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Interleukin-1β and Reactive Oxygen Species Mediate Activation of c-Jun NH_2-Terminal Kinases, in Human Epithelial Cells, by Two Independent Pathways

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The c-Jun N terminal kinases (JNKs) are members of the mitogen activated protein kinases family, which have been shown to be preferentially activated either by cytokines or stress stimuli. In this study we identify a selective and potent antisense oligonucleotide to RhoA (ISIS 17131) and investigate its effect on JNK activation induced by IL-1β and H_2O_2 in A549 cells. The RhoA antisense oligonucleotide was able to inhibit JNK activation when A549 cells were stimulated by H_2O_2, but did not have any effect on IL-1β induced JNK activation. Consistent with the idea that the phosphatidylinositol 3-kinase (PI 3-kinase) activates the small G protein exchange factors, H_2O_2 activated the PI 3-kinase. Additionally, Wortmannin, a potent inhibitor of the PI 3-kinase and phospholipase A_2 (PLA_2), and $AACOCF_3$, also a PLA_2 inhibitor, were able to inhibit JNK activation induced by H_2O_2, but they had no effect on JNK activation when stimulated by IL-1β. These results suggest that, in A549, IL-1β and H_2O_2 induce JNK activation by two independent pathways.

The c-Jun N-terminal kinases (JNKs), or stress activated protein kinases (SAPKs), are members of the mitogen activated protein kinase (MAPK) family, which is composed of 3 subfamilies: the ERK (ERK 1, 2, 3, 4, and 5); p38 (p38α, β, δ, and γ) (1); and the JNKs (JNK 1, 2, and 3) (2). ERK 1 and 2 are activated by mitogenic stimuli (3) and viral proteins (4–6). The cascade of events that induces their activation has been extensively studied and includes the activation of Ras/Raf/Mek (3). ERK 3, 4, and 5 are less well characterized. JNK and p38 are preferentially activated by cytokines (IL-1β and TNFα) or stress stimuli such as osmotic shock, UV, and heat (7, 8). Activated JNK activates c-Jun by phosphorylation of two serine residues at the 63 and 73 position of the NH_2 terminus of c-Jun. Activated c-Jun then mediates AP-1 transcriptional activation (9). JNK can also phosphorylate and activate activating transcription factor-2 (ATF2) (10) and Elk-1(11), which in turn control the transcription of multiple genes. In addition, recent studies with the two hybrid system demonstrated that JNK directly binds to the nuclear factor of activated T cell (NFAT4) preventing its nuclear accumulation (12). JNK activation has been linked to cellular proliferation (13, 14) as well as apoptosis.

Little is known about the initial events in the cascade that leads to JNK activation. MKK4, also known as JNKK1, is a member of the mitogen activated protein kinase pathway which activates JNK (15) and is also involved in the regulation of p38 (16). On the other hand, JNKK2 phosphorylates and activates JNK, but it does not have any effect on p38 (17). Studies using constitutively active mutants of Cdc42 and Rac1 in COS-7, HeLa, and NIH3T3 cells identify these two small G proteins as upstream mediators of JNK activation (18, 19). However, different results were obtained in human kidney 293T cells where RhoA and Cdc42 were shown to mediate JNK activation (20).

RhoA, Rac1, and Cdc42 are members of the Rho subfamily, which also includes RhoB, RhoC, RhoD, RhoG, Rac2, and Rac3 (21). They are small G proteins which are present in cells in both the GTP-bound active form and the GDP-bound inactive form. The activity of these proteins is regulated by exchange factors (GEF), which catalyze the release of GDP, and GTPases (GAP), which facilitate the hydrolysis of GTP to GDP. Recent studies showed that activation of the exchange factor Vav is dependent on PI 3-kinase, since the product [$PI(3,4,5)P_3$] is able to activate Vav by direct binding, whereas its substrate [$PI(4,5)P_2$] inhibits the exchange factor. *In vitro,* Vav was able to activate RhoA, Cdc42, and Rac1 (22).

Under certain conditions, activation of RhoA, Cdc42, and Rac1 is necessary for cytoskeleton reorganization.

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Rac1 mediates the formation of membrane ruffles and lamellipodia induced by PDGF, insulin, and bombesin, RhoA mediates the formation of stress fibers and focal adhesions induced by lysophosphatidic acid (LPA) and bombesin (23, 24), and Cdc42 mediates the formation of filopodia (25). RhoA, Cdc42, and Rac1 are also necessary for Ras mediated transformation of NIH-3T3 cells and Rat-1 fibroblasts (26–29).

