Selection of phage-displayed Fab antibodies on the active conformation of Ras yields a high affinity conformation-specific antibody preventing the binding of c-Raf kinase to Ras

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Abstract The Ras proteins cycle in the cell between an inactive state and an active state. In the active state, Ras signals via the switch I region to effectors like c-Raf kinase, leading to cell growth. Since Ras mutations in cancer are often associated with the presence of permanently active Ras, molecules that prevent downstream signaling may be of interest. Here, we show that by selection on the active conformation of Ras, using a recently described large phage antibody repertoire [de Haard et al. (1999) J. Biol. Chem. 274, 18218–18230], a Fab antibody (Fab H2) was identified that exclusively binds to active Ras, and not to inactive Ras. Using surface plasmon resonance (SPR) analysis, the interaction was demonstrated to be of high affinity (7.2 nM). In addition, the interaction with Ras is specific, since binding to the homologous Rap1A protein in BIAcore analysis is at least three orders of magnitude lower, and undetectable in an enzyme-linked immunosorbent assay. The antibody fragment prevents the binding of active Ras to the immobilized Ras-binding domain of c-Raf kinase (Raf-RBD) at an IC_{50} value of 135 nM. This value compares well to the K_D of active Ras-binding to immobilized Raf-RBD using SPR, suggesting identical binding sites. Like the IgG Y13-259, which does not demonstrate preferential binding to either inactive or active Ras, Fab H2 inhibits intrinsic GTPase activity of Ras in vitro. Mapping studies using SPR analysis demonstrate that the binding sites for the antibodies are non-identical. This antibody could be used for dissecting functional differences between Ras effectors. Due to its specificity for active Ras, Fab H2 may well be more selective than previously used anti-Ras antibodies, and thus could be used for gene therapy of cancer with intracellular antibodies.

Key words: Anti-GTP-Ras antibody; Antibody selection; Phage display; c-Raf kinase; Surface plasmon resonance analysis; Ras conformation; GTPase activity

1. Introduction

The Ras proteins are guanine nucleotide binding molecules, essential for normal cellular proliferation and differentiation [1,2]. These proteins belong to a superfamily of proteins termed GTPases that cycle between on and off states triggered by binding and hydrolysis of the guanosine triphosphate (GTP) nucleotide [3,4]. Because of the ability to bind to different guanine nucleotides, leading to structural changes in the molecule, Ras serves as a molecular switch in signal transduction pathways [5,6]. The importance of Ras in normal cell physiology is underscored by the fact that in many types of human cancer the protein is mutated. In particular, oncogenic mutations are found in both glutamine 61 and glycine 12 [7]. Such mutations affect the intrinsic GTPase activity of Ras, rendering the molecule to be trapped in the active form [8]. The oncogenic Ras species resemble the active form of Ras on a biochemical as well as on a structural level [9,10], however, structural differences account for the loss of inactivation in mutated Ras by the GTPase activating protein $p120^{GAP}$ [11].

Crystal structures of the catalytic sites of both the active and inactive forms of Ras in complex with nucleotides have been determined as well as the structures of nucleotide bound Ras [10,12–16]. These studies have demonstrated that the switch I (residues 30–37) and switch II (residues 60–76) regions of Ras are conformationally affected by exchange of the guanine nucleotides. Particularly switch I appears to be involved in the functional activity of Ras, since this domain overlaps the region generally referred to as the effector loop (residues 32–40) [17,18]. The effectors of Ras, amongst others c-Raf kinase, function as downstream signal transducing molecules, which only bind to the active form of Ras.

Extensive studies on the interaction of Raf-1 with Ras have demonstrated that on Raf-1 the binding residues are located within residues 55–131, generally referred to as Raf-RBD (Ras binding domain) [19]). The affinity of Raf-RBD for GTP-Ras has been determined to be 18 nM, whereas the affinity for guanosine diphosphate- (GDP)-Ras was shown to be in the micromolar range [20]. Determination of the crystal structure of the complex of the Ras-related protein Rap1A in the active form and Raf-RBD and by inference that of the complex of Ras with Raf-RBD has provided detailed insight into the switch I residues involved in the interaction [21]. Since it has been suggested that oncogenic Ras is insensitive to GAP activity, targeting the binding site of the effectors may be an option to obtain reagents to treat Ras induced tumors [22]. The effector binding site has been suggested to be highly antigenic, based on peptide studies, and therefore may be a suitable target to select antibodies against Ras [23].

