Identification of Radicicol as an Inhibitor of *In Vivo* Ras/Raf Interaction with the Yeast Two-hybrid Screening System

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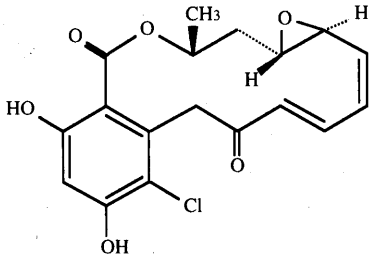
Activation of cytoplasmic serine/threonine kinase Raf-1, an important effector of Ras, requires direct binding to Ras. The yeast two-hybrid screening system used for identification of inhibitors of Ras/Raf-1 interaction showed radicicol to be an inhibitor. Radicicol has been shown to induce morphological reversion of transformed cells. Immunoprecipitation with an anti-Ras antibody revealed that the *in vivo* Ras/Raf-1 binding in v-Ha-*ras*-transformed cells was also blocked by low concentrations of radicicol (0.1 ~ 1 μg/ml), while degradation of Raf-1 was induced at concentrations higher than 2 μg/ml. However, *in vitro* binding of glutathion *S*-transferase-fused Ras to a maltose binding protein-fused RIP3 containing the Ras-binding domain (RBD) of Raf-1 was not inhibited by radicicol. Similar two-hybrid assays with several truncated forms of Raf-1 showed that both the conserved serine/threonine-rich domain (CR2) and the *C*-terminal protein kinase domain (CR3) were required for the full inhibition by radicicol. These results suggest that radicicol interacts directly or indirectly with the region except with RBD of Raf-1, thereby inhibiting a conformational change of Raf-1 prerequisite for binding to Ras.

The *ras* proto-oncogene is a central component of mitogenic signal-transduction pathways, and is essential for cells to progress the cell cycle through a quiescent state (G0) to S phase[1~4]. Raf-1, a serine/threonine kinase, is part of a highly conserved kinase cascade that mediates signaling from extracellular growth factors to mitogen-activated protein kinases. Raf-1 functions downstream of Ras, which in its active, GTP-bound state binds directly to the *N*-terminal regulatory domain of Raf-1[5]. This interaction serves to recruit Raf-1 to the cell membrane, which is necessary for Raf-1 activation[6,7]. Thus, activation of Raf-1 requires direct interaction with Ras. It is therefore likely that a specific inhibitor of Ras/Raf binding would be a promising chemotherapeutic agent against Ras-activated cancers. To identify such inhibitors, we employed the yeast two-hybrid system[8], since it seems to be a powerful strategy for identifying not only a protein that is physically associated with a certain target protein but also an agent that blocks protein-protein interaction. In this system, the interaction of Ras and Raf-1 can be monitored by transcriptional activation of two reporter genes, *HIS3* and *lacZ*[9]. We screened a variety of microbial metabolites for inhibitors blocking Ras/Raf interaction by this system, and identified radicicol (Fig. 1) as a candidate for such an inhibitor.

Radicicol, a macrocyclic antifungal antibiotic originally isolated from the fungus *Monosporium bonorden*[10], is known to be a potent tranquilizer of low toxicity[11] and suppreses morphological transformation by v-*src*, v-*ras*, as well as other oncogenes[12~15]. Although we and others found that radicicol inhibited the phosphorylation and protein kinase activity of pp60$^{v\text{-}src}$ both *in vivo* and *in vitro*[12,16,17], the inhibition by radicicol of protein-tyrosine kinases alone cannot account for morphological reversion of *ras*-transformed cells. Recently, we found that increased expression of gelsolin, an actin regulatory protein, is involved in the radicicol-induced morphologi-

Fig. 1. Chemical structure of radicicol.



cal changes of transformed cells[18]. Here we show that radicicol blocks Ras/Raf-1 interaction in the yeast two-hybrid system and inhibits *in vivo* binding of Ras and Raf-1 in not only yeast but also in v-Ha-*ras*-transformed cells, which may be associated, at least in part with suppression by radicicol of *ras*-mediated transformation. However, the experiments using the N-terminal Ras-binding domain of Raf-1 showed that radicicol did not inhibit *in vitro* direct binding of Ras and Raf-1. We suggest that *in vivo* inhibition of Ras/Raf-1 interaction is due to an allosteric effect of radicicol on the Raf-1 protein structure.

