Nox1 Redox Signaling Mediates Oncogenic Ras-induced Disruption of Stress Fibers and Focal Adhesions by Down-regulating Rho*

Received for publication, October 5, 2006, and in revised form, March 16, 2007. Published, JBC Papers in Press, April 15, 2007, DOI 10.1074/jbc.M609450200

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Generation of reactive oxygen species (ROS) by Ras oncogene-induced NADPH oxidase (Nox) 1 is required for Ras transformation phenotypes including anchorage-independent growth, morphological transformation, and tumorigenesis, but the signaling mechanism downstream of Nox1 remains elusive. Rho is known to be a critical regulator of actin stress fiber formation. Nonetheless, Rho was reported to no longer couple to loss of actin stress fibers in Ras-transformed Swiss3T3 cells despite the elevation of Rho activity. In this study, however, we demonstrate that Rho is inactivated in K-Ras-transformed normal rat kidney cells, and that abrogation of Nox1-generated ROS by Nox1 small interference RNAs or diphenyleneiodonium restores Rho activation, suggesting that Nox1-generated oxidants mediate down-regulation of the Rho activity. This down-regulation involves oxidative inactivation of the low molecular weight protein-tyrosine phosphatase by Nox1-generated ROS, which in turn inactivates LMW-PTP through oxidative modification of target proteins. Recent studies revealed that membrane-associated flavoprotein complexes NADPH oxidases are, at least in part, responsible for these ROS productions and that their catalytic subunits constitute a family of Nox enzymes (Nox1–5 and Duox) homologous to gp91phox of a phagocytic oxidase. The Nox proteins catalyze transfer of electrons from NADPH to oxygen to generate superoxide $O_2^-$, which is rapidly dismutated to $H_2O_2$. Each Nox homologue exhibits a distinct cellular and tissue distribution pattern, suggesting that Nox isoforms may possess distinct, specific functional roles in cells. In terms of relationships with cancer development, elevated ROS production has been recognized in various malignant cells and Nox4 and Nox5 have been implicated in survival of tumor cells (4–7). As for Nox1, we demonstrated that the Ras oncogene induces constitutive expression of Nox1 through the Raf-MEK-MAPK pathway and that Nox1-generated ROS exert an essential mediating role for Ras oncogene transformation phenotypes including augmented cell growth, altered cell morphology, anchorage-independent growth, and tumorigenesis (8). This appears to account for the biological significance of elevated superoxide production observed in Ras-transformed cells (9).

The signaling pathway downstream of Nox1 involved in transformation remains to be established. Regarding actin cytoskeleton organization, ROS have long been implicated in cell migration and adhesion of endothelial cells (10, 11), but the detailed mechanism has not been clarified at the molecular level. Recent study has shown that during integrin-induced cell spreading in HeLa cells, integrin triggers Rac1-dependent ROS production, which in turn inactivates LMW-PTP through oxidation, accumulates the tyrosine-phosphorylated, active form of p190RhoGAP, its direct target, and leads to down-regulation of Rho (12). In the Nimunal et al. (12) study, they showed the involvement of Rac1-dependent ROS generation in regulation of Rho by utilizing the Rac1-Δns mutant lacking a carboxy-terminal effector-binding site that is required for activation of Nox2 (gp91phox) (13). The observation therefore seemed to indicate Nox2 as a critical oxidase in the cell spreading process. Recently, Rac1 was found to interact with not only Nox2 but also Nox1 among the Nox family enzymes (14, 15). The mode of act to act as signaling molecules in various physiological processes because of their regulated production in response to ligands, the existence of catalytic metabolism to terminate their signaling, and redox-dependent reversible modification of target proteins (1, 2). Recent studies revealed that membrane-associated flavoprotein complexes NADPH oxidases are, at least in part, responsible for these ROS productions and that their catalytic subunits constitute a family of Nox enzymes (Nox1–5 and Duox) homologous to gp91phox of a phagocytic oxidase. The Nox proteins catalyze transfer of electrons from NADPH to oxygen to generate superoxide $O_2^-$, which is rapidly dismutated to $H_2O_2$. Each Nox homologue exhibits a distinct cellular and tissue distribution pattern, suggesting that Nox isoforms may possess distinct, specific functional roles in cells. In terms of relationships with cancer development, elevated ROS production has been recognized in various malignant cells and Nox4 and Nox5 have been implicated in survival of tumor cells (4–7). As for Nox1, we demonstrated that the Ras oncogene induces constitutive expression of Nox1 through the Raf-MEK-MAPK pathway and that Nox1-generated ROS exert an essential mediating role for Ras oncogene transformation phenotypes including augmented cell growth, altered cell morphology, anchorage-independent growth, and tumorigenesis (8). This appears to account for the biological significance of elevated superoxide production observed in Ras-transformed cells (9).

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interaction with Rac1 could be different between the two Nox proteins because of the following reasons. First, Nox1 is structurally diverged from Nox2 although the catalytic domain is well conserved (16). Second, Nox1 and Nox2 require structurally distinct accessory proteins for their activities: NOXO1 and NOXA1 assemble with Nox1, whereas p40phox, p47phox, and p67phox with Nox2 (17). Finally, Nox1 is constitutively activated by Rac1, whereas Nox2 is activated through Rac1 in response to ligand stimulation (18). Therefore, direct identification of the Nox protein involved is of critical importance for understanding the mechanism of Rac1-regulated redox signaling.

