RESEARCH COMMUNICATION

The Ras-related protein R-ras interacts directly with Raf-1 in a GTP-dependent manner

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R-ras is a member of the *ras* family of small GTPases that associates with the apoptosis-suppressing proto-oncogene product Bcl-2. Using the yeast two-hybrid system we provide evidence for an interaction between R-ras and the Raf-1 kinase. This interaction requires only the N-terminal regulatory domain (amino acids 1–256) of Raf-1, and is observed with both the wild type and a constitutively active R-ras mutant, but not with a deletion mutant that lacks the potential effector domain or a

mutant of R-ras impaired for GTP binding. Moreover, using an *in vitro* binding assay we show a direct GTP-dependent interaction of purified R-ras with a purified Raf-1 fragment corresponding to the proposed 81-amino-acid H-Ras-binding domain of Raf-1 (amino acids 51–131). Taken together, these data indicate that R-ras may exert its biological effect by means of modulating the activity of the Raf-1 kinase as its direct downstream effector.

INTRODUCTION

Members of the *ras* superfamily of small GTPase proteins are involved in a wide variety of cellular processes, including the regulation of cell proliferation, differentiation and possibly apoptosis [1–3]. Ras and Ras-like proteins cycle between a biologically inactive GDP-bound state and an active GTP-bound state. The rate of cycling between these states is regulated by GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity resulting in the inactive GDP-bound state, or by guanine nucleotide dissociation stimulators (GDSs), also known as exchange factors, which can induce the release of GDP thus enabling the binding of GTP [4].

The *ras* oncogene is one of the most frequently occurring oncogenes in human cancer [5]. Activation of Ras is implicated in many growth factor- and cytokine-stimulated signal-transduction pathways involved in the regulation of cell proliferation [4,6]. Receptor tyrosine kinase-induced activation of membrane-bound Ras appears to involve the recruitment of the exchange factor Sos1 to phosphorylated receptors by means of the adaptor protein Grb2 [6]. Many studies have shown the requirement of Ras activation in growth-factor-induced activation of Raf-1, MEK and mitogen-activated protein kinase (MAP-kinase) [7–9], as well as the involvement of Raf-1 in downstream signalling by Ras [10–12]. The activation of Raf-1 appears to be the first step in a kinase cascade in which Raf-1 phosphorylates and activates MEK, which then phosphorylates and activates MAP-kinase [13–16]. Recently, several studies have shown the direct interaction between H-Ras and Raf-1 [17–21], which is probably necessary for the activation of Raf-1 by an as yet unknown factor.

Based on homology, the *ras* superfamily can be subdivided, thus giving rise to the *rho*, *rab*, and *ras* families. The last includes, among others, the genes for H-*ras*, K-*ras*, N-*ras*, *rap*, *ral* and also the gene for R-*ras* (related-*ras*). R-*ras* encodes a 23 kDa protein

that has approx. 55% amino acid identity to H-*ras*, and has a 26-amino-acid N-terminal extension compared with H-*ras* [21]. Furthermore, it contains a domain (amino acids 58–66) identical with the effector domain of H-Ras (amino acids 32–40). R-*ras* appears to be non-transforming [22,23], and is sensitive to p120-GAP [24,25] and a 100 kDa GDS [26], but not to the exchange factor Sos [27]. In a recent study we have established that the hypervariable C-terminus of R-*ras* associates with Bcl-2 [3]. The *bcl-2* proto-oncogene is able to suppress naturally occurring or induced apoptosis [28–30]. Apoptosis (programmed cell death) is an important mechanism for cell regulation, being involved in tissue development, homeostasis and oncogenesis. These processes are all determined by the subtle balance between cell proliferation, differentiation and apoptosis. Several oncogenes such as *bcl-2*, *c-myc*, *E1A*, and *E1B*, as well as the tumour suppressor gene *p53*, have been implicated in apoptosis; however, many of the cellular and biochemical aspects underlying the mechanism of apoptosis are still unknown [28–30]. The interaction between R-*ras* and Bcl-2 strongly suggests a role for R-*ras* in the regulation of apoptosis [3]. This intriguing potential function of R-*ras* demands the identification of regulatory proteins (GDSs and GAPs) and effector proteins of R-*ras*. These proteins may very well be important components of a signal-transduction pathway involved in the regulation of apoptosis.

We have used the *in vivo* yeast two-hybrid system and an *in vitro* binding assay using purified proteins to investigate whether R-*ras* can interact with the Ras-effector Raf-1.

EXPERIMENTAL

Plasmid construction

All constructs were generated according to standard molecular biology procedures and sequenced to determine the proper reading frames of fusion proteins and to confirm mutations.