Materials and Methods

Strains and Plasmid Constructs

The genotype of the *Saccharomyces cerevisiae* reporter strain L40 is *MATa trp1 leu2 his3 LYS::lexA-HIS3 URA3::lexA-lacZ*[9]. Plasmids pLexA-RAS^{V12}, pVP16-RAF and pVP16-CDC25 for two-hybrid assays are described[9]. pVP16-ΔCR2ΔCR3 and pVP16-ΔCR3 were constructed by deleting the *Sal*I-*Not*I (Thr257 to Phe648 in Raf-1) and *Stu*I-*Not*I (Pro331 to Phe648 in Raf-1) fragments, respectively, from pVP16-RAF. pGST-Ras^{V12} and pMBP-RIP3 were used for production of glutathione *S*-transferase (GST)-fused RAS^{V12} and a maltose-binding protein (MBP)-fused RIP3 corresponding to Raf-1 Cys26 to Cys175, respectively, in *E. coli* DH5α.

Yeast Two-hybrid Screening

The L40 cells transformed with both pLexA-RAS^{V12} and pVP16-RAF (strain LZ) were able to grow on an agar plate lacking histidine and express β-galactosidase, since transcription of the reporter genes were stimulated by the formation of the complex of LexA-Ras^{V12} and VP16-Raf[9]. These cells were grown at 30°C in the SD medium lacking leucine, tryptophan and histidine, and 100 μl of this culture was inoculated into 100 ml of the medium containing 1% agar supplemented with (His$^+$) or without (His$^-$) 130 μM histidine. To increase the drug permeability and sensitivity in the screening, we also added 0.001% SDS and 1 mM 3-aminotriazole to the medium. The paper disks soaked in various drugs or microbial extracts were placed on agar plates. The plates were incubated at 30°C for 3 days, and the diameters of the growth inhibition zones were measured and compared between those on His$^-$ and His$^+$. The cells containing pLexA-RAS^{V12} and pVP16-CDC25 (strain LC) were used as control, since the domain of Ras required for interaction with CDC25 is different from that for the Raf-1 interaction.

Isolation of Radicicol

The producing strain No. 2348, probably a fungus, was inoculated into 50 ml of YMPG medium containing 2% glucose, 0.5% yeast extract, 0.5% malt extract (Difco), and 0.5% Bactopepton (Difco), and cultivated for 2 days at 26.5°C. Ten ml of the culture broth was inoculated into five flasks each containing 1 liter of YMPG medium and cultured for 6 days at 26.5°C on a rotary shaker operating at 115 rpm. For purification of the active compound, mycelium of strain No. 2348 was harvested by centrifugation. The active substance was extracted with 500 ml of acetone from the mycelia and concentrated by evaporation. The extract was then combined with the cultured supernatant. The active substance was extracted three times with ethylacetate from the mixture. The organic layer was evaporated *in vacuo*. The residue was dissolved in chloroform and fractionated by a silica gel column (50 × 100 mm, 200 ml) with a solvent system of a stepwise of chloroform-methanol. The active fractions eluted with chloroform-methanol (99 : 1) were collected and evaporated *in vacuo*. The residue was redissolved in chloroform and further separated by preparative HPLC on silica gel (AQUASIL SS-852N, Senshu, Tokyo), using a solvent system of chloroform-formic acid (99.5 : 0.5). The active fraction was evaporated, leaving us with 25 mg of an active compound of yellowish crystals.

Physico-chemical Measurements

UV spectra were recorded using methanol as a solvent with a Shimadzu UV-160A spectrophotometer. IR spectra were recorded on a Jasco IRA-103 spectrometer. Optical rotation was measured in chloroform with a Jasco DIP-371. ^{13}C (125 MHz) and ^{1}H NMR (500 MHz) spectra were measured in $CDCl_3$ with a JEOL JMN-

500N. FAB-mass spectra were measured on a JEOL JMS-SX102A mass spectrometer.

