The small GTPase RhoB is immediate-early inducible by DNA damaging treatments and thus part of the early response of eukaryotic cells to genotoxic stress. To investigate the regulation of this cellular response, we isolated the gene for rhoB from a mouse genomic library. Sequence analysis of the rhoB gene showed that its coding region does not contain introns. The promoter region of rhoB harbors regulatory elements such as TATA, CAAT, and Sp1 boxes but not consensus sequences for AP-1, Elk-1, or c-Jun/ATF-2. The rhoB promoter was activated by UV irradiation, but not by 12-O-tetradecanoylphorbol-13-acetate treatment. rhoB promoter deletion constructs revealed a fragment of 0.17 kilobases in size which was sufficient in eliciting the UV response. This minimal promoter fragment contains TATA and CAAT boxes but no other known regulatory elements. Neither MEK inhibitor PD98059 nor p38 kinase inhibitor SB203580 blocked stimulation of rhoB by UVC (UV light, 254 nm) which indicates that ERK or p38 mitogen-activated protein (MAP) kinase are not involved in the UV induction of rhoB. Also, phosphatidylinositol 3-kinase inhibitor wortmannin, which blocks UV stimulation of both JNK and p38 MAP kinase, did not inhibit rhoB activation. Furthermore, activation of JNK by interleukin-1β did not affect rhoB expression. These data indicate that JNK is not involved in the regulation of rhoB. Overexpression of wild-type Rac as well as the Rho guanine-dissociation inhibitor caused activation of rhoB. Wild-type RhoB inhibited both basal and UV-stimulated rhoB promoter activity, indicating a negative regulatory feedback by RhoB itself. The data provide evidence both for a signal transduction pathway independent of JNK, ERK, and p38 MAP kinase to be involved in the induction of rhoB by genotoxic stress, and furthermore, indicate autoregulation of rhoB. The Rho subfamily of small GTP-binding proteins play an important regulatory role in diverse cellular functions, including endocytosis (1), membrane ruffling (2, 3), cell cycle progression (4), transformation (5, 6), and the organization of the cytoskeleton (7–10). Recently the small GTPases Rac and Cdc42 have been shown to selectively activate c-Jun N-terminal kinase (JNK) signaling without affecting MAP kinase (MAPK),1 whereas RhoA does not act on JNK nor MAPK (11–13). JNK represents a family of closely related enzymes which are activated by cellular stress; they are therefore also named as stress-activated protein kinases (p46/p54 SAPK) (14, 15). JNK activity is strongly stimulated by inhibitors of protein biosynthesis, such as cycloheximide and anisomycin, by inflammatory cytokines such as TNF-α and IL-1 and by ultraviolet (UV) light or other DNA-damaging agents (14–16). Activated JNKs are thought to phosphorylate the transactivation domain of c-Jun, thereby strongly increasing the transactivating activity of AP-1. This is the way the expression of various AP-1 dependent genes, including c-jun itself, is controlled (17).

Recently, it turned out that JNK-dependent phosphorylation of ATF-2, which can dimerize with c-Jun, is the major mechanism mediating c-jun induction upon genotoxic stress (18). The finding that UV-induced expression of c-fos is regulated by JNK-mediated phosphorylation of TCF/Elk-1 (19) indicates a central role of JNK (stress-activated protein kinases) in the regulation of DNA damage-induced expression of the immediate-early genes c-jun and c-fos. It has been supposed that the protein kinase phosphorylating c-Jun in response to TPA treatment is distinct from JNK and does not act on ATF-2 (18). Another important mechanism involved in the regulation of early mammalian responses to genotoxic stress is based on the activation of tyrosine kinases, c-Ha-ras and MAPK (20, 21). Since overexpression of activated Ha-Ras elicits JNK activity (15), a cross-talk between JNK and MAPK signaling appears to be possible. JNK are not activated by TPA, and to a much lesser extent by growth factors than ERK1/2 (14, 16). Furthermore, induction of c-fos and c-jun by inhibitors of protein biosynthesis is thought to be mainly due to the activation of JNK (22, 23). Recently it was shown that, beside JNK and ERK, p38 MAP kinase cascade also interferes with stress-induced signaling (24–27). This signaling pathway can also be stimulated by Cdc42 and Rac (28, 29).

