Ras-related GTPase RhoB Represses NF-κB Signaling*

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RhoB encoding a Ras-related GTPase is immediately early inducible by genotoxic treatments, indicating that it is part of the cellular stress response. Here, we investigated the influence of RhoB on signal pathways that are rapidly evoked by genotoxic compounds. The data obtained show that wild-type RhoB neither affects activation of mitogen-activated protein kinases nor AP-1-dependent gene expression. However, RhoB inhibited both basal and genotoxic agent-stimulated activity of the transcription factor nuclear factor κB (NF-κB). Thus, RhoB attenuated alkylation-induced increase in the DNA binding activity of NF-κB and abrogated NF-κB-driven gene expression. Furthermore, RhoB inhibited decrease in the cellular amount of IκBα after genotoxic stress as well as after tumor necrosis factor α and 12-O-tetradecanoylphorbol acetate treatment. This indicates that RhoB represses NF-κB activation by inhibiting dissociation and subsequent degradation of IκBα. On the basis of the data, we suggest that RhoB is a novel negative regulator of NF-κB signaling.

Ras-related small GTPases (molecular mass, ~21 kDa) of the Rho family (e.g. Rho, Rac, and Cdc42) are known to be involved in a large variety of cellular processes, such as the organization of the microfilament network (1–5), cell cycle progression (6, 7), cellular transformation (8–10), and apoptosis (11–13). Furthermore, Rac and Cdc42 have been shown to interfere with genotoxic stress-induced signaling by regulating the activation of N-terminal c-Jun kinases (JNKs)/stress-activated protein kinases (SAPKs) and p38 mitogen-activated protein (MAP) kinase (14–16). This regulatory function of Rac and Cdc42 is independent of their influence on cytoskeleton or cell cycle (17). After exposure of cells to genotoxic stress, Rac and Cdc42 trigger, via activation of MAP kinases, the activity of transcription factors such as c-Jun, c-Fos, Elk-1, and ATF-2 (18). Thereby, the pattern of gene expression in cells exposed to DNA damage is controlled. Another transcription factor that is activated by different types of cellular stress is nuclear factor κB (NF-κB; Refs. 19, 20). A prerequisite for activation of NF-κB by tumor necrosis factor α (TNFα) is IKK-dependent phosphorylation of the inhibitory molecule IκBα (19). On its phosphorylation on Ser32, NF-κB is released for nuclear translocation, and free IκBα is proteosomally degraded (19). In the case of treatment of cells with ionizing radiation and UV light, activation of NF-κB occurs independent of phosphorylation of IκBα on Ser32 (21, 22). Moreover, UV-induced activation of NF-κB is even independent of IKK (22). Thus, overall, activation of NF-κB by genotoxic stress is different from activation triggered by TNFα. Previously, it was reported that Rho family GTPases interfere with the TNFα-induced activation of NF-κB (23). Also, different members of nuclear exchange factors for Rho proteins are able to stimulate NF-κB (24), indicating that multiple, Rho-regulated pathways are involved in NF-κB regulation.

Recently, we have shown that the gene encoding the small GTPase RhoB belongs to the group of immediate-early inducible genes (25). It is activated by genotoxic stress (25, 26) as well as by growth factors (25, 27). The regulation of RhoB induction appears to be different from that of c-jun and c-fos (25–27). The physiological function of RhoB is largely unknown. One interesting feature of RhoB is that it is essentially required for Ras-mediated transformation (8). Therefore, and because RhoB is subject to modification by farnesylation (28), RhoB is discussed as a further physiologically relevant target of farnesyltransferase inhibitors (29). Originally, this type of drug was described as a promising tumortherapeutic acting on Ras (30, 31). A variety of evidence strongly indicates that the function of RhoB is distinguished from that of other Rho species such as RhoA and RhoC. Thus, RhoB is distinguished from RhoA/RhoC in its C-terminal modification by farnesylation (28), intracellular localization (32) and concentration (25), association with regulatory proteins (33), cell cycle-specific expression (34), endosomal targeting of PRK1 (protein kinase C-related kinase 1) (35), and inducibility by growth factors and genotoxic stress (25–27). The latter finding is of particular interest because the rapidly increased expression of RhoB in cells exposed to a genotoxic agent indicates that RhoB plays a role in the cellular response to induced DNA damage. This view gains support from the recent finding that RhoB forces cells to alkylation-induced apoptotic cell death (36). Because of its GTPase function, it is reasonable to assume that RhoB can influence signal transmission in a very fast manner. Having this in mind, we wondered whether RhoB interferes with early steps of cellular stress response, in particular with stress-induced gene expression participating in the regulation of apoptosis. To address this question, we analyzed the effect of RhoB on the activation of MAP kinases and NF-κB signaling by genotoxic agents. Here, we show that RhoB represses NF-κB signaling without influencing MAP kinase-regulated pathways.