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; Fab, antigen binding fragment; GAP, GTPase activating protein; GAP-334, catalytic fragment of $p120^{GAP}$; GDP, guanosine diphosphate; GST, glutathione *S*-transferase; GTP, guanosine triphosphate; GTPγS, guanosine thiotriphosphate; Raf-RBD, Ras-binding domain of c-Raf kinase; scFv, single chain Fv fragment; SPR, surface plasmon resonance analysis; TBS, Tris-buffered saline

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The neutralizing monoclonal antibody Y13-259 has been shown to inhibit the GTPase activity of Ras, binding to residues constituting the switch II region [24,25]. In vitro, the antibody either sterically hinders the interactions of effector molecules binding to switch I, or prevents the exchange of GDP for GTP by compromising the conformational flexibility of Ras [26]. In vivo, the antibody has been demonstrated to inhibit Ras-mediated pathways using microinjection assays [27]. The variable domains of heavy and light chains of Y13-259 have been expressed as single chain Fv fragment (scFv) inside eukaryotic cells [28,29] (a technology generally referred to as intracellular immunization or intrabody expression, [30,31]). Upon intracellular expression, the scFv Y13-259 inhibits transforming activity in a number of studies [32,33], and even specifically induces apoptosis in human cancer cells but not in untransformed cells [34]. Nevertheless, the Y13-259 monoclonal antibody has been shown to be largely insoluble when expressed at 37°C cells, leading to intracellular aggregation [35].

Phage display technology has been demonstrated to allow for rapid selection of human antibodies from large repertoires to any protein of interest (for a recent review, see [36]). Persic et al. used this approach to obtain human antibody scFv fragments to the switch II region of Ras using a peptide representing this domain for selection [37]. Anti-Ras antibodies were isolated, many of which were shown to inhibit cellular functions in mammalian cells (unpublished). In this study, we attempted to derive antibodies towards epitopes which are conformationally changed upon exchange of nucleotides, in particular the effector loop, using the recently constructed large non-immunized phage-displayed antigen binding fragment (Fab) antibody repertoire [38]. We report here the isolation of a panel of human antibodies to Ras, some of which could be used either for dissecting the role of Ras-effector interactions in cell physiology or eventually for cancer gene therapy, using intracellularly expressed antibodies.

2. Materials and methods

2.1. Proteins and chemicals

Glutathione *S*-transferase fused H-Ras (GST-Ras), the catalytic fragment of $p120^{Gap}$ (GAP334) and the Raf-RBD were purified as described [11,20,39,40]. The monoclonal IgG antibodies Y13-259 and F111-85 were purchased from Oncogene Sciences and polyclonal goat anti-GST as well as the pGEX4T2 vector were from Amersham Pharmacia Biotech. GST, encoded by the latter vector, was purified after expression in *E. coli* strain TG1 and subsequent purification using glutathione beads, according to the instructions of the supplier. Guanine thiotriphosphate (GTPγS) was purchased from Boehringer Mannheim. All other reagents and chemicals used were reagent grade (Sigma or Merck).

2.2. Selections on conformationally restricted Ras

Immunotubes (NUNC) were coated overnight at 4°C with 100 μg anti-GST. After washing three times with 20 mM Tris containing 150 mM NaCl, pH 7.4 (TBS), tubes were blocked with TBS containing 2% w/v skimmed milk powder for 1 h at 37°C. 1 μM GST-Ras was first bound to GDP or GTPγS by incubation in TBS containing 1 mM EDTA and the relevant nucleotide for 20 min at 37°, followed by addition of 5 mM $MgCl_2$. Subsequently, GST-Ras was captured by coated anti-GST for 30 min at 37°C. Unbound GST-Ras was removed by performing three washing steps with TBS. Prior to selection on GST-Ras, 2×10^{12} phage from the non-immunized Fab repertoire [38], was depleted on coated goat serum for 30 min at 37°C. Unbound phage was suspended into TBS containing 2% marvel, and allowed to bind for 90 min at room temperature to the captured GST-Ras. Subsequently, tubes were washed extensively using TBS with and without 0.1% Tween 20 and bound phage was eluted using glycine-adjusted 50 mM hydrochloric acid, pH 2.0. Phages were rescued and amplified as described [41], followed by another two rounds of selections. Phage repertoires were alternately depleted either by preincubation on coated goat serum or by adding 25% goat serum to the incubation mixture during the selection. During the third round of selection, the library was also depleted on anti-GST captured GST, prior to selection on GST-Ras.