Overexpression of RhoA in NIH-3T3 induces activation of NFkB (30); in addition, activated RhoA regulates transcription via the serum response factor (31). Studies using the two hybrid system identified p160ROCK, a serine/threonine kinase, as a potential direct downstream Rho target (32); in addition PRK2, which is a target of both Rho and Rac, has been shown to mediate the formation of actin stress fibers (33). Other proteins which bind to activated Rho are p120 PKN, and p150 ROKα and ROKβ (34).

These studies were undertaken to help elucidate the role of RhoA in JNK activation. JNK was stimulated using a cytokine (IL-1β) or a stress signal (H_2O_2). Using antisense oligonucleotides we demonstrated that, in A549 cells, the activation of the small G protein RhoA is crucial for JNK activation induced by H_2O_2, but it does not play a role in IL-1β induced JNK activation. In accordance with this result, inhibitors of PI 3-kinase and PLA_2 (35–37), which have been shown to be involved in RhoA mediated signaling pathways (22, 38, 39) specifically inhibited only the peroxide mediated pathway supporting the data obtained with the antisense oligonucleotide.

EXPERIMENTAL PROCEDURES

Cell culture. A549 lung carcinoma cells, purchased from American Type Collection, were grown in DMEM low glucose (Gibco/BRL) containing 10% FCS and penicillin and streptomycin. Cell were passaged when 90–95% confluent.

Inhibition studies. A549 cells, incubated overnight in 0.1% FCS, were pretreated for 2h with either 30 nM Wortmannin or 40–160 μM LY294002 or for 30 min with 20 μM $AACOCF_3$. All the inhibitors were obtained from Calbiochem and dissolved in DMSO.

Measurement of JNK activation. Cells, plated in 10% FCS, were switched to 0.1% FCS after attachment. The next day they were stimulated for the indicated time with interleukin 1-β (3 ng/ml) or H_2O_2 (1 mM). After treatment, the cells were washed twice in PBS, and lysed in 25 mM Hepes pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate (Na_3VO_4), 0.5 mM PMSF, and 10 μg/ml of aprotinin and leupeptin. After 20 min incubation on ice the lysates were microfuged at maximum speed for 20 min and the protein concentration in supernatant was determined using the Bradford method (Bio-Rad Laboratories). To 150 μg of protein lysates were added 25 μl of c-Jun fusion protein beads (New England BioLabs) and incubated at 4°C on a rotating wheel overnight. The samples were then washed four times in 20 mM Hepes pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, and 0.05% Triton X-100 (HIBI buffer). The kinase reaction was run for 20 min at 30°C in 20 mM Hepes pH 7.7, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 0.1 mM Na_3VO_4, 2 mM DTT, 20 μM ATP, and 5 μCi of γ [^{32}P]-ATP. The reaction was stopped with 500 μl of ice cold HIBI buffer, the beads were pelleted, resuspended in PAGE loading buffer, boiled for 5 min, and the products were separated on a 12% SDS gel (Novex) and quantitated using the PhosphoImager.

Antisense oligonucleotide treatment. A549 cells were plated in 6 well plates at the concentration of 2.10^5 cells/well. After 24 hours, the cells were washed twice in Opti-MEM (Gibco/BRL), and the oligonucleotide formulated in Lipofectin (Gibco/BRL) and Opti-MEM, at a constant ratio of 2.5 μg/ml lipofectin per 100 nM oligonucleotide, was added, and the cells incubated for 4 hours. Following the incubation period, the oligonucleotide treatment solution was removed and replaced with DMEM, 0.1% FCS and incubated for either 24 or 48 hours.

Northern blots. 24 h after the oligonucleotide treatment, mRNA was purified using the Micro Fast Track Kit (InVitrogen), separated on a 1 % agarose/formaldehyde gel, transferred to Hybond-N+ membrane (Amersham), and probed. A PCR generated fragment (Fp 5′-TGC AAG CAC AGC CCT TAT G-3′; Rp 5′-TGT CAA AAG GAC CCT GGT G-3′) in the 5′-UTR of RhoA was used as a template for asymmetric PCR, in the presence of [α-^{32}P]dCTP (Amersham), to generate the RhoA probe. The G3PDH cDNA was purchased from Clontech and labeled by random primer using the Large (Klenow) Fragment (Gibco/BRL).

