Recruitment and Activation of Raf-1 Kinase by Nitric Oxide-Activated Ras[†]

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ABSTRACT: Nitric oxide (NO) and related species serve as cellular messengers in various physiological and pathological processes. The monomeric G protein, Ras, transduces multiple signaling pathways with varying biological responses. We have previously reported that NO triggers Ras activation and recruitment of an effector, phosphatidylinositol 3′-kinase (PI3K) and Ras-dependent activation of mitogen-activated protein (MAP) kinases which include extracellular signal regulated kinases (ERKs), c-Jun NH_2-terminal kinase (JNK), and p38 MAP kinase. In this study, we further defined NO-activated Ras signaling pathways. We have identified Raf-1 as another effector recruited by NO-activated Ras in T lymphocytes. NO activation results in association of Ras and Raf-1 and is biologically significant, as we observe an NO-induced increase in Raf-1 kinase activity. Downstream to Raf-1 kinase lie MAP kinases and their subsequent downstream targets, transcription factors. We found that treatment of T lymphocytes with NO yielded phosphorylation of the transcription factor, Elk-1. This phoshorylation is dependent on NO binding to the cysteine 118 residue of Ras. By further delineating the pathway with pharmacological inhibitors, Elk-1 phosphorylation was also found to be dependent on PI3K and ERK. Moreover, NO triggered an increase in mRNA levels of the proinflammatory cytokine, tumor necrosis factor-α (TNF-α), which was ERK dependent. Thus, we have defined an NO-induced signaling pathway in T lymphocytes arising at the membrane where NO-activated Ras recruits Raf-1 and culminating in the nucleus where Elk-1 is phosphorylated and TNF-α messenger RNA is induced. This NO-activated Ras-mediated signaling pathway may play a critical role in Elk-1-induced transcriptional activation of T lymphocytes, host defense and inflammation.

Nitric oxide is synthesized in most tissues and plays a central role in various physiological and pathological conditions (*1*−*6*). It can exist in various redox forms depending on its interaction with redox modulators and the redox milieu of the cell. Therefore, rather than NO, we will use the term reactive nitrogen species (RNS)[1] which refers to any chemical form derived from NO (*2*).

Redox chemistry is at the core of RNS-induced cell signaling. In appropriate redox environments, RNS signal by nitrosylation of cellular targets. Nitrosylation of either protein thiol or iron is the major chemical modification. This interaction can modulate the function of target proteins and lead to biological responses such as vasodilation, synaptic plasticity and inflammation (*2*, *3*, *7*). However, for many physiological events influenced by RNS, the detailed signaling pathways are largely unknown.

The monomeric G protein, Ras, is one of the cellular targets of RNS. Nitrosylation of the cysteine 118 thiol residue of Ras triggers GDP/GTP exchange (*8*−*11*). Activated Ras is known to interact with signaling proteins, termed effectors. Ras utilizes multiple functionally diverse effectors which include Raf, PI3K, protein kinase C-ζ, Ral-GDS family, Rin1, AF6, diacylglycerol kinase, and mitogen-activated protein kinase kinase kinase (*12*).

Previously, we have identified PI3K as one of the effectors recruited by RNS-activated Ras. Akt kinase and members of the MAP kinase family were found to further propagate this RNS signal (*13*). In an attempt to identify other effectors of Ras involved in RNS signaling in T lymphocytes, we examined the role of Raf-1. Raf, a threonine/serine kinase, is a key effector of Ras function (*14*−*20*). It exists in three isoforms, Raf-1, B−Raf, and A-Raf (*21*−*23*). Raf-1 is the predominant isoform present in T lymphocytes (*23*). In an inactive state, Raf-1 is localized in the cytosol. A stimulus which signals via the Ras-Raf pathway, initially triggers Ras activation and promotes translocation of Raf-1 to the plasma membrane. Raf-1 is subsequently phosphorylated which results in increased Raf-1 threonine/serine kinase activity. Raf-1 kinase then phosphorylates and activates the dual specificity kinases, MAPK/ERK kinases (MEK 1 and 2). MEKs phosphorylate tyrosine and threonine residues of its downstream target, ERKs (*17*). Other members of the MAP kinase family such as JNKs and p38 kinases, are phosphorylated by different members of the MAP kinase kinase family and may be Ras-dependent or independent depending

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[1] Abbreviations: RNS, reactive nitrogen species; NO, nitric oxide; PI3K, phosphotidylinositol 3-kinase; MAP kinase, mitogen activated protein kinase; ERK, extracellular signal regulated kinases; JNK, c-Jun NH_2-terminal kinase; MEK, MAPK/ERK kinase; TNF-α, tumor necrosis factor-α; SNP, sodium nitroprusside; SNAP, S-nitroso-*N*-acetyl penicillamine; PMA, phorbol myristate acetate; H_2O_2, hydrogen peroxide.