2.3. Screening of the selected anti-GDP/GTPγS-Ras repertoires

To identify GDP or GTPγS-Ras binding clones, an enzyme-linked immunosorbent assay (ELISA) was performed in which GST-Ras, after treatment with the relevant nucleotides, was captured by polyclonal anti-GST. Coated wells were blocked using TBS containing 3% w/v bovine serum albumin and Fab-phage expressed by single colonies were allowed to bind for 1 h at room temperature. Subsequent ELISA washing and staining procedures were essentially as described [42]. The number of unique Fabs was determined by PCR fingerprinting using *Bst*NI as described [41] followed by DNA sequencing.

2.4. Purification of Fab fragments and surface plasmon resonance affinity measurements

Selected anti-Ras Fab fragments were expressed upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG), harvested from the periplasmic space of *E. coli* TG1 cells and purified by immobilized metal affinity chromatography. Briefly, IPTG induced 500 ml cultures (4 h at 30°C), expressing relevant anti-Ras antibodies, were spun at $4600\times g$ for 20 min at 4°C. Bacteria were subsequently resuspended in phosphate buffered saline containing protease inhibitors (phenylmethyl-sulfonyl fluoride and benzamidine) and sonicated using an ultrasonic disintegrator (MSE Scientific Instruments). Suspensions were then centrifuged at $50\,000\times g$ for 30 min at 4°C, and supernatant fractions were incubated with TALON resin and eluted from the beads according to the instructions of the manufacturer (Clontech). Fab fragments were further purified by gel filtration using a Superdex 75 column (Amersham Pharmacia Biotech) connected to a Biologic instrument (Bio-Rad). Fab concentrations were quantitated using the bicinchoninic acid kit (Pierce).

To determine equilibrium dissociation constants, anti-GST antibodies were immobilized onto a CM5 sensorchip in a BIAcore2000 instrument (Biacore AB) by amine coupling at a high density of 120–140 fmol/mm^2. Using TBS containing 2 mM $MgCl_2$ and 0.01% v/v Tween 20 as a running buffer, the anti-GST was either loaded with GST or GST-Ras bound to GDP, GTP or GTPγS at a flow rate of 5 μl/min at 25°C, resulting in approximately 15 fmol captured protein, allowing for proper kinetic determinations. After increasing the flow rate to 20 μl/min, Fab antibodies were passed over the sensorchip at multiple concentrations around apparent K_D values. Binding to either GST or anti-GST was subtracted from specific binding responses to Ras. The rate constants k_{on} and k_{off} were obtained by direct fitting and from secondary plots (k_s versus concentration), respectively, and fitted to the data according to a single-site model, using the BIAevaluation 2.1 software (Biacore AB). K_D values that were calculated from k_{on} and k_{off} rate constants, fulfilled the criteria for self-consistency [43].

2.5. Competition studies using surface plasmon resonance analysis

Raf-RBD was immobilized onto a CM5 sensorchip (Biacore AB) up to approximately 4500 resonance units (RU; corresponding to 4.5 ng of protein). Prior to the analyses, Ras was loaded with GDP, GTP or GTPγS as described above. Using TBS containing 0.01% v/v Tween 20 and 2 mM $MgCl_2$ as running buffer using flow conditions of 10 μl/min, 100 nM of GTPγS loaded Ras, either untreated or preincubated with 1 μM concentrations of relevant antibodies, was passed over multiple Raf-RBD immobilized channels and residual binding was measured after 2 min association time. Fab antibodies were incubated over a range of concentrations. In case of no inhibition of Ras binding, results are indicated in the figure as 100% residual binding at the highest tested concentration.

