A Redox-triggered Ras-Effector Interaction

RECRUITMENT OF PHOSPHATIDYLINOSITOL 3'-KINASE TO Ras BY REDOX STRESS*

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Free radical signal transduction is thought to play a crucial role in diverse physiological and pathological processes. These include receptor signaling, such as advanced glycation end product (AGE)1 (1) and platelet-derived growth factor-mediated signaling in smooth muscle cells, and the regulation of cytoplasmic and nuclear signals, such as stimulation of guanylyl cyclase, ion channels, and transcription factors (1–7). These events, particularly those regulated by the free radical nitric oxide, result in vasodilation, host defense, synaptic plasticity, and inhibition of smooth muscle cell proliferation (4, 8–11). The mechanisms by which free radicals participate in these processes remain largely unknown. Hence, investigating the various molecular targets that are involved in these processes will play a pivotal role in our mechanistic understanding of redox signaling.

We have reported a reversible interaction between redox modulators and the monomeric G protein Ras, resulting in Ras activation via GDP/GTP exchange. Redox modification of amino acid residue Cys118 of the Ras protein was found to be crucial for its activation (12–14). Ras has been reported to be activated by endogenous NO in endothelial cells and primary cortical cultures and exogenous NO in T cells and PC12 cells (13, 15, 16).

Activation-induced conformational changes in Ras result in its interaction with various signaling proteins, termed effectors. Ras has multiple effectors and, depending on the stimuli, one or more effector and downstream signals propagated by it are utilized. These effectors include phosphatidylinositol 3'-kinase, Raf-1, protein kinase C-ζ, Raf-GDS family, Rin1, AF6, diacylglycerol kinases, and mitogen-activated protein kinase kinase (17).

Here we examined signaling events immediate to redox-activated Ras by identifying Ras effectors. PI3K is an effector of GTP-bound Ras (18, 19) and is implicated in the regulation of many biological responses including cell survival, mitogenesis, differentiation, the oxidative burst, membrane ruffling, and glucose uptake (20–22). We have observed that NO induces a dramatic increase in glucose uptake in human peripheral blood mononuclear cells (23). Hence, we examined whether PI3K was regulated by redox signals.

Class IA PI3Ks are heterodimeric enzymes composed of a 110-kDa catalytic subunit (p110) and a SH2 domain-containing adaptor protein. Mammals have genes for three adaptor subunits (p85α, p85β, and p55γ) and three p110 subunits (p110α, p110β, and p110δ; Refs. 22, 24, and 25). Interaction of the adaptor subunit with phosphorylated tyrosine residues on receptors or membrane-localized docking proteins recruits PI3K to the plasma membrane. In addition, the class IA PI3Ks can also interact with GTP-bound Ras. p110 catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate at the 3’ position of the inositol ring (22). The lipid products have various downstream targets, including the serine-threonine kinase Akt (22, 26–28) and possibly the extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase (29).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The human T-cell line Jurkat was maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum in a 37 °C incubator with 5% CO2. Rat pheochromocytoma PC12 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 5% horse serum. Jurkat cells were transfected with the mutant RasC118S cDNA as described previously (14).

Immunoprecipitation and Western Blotting—Serum-starved cells (16–24 h) were treated with anti-CD3 antibody, sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP; Sigma), AGE-albumin (7), or phorbol myristate acetate (100 ng/ml) plus ionomycin (1 μM) for

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10 min. Cells were lysed in a buffer containing 20 mM Tris, pH 7.5, 137 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. 2 mg of total cell lysate were subjected to immunoprecipitation by adding agarose-conjugated antibodies to either p85α or Ras (Y13-259; Santa Cruz Biotechnology). Western blots were performed on the immunoprecipitates using polyclonal antibodies against either p85α, p110α, p110β, or p110δ or monoclonal antibodies against Ras or phosphotyrosine (PY20; Santa Cruz Biotechnology) using a standard procedure with development by enhanced chemiluminescence (Amersham). In all such experiments, blots were stripped and reprobed with the immunoprecipitating antibody to control for immunoprecipitating efficiency. All blots shown had equal amounts of specific protein in each lane.