EXPERIMENTAL PROCEDURES

Materials—Rat RhoB cDNA was provided by T. Hunter. Hemagglutinin (HA) antibody was purchased from Roche Molecular Biochemicals, and IKKα antibody was from Pharmingen (Hamburg, Germany). Other antibodies used in the present study were from Santa Cruz Biotechnol-
ogy Inc. (Santa Cruz, CA). Methyl methanesulfonate (MMS) was purchased from Sigma. Mafosfamide was from ASTA Medica (Frankfurt, Germany). The NF-κB-specific reporter gene construct (3xNF-κB-luciferase) was kindly provided by U. R. Rapp. The human collagenase promoter-chloramphenicol acetyltransferase (Call-CAT) construct containing the AS2-regulated fragment of human intestinal collagenase gene (37) was obtained from H. J. Rahmsdorf, and the HA-JNK1 expression construct originates from M. Karin. Expression vectors encoding Rho GTPases Rhoa, Cdc42, and Rac were a gift from A. Hall.

**Cell Culture and Transfection Experiments**—NIH 3T3 cells were routinely grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Isolation of RhoB-overexpressing cells has been described previously (36). For determination of NF-κB-dependent gene expression, an NF-κB-specific reporter gene construct was used (3xNF-κB-luciferase; Ref. 38). 24 h after transfection of NIH 3T3 cells together or without an expression vector encoding the corresponding Rho GTPases, cells were exposed to a given genotoxic compound. After an incubation period of 24 h, cells were harvested, and luciferase activity was measured using a luciferase assay (Promega). To analyze the effect of Rho GTPases on the activity of the AP-1-regulated collagenase promoter, a human Coll-CAT construct (−73/+63; Ref. 37) was used for transfection experiments. 24 h after transfection, cells were harvested, and the amount of CAT protein was determined by the use of an enzyme-linked immunosorbent assay-based method (Roche Molecular Biochemicals).

**Western Blot Analysis**—20–30 μg of protein from nuclear or total cell extracts (1000 x g supernatant) were separated on 10% SDS-polyacrylamide gels. Proteins were wet-blotted to nitrocellulose filters and were detected using the corresponding primary antibodies and subsequently a peroxidase-coupled secondary antibody. Incubation of filters with antibodies was performed for 2 h at room temperature in 5% dry milk in phosphate-buffered saline and 0.1% Tween 20. For visualization of proteins, an ECL detection system was used (PerkinElmer Life Sciences).

**Gel Retardation Analysis**—32P labeling of oligonucleotides was performed using T4 kinase. Total cell extracts were prepared as described (39). Proteins were separated by electrophoresis of 10 μg of protein from NIH 3T3 extracts with ~5 fmol of 32P-labeled oligonucleotide for 30 min at room temperature (binding buffer, 10 mM Heps, pH 7.9, 60 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 μM/ml bovine serum albumin, 10% glycerol, 0.5 μg poly(dI-dC)). Subsequently, reaction mixtures were separated on 4% polyacrylamide gels at room temperature. After electrophoresis, gels were dried and subjected to autoradiography. A peroxidase-coupled secondary antibody resulted in a strong supershift in control cells (Fig. 2A). When using the anti-c-Jun antibody as a control, a supershifted, 32P-labeled complex was not observed (Fig. 2B). Therefore, quantitative differences in p50 and p65 were determined (Fig. 2C).

**Kinase Assays**—JNK, p38, and extracellular signal-regulated kinase (ERK) kinase activities were measured by the use of an anti-p65 antibody (data not shown). When using the anti-c-Jun antibody as a control, a supershifted, 32P-labeled complex was not observed (Fig. 2B). As determined by Western blot analysis, p50 and p65 protein levels are not changed in RhoB-overexpressing cells compared with control cells (Fig. 2C). Therefore, quantitative differences in p50 and p65 expression cannot be responsible for the differences in DNA binding activity of NF-κB in RhoB transfectants versus control cells.