Mammalian Cells and Culture Conditions

v-Ha-*ras*-transformed NIH3T3 cells were provided by Dr. T. YAMAMOTO. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2-95% air.

Immunoprecipitation and Western Blotting

v-Ha-*ras*-transformed NIH3T3 cells treated with various concentrations of drugs were lyzed with lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% (v/v) glycerol, 0.1 mM PMSF, 10 ng/ml aprotinin, 10 ng/ml leupeptin, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM $NaVO_3$. One μg of anti-Ras antibody (Y13-238, Oncogene Science, Inc.) was added to the lysate (2 mg protein) and incubated for 1 hour at 4°C. The immune complex was absorbed with protein G Plus/protein A agarose beads (Oncogene Science, Inc.) at constant rotation for 30 minutes at 4°C. The agarose beads were washed 5 times with the lysis buffer, and the bound proteins were eluted from the beads by boiling with the sample buffer for SDS polyacrylamide gel electrophoresis. Proteins were separated by slab gel electrophoresis on an SDS-12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The blots were blocked with 5% skim milk in 10 mM Tris-HCl (pH 8), 2.5 mM EDTA, 50 mM NaCl, and 0.1% Tween-20, and then incubated for 1 hour at room temperature with anti-Raf (C-12, Santa Cruz Biotech.) or anti-Ras (Y13-259, Oncogene Science, Inc.) antibody. For detection, the blots were incubated with the horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The antigen-antibody complexes were visualized with an ECL detection kit (Amersham Corp.) according to the manufacturer's instructions.

Assay for *In Vitro* Protein Binding

Bacterial production of GST-Ras^{V12} and MBP-N-terminal region of Raf-1 (RIP3) and their *in vitro* binding were performed according to the method described by VOJTEK *et al.*[9] For binding assays, GST or GST-Ras was incubated with the resin-bound MBP or MBP-RIP3 in 85 mM NaCl, 10% glycerol, 0.6 mM guanine nucleotide, and 6 mM free magnesium. The resin was washed with buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF, 10% glycerol and the bound proteins were eluted from the resin with 10 mM maltose in the same buffer. The proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using an anti-GST antibody (Santa Cruz Biotech), followed by ECL detection.

β-Galactosidase Assay

The filter assay was carried out according to the method of VOJTEK *et al.*[9] For quantitative assays, 0.1 ml of cell culture grown in the liquid medium at $OD_{600}=1$ was added to 0.7 ml of Z buffer (Miller) containing 0.75 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside, and 0.27% β-mercaptoethanol. The cells were permealized by mixing with 100 μl of 50% $CHCl_3$ in 0.05% SDS, and then incubated with 0.16 ml of an *o*-nitrophenyl-β-D-galactoside (ONPG) solution (4 mg/ml ONPG in 100 mM phosphate buffer) for 1 hour at 30°C. The reaction was terminated by adding 0.4 ml of 1 M Na_2CO_3, and the optical density of at 420 nm was measured. The specific activity was calculated according to the method of GUARENTE[19].