**In Vitro PI3K Activity**—Serum-starved cells were treated with SNP, SNAP, or NO gas for 10 min. 1 mg of total cell lysate was immunoprecipitated with agarose-conjugated antibodies against p85α or Ras (Y13-259; Santa Cruz Biotechnology). Washed immunoprecipitates were incubated for 20 min with sonicated phosphatidylinositol (0.2 mg/ml) and kinase buffer containing 30 mM Heps, pH 7.4, 30 mM MgCl₂, 50 μM ATP, 200 μM adenosine, and 20 μCi of [γ-32P]ATP. The reaction was terminated by the addition of 1 M HCl. The phospholipids were then extracted by chloroform:meanol (1:1) and separated by thin-layer chromatography, and the radioactive spots were detected by autoradiography as described previously (30).

**Mitogen-activated Protein Kinase Assay**—Serum-starved cells (24 h, 5 × 10⁶ cells) were either untreated or pretreated with wortmannin (100 ng/ml for 2 h; Sigma), and then NO donors were added for 10 min at 37 °C. Afterward, the mitogen-activated protein kinase assay was performed with immunoprecipitates of agarose-conjugated anti-ERK1/ERK2 antibody (Santa Cruz Biotechnology) as described previously (13).

**Akt Kinase Assay**—Serum-starved cells were treated with SNP, SNAP, or AGE-albumin for 15 min. Before the addition of redox agents, some cells were pretreated with wortmannin (100 ng/ml) for 2 h at 37 °C. Cell lysates were subjected to ultracentrifugation at 100,000 × g for 1 h, and the membrane pellet obtained was dissolved in 1% Nonidet P-40. This was again subjected to ultracentrifugation at 100,000 × g for 1 h, and the supernatant was used as membrane preparation. It was immunoprecipitated with carboxyl-terminal specific anti-Akt antibody (Santa Cruz Biotechnology). Washed immunoprecipitates were subjected to an in vitro kinase assay using histone 2B (1 mg/ml) as a substrate and kinase buffer containing 20 mM Heps, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, 2 mM ATP, and 40 μCi of [γ-32P]ATP. The kinase assay was performed at room temperature for 30 min and terminated by the addition of 5× Laemmli buffer. Samples were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis. The dried gel was analyzed with a phosphorimager (31).
RESULTS

Co-immunoprecipitation of Ras and PI3K—Direct association of PI3K with Ras in response to reactive free radicals was assessed by immunoprecipitating Ras with monoclonal anti-Ras antibody (19) from total cell extract of Jurkat and PC12 cells and by immunoblotting these immunoprecipitates with antibodies to p85α. Treatment of Jurkat cells with the NO donors SNP, SNAP, and NO gas increased the amount of p85α co-immunoprecipitated with Ras in a concentration-dependent manner (Fig. 1, A and B). Similar results were obtained when PC12 cells were treated with AGE-albumin (Fig. 1C). AGE-albumin is known to trigger redox stress upon binding to its receptor (7). The level of association of Ras with PI3K upon redox stimulation was similar to that observed upon “classical” activation of T cells, such as via an antibody against the T-cell receptor-associated molecule CD3 (Fig. 1D). To confirm our results, we reversed the immunoprecipitating and the immunoblotting antibodies (Fig. 1E, top panel). To verify that the propagated signal was indeed redox regulated, we also pre-treated cells with L-buthionine-(S,R)-sulfoximine, an agent that depletes the major intracellular antioxidant, glutathione (13). Pretreatment with L-buthionine-(S,R)-sulfoximine dramatically enhanced the levels of Ras associated with p85α (Fig. 1E, bottom panel).

Having identified p85α as one component of the redox-triggered Ras-effector complex, we sought to identify the associated p110 isoforms. We found that SNP and SNAP induce p110β and p110δ to bind to Ras, with an optimal concentration in the range of 30–100 μM (Fig. 2). Interestingly, although p110α was clearly present in the cells, it did not associate with redox-activated Ras (Fig. 2). To examine the possibility that p110α was not found to be associated with redox-activated Ras because of disruption of a low-affinity interaction during immunoprecipitation, we utilized a different T-cell stimulus (32) and examined whether p110α associated with Ras. When cells were treated with the combination of phorbol myristate acetate and ionomycin for 10 min, a clear association of p110α, as well as p110β and p110δ, was found in Ras immunoprecipitates (Fig. 2, right panel). This indicates that redox-activated Ras has a selective preference for p110α and p110δ.

