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Nitric oxide activates Rap1 and Ral in a Ras-independent manner

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Abstract

Rap1 and Ral, the small GTPases belonging to the Ras superfamily, have recently attracted much attention; Ral because of Ral-specific guanine nucleotide exchange factors which are regulated by direct binding to Ras and Rap1 because of its proposed role as an antagonist of Ras signaling. We have previously demonstrated that nitric oxide (NO) activates Ras and proposed the structural basis of interaction between NO and Ras. In the present study we have shown that NO activates Rap1 and Ral in a time- and concentration-dependent manner. Using activation-specific probes for Rap1 and Ral, it was found that the NO-generating compounds SNP and SNAP could activate both Rap1 and Ral in Jurkat and PC12 cell lines. To investigate the involvement of Ras in NO mediated activation of Rap1 and Ral, we used PC12 cell lines expressing either the Ras mutant C118S (Cys118 mutated to Ser) or N17 (GDP-locked and inactive). We had previously shown that NO fails to activate Ras in these mutant cell lines. However, here it was found that Rap1 and Ral were activated by NO in these cell lines. The evidence presented in this study unambiguously demonstrates the existence of Ras-independent pathways for NO mediated activation of Rap1 and Ral.

Keywords: Rap1; Ral; Nitric oxide; GTPase

Rap1 and Ral are small GTPases which belong to the Ras superfamily. These proteins are classified in this group due to similarities in their effector domains. These GTPases cycle between an active GTP-bound form and an inactive GDP-bound state, via reactions controlled by exchange factors and GTPase activating proteins. Both Rap1 and Ral have recently attracted much attention: Ral because Ral-specific GEFs are regulated by direct binding to Ras, and Rap1 because of its proposed role as an antagonist of Ras signaling [1].

Rap1 is expressed as two isoforms, Rap1A and Rap1B, which are 95% identical. Rap1's effector domain is virtually identical to that of Ras, indicating that both GTPases may interact with similar effectors. Based on the findings that Rap1 shares the effector domain with Ras and binds to several Ras effector molecules, it has been proposed that GTP-bound Rap1 antagonizes Ras signaling pathways [2]. The first evidence that Rap1 is involved in signal transduction came from the observation that cAMP induces an increase in the GTP-bound form of epitope-tagged Rap1 [3]. Subsequently, a number of stimuli were found to induce the activation of Rap1; e.g., in platelets by thrombin and thromboxane A2 [4] and low density lipoprotein [5], in lymphocytes by T-cell receptor and B-cell antigen receptor activation [6–8], in neutrophils by fMet-Leu-Phe (fMLP) and platelet activating factor [9], and in fibroblasts by EGF, platelet derived growth factor, endothelin, and lipopolysaccharide. In most cases, this activation occurs within seconds or minutes of stimulation, suggesting that Rap1 activation is a receptor proximal event.

Ral proteins exist as two isotypes, RalA and RalB, which are 24 kDa proteins and share >50% sequence

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identity with Ras [10]. The nucleotide binding and GTP hydrolysis activities of Ral are also very similar to those of Ras. Thus like Ras, Ral proteins have very high affinities for GTP and GDP and low intrinsic GTPase activity. It has been shown that Ral could be activated by various growth factor receptors (including receptor tyrosine kinase and serpentine receptors) and it involves multiple signaling pathways. However, there is still a controversy about whether Ral activation by these growth factors is dependent on Ras.

Nitric oxide (NO) is a short-lived free radical, which serves as a cellular messenger in many physiological and pathological processes such as vasodilation, host defense, synaptic plasticity, and inhibition of smooth muscle growth. The oxidized form of NO, the nitrosonium ion (NO^+), is known to react predominantly with cysteine residues of proteins forming nitrosothiols (R-S-NO). Some of the proteins whose functions are regulated by modification of cysteine residues are Ras, calcium-dependent potassium channels, *N*-methyl-D-aspartate receptor, caspases, and mammalian and bacterial transcription factors.

Previous work in our laboratory focused on identifying signaling cascades responsible for activation of Ras by free radicals. We have reported a reversible interaction between NO and other redox modulators and Ras resulting in its activation. S-nitrosylation of amino acid residue Cys118 of Ras was found to be crucial for its activation [11]. Ras has been reported to be activated by endogenous NO in endothelial cells and primary cortical cultures and by exogenous NO in T cells and PC12 cells [12].