In the present study, we focus on the role of Nox1-generated ROS production in changes of stress fiber formation and stabilization of cell adhesions upon Ras oncogene transformation. Two issues have to be considered. Contrasting with cell spreading in which integrin engagement to fibronectin induces ROS generation (12), Ras transformation prevents cell adhesions to the substrates by down-regulating integrins (19). As far as integrin action is concerned, the opposing events proceed between the two biological systems. Thus, a distinct mode of intracellular ROS-mediated signaling could be expected for Ras transformation compared with cell spreading.

Another issue is contribution of Rho family GTPases to loss of both stress fibers and cell adhesions characteristic of Ras-transformed cells. Despite a number of reports on morphological transformation, it is not well understood how Rho signaling is required for altered control of cytoskeletons in the transformed cells. Recently, Sahai et al. (20) have reported that H-Ras transformation activated Rho in Swiss-3T3 cells, and that activated Rho subsequently suppressed the cell cycle inhibitor p21
cdc4
d in growth control (21). Activated Rho no longer coupled to formation of actin stress fibers in the transformed cells. Instead, sustained ERK-MAPK signaling resulting from Ras activation down-regulated ROCK, leading to loss of stress fibers. Whereas the discovery unravels the unique cross-talk between Ras and Rho signaling, there might exist a different mechanism in K-Ras transformation of normal rat kidney cells where Rho is inactivated (the present study).

We demonstrate that K-Ras transformation reduces the level of active RhoGTP, and that depletion of Nox1-generated ROS reverts the reduction of the RhoGTP amount, suggesting the mediating role of Nox1-generated ROS in down-regulation of Rho. Furthermore, Nox1-generated ROS suppress the LMW-PTP-p190RhoGAP pathway and thereby inactivate Rho, leading to loss of stress fiber formation and focal adhesions. Unexpectedly, our study demonstrates that despite the loss of integrin dependence, oncogenic K-Ras transformation down-regulates Rho via a LMW-PTP and p190RhoGAP-dependent mechanism. In addition, our discovery, unlike the model described by Sahai et al. (20), reveals a new mechanism involving the Nox1-redox signaling-mediated down-regulation of Rho for altered stress fibers and focal adhesions in Ras-transformed cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—Normal rat kidney fibroblast (NKR) cells and Kirstein-Ras-transformed NRK (KNRK) cells were purchased from American Type Culture Collection (Manassas, VA). KNRK cell lines stably expressing scrambled RNAi (KNRKscrambled) and Nox1 siRNA (KNRKsiNox1 and KNRKsiNox1-M) have been isolated, characterized extensively, and designated as neg-1, siNox1-N-7, and siNox1-M-19 cell lines, respectively, in our previous study (8). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Rabbit anti-Rho antibodies and glutathione S-transferase (GST)-PAK1 agarose were purchased from UBI (Lake Placid, NY) and rabbit anti-p190RhoGAP from R&G, Minneapolis, MN). Mouse anti-HA antibodies, mouse anti-GFP antibodies, rabbit anti-vinculin antibodies, rabbit anti-cofilin antibodies, luminol, horseradish peroxidase, and rhodamine-phalloidin were obtained from Sigma. Diphenyleneiodonium chloride (DPI) was purchased from Calbiochem (La Jolla, CA).

**Plasmids**—pCDNA3.0-Nox1 (14) and pGEX-RBD derived from rhotekin (22) were previously described. Adenovirus vectors carrying HA-tagged wild-type LMW-PTP (Ad-HA-wt-LMW-PTP) and the HA-tagged catalytically inactive Cys
to Ser mutant (Ad-HA-LMW-PTP(C12S)) were previously described (23). pS3CAT carrying human catalase was provided by Dr. Finkel (24).

**RNA Interference of Rac1**—Rac1 siRNA was designed based on rat Rac1 nucleotide sequence, 5’-GGTCTAATTTCGCTTTCATCCATGATGAGG-3’, 5’-GCTTTTCC, and scrambled RNAi was from Ambion (Austin, TX). 250 pmol of Rac1 siRNA or scrambled RNAi was introduced into cells by using Oligofectamine (Invitrogen) according to the manufacturer’s instructions.

**Transfection and Immunoblotting**—Cells were transfected with the indicated vectors by using Lipofectamine2000 (Invitrogen) according to the manufacturer’s protocol and harvested for various assays 36–48 h after transfection. For immunoblotting, cells were lysed in extraction buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml aprotinin), lysates were clarified by centrifugation at 10,000 × g for 10 min and proteins were separated by SDS-PAGE. The protein blots were incubated with appropriate antibodies and protein bands were visualized by ECL (Amersham Biosciences) as described previously (7).

**Immunostaining**—Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained for actin with rhodamine-phalloidin or for vinculin and HA-LMW-PTP(C5) with the indicated first antibodies and fluorescein isothiocyanate-conjugated anti-rabbit or antmouse IgG. Stained cells were observed by a Zeiss confocal microscope.