Western blots. The cells were washed twice in PBS and lysed in 25 mM Tris-HCl pH 7.5, 1 % Triton X-100, 0.2% SDS, 0.5 % sodium deoxycholate, 450 mM NaCl, and 10 μg/ml aprotinin and leupeptin. After 15 min on ice the samples were microfuged at maximum speed and the protein concentration in the supernatant was determined. 50 μg of protein was separated by SDS-PAGE (15 % gel). Following electrophoresis, proteins were transferred to ImmobilonP membrane (Millipore). The membrane was blocked in 5 % fish gelatin (Sigma) and RhoA and RhoB specific antibodies (Santa Cruz Biotechnology) were used to visualize the proteins. After incubation with the appropriate secondary antibody, the proteins were visualized using either Lumiglo Reagent (New England BioLabs) or ECL+Plus (Amersham).

Phosphatidylinositol 3-kinases assay. After overnight incubation in 0.1% FCS, cells were stimulated for the indicated times with 1 mM H_2O_2, and lysed in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 10 μg/ml of aprotinin and leupeptin. The enzyme was immunoprecipitated, for 2 hours at 4°C, from 150 μg of cell lysate using anti-phosphotyrosine antibody (4G10) conjugated to protein A beads (Upstates Biotechnology). The immunoprecipitates were washed three times in lysis buffer, once in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, once in 100 mM Tris-HCl pH 7.5, 0.5 M LiCl, and twice in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA. All the washes were done in the presence of 0.1 mM Na_3VO_4. The beads were then resuspended in 50 μl of Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 0.1 mM Na_3VO_4 and 20 μg of either phosphatidylinositol (PI), or 10 μg of (PI4P), or 10 μg of (PI4,5,P_2) (Sigma). After 10 min at room temperature, the reaction was initiated by adding 1 μl of 1M $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP (Amersham). The reaction was stopped after 15 min at room temperature with 100 μl 1N NaCl and 200 μl of a 1:1 solution of methanol/chloroform. The lipid phase was then washed in a 1:1 solution of methanol/1 N HCl, dried, and resuspended in 50 μl of chloroform. The product was separated on silica gel thin layer chromatography plates (MERK), in chloroform/methanol/28 % ammonium hydroxide/water (90:90:9:19) and quantitated using the PhosphoImager (Molecular Dynamics).

RESULTS

H_2O_2 and IL-1β activate JNK in A549 cells. A dose response of H_2O_2 and IL-1β driven JNK activation following serum starvation was completed to determine optimal conditions. The concentrations that gave

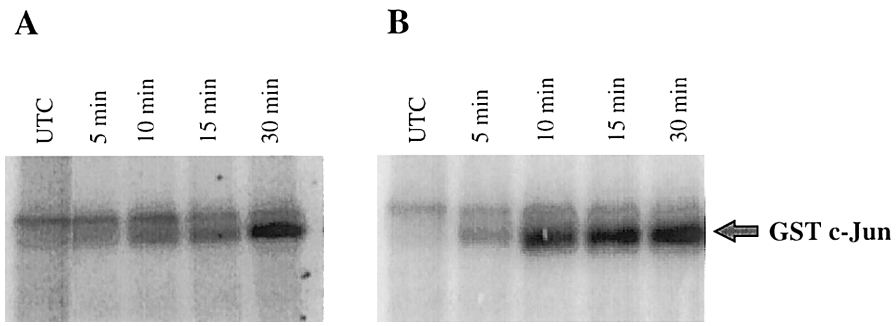

FIG. 1. H_2O_2 and IL-1β increase JNK activity. After overnight starvation in 0.1% FCS, A549 cells were either left unistimulated (UTC) or stimulated for the indicated times with 1mM H_2O_2 (panel A) or 30 ng/ml IL-1β (panel b). The JNK activity was measured as phosphorylation of GST-cJun.

optimal JNK activation were used to determine the kinetics of full activation. As shown in Fig. 1, both H_2O_2 (panel A) and IL-1β (panel B) were able to activate JNK. However, the kinetics of JNK activation were different. Whereas, 1mM H_2O_2 induces measurable levels of activation of the enzyme after 30 min stimulation, 30 ng/ml IL-1β induces maximal JNK activation by 10 min and remains active up to 30 min. Different experiments showed a 2 to 11 fold activation with H_2O_2 and a 3 to 16 fold activation with IL-1β.