Our earlier studies identified Cys118 on Ras as a molecular target of reactive free radicals; hence, we created a mutant Jurkat cell line expressing Ras with a cysteine to serine mutation at position 118 (12, 14). As shown in Fig. 3, top panel, overexpression of the RasC118S mutant abrogates any NO-induced increase in p85α levels in Ras immunoprecipitates, indicating that NO binding to Ras at Cys118 is required to trigger a Ras-effector interaction. In addition, there was no increase in the levels of p110β and p110δ associated with Ras upon NO treatment of the RasC118S-expressing cells (data not shown). However as seen in Fig. 3B, the RasC118S mutant responds to a classical Ras activator (32). There was no significant difference in ERK1/2 activity when the wild type and mutant cells were stimulated with phorbol myristate acetate and ionomycin. But unlike wild type cells, the RasC118S mutant cells did not respond to NO stimulation. This implies that the RasC118S cells are insensitive to NO stimulation but not to other stimuli. We also tested Jurkat cells overexpressing Ras effector mutants in which amino acids 35 and 37 have been mutated to Ser and Gly (33). These are mutations that disrupt the ability of Ras to interact with downstream effector molecules. Ras immunoprecipitates from Ras effector mutant-expressing cells contained very little p85α when treated with NO (Fig. 3, middle and bottom panels).

Effect of NO on Lipid Kinase Activity of PI3K—We next investigated whether the redox-triggered association of PI3K to Ras results in activation of the lipid kinase activity. Using an in vitro assay on p85α immunoprecipitates, we observed a NO-induced increase in lipid kinase activity by measuring the conversion of phosphatidylinositol to phosphatidylinositol phosphate (Ref. 30; Fig. 4A). The concentration-dependent increase in lipid kinase activity in p85α immunoprecipitates was similar to that observed in Ras immunoprecipitates, indicating that the increase in the kinase activity was due to a Ras-PI3K interaction (Fig. 4B). This activation of PI3K was not accompanied by altered Tyr phosphorylation of p85α (data not shown). Furthermore, NO was unable to elicit any response in RasC118S mutant cells (Fig. 4C).

PI3K-regulated Downstream Signaling—We next investigated the impact of redox-activated PI3K on downstream signaling pathways. One of the known downstream targets of PI3K is the serine-threonine kinase Akt (22, 26–28). As seen in Fig. 4D, SNP and AGE-albumin treatment of cells resulted in enhanced Akt kinase activity, an effect that could be com-
potassium channels, N-methyl-D-aspartate receptor, caspases, the mammalian transcription factors nuclear factor κB and activator protein 1, and the bacterial transcription factors OxyR and SoxR (4). Unlike Ras, most redox-sensitive proteins are not involved in generating divergent cellular outcomes. Ras is a key element of various signaling pathways and is implicated in the regulation of proliferation and differentiation by tyrosine kinase and G protein-coupled receptors (17); thus, we attempted to identify the effectors involved in the signaling cascade propagated by redox-activated Ras.

In this report, experimental evidence suggests that one of the effectors recruited by redox-activated Ras is PI3K. SNP and AGE-induced an increase in levels of Ras and p85α in the immunoprecipitate complex of anti-p85α antibody and anti-Ras antibody, respectively, suggesting a redox-induced interaction of Ras and PI3K (Fig. 1, A–C). Augmentation of this interaction was observed when cells were pretreated with L-buthionine-(S,R)-sulfoximine, confirming the redox nature of the signal. Of interest is that we observed the Ras-effector complex best using the anti-Ras antibody Y13-259. This antibody is known to bind to the Ras effector region. In contrast, the anti-Ras antibody Y13-238 is a non-neutralizing antibody but did not immunoprecipitate a NO-dependent Ras-PI3K complex. It is possible that Y13-238 binds to a region of Ras at which PI3K binds and is thus neutralizing for the Ras-PI3K interaction.

Intriguingly, we observed a selective association of the catalytic subunit of PI3K. NO donors increased p110β and p110δ levels in co-immunoprecipitates of Ras with unaltered levels of p110α. The fact that phorbol ester and ionomycin can recruit all three catalytic isoforms of PI3K rules out the possibility that different affinities for each of the p110 isoforms for Ras in the co-immunoprecipitate was responsible for the apparent selectivity. The reason for such a differential interaction of p110 isoforms with Ras is unclear. It is in line, however, with the fact that the Ras-binding domain of the p110 catalytic subunits is the most divergent region in these otherwise highly homologous molecules. This may allow for a variety of interactions with small GTP-binding proteins (24). It is worthy of mention that p110α is a proto-oncogene involved in transformation (34), and although NO activates Ras, it does not have any mitogenic effect (23). The functional role of the p110β and p110δ isoforms is not yet clear. However, it would be consistent with our studies if these subunits played a role in the regulation of transcription factor activation.