**Virus Infection**—Cells were seeded and infected with adenovirus vectors (a multiplicity of infection of 100–400) with HA-wild type LMW-PTP (Ad-HA-wt-LMW-PTP), HA-LMW-PTP(C125) mutant (Ad-HA-LMW-PTP(C12S)) or without insert the next day as described previously (23). Cells were harvested 24–36 h after infection and subjected to various assays.

**Pull-down Assay**—Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 1 mM dithiothreitol, 10% glycerol, 1 mM PMSF) and the lysates were incubated with GST-RBD or GST-PAK1 (2 mg) coupled
to glutathione-Sepharose for 90 min at 4 °C after extensive washing with lysis buffer, the proteins retained to the resins were analyzed by immunoblotting with anti-Rho or anti-Rac1 antibodies.

5′-Fluorescein Iodoacetamide Labeling—Labeling was performed by the slight modification of the published method (25). Cells (5 × 10^5) were infected with Ad-HA-tagged wt-LMW-PTP for 24 h and lysed in lysis buffer (50 mM MES-NaOH, pH 7.5, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF) under anaerobic conditions. Lysates were clarified by centrifugation at 100,000 × g for 15 min. The labeling reaction was performed by the addition of 10 μM iodoacetamide-fluorescein (Molecular Probes, Eugene, OR) to lysates for 60 min at 4 °C. The labeled HA-wt-LMW-PTP proteins were immunoprecipitated with anti-fluorescein antibodies and the immunoprecipitates were analyzed by Western blotting with anti-HA antibodies.

Measurement of Reactive Oxygen Species—Production of reactive oxygen species was measured by nitro blue tetrazolium reduction assay as described before (8). In addition, luminol luminescence assay was used in some experiments. Cells were inoculated into 24-well plates and transfected with 250 pmol of scrambled RNAi or Rac1 siRNA. After 48 h, cells were washed once with Hanks’ buffer and incubated with Hanks’ buffer containing 0.8 unit of horseradish peroxidase and 200 μμ mol linolin for 20 min at 37 °C. Luminescence was quantified using luminescencer-PSN (Atto, Tokyo).

Phosphatase Activity Assay—The assay was performed by modification of the published method (26). Cells (1 × 10^5) were infected with Ad-HA-wt-LMW-PTP for 24 h and harvested cells were homogenized in lysis buffer (20 mM PIPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF) by passing through a 27-gauge needle in an anaerobic chamber. After clarification by high speed centrifugation, lysates were immunoprecipitated with anti-HA antibodies and immunoprecipitates of LMW-PTP were incubated with 100 μl of reaction buffer (0.1 M sodium acetate, pH 5.5, 10 mM EDTA) containing p-nitrophenyl phosphate (5 mM) as a substrate for 60 min at 37 °C. The production of p-nitrophenol was measured colorimetrically at 410 nm.

Isolation of Membranes—Cells were sonicated in hypotonic buffer (5 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM PMSF) for a few seconds. The lysates were fractionated by low speed centrifugation at 600 × g for 10 min, followed by a high speed centrifugation at 100,000 × g for 40 min. Isolated crude membrane pellets were solubilized in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) and clarified by high speed centrifugation. The samples were analyzed by immunoblotting.

RESULTS

Oncogenic Ras Signaling Down-regulates the Rho Activity through Nox1—We first determined whether Nox1 affects the activity of Rho in KNRK cells. The Rho activity was monitored by the GST-rhotekin pull-down assay in which active GTP-Rho complexes are selectively bound to GST proteins fused with the Rho-binding domain of rhotekin (GST-RBD). The Rho activity was down-regulated in KNRK cells compared with untransformed NRK cells (Fig. 1A). There are discrepant reports about the changes in the activity of Rho GTPase observed in Ras-transformed cells. Whereas the Rho activity is increased in Ras-transformed MDCK cells (27) and Ras-transformed Swiss-3T3 cells (20), Rho appears to be inactivated in HB4A human mammary epithelial cells (20) and Ras-transformed Rat1 fibroblasts (28). The reason for this discrepancy is not exactly understood at present and might reflect the difference in cellular and genetic backgrounds. When KNRK cells were treated with DPI, a specific flavoprotein inhibitor, an increase in Rho activity was detected (Fig. 1B). Consistent with this, Rho activity was enhanced in the KNRK cell lines stably expressing different small interfering Nox1 RNA sequences (KNRKsiNox1 and KNRKsiNox1-M) compared with a KNRK cell line carrying scrambled siRNA (Fig. 1C). Consistent with this, actin stress fibers and focal adhesions were restored in both clones (see below). As the results make it very unlikely that the observed effects of siRNAs are the results of an unusual cell clone or off-target effect from the siRNAs used, we mostly focus on KNRKsiNox1 cells in the subsequent study. Our previous study demonstrated that generation of superoxide is augmented in KNRK cells compared with NRK cells due to constitutive induction of Nox1 expression by Ras oncogenes via the Raf-MEK-MAPK pathway (8). Furthermore, this increased oxidant production was abolished by silencing Nox1 in KNRKsiNox1 cells (Fig. 1, D and E). We therefore conclude that Nox1-produced ROS are required for down-regulation of Rho by activated Ras.

