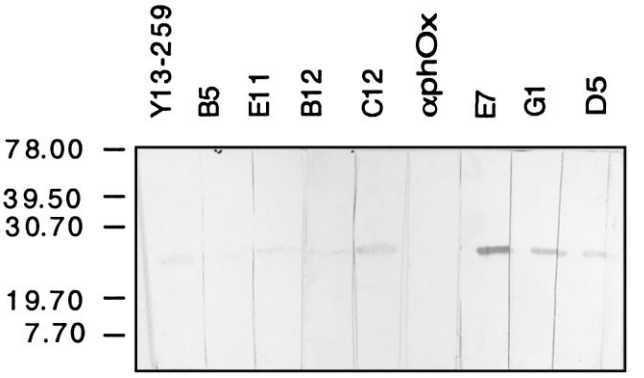

Fig. 1. Recognition of the bacterially produced p21Ras by the selected anti-Ras(57–76) scFvs. αphox: anti-phenyloxasolone scFv which does not recognise p21Ras The molecular weight markers are given in kDa.

In order to produce sufficient quantities of the scFvs for further analysis, each of the anti-Ras(57–76) scFvs was subcloned into the vector pUC119 Sfi-NotmycHis, which eliminates g3p and adds a hexa-histidine tag at the C-terminus of the scFv [51]. After purification by metal affinity chromatography (IMAC) and fractionation by gel filtration, the monomeric scFv fractions were subjected to affinity (K_d) measurements using surface plasmon resonance in a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). It proved impossible to carry out this technique with native p21Ras, as the epitope recognised by these antibodies (including Y13-259) appears to be masked when p21Ras is immobilised on the BIAcore chip (data not shown), therefore measurements were performed using immobilised BSA-Ras(57–76) conjugate. As shown in Table 2, the K_d of the anti-Ras(57–76) scFvs ranged from 5.4 to 803 nM.

In order to test the cross-reactivity of the selected anti-Ras(57–76) scFvs proteins with full-length p21Ras protein two approaches were taken: Western blotting with p21Ras and an ELISA using native p21Ras. The Western blot was performed with p21Ras expressed in *E. coli* (clone BW2029) and using soluble scFv proteins harvested from the periplasm [40]. As shown in Fig. 1, all scFvs analysed (except E3 and A1) are able to recognise a band corresponding to a molecular weight of 21 kDa in Western blots. While these experiments indicated that the selected scFvs could recognise denatured

Table 2
Affinity measurements of the anti-Ras(57–76) antibodies on BSA-Ras(57–76) using BIAcore 2000

Selector	Antibody	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (nM)
BSA-Ras(57–76)	Y13-259 scFv	1×10^{5}	1.6×10^{-2}	160
	A1	1.8×10^{6}	9.7×10^{-3}	5.4
	B12	3.1×10^{6}	3.3×10^{-2}	10.7
	C12[a]	ND	2.1×10^{-3}	ND
	D5	1.6×10^{6}	7.5×10^{-2}	46.9
	E7	8.5×10^{4}	2.7×10^{-2}	318
	E11	1.3×10^{6}	1.1×10^{-2}	8.5
	G1	5.5×10^{4}	1×10^{-2}	182
LamB-Ras(57–76) (without depletion)	B5	7.1×10^{4}	5.7×10^{-2}	803
LamB-Ras(57–76) (with depletion)	E3	3.5×10^{4}	9.1×10^{-3}	260

The table summarizes k_{on} and k_{off} constants, together with the calculated equilibrium dissociation constants (K_d).
[a]The clone demonstrates atypical kinetics during the association phase, therefore it was not possible to determine the association rate constant.

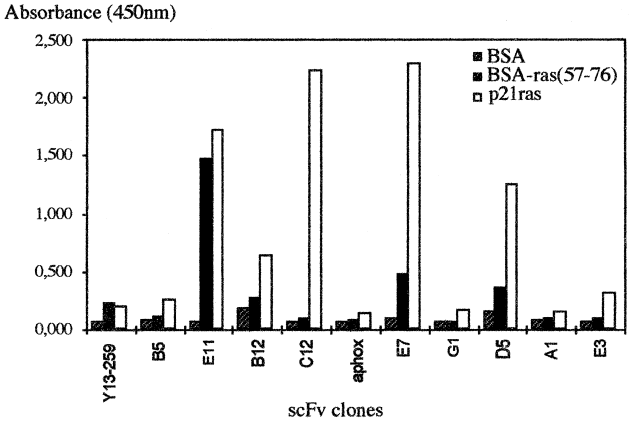

Fig. 2. Solid-phase p21Ras ELISA. Absorbance signals given by periplasmic space preparations of single scFv clones on solid-phase coated p21Ras (grey bar), BSA-Ras(57–76) (black bar) and BSA (white bar) are given. αphox: anti-phenyloxasolone scFv.

p21Ras in a Western blot, it gives no indication of their ability to recognise native p21Ras. This was shown for most clones by ELISA on native p21Ras (Fig. 2), in which some scFv (C12, D5, E7 and E11) gave ELISA signals far stronger than the positive control scFv Y13-259. Surprisingly, even though the scFv A1 is able to recognise the Ras peptide in the BIAcore, it does not interact with p21Ras or the Ras peptide in ELISA.

