Transcriptional activation of the small GTPase gene $\text{rhoB}$ by genotoxic stress is regulated via a CCAAT element

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ABSTRACT

The gene encoding the Ras-related GTPase $\text{RhoB}$-specific is immediate-early inducible by genotoxic treatments. Regulation of transcriptional activation of $\text{rhoB}$ is still unclear. Here we show that cells lacking either p53 or c-Fos are not different from wild-type cells with respect to the level of $\text{rhoB}$ induction upon UV irradiation, indicating that these transcription factors are not crucial for stimulation of $\text{rhoB}$ mRNA expression. Extracts from UV-irradiated and non-irradiated cells revealed similar DNA-binding activities to a 0.17 kb $\text{rhoB}$ promoter fragment harboring the functional element(s) necessary for stimulation of $\text{rhoB}$ by UV light. By means of immunoprecipitation we found that an ATF-2-specific antibody co-precipitates the $^{32}$P-labeled 0.17 kb $\text{rhoB}$ fragment, whereas an anti-AP1 antibody did not. Since no consensus sequence for binding of ATF-2 is present within the $\text{rhoB}$ promoter, ATF-2 is likely to be associated with another factor that binds to the minimal promoter. Deletion analysis and site-directed mutagenesis of the 0.17 kb $\text{rhoB}$ fragment revealed a CCAAT box to be an essential requirement for stimulation of $\text{rhoB}$ by UV light and methyl methanesulfonate. Moreover, immunoprecipitation experiments showed that the CCAAT-binding factor NF-YA is complexed with ATF-2. Overall, the data strongly indicate that transcriptional activation of the $\text{rhoB}$ gene by genotoxic stress is regulated via a CCAAT box and that interaction of CCAAT-binding factor and ATF-2 triggers the stress-inducible expression of $\text{rhoB}$.

INTRODUCTION

Exposure of mammalian cells to DNA-damaging agents elicits a variety of cellular responses, including stimulation of gene expression among others. Growth factor receptors such as the EGF receptor (1), cytokine receptors (2) and PI 3-kinase coupled receptors (3) have been suggested as primary targets for UV-stimulated signaling leading to activation of protein kinase cascades involving ERKs, N-terminal c-Jun kinases/ stress-activated protein kinases (JNKs/SAPKs) (4) and p38 MAP kinases (4–6). Phosphorylation of transcription factors by these MAP kinases finally results in transcriptional activation of genes (7). Prototypes of genes that are transcriptionally activated within minutes after exposure encode the proto-oncoproteins c-Fos and c-Jun (8–11), which form part of the heterodimeric AP1-like transcription factors (e.g. c-Jun/c-Fos or c-Jun/ATF-2). Via trans-activation of the corresponding target genes, these transcription factors control the expression of proteins involved in the regulation of cell cycle progression and cell death (7). For example, as compared to wild-type cells, fibroblast cells derived from c-fos$^{-/-}$ mice are hypersensitive to the cytotoxic and apoptotic effects of a large variety of genotoxic agents (12). This shows that c-Fos protects cells from cell killing induced by DNA-damaging treatments.

Another type of ‘immediate-early’ inducible gene that is also transcriptionally activated by DNA-damaging treatments encodes the Ras-related GTPase RhoB (13,14). Transcriptional activation of $\text{rhoB}$ by genotoxic stress is of particular interest because RhoB is a GTP-binding protein and is, therefore, capable of modulating the activity of downstream targets in a very fast manner via GTP binding and GTP hydrolysis. In comparison to other members of the Rho protein family, the physiological function of RhoB has been poorly investigated. Data available so far point to a potential regulatory function of RhoB in S phase cells (15) and indicate that RhoB participates in the regulation of EGF receptor trafficking (16). Other interesting features of RhoB are that it is essentially required for Ras-mediated transformation (17), is subject to negative autoregulation (18) and represses NF-κB signaling (19). The identification of RhoB as a gene that is rapidly inducible by DNA-damaging treatments further indicates that RhoB is part of the cellular response to genotoxic stress. In line with this hypothesis, overexpression of RhoB renders cells hypersensitive to the apoptosis-inducing effects of alkylating agents, including antineoplastic drugs (20). Notably, the regulation of $\text{rhoB}$ induction by genotoxic stress appears to be different from that of other rapidly inducible genes such as c-fos and c-jun. One argument supporting this hypothesis is that the phorbol ester TPA elicits an increase in expression of c-fos and c-jun mRNA but does not affect $\text{rhoB}$ mRNA expression (13,14). Furthermore, MAP kinases (i.e. JNKs and p38 kinase) and AP1-like transcription factors are believed to be most relevant