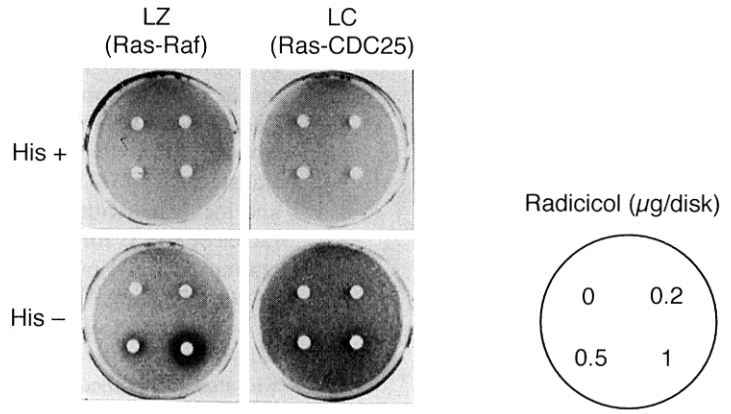
Results

Screening for Inhibitors of Ras/Raf Interaction with the Yeast Two-hybrid System

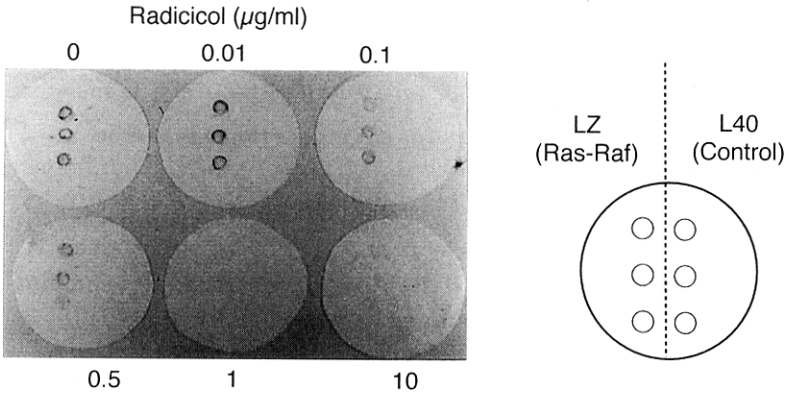
In 1993, VOJTEK *et al.*[9] identified c-Raf-1 as an important effector of Ras by the yeast two-hybrid system. The *N*-terminal domain of Raf-1 containing the Ras-binding domain (RBD) directly binds to Ras in an active GTP-bound state. Since the association of Ras and Raf-1 is therefore a critical step for Ras-mediated signal transduction, a drug blocking the association could be a potential chemotherapeutic agent against *ras*-activating cancers. We applied the yeast two-hybrid system to screening for such inhibitors from a variety of microbial metabolites. If the interaction between oncogenic Ras^{V12} and Raf-1 was inhibited in LZ cells carrying both pLexA-RAS^{V12} and pVP16-RAF, then the cells would grow in the presence of histidine but not in the absence of histidine. On the other hand, although pVP16-CDC25 with pLexA-RAS^{V12} can also confer the His^+ phenotype, specific inhibitors blocking Ras/Raf interaction would not inhibit the growth of this strain even in the absence of histidine, since the domain of Ras required for interaction with CDC25 is different from that for the Raf-1 interaction. As a result of screening about 10,000 culture extracts of newly isolated microbes for compounds blocking the Ras/Raf interaction in yeast,

Fig. 2. Specific inhibition of Ras/Raf-1 interaction by radicicol in the yeast two hybrid assays.

(A) Paper disk assay of histidine synthesis. Ras/Raf-1 binding was monitored by cell growth of the histidine auxotrophs in the presence or absence of histidine. Growth inhibition of the LZ (Ras-Raf) and LC (Ras-CDC25) strains around the paper disks soaked with a various concentrations of radicicol was compared between the presence and absence of 130 μM histidine.



(B) Assay of β-galactosidase. Ras/Raf-1 interaction was monitored by β-galactosidase activity. LZ (Ras-Raf) and L40 (control) cells grown on agar plates in the presence of various concentrations of radicicol were transferred to nitrocellulose filters, fixed and stained with 5-bromo-4-chloro-3-indolyl-β-galactoside.



we found a fungus producing such an activity.

Isolation of Radicicol

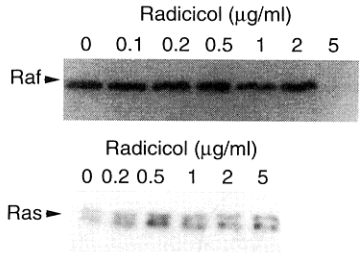
We isolated 25 mg of the active compound by silica gel column chromatography and HPLC from a 5-liter culture of the fungal strain No. 2348. UV, IR, and NMR spectra indicated that it was identical to radicicol (data not shown). Radicicol (1 μg/disc) caused growth inhibition of LZ cells in the medium lacking histidine but not in the medium containing histidine (Fig. 2A). On the other hand, growth of LC cells carrying pVP16-CDC25 with pLexA-RAS^{V12} was scarcely inhibited by radicicol in the absence of histidine. Expression of the *lacZ* reporter gene was decreased in a dose-dependent manner when LZ cells were grown in the medium containing radicicol (Fig. 2B). However, radicicol did not inhibit β-galactosidase directly *in vitro* (data not shown). These results indicate that radicicol inhibits Ras/Raf-1 interaction in the yeast cells.