RhoB, which belongs to the Rho family of small GTPases is, similar to c-fos and c-jun, very rapidly inducible by DNA damaging treatments (30). RhoB distinguishes from other Rho species (e.g. RhoA and RhoC) in its inducibility by both growth factors and DNA damaging agents, such as UV light and alkylating agents (30, 31), as well as by its intracellular localization (32). Thus, the physiological function of RhoB appears to be different from RhoA and RhoC which are thought to function mainly in the regulation of the actin cytoskeleton (10). Similar to c-fos and c-jun, the amount of rhoB mRNA is very rapidly enhanced after treatment of cells with cycloheximide, growth factors, and genotoxic agents. However, in contrast to c-fos and

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rhoB Encoding a UV-inducible Ras-related Small GTP-binding Protein Is Regulated by GTPases of the Rho Family and Independent of JNK, ERK, and p38 MAP Kinase*

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c-jun, the phorbol ester TPA did not elicit increase in rhoB mRNA expression (30, 31). This lack of TPA response prompted us to hypothesize that the regulation of rhoB expression is different from that of c-jun and c-fos.

In the present study, we aimed at elucidating the regulation of rhoB on the level of the promoter. Having cloned the mouse gene for rhoB and analyzed its promoter region, we demonstrate that rhoB promoter activity is autoregulated by small GTPases of the rho family. Furthermore, based on sequence data as well as inhibitor studies, we suggest a signal transduction pathway which is independent from JNK, ERK, and p38 MAP kinase to be involved in the UV stimulation of the rhoB promoter.

EXPERIMENTAL PROCEDURES

Materials—MEK inhibitor PD98059 and p38 kinase inhibitor SB203580 were purchased from Calbiochem (San Diego, CA). PI 3-kinase inhibitor wortmannin was obtained from Sigma (Germany). All antibodies used in this study originate from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids and Cell Lines—The RhoB expression plasmid pcDNA3-rhoB was constructed by cloning of a 1.6-kb EcoRI fragment from rat rhoB cDNA (31) into pcDNA3neo (Invitrogen). Complete cDNAs of human rhoB, rhoA, and V14 rhoA, and bovine rho-GDI were cloned as BamHI/EcoRI fragments from the corresponding bacterial pGEX expression vectors (obtained from K. Aktories, Freiburg, Germany) into pcDNA3neo. Eukaryotic expression vectors pEYV-cdc42, pEYV-N17cdc42, pEYV-V12cdc42, and pEYV-V12rac1 and pEYV-V14rhoA were generously provided by A. Hall (London, United Kingdom). Dominant-negative rac as well as dominant-negative rhoB were generated by replacing theronine for aspartagine at codon 17 of rac(N17rac) and codon 19 of rhoB(N19rhoB), respectively, using PCR-directed mutagenesis. Human Coll-CAT construct (Invitrogen) was generously provided by A. Balmain (Glasgow, UK) and E. Wagner (Vienna, Austria), respectively.

Cell Culture and Transfection Experiments—Fibroblast cell lines were routinely grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. For transient transfection experiments, 106 cells were seeded per 10-cm dish and transfected 16–24 h later with 5 μg of the promoter-CAT constructs and identical amount of indicated expression plasmids using the calcium phosphate coprecipitation technique (33). Salmon sperm DNA was added to a final DNA concentration of 20 μg/μl to each reaction. If not otherwise stated, cells were harvested after an incubation period of 24–48 h for determination of the level of CAT expression using an enzyme-linked immunosorbent assay (Boehringer Mannheim). Determination of protein concentration was done according to the method of Bradford (34). Data obtained from transfection and subsequent CAT analysis of rhoB promoter CAT constructs were reproduced at least twice. If not stated otherwise, representative experiments are shown. Induction factors of up to 1:5-fold were considered as nonsignificantly different from control which was set to 1.0.

