Inhibition of yeast adenylate cyclase by antibodies to *ras* p21

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Monoclonal antibody Y13-259 to *ras* p21 was shown to bind to the highly conserved residues in the region 63–73 and to neutralize *ras* action in the *Saccharomyces cerevisiae* adenylate cyclase system. Inhibition of adenylate cyclase activity in isolated membranes by antibody Y13-259 occurred after a lag period of 6 min. This lag corresponded to the time necessary for binding of antibody Y13-259 to the membranes in a *ras*-dependent manner. The mechanism of inhibition appeared to be steric in nature because antibody Y13-259 neutralized *ras* p21 bound to a stable GTP analogue. Monoclonal antibodies Y13-4 and Y13-128 also inhibited yeast adenylate cyclase activity, and the epitopes for both these antibodies were localized to *ras* region 65–75. However, the *ras* residues essential for binding of antibodies Y13-4 and Y13-128 to *ras* p21 (positions 65, 66, 68 and 75) were different from those essential for binding of antibody Y13-259 (positions 63, 65, 66, 67, 70 and 73). These results indicate that residues 63–75 constitute a major neutralizing epitope on *ras* p21.

INTRODUCTION

The mammalian *ras* oncogenes encode highly homologous proteins of M_r 21 000 (p21) that are membrane-bound and that bind and hydrolyse GTP [1]. These proteins are biochemically and biologically active when bound to GTP and inactive when bound to GDP [2–5]. The presence of *ras* in normal mammalian cells and in evolutionarily diverse organisms suggests that *ras* is fundamentally important for the maintenance of normal cell physiology [1,6]. Moreover, constitutively activated forms of the *ras* genes have been identified in up to 30 % of human cancers [7]. The original panel of monoclonal antibodies directed against p21 [8] has been critical in identifying the biochemical properties of the *ras* proteins. Recently it was observed that one of these antibodies, Y13-259, neutralizes *ras* biological function [9]. Furthermore, antibody Y13-259 inhibits the *ras*-dependent adenylate cyclase activity in isolated membranes derived from *Saccharomyces cerevisiae* cells [10].

The use of neutralizing antibodies is important for probing the biochemical function of p21 in mammalian cells. However, the mechanism by which antibody Y13-259 neutralizes *ras* activity is unclear. The epitope for antibody Y13-259 encompasses residues 63–73, which are highly conserved among all members of the *ras* gene family [10]. Mutations in the *ras* region 63–73 that abolish binding of antibody Y13-259 do not impair biological activity, suggesting that this region is not the point of *ras* interaction with a target protein [10,11]. In contrast, mutations in another highly conserved region of *ras*, residues 31–43, can abolish biological activity without altering the known biochemical properties of p21 [10,11]. This putative ‘effector’ region may be the site of protein–protein interaction or may directly influence a neighbouring region that interacts with a target protein.

In the present study we have analysed in more detail the mechanism by which yeast adenylate cyclase activity

is inhibited by antibody Y13-259. Furthermore, we report that monoclonal antibodies Y13-4 and Y13-128, which also inhibit yeast adenylate cyclase activity, bind to an epitope in the region 65–75.

MATERIALS AND METHODS

Yeast membranes

S. cerevisiae cells were grown in rich YEPD medium or a selective minimal medium [4] at 30 °C to $A_{600} = 0.5$ – 1.0 . For the expression of *ras* proteins, cells were transformed with plasmids derived from the AAH5 vector [4]. Membranes were prepared as described previously [4] after glucuronidase digestion in 50 mM-Mes/NaOH buffer, pH 6.0, containing 0.1 mM-EGTA, 0.1 mM-MgCl₂ and 1 mM-dithiothreitol. Adenylate cyclase assays [12] were performed at 22 or 30 °C in the presence of 10 mM-MgCl₂ with 50–100 µg of membrane protein. GTP[S] was purchased from Boehringer Mannheim. Strain genotypes are: 112.699 (α *leu2-3,112 ura3-1 can1-100 ade2-1 ras2-699*), MY1186 (α *leu2-3,112 can1-100 ade2-1 ras1-545 ras2-699* AAH5-[Arg¹²,Thr⁵⁹]Ha) and JC377-10B (α *can1 lys2-801 his4-539 ras1-545 ras2-530 SRA4-6*).

