Radicicol Leads to Selective Depletion of Raf Kinase and Disrupts K-Ras-activated Aberrant Signaling Pathway*

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Activation of Ras leads to the constitutive activation of a downstream phosphorylation cascade comprised of Raf-1, mitogen-activated protein kinase (MAPK) kinase, and MAPK. We have developed a yeast-based assay in which the Saccharomyces cerevisiae mating pheromone-induced MAPK pathway relied on co-expression of K-Ras and Raf-1. Radicicol, an antifungal antibiotic, was found to inhibit the K-ras signaling pathway reconstituted in yeast. In K-ras-transformed, rat epithelial, and K-ras-activated, human pancreatic carcinoma cell lines, radicicol inhibited K-Ras-induced hyperphosphorylation of Erk2. In addition, the level of Raf kinase was significantly decreased in radicicol-treated cells, whereas the levels of K-Ras and MAPK remained unchanged. These results suggest that radicicol disrupts the K-Ras-activated signaling pathway by selectively depleting Raf kinase and raises the possibility that pharmacological destabilization of Raf kinase could be a new and powerful approach for the treatment of K-ras-activated human cancers.

The Ras and Raf protooncogene products are key proteins involved in the transmission of many proliferative signals. They serve as intermediates in this signaling pathway by connecting upstream tyrosine kinases with downstream serine/threonine kinases such as mitogen-activated protein kinase (MAPK) or MAPK kinase (MAPKK). A similar MAPK signaling pathway also exists in the mating pheromone-responding signaling cascade of Saccharomyces cerevisiae. This signaling pathway consists of the Ste11, Ste7, and Fus3/Kas1 kinase, which are homologs of MAPKK kinase, MAPKK, and MAPK, respectively (1). We have previously reported that expression of mammalian H-Ras and Raf-1, together with Ste7P368, rescued the defect in mating pheromone signal transduction because of STE11 deficiency (2). In the present study, we modified this in vivo system by substituting the K-Ras for H-Ras, because K-ras is the most frequently activated ras gene in human cancer (3) and, therefore, more important as a target for cancer therapy than H-ras or N-ras. Using this modified yeast assay, we screened for inhibitors that block the Ras–Raf pathway reconstituted in S. cerevisiae and have identified radicicol (Fig. 1) as a candidate inhibitor of the Ras–Raf signaling pathway.

Radicicol (Fig. 1), a macrocyclic antifungal antibiotic originally isolated from the fungus Monosporium bonorden (4), is a potent tranquilizer of low toxicity (5, 6) and an inhibitor of in vitro angiogenesis (7). Radicicol induces reversal of the transformed phenotype of src-transformed cells (8) and has also been reported to inhibit the phosphorylation and protein kinase activity of pp60 src (9). It does not inhibit the serine/threonine kinases, such as protein kinase C and protein kinase A (9–11). In addition to the inhibitory activity of radicicol against Src kinase, there is evidence that radicicol suppresses transformation by the ras oncogene. Decreased MAPK activity accompanies morphological reversion of H-ras-transformed cells by radicicol (12). However, radicicol does not inhibit the kinase activity of MAPKK or MAPK in vitro (13). These results suggest that radicicol does not directly inhibit the MAPK or MAPKK activity in ras-transformed cells but inhibits MAPK activation by unknown mechanisms.

Our findings pertaining to radicicol as an inhibitor of the K-Ras signaling pathway in yeast mutants prompted us to examine its effect on K-ras-transformed mammalian cell lines. Our present data reveal that radicicol inhibits ras-dependent phosphorylation of MAPK in K-ras-transformed rat epithelial cell lines and, further, that disruption of the K-Ras signaling pathway is due to the destabilization of Raf protein by radicicol.