Isolation of the Gene for rhoB and Sequence Analysis—For isolating the gene for rhoB, a AFIX II SVJ 129 mouse genomic library (Stratagene) was screened using a 0.8-kb ScaI fragment from rat rhoB cDNA as hybridization probe. This fragment covers the whole coding region and about 200 bp of the 3′-noncoding region of the rat rhoB cDNA. In total, 5 × 109 λ-clones were plated and screened according to a standard plaque screening protocol (35). After transfer and fixation of plaques to gene screen nylon filters (Amersham), the filters were hybridized with the indicated rhoB cDNA probe. 32P-labeled DNA was produced by random priming and hybridization was performed as described below. Genomic BamHI fragments (0.8 and 4.5 kb) which were shown to hybridize to the 32P-labeled rat rhoB cDNA probe were subcloned into Bluescript (Stratagene). Sequence analysis was performed by T7 sequencing (T7 sequencing kit, Pharmacia) as well as by automated sequencing (373A DNA Sequencer from ABI). Oligonucleotide primers for sequencing were purchased from MWG Biotech (Germany). The whole coding region as well as about 2 kb upstream from the ATG start codon were subcloned into double-strand sequencing for search of consensus sequences for putative binding of transcription factors, signalscan software (from the software library disposed by EMBL) and a list of consensus sequences of transcription factors (36) were used.

Southern and Northern Blot Analysis—For Southern blot analysis, DNA was digested with the restriction enzymes indicated and separated on a 0.8% agarose gel. Subsequently DNA was transferred to Hybond N’ membrane (Amersham) under alkaline conditions. For prehybridization, filters were incubated for 2 h at 60 °C in solution containing 7% SDS, 1 m EDTA, 0.5 M phosphate buffer. Hybridization was done overnight in the same solution containing additionally 1% bovine serum albumin and 32P-labeled probe. Afterward, filters were washed with decreasing concentrations of salt (2 × SSC, 0.5 × SSC, 1 × SSC; 0.1 × SSC) and subjected to autoradiography. To analyze rhoB expression on mRNA level, total RNA was prepared according to the method of Chomczynski and Sacchi (37). RNA was separated on a 1% agarose gel and transferred overnight on Hybond N’ filters. For rhoB-specific hybridization, a 0.95-kb EcoRI/Stul fragment from the 3′-region of rat rhoB cDNA was used which does not hybridize with other rho species (30). As internal standard for the amounts of RNA loaded, filters were rehybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA probe as described previously (38). For quantification, densitometrical analysis of the autoradiograms was performed. Relative gene expression was calculated by referring rhoB mRNA to the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA and by relating to control cells included.

Primer Extension—5 μg of total RNA was used for primer extension reaction with avian myeloblastosis virus reverse transcriptase (35). The primer used for primer extension reaction was located 50–80 bp 3′ from the TATA box-like element identified by sequencing (primer sequence: 5′-GACAAACGAACTGGGCGTGCA-3′). 32P labeling of the primer was done by use of T4 kinase. In parallel to primer extension reaction, T7 sequencing of cloned genomic 4.5 kb BamHI rhoB fragment with the identical primer was performed. Products of primer extension reaction and sequencing reaction were separated on a 8% sequencing gel and visualized by autoradiography.

PCR Analysis—To analyze the rhoB gene for the existence of intron sequences, 0.1 μl of the isolated genomic rhoB A DNA and 0.1 μl of rat rhoB cDNA were used for standard PCR reaction with Taq polymerase (35). The 5′-primers used correspond to position 42–63 (5′-GCTTGCGCGAAGACGT-GCTGT-3′) and 272–293 (5′-CTTCTGAAAACTCCCCCAGA-3′) of rat rhoB cDNA, respectively. Reverse primer starts at the stop codon of the rat rhoB cDNA (5′-CTGATAGCCTTGGCGATTT-3′). PCR products were
FIG. 2. Sequence of the 5′-noncoding region of rhoB. A, about 1.9 kb 5′ to the translation initiation site (ATG) were subjected to sequence analysis (EMBL accession number Y099248). Underlined are the two putative TATA box elements. Underlined and in bold are the putative start sites for transcription identified by primer extension analysis described in B. B, total RNA isolated from logarithmically growing NIH 3T3 cells was subjected to primer extension (PE) analysis using the following primer: 5′-GACAACCGAATTCGGTGCA-3′ (located 353–373 bp 5′ to the ATG start codon). In parallel, T7 sequencing was performed using the same primer. Products of PE reaction and sequencing reaction were separated on a 8% sequencing gel and subsequently visualized by autoradiography. Arrows indicate the position of the two putative start sites for transcription. The bases underlined and in bold represent the putative start sites of transcription.

analyzed on a 1.2% agarose gel. Conditions for PCR reaction were the following: 1 min, 95°C; 2 min, 55°C; 2 min, 72°C. Dominant-negative Rac (N17Rac) as well as dominant-negative RhoB (N17RhoB) were generated using PCR-based site-directed mutagenesis system as described (39). 