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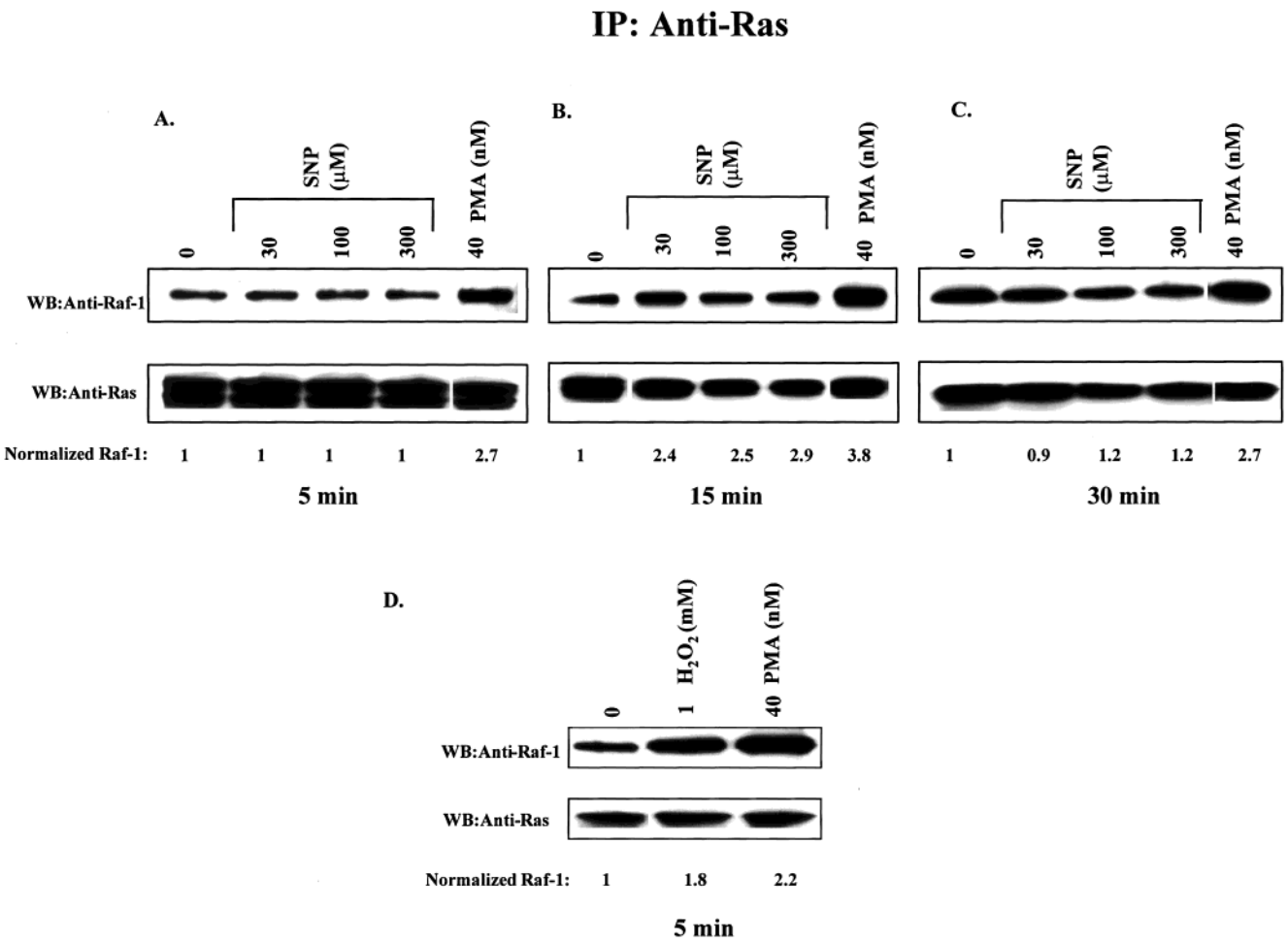

FIGURE 1: SNP triggers a Ras-Raf-1 interaction. Serum-starved Jurkat cells (10^7cells/mL) were treated with the indicated concentrations of SNP, H_2O_2, or PMA at different time intervals. Cell lysates were subjected to immunoprecipitation with an anti-Ras (Y13−238) antibody. Immunoprecipitates were western blotted with an anti-Raf-1 antibody and the total amount of Ras was confirmed with an anti-Ras (Y13−259) antibody. The blots are representative of three experiments. Normalized Raf-1 indicates the densitometric ratio of Raf-1 to Ras where the lane with no additions was set to 1.

on the cell type and the stimuli (*24*). We have previously reported activation of ERK, JNK, and p38 MAP kinases simultaneously by RNS-activated Ras in Jurkat cells, a human T cell leukemia line (*25*). The activated MAP kinases are known to translocate to the nucleus and phosphorylate various substrates. One of the primary targets is Elk-1, a member of the Ets class of transcription factors. Elk-1 can either autonomously interact with the Ets target sequence or form a complex with serum response factor (SRF) at the serum response element (SRE) present in different promoters (*26*−*30*). Hence we examined the RNS-induced phosphorylation status of Elk-1 in Jurkat cells and made an attempt to identify various signaling proteins utilized by RNS to achieve Elk-1 phosphorylation.

TNF-α is a central cytokine in inflammation and host defense (*31*). We have previously observed that RNS donors can induce secretion of TNF-α in human peripheral blood mononuclear cells (*32*). In monocytes, macrophages, mast cells, and A3.01 T cells, TNF-α expression involves MAP kinase activation (*33*−*36*). Therefore, we also determined the effect of RNS on TNF-α gene expression in Jurkat cells and the involvement of the MAP kinase pathway.

Although RNS are among the most potent cellular messengers, the signaling pathways they utilize are ill-defined. In this study, we have further identified the cellular events initiated by RNS-activated Ras in T lymphocytes beginning at the plasma membrane and terminating with specific nuclear events.

