Oncogenic H-Ras Enhances DNA Repair through the Ras/Phosphatidylinositol 3-Kinase/Rac1 Pathway in NIH3T3 Cells

EVIDENCE FOR ASSOCIATION WITH REACTIVE OXYGEN SPECIES*

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This study investigated the role of oncogenic H-Ras in DNA repair capacity in NIH3T3 cells. Expression of dominant-positive H-Ras (V12-H-Ras) enhanced the host cell reactivation of luciferase activity from UV-irradiated and cisplatin-treated plasmids and also increased the unscheduled DNA synthesis following cisplatin or UV treatment of cells. This observed enhancement of DNA repair capacity was inhibited by transient transfection with dominant-negative H-Ras (N17-H-Ras) or Rac1 (N17-Rac1) plasmids. Moreover, stable transfection of dominant-positive Rac1 (V12-Rac1) further enhanced DNA repair capacity. Because reactive oxygen species (ROS) are known to be a downstream effector of oncogenic Ras, we examined the role of ROS in DNA repair capacity. We found that ROS production by V12-H-Ras expression was mediated by the Ras/phosphatidylinositol 3-kinase (PI3K)/Rac1/NADPH oxidase-dependent pathway and that pretreatment of V12-H-Ras-transfected cells with an antioxidant (N-acetylcysteine) and an NADPH oxidase inhibitor (diphenyleneiodonium) decreased DNA repair capacity. Similarly, treatment with PI3K inhibitors (wortmannin and LY294002) inhibited the ability of oncogenic H-Ras to enhance DNA repair capacity. Furthermore, inhibition of the Ras/PI3K/Rac1/NADPH oxidase pathway resulted in increased sensitivity to cisplatin and UV in V12-H-Ras-expressing NIH3T3 cells. Taken together, these results provide evidence that oncogenic H-Ras activates DNA repair capacity through the Ras/PI3K/Rac1/NADPH oxidase-dependent pathway and that increased ROS production via this signaling pathway is required for enhancement of the DNA repair capacity induced by oncogenic H-Ras.

The cellular Ras protein, which is normally activated by growth factor receptors, is a mediator of those intracellular signaling pathways that are responsible for regulating cell proliferation (1, 2) and differentiation (3). Point mutation in the ras gene occurs at high frequency in mammalian cells, resulting in transformation and malignant progression to cancer, with oncogenic Ras mutations occurring in ∼30% of all human tumors (4). This active mutant form of Ras may induce drug resistance mechanisms, including enhanced DNA repair activity (5). Although a number of studies concerning the effect of Ras on DNA repair activity have been performed, the precise role of Ras in the regulation of DNA repair activity has not been fully elucidated. Several prior studies have provided evidence indicating that the Ras signaling pathway is involved in the down-regulation of DNA repair capacity (6, 7). However, others have suggested that Ras activation exhibits a resistance to cisplatin (8–10), which is associated with an increased DNA repair capacity for cisplatin-induced lesions (5). In this study, we therefore directly addressed the question of whether oncogenic Ras contributes to the regulation of DNA repair capacity. Because oncogenic Ras is known to participate in the development of carcinogenesis in many human cancers, understanding the molecular basis of oncogenic Ras-regulated DNA repair capacity could lead to strategies that improve anticancer therapeutic benefits.

Oxygen free radicals (ROS),¹ shown to participate in a number of human diseases such as cancer, neurodegeneration, and aging (11–14), have therefore been generally considered toxic to cells. However, recent studies have demonstrated that ROS play a role as second messengers in regulating mitogenic signal transduction in various cell types (15–18). More recently, ROS have been demonstrated to control a variety of Ras-mediated cellular effects, including cell transformation (19, 20), and have been shown to be involved in the modulation of DNA repair capacity (21–23).

In this study, we sought to determine whether oncogenic Ras is involved in the regulation of DNA repair capacity in NIH3T3 cells. The results show that expression of dominant-positive V12-H-Ras both protects NIH3T3 cells from UV- and cisplatin-induced cytotoxicity and enhances DNA repair capacity through the Ras/PI3K/Rac1/NADPH oxidase pathway, and increased ROS production via this signaling pathway is required for enhancement of the DNA repair capacity induced by oncogenic H-Ras.