Protein Kinase Assays—JNK, p38 MAP kinase, and ERK2 activity was determined by an immune complex kinase assay. The kinases were immunoprecipitated with the corresponding antibody (Santa Cruz) and in

Protein Kinase Assays—JNK, p38 MAP kinase, and ERK2 activity was determined by an immune complex kinase assay. The kinases were immunoprecipitated with the corresponding antibody (Santa Cruz) and subsequently visualized by autoradiography. Arrows indicate the position of the two putative start sites for transcription. The bases underlined and in bold represent the putative start sites of transcription.

RESULTS

Cloning of the Mouse Gene for rhoB—To analyze the regulation of induction of rhoB by genotoxic stress on the level of the promoter, we isolated the gene for rhoB from a FIX II 129/1IX mouse genomic library using a 0.8-kb SacI fragment from rat rhoB cDNA as a hybridization probe. After screening of 5 x 10^6 phages, we isolated one positive clone. Southern blot analysis of various restriction digests of cloned DNA is shown in Fig. 1A. Interestingly, both after SacI and BamHI digestion a fragment of ~0.8 kb in size hybridized with the rat rhoB cDNA hybridization probe. Fragments of identical size were generated upon cleavage of rat rhoB cDNA and the corresponding genomic DNA (not shown), indicating that the coding region of the mouse rhoB gene does not contain intron sequences. 0.8- and 4.5-kb BamHI fragments which hybridized with the rat cDNA probe (see Fig. 1A, indicated by an arrow) were subcloned and subjected to DNA sequence analysis. No intron sequences were detected within the coding region of rhoB (Fig. 1B; sequence data not shown). To check whether intron sequences are present exactly within the BamHI site, we performed PCR analysis with primers covering the BamHI site. As shown in Fig. 1C, PCR products obtained with rat rhoB cDNA (taken for a control) and the cloned mouse rhoB λ genomic DNA exhibited identical length, thus proving lack of intron sequences within the BamHI site of rhoB. Comparison of the rhoB cDNA sequences from mouse and rat revealed 98.8% homology. On the level of the protein the homology was 100%.

To characterize the rhoB promoter, the nucleotide sequence of a ~1.9-kb fragment upstream of the start of transcription was determined (Fig. 2A). In the region 402–432 bp 5′ to the ATG start codon, two TATA box-like elements (TATATTA and TTTAAA) were identified (Fig. 2B). To determine the initiation site for transcription, primer extension analysis was performed using a primer located 50–80 bp 3′ to the presumed TATA boxes. Using this method, two possible initiation sites for transcription were detected (Fig. 2B). We infer from these data that the 5′ located TATA-like element (TATATTA) very likely represents a functional TATA box of the rhoB gene. The initiation site located more 5′ was defined as +1 site for the start of transcription.

rhoB Promoter Activity Is Stimulated by Genotoxic Stress—For functional analysis of the rhoB promoter, a 3.5-kb XhoI fragment (3′-end at position +88) from the 5′-flanking region of rhoB was cloned into pCAT basic (Fig. 3, see also Fig. 1B). NIH
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**FIG. 3.** The rhoB promoter is inducible by DNA damage and treatment with cycloheximide, but not by TPA. A 3.5-kb XhoI fragment from the 5'-noncoding region of the rhoB gene was cloned into pCAT basic and was used for transfection experiments. Logarithmically growing NIH 3T3 cells were transfected with 5 μg of the 3.5-kb XhoI rhoB promoter-CAT construct (designated as RhoB-CAT1) and, for control, a human collagenase-promoter-CAT construct (Coll-CAT). Cells were left untreated (control) or were treated 24 h later with UVC light (10–60 J/m²), methyl methanesulfonate (MMS; 1 mM), H₂O₂ (1 mM), cycloheximide (Chx; 5 μg/ml), or TPA (2 × 10⁻⁷ m). Methyl methanesulfonate and H₂O₂ treatment was done for 1 h, Chx treatment for 30 min. Thereafter, the medium was replaced by fresh medium. After a further incubation period of 24 h, cells were harvested for determination of the amount of CAT protein using an enzyme-linked immunosorbent assay. Transfection with pSV-CAT served as control. Induction factors were calculated by referring the amount of CAT protein in untreated cells, which was set to 1.0, to the amount of CAT in treated cells.