Inhibition of RhoA mRNA and protein expression by antisense oligonucleotides. To investigate the importance of the small G protein RhoA in JNK activation, we designed antisense phosphorothioate oligonucleotides targeted against RhoA mRNA. Fourteen different oligonucleotides, all designed to target the coding region, were tested for their ability to specifically decrease the mRNA level (not shown). Dose response experiments indicated the most potent and specific inhibitor as ISIS 16201 (Table 1). To improve nuclease stability, its sequence was used to generate 2′-methoxyethyl phosphorothioate gapmer oligonucleotides (40); the effect of the position of the modification on the oligonucleotide sequence on the RhoA mRNA levels was investigated (table 1). All the modified oligonucleotides were able to inhibit RhoA mRNA, however ISIS 17131 was the most active (Fig. 2a); here ISIS 17134, a fully modified ASO, is used as a control oligonucleotide since 2′-methoxyethyl fully modified oligonucleotides are not RNase H substrates (40). ISIS 17131 was also able to specifically reduce the RhoA protein level (Fig. 2b). RhoA protein is reduced with 24h oligo treatment, but is still present at high concentrations, while protein levels were further decreased with 48–72 h treatment. ISIS 17163, here used as a control, is a 2′-methoxyethyl oligonucleotide design to reduce Rac1 mRNA levels.

TABLE 1

Sequence of the ASO Used in This Study

Target	ISIS no.	Antisense oligonucleotide sequence
RhoA	16201	5′-GGC TGT TAG AGC AGT GTC AA-3′
RhoA	17130	5′-GGC TGT TAG **AGC AGT GTC AA**-3′
RhoA	17131	5′-**GGC** TGT TAG AGC **AGT GTC AA**-3′
RhoA	17132	5′-**GGC TGT** TAG AGC AGT **GTC AA**-3′
RhoA	17133	5′-**GGC TGT TAG** AGC AGT GTC **AA**-3′
RhoA	17134	5′-**GGC TGT TAG AGC AGT GTC AA**-3′
cnt RhoA	18550	5′-**TGC** GGT AAG TGC **GGT ATC AA**-3′
Rac1	17163	5′-**ATA AGC CCA** GAT TCA CCG-3′
Ha-Ras	13920	5′-**TCC** GTC ATC GCT **CCT CAG GG**-3′

Note. The 2′-methoxyethyl sugar modification is indicated by the bold character. All the oligonucleotides are full phosphorothioates.

To further investigate the specificity of the oligonucleotide, dose response experiments were performed at 48 h (Fig. 2c). The mismatch control oligonucleotide, ISIS 18550, did not have any effect on RhoA protein level at any concentration tested. In addition, we measured the effect of ISIS 17131 on RhoB, which is highly homologous to RhoA. The cells were treated with 150 nM oligonucleotide and after 48 h re-treated with the same oligo concentration. Cells were left in culture for additional 72 h before harvesting. As shown in Fig. 2d, the antisense oligonucleotide is highly specific since it does not decrease RhoB protein levels.

Inhibition of JNK activation by RhoA antisense oligonucleotides in A549 cells stimulated with H_2O_2. A549 cells were treated with 150 nM oligonucleotide for 4 hours. The media was then replaced with 0.1% FCS-DMEM and the cells were left in culture for 48 h before stimulation because of the long half life of RhoA protein. The cells were stimulated with IL-1β or H_2O_2 for 30 min. As shown in Fig. 3a, the RhoA antisense oligonucleotide was able to specifically inhibit JNK activation (30–37.5 % inhibition in different experiments) when the cells were stimulated with H_2O_2. However, no inhibition of full activities was observed when the cells were stimulated with IL-1β (Fig. 3b). At higher concentrations (300 nM) the ASO inhibited IL-1β induced JNK activity in a non-specific manner,