Our earlier studies identified Cys118 on Ras as a molecular target of reactive free radicals (12, 14). To test our hypothesis that the binding of free radical species to Cys118 activates Ras and initiates signal transduction by recruiting PI3K, we created a mutant of Ras in which Cys118 was mutated to a serine residue (RasC118S; Ref. 14). Jurkat cells overexpressing RasC118S exhibited normal Ras-dependent signaling when stimulated with the classical Ras activators, phorbol ester and ionomycin (IONO; 1 μM) or SNP for 10 min and then subjected to the ERK1/2 assay. Data points are the mean and standard deviation of four to six experiments.

**DISCUSSION**

Post-translational modifications of proteins are crucial events in cell signaling. They include glycosylation, phosphorylation, and redox modifications such as the nitrosylation of cysteine residues. Oxidative and nitrosative agents are known to modulate the functions of proteins by modifying cysteine residues that are strategically located at catalytic or allosteric sites. Some of the proteins whose functions are regulated by modification of cysteine residues are Ras, calcium-dependent

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**Fig. 3.** A, NO-mediated PI3K interaction in cells expressing Ras mutants. Jurkat cells were stably transfected with plasmids expressing either wild type Ras (Ras-wt) or Ras with mutations in amino acids 35, 37, or 118 (RasT35S, RasE37G, and RasC118S). Cells were treated for 10 min before lysis and immunoprecipitation. Two experiments were performed for each condition with similar results. Representative blots are shown. B, selective response to Ras stimuli by RasC118S cells. Wild type and mutant cells were treated with phorbol myristate acetate (PMA; 100 ng/ml) and ionomycin (IONO; 1 μM) or SNP for 10 min and then subjected to the ERK1/2 assay. Data points are the mean and standard deviation of four to six experiments.
FIG. 4. Effect of redox stimuli on PI3K lipid kinase activity and downstream signaling molecules. Jurkat cells were treated with SNP for 10 min, and lipid kinase activity in p85α immunoprecipitates was measured (A) and compared with that in Ras immunoprecipitates (B). A comparison of PI3K activity in p85α immunoprecipitates from Jurkat cells expressing wild type or C118S mutant Ras is shown (C). A representative blot from three experiments (D) shows the effect of SNP and wortmannin (top panel, Jurkat cells) and AGE-albumin (lower panel, PC12 cells) on Akt activity. The effect of SNP and wortmannin on ERK1/2 activity in Jurkat cells is shown (E). Data points shown are the mean and standard deviation of four to six experiments.
The SNP-induced increase in lipid kinase activity of PI3K suggested that the physical interaction between Ras and PI3K was biologically significant (Fig. 4A). The increase in lipid kinase activity was similar in immunoprecipitates of both anti-p85α and anti-Ras antibodies, indicating that the stimulation in the kinase activity was due to a redox-activated Ras and PI3K interaction (Fig. 4B). The SNP-induced increase in lipid kinase activity was not observed in the cell line overexpressing RasC118S, again confirming a requirement for a NO-Ras in kinase activity was not observed in the cell line overexpressing PI3K interaction (Fig. 4C).

Akt kinase is a very important downstream target of PI3K. SNP and AGE both activated Akt kinase and recruited it to the membrane. The PI3K inhibitor, wortmannin, completely abrogated activation by SNP and AGE (Fig. 4D). SNP also activated ERK1/2 activity, another downstream target of PI3K; however, wortmannin could only partially inhibit this activation (Fig. 4E). These data suggest that PI3K is solely responsible for the redox signal between Ras and Akt kinase and is partially responsible for the signal between Ras and ERK1/2. It is likely that other effector pathways, such as Raf kinase, also contribute to the signaling between Ras and ERK1/2.

To date, the precise biochemical events triggered by redox agents have been ill-defined. In this report, we identify a Ras effector, PI3K, which orchestrates redox-triggered downstream signaling events. The rapid progress being made in understanding PI3K signaling will likely also have a major impact on the elucidation of crucial events in redox signaling. It will be very interesting to identify other effectors that redox-activated Ras may selectively recruit. This will help in understanding various physiological processes such as long-lasting neuronal responses and the cellular response to redox stress during host defense. Clinically, these studies may have far-reaching implications in intervention directed at various pathophysiological disorders in which redox signaling is thought to play crucial roles. These may include hypertension, diabetes, renal failure, atherosclerosis, and Alzheimer’s disease.

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