2.6. GTPase assays

Hydrolytic activity of Ras either in the presence or absence of competitors was determined by performing GTPase assays according to the charcoal method of Bollag and McCormick [44]. In these experiments, Ras was incubated either in the presence or absence of the

anti-Ras antibodies Fab H2, Fab A8 or IgG Y13-259 and GAP334 at concentrations of 1 μM. Experiments were performed in duplicate.

2.7. Mapping studies

Mapping studies using surface plasmon resonance analysis (SPR) were performed by loading the anti-GST immobilized chip with 8.3 fmol (400 RU) of Ras-GTP, followed by injections well over K_D of IgG Y13-259 immediately followed by saturating amounts of Fab H2. First, saturating amounts of either antibody was determined to be maximally 150 RU for IgG Y13-259 and 350 RU for Fab H2, respectively. Next, in the actual experiment, responses obtained were compared to these values.

3. Results

3.1. Selections of Fab antibodies on active and inactive GST-Ras

Phage antibodies against Ras from large phage single chain Fv antibody libraries have been selected previously by a number of methods, either selecting on peptides representing functional epitopes [37], or selecting on the full-length protein (unpublished results). However, to our knowledge antibodies have never been identified specific for the conformational state of Ras, neither by hybridoma nor phage antibody technology. Since it has been suggested in literature [23] that particularly residues in the switch I region of the protein may be highly antigenic and that these residues appear to be either exposed or cryptic upon conformational changes induced by guanine nucleotide exchange, we have set out to identify antibodies with exquisite specificity by selection on GDP or GTP bound Ras. GTP specific antibodies may later be used for intracellular immunization, in order to block Ras function in tumor cells. In addition, selection on conformational Ras and isolation may serve as a paradigm for the superfamily of GTP-binding proteins, the members of which are considerably homologous and, most importantly, contain switch regions determining the specificity for intracellular effector molecules [45].

We chose to select antibodies to GST-Ras from the 37 000 000 000 clones Fab antibody library, that has recently been constructed in our laboratory [38]. Ras was not immobilized to prevent immobilization-induced conformational changes but was captured by antibodies directed to the GST

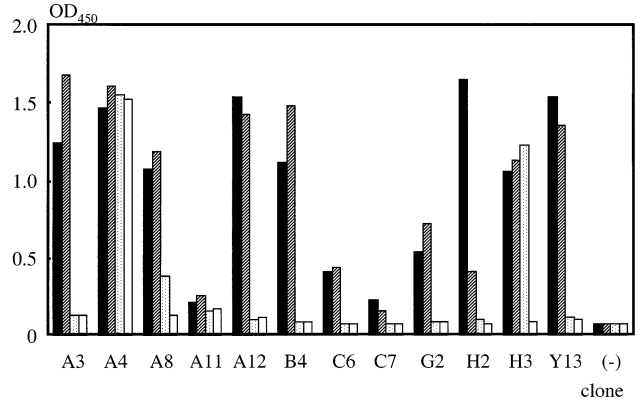

Fig. 1. Phage ELISA screening of Ras selected Fab phage. Binding to GTPγS-Ras is indicated by the black bars, binding to GDP-Ras by the hatched bars and binding to either captured GST or anti-GST by the dotted and open bars, respectively. A3, A8, A11, A12, B4 and G2 Fab phage were all selected on GDP-Ras, whereas A4, C6, C7, H2 and H3 were derived from GTPγS-Ras selections. Y13 refers to the cloned Y13-259 scFv form [28].