The *Xba*I-*Eco*RI fragment of plasmid pTRGA [22], which

Abbreviations used: ABTS, 2,2-azino-di-(3-ethylbenz-thiazolinesulphonate); GAP, GTPase-activating protein; GDS, guanine nucleotide dissociation stimulator; GST, glutathione S-transferase; GTP γ S, guanosine 5'-[γ -thio]triphosphate; HRP, horseradish peroxidase; MAP-kinase, mitogen-activated protein kinase; MEK, MAP-kinase or extracellular signal-regulated kinase kinase; ONPG, *O*-nitrophenyl- β -D-galactoside; PBST, PBS + 0.1% Tween-20; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside.

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contains a full-length human R-ras coding sequence in which the first 21 codons have been modified, was subcloned into a convenient cloning vector. The resulting plasmid was *HindIII*–*SmaI*-digested to replace the sequence encoding the modified 21 codons for the wild-type human R-ras sequence by means of four oligonucleotides. Two double-stranded oligonucleotide fragments were cloned into the *HindIII*- and *SmaI*-digested plasmid by means of a three-point ligation. The *NcoI*–*SalI* fragment of the resulting plasmid was used for in-frame cloning into the *NcoI*- and *SalI*-digested pGBT8 (a derivative of pMA424; [31,32]), resulting in plasmid pGBT8-R-ras. The R-ras point mutations G³⁸V and S⁴³N, were generated by oligonucleotide-directed mutagenesis of the subcloned pTRGA-derived R-ras sequence *in vitro* according to the manufacturer's instructions (Amersham International, Amersham, Bucks., U.K.). The resulting plasmids were digested with *HindIII* and *SmaI* and synthetic oligonucleotides were used to generate the wild-type first 21 codons of R-ras, exactly as described for pGBT8-R-ras. Subsequently the *NcoI*–*XhoI* fragments of the resulting plasmids were cloned into pGBT8 *NcoI*–*SalI*-digested in order to generate pGBT8-V³⁸-R-ras and pGBT8-N⁴³-R-ras. The plasmid pGAD-R-ras was made by subcloning the *BamHI*–*SalI* fragment of pGBT8-R-ras into *BamHI*- and *SalI*-digested pGADGH (a derivative of pGAD1; [31,32]). The pGAD-R-rasΔN plasmid was obtained from a two-hybrid HeLa cDNA library screening and lacks amino acids 1–70 [3]. The plasmid pAcOG-R-ras for the baculovirus system was made by cloning the *EcoRI*–*SalI* fragment of pGBT8-R-ras into *EcoRI*–*NheI*-digested pAcOG-1.

The full-length human *c-raf-1* sequence was obtained by PCR cloning from a placental cDNA library, and subcloned to generate pGEM-Raf [16]. pGBT8-Raf was obtained by subcloning the *NcoI*–*SacI* fragment of pGEM-Raf into pGBT8 digested with *NcoI* and *SacI*. pGAD-Raf was made by subcloning the *SmaI*–*PstI* fragment of pGBT8-Raf into *SmaI*-digested pGADGH. To generate pGAD-RafΔC (amino acids 1–256), a RafΔC fragment was obtained from pGEM-Raf by PCR. The resulting PCR product was *BamHI*–*SalI* digested and cloned into *BamHI*- and *SalI*-digested pGADGH. The plasmid pGEX-Raf-RBD (amino acids 51–131) was obtained by PCR cloning using pGEM-Raf as template, and the PCR product was subcloned between the *BamHI* and *EcoRI* sites of pGEX 2T (Pharmacia).

Yeast two-hybrid system

All yeast cultures were grown in standard liquid or on solid media, either based on rich YPD medium [1 % (w/v) bacto-yeast extract/2 % (w/v) bacto-peptone/2 % (w/v) glucose], or minimal SC medium [0.67 % yeast nitrogen base without amino acids (DIFCO)/ 0.075 % complete supplement medium (lacking the indicated amino acids; BIO101)/2 % (w/v) glucose], both in the presence of 100 mg/l adenosine hemisulphate. For the yeast two-hybrid system the *Saccharomyces cerevisiae* strain YGH-1 (genotype: *MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 Can^r gal4-542 gal80-538 LYS2::gal1_{UAS}-gal1_{TATA}-HIS3 URA3::gal1-lacZ*) was co-transformed with the different pGBT8 and pGAD plasmid constructs [31]. These yeast expression vectors contain the 2μ origin of replication *trp1* (pGBT8) or *leu2* (pGAD) gene for selection in yeast, and have under control of the strong ADH1 promoter either the GAL4 DNA-binding domain (amino acids 1–147) (pGBT8) or the GAL4 activation domain (amino acids 768–881) (pGAD), which are followed by a multiple cloning site for the in-frame generation of fusion proteins and a nuclear localization signal [31–33].