Given that Ral and Rap1 share >50% homology with Ras and, specifically, that Rap1 also contains Cys118 identical to Ras, we set out to analyze whether NO can promote GTP loading of Ral and Rap1. Further, we explored whether NO-dependent activation of Rap1 and Ral is mediated by Ras activation.

Materials and methods

Materials. The pGEX-RalGDS plasmid encoding a glutathione *S*-transferase fusion protein containing 97 amino acid Rap1 binding domain (RBD) of Ral GDS protein and Gst-RalBD construct containing amino acids 397 to 518 of human RLIP76 cloned in pGEX4T3 were kindly provided by Dr. Johannes L. Bos, Laboratory for Physiological Chemistry, Utrecht University, The Netherlands. Anti-Rap1A and Anti-Ral monoclonal antibodies were from Transduction laboratories (Lexington, KY). Anti-mouse IgG POD was from Roche Applied Science (Indianapolis, IN). PMA, SNP, and SNAP were purchased from Sigma (St. Louis, MO).

Cell culture and transfection. The human T-cell line Jurkat was maintained in RPMI1640 medium containing 10% heat-inactivated fetal calf serum in a 37 °C incubator with 5% CO_2. Rat pheochromocytoma PC12 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 5% horse serum. Both cell lines were transfected with mutant Ras C118S DNA, N17 DNA, and wt Ras DNA as described previously [13].

Treatments. Jurkat cells were serum starved overnight and treated (10^7 cells in 1 ml medium) with SNP, SNAP, PMA, and ionomycin for 10 min at 37 °C at the indicated concentrations. After treatments, the cells were lysed in 1 ml ice-cold lysis buffer (10% glycerol, 1% NP-40, 50 mM Tris–Cl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor) for 30 min on ice. Lysates were clarified by centrifugation at 13,000 rpm for 10 min.

Preparation of Gst-RalGDS and Gst-RalBD. Plasmids containing Gst-RalGDS and Gst-RalBD constructs were transformed in AD202 protease negative *Escherichia coli* and the protein production was initiated by adding 1 mM IPTG to the cultures grown up to OD_{600} 0.6–0.8. Bacteria were resuspended in PBS containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, and 0.5 mM dTT and subjected to sonication. Triton X-100 was added to a final concentration of 1% and the lysate was incubated on ice for 20 min. The lysate was centrifuged at 20,000 rpm for 40 min in the cold. One milliliter of 50% glutathione–Sepharose 4B bead slurry was added to the supernatant and incubated at 4 °C for 1 h with mild shaking. The beads were recovered by centrifugation at 500*g* for 5 min and washed three times with 1× PBS. The beads were stored at 4 °C no longer than 2 weeks.

Rap and Ral activation assay. To the treated cell lysates (protein normalized to 1.0 mg/ml) were added 5–10 μl of Gst-RalGDS or Gst-RalBD beads (5–10 μg protein) and incubated on a nutator at 4 °C for 1 h. Beads were recovered by centrifugation at 500*g* for 5 min and washed 4× with lysis buffer. After the final wash, Laemmli buffer was added to the sample. The proteins were separated by 12.5% SDS–PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk in 1× PBS with 0.1% Tween 20 for 1 h. A primary antibody directed against Rap1 or Ral was used and secondary antibody (anti-mouse IgG peroxidase conjugated) was used. The immune complexes were detected by enhanced chemiluminescence (ECL+, Amersham). All experiments shown here were performed at least three times with the same results.

Results

Based on the knowledge about GTPase effector molecules, activation-specific probes for Rap1 and Ral have recently been developed [14]. These constructs preferentially recognize and precipitate GTP-loaded forms of specific GTPases. This allows determination of the activity of endogenous Rap1 and Ral without radioactive in vivo labeling. Using these non-radioactive GTPase activation assays, we first tested whether NO activates endogenous Rap1 in Jurkat and PC12 cell lines.