**EXPERIMENTAL PROCEDURES**

Plasmid and Yeast Strains—The plasmid pVT-VKR expresses Kirsten sarcoma virus ras gene from the ADH promoter. It was constructed by ligating the Stu-BamHI fragment of pHN12 (14) into the pPla-BamHI site of pVT101-L in which the pPla site was blunt-ended. pVT101-L is a Ycp-based plasmid containing the LEU2 selectable marker (15). pADU-Raf contains c-Raf-1 controlled from the ADH1 promoter. pNC318-P368 carries the STE7P368 allele controlled from the CYC1 promoter.

Strain SY1984-RP is SY1984 (MATa leu2 ura3 trpl1 ste11Δ his3Δ fus1–His3) transformed with pADU-Raf and pNC318-P368 (12). Yeast strains expressing K-Ras and Raf-1 were obtained by transducing pVT-VKR into strain SY1984-RP.

Yeast cells were grown in the synthetic medium SC, which is SD 2% glucose, 0.7% yeast nitrogen base without amino acid containing appropriate auxotrophic supplements (16). SC lacking amino acids or other nutrients (e.g., SC-ura, which lacks uracil) was used to select transformants. Yeast transformation was performed by the method of Itoh et al. (17).

Inhibition of Growth of SY1984-RP Expressing K-Ras—The yeast strain SY-1984-RP carrying pVT-VKR was grown at 30 °C to stationary

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† The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, same protein as MAPK kinase; Erk, extracellular signal-regulated protein kinase (same protein as MAP kinase); hsp90, heat shock protein 90; GAP, GTPase-activating protein.
phase in SC-Ura-Trp-Leu. Agar plates were prepared by adding 50 μl of the above culture to 50 ml of SC agar lacking Trp, Leu, His, and Ura. Paper discs soaked in drugs were placed on agar plates, the plates were inoculated at 30 °C for 3 days, and the diameters of the zones of growth inhibition were measured.

**Antibodies and Cell Culture**—The antibodies used were phospho-specific MAPK antibody, phospho-specific MEK1/2 (Ser217/221) antibody, pan-MEK1/2 antibody (New England Biolabs), Erk2 antibody (Upstate Biotechnology Inc.), MEK-1 and MEK-2 antibodies (Transduction Laboratories), c-Ras(Ab-1) antibody (Oncogene Science), and Raf-1(C-12), A-Raf(C-20), and B-Raf(C-19) antibodies (Santa Cruz Biotechnology).

NRK and KNRK5.2 cells were obtained from Dr. David A. Johnson. PSN-1 cells were obtained from Dr. Ken Yamaguchi. These cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

**Radicicol Treatment of Cells**—KNRK5.2 cells (1 × 10⁶) or PSN-1 cells (1 × 10⁶) were plated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a 24-well tissue culture plate. Approximately 8 h later, various amounts of radicicol were added to each well without adding any fresh medium, and the cells were cultured for a further period of 40 h. To examine the growth inhibitory effect of radicicol, cells were dispersed by trypsin-EDTA, and cells in the suspension were counted by an F-520 cell counter.

**Cell Lysis and Western Blotting**—Generally, 24-well tissue culture plate cells were used for each sample. Cells were washed once with phosphate-buffered saline. The cells were lysed for 10 min on ice and clarified by centrifugation. 10 μg of total cell lysate from each sample was electrophoresed on a 10% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes, and immunoblotted with appropriate antibodies. For detection, the blots were incubated with the appropriate ECL secondary antibody (horseradish peroxidase-conjugated anti-Ig antibody) and developed using the ECL detection system (Amersham Corp.), according to the instructions of the manufacturer. Films were scanned into a Macintosh computer and analyzed by using NIH Image software.