**RESULTS**

To analyze whether RhoB interferes with the early cellular response to genotoxic stress, we made use of NIH 3T3 cells stably and transiently overexpressing wild-type RhoB protein. For stable transfection, a pCDNA3neo expression vector containing the DNA sequence for His-tagged wild-type RhoB was used. Cell clones overexpressing RhoB were identified by Western blot analysis using a His-specific antibody (36). Activation of MAP kinases represents one type of early cellular response to genotoxic stress that affects gene expression (18). Comparing parental and RhoB-overexpressing cells with regard to the activation of MAP kinases, we observed a similar level of activation of JNK1, p38 MAP kinase, and ERK2 on treatment with MMS and UV light (Fig. 1A). In line with this, transient overexpression of RhoB (V14RhoB) also failed to stimulate JNK1 activity (Fig. 1B). On the other hand, as one would expect from data previously reported (15, 16), Cdc42 (V12Cdc42) was able to activate JNK1 (Fig. 1B). The UV- and MMS-stimulated increase in the DNA binding activity of AP-1 was similar in RhoB-overexpressing and control cells (Fig. 1C). In accordance, the activity of an AP-1-regulated human collagenase promoter fragment (−73/+63; Ref. 37) was not affected by transient overexpression of RhoB, whereas overexpression of Rac and Rhoa caused its activation (Fig. 1D). A stimulatory effect of Rac and Rhoa on AP-1 transcription was reported recently for T cells (41). Overall, the findings strongly indicate that RhoB is unable to influence activation of MAP kinases and AP-1-regulated gene expression.

Another important element involved in the regulation of gene expression induced by cellular stresses is the transcription factor NF-κB (19). To analyze whether RhoB affects NF-κB activity, a 32P-labeled oligonucleotide containing an NF-κB-specific consensus sequence was incubated with extracts from wild-type and RhoB-overexpressing cells. After gel electrophoresis, two 32P-labeled complexes, the specificity of which was shown by competition experiments, were detected in wild-type cells (Fig. 2A). These complexes are known to consist of p50/p65 heterodimers (complex 1) and p50/p50 homodimers (complex 2), respectively (21, 42). In contrast to wild-type cells, extracts from RhoB-overexpressing cells predominantly showed expression of complex 2 (i.e. p50/p50; Fig. 2A), whereas complex 1 (i.e. p50/p65) was only very poorly detectable (Fig. 2A).

Preincubation of the binding reaction with p50-specific antibody resulted in a strong supershift in control cells (Fig. 2B). Appearance of the p50-induced supershift is accompanied mainly by a decrease in the intensity of complex 1 (i.e. p50/p65) Under conditions of RhoB overexpression, this supershift was largely reduced (>80%; Fig. 2B). Similar results were obtained by the use of an anti-p65 antibody (data not shown). When using the anti-c-Jun antibody as a control, a supershifted, 32P-labeled complex was not observed (Fig. 2B). As determined by Western blot analysis, p50 and p65 protein levels are not changed in RhoB-overexpressing cells compared with control cells (Fig. 2C). Therefore, quantitative differences in p50 and p65 protein expression cannot be responsible for the differences in DNA binding activity of NF-κB in RhoB transfectants versus control cells.

The question arising next was whether RhoB is able to interfere with drug-induced activation of NF-κB. As analyzed 2 h after exposure of control cells to the alkylating mutagen MMS, DNA binding activity of NF-κB was clearly enhanced. In contrast, MMS failed to stimulate NF-κB binding activity in RhoB-overexpressing cells (Fig. 3A). A mechanism behind the RhoB-triggered inhibition of NF-κB activation on alkyla could be blockage of nuclear translocation of NF-κB. To test this hypothesis, we analyzed the appearance of p50 protein in the nuclear fraction after MMS treatment. As shown in Fig. 3B, an ~11-fold increase in the amount of p50 protein in nuclear extracts of control cells was detected within 8 h after MMS treatment. RhoB-overexpressing cells, however, did not show elevation of p50 protein level (Fig. 3B). Basically the same results were
obtained when translocation of p65 was measured (data not shown). Thus, on the basis of data obtained from both gel retardation experiments and Western blot analysis, we conclude that RhoB impairs the activation of NF-κB in cells exposed to alkylating agents.