NO activates Rap1 in Jurkat and PC12 cells

Jurkat cells were treated with different concentrations of the NO-generating compounds SNP and SNAP for 10 min. Activated Rap1 was precipitated with the Gst-tagged Rap binding domain of RalGDS bound to glutathione–Sepharose beads and identified by Western blotting using a monoclonal antibody directed against Rap1. As shown in Fig. 1, the basal level of GTP-bound Rap1 detected in cells maintained in serum-free medium was very low. The amount of GTP-bound Rap1 increased in a concentration-dependent manner with both SNP and SNAP treatment. GTP loading was maximal

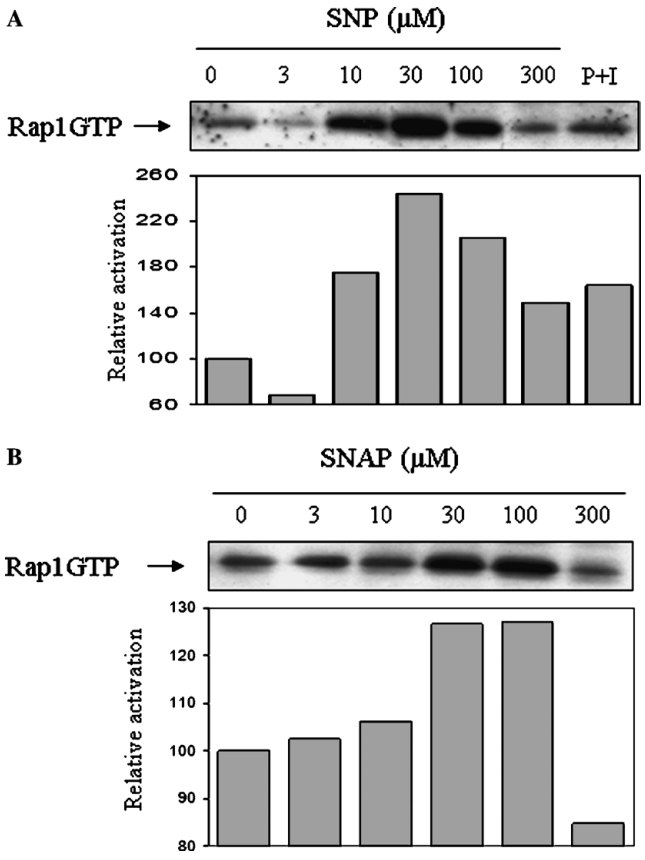

Fig. 1. Nitric oxide-induced activation of Rap1 in Jurkat cells. (A) Jurkat cells were stimulated with SNP (0–300 μM) or PMA (100 ng/ml) + ionomycin (1 μM) for 10 min. Rap1 activity was determined in the cell lysates by precipitating with the activation-specific probe, RalGDS. After gel electrophoresis and Western blotting, Rap1 was detected with mouse monoclonal antibody against Rap1. (B) SNAP-induced activation of Rap1 in Jurkat cells. Stimulation and activation assays were done as described in (A).