3T3 cells were transiently transfected with this construct and irradiated with UVC light 24 h later. After an additional incubation period of 24 h, cells were harvested for determination of the amount of CAT protein produced. As shown in Fig. 3, the amount of CAT protein increased upon UV irradiation (10–60 J/m²) in a dose-dependent manner up to 3.3-fold. This increase is similar to the induction level of the endogenous gene (30).

Stimulation of rhoB expression was not limited to UVC light. It pertained also to other DNA damaging agents such as methyl methanesulfonate and hydrogen peroxide (H₂O₂). Besides these genotoxic agents, increase in rhoB promoter activity was also observed after transient treatment with the protein biosynthesis inhibitor cycloheximide. TPA induced no effect on rhoB promoter activity, whereas it stimulated the collagenase-CAT promoter construct (Coll-CAT), which was included as a control, by ≈10-fold.

**Rho GTPases Affect the Activity of the rhoB Promoter**—Since Rho GTPases are known to interfere with transcriptional activation of c-jun and c-fos via stimulation of JNK's (11, 14, 16, 19) as well as with p38 MAP kinase signaling (28, 29), we analyzed whether or not also the rhoB promoter is regulated by small GTPases of the Rho family. As shown in Fig. 4, coexpression of wild-type Rac caused a 3.5-fold increase in rhoB-driven CAT expression as compared with mock-transfected control cells, whereas coexpression of dominant negative Rac (N17Rac) exerted no significant effect on rhoB (Fig. 4). Furthermore, UV-stimulated rhoB activity in cells coexpressing wild-type Rac was very similar to non-cotransfected cells, and coexpression of N17Rac did not inhibit activation of rhoB by UV irradiation (data not shown). Coexpression of constitutively activated RhoA (V14RhoA) as well as wild-type RhoB strongly inhibited rhoB promoter activity (Fig. 4). The dramatic inhibitory effect of RhoB on rhoB promoter activity was not observed if dominant-negative RhoB (N19RhoB) was used for cotransfection (Fig. 4). Coexpression of wild-type RhoB not only clearly reduced basal rhoB activity but also blocked activation of the rhoB promoter by UV. This effect was not observed if N19RhoB was used for cotransfection (data not shown). The data indicate that the rhoB promoter is regulated by Rho GTPases and is subject for autoregulation by RhoB itself. Interestingly, coexpression of either wild-type Cdc42 or dominant-negative Cdc42 (N17Cdc42) affected rhoB promoter activity (Fig. 4), indicating that Rac and Cdc42 are not completely identical with respect to the signal pathways they induce. Notably, as compared with wild-type Rac, constitutively activated Rac (V12Rac) was ≈2-fold more effective in stimulating rhoB activity, whereas V12Cdc42 again failed to exert any activating effect on rhoB expression (data not shown). We also analyzed the effect of cotransfection of Rho-GDI, which is an inhibitory molecule for Rho proteins (40, 41) on rhoB expression. As shown in Fig. 4, Rho-GDI exerted a very strong stimulatory effect on rhoB promoter activity. This finding supports the hypothesis of a negative regulation of rhoB by Rho proteins (e.g. RhoA and RhoB).