4. Discussion

Antibodies have been used in a wide range of applications such as in vitro and in vivo diagnostics, in passive immunisation, as therapeutics, in research, and more recently in intracellular immunisation. They have been routinely obtained by animal immunisation or by hybridoma technology. The use of phage antibody libraries has proved to be a quick and reliable alternative source of monoclonal antibodies, specific for a wide range of antigens, from diverse species, including self-antigens [12,14,15,17]. However, a successful animal immunisation or library selection relies on the availability of a purified and conformationally correct antigen. This is usually obtained after purification of cloned fusion proteins. Although antibodies against cloned gene products have been successfully raised in animals and selected from libraries [13,14,52], some proteins are poorly expressed in bacteria and may precipitate, causing problems in subsequent selection. This is likely to be particularly true for proteins such as integral membrane proteins, or cytoplasmic proteins, such as p21Ras, residing in specific cellular environments. These problems are likely to be especially difficult to overcome when faced with the need to select antibodies destined for intracellular immunisation.

The purpose of this work was to derive antibodies against p21Ras, to be used for intracellular expression to interfere with its function. As p21Ras is a highly conserved self-protein, the attempt to isolate antibodies against it was done by selection from a large naive and diverse phage antibody library [13], using the peptide 57–76, in different forms, as selector. It has been postulated that peptides in solution can assume a wide number of conformations, of which one predominates [1]. By molecular dynamics and mechanics it was shown that the 57–76 free peptide was expected to mimic the α-helix it adopts within the protein (discussed in [45]). For library selections, two approaches to selection were taken: the free peptide was conjugated to BSA and coated on immunotubes, and the peptide was displayed on bacterial cell surfaces within the context of the outer membrane protein LamB.

Antibodies were selected against the Ras peptide on both formats (Table 1A) and in all selections anti-Ras peptide binders were identified (Table 1B). Many more different antibodies (as manifested by different PCR fingerprints) were obtained when using BSA-Ras(57–76) as selector rather than LamB-Ras(57–76) bacteria (Table 1B).

The binding affinities of the selected scFvs measured on the BIAcore ranged from 5.4 to 803 nM (Table 2). Unfortunately, affinities on p21Ras could not be measured as the epitope is masked upon binding to the chip (data not shown).

Anti-peptide antibodies are often able to recognise the denatured parental protein very well [1] and this is true for the majority of the isolated anti-peptide antibodies which were able to reveal p21Ras in Western blots in which whole bacterial extracts containing p21Ras were loaded (Fig. 1). The clear signal obtained in Western blots indicates the high specificity of these antibodies for their antigen, as no other bands are visualised.

The majority of the scFvs selected were also able to recognise native p21Ras in an ELISA in which native p21Ras was coated on the plastic surface, with some of the scFvs (C12, D5, E7 and E11) giving signals far higher than the positive control scFv Y13-259 (Fig. 2). There is no correlation between the affinities for the BSA-Ras peptide as determined by BIAcore and the ELISA signals obtained on native p21Ras. This is likely to be due to a number of different factors, including susceptibility of the detection tag to proteolysis, the use of different antigens and different immobilisation protocols.

These scFvs were selected for use in intracellular studies. Preliminary data have shown that some of these clones (S. Biocca et al., manuscript in preparation), as well as Y13-259 [31], are able to inhibit DNA synthesis in 3T3 NIH transformed cells when expressed within the cytoplasm, analogous to similar results obtained with microinjection of Y13-259 scFv [25]. Should intracellular antibodies find a role in cancer therapy, the fact that they are of human origin is likely to be of great advantage.

We have shown that it is possible to use peptides to select antibodies from phage antibody libraries which recognise the native protein. This is a result analogous to those which have been previously obtained with peptide immunisation in experimental animals. However, the use of phage antibodies avoids the involvement of cell-mediated immunity. This is likely to play a role in the composition and sequence of peptides which are able to select antibodies recognising the native protein. An analysis of the factors which determine the 'immunogenicity' of a peptide within the context of phage antibody selection is likely to be very important in the selection of multiple antibodies against the protein products of genes identified in the human genome project. In most cases, the protein is not available in sufficient quantity for conventional chemical studies or its presence in the cell may even be in doubt. By synthesising peptide fragments of the putative protein and selecting antibodies against it, it may be possible to isolate and characterise the native protein using appropriate immunoassays. Selection using recombinant bacterial columns may also be useful in

those cases when synthetic peptides cannot be easily synthesised.

Restricted phage-antibody libraries derived by selection against peptides may be screened against native antigens in situ by intracellular expression, using the yeast two-hybrid system or by conferring a phenotype after intracellular expression. The latter case is elegantly described in [53], where the expression of an antibody fragment against the Moloney mouse leukaemia virus reverse transcriptase leads to selective survival of the virus infected cells.

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