Inhibition of *In Vivo* Ras/Raf Interaction in Ras-transformed Mammalian Cells

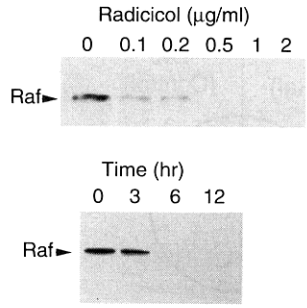
To examine whether radicicol inhibits Ras/Raf-1 interaction in mammalian cells as well, we determined the total Ras and Raf-1 levels and the amount of Ras-bound Raf-1 upon treatment of v-Ha-*ras*-transformed NIH3T3 cells by radicicol. The amounts of Ras in the total lysate of cells that had been treated with radicicol at various concentrations were essentially unchanged even at a high concentration (5 μg/ml) (Fig. 3A). Although a marked decrease in Raf-1 was observed in cells treated with 5 μg/ml radicicol, the amount of

Fig. 3. Inhibition by radicicol of *in vivo* binding of Ras/Raf-1 in v-Ha-*ras*-transformed NIH3T3 cells.

(A) Effects of radicicol on total amounts of Ras and Raf-1. The amounts of Ras and Raf-1 in the cell extract were determined by SDS-PAGE and Western blotting.



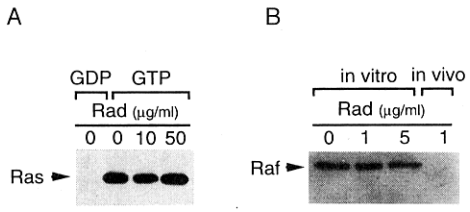
(B) Ras/Raf-1 binding in cells treated with various concentrations of radicicol. Cells exposed to radicicol at various concentrations for 40 hours (upper panel) or at 0.5 μg/ml radicicol for the indicated lengths of time (lower panel) were lyzed and the Raf-1 in the immune complex precipitated with an anti-Ras antibody was quantified by SDS-PAGE and Western blotting.



Raf-1 was unaffected by radicicol in the concentration range between 0.1 and 2 μg/ml, in which radicicol effectively induced morphological change[14,15]. We extracted the Ras-containing protein complex from v-Ha-*ras*-transformed NIH3T3 cells by immunoprecipitation and quantified the Raf-1 protein in the complex by Western blotting (Fig. 3B). The amount of Raf-1 in the immune complex precipitated with an anti-Ras antibody was greatly reduced in the cells treated with low concentrations of radicicol (0.1 ~ 0.2 μg/ml) and was almost undetectable by treatment at more than 0.5 μg/ml. The results clearly show that binding of Ras to Raf-1 was blocked in radicicol-treated cells. Time course experiments showed that the blockage caused by 0.5 μg/ml radicicol was observed within 6 hours.

Fig. 4. Effects of radicicol on *in vitro* binding of Ras/Raf-1.

(A) Binding of bacterially produced GST-Ras to immobilized MBP-RIP3. Binding of GST-Ras to MBP-RIP3 in the presence of various concentrations of radicicol was monitored by Western blot analysis using an anti-GST antibody. GTP-dependent binding was detected even in the presence of high concentrations of radicicol. (B) Effects of radicicol on the immune complex containing Ras and Raf-1. The Ras-containing immune complex was precipitated from the extract of untreated v-Ha-*ras*-transformed NIH3T3 cells by the anti-Ras antibody and incubated with the indicated concentrations of radicicol at 4°C for 1 hour (*in vitro* treatment). The amount of Raf-1 in the complex was determined by Western blotting. The Raf-1 content in the complex from the extract of cells that had been treated with 1 μg/ml radicicol for 40 hours (*in vivo* treatment) was also determined. Although the *in vivo* association between Ras and Raf-1 was inhibited by radicicol in v-Ha-*ras*-transformed NIH3T3 cells, the complex that had been formed in the absence of radicicol was not dissociated by radicicol *in vitro*.