EXPERIMENTAL PROCEDURES

Materials. The construct for kinase-inactive GST-MEK1 was kindly provided by Dr. Joseph Avruch (*37*). The MEK inhibitor PD98059 and antibodies to Elk-1 and phospho-Elk-1 (ser-383) were purchased from New England Biolabs. p38 MAP kinase inhibitor, SB202190, was from Calbiochem. Nitric oxide donors, sodium nitroprusside (SNP), and S-nitroso-*N*-acetyl penicillamine (SNAP), hydrogen peroxide, phorbol myristate acetate (PMA), ionomycin, phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, sodium vanadate, wortmannin, LY294002, and protein A were all obtained from Sigma. α-Hydroxyfarnesylphosphonic acid was from BioMol. The radioisotopes were from NEN Life Science Products and ECL detection system from Amersham Pharmacia Biotech. Antibodies to Raf-1 and Ras (Y13−238, Y13−259) were purchased from Santa Cruz Biotechnology. Trizol reagent was from Gibco BRL. QuickHyb and Prime-it Rmt random primer labeling kit was obtained from Stratagene and Qiaquick PCR purification kit was from Qiagen.

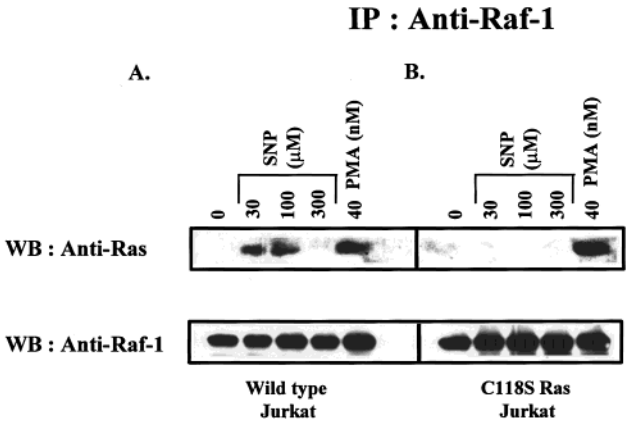

FIGURE 2: Presence of Ras in the immunoprecipitates of Raf-1. Serum-starved wild-type and C118S Ras Jurkat cells (10^7cells/mL) were treated with the indicated concentrations of SNP or PMA for 15 min. Cell lysates were immunoprecipitated with an anti-Raf-1 antibody and western blotted with an anti-Ras (Y13−259) antibody to detect the presence of Ras. Equal amounts of immunoprecipitated Raf-1 was confirmed in the lower panel.

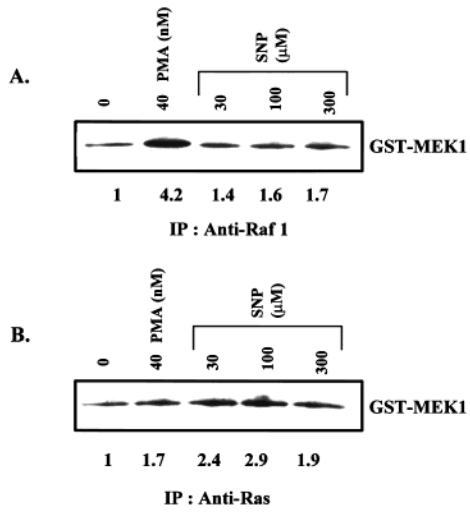

FIGURE 3: SNP induces Raf-1 kinase activity. Serum-starved Jurkat cells (10^7cells/mL) were treated with SNP or PMA for 15 min. Cell lysates were subjected to immunoprecipitation with either an anti-Raf-1 antibody (panel A) or an anti-Ras (Y13−238) antibody (panel B). Immunoprecipitates were used to detect Raf-1 kinase activity toward the substrate GST-MEK1. SNP-triggered activation was observed in several repeated experiments. Values below the panel were obtained after densitometric analysis.

Cell Culture and Transfection. The human T-cell line Jurkat was maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Jurkat cells were stably transfected with the mutant Ras C118S cDNA as previously described (*10*).

Immunoprecipitation and Western Blotting. Cells were serum-starved for 16−24 h and 10^7 cells/mL were treated with SNP, SNAP, or PMA. Cells were lysed in RIPA buffer (20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 μg/mL each of aprotinin and leupeptin, and 5 mM sodium vanadate). A total of 1−2 mg of total cell lysate proteins was subjected to immunoprecipitation for 1 h by either anti-Raf-1 and protein A or agarose-conjugated anti-Ras (Y13−238). Western blots were performed on the immunoprecipitates using polyclonal antibodies to Raf-1 or rat monoclonal antibody to Ras (Y13−259). Blots were developed using an enhanced chemiluminescence plus kit. For each figure, densitometric analysis was performed on each blot and the data pooled from three experiments. In all cases, the standard deviation was less than 15%.

Raf-Kinase Assay. The kinase assay was performed essentially as previously described (*38*). Briefly, cell lysates were obtained as described above. One milligram of total protein was subjected to immunoprecipitation with either anti-Raf-1 and protein A or agarose-conjugated anti-Ras (Y13−238) for 2−3 h. Washed immunoprecipitates were subjected to kinase assay at 25 °C for 20 min in 40 μL of assay buffer containing 30 mM Hepes, pH 7.4, 7 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 15 μM ATP, 20 μCi of [γ -^{32}P]-ATP. A total of 0.1 μg of kinase-inactive GST-MEK1 was used as a substrate. The reaction was terminated with 5× Laemmli buffer. Samples were boiled for 5 min and subjected to SDS−polyacrylamide gel electrophoresis. The proteins were transferred onto the nitrocellulose membrane and analyzed with a Phospho-Imager. Re-immunoprecipitation of the supernatants with anti-Raf-1 antibody yielded <10% of the total Raf-1 kinase activity indicating the near-quantitative nature of the assay.