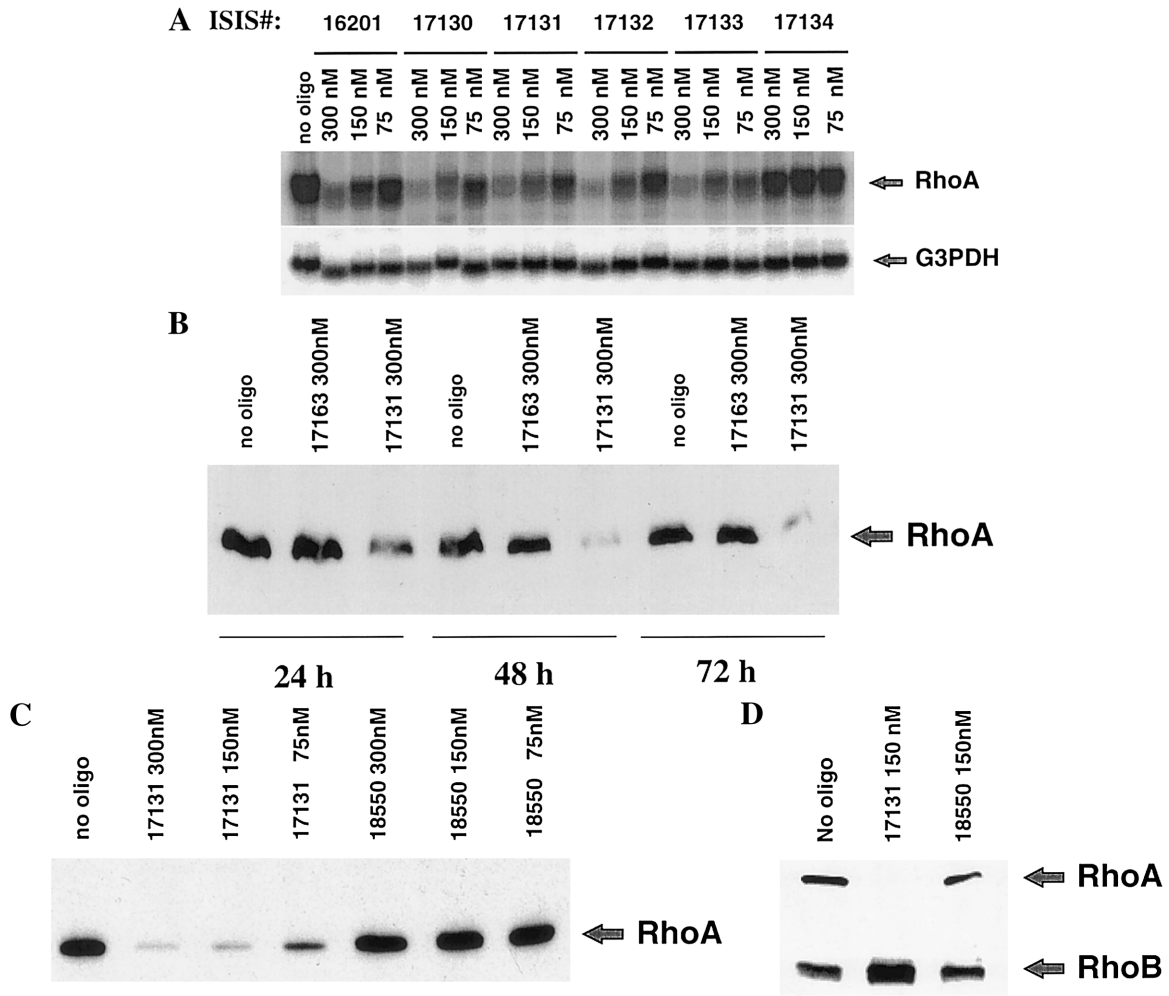

FIG. 2. Antisense oligonucleotide specifically reduce RhoA mRNA and protein levels. **(A):** A549 cells were treated with different antisense 2′-methoxyethyl phosphorothioate oligonucleotides targeting RhoA; 24 h after ASO treatment, RhoA mRNA levels were analyzed by Northern blotting. **(B):** The effect of ISIS 17131 on RhoA protein levels was determined 24 h, 48 h, and 72 h after ASO treatment. ISIS 17163, here used as a control, is a ASO design to inhibit Rac1 expression. **(C):** 48 h after treatment with different oligonucleotide concentrations (300 nM, 150 nM, and 75 nM), the cells were harvested and the RhoA protein levels were determined by western blotting. ISIS 18550 is a mismatch control ASO for ISIS 17131. **(D):** Cells were treated twice with 150 nM of either ISIS 17131 or ISIS 18550, as described in the experimental procedures. Five days after the first ASO treatment the cells were lysed and RhoA and RhoB protein levels were determined by western blotting.

since the control mismatch ASO (ISIS 18550) had the same effect (not shown).

