p21ras as a Common Signaling Target of Reactive Free Radicals and Cellular Redox Stress*

(Received for publication, April 24, 1995, and in revised form, June 26, 1995)

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Reactive free radicals have been implicated in mediating signal transduction by a variety of stimuli. We have investigated the role of p21ras in mediating free radical signaling. Our studies revealed that signaling by oxidative agents which modulate cellular redox status, such as H2O2, hemin, Hg2+, and nitric oxide was prevented in cells in which p21ras activity was blocked either through expression of a dominant negative mutant or by treating with a farnesyltransferase inhibitor, as assessed by NF-κB binding activity. Furthermore, the NF-κB response to these oxidative stress stimuli was found to be enhanced when cells from the human T cell line, Jurkat, were pretreated with L-buthionine-(S,R)-sulfoximine, an inhibitor of glutathione synthesis. We directly assayed p21ras and mitogen-activated protein kinase activities in Jurkat cells and found both of these signaling molecules to be activated in cells treated with the redox modulating agents. Blocking glutathione synthesis made cells 10- to 100-fold more sensitive to these agents. Finally, using recombinant p21ras in vitro, we found that redox modulators directly promoted guanine nucleotide exchange on p21ras. This study suggests that direct activation of p21ras may be a central mechanism by which a variety of redox stress stimuli transmit their signal to the nucleus.

Free radicals have been shown to play important roles in carcinogenesis by directly damaging DNA and acting as tumor promoters (1–4). Free radicals and redox stress are now thought to participate in cellular signaling (5–9), and, thus, additional targets may exist. The transcription factor NF-κB has been demonstrated to mediate signaling by reactive oxygen (5) and reactive nitrogen (10). The exact target of these species is unknown, although it has been postulated to be upstream of p21ras and involve tyrosine phosphorylation (8, 9, 11). We have previously identified G proteins (12), and particularly p21ras (13), as central targets by which nitric oxide transmits signals. Therefore, we explored whether p21ras is a more general target for reactive free radicals and senses cellular redox status.

EXPERIMENTAL PROCEDURES

Materials—The farnesyltransferase inhibitor, α-hydroxyfarnesylphosphonic acid, was obtained from Biomol (Plymouth Meeting, PA), and L-buthionine-(S,R)-sulfoximine, H2O2, phorbol 12-myristate 13-acetate (PMA), and L-buthionine-(S,R)-sulfoximine were generously provided by Dr. Daniel Manor, Dept. of Pharmacology, Cornell University.

Cell Culture and Treatment—The rat pheochromocytoma PC12 cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% horse serum. The p21ras(WT)-transfected PC12 line (14) was generously provided by Dr. Geoffrey Cooper, Dana Farber Cancer Institute, Boston, MA. The Jurkat line was maintained in RPMI 1640 containing 10% fetal bovine serum. Cells treated with inhibitors were resuspended in serum-free medium containing α-hydroxyfarnesylphosphonic acid (10 μM) or L-buthionine-(S,R)-sulfoximine (100 μM) 24 h prior to redox stress.

RESULTS AND DISCUSSION

Requirement of p21ras for Reactive Radical and Redox Signaling—We investigated the role of p21ras in mediating NF-κB transcription factor activation by reactive free radicals and redox modulators. We treated either wild type rat pheochromocytoma PC12 cells or PC12 cells expressing a dominant negative mutation in p21ras (14) with Hg2+, hemin, or H2O2. We found that wild type cells responded by activating NF-κB after 4 h of treatment, whereas p21ras-defective cells showed a greatly diminished or no response (Fig. 1A). Furthermore, p21ras-defective cells showed a greatly diminished or no response (Fig. 1A). Furthermore,
blocking p21\textsuperscript{ras} localization, and therefore function (17), by inhibiting farnesytransferase activity with \(\alpha\)-hydroxyfarnesylphosphonic acid (10 \text{ mM}) (18), led to a 50\% reduction of the NF-\(\kappa\)B response to Hg\(\text{II}\)\textsubscript{2} and sodium nitroprusside in the human Jurkat T cell line (Fig. 1B). These data suggest that p21\textsuperscript{ras} activity is essential for mediating NF-\(\kappa\)B binding activity. Bands were quantified via PhosphorImage analysis, and their relative counts/min are indicated (Rel. CPM). Arrow denotes migration of the NF-\(\kappa\)B-DNA protein complex.