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for regulation of c-jun expression upon UV irradiation (21). In contrast, analysis of the rhoB promoter indicated that activation of rhoB by UV irradiation is independent of MAP kinases and so far known UV-stimulated transcription factors and regulatory elements (18). Therefore, a novel mechanism was hypothesized to be involved in the regulation of rhoB induction by UV light. Here we present evidence that a CCAAT box is an essential requirement for the transcriptional activation of rhoB by genotoxic stress.

MATERIALS AND METHODS

Materials

Isolation and characterization of the rhoB promoter as well as the generation of a 0.17 kb rhoB promoter fragment that is still inducible by UV irradiation were described previously (18). Mutational analysis of the 0.17 kb rhoB promoter fragment was performed by use of a standard PCR-based method. Anti-ATF-2 (sc-187x) and anti-c-Jun/AP1 (sc-45x) antibodies used in this study were obtained from Santa Cruz (San Diego, CA). Anti-NF-YA antibody was purchased from Pharmingen (San Diego, CA). c-Fos-deficient cells originated from A. Wagner (Vienna, Austria); p53-deficient cells were generously provided by A. Balmain (Glasgow, UK). Mutational inactivation of CCAAT boxes in the rhoB promoter was done by standard PCR-based methods.

Cell culture and transfection experiments

NIH 3T3 fibroblast cells were routinely grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. For transient transfection experiments, 5 × 10⁵ cells were seeded per 5 cm dish and transfected 16–24 h later with 5 µg promoter–CAT constructs using the calcium phosphate co-precipitation technique (22). Salmon sperm DNA was added to a final concentration of 10 µg/assay. If not stated otherwise, cells were treated 16–24 h after transfection. After a further incubation period of 24 h, cells were harvested for determination of the level of CAT protein expression using an ELISA-based assay system (CAT-Elisa Kit; Roche Diagnostics GmbH). Determination of protein concentration was done according to the method of Bradford (23).

Northern blot analysis

In order to analyze rhoB expression at the mRNA level, total RNA was prepared according to the method of Chomczynski and Sacchi (24). RNA was separated on a 1% agarose gel and transferred overnight onto Hybond N+ filters. For prehybridization filters were incubated for 2 h at 60°C in a solution containing 7% SDS, 1 mM EDTA, 0.5 M phosphate buffer pH 7.4. Hybridization was done overnight in the same solution additionally containing 1% BSA and 32P-labeled probe. For rhoB-specific hybridization a 0.95 kb EcoRI–XhoI fragment from the 3′-region of the rat rhoB cDNA was used. This rhoB fragment does not cross-hybridize to other rho mRNA species (13). After washing with decreasing concentrations of salt (2–0.5x SSC, 1 mM EDTA, 0.5% SDS) filters were subjected to autoradiography. As an internal standard for the amount of RNA loaded, filters were rehybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe as described previously (13). For quantitation, densitometric analysis of the autoradiograms was performed. Relative gene expression was calculated by referring the level of rhoB mRNA to the amount of GAPDH mRNA. The relative rhoB mRNA level in treated cells was related to that of control cells, which was set to 1.0.

Gel retardation analysis

32P-labeling of the 0.17 kb rhoB promoter fragment as well as of shorter oligonucleotides was done by use of T4 kinase. Total cell extract was prepared as described (25). Binding reactions were performed by incubation of 5–10 µg protein with ~5 fmol 32P-labeled oligonucleotide for 30 min at room temperature [binding buffer, 10 mM HEPES pH 7.9, 60 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 µg/ml BSA, 10% glycerol, 0.5 µg poly(dI·dC)]. For competition experiments the reaction mixture was preincubated with 10–50-fold molar excess of unlabeled oligonucleotide for 10 min at room temperature before 32P-labeled probe was added. After the binding reaction the reaction mixture was separated on 4% polyacrylamide gels at room temperature. After electrophoresis, gels were dried and subjected to autoradiography. The following oligonucleotides were used for DNA binding analyses: NF-YA (5′-GGCTTTC-CAATTCGTTGCTAT-3′); AP-1-specific oligonucleotide (5′-AGTGGTGACTCA-3′); two different oligonucleotides for detection of binding of C/EBP proteins, C/EBPα consensus sequence (sc-2525; Santa Cruz) (5′-TGCAATTGCGCAATCGTCA-3′) and C/EBPβ (5′-ATCCATTGGCGCAATCG-3′) (26); ATF/CREB (5′-AGAGATTCCTGACGTCAGAGCCA-3′).