**RESULTS**

**Identification of a K-Ras Signaling Inhibitor Using Yeast Mutants Expressing K-Ras and Raf-1**—Kirsten sarcoma virus ras gene was expressed together with c-Raf-1 and Ste7P368 in a ste11-disrupted mutant strain having a mating pathway-responsive reporter gene (FUS1::HIS3). Consistent with our previous results obtained for H-Ras (2), expression of K-Ras in this strain conferred a His+ phenotype, indicating that Raf was activated by K-Ras in S. cerevisiae and transmitted the signal to the yeast phosphorelay-MAPK pathway. Using this K-Ras expressing mutant strain, we screened our chemical library for compounds that could inhibit the K-Ras signaling pathway reconstituted in yeast. One compound, radicicol (Fig. 1), was found to have such an activity. As shown in Fig. 2A, the yeast strain expressing K-Ras and Raf-1 could grow in agar medium lacking exogenous histidine, but its growth was inhibited by radicicol as evidenced by a halo of growth inhibition around the paper disc soaked with radicicol. When histidine or alanine was added to the drug-containing paper disc and incubated for 2 days, the halo of growth inhibition by radicicol disappeared upon exogenous addition of histidine but not alanine. One simple explanation of these results was that growth inhibition by radicicol was due to the inhibition of the K-Ras-MAPK pathway in the yeast strain. We could exclude the possibility that radicicol inhibited some enzyme associated with histidine biosynthesis because radicicol was not active against a yeast two-hybrid strain that also had a HIS3 reporter gene and has been used previously to detect the interaction between SNF1 and SNF4 (18).

Previous studies have shown that Raf-1 required the co-expression of activated Ras for rescuing Ste11 deficiency but that an activated form of Raf-1 (RafLN) alone could compensate for Ste11 deficiency (2). We therefore examined the effect of radicicol on the growth of the mutant expressing RafLN (Fig. 2B). Radicicol was effective in growth inhibition of this mutant strain. Similar results were obtained for two different mutant strains and suggested that the molecular target of radicicol in yeast could be a molecule that is uniquely common between the two strains. Such a molecule might include, for example, Raf-1 itself, factors downstream of Raf-1 in the pathway, or a protein that could interact with and regulate Raf-1 kinase.

**Radicicol Inhibits K-ras-dependent MAPK Phosphorylation in a K-ras-transformed Cell Line**—Based upon the effects of radicicol on yeast signal transduction, we decided to examine its effect on the K-ras-MAPK signal transduction pathway in cultured mammalian cells. To this end, the cell line KNRK5.2 was selected for further analysis. KNRK5.2 was derived from the rat kidney epithelial cell line, NRK, by transfection of an activated K-ras gene cloned from the human colon carcinoma cell line, SW480. Immunoblot analysis with anti-phosphotyrosine-specific Erk2 antibody revealed that the K-ras-trans-
formed cell line, KNRK5.2, had a marked elevation in the level of phosphorylation of Erk2, whereas NRK cells had no phosphorylation signal (compare Erk2 and phospho-Erk2 signal in lanes 1 and 2, Fig. 3A). Thus, the K-Ras signaling pathway was constitutively activated in this cell line. We examined the effect of radicicol on K-ras-induced phosphorylation of Erk2. Cells were treated with varying amounts of radicicol for 40 h, and cell lysates were subjected to immunoblot analysis with anti-phosphotyrosine-specific Erk2 antibody. Radicicol inhibited phosphorylation of Erk2, as revealed by the decrease in the phospho-Erk2 band. This was also evident from the decrease in the phospho-Erk2 band, which migrated slightly more slowly than unphosphorylated Erk2 in gels immunoblotted with anti-Erk2 antibody (Fig. 3). Intracellular amounts of Erk2 protein remained relatively unchanged with increasing concentrations of radicicol. Inhibition of Erk2 phosphorylation by radicicol was quantitated by calculating the relative amount of phospho-Erk2 band, which migrated slightly more slowly than the phospho-Erk2 band. This was also evident from the decrease in the phospho-Erk2 band. The simplest interpretation of these results was that the inhibition of MEK phosphorylation was due to the inhibition of phosphorylation of MAPK by radicicol.