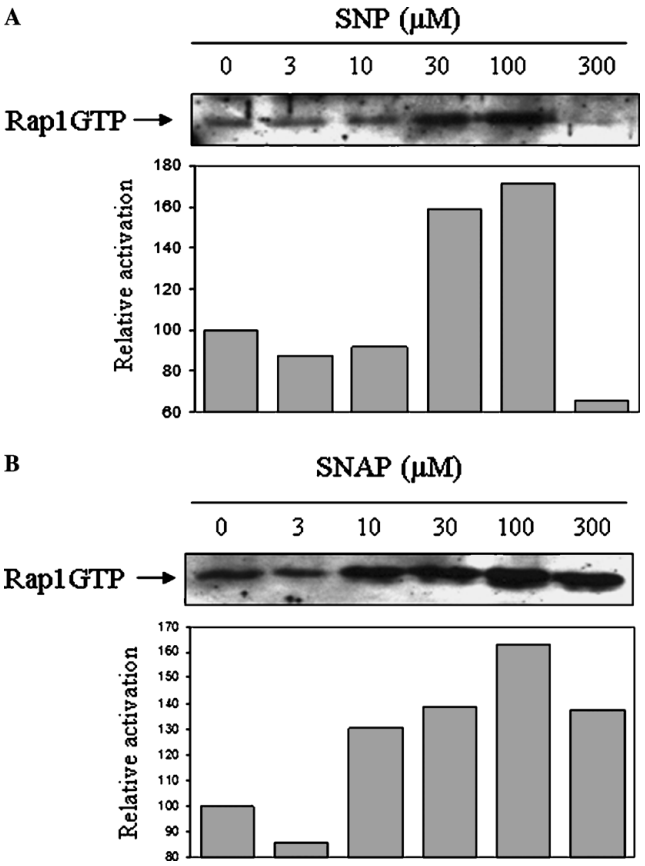

Fig. 2. Nitric oxide-induced activation of Rap1 in PC12 cells. PC12 cells were stimulated with different concentrations of SNP (A) or SNAP (B) for 10 min. Rap1 activity assays were done as described in Fig. 1A.

at 30 and 100 μM SNP and SNAP, respectively. PMA (100 ng/ml) and ionomycin (1 μM) also activated Rap1 significantly in Jurkat cells (Fig. 1A). The activation of Rap1 was lower at high concentrations of both SNP and SNAP (300 μM). In parallel, we performed the same experiment with pheochromocytoma PC12 cells using the same concentrations of SNP and SNAP (Fig. 2). A similar concentration-dependent increase in GTP-bound Rap1 was observed with maximal activity at 100 μM SNP and SNAP. The positive controls PMA and ionomycin for Jurkat and NGF for PC12 cells were also used as they are known to activate GTPases such as Ras.

NO activates Ral in Jurkat and PC12 cells

The activation of Ral was then examined in Jurkat and PC12 cell lines upon treatment with SNP and SNAP. Activated Ral was precipitated with the Gst-tagged Ral-binding domain of RLIP76 bound to glutathione–Sepharose beads and detected by Western blotting using a monoclonal antibody against Ral. As shown in Fig. 3, there is some basal GTP-bound Ral detected in cells maintained in the control medium, but the activation is again concentration dependent in Jurkat cells. With SNP, the GTP-bound Ral peaked at 100 μM after 10 min of treatment. However, in the case of SNAP treatment, the activity peaked at 30 μM and remained high. Similarly in PC12 cells the basal level of GTP-bound Ral was barely detectable in cells maintained in serum-free medium (Fig. 4). The activity peaked at 100 μM SNP after 10 min whereas with SNAP maximal activity was reached at 30 μM and remained high. NGF treatment for 10 min was used as a positive control and it showed an increase in GTP-bound Ral as compared to control cells (data not shown).

Since RalGDS and RalBD associate exclusively with the GTP-bound forms of Rap1 and Ral in vitro, with no detectable affinity for GDP-bound forms [14], we conclude that NO induces a rapid conversion of the majority of Rap1 and Ral to their GTP-bound active states. To confirm this observation, after precipitating with RalGDS and RalBD, the remaining supernatant was subjected to immunoprecipitation with anti-Rap1