Monoclonal antibodies

Rat hybridoma lines Y13-259, Y13-238, YA6-172, Y13-4 and Y13-128 were grown at 37 °C in Dulbecco's modified Eagle's medium (GIBCO) containing 10 % (v/v) fetal bovine serum [8]. Antibody secreted into the medium was prepared from cultures with less than 20 % viable cells by precipitation with 50 %-saturated (NH₄)₂SO₄ (Bio-Rad Laboratories) followed by dialysis against 10 mM-sodium phosphate buffer, pH 7.2, containing 150 mM-NaCl. Antibodies prepared in this manner were 20 mg/ml and contained 50 % albumin as detected by SDS/polyacrylamide-gel electrophoresis and Coomassie Blue staining.

Abbreviations used: GTP[S], guanosine 5'-[γ-thio]triphosphate; Ha, Harvey *ras*; p21, the 21 000- M_r protein encoded by Ha *ras*.

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Immunoblot analysis

For the detection of antibodies bound to yeast membranes, 100–200 μg of antibody preparation was incubated with 100 μg of yeast membranes under the conditions of the adenylate cyclase assay. At appropriate times the mixture was diluted with 1 ml of ice-cold buffer (50 mM-Mes/NaOH buffer, pH 6.0, containing 150 mM-NaCl) and centrifuged at 8000 g for 60 s. The supernatant was aspirated and the pellet was washed three times by resuspension and centrifugation. A sample of the collected membranes (20 μg) was subjected to SDS/polyacrylamide-gel electrophoresis on 13.5% acrylamide gels and then transferred on to nitrocellulose (Schleicher and Schuell BA83) as described previously [4,5] at 4 °C. The rat anti-*ras* monoclonal antibodies were detected by incubation as described previously [4,5] with ^{125}I -labelled anti-(rat immunoglobulin) antibody (7 $\mu\text{Ci}/\mu\text{g}$; Amersham International) followed by autoradiography.

Immunoprecipitation reactions

Mutant *ras* proteins were created by oligonucleotide-directed mutagenesis and purified as described previously [10]. For binding reactions the *Escherichia coli*-expressed *ras* proteins were pre-equilibrated with radiolabelled GTP in 200 mM-sodium phosphate buffer, pH 6.8, containing 100 mM-NaCl, 0.5 mM-MgSO₄, 1 mM-dithiothreitol, 0.01% sodium cholate, 200 nM- $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (1500 Ci/mmol; ICN) and 20 μg of p21 for 15 min at 30 °C in a 200 μl reaction volume. Covalent ^{32}P -labelling of p21 was performed in an autophosphorylation reaction (in 50 μl of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mM-MgSO₄, 2 mM-dithiothreitol, 100 mM-NaCl, 0.1% Nonidet P-40, 10 μM -GTP, 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and 4 μg of p21) by incubation at 37 °C for 2½ h. A sample (5 μl) was incubated for 1 h at 4 °C in 25 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mM-MgCl₂ and 100 mM-NaCl with Protein A-Sepharose resin (Pharmacia) previously coupled with anti-(rat immunoglobulin G) antibody (Cappel) and anti-*ras* monoclonal antibody. The immunoprecipitate was washed three times in the same Tris buffer and then collected on nitrocellulose filters. Radiolabelled GTP bound to p21 in the immunoprecipitate was determined quantitatively by Cerenkov counting.