Elk-1 Phosphorylation. ELK-1 phosphorylation was determined in nuclear extracts of serum-starved Jurkat cells. Nuclear extracts were prepared by a mini-extraction protocol with some modification (*39*). Briefly, after appropriate treatment, serum-starved cells (10^7 cells/mL) were washed with PBS and suspended in cold 1 mL of buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM DTT, 0.5 mM PMSF, and 1 mM sodium vanadate) by gentle pipetting on ice for 15 min resulting in cell swelling. Cells were lysed by addition of 40 μL of 2.5% Nonidet P40. The homogenate was centrifuged at 12000*g* for 10 min. The nuclear pellet obtained was resuspended in 15 μL of ice-cold buffer C (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 0.1% Nonidet P40, 0.1 mM DTT, 0.5 mM PMSF, and 1 mM sodium vanadate) for 15 min with intermittent vortexing. The nuclear extract was centrifuged for 10 min at 12000×*g* and the supernatant containing nuclear proteins was diluted with 75 μL of ice-cold buffer D (10 mM Hepes, pH 7.9, 50 μM KCl, 0.2 mM EDTA, 20% glycerol, 0.1 mM DTT, 0.5 mM PMSF, 1 mM sodium vanadate). Nuclear proteins (100−150 μg) were subjected to SDS−polyacrylamide gel electrophoresis and subsequent western blotting for phospho-Elk-1 and Elk-1. MAP kinase inhibitors were used at the concentrations specific for their target with no inhibitory activity toward nontarget MAP kinases (*36*).

Northern Blot Analysis. Total RNA was isolated using Trizol reagent according to the manufacturer's protocol. A total of 20 μg of total RNA was subjected to Northern blot analysis using QuickHyb reagent. Prehybridization and hybridization were performed at 65 °C. The membranes were probed with 710 bp fragment of human TNF-α cDNA and 300 bp fragment of human β-actin cDNA. The fragments were obtained by PCR and purified by Qiaquick PCR purification kit. The purified fragments were labeled with [α-^{32}P]dCTP using a Prime-it Rmt random primer labeling kit. Membranes were subjected to autoradiography and phosphoimaging.