A 0.17-kb Fragment of the rhoB Promoter Is Sufficient for UV Induction—Based on the partial sequence analysis of the rhoB promoter, diverse consensus sequences for the binding of transcription factors were identified (Fig. 5A). Thus, besides the already mentioned TATA box, the rhoB promoter also contains consensus sequences for CAAT, SP1, p53, AP-2, AP-4, PEA3, XRE, PPAR, and C/EBP. To exclude a possible interference of distant 5'-regulatory elements with the UV induction of rhoB, diverse rhoB promoter deletion constructs (ranging from 3.5 kb up to 0.17 kb in size) were generated (Fig. 5B). All the promoter fragments generated, including the smallest one covering the region of −85 up to +88 (0.17-kb XbaI/PstI fragment), were
subject to activation by UV irradiation. Coexpression of wild-type Rac (Fig. 5A) stimulated the 0.17-kb fragment to a similar extent as the largest rhoB promoter fragment tested (3.5-kb XbaI fragment) did (Fig. 5C). Under the same conditions, the activity of a TATA-CAT construct which was included as control, was not stimulated (Fig. 5C). Notably, the inhibitory effect of RhoB was similar for the large 3.5 kb and the small 0.17-kb promoter fragment tested (3.5-kb XbaI fragment) did (Fig. 5C). From the data we conclude that the 0.17-kb XbaI/PstI promoter fragment is sufficient for activation of rhoB by UV light and Rac as well as for inhibition by RhoB. It apparently contains all the regulatory elements which are required for both stimulation and repression. Interestingly, this fragment does not contain any putative binding site for one of the transcription factors which are known to activate gene expression upon UV irradiation (e.g. AP-1, ATF-2, Elk-1, and CHOP). It should be noted that UV irradiation results in an increase in the amount of rhoB mRNA both in c-Fos- and p53-deficient cell lines (data not shown), indicating that c-Fos and p53 are not necessary for UV-stimulated rhoB expression.

**Effect of Inhibition of MEK, p38 MAP Kinase, and PI 3-Kinase on the Activity of the rhoB Promoter**—Based on the finding that the minimal rhoB promoter fragment sufficient for the UV-response lacks any consensus sequences for transcription factors known to be involved in UV signaling, we addressed the question whether a novel signal transduction pathway and target sequence(s) are involved in rhoB regulation. To this end, we used the pharmacological protein kinase inhibitors PD98059 (MEK inhibitor) and SB203580 (p38 kinase inhibitor), which are known to specifically block activation of ERK and p38 MAP kinase upon UV irradiation (42–46). Both inhibitors do not interfere with JNK activation upon UV irradiation (Fig. 6A). Treatment of cells with the MEK inhibitor PD98059 slightly increased basal rhoB activity but did not block stimulation of rhoB by UV irradiation (Fig. 6B). Furthermore, the p38 MAP kinase inhibitor SB203580 had no effect on the extent of rhoB stimulation by UV (Fig. 6B). Since Rac-regulated signaling appears to be involved in rhoB regulation, which was indicated by rhoB activation upon Rac overexpression (see Fig. 4), we analyzed whether or not inhibition of PI 3-kinase which is involved in the regulation of Rac (47–49), affects the UV-stimulated rhoB expression. We made use of wortmannin which is a specific inhibitor of PI 3-kinase. The experiments revealed that wortmannin rather inhibited UV stimulation of JNK and p38 MAP kinase upon UV irradiation (Fig. 7A). Treatment with different kinase inhibitors, we suggest that JNK, ERK, and p38 MAP kinase are not involved in rhoB regulation upon UV treatment.

**IL-1 Activates JNK (Stress-activated Protein Kinases) But Does Not Affect rhoB Expression**—Both our sequence data as well as the data obtained with the PI 3-kinase inhibitor wortmannin indicate that JNK is not involved in rhoB regulation. To further verify this hypothesis, physiological activators of
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JNK were analyzed as to their capacity to activate rhoB expression. As shown in Fig. 8A, IL-1β as well as a cytokine mixture containing INF-γ, TNF-α, and IL-1β were potent activators of JNK. They did not, however, exert a significant stimulatory effect on the activity of the rhoB promoter (Fig. 8B). This observation again indicates that JNK is not decisively involved in the regulation of rhoB expression upon UV irradiation.

DISCUSSION

Exposure of eukaryotic cells to genotoxic agents induces a variety of responses including the activation of cellular growth factor receptors and Src tyrosine kinases (20, 21, 50), the accumulation of p53 and subsequent blockage in cell cycle progression (51–55), and the transcriptional activation of a variety of genes (56, 57). Among the DNA damage inducible genes, most interest has focused on the immediate-early genes c-fos and c-jun. It is believed that both the receptor mediated activation of MAPK (20, 21, 58) and Rac/Cdc42-mediated activation of JNK participate in the regulation of these genes (19, 50). The induction of the rhoB gene, which codes for a Ras-related small GTPase, is another very early event after exposure of cells to DNA damaging agents (30). Induction of RhoB appears to be of particular interest because GTP-binding proteins can induce changes in the activity of their downstream targets very rapidly. Thus they may quickly trigger subsequent responses which are supposed to be protective. In contrast to c-fos and c-jun, rhoB expression is not stimulated by the phorbol ester TPA (30, 31) which indicates that different signal transduction pathways are involved in the regulation of these immediate-early genes. In this study, we aimed at elucidating mechanisms controlling rhoB expression.