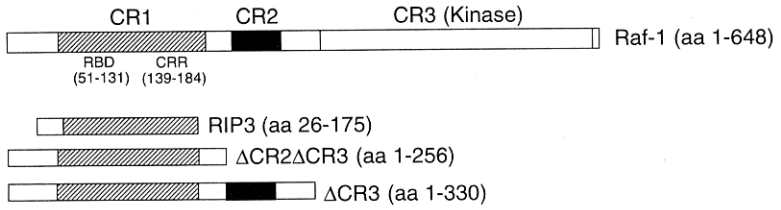


Indirect Inhibition of Ras/Raf-1 Interaction by Radicicol

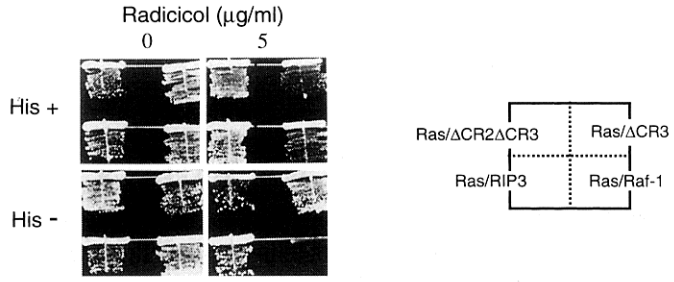
We next examined whether radicicol directly inhibits association of Ras and Raf-1. To this end, we purified bacterially produced GST-Ras and the MBP-fused Ras-binding domain of Raf-1 (MBP-RIP3), and analyzed the effect of radicicol on *in vitro* binding of these proteins. Purified GST-Ras was loaded on the MBP-RIP3-bound amylose resin in the presence of GDP or GTP, and the fusion protein associated with the resin was eluted and detected by Western blotting using an anti-GST antibody. As shown in Fig. 4A, *in vitro* GTP-dependent association of Ras and RIP3 was observed. Addition of radicicol in the reaction mixture did not cause any decrease in the amount of GST-Ras bound to MBP-RIP3 even at a high concentration (50 μg/ml). This observation suggests that the *in vivo* inhibition of Ras/Raf interaction by radicicol is not due to simple competition of the binding of the two proteins. We then examined whether radicicol releases Raf-1 from the Ras/Raf-1 complex that had been formed in the absence of radicicol by the

Fig. 5. Raf-1 region required for inhibition by radicicol of Ras interaction.

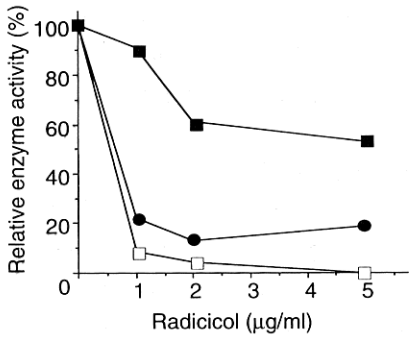
(A) Schematic representation of Raf-1 and its truncated mutants expressed as VP16 fusions in yeast.



(B) Assay of histidine synthesis. Interaction of Ras with Raf-1 or its truncated mutants was monitored by cell growth of the histidine auxotrophs in the presence or absence of histidine.



(C) Quantitative assay of β-galactosidase. Interaction of Ras with Raf-1 (□), ΔCR3 (●), or ΔCR2ΔCR3 (■) was monitored by β-galactosidase activity. The total β-galactosidase activity was quantitatively determined as described in Materials and Methods.