In the control experiment, treatment of KNRK cells with a MAPKK inhibitor, PD98059, increased the amount of GTP-Rho (Fig. 1F), indicating that inhibition of MAPK signaling restores Rho activity, which is in agreement with our speculation (Fig. 6) that Ras-MAPK signaling down-regulates Rho activity through induction of Nox1. In an attempt to relate Rac to the regulation of Rho activity, we examined the level of GTP-Rac1 in KNRK cells by using the GST-PAK-1 pull-down assay. The data show that the level GTP-Rac1 decreased in KNRK cells compared with NRK cells (Fig. 1G), demonstrating that oncogenic Ras down-regulates the Rac1 activity. We next sought to determine whether this low level of GTP-Rac1 still affects the regulation of Rho activity. siRNA-mediated inhibition of Rac1 restored the Rho activity in parallel with suppression of ROS generation in KNRK cells (Fig. 1, H and I). These data suggest that Rac1, although reduced in the active form, participates in down-regulation of the Rho activity together with oncogenic Ras-induced induction of Nox1.

We next examined the biochemical signaling pathway linking Nox1 to negative regulation of Rho. Accumulating evidence indicates that ROS affect the steady-state level of tyrosine phosphorylation of receptor tyrosine kinases and their substrates through reversible inhibition of protein-tyrosine phosphatases (29). As for Rho regulation, p190RhoGAP, a GTPase activating protein for Rho is activated following tyrosine phosphorylation, resulting in down-regulation of Rho (30). We therefore explored the effect of Nox1 on tyrosine phosphorylation of p190RhoGAP by estimating the level of tyrosine-phosphorylated p190RhoGAP. Immunoblotting analysis showed that DPI
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FIGURE 1. Suppression of the Rho activity by oncogenic Ras involves Nox1. A, comparison of Rho activity between NRK and KNRK cells. Cells (2 × 10⁶) were harvested 12 h after plating, cell lysates were prepared and the level of GTP-Rho was determined by using GST-RBD affinity resins. B, effects of DPI on Rho activity in KNRK cells. Cells (2 × 10⁶) were treated with DPI (1 or 5 µM) for 1 h and the level of GTP-Rho was determined as in A. C, Nox1 inhibition elevates Rho activity. Cell lysates were prepared from NRK cells, KNRK cells stably transfected with scrambled RNAi (KNRKscrambled), Nox1siRNA N-7 (KNRKsiNox1), or siNox1-M-19 (KNRKsiNox1-M) and subjected to a GST-RBD pull-down assay, followed by immunoblotting with anti-Rho antibodies. D, superoxide production in the indicated cell lines. The level of superoxide production was determined by utilizing the nitro blue tetrazolium reduction assay. E, immunoprecipitation and the immune complexes were subjected to immunoblotting with mouse monoclonal anti-Nox1 antibodies (provided by Dia Deus). Protein levels were normalized by immunoblotting with anti-β-actin antibodies. F, KNRK cells were treated or untreated with 100 µM PD98059 for 1 h and cell lysates were subjected to GST-RBD pull-down assay. The amount of total Rho and GST-RBD used were monitored as loading controls. G, Rac1 bound to the resins was analyzed by immunoblotting with mouse anti-Rac1 antibodies (UBI). H, KNRK cells (1.5 × 10⁶) were transfected with 250 pmol of scrambled RNAi or Rac1 RNAi for 48 h and lysates were subjected to GST-RBD pull-down assay. Total Rac1 was determined by anti-Rac1 antibodies. I, KNRK cells were incubated into a 24-well plate, transfected with scrambled RNAi or Rac1 RNAi for 48 h, and subjected to luminol luminescence assay. Data represent mean ± S.E. (n = 3).

FIGURE 2. DPI treatment and Nox1siRNA decrease tyrosine phosphorylation of p190RhoGAP in KNRK cells. Lysates were prepared from KNRK cells treated with DPI (5 µM) for 1 h or KNRKsiNox1 cells. p190RhoGAP was immunoprecipitated (IP) with mouse anti-phosphotyrosine antibodies (4G10). The immunoprecipitates were subjected to immunoblotting (IB) with anti-p190RhoGAP antibodies to detect tyrosine phosphorylation of p190RhoGAP. The p190RhoGAP protein levels of extracts were measured by immunoblotting. DPI treatment and Nox1siRNA decrease tyrosine phosphorylation of p190RhoGAP, and that the level of tyrosine phosphorylation of p190RhoGAP was reduced in KNRKsiNox1 cells compared with KNRKscrambled cells (Fig. 2). Thus, Nox1-derived ROS could augment tyrosine phosphorylation of p190RhoGAP, which presumably leads to down-regulation of Rho.