To this end, we cloned and analyzed the promoter of the mouse rhoB gene. We demonstrate here that the activity of the rhoB promoter was stimulated by genotoxic treatments such as UV, methyl methanesulfonate, and hydrogen peroxide. The sequence data obtained revealed that the rhoB promoter contains TATA, Sp1, and CAAT box elements as well as consensus sequences for AP-2, AP-4, and p53. Interestingly, consensus sequences for known, UV-activated transcription factors (e.g. AP-1, c-Jun/ATF-2, Elk-1, or CHOP (gadd153)) were not present within the rhoB promoter. Generation of a set of rhoB promoter deletion constructs let us to identify a 0.17-kb rhoB promoter fragment which still retained activator activity upon UV irradiation. Furthermore, this fragment was also subject of stimulation by coexpression of Rac and inhibition by coexpression of RhoB to a similar extend as the original 3.5-kb fragment. Thus we suggest that the regulatory elements required for both positive and negative regulation of rhoB are located within this minimal 0.17-kb rhoB promoter fragment. With the exception of the CAAT and TATA box, the 0.17-kb fragment lacks any binding site for known transcription factors. As indicated by the data obtained from sequence and deletion analysis, two mediators of the UV response, namely AP-1 and p53, appear not to be involved in rhoB regulation. This hypothesis is supported by the finding that cells deficient in either c-fos or p53 responded in the same way as wild-type cells do with regard to the extent of UV-induced increase in the amount of rhoB mRNA (data not shown). Thus, our data give evidence for the existence of a novel regulatory element within the rhoB promoter, mediating UV induction according to a yet not described mechanism.
UV response of rhoB. (iii) IL-1β rather activated JNK but failed to increase rhoB expression. (iv) p38 inhibitor SB203580 did not inhibit UV stimulation of rhoB and (v) wild-type Rac is sufficient to activate rhoB but even V12Cdc42 fails to do so. Bearing in mind that TPA is unable to increase rhoB expression and that the MEK inhibitor PD98059 did not block UV stimulation of rhoB, it further appears that ERK also do not participate in rhoB regulation upon UV irradiation. The observation that down-modulation of signaling by TPA pretreatment results in a partial block of UV-induced rhoB mRNA expression (30) and also rhoB promoter activity (data not shown) does not contradict this hypothesis because TPA induced PKC signaling is not limited to ERK. Furthermore, the finding that INF-γ failed to increase rhoB expression may be taken as an indication that JAKs, which are generally activated by INF-γ, are also not involved in rhoB expression.

A further interesting feature of rhoB regulation is its negative feedback regulation by rhoB itself. In this context we would like to emphasize that the inhibitory effect of RhoB on its own expression is already seen with wild-type RhoB. This is important to notice, because it reflects the physiological situation after UV-induced increase in the amount of RhoB. Thus, the responsiveness of rhoB to wild-type RhoB also provides strong evidence that the observed autoregulation of rhoB is physiologically relevant. Since inhibition of rhoB expression was already 90% with wild-type RhoB, it was difficult to detect stronger effects even by use of activated RhoB. Thus it was not surprising that we observed V14RhoB to elicit a similar inhibition of rhoB expression as wild-type RhoB did (data not shown). The same is true if expression vectors coding for either V14RhoA or wild-type RhoA were used (data not shown).

The activity of RhoA and RhoB as inhibitors of rhoB expression is also supported by the finding that blockage of Rho (RhoA, RhoB) activity by overexpression of the Rho inhibitory molecule Rho-GDI resulted in a strong increase in the activity of the rhoB promoter. Rho-GDI was also shown to be a negative regulator of Rac (41). However, its inhibitory effect on Rho proteins (both RhoA and RhoB) obviously becomes predominant under conditions of overexpression, resulting in activation of the rhoB promoter. The assumption of a preferential action of Rho-GDI on Rho, at least under conditions of Rho-GDI overexpression, is in agreement with the observation that microinjection of Rho-GDI causes cell rounding which is supposed to be mainly due to inactivation of RhoA (62).