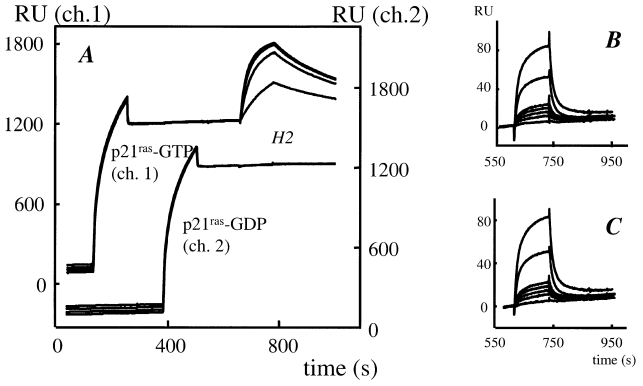

Fig. 2. Binding of Fab H2 and Fab A8 to captured GTP- or GDP-Ras using SPR. A: Dose-dependent binding of Fab H2 to captured Ras. The figure shows initial loading of anti-GST with GST-Ras, either bound to GTP (1) or GDP (2), and subsequent binding of the antibody. B and C: Dose-dependent binding of Fab A8 to captured Ras, either to GTP- or GDP-Ras, respectively.

moiety. By selection on GDP or GTPγS GST-Ras, while extensively depleting for GST binders and binders specific for anti-GST antibodies as described in Section 2, we obtained a panel of Fab fragments binding to Ras. During the four consecutive rounds of selection, we found an increase in the ratios of output phage over input, indicative of enrichment for antigen binders, mounting to three orders of magnitude for both selections on GDP and GTPγS GST-Ras. To demonstrate binding of individual Ras Fabs, we performed a phage ELISA, in which we captured GST-Ras either bound to GDP or GTP. From both pannings, after the third round of selection, we obtained anti-Ras binders as indicated by this ELISA (70–80% positives, data not shown). To be able to demonstrate specificity for a defined Ras conformation, we next performed an ELISA, in which we either captured GDP-Ras, GTP-Ras, GST or no protein at all, and bound phage antibodies were detected. The results are shown in Fig. 1. From both selections, Fabs were selected binding to either form of Ras. The Fabs A3, A8, A11, A12, B4 and G2 were all obtained from the GDP-Ras selections. The Fabs A4, C6, C7, H2 and H3 were derived from the GTP-Ras selections. The selections also yielded a low percentage ($<10\%$) non-specific binders and GST binders. As can be seen in Fig. 1, Fab H2 demonstrated specificity for the active conformation of Ras. During the selections on GTP-Ras without depleting on captured GST, anti-GTP-Ras binders (Fab H2) were dominantly enriched (48.9% of binding Fabs). By depletion on GST, this number increased to 75% (see also Table 1). The percentage of GST binders (e.g. Fab H3) was reduced concomitantly from 40 to 5.5%. Selections on GDP-Ras yielded Ras binders which do not discriminate between active and inactive Ras. Using *Bst*NI fingerprinting and DNA sequencing, we could identify nine different anti-Ras clones.

Table 1
GST depletion prior to pannings on GST-GTPγS-Ras increases the number of Ras-specific clones after three rounds of selection

Specificity	No GST depletion %	GST depletion %
Anti-GTP-Ras	48.9	75.0
Anti-Ras	2.2	5.6
Anti-GST	40.0	5.5
Negative	8.9	13.9

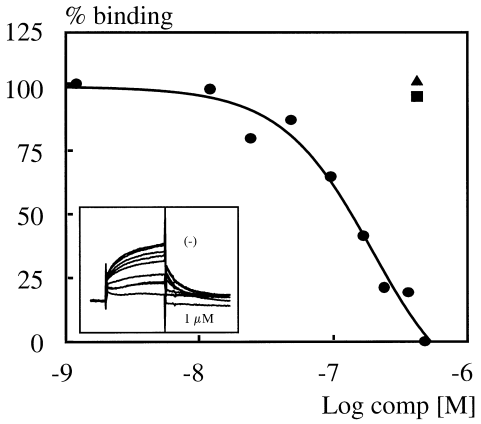

Fig. 3. BIAcore competition experiment. Measurements of competition of Ras binding to Raf-RBD by scFv anti-Ras 3 (▲), Fab A11 (■), or Fab H2 (●), using SPR. The binding curves, representing Ras binding in the absence of Fab H2 (upper curve), or increasing amounts up to 1 μM (lower curve), are shown in the inset figure.