Transformation was performed by the lithium acetate method essentially as described in [34]. Transformants were plated on solid SC medium lacking Trp and Leu for selection of double transformants and determination of transformation efficiency, and on SC plates lacking Trp, Leu and His to select for His⁺ colonies. Qualitative determination of β-galactosidase activity was performed on His⁺ yeast colonies, patches or streaks on SC-Trp/-Leu/-His plates as indicated, by a filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as substrate, essentially as described previously [31]. Quantitative analysis was performed on yeast liquid cultures grown in SC-Trp/-Leu/-His using *O*-nitrophenyl-β-D-galactoside (ONPG) as substrate, essentially as described in [35].

In vitro Raf-binding assay

The production and purification of Glu-Glu-tagged R-ras from recombinant baculovirus-infected Sf9 cells and bacterially expressed glutathione S-transferase (GST)-tagged Raf-RBD, was performed according to standard procedures. A 96-well plate was coated overnight with the GST–Raf-RBD fusion protein (2 μg/50 μl per well) in PBS + 1 mM dithiothreitol at 4 °C, washed with PBS + 0.1 % Tween-20 (PBST), blocked with 100 μl PBS + 1 % (w/v) BSA/well at 4 °C for 2 h, and finally washed again with PBST. The loading of R-ras with either guanosine 5'-[γ-thio]triphosphate (GTPγS) or GDP was done by the incubation of R-ras in 50 mM Tris (pH 8) containing 50 mM NaCl, 5 mM EDTA, 0.1 % BSA and 1 mM nucleotide, in the absence of MgCl₂ for 30 min at 30 °C, followed by the addition of 5 mM MgCl₂ at 4 °C. Subsequently, R-ras was serially diluted in 50 mM Tris/HCl (pH 8) containing 50 mM NaCl, 5 mM MgCl₂, 1 % Nonidet P-40 and 0.1 % BSA, and 50 μl was added to the wells and incubated for 2 h at room temperature. The wells were washed three times with PBST, and then 50 μl of a 1:1000 dilution of anti-(Glu-Glu) antibody solution [36], linked to horseradish peroxidase (HRP), in PBST + 0.1 % BSA was added and incubated for 1 h at room temperature. After three washes with PBST the HRP substrate 2,2-azino-di-(3-ethylbenzthiazolinesulphonate) (ABTS; Boehringer–Mannheim) was added to the wells and incubated at 37 °C for 10 min. ABTS colour development was assessed by reading of the plate on a microplate reader (Molecular Devices) at 405 nm.

RESULTS AND DISCUSSION

Previous studies have demonstrated the usefulness of the yeast two-hybrid system for the detection, *in vivo*, of protein–protein interactions in general [31–33,37], and for the detection of the interaction of H-ras and Raf-1 in particular [17,18,20]. Therefore, we used this technique to investigate whether there is an interaction between R-ras and Raf-1. The two-hybrid system we used is based on the observation that if the activation domain and DNA-binding domain of the GAL4 transcription factor are separated and fused with two other proteins, the activity of the GAL4 transcription factor will be restored if these two proteins are able to interact [31–33,37]. The *S. cerevisiae* strain lacks endogenous GAL4 activity and has two genome-integrated reporter genes, i.e. the *HIS3* and *lacZ* genes, both under control of the GAL4-binding upstream activating sequence GAL1, with different flanking sequences. The interaction of tested proteins will allow growth of the yeast in the absence of histidine and will result in the production of β-galactosidase which is easily detected.

Using the two-hybrid system, full-length wild-type human R-ras and full-length human *c-Raf-1*, as well as different deletion

Figure 1 Schematic representation of the different R-ras and Raf proteins used as fusion proteins in this study

Shown are the schematic representations of the human wild-type R-ras protein compared with H-ras, and the mutant R-ras proteins V³⁸-R-ras, N⁴³-R-ras, and R-rasΔN (amino acids 71–218). In the different Ras proteins the 26-amino-acid N-terminal extension in R-ras is represented by the dotted box, the effector domain (H-ras: amino acids 32–40, R-ras: amino acids 58–66) by the black box, and the C-terminal highly variable domain by the differently hatched boxes. Furthermore, the human wild-type Raf-1 protein is shown as well as the deletion mutants RafΔC (amino acids 1–256), and Raf-RBD (amino acids 51–131). The black box represents the Cys-rich conserved region 1, the dotted box the Ser/Thr-rich conserved region 2, and the hatched box the kinase-containing conserved region 3.