Southwestern analysis, cross-linking and immunoprecipitation

For southwestern analysis 50 µg protein from total NIH 3T3 cell extract was separated by SDS–PAGE (10% gel). After transfer onto nitrocellulose, nitrocellulose-bound proteins were renatured and hybridized with the 32P-labeled 0.17 kb rhoB promoter fragment as described (27). Proteins binding to the 32P-labeled DNA were detected by autoradiography. For UV cross-linking experiments the 32P-labeled rhoB fragment was incubated with nuclear extract under identical conditions to those described for the gel retardation analysis. Afterwards the reaction mixture was irradiated with a 254 nm hand lamp for 10 min. The lamp was placed on top of an unscrewed tube which was kept on ice during irradiation. After cross-linking the products were separated by SDS–PAGE and cross-linked 32P-labeled protein–DNA complexes were visualized by autoradiography. Immunoprecipitation of UV cross-linked protein was performed basically as described (28).

RESULTS AND DISCUSSION

Expression of the gene encoding the Ras-related GTPase RhoB is rapidly inducible by genotoxic agents such as UV irradiation and alkylating compounds (13,18,20). Data obtained by cloning and analysis of the rhoB promoter indicated that the regulatory mechanism involved in rhoB induction is different from that triggering expression of other genotoxic stress-inducible genes such as c-fos and c-jun (18). As shown in Figure 1, the increase in the level of rhoB mRNA by UV irradiation in mouse fibroblasts deficient in c-Fos or p53 protein is similar to that of wild-type cells. This finding
demonstrates that the stimulation of rhoB mRNA expression by UV light is independent of c-Fos and p53. NIH 3T3 wild-type cells and c-fos–/– and p53–/– fibroblast cells were left untreated (Control) or were irradiated with UV light (40 J/m²). Thirty minutes after treatment, total RNA was isolated and the expression of rhoB mRNA was analyzed by northern blot analysis as described in Materials and Methods. The autoradiogram is shown. The relative amount of rhoB mRNA in untreated cells was set to 1.0.

Figure 1. Induction of rhoB mRNA expression by UV irradiation is independent of c-Fos and p53. NIH 3T3 wild-type cells and c-fos–/– and p53–/– fibroblast cells were left untreated (Control) or were irradiated with UV light (40 J/m²). Thirty minutes after treatment, total RNA was isolated and the expression of rhoB mRNA was analyzed by northern blot analysis as described in Materials and Methods. The autoradiogram is shown. The relative amount of rhoB mRNA in untreated cells was set to 1.0.

additional [32P]DNA–protein complex emerged upon UV irradiation (Fig. 3B). Thus it appears unlikely that transcriptional activation of rhoB is due to additional binding of a novel factor to a particular sequence motif within the 0.17 kb fragment. Both AP1 and ATF-2-specific antibodies failed to induce supershifts (Fig. 3C), suggesting again that these factors are not involved in [32P]DNA–protein complex formation. In order to characterize the proteins which bind constitutively to the 0.17 kb rhoB promoter fragment, DNA cross-linking and southwestern analyses were performed. Upon UV cross-linking of the DNA–protein complex and subsequent SDS-PAGE a ~36 kDa complex was observed (Fig. 4A). Taking into account the DNA within the cross-linked complex, which contributes to an overestimation of the actual size of the protein bound, the molecular weight of the UV cross-linked protein can be estimated to be ~48 kDa. As determined by southwestern analysis, the molecular weight of proteins which predominantly bind to the 0.17 kb rhoB fragment is ~36 kDa (Fig. 4B). This is similar to the molecular weight of TATA-binding factors. Because the 0.17 kb rhoB promoter fragment contains TATA boxes, it is rational to assume that TATA-binding factors were UV cross-linked to the 32P-labeled rhoB fragment.