3.2. Affinity determination of anti-Ras Fab antibodies

Next, we purified the Fab antibodies H2 (active Ras specific), A8 and A11 (both pan-Ras reactive) as three representative clones and determined the affinity of the clones for Ras using SPR. To compare binding to different Ras-forms, we developed a BIAcore assay as follows. After coupling a high amount of polyclonal anti-GST to a CM5 BIAcore sensorchip, we captured Ras either treated with GDP or GTPγS and subsequently measured the interaction of the antibodies with the captured Ras. Fig. 2 shows two sensorgrams run synchronously in different channels. In channel 1, a low amount of GTPγS-Ras and in channel 2 a low amount of GDP-Ras was captured. After capturing per channel individually at a flow rate of 5 μl/min, flow rates were increased to 20 μl/min to allow for kinetic determinations and antibodies were passed synchronically over both channels and a control background channel. As can be seen in Fig. 2A, Fab H2 only binds to the active form of Ras. The calculated K_d for the interaction is 7.2 nM (k_a, 3.6×10^5 M^{-1} s^{-1}, k_d, 2.6×10^{-3} s^{-1}). The specificity for Ras was demonstrated by the fact that Fab H2 does not interact with the related Rap1A protein (not shown). The K_d value of the interaction of Fab H2 with active Ras compares well to a calculated K_d value determined in an ELISA (6 nM, data not shown). Fab A8 bound to both forms of Ras (GTPγS-Ras, Fig. 2B and GDP-Ras, Fig. 2C), although with lower affinity (calculated K_d values 197 nM and 211 nM for GTPγS-Ras and GDP-Ras, respectively). The interaction of A11 with Ras was not quantitated accurately, however, the apparent affinity was in the same order of magnitude as that of Fab A8. The reverse experiment, in which the antibodies Fab H2 and A8 were immobilized to CM5 sensorchips and subsequent binding of either GDP, GTP or GTPγS-Ras was measured, yielded similar results (data not shown).

3.3. Competition of Fab antibodies for Ras binding to Raf-RBD

Recently, using SPR, we measured binding of GTPγS-Ras and GTP-Ras, but not GDP-Ras to immobilized Raf-RBD and determined possible interference of selected scFv's on this interaction (unpublished results). None of the tested antibodies, selected on directly coated Ras, were capable of inhibition. Only Y13-259, either in the IgG or scFv format, was capable of completely inhibiting the binding at micromolar concentrations. We used the same experimental setup to determine if our newly selected antibodies, directed to conformational epitopes, may interfere with the Ras-Raf-RBD interaction. As shown in Fig. 3, the Fab H2 antibody inhibits the interaction in a dose-dependent fashion, at an IC_{50} value of 135 nM. The obtained BIAcore curves are shown in the inset figure. A control antibody which demonstrates a good affinity for Ras (anti-Ras 3, K_D 52 nM, unpublished), as well as the Fab A11 did not inhibit the interaction up to micromolar concentrations. Because of the observed inhibition of GTPγS-Ras binding to Raf-RBD by Fab H2, we compared the DNA encoding VH and VL regions of Fab H2, with the primary sequence of Raf-RBD, but were unable to find similarities or significant homologies (data not shown).

3.4. GTPase assays

Since the IgG Y13-259 antibody can inhibit Ras mediated signal transduction in in vivo assays, preventing GTPase activity in in vitro systems and interacting with the conformationally flexible switch II region, we were curious to see if the H2 antibody could also exert GTPase inhibitory activity. Therefore, we performed standard GTPase assays as described by Bollag and McCormick [44]. As controls, we performed incubations with Y13-259, either in the IgG or scFv format, with irrelevant antibodies (anti-MHC Fab G8, Châmes et al., unpublished) and with GAP334. As expected, the incubations with irrelevant antibodies did not influence the rate of intrinsic GTPase activity, whereas the incubations with Y13-259 or GAP334 completely inhibited or strongly stimulated the activity, respectively (Fig. 4). Upon incubation with Fab H2, the GTPase activity of Ras was completely blocked. scFv anti-Ras antibodies directed to regions which are unaffected by conformational changes did neither inhibit nor stimulate GTPase activity (unpublished results).