RESULTS AND DISCUSSION

When *ras* monoclonal antibody Y13-259 is added to membranes derived from *S. cerevisiae* cells, adenylate cyclase activity is inhibited by more than 85% [10]. All evidence suggests that this inhibition is due to neutralization of p21 action. First, the effect of antibody Y13-259 is blocked by *ras* peptide 60–76, which encompasses the epitope [10]. Secondly, antibody Y13-259 has poor neutralizing activity on membranes expressing *ras* mutants that do not effectively bind antibody Y13-259 [10]. Thirdly, antibody Y13-259 has diminished neutralizing activity on membranes derived from a mutant yeast strain containing the *SRA4-6* allele that suppresses *ras* function with a constitutive adenylate cyclase ([13]; J. B. Gibbs, J. D. Marsico-Ahern, E. M. Scolnick & I. S. Sigal, unpublished work). To examine the mechanism of this response in more detail, we tested the time-dependence of antibody-Y13-259-mediated inhibition of adenylate cyclase activity using yeast mem-

Fig. 1. Time course of adenylate cyclase activity

Membranes were prepared from yeast strain MY1186, which expresses only mammalian $[\text{Arg}^{12}, \text{Thr}^{59}]\text{Ha}$. Antibody Y13-259 to p21 was preincubated with the membranes for 15 min ($t = -15$) (■) or added to the membranes at the start of the assay at 22 °C ($t = 0$) (▲). Antibody was not present in the control (●). Samples were removed at the indicated times and quenched as described previously [12].

branes that expressed only viral Harvey ($[\text{Arg}^{12}, \text{Thr}^{59}]\text{Ha}$) *ras* p21 and that were devoid of yeast *RAS* proteins. As shown in Fig. 1, yeast adenylate cyclase activity was linear for at least 40 min (control). When the membranes were preincubated with antibody Y13-259 for 15 min before the assay ($t = -15$), inhibition of adenylate cyclase was observed at all time points. In contrast, a lag period of 6 min was observed when antibody was added at the start of the adenylate cyclase assay ($t = 0$) or at subsequent times during the assay (results not shown).

To test whether this lag was due to the time required for antibody binding to p21 in the membranes, the time course of antibody Y13-259 binding to yeast membranes expressing p21 was analysed by immunoblot with ^{125}I -labelled anti-(rat IgG) antibody. As shown in Fig. 2(a), antibody binding to the membranes was barely detectable until 6 min, after which the amount remained constant. An equal amount of antibody Y13-259 was associated at all time points in the membranes preincubated with the antibody for 15 min before initiation of the assay (Fig. 2b). Antibody did not bind to membranes that were genetically devoid of *ras*-encoded proteins (Fig. 2c). These results indicated that inhibition of yeast adenylate cyclase by antibody Y13-259 occurred only after a maximal amount of antibody had associated with the membranes. The inhibition presumably correlated with neutralizing the *ras* protein in the membrane, since antibody did not bind to membranes that lacked p21 (Fig. 2c) and antibody Y13-259 did not inhibit adenylate cyclase (results not shown) in the *ras*-minus JC377-10B strain that has a constitutive *SRA4-6* adenylate cyclase activity [13].

The binding of antibody Y13-259 to p21 impairs the dissociation of guanine nucleotides [14]. It has been postulated that this effect of antibody Y13-259 might interfere with nucleotide exchange mechanisms and therefore might neutralize *ras* action simply by preventing GTP binding. To test this idea, we pre-equilibrated $[\text{Asn}^{16}]\text{Ha}$ p21 in yeast membranes first with the hydrolysis-resistant GTP analogue GTP[S] followed by a second incubation with antibody Y13-259 and then

Fig. 2. Antibody binding to yeast membranes

Membranes were incubated at 22 °C with antibody Y13-259 for various times and then washed to remove free antibody as described in the Materials and methods section. The amount of antibody bound was analysed by autoradiography after SDS/polyacrylamide-gel electrophoresis and immunoblot with ¹²⁵I-labelled anti-(rat immunoglobulin) antibody. Membranes were prepared from strains that expressed only mammalian [Arg¹²,Thr⁵⁹]Ha (a and b) or that were genetically devoid of *ras* (c). (a) Antibody Y13-259 added at *t* = 0. (b) and (c) Antibody Y13-259 preincubated for 15 min at 4 °C before 22 °C incubation.