Nox1 Signaling Causes Oxidative Inactivation of LMW-PTP—We next sought to identify a sensor for Nox1-generated ROS that transmits an activation signal to p190RhoGAP. Given recent observations that LMW-PTP deactivates p190RhoGAP, we investigated whether LMW-PTP is regulated by Nox1-produced ROS. To this end, we used the 5′-iodoacetamide fluorescein labeling approach in which the iodoacetamide derivative specifically alkylates a Cys-SH residue that is susceptible to oxidation by intracellular ROS (25). The labeling reaction was performed following infection of adv-HA-LMW-PTP into cells. The significant amount of labeled LMW-PTP was detected in KNRKsiNox1 cells, whereas little or no labeling of the protein was observed in KNRKscrambled cells (Fig. 3A), suggesting that the Cys-SH of LMW-PTP was oxidized by Nox1-generated ROS. To investigate whether this oxidation alters the activity of LMW-PTP, exogenously expressed HA-LMW-PTP was immunoprecipitated and the immune complexes were sub-
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LMW-PTP Mediates Nox1-induced Down-regulation of Rho—To further assess the involvement of LMW-PTP in regulation of Rho by Nox1-redox signaling, we examined the effect of LMW-PTP overexpression on the Rho activity. KNRK cells overexpressing LMW-PTP reduced the active, tyrosine-phosphorylated form of p190RhoGAP (Fig. 3C) and in parallel increased the amount of active Rho-GTP (Fig. 3D), suggesting that overexpression of LMW-PTP bypassed the Nox1-dependent inactivation and reactivated Rho possibly through dephosphorylation of p190RhoGAP in KNRK cells.

Our above experiment demonstrated Nox1-dependent oxidative inactivation of LMW-PTP (Fig. 3, A and B). Because H$_2$O$_2$ is known to cause oxidation of Cys$^{12}$ and Cys$^{17}$, the two vicinal cysteines in the catalytic pocket of LMW-PTP, to form a disulfide bond and thereby suppress its enzymatic activity (31), it is most likely that a dominant-negative mutant at the active Cys residues of LMW-PTP mimics the effect of Nox1 on Rho signaling. When KNRKsiNox1 cells were transfected with LMW-PTP$^{C12S}$, a catalytically inactive, Cys$^{12}$ to Ser mutant of LMW-PTP, the Rho activity was significantly reduced (Fig. 3E). In contrast, transfection of LMW-PTP$^{C12S}$ into KNRKscrambled cells did not alter Rho activity (Fig. 3E), because endogenous LMW-PTP has already been inactivated by Nox1-generated ROS in the cells. As Nox1 expression is increased in KNRK cells (8), these results indicate that inactivation of LMW-PTP by Nox1-derived ROS-induced down-regulation of Rho.

Nox1 Signaling Mediates Ras Oncogene-induced Suppression of Stress Fibers—Cell transformation by Ras oncogene is accompanied by profound alteration in actin cytoskeleton organization, in which actin stress fibers are disassembled and the cell adhesion activity is reduced, as manifested in morphological changes and augmented anchorage-independent growth (28). This process is triggered by down-regulation of Rho, a pivotal factor in the formation of stress fibers and focal

j ected to protein-tyrosine phosphatase assay. As expected, the protein-tyrosine phosphatase activity of LMW-PTP immunoprecipitates from KNRKsiNox1 cells was greater than that from KNRKscrambled cells (Fig. 3B). Together, these data support the notion that Nox1-generated ROS inactivate LMW-PTP through oxidation of the Cys-SH residue.

In additional experiments, LMW-PTP was exogenously overexpressed in KNRKscrambled or KNRKsiNox1 cells and cell lysates were incubated with iodoacetamide. LMW-PTP that is not exposed to ROS in KNRKsiNox1 cells will be irreversibly alkylated at the SH group of active Cys residues with iodoacetamide. By contrast, LMW-PTP in which active Cys residues have been oxidized by Nox1-generated ROS in KNRKscrambled cells could be resistant to alkylation. When after recovery by dithiothreitol reduction, the activities of PTPs in cell lysates were assayed using poly(Glu$_4$-Tyr(P)) peptides as the substrate, PTPs oxidation in KNRKscrambled cells increased compared with KNRKsiNox1 cells by approximately 50%. This also suggests that Nox1-generated ROS are involved in regulation of PTPs.

LMW-PTP by using anti-HA antibodies or control IgG and the immune complexes were precipitated from KNRKscrambled or KNRKsiNox1 cells infected with Ad-HA-wt-LMW-PTP by using anti-HA antibodies. LMW-PTP was exogenously overexpressed in KNRKscrambled or KNRKsiNox1 cells and cell lysates were incubated with iodoacetamide. LMW-PTP that is not exposed to ROS in KNRKsiNox1 cells will be irreversibly alkylated at the SH group of active Cys residues with iodoacetamide. By contrast, LMW-PTP in which active Cys residues have been oxidized by Nox1-generated ROS in KNRKscrambled cells could be resistant to alkylation. When after recovery by dithiothreitol reduction, the activities of PTPs in cell lysates were assayed using poly(Glu$_4$-Tyr(P)) peptides as the substrate, PTPs oxidation in KNRKscrambled cells increased compared with KNRKsiNox1 cells by approximately 50%. This also suggests that Nox1-generated ROS are involved in regulation of PTPs.