3.5. Mapping study

Because of comparable features of antibody Y13-259 and Fab H2, we performed a mapping study using SPR. In this assay, we captured GTPγS-Ras with anti-GST and subse-

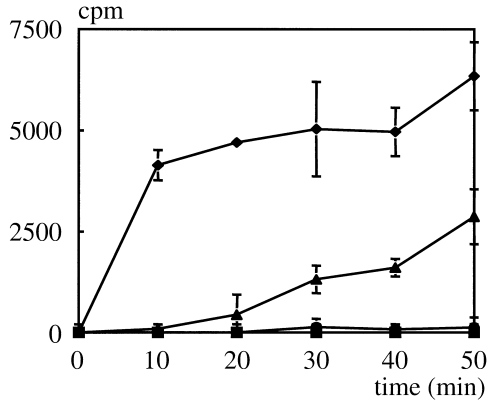

Fig. 4. GTPase assay. Triangles (▲) indicate the hydrolysis of Ras either in the presence or absence of irrelevant (G8) or anti-Ras Fab A11 antibodies. Influences on the GTPase activity by GAP-334 and by IgG Y13-259 are represented by the diamonds (◆) and by the circles (●), respectively. Incubations in the presence of Fab H2 are represented by the squares (■).

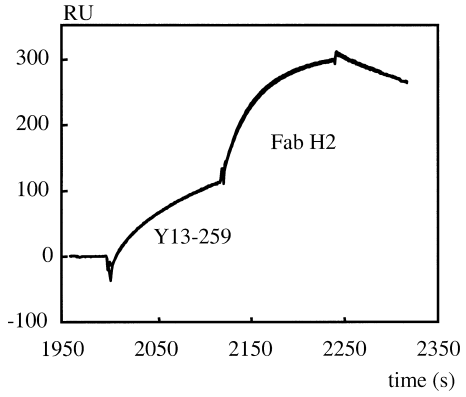

Fig. 5. Epitope mapping of Fab H2 binding to Ras using SPR. Captured GTPγS-Ras was first saturated with monoclonal antibody Y13-259 and subsequently incubated with Fab H2.

quently determined maximal binding responses of antibodies binding to Ras. Taking into account the molecular mass of the antibody, maximal binding of Y13-259 was lower than expected, possibly due to a low accessibility of the epitope in this experimental setup. After determining the maximal binding responses, we saturated the Ras with IgG Y13-259 and then injected a saturating amount of Fab H2. The results of this experiment are depicted in Fig. 5. As can be deduced from this figure, Y13-259 and Fab H2 can both bind to the captured Ras, indicative of independent binding sites. The opposite experiment, in which Ras was first saturated with Fab H2 and then with Y13-259, yielded similar results. Because of the apparent low accessibility of the Y13-259 epitope in the SPR assay, we performed a competitive ELISA in which we competed with Y13-259 for Fab H2 binding and reverse under conditions of half-maximal saturation. Since competition was observed only at high concentrations of Y13-259 for Fab H2 binding, whereas the reverse could not be shown, we conclude that the binding sites are non-identical.

4. Discussion

In the present study, we have used phage display technology to rapidly select antibodies to the active conformation of Ras. In this way, we attempted to increase the chance of isolating antibodies capable of inhibiting the interaction of downstream effectors like c-Raf kinase with Ras. We have used a large Fab antibody library, which in our lab has yielded a number of antibodies to diverse antigens binding to their targets with nanomolar affinities [38]. This library was selected on captured GST-Ras, bound to either GDP or GTPγS. This approach yielded a Fab antibody that interacts with Ras with high affinity and inhibits the intrinsic GTPase activity and the binding of Ras to Raf-RBD. Surprisingly, the selections on GDP-Ras did not yield any clones specific for the inactive conformation. In fact, several antibodies were obtained that interact with Ras irrespective of the conformational state. The reason for these findings may be the fact that the effector loop is highly flexible in the GDP-bound state, but rather fixed in the GTP-bound state, when it exists in only two conformations due to the fact that the γ-phosphate interacts with a tyrosine residue in the loop [46]. It also suggests that the effector binding domain, to which Fab H2 seems to bind, only contains selection dominant epitopes in the active conformation. The latter suggestion is strengthened by the fact that in addition to a panel of Ras binders, the GTP-Ras specific H2 clone was dominantly selected (see also Table 1) and the affinities of antibodies to Ras indifferent of the conformation, were rather low (10^{-7} M). Studies undertaken by Wang and colleagues, who made Ras peptides and isolated antibodies against them, have indicated that particularly the effector binding region may be highly antigenic in vivo [23]. Selection-dominant epitopes may often be overlapping with immunogenic epitopes, as demonstrated in a study by Hoogenboom et al. [47]. The possible immunogenicity of the effector loop may as well explain the strong selection of the high affinity Fab H2 during the stringent selections.