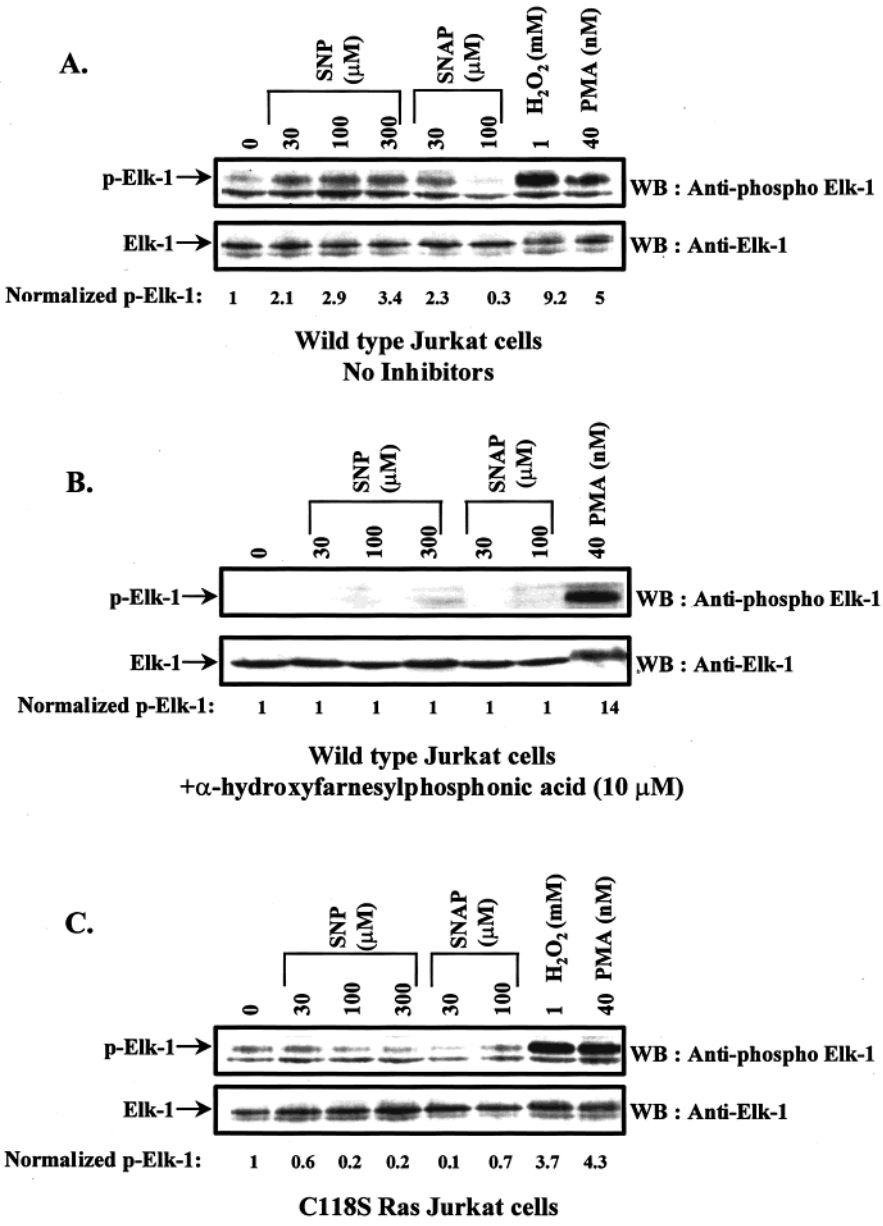

FIGURE 4: RNS-induced Elk-1 phosphorylation is dependent on Ras and its C118 residue. Serum-starved wild-type Jurkat cells (panels A and B) and C118S Ras Jurkat cells (panel C) were treated with SNP, SNAP, H_2O_2 or PMA for 15 min. 100−150 μg of nuclear extract were subjected to western blotting with either anti-phospho-Elk-1 (p-ELK-1) or anti-Elk-1 antibodies. Cells in panel B were pretreated with α-hydroxyfarnesylphosphonic acid (10 μM) overnight. Blots are representative of three experiments. Normalized p-ELK-1 indicates the densitometric ratio of p-ELK-1 to ELK-1 where the lane with no additions was set to 1.

RESULTS

Co-immunoprecipitation of Raf-1 and Ras. To determine whether RNS induce the direct interaction of Ras and Raf-1, we performed co-immunoprecipitations of total cell extracts obtained from serum-starved Jurkat cells treated with the RNS-donor, SNP. Cells were treated with SNP for different time intervals and a concentration range of 30−300 μM (Figure 1). After 5 min of treatment with SNP, there was no detectable increase in the level of Raf-1 in the immunoprecipitates of Ras (Figure 1A). However, a significant amount of Raf-1 was recruited by Ras after 15 min of SNP treatment (Figure 1B). The interaction of Ras and Raf-1 returned to basal levels after 30 min of SNP treatment (Figure 1C). Cells were treated with PMA to simulate activation in T lymphocytes and these cells exhibited increased Ras-Raf-1 interactions rapidly within 5 min which persisted until 30 min (Figure 1). Furthermore, the reactive oxygen generator, H_2O_2, was found to similarly trigger a Ras/Raf-1 interaction (Figure 1D).

To confirm the above observation of an RNS-induced Ras-Raf-1 association, we reversed our immunoprecipitating and western blotting antibodies. We observed the presence of Ras in the immunoprecipitates of Raf-1 when Jurkat cells were treated with SNP for 15 min in the concentration range of 30−100 μM (Figure 2A). Thus, it is evident from co-immunoprecipitation experiments that RNS-activated Ras recruits Raf-1. Similar observations were made with another RNS donor, SNAP (data not shown).

We have previously identified the cysteine 118 residue of Ras as a molecular target of RNS. To determine the specificity of RNS signaling, we used a Jurkat cell line stably transfected with a Ras mutant containing a serine instead of