FIGURE 3. Nox1 causes oxidation and inactivation of LMW-PTP resulting in down-regulation of Rho. A, KNRKscrambled or KNRKsiNox1 cells were infected with Ad-HA-tagged wt-LMW-PTP for 24 h, cell lysates were prepared, and proteins were labeled with S'-iodoacetamide fluorescein. The labeled HA-wt-LMW-PTP was immunoprecipitated (IP) with anti-fluorescein antibodies (anti-Fluo), followed by immunoblotting (IB) with mouse anti-HA antibodies. The expression of transfected HA-wt-LMW-PTP in the cells was analyzed by immunoblotting with anti-HA antibodies. B, LMW-PTP was immunoprecipitated from KNRKscrambled or KNRKsiNox1 cells infected with Ad-HA-wt-LMW-PTP by using anti-HA antibodies or control IgG and the immune complexes were subjected to protein-tyrosine phosphatase assay. The data indicate the activity relative to the one in anti-HA immunoprecipitates from KNRKsiNox1 cells (mean ± S.E. (n = 3)). The presence of an equal amount of LMW-PTP in immunoprecipitates was verified by immunoblotting with anti-HA antibodies. C and D, overexpression of LMW-PTP inhibits tyrosine phosphorylation of p190RhoGAP and induces Rho up-regulation in KNRK cells. KNRK cells were infected with Ad-HA-tagged wt-LMW-PTP or control adenovirus and lysates were subjected to either immunoblotting with anti-p190RhoGAP antibodies after immunoprecipitation with anti-phospho-tyrosine antibodies (C) or the Rho activity assay by using GST-RBD affinity resins (D). The amount of GTP-Rho and total Rho was measured by immunoblotting with anti-Rho antibodies. Expression of wt-LMW-PTP was verified by immunoblotting with anti-HA antibodies. E, cell lines, KNRKsiNox1, and KNRKscrambled were infected with increasing amounts (100, 200, or 400 multiplicity of infection) of Ad-HA-tagged LMW-PTP$^{C12S}$ or control adenovirus (200 multiplicity of infection) and lysates were subjected to Rho-GTP pull-down assay as in A. For A–E, expression of HA-tagged wt-LMW-PTP and HA-tagged LMW-PTP$^{C12S}$ was detected by immunoblotting with anti-HA antibodies.
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adhesions (32). As Nox1-redox signaling negatively regulated Rho in our study, we then determined the role of the signaling pathway in regulation of these two cytoskeletal dynamics following Ras transformation. Actin stress fiber formation was evident in NRK cells, but transformation of NRK cells by the K-Ras oncogene inhibited the formation of stress fibers (Fig. 4A). When the Nox1 activity in KNRK cells was suppressed by Nox1 RNAi or DPI treatment, there was an increase in stress fiber formation, suggesting that depletion of Nox1-generated ROS led to promotion of actin stress fibers through reactivation of Rho (Fig. 4A). To assess the ROS requirement for disruption of actin stress fibers, we co-transfected catalase, a scavenger of hydrogen peroxide and GFP as a transfection indicator into KNRK cells. Overexpression of catalase reversed the suppressive effect of Nox1 on stress fiber formation (70 ± 2.0% (n = 3) of transfected cells when stress fiber-positive, implicating H₂O₂, a product of superoxide as a potential mediator (Fig. 4B).

Because previous studies demonstrate that ROCK, a Rho-associated, coiled-coil forming protein kinase acts downstream of Rho to induce formation of actin stress fibers (33), the involvement of ROCK in Nox1-mediated rearrangement of actin cytoskeleton was examined. Whereas treatment of NRK cells with a ROCK inhibitor Y27632 blocked stress fiber formation, treatment of KNRK cells with the ROCK inhibitor had little or no effect (Fig. 4C). Stress fibers were transiently restored with DPI treatment (40 ± 2.0%, n = 3) in KNRK cells as compared with untreated cells (3.1 ± 0.5%, n = 3) and this DPI effect was attenuated by inhibition of ROCK with Y27632 (2.0 ± 0.1%, n = 3) (Fig. 4C). Likewise, the increased stress fiber formation in KNRKsiNox1 cells (85 ± 3.5%, n = 3) as shown in Fig. 4A was abolished by Y27632 treatment (7.0 ± 1.0%, n = 3) (Fig. 4C). These results argue that down-regulation of actin stress fiber formation by Nox1-generated ROS, at least in part, involves ROCK as a downstream effector for Rho. Because LIM kinase is thought to inactivate the actin-destabilizing protein cofilin through phosphoryl-