As previously reported for H-ras-transformed fibroblastic cell lines (12, 13), radicicol caused a morphological reversion of K-ras-transformed epithelial cells to a more normal phenotype (Fig. 4). Morphological reversion was apparent at drug concentrations as low as 0.625 \( \mu \text{M} \). At this drug concentration, radicicol was required to attenuate Raf-1 protein expression. Removal of radicicol restored both Erk2 phosphorylation and Raf depletion in KNRK5.2 cells, as determined by immunoblot analysis (data not shown). A-Raf was expressed and its levels were significantly decreased with increasing concentrations of radicicol (Fig. 5). IC50 values for A-Raf and Raf-1 depletion were 5.7 and 5.0 \( \mu \text{M} \), respectively. Thus, radicicol induced the destabilization of all isoforms of Raf protein expressed in KNRK5.2 cells.

To clarify the relationship between the inhibition of phosphorylation of Erk2 and Raf depletion, we examined the time dependence of both events in the presence of 10 \( \mu \text{M} \) radicicol. Raf-1 depletion and Erk2 phosphorylation inhibition occurred with similar kinetics (Fig. 6). Taken together, these results strongly suggested that radicicol inhibited Erk2 phosphorylation by depleting Raf kinase in KNRK5.2 cells. As evidenced by the decrease in the MEK signal detected by the anti-MEK antibody (which can recognize MEK doubly phosphorylated at Ser-217 and Ser-221), radicicol also inhibited the phosphorylation of MEK without affecting the level of MEK protein significantly (Fig. 3). The simplest interpretation of these results was that the inhibition of MEK phosphorylation was due to the depletion of Raf kinases.

The effect of radicicol on Raf depletion in KNRK5.2 cells was reversible (Fig. 7). Cells were treated initially for a period of 24 h with radicicol, which significantly depleted Raf-1 protein and inhibited Erk2 phosphorylation in a dose-dependent manner. Radicicol was then removed, and the cells were washed and incubated in drug-free media for a further 24-h period. Removal of radicicol restored both Erk2 phosphorylation and levels of protein Raf-1. Therefore, continuous exposure to radicicol was required to attenuate Raf-1 protein expression.

**Effect of Radicicol on the Ras Signaling Pathway in Activated K-ras Human Pancreatic Carcinoma Cell Line, PSN-1—** Previous studies have demonstrated the ability of radicicol to inhibit the anchorage-independent growth in soft agar of the

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**Fig. 3.** Radicicol inhibits K-Ras-induced Erk2 phosphorylation by depleting Raf-1 kinase in KNRK5.2 cells. A, KNRK5.2 cells were grown for 40 h in the presence of various concentrations of radicicol. 10 \( \mu \text{g} \) of cell lysate was analyzed by immunoblotting with an antibody against Raf-1, the phosphorylated form of MEK1/2, MEK1/2, the phosphorylated form of Erk1/2, or Erk2, respectively (from top to bottom). B, immunoblotted gel was analyzed as described under “Experimental Procedures.” The levels of Raf-1, phosphorylated Erk2, and phosphorylated MEK1/2 were plotted as a percentage of the control.
human bladder carcinoma cell line, EJ, that harbors a point mutational activated allele of endogenous H-ras oncogene and the human fibrosarcoma cell line, HT1080, with a point mutational activated allele of N-ras oncogene (12). Given our present findings in K-ras-transformed NRK cells, we wanted to extend our observation to human tumor cell lines in which the endogenous K-ras gene was activated by a point mutation. The human pancreatic carcinoma cell line, PSN-1, has a Gly to Val mutation at amino acid 12 in its K-ras gene (19). Immunoblot analysis of PSN-1 cell lysate revealed the presence of the phosphorylated form of Erk2, indicating the activation of the K-Ras signaling pathway in cells (Fig. 8). Radicicol inhibited the tyrosine phosphorylation of Erk2 with an IC50 of 1.2 μM. Interestingly, Erk1 was minimally phosphorylated in the PSN-1 cell line. Depletion of Raf-1 was also observed in radicicol-treated PSN-1 cells. Although the level of MEK1/2 remained unchanged in radicicol-treated KNRK5.2 cells (Fig. 3), a slight decrease in the level of MEK1/2 was observed in PSN-1 cells.