EXPERIMENTAL PROCEDURES

Reagents—Cisplatin, N-acetylcysteine, and diphenyleneiodonium (DPI) were purchased from Sigma. 2’,7’-dichlorofluorescein diacetate (DCF-DA) was from Molecular Probes, Inc. (Eugene, OR). Anti-H-Ras and anti-Rac1 polyclonal antibodies were from BD Biosciences (San Diego, CA). PD98059, U0126, wortmannin, and LY294002 were ob-

¹ The abbreviations used are: ROS, reactive oxygen species; PI3K, phosphatidylinositol 3-kinase; DPI, diphenyleneiodonium; DCF-DA, 2’,7’-dichlorofluorescein diacetate; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase.
transfected from Calbiochem. Ponasterone A was purchased from Sigma.

**Cell Culture**—The NIH3T3 mouse embryo fibroblast line was obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen). The cells were cultured at 37 °C in a humidified chamber containing 5% CO2.

**Plasmid Constructs**—The wild-type H-ras and rac1 cDNAs were cloned by reverse transcription-PCR from human Jurkat cells. The dominant-positive and dominant-negative forms of H-Ras (V12-H-Ras and N17-H-Ras, respectively) and DNA-LipofectAMINE Plus reagent complexes at room temperature for 15 min; 2 μl of LipofectAMINE reagent was added; and the mixture was incubated at room temperature. After 15 min, semiconfluent NIH3T3 cells were washed twice with 1× PBS and then incubated with DNA-LipofectAMINE Plus reagent complexes at 37 °C in a humidified chamber containing 5% CO2 for 4 h. After transfection, the mixture was aspirated, and the cells were cultured in Dulbecco’s modified Eagle’s medium with 15% fetal bovine serum for an additional 24 h. Subsequently, cells were incubated with complete medium containing 400 μg/ml G418 for 5 weeks. Cell clones resistant to G418 were isolated and maintained with trypsin/EDTA, washed once with PBS, and resuspended in regular medium with 15% FBS for an additional 24 h. The HIV-LTR (Invitrogen) was used as an internal control for transfection efficiency. For host cell reactivation, transfected cells were cultured in complete medium containing 400 μg/ml G418 for 5 weeks. Cells resistant to G418 were isolated and analyzed.

**Transfection and Selection**—Cells were transfected by the LipofectAMINE method (Invitrogen) according to the manufacturer’s instructions. Briefly, 1 μg of pIND-V12-H-Ras or pIND-V12-Rac1 plasmid was mixed with 6 μl of LipofectAMINE Plus reagent for 20 min at room temperature for 15 min; 2 μl of LipofectAMINE reagent was added; and the mixture was incubated at room temperature for 24 h. After transfection, the cells were cultured in complete medium containing 400 μg/ml G418 for 5 weeks. Cells resistant to G418 were isolated and analyzed.

**Host Cell Reactivation Assay**—Host cell reactivation of luciferase activity was determined as described by Zeng et al. (24). The pGL3-Luc plasmid (Promega), in which the firefly luciferase gene is driven by the cytomegalovirus promoter, was used as an internal control for transfection efficiency. For host cell reactivation assays, the pGL3-Luc plasmid was damaged in vitro by either exposure to 200, 400, or 800 J/m² UV-C or treatment with 500, 750, or 1000 μmol cisplatin. V12-H-Ras- or V12-Rac1-transfected and nontransfected NIH3T3 cells were then transiently transfected with 1 μg of treated pGL3-Luc plasmid/well and 0.1 μg of pRL-CMV plasmid/well using the LipofectAMINE Plus method following the manufacturer’s instructions. After 4 h, cells were incubated in fresh medium with or without ponasterone A. The pRL-CMV plasmid was used to normalize for the total DNA transfected. After 4 h of transfection, the transfection medium was replaced with regular complete medium. In all cases, cells were collected 48 h after transfection, and cell extracts were used to determine luciferase activity. Luciferase activity values were quantified with a luminometer (Lumat LB9507, EG&G). To investigate whether the oncogenic H-Ras-coupled signaling pathway is required for the modulation of DNA repair, cells were cotransfected with either cisplatin-damaged or UV-irradiated pGL3-Luc and pRL-CMV in the presence of dominant-negative mutant N17-H-Ras, N17-H-Ras3, or N17-H-Ras3-N17-H-Ras plasmids. Cotransfection experiments were performed using a concentration of 2 μg of each plasmid/well. pRL-CMV was used to normalize for transfection efficiency. After 4 h of transfection, the transfection medium was replaced with regular complete medium.