Furthermore, overexpression of Rho-GDI exerted no effect on Rac-mediated activation of JNK (13). Rho proteins are known to participate in the regulation of the actin cytoskeleton (7, 8, 10) and cooperative action of Rho and Rac on actin cytoskeleton has been described (63). On the other hand, opposite effects of Rac/Cdc42 and RhoA have been shown on neurite outgrowth (64). This finding indicates that RhoA and Rac inversely interfere with signal mechanisms involved in neurite development and collapse, respectively. Thus, it appears likely that Rac has stimulating effects whereas RhoA,B exerts inhibitory activity on the signaling which is involved in the regulation of rhoB expression upon UV irradiation.

In summary, having cloned and analyzed the promoter of the mouse rhoB gene, we present evidence that the rhoB promoter is activated by UVC and other forms of genotoxic stress, and that UV activation of rhoB does not require JNK, ERK, and p38 MAP kinase. Therefore, the UV activation of the rhoB gene is different from other immediate-early genes such as c-jun and c-fos. Furthermore, rhoB activity is stimulated by Rac but not by Cdc42 and is down-modulated by its own product RhoB and the related RhoA protein. This negative regulatory feedback might be functionally important in restoring the steady-state.

FIG. 8. Cytokine-mediated activation of JNK does not affect rhoB promoter activity. A, NIH 3T3 cells, which were serum-depleted by overnight incubation with medium containing 0.2% fetal calf serum, were stimulated or not (Con) by addition of the cytokines INF-γ (100 units/ml), IL-1β (50 units/ml), and TNF-α (10 ng/ml). Cytokines were added either separately or as a mixture (Cytokine Mix). After an incubation period of 30 min, cells were harvested for determination of JNK activity as described under “Experimental Procedures.” The autoradiography is shown. B, 24 h after transfection with the 3.5-kb XhoI rhoB promoter CAT construct (rhoB-CAT1), cells were serum-depleted as described under A. Subsequently cytokines (INF-γ, 100 units/ml), IL-1β (50 units/ml), TNF-α (10 ng/ml), and the Cytokine Mix were added and, after a further incubation period of 24 h, the amount of CAT protein was determined. Promoter activity was related to that of the untreated control (Con) which was set to 1.0. Data shown are mean values from at least two independent experiments.

To address the question of what kind of signaling might be involved in rhoB induction, we analyzed the effect of overexpression of various small GTPases of the Rho family, which have been shown to participate in genotoxic stress signaling (11, 13), on the activity of the rhoB promoter. Interestingly, overexpression of wild-type Rac was sufficient to strongly stimulate rhoB promoter, whereas wild-type Cdc42 had no effect. As might have been expected, V12Rac exerted a stronger stimulatory effect on rhoB than the wild-type form did. However, even the use of V12Cdc42 did not elicit rhoB promoter activity. Thus, although Rac and Cdc42 share the same signaling activities in respect to activation of JNK and p38 MAP kinase, they obviously distinguish from each other in respect to rhoB regulation. Notably, despite their identical signaling activities on JNK and p38, different physiological activities of Rac and Cdc42 have also been described. For example, Rac has been demonstrated to be involved in the formation of lamellipodia, whereas Cdc42 interferes with the generation of filopodia (60). Furthermore, TNF-α-dependent activation of NF-κB is reported to depend on Cdc42 but not on Rac (61). These reports agree with our data in that they clearly show the involvement of Rac and Cdc42 also in different signaling pathways. Although JNK and p38 kinase are known as important kinases in stress-induced signaling, it appears unlikely that these kinases are involved in rhoB regulation because of the following reasons. (i) As mentioned above, no target sequences for transcription factors regulated by these kinases (e.g. c-Jun, ATF-2, Elk-1, and CHOP) were detected within the rhoB promoter. (ii) Inhibition of PI 3-kinase by wortmannin rather blocked UV-mediated activation of JNK and p38 kinase but did not affect
level of RhoB upon exposure of cells to UVC and other genotoxic agents.

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