and point mutants of the R-ras and Raf-1 proteins (Figure 1), were tested for their ability to interact. As shown in Figure 2, full-length wild-type R-ras and Raf-1, as fusion proteins expressed by pGBT8-R-ras and pGAD-Raf, interacted specifically as determined by the growth of yeast in the absence of histidine and by the blue colour development, indicating β -galactosidase activity. The N-terminal deletion mutant R-rasΔN (amino acids 71–218), which lacks its putative effector domain, is unable to bind Raf (Figure 2). This observation is consistent with previous studies, indicating that the effector domain of H-ras is involved in complex formation with Raf [17–20]. Furthermore, R-ras was also able to interact with RafΔC, which encodes the N-terminal region of Raf (amino acids 1–257). This interaction was much stronger than with full-length Raf (Figure 2). The quantitative β -galactosidase assay indicated that the apparent affinity of the interaction with full-length Raf-1 was approx. 70-fold lower than the interaction of R-ras with RafΔC (results not shown). The reversed fusion proteins (pGBT8-Raf and pGAD-R-ras) associated with a similar strength, indicating that the position of the tested proteins relative to GAL4 does not influence the strength

Figure 2 R-ras interacts with Raf-1 in the two-hybrid system

The two-hybrid system was used to determine the possible interaction between the indicated constructs. The proteins (see Figure 1) were either expressed as fusion proteins with the GAL4 DNA-binding domain (in pGBT8), or the GAL4 activation domain (in pGAD). C indicates 'empty' pGBT8 or pGAD without insert. (a) HIS3 expression. Yeast were co-transformed with the indicated pGBT8 and pGAD constructs and plated on SC-Trp/-Leu. After 4 days a colony was picked that was patched and re-patched on SC-Trp/-Leu/-His plates to test for transcriptional activation of the *HIS3* gene (each patch is representative for 2–10 independent transformations). (b) LacZ expression. On co-transformation yeast were directly plated on SC-Trp/-Leu/-His. After 6 days this was followed by a qualitative β -galactosidase assay. +++ indicates a strong interaction (specific His⁺ colonies observed within 3 days and blue-colour development within 1 h), ++ an intermediate interaction (His⁺ colonies after 4 days and colour development from 2–4 h), + a weak interaction (His⁺ colonies after 5 days and colour development after 5 h), and – means no interaction. Each score is representative for 2–10 independent transformations.

of the signal (Figure 2). It has been reported that the interaction of H-ras with Raf-1 also does not require the C-terminal catalytic domain of Raf [17–20], and that, at least in the two-hybrid system, the interaction with an N-terminal region of Raf (amino acids 48–178) is of a higher apparent affinity compared with the interaction with full-length Raf [18]. One explanation for this weaker interaction is that R-ras (and H-ras) competes for binding to the N-terminal regulatory domain of Raf with the C-terminal catalytic domain of full-length Raf. This is supported by the observation that a pGBT8-Raf and pGAD-RafΔC co-transformation resulted in a positive, albeit weak, signal (Figure 2). The N-terminal regulatory domain of Raf may function as a suppressor of the catalytic activity [38], which may be released upon binding of R-ras (and H-ras) to the N-terminal region, thus allowing the activation of Raf.

In order to further characterize and establish the biological relevance of the interaction between R-ras and Raf, two point mutants of R-ras were made: Gly³⁸ was replaced with Val or Ser⁴³ was replaced with Asn. These mutants are analogous to known mutants of H-ras, where the Val¹² mutation results in a constitutively active GTP-bound H-ras protein, and Asn¹⁷ in an inactive dominant negative GDP-bound H-ras protein. As shown in Figure 3, the active V³⁸-R-ras mutant strongly binds RafΔC

Figure 3 GTP-dependent interaction of R-ras with Raf-1 in the two-hybrid system

(a) and (b) are exactly as described in the legend of Figure 2

Figure 4 Direct GTP-dependent interaction of R-ras and Raf-1 *in vitro*

The amount of baculovirus-produced and purified Glu-Glu-tagged R-ras that binds to GST-tagged Raf-RBD is determined at A_{405} and plotted against the concentration of the serially diluted R-ras, which was either pre-loaded with GTP γ S (■) or GDP (□). Binding of GTP γ S-loaded R-ras to the GST alone was included as a control (○). The data shown represent three independent experiments.

and to a lesser extent full-length Raf, whereas the inactive N⁴³-R-ras mutant is impaired in its ability to interact with both proteins. The affinity of the interaction between Raf and the wild type or active Val³⁸ mutant of R-ras appears to be similar as determined by growth rate in the absence of histidine and the qualitative β -galactosidase activity (Figure 3), as well as by quantitative β -galactosidase activity (results not shown). This suggests that *S. cerevisiae* does not contain a GAP that is able to stimulate the GTPase activity of R-ras.