Recently, it was shown that the DNA binding activity of NF-κB and nuclear translocation are not necessarily related to NF-κB-dependent gene expression (43). Therefore, we investigated whether RhoB exerts an effect on NF-κB-driven gene expression. To this end, transfection experiments using an NF-κB-specific minimal reporter gene construct (3xNF-κB-luciferase) were performed. These studies revealed that both basal and MMS-induced activation of gene expression triggered by NF-κB are largely blocked in RhoB-overexpressing cells
We point out that the same is true when the NF-κB reporter construct was transiently coexpressed with wild-type RhoB (Fig. 4B). Whereas control cells showed a 3–5-fold increase in luciferase activity on MMS exposure, stable as well as transient overexpression of wild-type RhoB largely blocked this response (Fig. 4C). Neither the coexpression of wild-type Rac nor that of RhoA blocked NF-κB signaling (Fig. 4D). Overall, the data indicate that RhoB acts as an inhibitory component of both basal and MMS-induced NF-κB-regulated gene expression.

A prerequisite for NF-κB activation, at least on treatment with TNFα, is IKK-dependent phosphorylation of the inhibitory molecule IκBα (19). On phosphorylation on Ser32, IκBα dissociates from NF-κB and subsequently becomes degraded (19). Genotoxic stress-induced activation of NF-κB appears to be regulated differently from that of TNFα (21, 22). This is
illustrated by the finding that stimulation of NF-κB by UV light results in IKK-independent degradation of IκBα (21, 22). A possible mode of action of RhoB would be that it inhibits MMS-induced IKK activation. However, similarly, as reported for UV light (22), the alkylating agent MMS failed to activate IKK (Fig. 5). Thus, the inhibitory effect of RhoB on MMS-stimulated NF-κB activity cannot be explained by inhibition of IKK. As observed 8 h after MMS exposure, the IκBα protein level was reduced by ~40% in control cells but remained unchanged in RhoB-overexpressing cells (Fig. 6). Obviously, RhoB attenuates the MMS-induced decrease in IκBα.

To address the question of whether the inhibitory effect of RhoB on NF-κB is specific for alkylating agents, we investigated the effect of RhoB on other NF-κB-activating compounds. As shown in Fig. 7A, RhoB attenuates both UV- and 12-O-tetradecanoylphorbol acetate (TPA)-induced increase in the DNA binding activity of NF-κB. Also, UV- and TPA-driven reduction in IκBα was abolished by RhoB (Fig. 7, B and C), yet RhoB did not impair activation of ERK2 by TPA, (Fig. 7D) as analyzed in identical extracts. This finding further supports the view that RhoB specifically interferes with NF-κB signaling without affecting MAP kinase-regulated pathways. An inhibitory effect of RhoB on NF-κB-driven gene expression was observed not only for MMS but also for other genotoxic compounds, such as mafosfamide, which is a cyclophosphamide analogue, and UV light (Fig. 8). This was shown both by the use of stably transfected cells (Fig. 8A) and by transient overexpression of wild-type RhoB (Fig. 8B). Compared with MMS and mafosfamide, the repressive effect of RhoB on UV-induced NF-κB activation was only partial (Fig. 8). This is in line with data obtained from gel retardation experiments shown before (see Fig. 7). Finally, we analyzed whether RhoB interferes with activation of NF-κB by TNFα. As shown in Fig. 9, the TNFα-induced increase in the DNA binding activity of NF-κB (Fig. 9A) as well as the TNFα-driven degradation of IκBα (Fig. 9B) were partially blocked by RhoB. Obviously, compared with genotoxic agents and TPA, RhoB impairs TNFα-mediated signaling to NF-κB to a lesser extent. Table I summarizes the inhibitory function of RhoB on the effectiveness of different types of agents to evoke IκBα degradation. It turns out that RhoB exerts an inhibitory effect on IκBα degradation stimu-

![Fig. 5. MMS does not stimulate IKK activity.](image)

![Fig. 6. RhoB inhibits MMS-induced degradation of IκBα.](image)

![Fig. 7. RhoB impairs signaling to NF-κB stimulated by TPA or UV light.](image)
lated by various types of NF-κB-activating agents, including genotoxic and nongenotoxic compounds. Thus, RhoB appears to interfere with the regulation of different signal pathways converging in the activation of NF-κB.