Table 1. Effect of nucleotides and antibody Y13-259 on yeast adenylate cyclase

Membranes were prepared from yeast strain 112.699 transformed with AAH5-[Asn¹⁶]Ha. This *ras* protein exchanges guanine nucleotide rapidly at 4 °C [4]. Membranes were at 4 °C for sequential 15 min incubations before assay at 30 °C. GDP and GTP[S] were at 50 μM. Data are the average of duplicate determinations that deviated by less than 10 %.

Incubation addition		Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)
First	Second	
GDP	–	0.45
GDP	Y13-259	0.30
GTP[S]	–	4.4
GTP[S]	Y13-259	0.53
GTP[S]	Rabbit Ig	3.4

performed the adenylate cyclase assay. As shown in Table 1, [Asn¹⁶]Ha stimulated adenylate cyclase activity when bound to GTP[S], and antibody Y13-259 readily inhibited this effect by 88 %. Adenylate cyclase activities assayed with GDP plus antibody Y13-259 or with GTP[S] plus non-immune antibody were 66 % and 70 % of the respective control values. It is unlikely that antibody Y13-259 altered the nucleotide bound to p21 because we have observed that this antibody forms stable immune complexes with p21–GTP [5]. These results suggest that the neutralizing effect of antibody Y13-259 on yeast membrane adenylate cyclase does not occur by promoting an inactive p21–GDP complex. Since antibody Y13-259 binding to membranes is stable to washing procedures as described in the Materials and methods section, antibody Y13-259 cannot be inhibiting *ras* action by extracting p21 from the membrane. The mechanism of inhibition is probably steric in nature. The binding of a 150 000-*M_r* immunoglobulin to the 21 000-*M_r* *ras* protein might block other critical regions on p21 or prevent a target protein from coming in close proximity to p21.

Antibodies Y13-4 and Y13-128 to p21 also inhibit

yeast adenylate cyclase activity by more than 75 % [10] and bind to yeast membranes in a *ras*-dependent manner (results not shown). Like antibody Y13-259, these antibodies recognize both mammalian and yeast *ras* proteins (J. B. Gibbs, J. D. Marsico-Ahern, E. M. Scolnick & I. S. Sigal, unpublished work). The regions corresponding to Ha residues 31–43 and 61–75 are highly conserved among the *ras* genes from these evolutionarily divergent organisms. In order to identify the epitope for the binding of antibodies Y13-4 and Y13-128 to p21, we tested the ability of these antibodies to bind *ras* mutants with substitutions in these conserved regions. We have previously observed that mutations at 35–36, 38 and 40 inhibit *ras* biology [10]. First, [Val¹²,Thr⁵⁹]Ha *ras* proteins containing mutations at positions 35–36, 38, 39, 40, 65, 66 or 73 were ³²P-labelled by autophosphorylation and then were analysed for reactivity with antibody Y13-4 or Y13-128 by immunoprecipitation. Both antibodies recognized proteins with mutations in the region 35–40 and at residue 73; however, poor or no binding was observed with proteins having substitutions at residues 65 and 66 (Figs. 3a and 3b). As controls, we observed that antibodies YA6-172 (Fig. 3c) and Y13-238 (results not shown) effectively immunoprecipitated all of these *ras* proteins.

The amino acid residues in the region 61–75 required for the binding of antibodies Y13-4 and Y13-128 were examined in more detail by immunoprecipitating *ras* mutants bound to [γ-³²P]GTP. Immunoprecipitations were performed at 4 °C to prevent significant GTP hydrolysis. For controls, we tested antibody Y13-238, which does not bind to the region 61–75, and antibody Y13-259, which binds to residues 63, 65, 66, 67, 70 and 73 as determined by immunoblot analysis [10]. As shown in Table 2, neither Y13-4 nor Y13-128 antibody effectively recognized *ras* proteins having single substitutions at residues 65, 66, 68 or 75. The residues found by this analysis to be critical for the binding of antibody Y13-259 were identical with those determined under denatured conditions [10]. With some mutants a greater amount of [γ-³²P]GTP was immunoprecipitated by antibody Y13-4, Y13-128 or Y13-259 relative to that observed with antibody Y13-238. The mechanism of this effect is not clear at present.