FIGURE 4. Nox1 is required for suppression of actin stress fibers in KNRK cells. A, NRK cells, KNRK cells, KNRKSscrambled cells, KNKRKsiNox1, or KNKRKsiNox1-M cells were cultured for 24 h. In some experiments, KNRK cells were treated with DPI (5 μM) for 24 h. Cells were fixed, permeabilized, and stained with rhodamine-phalloidin (Sigma). Arrowheads indicate actin fibers. KNKRKsiNox1 and KNKRKsiNox1-M cells exhibit 85 ± 1 and 79 ± 8% (n = 5) stress fiber positive, respectively. B, KNRK cells were transfected with pS3CAT (1.5 μg) or a control vector pS3 together with pEGFPN1 (0.5 μg) as a transfection indicator for 48 h. Cells were fixed and stained with mouse anti-GFP antibodies and rhodamine-phalloidin. Transfected cells are indicated by arrowheads. C, ROCK mediates restoration of actin stress fibers by Nox1 inhibition. NRK cells, KNRK cells, KNRK-scrumbled cells, or KNKRKsiNox1 cells were cultured and treated with or without Y27632 (10 μM) for 24 h. In some experiments, KNRK cells were treated with Y27632 (10 μM) plus DPI (5 μM). Cells were fixed and stained with phalloidin. D, cofilin phosphorylation was suppressed upon Ras transformation and siNox1 expression increases cofilin phosphorylation in KNRK cells. Lysates were prepared from NRK cells, KNRK cells, KNRK-scrumbled cells, or KNKRKsiNox1 cells. Levels of cofilin phosphorylation and the amount of total cofilin were analyzed by immunoblotting with anti-phospho-cofilin antibodies and anti-cofilin antibodies (Sigma), respectively. E, down-regulation of LMW-PTP by Nox1 is required for actin stress fibers. KNRKsiNox1 cells were infected with the Ad-HA-tagged LMW-PTP(Δ5) mutant for 36 h, fixed, permeabilized, and stained with rhodamine-phalloidin. The infected cells expressing LMW-PTP(Δ5) (arrowheads) were identified by staining with mouse anti-HA antibodies and fluorescein isothiocyanate-conjugated anti-mouse IgG.
expression of the LMW-PTP<sup>C125</sup> mutant blocked restoration of stress fiber formation in KNRKsiNox1 cells, indicating that down-regulation of LMW-PTP by Nox1 is a necessary step for disruption of action stress fibers in KNRK cells (Fig. 4E).

**Nox1 Signaling Is Required for Ras Oncogene-induced Suppression of Focal Adhesion**—In addition to deregulation of stress fiber formation, oncogenic Ras reduces cell adhesion to the extracellular matrix. To determine whether Nox1 participates in the control of focal adhesion assembly, we performed immunostaining utilizing antibodies against vinculin, a marker for formation of focal adhesions. Whereas the characteristic staining of punctuate vinculin was observed at focal contacts in NRK cells, no vinculin accumulation was found in KNRK cells (Fig. 5A). In contrast, either DPI treatment (46 ± 3.0%, n = 3) or silencing of Nox1 expression (76 ± 10% for KNRKsiNox1 and 82 ± 5% for KNRKsiNox1-M, n = 5) induced focal adhesion-like structures in KNRK cells as evidenced in vinculin enrichment at the peripheral region (Fig. 5A). Additionally, restoration of vinculin clustering in KNRKsiNox1 cells was prevented by the ROCK inhibitor (16 ± 3.0%, n = 3) (Fig. 5B). Because the

Rho activity was suppressed in KNRK cells, the data indicate that Nox1-produced ROS inhibit ROCK-dependent formation of focal adhesions by decreasing the Rho activity in KNRK cells. Moreover, accumulation of vinculin at focal points detectable in KNRKsiNox1 cells was abrogated by expression of a LMW-PTP<sup>C125</sup> mutant (10 ± 5%, n = 3) (Fig. 5B), suggesting that focal adhesion complexes are disrupted by the redox-dependent inactivation of LMW-PTP.

**DISCUSSION**

In conclusion, we have demonstrated that Nox1-induced ROS production is a necessary step for the signaling cascade controlling alteration of stress fibers and focal adhesions associated with Ras transformation. This process involves oxidative inactivation of LMW-PTP by Nox1-generated ROS and a subsequent activation of p190RhoGAP that causes down-regulation of Rho, leading to the dysregulation of action stress fibers and focal adhesions (Fig. 6). This redox signaling is augmented by Ras oncogene-induced up-regulation of Nox1 via the MEK-MAPK pathway (8), which is supported by the fact that inhibition of MAPK signaling by PD98059 restored the Rho activity in KNRK cells. Similarly, we found that Nox1-generated ROS mediate epidermal growth factor-induced inhibition of the Rho activity in human colon cancer CaCo-2 cells that may be required for cell migration.5

During the course to explore the role of Rac1 in Rho regulation, we found that oncogenic Ras lowers the Rac1 activity in KNRK cells. This is not surprising because the previous study (27) reported a similar observation that oncogenic H-Ras down-regulates Rac1 activity in Madin-Darby canine kidney cells, in which the suppression of Rac1 is caused by transcrip-

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5 M. Shinohara, S. Harada, J. Mitsushita, and T. Kamata, unpublished data.
Nox1 Redox Signaling Mediates Ras-induced Morphological Transformation

Whereas our study demonstrated that LMW-PTP is inactivated by ROS production that in turn deactivates LMW-PTP fibronectin, integrin has been reported to induce Rac-1-mediated cell spreading. In response to ligation by oncogenic Ras, platelet-derived growth factor signaling requires oxidative modification of PTP1B through its oxidation in A431 cells (35). This observation is consistent with the idea that redox regulation of PTPs constitutes critical intra-cellular signaling pathways for ROS sensors because Cys residues near the active center of the enzymes are susceptible to sulfenic acid (Cys-SOH) modification with H₂O₂. This modification is reversible and allows PTPs to respond to swift changes of cell signaling. Recent studies have revealed some aspects of oxidative regulation of PTPs in elegant ways. Epidermal growth factor stimulation leads to production of ROS and concomitant suppression of PTP1B through its oxidation in A431 cells (35). Platelet-derived growth factor signaling requires oxidative inactivation of the Src homology 2 domain containing PTP, SHP-2 (36). Thus, we found that LMW-PTP is a physiological target for Nox1-generated ROS provides further support to the notion that redox regulation of PTPs constitutes critical intra-cellular signalings through controlling tyrosine phosphorylation of proteins.