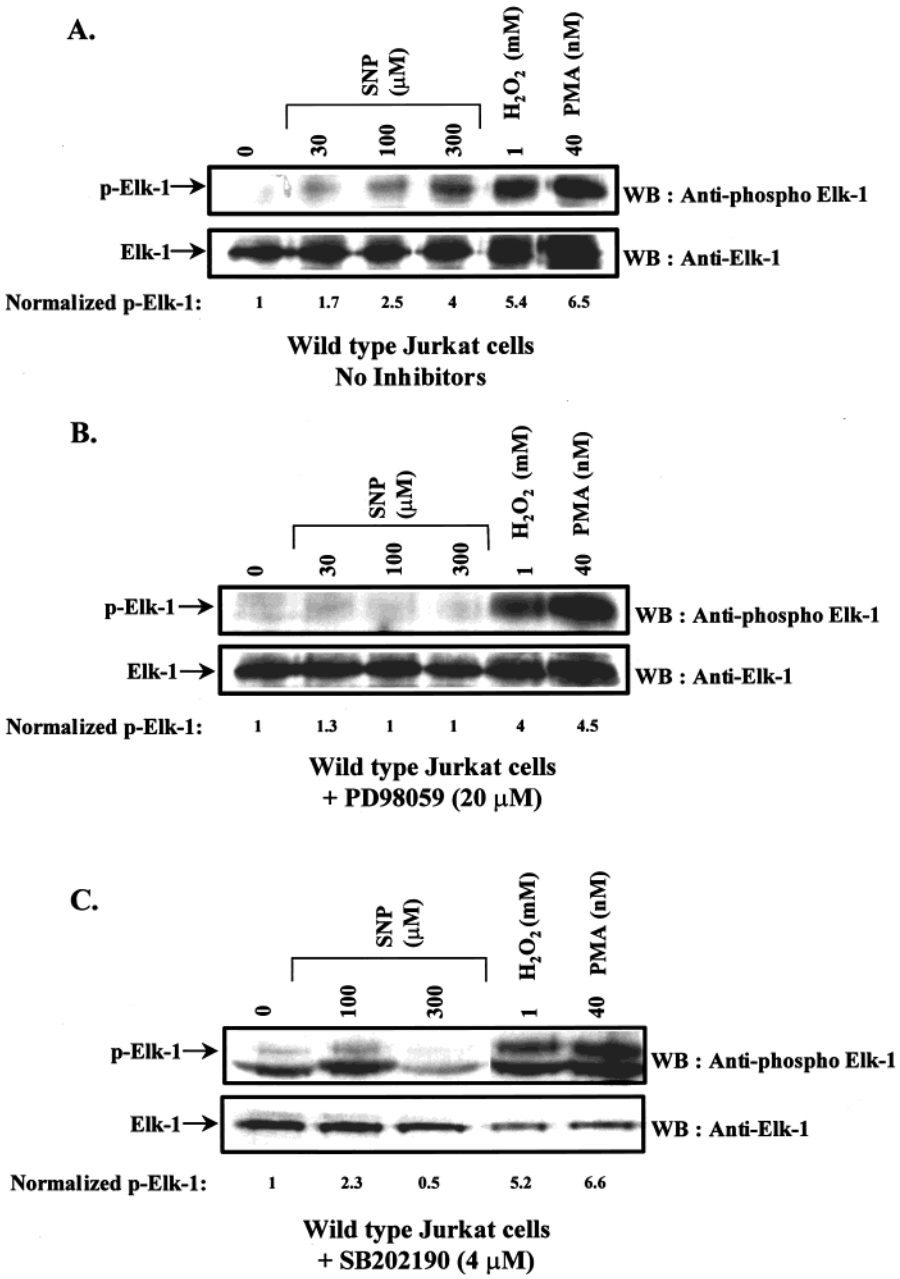

FIGURE 5: Involvement of ERK and p38 MAP kinase in Elk-1 phosphorylation. Serum-starved Jurkat cells were pretreated with either (A) DMSO (0.5%); (B) PD98059 (20 μM); or (C) SB202190 (4 μM) for 45 min, then treated for 15 min with SNP, SNAP, H_2O_2, or PMA and nuclear proteins were extracted. Nuclear extract was subjected to western blotting with anti-phospho-Elk-1 or anti-Elk-1 antibodies. Blots are representative of three experiments. Normalized p-ELK-1 indicates the densitometric ratio of p-ELK-1 to ELK-1 where the lane with no additions was set to 1.

cysteine residue at position 118 (C118S) (*8*, *10*). As seen in Figure 2B, immunoprecipitates of Raf-1 obtained from these cells did not contain Ras after SNP treatment. Moreover, only RNS signaling is blocked in these mutant cells and not PMA-induced recruitment of Raf-1 by Ras.

RNS-induced Raf-1 kinase activity. Raf-1 activation is a multistep process where translocation and interaction with Ras is an initial step of activation (*17*). To determine whether an RNS-propagated signal could successfully trigger kinase activity of Raf-1, we performed an in vitro kinase assay using kinase inactive GST-MEK1 as a substrate. As seen in Figure 3A, Raf-1 immunoprecipitates from Jurkat cells treated with SNP showed enhanced kinase activity. In addition, enhanced Raf-1 kinase activity was observed in Ras immunoprecipitates of SNP treated cells (Figure 3B). Both RNS donors, SNP and SNAP, were unable to augment Raf-1 kinase activity in C118S Ras Jurkat cells (data not shown). These data strongly suggest that the RNS-triggered Ras-Raf-1 interaction leads to enhanced Raf-1 kinase activity.

Elk-1 Phosphorylation. ERKs are the downstream members of the Raf-1 kinase signaling cascade. We have previously reported that RNS trigger ERK activation in Jurkat cells in a Ras-dependent manner (*25*). Moreover, RNS-activated Ras also upregulates kinase activity of JNK and p38 MAP kinase (*25*). Transcription factors are important substrates of MAP kinases. Depending on the cell type and stimuli, Elk-1 can be phosphorylated by each of the three MAP kinases (*28*, *29*). Hence, we examined the effect of RNS on the phosphorylation state of the transcription factor Elk-1 in nuclear extracts by using an anti-phospho-Elk-1 (ser