Antisense methoxyethyl gapmer oligonucleotides to Rac1 and Ha-Ras did not have any effect on either H_2O_2 or IL-1β induced JNK activation (not shown).

Activation of PI 3-kinase in A549 cells stimulated with H_2O_2. Since recent studies *in vitro* suggested a possible role for PI 3-kinase for the activation of small G proteins, we investigated whether H_2O_2 could activate PI 3-kinase. The activity was measured after stimulation with 1mM H_2O_2, the same concentration used to stimulate JNK, at different times. As shown in Fig. 4a, PI 3-kinase is rapidly activated, as measured by phosphorylation of phosphatidylinositol (PI), and reaches its maximum after 2.5 min. At 10 min, the activity has returned to background level. Similar results were obtained using phosphatidylinositol 4,5-diphosphate ($PI(4,5)P_2$) as a substrate (Fig. 4b). Fig. 4c and 4d represent a quantitative analysis of the products, PIP and ($PI(3,4,5,)P_3$). Similar kinetics of activation were obtained using phosphatidylinositol 4-monophosphate as substrate (not shown). In different experiments, the enzyme was activated 1.7–2 fold.

Inhibition of JNK activity by Wortmannin and $AACOCF_3$. A549 cells, incubated overnight in 0.1 % FCS, were pretreated for 2 h with 30 nM Wortmannin and for 30 min with 20 μM $AACOCF_3$ prior to stimulation with either IL-1β or H_2O_2. Both inhibitors decreased H_2O_2 induced JNK activation (Fig. 5a and 5b). In different experiments, Wortmannin inhibited JNK

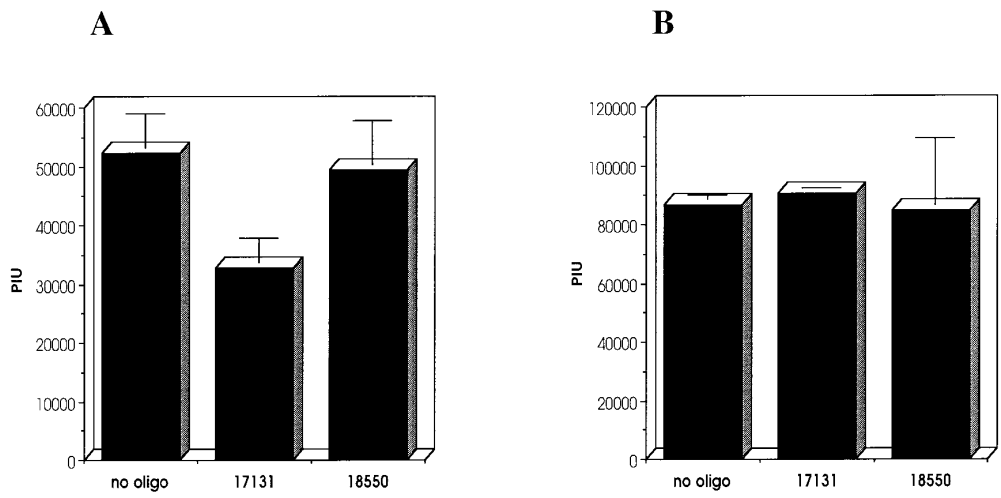

FIG. 3. Antisense oligonucleotide to RhoA reduces H_2O_2 mediated JNK activity. **(A):** Lysates of A549 cells pretreated with ASO and stimulated 48 h later with H_2O_2 for 30 min, were assayed for JNK activity. The graph represents the average of the amount of phosphorylated GST-jun expressed in PhosphoImager Units (PIU). **(B):** The cells, treated as in panel A, were stimulated for 30 min with IL-1β before measuring the JNK activity.

activity by 43–53% and AACOCF3 inhibited the activity of the enzyme by 40–57%. DMSO, used at the same concentrations and time points, did not have any effect on H_2O_2 induced JNK activation (not shown). Neither inhibitor had any effect on IL-1β induced activation of the enzyme.

DISCUSSION

The Rho family of small G proteins has been implicated in multiple cellular processes which include cytoskeleton rearrangements, cellular transformation, and JNK and NFkB activation. Here, we investigated the role of RhoA in JNK activation induced by different stimuli using antisense oligonucleotides. ASO allowed us to determine their function without overexpressing the protein, which can potentially lead to misleading conclusions, since often times overexpressed proteins are not localized in their natural cellular compartments. Therefore, the results obtained with the overexpressed plasmids may not represent the true physiological functions of proteins. In addition, antisense oligonucleotides are more specific than the dominant negative mutants, since these constructs have inhibitory effects on highly homologus proteins (for example, the Rac dominant negative mutant inhibits the activity of both Rac1 and Rac2) (41).