Since members of the superfamily of GTP-binding proteins are all characterized by the presence of the switch regions, which are affected conformationally upon nucleotide binding, the method described could well serve as a general way to rapidly select antibodies against these conformationally flexible regions. Particularly, if the aim would be to discriminate between different GTP-binding proteins, mediating different cellular events by binding to different effectors, this method may be applicable, since the Fab H2 antibody demonstrates a much higher affinity for Ras than for Rap1A. The difference in affinity is at least three orders of magnitude in BIAcore, and binding of Fab H2 to Rap1A in ELISA could not be detected at all.

The Fab H2 antibody could be an interesting candidate for intracellular antibody expression studies. Work is currently performed to clone the antibody in a suitable eukaryotic expression vector [29] and express it in the smaller scFv format. Antibodies have been intracellularly expressed in a number of studies [30,31]. However, attempts to express antibodies that were obtained by classical hybridoma technology and subsequently cloned have not always been successful. The antibody may not fold correctly in certain compartments of eukaryotic cells and expression levels may be poor. This may be corrected by mutation and selection as was proposed by Martineau et al. for cytoplasmic antibody expression inside bacteria [48]. On the other hand, a new mechanism of action may be observed upon intracellular expression of an antibody in a different molecular format. This was proven to be the case for the Y13-259 antibody by Cardinale and co-workers when they expressed the scFv format of this antibody in a eukaryotic cell system [35]. The scFv was highly aggregating intracellularly, thereby trapping the intracellular Ras in an insoluble complex which can be subsequently degraded by the cell.

The question remains if a targeted approach is actually required for inactivation of intracellular targets. In a recent study, Lener and coworkers have shown that expression of non-inhibitory antibodies intracellularly may very well lead to aggregation resulting in inhibition of intracellular functions or pathways (unpublished). In this study, the measured low affinity did not appear to affect the efficiency of intracellular target inactivation. The Fab H2 antibody, however, demonstrates specificity for Ras and can inhibit a particular protein-protein interaction, thereby most likely allowing us to discriminate between inhibition of certain intracellular pathways. Because of its specificity, it may also be used to quantitate intracellular active Ras levels. Alternatively, molecules (either antibodies or alternative scaffolds) inhibiting particular intracellular interactions or signal transduction routes, could also

be selected using an in vivo approach. Screening could subsequently be performed based on for instance phenotypic changes, apoptosis, cell morphology or surface protein expression. The initial proof of concept of an in vivo approach has been reported by Gargano and Cattaneo in a study in which cells expressing anti-retroviral antibodies were rescued [49]. An in vivo selection may be done using a previously selected antibody repertoire, directed against a known antigen, as well as using a completely naive repertoire. In the latter case, the unknown target will be identified in a later instance, possibly yielding new insights in importance of molecules involved in signal transduction pathways or leading to the discovery of new signal transduction molecules. Such antibodies will per definition be antibodies that can be expressed intracellularly, avoiding the possible problems associated with expression of in vitro selected antibodies. Well expressed antibodies, for example selected for high level expression inside the cell using procedures as described by Martineau, may later be used as a scaffold to create new libraries [48]. We conclude that high affinity antibodies directed to the active conformation of Ras can rapidly be selected from large phage-displayed antibody libraries. As shown in this paper, binding of the antibodies to conformational epitopes is very likely to be associated with functional inhibition of effector binding, since interaction of such molecules is governed by the conformational state of Ras. We propose this method to be generally applicable for selection of antibodies to conformational regions in related GTP-binding proteins.

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