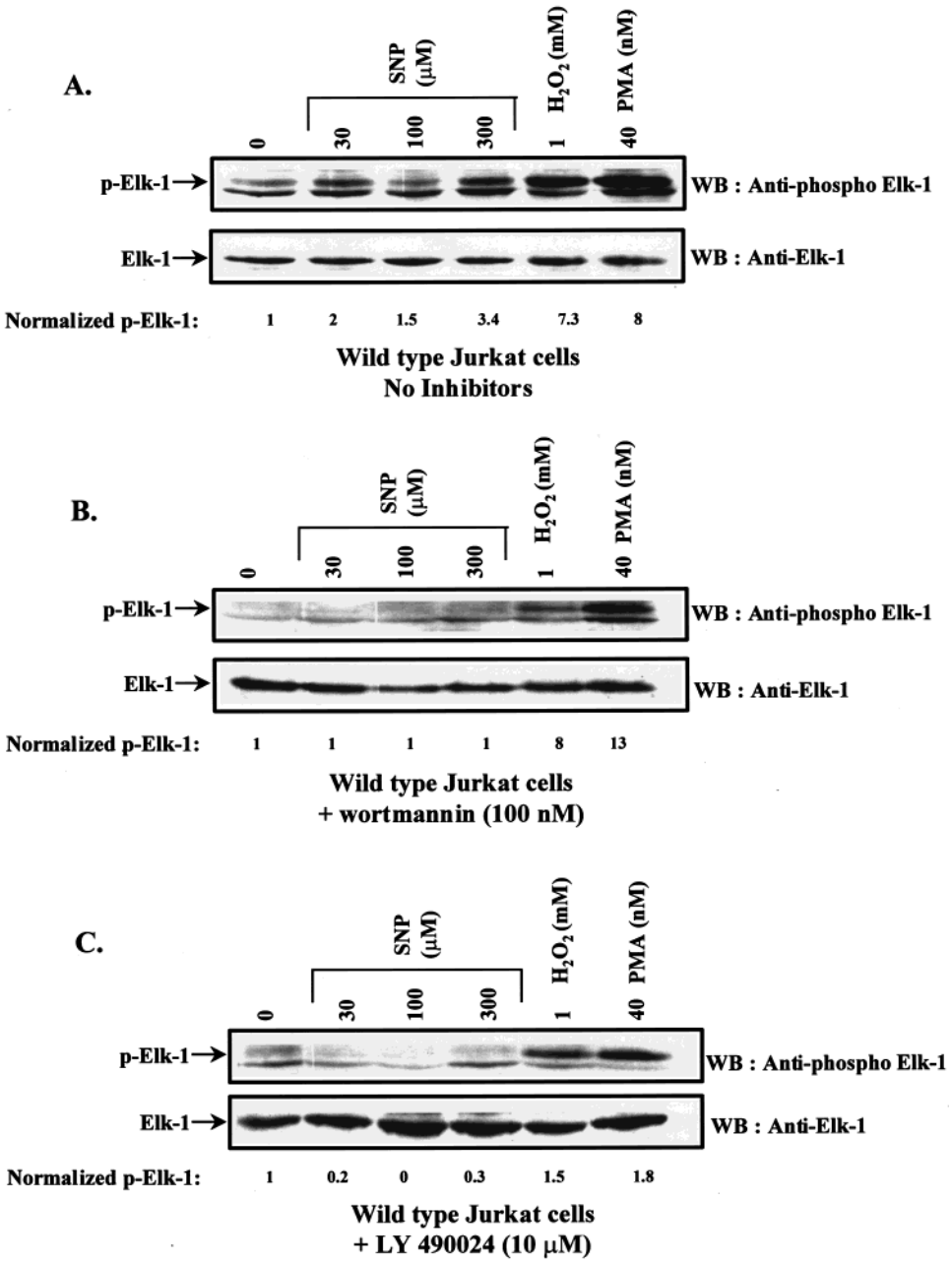

FIGURE 6: PI3K is another upstream component involved in phosphorylating Elk-1. Serum-starved Jurkat cells were pretreated with either (A) DMSO (0.5%), (B) wortmannin (100 nM); or (C) LY294002 (10 μM) for 45 min and then treated for 15 min with SNP, SNAP, H_2O_2, or PMA before Western blotting the nuclear extracts. Blots represent the results of three experiments. Normalized p-ELK-1 indicates the densitometric ratio of p-ELK-1 to ELK-1 where the lane with no additions was set to 1.

383) antibody. As seen in Figure 4A, SNP and SNAP significantly enhanced the level of phosphorylated Elk-1 in a concentration range of 30−300 μM. PMA and H_2O_2 induced very strong phosphorylation of Elk-1. To identify upstream signaling molecules which result in phosphorylated Elk-1, we utilized specific pharmacological inhibitors and the C118S Ras Jurkat cell line. Involvement of the Ras pathway in RNS signaling is confirmed in Figure 4B, since a farnesyl transferase inhibitor, α-hydroxyfarnesylphosphonic acid, abrogated phosphorylation of Elk-1 by SNP and SNAP. This inhibitor prevents Ras from localizing to the plasma membrane. Furthermore, C118 of Ras is the likely target as the C118S Ras Jurkat cells only blocks RNS-induced Elk-1 phosphorylation without affecting H_2O_2 or PMA signaling (Figure 4C). In addition, only the MEK1 inhibitor, PD98059 (*40*), and not the p38 MAP kinase inhibitor, SB202190 (*41*), inhibited the signal (Figure 5). This suggests that ERK and p38 MAP kinase signaling diverge upstream of ELK-1 phosphorylation. PI3K was found to be involved in this signaling cascade, since the PI3K inhibitors, wortmannin and LY294002, abrogated RNS-induced phosphorylation completely and H_2O_2 and PMA signaling partially (Figure 6). The above inhibitor studies suggest that Ras, PI3K, and ERK are required for RNS-induced Elk-1 phosphorylation.

Induction of TNF-α. To determine whether nuclear transcriptional events were triggered by RNS, we performed Northern analysis of total RNA obtained from SNP-treated cells which had been pretreated with PD98059 or the solvent DMSO for 45 min. No RNS-induced increase of TNF-α mRNA levels were observed after 1 h of treatment (data not

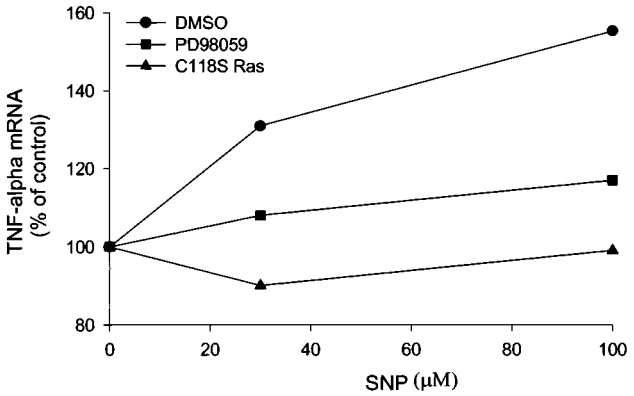

FIGURE 7: SNP modulates TNF-α mRNA levels. Wild-type Jurkat cells were pretreated with either DMSO (0.5%) or PD98059 (20 μM) for 45 min. RNA was extracted from serum-starved wild-type and C118S Ras Jurkat cells after 4 h of SNP treatment and Northern analysis was performed on 20 μg of total RNA. The TNF-α mRNA levels plotted represent values obtained after normalizing with β-actin mRNA levels. Values are the mean of two independent experiments and variability ranged to 15%.