Modified oligonucleotides were designed to increase the affinity and the stability of the phosphorothioate oligonucleotide. Among those tested, the 2′-methoxyethyl modifiefication showed higher stability compared to the phosphorothioate (40). This allows longer exposure to the cells and to achieve good inhibition of proteins even with relatively long half lives (a recent report stated that RhoA half life in a macrophage cell line was 31 h) (42). Therefore, we believe antisense oligonucleotides are an efficient tool to dissect signaling pathways and they will allow us to understand the exact role of different proteins which may share common effector molecules.

In this study, we stimulated JNK with a cytokine (IL-1β) or oxidative stress (H_2O_2) and determined the effect of antisense oligonucleotides to RhoA on JNK activation. We also tested a Rac1 antisense oligonucleotide (ISIS 17163). Interestingly, the Rac1 oligo did not have any effect on the activity of the JNK when stimulated either with IL-1β, or H_2O_2, or UV, or high temperatures (45°C for 30′) suggesting that Rac1 is not necessary for JNK activation in A549 when these stimuli are used (not shown).

Contrary to this, the RhoA ASO (ISIS 17131) was able to effectively inhibit JNK activation by H_2O_2, but did not have any effect on IL-1β induced JNK activation. In additional studies, RhoA antisense oligonucleotide also inhibits JNK activation induced by UV (not shown). The decrease in JNK activity by ASO to RhoA was small (30–37.5 %), but significant. Higher inhibition might not expected since the cells express different Rho genes which may have redundancy in function. In addition, after treatment with ISIS 17131 the cells seem to compensate the RhoA decreased level by increasing, at least to a certain extent, RhoB expression. Furthermore, some RhoA protein is still present in the cells treated with the ISIS 17131. The results described in this report may be somewhat contradictory to other published studies on the role of Rac and Rho as upstream activators of JNK (18, 19). One explanation of this discrepancy could be the cell line used. Another group reported that RhoA or Cdc42 overexpression, but