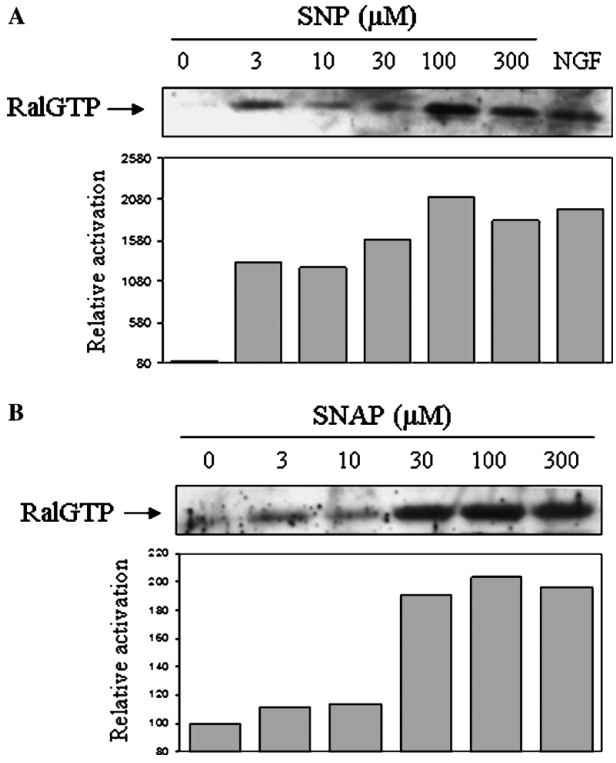

Fig. 3. Nitric oxide-induced activation of Ral in Jurkat cells. (A) Jurkat cells were stimulated with SNP for 10 min. Ral activity was determined in cell lysates by precipitating with the activation-specific probe, RalBD. After gel electrophoresis and Western blotting, Ral was detected with mouse monoclonal antibody against Ral. (B) SNAP-induced activation of Rap1 in Jurkat cells. Stimulation and activation assays were done as described in (A).

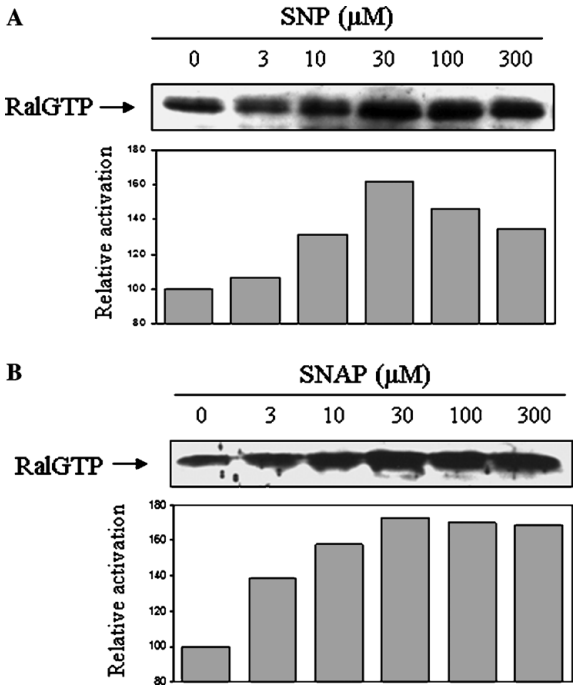

Fig. 4. Nitric oxide-induced activation of Ral in PC12 cells. PC12 cells were stimulated with either SNP (A) or SNAP (B) for 10 min. Ral activity assays were done as described in Fig. 2A.

antibody and anti-Ral antibody. Supernatants from cells treated with 30 and 100 μM SNP and SNAP had more than a 50% reduction in the amount of total Rap1 and Ral (data not shown).

NO-dependent activation of Ral and Rap1 is independent of Ras activation

Activation of Rap1 and Ral by NO and growth factors may serve one of several functions. First, they may act in parallel with Ras to activate common signaling pathways. Second, they may generally interfere with the action of Ras as it is known that Rap1 can bind to Ras effectors. To test these different possibilities and to evaluate whether Rap1 and Ral activation by NO is dependent on Ras activation, we utilized mutant/transfected PC12 cell lines. Previous studies in our laboratory on the activation status of Ras have identified Cys118 as the molecular target of NO [16]. To study the mechanism by which NO triggers activation of Ras, we generated two forms of mutant Ras, one identical to wild type except that Cys118 was modified to a serine residue (Ras C118S) and the other constitutively expressing a GDP-locked inactive Ras (Ras N17). We have previously shown that NO fails to activate Ras and MAP kinase pathways in C118S cells [15]. Ras N17 is always GDP-locked, and thus Ras activation does not occur in these cells. The controls for these transfected PC12 cells were PC12 cells with overexpressed wild type Ras. We hypothesized that if Ral or Rap1 activation by NO is through Ras, then in Ras Cys118S and Ras N17 mutants, there should not be any activation of Ral or Rap1. But when these mutant cells were treated with SNP (100 μM), Ral and Rap1 activation was normal (Figs. 5 and 6). This clearly indicates that activation of Rap1 and Ral is independent of Ras activation. Of interest is the observation that basal Rap1 and Ral activity was dependent upon the cell density. It was found that when the PC12 cells were confluent, the basal Ral and Rap1 levels were higher (data not shown).