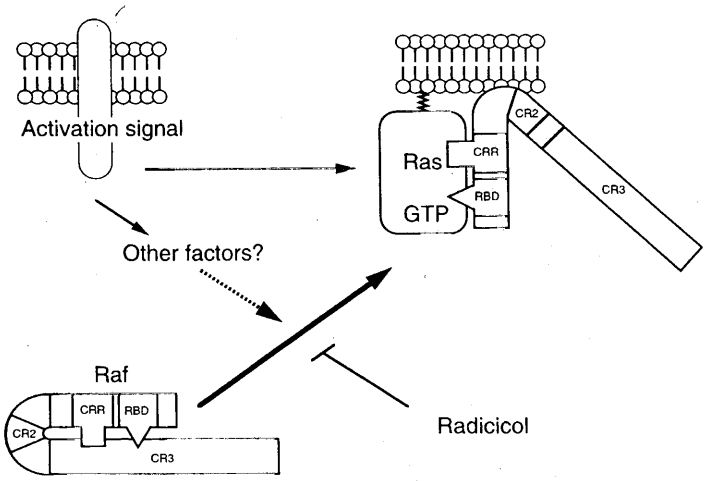


pull-down assay using the anti-Ras antibody (Fig. 4B). The immunoprecipitated Ras-containing complex was incubated with radicicol for 1 hour at 4°C, and then the Raf-1 protein in the complex was quantified by Western blotting. Essentially the same amount of Raf-1 was recovered from the complex even after the exposure to a high concentration of radicicol (5 μg/ml), indicating that radicicol does not dissociate the Ras/Raf complex *in vitro*. On the other hand, treatment of cells with 1 μg/ml of radicicol caused almost complete loss of the Raf-1 protein in the immune complex containing Ras.

Requirement of Raf-1 CR2 and CR3 for Full Inhibition by Radicicol

We further analyzed the mode of inhibition of Ras/Raf interaction by using the two-hybrid system. Raf-1 shares three regions of conservation, termed CR1, CR2 and CR3, with other Raf isomers and homologs (Fig. 5A). CR1 and CR2 are located in the *N*-terminal half and CR3 corresponds to the *C*-terminal kinase domain. RBD (amino acids 51-131) has been mapped in CR1. To identify the region required for inhibition by radicicol, we generated two truncated forms of Raf-1 lacking CR3 (ΔCR3) and both CR2 and CR3 (ΔCR2ΔCR3), which were fused to the VP16 transactivator, and examined the

Fig. 6. Model for *in vivo* inhibition of Ras/Raf-1 interaction by radicicol.



interaction with Ras in the two-hybrid strains. These truncated Raf-1 mutants can bind to Ras due to the presence of RBD when the interaction has been assessed by growth of the yeast strains in the absence of histidine (Fig. 5B). However, the expression of these Raf-1 mutants allowed cells carrying pLexA-Ras^{V12} to grow on the His$^-$ plate even in the presence of a high concentration (5 μg/ml) of radicicol, while wild-type Raf-1 did not. Their activity to interact with Ras was quantified by measuring intracellular β-galactosidase activity (Fig. 5C). The enzymatic activity in the cells carrying pVP16-RAF (wild-type) was markedly reduced by treatment with 1 μg/ml radicicol and was almost undetectable at 5 μg/ml. Radicicol also reduced the intracellular β-galactosidase activity in cells containing pVP16-ΔCR3 or pVP16-ΔCR2ΔCR3, but significant activity (20% for ΔCR3 and 50% for ΔCR2ΔCR3, respectively) still remained after treatment of 5 μg/ml radicicol. These results indicate that CR2 and CR3 are required for the full inhibition by radicicol.

Discussion

In order to discover inhibitors of Ras/Raf-1 interaction, we conducted a screening program using the yeast two-hybrid system. A microbial metabolite found in the course of screening was identical to radicicol. Radicicol has been shown to reverse the morphology of *ras*-, *src*-, and other oncogene-transformed cells to apparently normal ones[12~15]. We previously demonstrated that the drug inhibited intracellular activation of MAP kinase, a downstream mediator for the Ras-signal transduction pathway, without inhibiting kinase activity of MAPKK or decreasing the level of GTP-bound Ras[14]. Our present data suggest that radicicol inhibits *in vivo* Ras/Raf-1 interaction in mammalian cells, thereby decreasing MAP kinase activity.