Our previous work implicated G proteins, and particularly p21\textsuperscript{ras}, as targets of the reactive nitrogen species, nitric oxide (12, 13). Therefore, we directly assessed the activation state of p21\textsuperscript{ras} upon exposure of Jurkat cells to free radicals and redox modulators. We found that treatment of cells with \(\text{H}_2\text{O}_2\), Hg\(\text{II}\)\textsubscript{2}, or hemin led to recovery of an activated form of p21\textsuperscript{ras} as evidenced by an increase in GTP-bound p21\textsuperscript{ras} (Fig. 2). We then treated Jurkat cells for 24 h with \(\text{l}\)-buthionine (S,R)-sulfoximine, a specific inhibitor of \(\gamma\)-glutamylcysteine synthetase (19), the rate-limiting enzyme in glutathione synthesis, and thus depleted cells of this critical antioxidant. We found that depletion of intracellular glutathione with \(\text{l}\)-buthionine (S,R)-sulfoximine made cells 10- to 100-fold more sensitive to these agents (Fig. 2). Treatment of Jurkat cells with \(\text{l}\)-buthionine (S,R)-sulfoximine (100 \text{ \mu g/ml}) yields cells with less than 20\% of their original intracellular glutathione levels (8). We next examined whether a known downstream effector of p21\textsuperscript{ras} was also activated by redox stress. We found that MAP kinase

![Fig. 1. Effect of p21\textsuperscript{ras} inhibition on free radical signaling.](image1)

![Fig. 2. Effect of cellular redox stress on endogenous p21\textsuperscript{ras} activity.](image2)
immunoprecipitated from cells treated with hemin or H$_2$O$_2$ for 30 min had an enhanced ability to phosphorylate myelin basic protein (Fig. 3). Furthermore, pretreatment of cells with L-buthionine-(S,R)-sulfoximine greatly enhanced their MAP kinase activity (Fig. 3). The close correlation between p21$\text{ras}$ and MAP kinase activation in response to oxidative stimuli suggest that this pathway is indeed important in transmitting redox stress signals. Furthermore, downstream signaling in response to Hg$^{2+}$ and sodium nitroprusside, a nitric oxide-generating compound, was profoundly enhanced by L-buthionine-(S,R)-sulfoximine treatment, as assessed by the NF-$\kappa$B binding assay (Fig. 4). Therefore, depletion of intracellular glutathione resulted in enhanced free radical signaling through p21$^{\text{ras}}$, MAP kinase, and NF-$\kappa$B. The disparity in the dose-response relationship between the ability of Hg$^{2+}$ and hemin to activate p21$^{\text{ras}}$ and NF-$\kappa$B may lie in the chemical nature of these reagents. Hemin is a known free radical generator which can catalytically generate oxygen-free radicals, whereas Hg$^{2+}$, a thiol-specific reagent, will stoichiometrically bind thiols. Therefore, differences seen in dose-response relationships between cell types may likely reflect their differential intracellular thiol content. These data suggest that the p21$^{\text{ras}}$ pathway acts to transmit cellular redox stress signals to the nucleus.

**In Vitro Studies**—We have previously demonstrated that NO could directly activate p21$^{\text{ras}}$ in vitro by interacting with a critical Cys residue (13). Therefore, we examined whether H$_2$O$_2$, hemin, and Hg$^{2+}$ could directly induce guanine nucleotide exchange on pure, recombinant p21$^{\text{ras}}$. We found that p21$^{\text{ras}}$, which was preloaded with GDP, had an enhanced ability to hydrolyze [γ$^{32}$P]GTP in the presence of these agents (Fig. 5). These data suggest that p21$^{\text{ras}}$ is a direct target of reactive free radicals and thus may be responsible for sensing cellular redox status. We have previously reported the mitogenic properties of iron compounds such as hemin on lymphocytes and implicated free radicals in the cell signaling process.
radical generation as a mechanism of activation (20, 21). HgCl₂, a thiol-binding agent, was also shown to be mitogenic to lymphocytes (22, 23). The exact target of free radicals responsible for initiating the mitogenic event was unknown. Furthermore, the oncogenicity of free radicals is known to be mediated through both physical damage to DNA and their properties as tumor promoters (1–4). The recent identification of free radicals as second messengers (5–9) suggests that some of the pathophysiological consequences of free radical generation may be due to effects on the enzymes controlling signaling pathways. Our data suggest that free radicals can activate p21^{ras} and lead to a nuclear signal. Mutated and activated forms of p21^{ras} have been found in many human cancers (24) and, thus, it is possible that the apparent sensitivity of p21^{ras} to free radicals and redox status, which we have identified here, provide an additional mechanism by which free radicals promote oncogenesis.

A common mechanism by which cells respond to redox signals and initiate gene expression has been postulated (9, 11, 15), although the precise target remains unknown. Our data indicate that, under normal conditions, p21^{ras} may represent the cellular sensor for redox stress. Studies are currently underway to identify the exact molecular alteration on p21^{ras} generated by reactive free radicals.

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