shown). However, after 4 h of treatment, SNP (100 μM) induced a significant increase in TNF-α mRNA levels (Figure 7). Treatment with phorbol myristic acetate (40 nM) and ionomycin (500 nM) yielded a 1.9-fold increase in TNF-alpha mRNA levels, similar to those achieved with SNP. Interestingly, blocking ERK with PD98059 inhibits the SNP-induced increase in TNF-α mRNA levels (Figure 7). The specificity of SNP-induced signaling was confirmed by using the C118S Ras Jurkat cells, as SNP did not affect TNF-α mRNA levels in these cells (Figure 7).

DISCUSSION

Nitric oxide and its congeners are involved in the propagation of multiple signaling pathways which lead to crucial physiological events such as vasodilation, synaptic plasticity and inflammation. Recent studies have broadened our mechanistic understanding of signaling triggered by RNS. The most significant findings include identifying their cellular and molecular targets. Some of the cellular targets of RNS include guanylyl cyclase, aconitase, Ras, calcium release channel, calcium-dependent potassium channels, *N*-methyl-D-aspartic acid receptor, nuclear factor-κB, hemoglobin and cyclooxygenase. At the molecular level, major interactions of RNS involve either iron or a thiol moiety of the above targets (*2*, *7*, *42*). However, identifying multiple targets raised many questions, including how cells achieve selectivity while utilizing redox signaling. Thus, the detailed signaling cascades triggered by RNS-modified proteins need to be defined.

Our earlier studies have identified the monomeric G protein, Ras, as a cellular target of RNS. Direct interaction of RNS with the cysteine 118 residue of Ras leads to a conformational change and results in GDP/GTP exchange (*8*−*11*). We have identified PI3K as one of the effectors utilized by RNS-activated Ras to propagate its signal (*13*). The main focus of this study was to identify other effectors recruited by RNS-activated Ras and further delineate the pathway leading to nuclear events.

Our experimental data suggest that Raf-1 is a part of the signaling cascade triggered by RNS-activated Ras. The interaction of Raf-1 and Ras induced by RNS is transient, since increased interaction is seen 15 min following SNP treatment and reaches basal levels after 30 min. PMA triggered an interaction rapidly within 5 min which persisted until 30 min. The difference in kinetics between PMA and SNP most likely results from the long half-life of PMA, which is not metabolized, and the short half-life of RNS. Results obtained by interchanging both immunoprecipitating and western blotting antibodies confirms that RNS-induce an increased physical interaction of Raf-1 and Ras. Using a cell line stably transfected with Ras C118S is a very important tool in determining specificity of RNS signaling. In C118S Ras Jurkat cells, the absence of Ras in the immunoprecipitates of Raf-1 treated with SNP and a robust signal of Ras in cells treated with PMA indicates that the signal is generated by the interaction of RNS with the cysteine 118 residue of Ras.

Activation of Raf-1 is a multistep cascade and involves many cellular components. Translocation and interaction of Raf-1 with Ras is one of the prerequisites of this process (*17*). Hence, to determine whether the increased interaction of Raf-1 and Ras has biological significance, we performed an in vitro Raf-1 kinase assay utilizing its physiological substrate, MEK1. Increased phosphorylation of GST-MEK1 in the immunoprecipitates from cells treated with SNP of both Raf-1 and Ras suggests that translocation of Raf-1 has functional significance and is Ras-dependent. The activation maxima varied from 30 to 300 μM for SNP and 30 to 100 μM for SNAP. This variation can be attributed to the dynamic cellular redox milieu, and the different rates at which SNP and SNAP generate RNS.

Since we have previously reported that RNS induce activation of the three MAP kinases ERK, JNK, and p38 MAP kinase (*25*), we studied one of their downstream nuclear substrates, Elk-1. Elk-1 regulates transcription of various genes by either autonomously binding an Ets target sequence or as a component of the ternary complex that binds to the serum response element (*26*−*30*). By using pharmacological inhibitors, we were able to assign Ras and specifically its C118 residue, as well as PI3K and ERK as components of an upstream cascade responsible for Elk-1 phosphorylation by RNS. PI3K may be involved in the Elk-1 phosphorylation due to its influence on MAP kinase activation (*43*−*45*). This suggests that redox signaling utilizes pathways of classical signal transduction, which involves cross-talk, interconnection between signaling pathways and shared substrates among multiple enzymes.

RNS play a very important role in inflammation and host defense (*46*). Here, we observed that RNS trigger TNF-α transcription in Jurkat cells. After 4 h of treatment, SNP increased TNF-α mRNA levels 1.6-fold and was found to be ERK dependent. Once again, RNS-specific signaling was verified by using C118S Ras Jurkat cells.

We have thus unraveled some of the cellular signaling components utilized by the gaseous messenger NO and its congeners to achieve biological responses. We have identified a novel RNS−Ras pathway which includes Raf-1 kinase and Elk-1 and the involvement of TNF-α strongly suggests the importance of this RNS pathway in inflammation and host defense. This may aid in a mechanistic understanding and conceptualization of redox signaling. The cellular components identified can be pharmacologically targeted to intervene in the pathophysiological processes affected by RNS.

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