**Unscheduled DNA Synthesis**—Unscheduled DNA synthesis of a population of cells in 96-well plates was measured as described previously (7). Cells were seeded onto plates and then washed twice with PBS, and regular medium was substituted for arginine-free medium (MEM Select-Amine, Invitrogen). After 24 h, the medium was changed to fresh arginine-free medium containing 1% serum for an additional 48 h. Cells were washed with serum-free media and resuspended in regular medium with 15% FBS for an additional 4 h. Finally, cells were washed twice with cold PBS and resuspended in regular medium with 15% FBS. A 10 μl sample of 10 Ci/ml [3H]dThd (specific activity of 20 Ci/mmol; Amersham Biosciences) for 2 h, washed twice with PBS, and then further cultured in fresh arginine-free medium containing 1% serum and 10 μCi/ml [3H]dThd for an additional 4 h. The next day, cells were washed twice with cold PBS and then resuspended in fresh regular medium with 15% FBS. After 1 h, the medium was harvested following the manufacturer’s instructions. Unscheduled DNA synthesis was estimated as the ratio of (dpm/μg DNA in drug-treated cells) to (dpm/μg DNA incorporated in control cells) × 100.

**Luciferase Activity Assays**—Transfected cells were washed twice with PBS and lysed in lysis buffer (FL600, Promega) with gentle shaking at room temperature for 15 min. Cell lysates were cleared from debris by centrifugation at 12,000 rpm for 5 min at 4 °C. Dual luciferase activity in the cell extracts was determined according to the manufacturer’s instructions (Promega). Briefly, each assay mixture contained 20 μl of cell lysate and 100 μl of firefly luciferase-measuring buffer (LAR II®, Promega). Firefly luciferase activity was measured with the luminometer. The luminometer was programmed to perform a 2-s preincubation and a 1-s delay before the readout (22). Data were normalized with the total luciferase activity measured. After measuring the firefly luciferase activity, the reaction mixture was added to 100 μl of Renilla luciferase-measuring buffer (Stop & Glo®, Promega). Renilla luciferase activity was measured. Renilla luciferase activity was used to normalize for transfection efficiency, and the relative luciferase activity was then calculated as a percentage of the experimental luciferase value relative to control levels using untreated pGL3-Luc reporter plasmids. All luciferase assays were performed in duplicate and repeated at least three times.

**Measurement of ROS**—ROS were measured using a previously described method (25) with some modification. Briefly, cells were plated at 1 × 10⁵/well in 60-mm dishes, treated for the indicated times, harvested with trypsin/EIPA, washed once with PBS, and resuspended in 5 μg/ml DCF-DA in Hank’s balanced salt solution. Samples were incubated for 10 min at 37 °C, and the 2′,7′-dichlorofluorescein (DCF) fluorescence intensity was measured with a fluorescence plate reader (FL600, Bio-Tek) (excitation wavelength, 485 nm; emission wavelength, 530 nm).

**Western Blotting**—Cells were centrifuged, washed with PBS, and lysed at 0 °C for 30 min in lysis buffer (20 mM Heps (pH 7.4), 2 mM EGTA, 50 mM NaCl, 1% Triton X-100, 10% β-mercaptoethanol, 1 mM dithiothreitil, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, and 5 mM NaF). Protein content was determined using the Bio-Rad dye binding microassay, and 20 μg of protein/lane was electrophoresed on 12% SDS-polyacrylamide gels after boiling for 5 min in Laemmli sample buffer. Proteins were blotted onto Hybond ECL membranes (Amersham Biosciences). Colored molecular weight markers (Bio-Rad) were used as size standards. After electroblotting, the membranes were blocked with Tris-buffered saline and Tween 20 (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) containing 5% milk and incubated with the primary antibody diluted in blocking buffer for 1 h. Primary antibody dilutions were those recommended by the manufacturer. Membranes were then washed, incubated with the appropriate secondary antibody (1:3000) in blocking buffer for 1 h, and re-washed. Blotted proteins were detected using the enhanced chemiluminescence detection system (Amersham Biosciences).