These data strongly suggested a GTP-dependent interaction between R-ras and Raf-1; however, the possibility could not be excluded that the interaction was mediated by an endogenous bridging yeast protein. Furthermore, we wanted to investigate whether a small 81-amino-acid N-terminal Raf fragment (Raf-RBD; amino acids 51–131) was able to interact with R-ras. This region was proposed to contain the H-ras-binding domain, which was based on the observation that this region was shared among different Raf deletion mutants that were still able to interact with H-ras [18]. To address these questions we developed an Raf-binding assay *in vitro*. For this assay Raf-RBD was produced and purified as a GST fusion protein from *Escherichia coli* and then used to coat a 96-well microtiter plate. Purified Glu-Glu-tagged R-ras was obtained from recombinant baculovirus-infected Sf9 insect cells, loaded *in vitro* with either the non-hydrolysable GTP analogue GTP γ S or with GDP, and tested for direct interaction with the Raf fragment on the plates. Detection of bound R-ras was performed by means of an anti-(Glu-Glu) antibody coupled to HRP. As can be seen in Figure 4, purified R-ras interacts directly with the proposed Ras-binding domain of Raf, with an approx. 15-fold higher apparent affinity for GTP γ S-loaded R-ras compared with GDP-loaded R-ras. Therefore it can be concluded that R-ras interacts directly with Raf-1 *in vitro* in a GTP-dependent fashion, and that the amino acid domain 51–131 of Raf-1, which lacks the cysteine-rich zinc-finger motif, is sufficient for the interaction.

The observations reported here are intriguing given the different biological effects of the Ras, Rap and R-ras proteins: Ras being involved in the regulation of proliferation and able to transform cells; Rap being able to reverse the Ras-induced transformation [39]; and R-ras being non-transforming and possibly involved in the regulation of apoptosis [3]. The so-called effector domain, which is conserved between Ras and Rap, has been proposed to mediate their interactions with p120-GAP and Raf [17–20,40]. Since R-ras has an identical domain, our observation that R-ras is also able to interact with Raf-1 provides further support for that idea. In this respect it is also noteworthy that purified p120-GAP (and NF-1) is able to bind directly and enhance the GTPase activity of R-ras *in vitro* (M. J. Fernandez-Sarabia, unpublished work; [24,25]). In contrast, Rap also binds p120-GAP but is insensitive to it [40]. Given their identical effector domain sequences and p120-GAP-binding ability, this implies that an additional region is involved in defining sensitivity for p120-GAP. By comparing sequences of Ras and Rap a critical role in this has been suggested for the H-ras amino acid region 61–65 and in particular Gln⁶¹ [39,41]. The greater sequence similarity between Ras and R-ras in this region and the presence of the putative critical amino acid (Gln⁸⁷ in R-ras), may provide an explanation for the similarity in p120-GAP responsiveness between them compared with Rap. Thus whereas the effector domain may determine the binding with their effector proteins, it is the context of the flanking sequences which determines the functional outcome of the interaction. Therefore it will be interesting to determine whether R-ras behaves like Ras in that it enables the activation of Raf, or whether it acts like Rap in suppressing Ras signalling [42]. On the basis of sensitivity to p120-GAP of both Ras and R-ras, compared with the insensitivity of Rap, it is tempting to speculate that R-ras may act like Ras in activating Raf-1. We are currently addressing these questions, which will also reveal whether R-ras is able to reverse oncogenic Ras-induced transformation, and whether Raf-1 is the main effector of R-ras.

It will be interesting to establish whether Raf-1 and Bcl-2 can bind simultaneously to R-ras, or whether they are mutually exclusive. Although we have not been able to detect an interaction

between Raf-1 and Bcl-2 by means of the two-hybrid system (not shown), the possibility that Bcl-2 and Raf-1 bind to each other, or that Bcl-2, R-ras and Raf-1 can exist in the same complex, cannot be excluded. With respect to the interaction of R-ras with Bcl-2 [3], and a proposed synergistic effect of Raf and Bcl-2 in suppressing apoptosis [30], it will be interesting to determine what role R-ras and the complex formation of R-ras and Raf-1 may play in apoptosis.

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