The lack of consensus sequences for binding of known UV-activated transcription factors together with the failure to detect UV-induced changes in the DNA-binding activity of extracts from UV-treated cells to the 32P-labeled rhoB promoter fragment raises the question of whether CCAAT- or TATA-binding factors might be involved in the transcriptional activation of rhoB by UV irradiation. Previously, it was reported that the transcriptional activity of the c-fos gene can be enhanced via TATA-binding protein-associated factors (TAFs) (29). Moreover, CCAAT-binding proteins were also shown to increase gene expression upon treatment of cells with serum or calcium (30,31). Most interestingly, the CCAAT-binding factor NF-YA is reported to interact with ATF-2 in regulation of the fibronectin gene (28). Notably, the stimulatory effect of ATF-2 on fibronectin gene expression is supposed to be independent of DNA binding of ATF-2. Rather, it is assumed that ATF-2 exerts its stimulatory effect exclusively via binding to NF-Y, without contacting DNA (28). In this context it is important to note that the ATF-2/NF-Y
complex is believed to be unstable under conditions of gel electrophoresis (28). Therefore, the interaction of ATF-2 with NF-Y cannot be detected by gel retardation experiments. Overall, these data prompted us to investigate whether CCAAT-binding factors and ATF-2 might also be relevant for the transcriptional activation of rhoB by genotoxic stress. In a first step a possible involvement of ATF-2 in the regulation of rhoB expression was analyzed by immunoprecipitation experiments (28). After incubation of the 32P-labeled rhoB promoter fragment with nuclear proteins (under identical experimental conditions to those used in the gel retardation experiments) and

Figure 3. Gel retardation analysis using the 0.17 kb rhoB promoter fragment. (A) An aliquot of 5 µg protein from NIH 3T3 cell extracts was analyzed for DNA binding activity to the 32P-labeled 0.17 kb rhoB promoter fragment by gel retardation as described in Materials and Methods. For competition experiments the unlabeled fragment (SComp) was added in 10-fold molar excess. As a control, an AP-1-specific oligonucleotide was added in 10- and 50-fold molar excess. The arrow points to the specific band. (B) Up to 2 h after irradiation (40 J/m²) of NIH 3T3 cells extracts were prepared and analyzed for DNA binding activity using the 32P-labeled 0.17 kb rhoB promoter fragment. (C) Extracts obtained from cells left untreated (Control) and treated with UV light (40 J/m², 30 min) were used for supershift analysis using 1 µg ATF-2- and c-Jun/AP-1-specific antibodies.

Figure 4. An ∼48 kDa protein is UV cross-linked to the 0.17 kb rhoB promoter fragment. (A) 32P-labeled rhoB fragment was incubated with extracts from UV-irradiated cells under identical conditions to those used in the gel retardation experiments. Afterwards the reaction was irradiated with UVC light as described in Materials and Methods. Products were separated by SDS–PAGE and the cross-linked protein–DNA complex was visualized by autoradiography. (B) An aliquot of 50 µg protein from total cell extract was separated by SDS–PAGE. After blotting to nitrocellulose, southwestern analysis using 32P-labeled rhoB promoter fragment was performed as described in Materials and Methods. The autoradiogram is shown.

Figure 5. ATF-2 is associated with the 0.17 kb rhoB promoter fragment. The 32P-labeled 0.17 kb rhoB promoter fragment was incubated with cell extracts under the same experimental conditions as used in the gel retardation analyses. Afterwards UV cross-linking and subsequent immunoprecipitation with anti-ATF-2- and c-Jun/AP-1-specific antibodies were performed as described in Materials and Methods. As a further control antibodies were inactivated by heat (5 min, 95°C) prior to use for immunoprecipitation. After washing, the amount of immunoprecipitable radioactivity was determined by scintillation counting. Data shown are means ± SD from at least three independent experiments, each performed in duplicate.
subsequent UV cross-linking, immunoprecipitation with ATF-2-specific antibody was performed and the radioactivity in the precipitate was determined. The experiments showed that the ATF-2-specific antibody used was able to co-precipitate a significantly higher amount of $^{32}$P-labeled DNA than either heat-inactivated ATF-2 antibody or an AP1-specific antibody, which was included as a further control (Fig. 5). Overall, these data strongly indicate that ATF-2 is associated with factors that bind to the $0.17$ kb $\rho_B$ fragment.