**Clonogenic Cell Survival Assay**—Cells were seeded at 4 × 10⁴/25-cm² culture flask and incubated at 37 °C in a 5% CO2 atmosphere. Cells were then treated with cisplatin or UV-C, washed twice with PBS, trypsinized, and resuspended in fresh medium. They were counted with a Coulter counter, and the number of cells required for plating was obtained by successive dilutions in fresh complete medium. The cells were plated in triplicate in 100-mm Petri dishes over a homologous feeder layer formed 24 h previously by plating 5 × 10⁴ irradiated cells. Cells were then allowed to grow at 37 °C in a 5% CO2 atmosphere for 14 days. Fresh medium was added on day 7. On day 14, cultures were fixed with methanol and stained with Giemsa. The number of colonies exceeding 50 cells was counted under a microscope. Colony formation was determined as the ratio of the number of colonies observed after treatment to the number of cells seeded, adjusted to the plating efficiency.

**Data Analysis**—Data in all experiments are represented as means ± S.E. Statistical comparisons were carried out using two-tailed Student’s t test. p values <0.05 were considered to be statistically significant.
Experimental Procedures.

FIG. 1. Enhancement of host cell reactivation in oncogenic H-Ras-expressing NIH3T3 cells. NIH3T3 cells were transfected with the oncogenic H-Ras-inducible expression vector pIND-V12-H-Ras. Cells were then selected in the presence of G418 as described under “Experimental Procedures.” V12-H-Ras-expressing NIH3T3 cells were prepared following ponasterone A treatment for the indicated times. A total of 20 μg of cellular protein was separated by 12% SDS-PAGE, and then H-Ras was analyzed, transferred to Hybond ECL membrane, immunoreacted with anti-H-Ras antibody, and detected by ECL (A). V12-H-Ras-expressing NIH3T3 cells were transfected with 1 μg of either UV-irradiated (B) or cisplatin-treated (C) pGL3-Luc reporter plasmid and 0.1 μg of pRL-CMV. After 4 h of transfection, the cells were incubated either with or without 5 μM ponasterone A (Pon A) for an additional 48 h, and luciferase activities were measured. Cotransfection with the Renilla luciferase plasmid was used to normalize for transfection efficiency. Each point is the mean of at least three independent observations, and the error bars indicate ±S.D. All p values were <0.05 versus the specific parental control.

RESULTS

Expression of Oncogenic H-Ras Enhances the DNA Repair Capacity in NIH3T3 Cells—To understand the relationship between oncogenic H-Ras and the modulation of DNA repair capacity in NIH3T3 cells, the cells were stably transfected with oncogenic H-Ras through the pIND-V12-H-Ras plasmid construct under the control of the ecodyson-responsive minimal promoter. Following selection by G418 at 400 μg/ml for 5 weeks, we isolated several clones and analyzed H-Ras expression that was able to be turned on or off using ponasterone A. A particular NIH3T3-expressed V12-H-Ras clone, which expresses oncogenic H-Ras, was chosen for further study in this investigation. Western blot analysis demonstrated that the addition of 5 μM ponasterone A efficiently induced expression of V12-H-Ras. V12-H-Ras expression became detectable within 6 h of ponasterone A treatment, and the level of H-Ras protein was continuously elevated for the duration of ponasterone A treatment (Fig. 1A). To investigate whether expression of V12-H-Ras has any effect on DNA repair capacity, we used the host cell reactivation of luciferase activity, which reflects the capacity of cells to repair plasmids damaged by UV or cisplatin. The pGL3-Luc reporter plasmid was treated with 200, 400, or 800 J/m² UV-C or with 500, 750, or 1000 nM cisplatin. V12-H-Ras-expressing NIH3T3 cells were transfected with untreated, UV-irradiated, or cisplatin-treated pGL3-Luc plasmid and then added to ponasterone A to induce V12-H-Ras expression. As shown in Fig. 1B and C, V12-H-Ras-expressing cells significantly enhanced host cell reactivation of UV-irradiated or cisplatin-treated luciferase activity. This enhancement of host cell reactivation could be reduced by transient transfection with dominant-negative N17-H-Ras (data not shown), suggesting that enhancement of host cell reactivation results specifically from expression of V12-H-Ras. To further verify the effect of oncogenic H-Ras on DNA repair capacity, we performed unscheduled DNA synthesis, which reflects the cellular ability to repair damage to genomic DNA. V12-H-Ras-expressing NIH3T3 cells were treated with 20 μM cisplatin or 2 kJ/m² UV-C in medium containing 10 μCi/ml [3H]dTd in the presence of absence of ponasterone A. As shown in Fig. 2, V12-H-Ras-expressing NIH3T3 cells were found to increase DNA repair synthesis by ~60% compared with non-expressing cells.