Fig. 4. Morphological change of KNRK5.2 cells caused by radicicol. KNRK5.2 cells were treated with various concentrations of radicicol for 40 h, and then photographs were taken with a phase-contrast microscope.

Fig. 5. Radicicol destabilizes the A-Raf protein in KNRK5.2 cells. A, KNRK5.2 cells were grown for 40 h in the presence of various concentrations of radicicol. 10 μg of each cell lysate was analyzed by immunoblotting with antibody against Raf-1, A-Raf, or Erk2, respectively (from top to bottom). B, the immunoblotted gel was analyzed as described under “Experimental Procedures.” The levels of Raf-1 and A-Raf were plotted as a percentage of the control.
treated with radicicol. In PSN-1 cells, endogenous K-Ras protein was fully farnesylated, and it migrated to the same position as a standard sample of farnesylated K-Ras protein in SDS-polyacrylamide gel electrophoresis (data not shown). As shown in Fig. 8, the level of K-Ras protein in PSN-1 cells remained unchanged in the presence of different concentrations of radicicol. These observations further supported our conclusion obtained from the KNRK5.2 cell study that the primary target of radicicol in the K-Ras signaling pathway was, in fact, the Raf-1 kinase.

**DISCUSSION**

Genetic and biochemical studies demonstrate that Raf functions downstream of Ras in many signaling pathways, and activation of Ras leads to constitutive activation of a downstream phosphorylation cascade comprising Raf proteins,
MAPKK and MAPK. A small molecule inhibitor of Ras–Raf signal transduction could not only be a valuable tool in elucidating that signal transduction pathway but could also have a therapeutic potential. We have developed a yeast assay system in which the yeast phenomone-induced MAPK pathway is dependent upon co-expression of K-Ras and Raf-1. Using this system, we have screened for inhibitors of the Ras signaling pathway reconstituted in yeast and rediscovered radicicol as an inhibitor of the Ras pathway. Radicicol inhibited the K-Ras signaling pathway in mammalian epithelial cells as well.

Although radicicol has been shown to inhibit activation of MAPK in H-ras-transformed fibroblastic cell lines (12), the precise mechanism by which radicicol disrupts Ras signaling has not been unambiguously determined. Radicicol did not affect the biochemical function of Ras protein, such as GTP binding or membrane localization (13). Furthermore, it inhibited neither MAPKK nor MAPK in enzyme assays using purified enzyme from Xenopus oocytes (13). Our present results clearly demonstrate a molecular mechanism by which radicicol inhibits the Ras signaling pathway. Radicicol inhibits K-Ras-dependent phosphorylation of MAPK through the destabilization of Raf protein in a K-ras-transformed rat kidney epithelial cell line and in K-ras-activated human pancreatic carcinoma cells. The level of Ras and MAPK did not change in the cells treated with radicicol. Depletion of Raf-1 by radicicol can also explain why radicicol is active against the yeast mutant strains expressing Raf-1ΔN.