The mechanism by which radicicol inhibits Ras/Raf-1 interaction appears to be complicated. Raf-1 contains two Ras-binding domains[20]. The best-characterized one is RBD and is contained within residues 51~131[9,21,22]. The interaction between RBD and Ras appears to then allow for a second Ras binding domain in Raf-1 to contact Ras[23~25]. The second domain (residues 139~184) encompasses the conserved cysteine finger motif within CR1 and is referred to as the cysteine-rich region[24] (CRR, see Fig. 5A). In the full-length Raf-1 molecule, CRR does not interact with Ras unless RBD-mediated binding can occur. However, removal of the carboxy-terminal kinase domain (CR3) or mutations introduced into the serine/threonine-rich CR2 domain can enable CRR to contact Ras in the absence of RBD binding[25]. Thus, CRR in the full-length Raf-1 may be inaccessible for Ras binding, but either mutational events or RBD binding can unmask CRR and allow it to interact with Ras. Recently, CUTLER *et al.*[26] reported that the *N*-terminal half of Raf-1 containing both CR1 and CR2 domains could inhibit kinase activity of CR3 in trans, and that these two halves of Raf-1 expressed in *Xenopus* oocytes could interact with each other. They also showed that a mutation of CRR or a mutation mimicking an activating phosphorylation event (Y340D) in CR3 blocked this inhibitory effect. These results suggest that CR3 is masked intramolecularly by the *N*-terminal half of Raf-1, thereby stabilizing the inactive state, but this conformation can be disrupted upon binding to Ras (Fig.

6). In the present study, the *in vitro* binding assay using GST-Ras and MBP-RIP3 showed that radicicol did not compete with RBD binding to Ras. The yeast two-hybrid assays demonstrated that both CR2 and CR3 domains of Raf-1 were required for the full inhibition of the interaction by radicicol. These results suggest that radicicol prevents the association of Raf-1 with Ras-GTP by interacting directly or indirectly with Raf-1 in the region other than RBD or CRR. Furthermore, we showed that radicicol did not cause dissociation of the protein complex that had been formed in the absence of radicicol (Fig. 4B). Taken together, radicicol may distort the protein structure of newly synthesized Raf-1 or prevent the conformational change prerequisite for binding to Ras (Fig. 6).

Raf-1 is known to interact with some proteins other than Ras. Among them, members of the 14-3-3 protein family, the molecular chaperone Hsp90 and its kinase-targeting subunit p50, a homolog of CDC37, are suggested to function as regulators of the Raf-1 structure and activity[27~29]. Raf-1 contains two serine phosphorylation sites that are part of the consensus 14-3-3-binding motif, RXSXphosphoSXP[30], namely Ser259 in CR2 and Ser621 in CR3. Since 14-3-3 may be required for both maintaining Raf-1 in an inactive conformation prior to signaling events and stabilizing the active Raf-1 conformation after stimulation[27,30,31], it seems possible that radicicol modulates the function of 14-3-3. Association of Raf-1 with Hsp90 and p50 is constitutive and appears to be important for maintaining protein stability and for the proper localization of Raf-1 within the cell. SCHULTE *et al.*[32,33] reported that disruption of Raf-1-Hsp90 complex by a drug geldanamycin resulted in degradation of Raf-1 and loss of Raf-1-Ras association. Recently, radicicol was reported to inhibit the association of Hsp90 with geldanamycin[34]. These results strongly suggest that radicicol also interacts with Hsp90 at the site close to the geldanamycin-binding site and destablizes Raf-1. In fact, we (present study) and others[35] observed a decrease in the amount of Raf-1 upon radicicol treatment. However, loss of Raf-1 cannot fully account for the present results, since *in vivo* inhibition of Ras/Raf-1 interaction was detected at much lower concentrations than that for Raf-1 degradation (Fig. 3A). It seems possible that radicicol binds Hsp90 and causes a conformational change of Raf-1, which may result in inhibition of the Ras/Raf-1 interaction by radicicol at the concentrations lower than those for destabilizing Raf-1.

Although radicicol was not a direct inhibitor of Ras/Raf-1 interaction, the present study showed that the two-hybrid system was generally applicable to identifying not only a protein that is physically associated with a certain target protein but also an agent that blocks protein-protein interaction. Further screening may lead to discovery of new inhibitors that compete or disrupt the Ras/Raf-1 interaction. Several radicicol derivatives are currently in preclinical trials as antitumor agents. The antitumor activity of radicicol may come from its potent biological activities of morphological reversion and cell cycle arrest of tumor cells, which are apparently mediated by distinct mechanisms involving inhibition of Src-family protein tyrosine kinases and blockage of the Ras-mediated signal transduction. Identification of the target proteins directly associated with biological activity of radicicol is obviously needed for elucidation of the molecular mechanisms by which radicicol elicits *in vitro* and *in vivo* antitumor effects.

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