Enhancement of DNA Repair Capacity by Expression of Oncogenic H-Ras Is Mediated through the Ras/PI3K/Rac1 Pathway—It is known that Ras can trigger multiple signaling pathways. The Raf/MEK/ERK pathway is a major signal
transduction pathway activated by Ras, and PI3K is also one of the Ras effector molecules (26–30). To determine which pathway contributes to Ras-mediated DNA repair capacity, V12-H-Ras-expressing NIH3T3 cells were pretreated with the MEK inhibitors PD98059 and U0126 or the PI3K inhibitors wortmannin and LY294002. Pretreatment of V12-H-Ras-expressing NIH3T3 cells with wortmannin or LY294002 decreased host cell reactivation, whereas PD98059 or U0126 did not exert any effect on host cell reactivation (Fig. 3, A and B). These results suggest that PI3K activity may be involved, at least in part, in enhancement of the DNA repair capacity induced by oncogenic H-Ras in NIH3T3 cells. Because Rac1 is known to be the downstream effector of the Ras signal transduction pathway, we next examined whether Rac1 participates in enhancement of DNA repair capacity in V12-H-Ras-expressing NIH3T3 cells. As shown in Fig. 4, transient transfection of V12-H-Ras-expressing NIH3T3 cells with dominant-negative N17-Rac1 decreased the host cell reactivation. To investigate further the effect of Rac1 on DNA repair capacity, NIH3T3 cells were stably transfected with dominant-positive V12-Rac1. A particular NIH3T3-expressed V12-Rac1 clone was chosen, and then V12-Rac1 expression was confirmed by Western blot analysis (Fig. 5A). As shown in Fig. 5 (B and C), V12-Rac1 expression resulted in enhancement of host cell reactivation in NIH3T3 cells compared with that in non-expressing cells. Taken together, these results suggest that the Ras/PI3K/Rac1 pathway may contribute in part to enhancement of the DNA repair capacity induced by oncogenic H-Ras in NIH3T3 cells.
by treatment with the NADPH oxidase inhibitor DPI. To investigate a linkage between MAPK activity and ROS generation in V12-H-Ras-expressing NIH3T3 cells, the cells were preincubated with wortmannin, LY294002, PD98059, or U0126, after which ROS production was measured. As shown in Fig. 7, treatment of V12-H-Ras-expressing NIH3T3 cells with the PI3K inhibitors wortmannin and LY294002, but not with the ERK inhibitors PD98059 and U0126, decreased ROS production. These results suggest that enhancement of the ROS production induced by oncogenic H-Ras in NIH3T3 cells is mediated through the Ras/PI3K/Rac1/NADPH oxidase pathway. Interestingly, although treatment of V12-H-Ras-expressing cells with the ERK inhibitors did not have any effect on the enhanced host cell reactivation, the PI3K inhibitors were able to reduce the host cell reactivation in V12-H-Ras-expressing NIH3T3 cells (Fig. 3). Therefore, intracellular ROS, which are produced by the Ras/PI3K/Rac1/NADPH oxidase pathway in NIH3T3 cells, may be involved in enhancement of DNA repair capacity. To investigate whether enhancement of the ROS production induced by oncogenic H-Ras may be associated with enhancement of DNA repair capacity, N-acetylcysteine and DPI were used to remove intracellular ROS in V12-H-Ras- and V12-Rac1-expressing NIH3T3 cells. Pretreatment of V12-H-Ras- and V12-Rac1-expressing NIH3T3 cells with 20 mM N-acetylcysteine or 500 nM DPI was found to effectively inhibit host cell reactivation of UV-irradiated or cisplatin-treated luciferase activity (Fig. 8). These results strongly support the
suggestion that intracellular ROS are required for enhancement of the DNA repair capacity induced by oncogenic H-Ras in NIH3T3 cells.