Discussion

Small GTPases are activated by conversion of the GDP-bound conformation into the GTP-bound conformation and inactivated by GTP hydrolysis. Previous work in our laboratory studied the effect of NO on activation of Ras in human T cells, PC12 cells, and human endothelial cells. Nitrosothiol formation at a single cysteine residue of Ras (Cys118) was found to trigger GDP/GTP exchange [10,13]. The method to measure the active GTP-bound form of Ras was by immunoprecipitation of ^{32}P-labeled Ras followed by separation of the labeled GDP/GTP bound to Ras. This method allowed the determination of the percentage of total Ras that is

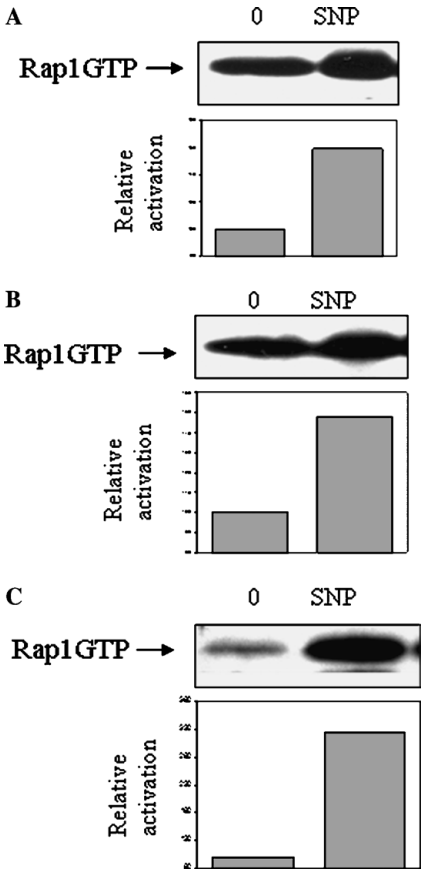

Fig. 5. Nitric oxide-induced activation of Rap1 is independent of Ras activation. (A) PC12 cells transfected with wt Ras were stimulated with SNP (100 μM) for 10 min. The Rap1 activation assay was done as described in Fig. 1. (B) PC12 cells transfected with Cys118 mutated to Ser (C118S Ras) were treated similarly. (C) PC12 cells transfected with N17 Ras were treated as described for wt Ras.

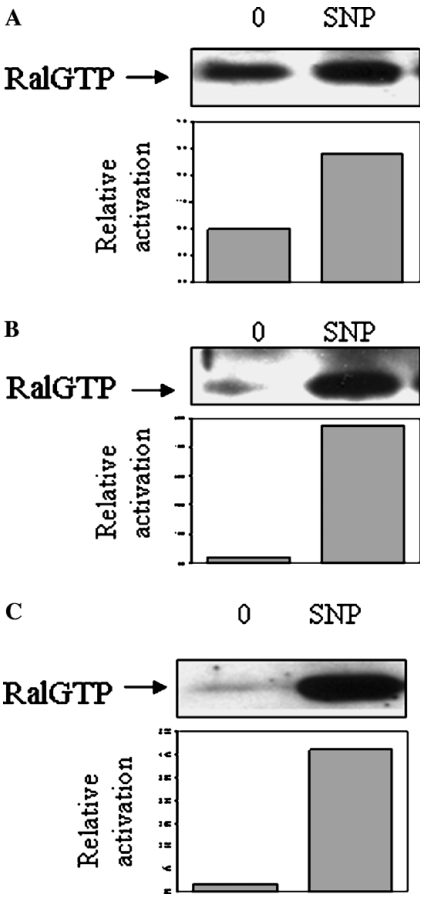

Fig. 6. Nitric oxide-induced activation of Ral is independent of Ras activation. (A) PC12 cells transfected with wt Ras were stimulated with SNP (100 μM) for 10 min. The Ral activation assay was done as described in Fig. 1. (B) PC12 cells transfected with Cys118 mutated to Ser (C118S Ras) were treated similarly. (C) PC12 cells transfected with N17 Ras were treated as described for wt Ras.

in the GTP-bound form. Although, this method is accurate and still used, it is technically demanding and needs relatively high levels of radioactivity. In the present study we have used a novel procedure which eliminated the need of radioactivity. The method is based on the large difference in affinity of the GTP- versus GDP-bound forms for specific binding domains of effector proteins in vitro. By using glutathione *S*-transferase (GST) fusion proteins containing these binding domains, the GTP-bound form of the GTPases can be precipitated from cell lysates. This method has been used here to identify the GTP-bound form of Rap1 and Ral from cell lysates [14]. Specifically, Rap1 binding domain (RBD) of Ral GDS (97 aa) and Ral binding domain (aa 397–518) of RLIP76 have been used as GST-fusion proteins to pull GTP-bound forms of Rap1 and Ral, respectively. These activation-specific probes were precoupled to glutathione–agarose beads and the detection was done by SDS–PAGE and Western blotting using antibodies specific for Rap1 and Ral.