Oncogenic Ras is known to attenuate cell adhesions to the extracellular matrix by down-regulating integrins (19). It is surprising that despite the loss of integrin dependence, K-Ras oncogene suppresses Rho by employing the LMW-PTP/p190RhoGAP pathway in a manner similar to the process of integrin-induced cell spreading. In response to ligation by fibronectin, integrin has been reported to induce Rac-1-mediated ROS production that in turn deactivates LMW-PTP and down-regulates Rho via activation of p190RhoGAP (12). Whereas our study demonstrated that LMW-PTP is inactivated by Nox1 in K-Ras-transformed cells, Nox2 (gp91phox) has been implicated in ROS production that causes deactivation of LMW-PTP during integrin-mediated cell spreading (12). These observations raise a possibility that Nox1 redox signaling leading to Rho inhibition is integrated into a membrane receptor system distinct from integrin. In this context, it is intriguing that epidermal growth factor stimulation of CaCo-2 cells also induces Nox1-mediated down-regulation of Rho as mentioned above. The Nox1-dependent cellular redox state could be a critical limiting factor for actin dynamics. Consistently, it has been suggested that reactive oxygen radicals contribute to the actin cytoskeleton reorganization required for cell migration and cell adhesion of endothelial cells (10, 11). In fact, our study revealed that functional inactivation of Nox1 by Nox1 RNAi impaired motility of KNRK cells as determined by Matrigel assay.⁶

Sahai et al. (20) proposed that Rho is uncoupled to both ROCK and stress fiber formation in Ras-transformed Swiss3T3 cells, and that sustained activation of MAPK suppresses ROCK leading to disassembly of stress fibers. This is based on the observation that stress fibers were lost despite of an increase in active Rho levels in Ras-transformed Swiss3T3 cells and restored by MAPK inhibition (20). In contrast, our data indicate that the amount of RhoGTP is reduced in K-Ras-transformed NRK cells, correlating with loss of stress fibers and focal adhesions. This Rho inhibition is likely to be a direct consequence of increased Nox1 expression in KNRK cells (8) because the Rho activity is restored when the Nox1 expression is inhibited by siRNAs. Restoration of Rho activation coincides with morphological reversion of KNRK cells such as formation of stress fibers and cell adhesions. This is mediated by ROCK signaling as treatment of KNRKsiNox1 cells with the ROCK inhibitor Y27632 blocks the morphological changes (Figs. 4C and 5B). Contrasting with the system described by Sahai et al. (20), Rho appears to couple to stress fiber loss in KNRK cells. Although ERK-MAPK activation, like their observation, was detectable in KNRK cells, ERK-MAPK kinase pathway was shown to up-regulate Nox1 expression in KNRK cells (8). Thus, it is possible to speculate an alternative scenario about the role of ERK-MAPK signaling in which ERK-MAPK activation contributes to loss of stress fibers by stimulating the Nox1-redox signaling, but not down-regulating ROCK.

With respect to a question of how general our finding with KNRK cells is, similar down-regulation of Rho by activated Ras has been detected in colon carcinoma cells containing K-Ras (37) and Ras-transformed Rat1 fibroblast cells (28). Whereas the underlying mechanism of Ras-dependent down-regulation of Rho is less clear in the latter case, activated Ras-MAPK signaling stimulated the Fra-1 transcription factor in the former case and Fra-1 subsequently promoted cell motility by inactivating β1-integrin and keeping the RhoA activity low. Because Nox1 is overexpressed in colorectal cancer cells with a high frequency and its expression is prevented by a MAPK inhibitor,⁶ it is possible that oncogenic Ras-MAPK signaling, in addition to Fra-1 activation, up-regulates Nox1 expression, which in turn results in down-regulation of Rho through a Nox1-redox reaction as described here. The mode of regulation of Ras, Rho, and Rac, which are involved in morphogenetic processes, seems to be dependent on cell type, possibly reflecting the developmental history of each cell line (27, 38). The interrelated issues stemming from this complexity remain to be addressed in a future study. Whatever the reason for diversity in the Ras-

⁶ J. Mitsushita and T. Kamata, unpublished data.
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Rho signaling mechanism, our study for the first time reveals that Nox1 is a critical regulator of cellular redox state coupled to Ras oncogene-induced actin cytoskeleton rearrangement through Rho signaling.

Acknowledgments—We thank Dr. T. Finkel for catalase plasmids. We thank Z. Pengyao and staffs at the Biomedical Research Center, Laboratory of Medicine, Shinshu University for technical help, and F. Ushiyama for assistance in manuscript preparation.

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