**Effect of Oncogenic H-Ras on Cellular Response to Cisplatin and UV**—To investigate the correlation between oncogenic H-Ras expression and the resistance to cisplatin and UV, V12-H-Ras- and V12-Rac1-expressing NIH3T3 cells were treated with various doses of cisplatin or UV in the presence or absence of ponasterone A, after which survival curves were obtained by clonogenic cell survival assays. As shown in Fig. 9 (A and B), the IC<sub>50</sub> values for H-Ras-non-expressing cells were ~26 μM cisplatin and 10 J/m<sup>2</sup> UV. However, V12-H-Ras- and V12-Rac1-expressing cells exhibited a significant resistance to cisplatin and UV. The IC<sub>50</sub> of cisplatin was 60 μM, and that of UV-C was 30 J/m<sup>2</sup>, representing 2.7- and 3-fold increases in resistance, respectively, compared with non-expressing cells. We next studied the effect of PI3K inhibitors and ROS scavengers on cell survival against cisplatin and UV. As indicated in Fig. 9, both PI3K inhibitors D1P and N-acetylcysteine were able to effectively inhibit resistance to cisplatin and UV in V12-H-Ras-expressing NIH3T3 cells. These results indicate that there is a direct correlation between activation of the Ras/PI3K/Rac1/NADPH oxidase pathway and cell survival.

**DISCUSSION**

The major findings of this study are that DNA repair is enhanced by oncogenic H-Ras expression in NIH3T3 cells; that this enhancement of DNA repair occurs via a Ras/PI3K/Rac1/NADPH oxidase-dependent pathway; and, most importantly, that stimulation of ROS generation via this signaling pathway is required for enhancement of DNA repair activity. To our knowledge, the findings of this study represent the first evidence demonstrating that ROS serve as a Ras effector to enhance DNA repair activity.

Several prior studies have reported that oncogenic Ras is associated with altered cellular response to DNA damage and DNA repair. However, the results of these studies are contradictory. Overexpression of activated Ras in NIH3T3 cells resulted in an increase in resistance to DNA-damaging agents such as UV light and cisplatin (8, 32). This result was later confirmed independently by several other groups using NIH3T3 cells (9), rat rhabdomyosarcoma (33), human epithelial HBL-100 cells (5), human breast adenocarcinoma (34), and human HT-1080 fibrosarcoma (35). The mechanisms of the observed increase in Ras-mediated resistance to DNA-damaging agents are unclear, but may involve the influence of oncogenic Ras on enhancement of the DNA repair activity because the higher survival of the Ras-transformed cells appears to be associated with a lower amount of cisplatin- or UV-induced DNA lesions and a higher efficiency of DNA repair capacity (5, 36–39). Consistent with such an enhanced DNA repair activity for oncogenic Ras, several groups have reported that the induction of activated Ras up-regulates Gadd45 and p53 (40), human ERCC-1 (excision repair cross-complementing gene-1) (41), ribonucleotide reductase (42), and human DNA helicase VII (43), which are believed to be involved in the DNA repair system. However, others have reported either no change or increased sensitivity to DNA-damaging agents in response to overexpression of active ras genes in NIH3T3 cells (44), Rat-1 fibroblast cell lines (45), and human ovarian carcinoma (46). In addition, Yen et al. (7) have shown that the modulation of ErbB-2 (receptor tyrosine kinase family) activity significantly enhances the cytotoxicity of cisplatin by mechanisms involving down-regulation of DNA repair, and this down-regulation of DNA repair is mediated by the Ras signaling pathway. More recently, two groups have reported that oncogenic Ras has no effect on the DNA repair activity (46, 47). In this study, we tried to determine the role of activated V12-H-Ras in the regulation of DNA repair activity in NIH3T3 cells. To better study the potential role of oncogenic H-Ras in the DNA repair capacity, NIH3T3 cells were stably transfected with the pIND-V12-H-Ras plasmid under the control of ponasterone A. In this study, we have demonstrated that expression of oncogenic H-Ras enhances host cell reactivation of luciferase activity from UV-irradiated and cisplatin-treated pGL3-Luc reporter plasmids (Figs. 1 and 2) and that dominant-negative N17-H-Ras blocks V12-H-Ras-mediated enhancement of host cell reactivation (data not shown). We also have shown that oncogenic H-Ras expression leads to an increase in the unscheduled DNA synthesis in UV-irradiated or cisplatin-treated NIH3T3 cells (Fig. 2). These results have provided evidence that expression of oncogenic V12-H-Ras is involved in enhancement of DNA repair activity in NIH3T3 cells.