Using these activation-specific probes it was found that exogenous NO provided by SNP and SNAP at a concentration range of 30–100 μM activates the small GTPases Rap1 and Ral in Jurkat and PC12 cells within 10 min. 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 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 [13]. We have reported a reversible interaction between NO and Ras, resulting in Ras activation via GDP/GTP exchange. Our earlier studies identified Cys118 on Ras as a molecular target of NO. Since Rap1 and Ral are close relatives of Ras with Rap1 also having a Cys residue at position 118, we tested whether these small GTPases are activated by NO.

The data presented here show that Rap1 activity is regulated by NO in two different cell lines (PC12 and Jurkat). It has already been reported that Rap1 activity is regulated by a variety of tyrosine kinase and G-protein coupled receptors in fibroblasts as well as in several cell lines such as PC12, Cos-7, and PAE [2]. Rap1 activation has also been shown following stimulation of the thrombin receptor in platelets and the T-cell receptor in T cells [4,8].

Ral is another GTPase which shares >50% sequence identity with Ras. The nucleotide binding and GTP hydrolysis activities of Ral are also very similar to those of Ras. Thus, like Ras, Ral proteins have very high affinities for GTP and GDP and low intrinsic GTPase activity. Structural studies have shown that the conformations of the two proteins are also very similar [16]. This prompted us to explore the effect of NO on Ral as well. Like Rap1, Ral was also activated after 10 min treatment of both Jurkat and PC12 cells with SNP and SNAP at a concentration of 30 μM. It has been previously shown that Ral could be activated by various growth factor receptors (including receptor tyrosine kinase and serpentine receptors) involving multiple signaling pathways. A rapid activation of Ral has been reported by EGF and NGF by various investigators [2,17,18].

It has been established that one of the effectors of Ras, RalGDS, has guanine nucleotide exchange factor activity for Ral [19]. Furthermore, Rap1 can block Ras mediated RalGDS signaling [20]. Given the interaction between Rap1, RalGDS, Ral, and Ras, we set out to determine whether or not NO-dependent activation of Ral and Rap1 is independent of Ras. Of interest, Rap1 and Ral could be activated in cell lines with mutant forms of Ras. These cell lines either had Ras expressed in a GDP-locked state or Cys mutated to Ser so it could not be activated by NO. This clearly demonstrates that Rap1 and Ral are two independent targets of NO. Ras-independent activation of Ral has already been reported for mitogens such as LPA and EGF in Rat-2 fibroblasts with mutant Ras N17 [18]. This is in contrast with stimulation of Ral with various growth factors including receptor tyrosine kinase and serpentine receptors which has been reported to be dependent on Ras [17]. Although Ral activation has been shown to be directly downstream of Ras activation, we found that NO bypassed Ras in activating Ral. This may be explained by understanding of the molecular targets of NO.

We have identified Cys118 of Ras as a molecular target of NO. A mechanistic understanding of how S-nitrosylated Cys118 leads to enhanced guanine nucleotide exchange will provide a general model which may be applicable to other GTPases. Cys118 is located within a highly conserved region (NKXD) of the Ras superfamily. This NKXD motif, in which Cys118 is the variable × residue in Ras, interacts directly with the guanine nucleotide ring of GTP and GDP and with other nucleotide binding loops of the protein [21]. The presence of a redox active residue in such a critical domain suggests that its conservation may reveal enzymes and transductional systems that may be similarly regulated. Rap1 also contains a Cys residue in this conserved region. In contrast, Ral does not have it. A further mechanistic understanding about this molecular redox trigger on Rap1 and Ral will provide insight into how cells respond to reactive free radicals.

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