DISCUSSION

The gene coding for the small GTPase RhoB is immediately-early-inducible by DNA-damaging agents such as UV irradiation and alkylating compounds (25, 26). On the basis of this and having in mind its GTPase function, which enables a very rapid modulation of signal mechanisms, we hypothesized that RhoB is important for the regulation of early cellular responses to genotoxic stress. This hypothesis was supported by our recent observation that overexpression of RhoB affects cellular sensitivity to induced DNA damage (36). In particular, overexpression of RhoB renders cells hypersensitive to alkylation-induced apoptotic cell death (36). Here, we analyzed the influence of RhoB on signal mechanisms that are quickly activated by DNA-damaging treatments, focusing on MAP kinase- and NF-κB-related mechanisms.

Analyzing the influence of RhoB on the stimulation of MAP kinases by UV light and MMS, we found that RhoB-overexpressing cells were not distinguished from the control. Genotoxic stress-induced activation of ERK2, JNK1, and p38 MAP kinase were not affected by RhoB. In line with this, the UV- and MMS-stimulated increase in the DNA binding activity of AP-1 was not changed by RhoB. Furthermore, transient overexpression of RhoB failed to stimulate JNK1 activity and did not increase the expression of the AP-1-regulated human collagenase promoter. Obviously, RhoB does not interfere with the genotoxic stress-induced activation of MAP kinases and AP-1-regulated gene expression. In contrast, as revealed by gel retardation experiments, RhoB overexpression causes consider-
able changes in the basal DNA binding activity of the transcription factor NF-κB. Thus, the NF-κB-specific DNA binding complex p50/p65 was only very poorly detectable in the transfectants. Furthermore, compared with control cells, an anti-p50-antibody-induced supershift was largely reduced under conditions of RhoB overexpression. Because the level of p50 and p65 protein was similar in wild-type and variant cells, we suppose that RhoB overexpression gives rise to posttranslational changes of p50, which in turn affect its interaction with other members of the NF-κB family, in particular Rel A (p65).

Most interestingly, RhoB overexpression inhibited stimulation of the DNA binding activity of NF-κB after exposure of cells to the alkylating compound MMS. In line with the current view of NF-κB activation, RhoB-mediated abrogation of NF-κB binding activity was found to be accompanied by inhibition of nuclear translocation of NF-κB in RhoB-overexpressing cells treated with MMS. As a consequence of the inhibition of the ability of NF-κB to enter the nucleus and to bind to DNA, basal and MMS-induced expression of NF-κB-regulated genes is blocked by RhoB. This was shown in reporter gene transfection experiments, which is important to note, because, as reported recently, DNA binding activity of NF-κB is not necessarily related to its capacity to stimulate promoter activity (43). The same inhibitory effect on NF-κB-regulated gene expression as observed in RhoB-overexpressing cell lines was found in cells transiently coexpressing wild-type RhoB. This shows that stable overexpression of wild-type RhoB is not associated with unphysiological side effects. Inhibition of NF-κB-driven gene expression appears to be very specific for RhoB because it was not observed with with Rac or RhoA. In line with another report, NF-κB activity was even stimulated by RhoA (23). Recently, we showed that overexpression of RhoB results in hyperstimulation of NF-κB, which in turn affects its interaction with one or various transcription factors that become activated by genotoxic stress. Thus, as already observed for genotoxic stress-induced signal transduction, we showed that overexpression of RhoB results in hypertrophy of NF-κB-driven gene expression back to the basal level. This may be achieved by inhibition of the activity of one or various transcription factors that become activated by genotoxic treatment. One of these factors is NF-κB. Therefore, we suggest that transient induction of RhoB in cells exposed to a genotoxic agent counteracts coactivated gene expression triggered by NF-κB. The hypothesis of a homeostatic role of RhoB in gene expression is supported by the finding that stimulation of gene expression by transforming growth factor β is accompanied by increase in RhoB protein level (because of RhoB stabilization), which in turn provokes abrogation of the transforming growth factor β response (48).

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