Recent research has suggested a linkage between MAPK and DNA repair systems. For example, ERCC-1, which is required for the excision step necessary to remove damaged DNA, is induced by the activation of the Ras/ERK-dependent pathway...
Hepatocyte growth factor significantly enhances the DNA repair of DNA strand breakage, and this enhancement is mediated by the PI3K and c-Akt signaling pathways (48). Moreover, the tumor suppressor p53, which is known to be involved in enhancement of DNA repair, including nucleotide excision repair and base excision repair (49–53), activates the Ras/Raf/MAPK and PI3K/Akt pathways through up-regulation of the heparin-binding epidermal growth factor and thereby increases cell survival after DNA damage (54, 55). Thus, we have asked whether enhancement of DNA repair capacity induced by oncogenic H-Ras is primarily mediated through activation of a single branch, being either the Raf/MEK/ERK pathway or the PI3K pathway. In this study, we have demonstrated that the PI3K inhibitors wortmannin and LY294002 effectively inhibit V12-H-Ras-induced host cell reactivation of luciferase activity from UV-irradiated or cisplatin-treated pGL3-Luc reporter plasmids, whereas the ERK/MAPK inhibitors PD98059 and U0126 do not (Fig. 3). These results suggest that PI3K activity is involved, at least in part, in the oncogenic H-Ras-mediated DNA repair capacity. We also observed that transient transfection of dominant-negative N17-Rac1 reduced DNA repair capacity in V12-H-Ras-expressing NIH3T3 cells (Fig. 4) and that expression of dominant-positive V12-Rac1 increased DNA repair capacity (Fig. 5). Taken together, these results demonstrate that enhancement of DNA repair capacity by oncogenic H-Ras is mediated through the Ras/PI3K/Rac1-dependent pathway.

ROS have been demonstrated to serve as a downstream effector of Ras (19). However, the role of ROS in cellular signal transduction remains unknown. Ras-transformed NIH3T3 cells produce intracellular ROS in NIH3T3 cells (19), human keratinocyte HaCaT cells (20), and human lung WI-38VA-13 cells (31). This ROS production is thought to be mediated by Rac-dependent activation of NADPH oxidase, a multicompartment enzyme, and localized to the cell membrane in non-phagocytic cells. We confirmed that expression of oncogenic H-Ras significantly enhanced ROS production and that this enhancement of ROS production was blocked by transient transfection of dominant-negative N17-Rac1 as well as by treatment with...
The correlation of enhanced DNA repair capacity with increased intracellular ROS. A role for ROS in enhancement of DNA repair capacity by activation of the Ras/Pi3K/Rac1 signaling pathway was suggested by the finding that transient transfection of dominant-negative N17-Rac1 and inhibition of PI3K activation with a PI3K inhibitor (wortmannin or LY294002) led to the attenuation of ROS production (Figs. 3 and 4) as well as a decrease in DNA repair activity (Figs. 6A and 7). Furthermore, overexpression of V12-Rac1 caused an increase in ROS generation (Fig. 6A) as well as an enhancement of DNA repair activity (Fig. 5). Direct evidence for the ability of ROS to enhance DNA repair was obtained in V12-H-Ras-expressing NIH3T3 cells using the antioxidant N-acetylcysteine (NAC), after which cells were treated with different doses of UV (A) or cisplatin (B). The percentage of cell survival was determined by clonal cell survival assays as described under “Experimental Procedures.” Each point represents the mean of four independent experiments. All p values were <0.05 versus the specific parental control.

DPI, an NADPH oxidase inhibitor (Fig. 6). Moreover, we found that expression of dominant-positive V12-Rac1 led to stimulation of ROS production (Fig. 6A). Thus, the results of our study, together with those of previous studies, suggest that ROS production by oncogenic H-Ras is mediated by the Ras/Rac1/NADPH oxidase pathway. NADPH oxidase-mediated bursts in neutrophil cells are mediated by p38 MAPK activity (56), and PI3K is required for the platelet-derived growth factor-induced production of hydrogen peroxide in non-phagocytic cells (57). More recently, Liu et al. (31) have established that protein-tyrosine kinase activity is required for superoxide production by activated H-Ras expression in human lung WI-38VA-13 cells. In this study, however, we found that treatment of V12-H-Ras-expressing NIH3T3 cells with a PI3K inhibitor (wortmannin or LY294002), but not with an ERK inhibitor (PD98059 or U0126), decreased V12-H-Ras-mediated ROS production (Fig. 7). These results suggest that enhancement of ROS production by oncogenic H-Ras is mediated through the Ras/Pi3K/Rac1/NADPH oxidase pathway.