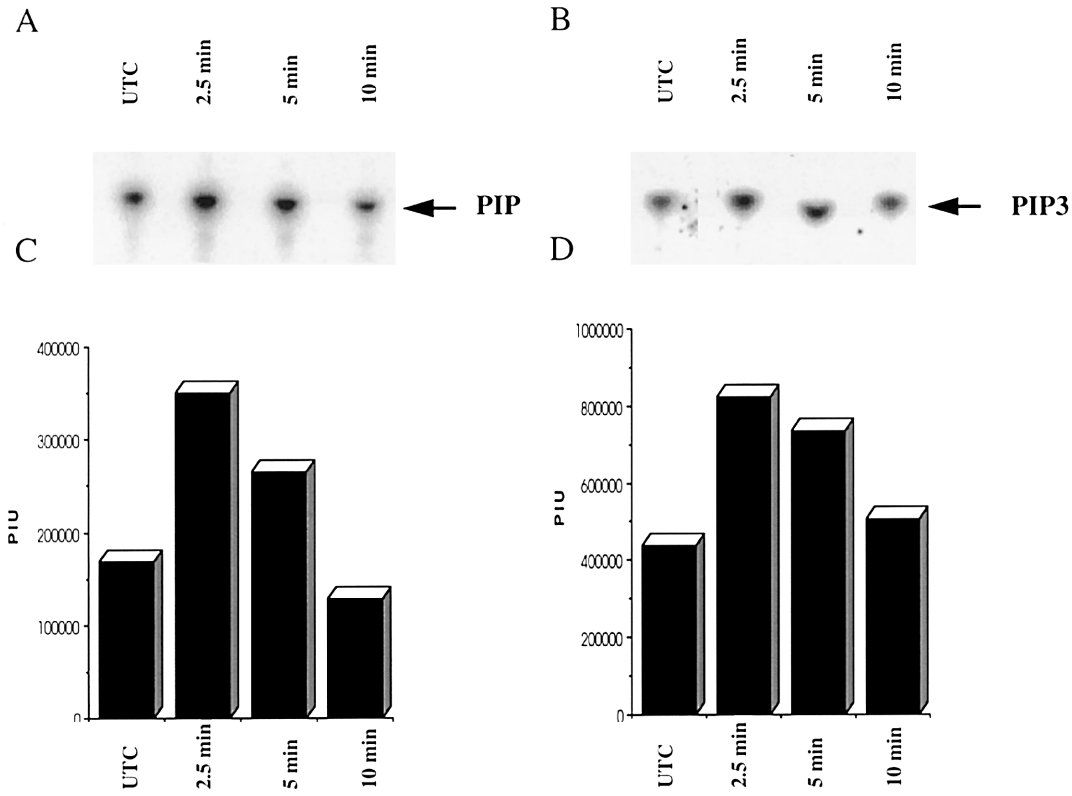

FIG. 4. H_2O_2 increases PI 3-kinase activity in A549 cells. A549 cells were treated with 1 mM H_2O_2 for the indicated times (2.5 min, 5 min, 10 min), and PI 3-kinase activity, recovered in the antiphosphotyrosine immunoprecipitates, was measured. Both phosphatidylinositol (PI) (panel A) and phosphatydilinositol 4,5 biphosphate (panel B) were used as substrates. The reaction products are indicated as phosphatidylinositol monophosphate (PIP), and phosphatidylinositol (3,4,5) phosphate (PIP3). Panel C and D represent the quantitative analysis of the gels in panel A and B, respectively.

not Rac1 overexpression, resulted in stimulation of JNK activity in 293T cells (20).

Surprisingly, RhoA antisense inhibition did not affect IL-1β induced JNK activation. Since it has been shown that activation of the IL-1 receptor stimulates JNK activation through the production of reactive oxygen species in bovine chondrocytes (43), we expected to see the same pattern of inhibition and similar kinetics of activation of the JNK. At the least, it would be expected that H_2O_2 activates JNK more rapidly than IL-1β. Instead, no common upstream molecule which regulates both IL-1β and peroxide induced JNK activation could be identified. However, RhoA, PLA_2, and possibly PI 3-kinase are playing a role in JNK activation mediated by H_2O_2. These results suggest that in this system H_2O_2 is not acting as an IL-1β induced second messenger. Consistent with previous published observations, Ha-Ras antisense inhibition did not have any effect on either IL-1β or H_2O_2 stimulated JNK activation (not shown).

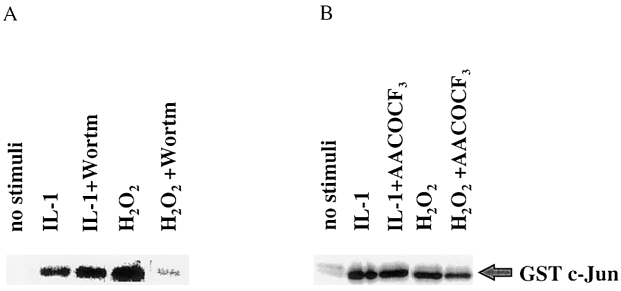

FIG. 5. H_2O_2 stimulated JNK activity is inhibited by Wortmannin and $AACOCF_3$. A549 cells were pretreated for 2 h with 30 nM Wortmannin (panel A) and for 30 min with 20 μM $AACOCF_3$ (panel B) and then stimulated with 30 ng/ml IL-1β and 1 mM H_2O_2 for 30 min. The cells were then lysed and the JNK activity was measured as the amount of phosphorylated GST-jun.

The importance of PLA_2 in JNK activation has already been demonstrated in rat astrocytes (44). Unfortunately, due to the lack of specificity of Wortmannin, it was not possible to conclude whether PI 3-kinase is playing a direct role in this signaling pathway. Micromolar concentrations of LY29004, a selective PI 3-kinase inhibitor (45), did not have any effect on either JNK activity or PI 3-kinase activity in A549 cells (not shown). However, this drug did inhibit the PI 3-kinase activity when added directly to the cell lysates, suggesting poor drug uptake by

A549 cells. We believe that antisense oligonucleotides to PI 3-kinase isoforms will help elucidate their role in this pathway.

In this report, we have identified selective and potent ASO to RhoA and demonstrated that ASO are useful tools to understand the role of specific members of a multigene family. In addition, we demonstrated that JNK activation by H_2O_2 and IL-1β is mediated by different pathways. JNK activation by peroxide requires functional RhoA and PLA_2.

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