Antibody Y13-259 but not Y13-4 or Y13-128 recognizes denatured *ras* proteins in an immunoblot

Fig. 3. Recognition of *ras* mutants by different antibodies

Purified [Val¹²,Thr⁵⁹]Ha mutants were ³²P-labelled by autophosphorylation as described in the Materials and methods section and then incubated with immune complexes of the indicated monoclonal antibody. Immunoprecipitations were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Numbers above each lane indicate the residue that was mutated. Lanes: C, control; 35–36, Thr-Ile to Ala-Leu; 38, Asp to Ala; 39, Ser to Ala; 40, Tyr to Lys; 65, Ser to Arg; 66, Ala to Thr; 73, Arg to Gln.

Table 2. Immunoprecipitation of *ras* mutants

The various purified *ras* proteins [10] were equilibrated with [γ -³²P]GTP and then incubated at 4 °C with the indicated antibody as described in the Materials and methods section. Results are expressed relative to the amount of ³²P-labelled nucleotide immunoprecipitated by antibody Y13-238 (10.2–31.5 fmol, 4440–13 700 c.p.m.). The subtracted blank values, determined by immunoprecipitation without *ras* antibody, were less than 670 c.p.m.

p21	Relative antibody immunoprecipitation			
	Y13-238	Y13-4	Y13-128	Y13-259
Wild-type	1.0	1.2	1.0	1.0
Gln ⁶¹ to Leu	1.0	1.1	0.82	1.7
Glu ⁶² to Gln	1.0	1.1	0.79	1.9
Glu ⁶³ to Gln	1.0	1.0	0.92	0.41
Ser ⁶⁵ to Arg	1.0	0.06	0.09	0.01
Ala ⁶⁶ to Thr	1.0	0.1	0.01	0.01
Met ⁶⁷ to Ile	1.0	0.7	0.94	0.01
Arg ⁶⁸ to Leu	1.0	0.25	0.09	0.96
Gln ⁷⁰ to Ser	1.0	0.94	0.62	0.23
Tyr ⁷¹ to Phe	1.0	2.1	1.2	1.5
Met ⁷² to Leu	1.0	1.9	1.2	4.2
Arg ⁷³ to Gln	1.0	1.2	1.6	0.01
Gly ⁷⁵ to Ser	1.0	0.03	0.01	0.94

analysis (J. B. Gibbs, J. D. Marsico-Ahern, E. M. Scolnick & I. S. Sigal, unpublished work). Although we have not rigorously determined the binding affinities of these three antibodies, we have observed efficient immunoprecipitation of *ras* proteins under the same detergent conditions used for immunoblot analysis. Therefore the inability of antibodies Y13-4 and Y13-128 to recognize denatured *ras* proteins may be due to the residues essential for binding. This observation suggests that the binding of antibodies Y13-4 and Y13-128 is dependent on the conformation of p21. Since the binding of antibodies Y13-4 and Y13-128 to *ras* proteins appears to be conformation-dependent, it is not clear whether the mutations that inhibit binding are the exact points of protein-protein interaction or whether the mutations affect the structure of this conserved domain. If secondary or tertiary structural parameters are critical to the

binding of antibodies Y13-4 and Y13-128, then the ability of these antibodies to immunoprecipitate *ras* proteins having substitutions in the putative effector region (35–40) indicates that the mutations that inhibit *ras* biological activity do not have major overall structural effects on *ras* conformation as assayed by immunorecognition of the region 65–75 with monoclonal antibodies Y13-4 and Y13-128. Although we have not necessarily identified the complete epitope for antibodies Y13-4 and Y13-128, antibodies Y13-4 and Y13-128 bind to an epitope that is shared with but not identical with the one for antibody Y13-259. These results indicate that residues 63–75 constitute a major neutralizing epitope on p21 as defined in the yeast adenylate cyclase assay. The identification of several antibodies binding to this region provides further immunological probes for *ras* action in mammalian cells.

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