The correlation of enhanced DNA repair capacity with induced oncogenic H-Ras expression as well as with elevated levels of ROS led us to hypothesize that raising the level of oncogenic H-Ras may enhance DNA repair capacity via increased intracellular ROS. A role for ROS in enhancement of DNA repair capacity by activation of the Ras/Pi3K/Rac1 signaling pathway was suggested by the finding that transient transfection of dominant-negative N17-Rac1 and inhibition of PI3K activation with a PI3K inhibitor (wortmannin or LY294002) led to the attenuation of ROS production (Figs. 3 and 4) as well as a decrease in DNA repair activity (Figs. 6A and 7). Furthermore, overexpression of V12-Rac1 caused an increase in ROS generation (Fig. 6A) as well as an enhancement of DNA repair activity (Fig. 5). Direct evidence for the ability of ROS to enhance DNA repair was obtained in V12-H-Ras-expressing NIH3T3 cells using the antioxidant N-acetylcysteine and the NADPH oxidase inhibitor DPI. Because the ras oncogene produces ROS via the NADPH oxidase-dependent pathway, inhibition of NADPH oxidase can inhibit the major source of ROS generation in oncogenic H-Ras-expressing NIH3T3 cells. In this experiment, removed intracellular ROS were associated with reduced DNA repair capacity (Fig. 8). These data allowed us to delineate the following relationship: oncogenic H-Ras → PI3K → Rac1 → NADPH oxidase → stimulates DNA repair capacity in NIH3T3 cells (Fig. 10).

Enhanced DNA repair capacity can contribute to drug resistance, and inhibition of DNA repair can enhance cytotoxicity and induce apoptosis (36–39). In our system, expression of oncogenic H-Ras significantly increased resistance to cisplatin and UV (Fig. 9). Upon blocking activated H-Ras-mediated ROS production, the observed cell resistance to cisplatin and UV treatment suggested that ROS act as a cisplatin and UV resistance signal to promote cell survival. However, N-acetylcysteine treatment prevented cytotoxicity in a large number of cisplatin- and UV-treated cells. The effects of ROS on cellular activity appear to depend on the dose and cell type. A high ROS concentration (100 μM to mM) results in a parallel up-regulation of poly(ADP-ribose) polymerase, which is a marker of apoptosis (58). The concentration of ROS acting as signaling agents in the regulation of cell proliferation is in the nanomolar to micromolar range and is therefore significantly lower than the concentration necessary to induce apoptosis (59). Thus, ROS may exert different biologic effects, which are dependent on their intracellular concentration. UV-C and cisplatin are known to stimulate the generation of intracellular ROS. Thus, the high
level of UV and cisplatin treatment results in the generation of a large amount of ROS. Under these conditions, the physiological role of ROS may be related to the induction of apoptosis. The use of such high levels of UV-C and cisplatin raises concerns regarding the biologic relevance of the responses, as such concentrations can be markedly toxic for cells and thus lead to apoptosis.

ROS play a regulatory role in the cellular signaling pathway. Although a large number of signaling pathways are regulated by ROS, the signaling molecules targeted by ROS are far from clear. There is growing evidence, however, that transiently increased ROS production is functionally associated with the regulation of gene expression and the activation of transcription factors (60, 61). For example, ROS have been implicated in the activation of transcription factors such as nuclear factor-kB, AP-1 (activator protein-1), Sp1, Nrf2, and p53. Interestingly, the promoters for many DNA repair genes contain redox-sensitive transcription factor-binding sites. The promoter for the nucleotide excision repair gene (xeroderma pigmentosum A, B, C, and D and Cockayne’s syndromes A and B) contains Sp1, Ets1 (AP-1-like family), and p53 sites; that for human OGG1 (oxidoguanine-DNA glycosylase) contains Ets1 and Nrf2 sites; that for NTH1 (human endonuclease III homolog) contains Ets1 and Sp1 sites; and that for MSH2 (mismatch repair-related gene) contains p53, Ets1, and AP-1 sites. According to such promoter analysis experiments, these redox-sensitive transcription factor-binding sites are essential for their gene expression (62–66). Thus, it is likely that increased ROS production may be involved in the regulation of DNA repair activity through the activation of redox-sensitive transcription factors. In conclusion, this work has demonstrated that oncogenic H-Ras enhances DNA repair capacity in NIH3T3 cells and that this enhanced DNA repair capacity is required, at least in part, for increased ROS production, which is mediated by the Ras/Pi3K/Racl/NADPH oxidase pathway.

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