Selective destabilization of Raf was also reported for the benzoquinone ansamycin family antibiotic, geldanamycin (20, 21). Although both geldanamycin and radicicol have a macrocyclic moiety in their structure, radicicol differs structurally from ansamycin family antibiotics. Raf is known to exist as part of a multimolecular complex that contains hsp90, Cdc37/p50, and other proteins (22, 23). This complex may function as a transportsome directing Raf to its proper subcellular localization. Geldanamycin has been shown to bind directly to hsp90 and disrupts the complex of which it is a part (20). Disruption of the Raf-hsp90 association by geldanamycin leads to destabilization of Raf protein. In KNRK5.2 cells, we also found that geldanamycin caused a depletion of Raf protein and inhibited the phosphorylation of Erk2 (data not shown). These results are similar to those observed for radicicol. Therefore, it is possible that radicicol may affect hsp90, which in turn regulates the stability of Raf kinases. This prompted us to study whether radicicol could bind hsp90. Our preliminary data show that radicicol binds specifically to the chaperone protein hsp90. Furthermore, radicicol is able to compete for binding with geldanamycin to hsp90. Taken together, destabilization of Raf kinases by radicicol may result from the ability of the drug to inhibit the binding of hsp90 to Raf kinases. Thus, radicicol is the first non-benzoquinone ansamycin capable of binding to hsp90 and interfering with its function.

Radicicol has been reported to inhibit Mos-induced MAPK activation (12). Since Raf is probably not involved in this activation, molecular target(s) for the inhibition of Mos-stimulated MAPK activation could be some other factor(s), which could potentially interact with hsp90. The present observation that radicicol induced morphological reversion of KNRK5.2 cells at concentrations that were lower than those that affected the Raf-MAPK pathway also implies the presence of molecular target(s) that are not involved in the linear pathway from Ras to MAPK but are associated with cell morphological changes.

There is increasing evidence that Ras may mediate its action by stimulating multiple downstream targets, of which Raf is only one. Oncogenic Ras activation of Rac1 and RhoA, coupled with activation of the Raf/MAPK pathway, is required to trigger the full morphologic and mitogenic consequence of oncogenic Ras transformation (24–26). Dominant inhibitory mutants of Rac1 and RhoA block oncogenic ras-transforming activity and partially reverse the morphology of ras-transformed NIH3T3 cells. On the other hand, activated Rac1 and RhoA further enhance oncogenic ras-mediated morphologic transformation and cell motility (27). Thus, Rac- and Rho-induced changes in actin cytoskeleton could contribute significantly to oncogenic transformation of fibroblastic cells. If the same were true in epithelial cells, morphological reversion of KNRK5.2 cells at lower concentrations of radicicol may be due to the inhibition of some molecule(s) in the signaling pathway involving Rac and/or Rho. One of the components that links Ras and Rho is p120 Ras-GTPase-activating protein (GAP). The N-terminal region of GAP has been shown to regulate cytoskeletal structure and cell adhesion (28), and it is the site of interaction with two cytoplasmic phosphoproteins, p190 and p62 (29, 30). GAP-associated p190 protein itself functions as a GTPase activation protein for Rho (31, 32). GAP-associated protein p62, which has recently been cloned and renamed as p62\textsuperscript{gsh}, is a novel protein with features of a signaling molecule in a pathway downstream of receptor tyrosine kinases (33, 34). It should be noted that radicicol has previously been shown to inhibit the tyrosine phosphorylation of p62\textsuperscript{gsh} in ras-transformed NIH3T3 cells (12). Taken together with previous studies, radicicol might cause morphological reversion of KNRK5.2 cells by interfering with the interaction of hsp90 with some tyrosine kinase that is involved in the phosphorylation of p62\textsuperscript{gsh}.

Several lines of evidence show that inhibition of the function of Raf could lead to the disruption of an aberrant mitogenic signal originating from an activated ras gene. A loss-of-function mutation of the ras gene in Caenorhabditis elegans suppresses the phenotype resulting from an activated ras gene (35). Anti-sense oligodeoxynucleotides targeted against ras have been shown to suppress the growth of ras-activated human tumors inoculated into nude mice (36, 37). Our present results support the notion that Raf is a crucial pharmacological target for human malignancy that is closely associated with ras gene activation and highlight the possibility that pharmacological destabilization of Raf protein could be one potential approach for inhibiting the function of Raf in K-ras-activated human cancers.

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