In a next step we analyzed whether or not CCAAT-binding factors might be relevant for $\rho_B$ induction by genotoxic stress. To this end we examined the consequences of mutational inactivation of the CCAAT boxes in the $\rho_B$ promoter on its inducibility by UV irradiation. As shown in Figure 6, deletion of CCAAT box 1 did not attenuate the induction of $\rho_B$ by UV irradiation or methyl methanesulfonate (MMS) treatment, indicating that this CCAAT element is not crucial for the activation of $\rho_B$ by genotoxic treatment. In line with this, inactivation of CCAAT box 1 by point mutation also failed to affect transcriptional stimulation of $\rho_B$ by UV light (Fig. 6). However, functional disruption of CCAAT box 2 by point mutation blocked the increase in $\rho_B$ gene expression induced by UV irradiation as well as by MMS treatment (Fig. 6). Obviously, CCAAT box 2 (i.e. CATTGGG), which is located close to the functional TATA box, is an essential requirement for induction of $\rho_B$ by UV light and MMS. Notably, based on computational sequence analysis, this CCAAT site is thought to be a NF-Y (CBF, CP1) binding site. Yet, besides NF-Y, many other factors are also described to bind to CCAAT elements. One important group is the CCAAT enhancer-binding proteins (C/EBP). Therefore, we analyzed whether members of the C/EBP family are able to bind to the functionally relevant CATTGGG sequence (= NF-Y$^{\rho_B}$) of the $\rho_B$ promoter. To this end, competition experiments were performed. As shown in Figure 7, binding of proteins to the NF-Y$^{\rho_B}$ oligonucleotide can only be competed by the non-labeled NF-Y$^{\rho_B}$ sequence, and not by an oligonucleotide harboring a C/EBP consensus sequence (Fig. 7A). Moreover, binding of proteins to two different types of $^{32}$P-labeled C/EBP-specific oligonucleotides (i.e. C/EBP$^A$ and C/EBP$^B$) was not competed with excess unlabeled NF-Y$^{\rho_B}$-specific oligonucleotide (Fig. 7B and C). Based on the data we suggest that proteins of the C/EBP family are very likely not involved in binding to the CATTGGG sequence present in the $\rho_B$ promoter. Having in mind a previous report showing an interaction of ATF-2 with the CCAAT-binding factor NF-YA (28), we speculate that NF-YA, together with ATF-2, is involved in the regulation of $\rho_B$ by genotoxic stress. To ascertain that association of CCAAT-binding factor NF-Y with ATF-2 really occurs in our cell system, immunoprecipitation experiments were performed (Fig. 8). Upon immunoprecipitation with anti-NF-YA antibody followed by western blot analysis using anti-ATF-2-specific antibody we found that anti-NF-YA antibody is able to co-precipitate proteins cross-reacting with...
Members of the C/EBP family are not involved in the regulation of rhoB expression (26) was used for band shift analysis (C/EBP b,5′-ATTCAATTGCGCAAT-3′). Specific competition for binding of C/EBP (C/EBPa, 5′-TGCAGATTGCGCAATCTGCA-3′) (sc-2525; Santa Cruz). SComp, specific competition. (B) For band shift analysis the 32P-labeled C/EBP-specific oligonucleotide (C/EBPa) described in (A) was used. Competition experiments were done as described using either unlabeled C/EBPa (SComp) or NF-Y rhoB-specific oligonucleotide. SComp, specific competition. (C) A second consensus sequence described to bind C/EBP proteins (26) was used for band shift analysis (C/EBPa, 5′-ATCCATTGGCAATCGA-3′). Specific competition (SComp) and competition with NF-Y rhoB-specific oligonucleotide was done as described before.

anti-ATF-2 antibody (Fig. 8A). As concluded from cloning and in vitro translation experiments, ATF-2 protein is ∼46 kDa in size, however, splice variants and post-translational modifications might lead to different migration on SDS gels (32). The amount of co-precipitated ATF-2 protein was not enhanced if extract from UV-irradiated cells was used (Fig. 8A). This indicates that NF-YA is constitutively associated with ATF-2. Complexation of NF-YA and ATF-2 was further confirmed by immunoprecipitation using an anti-ATF-2 antibody and subsequent analysis of co-precipitated NF-YA protein by western blot analysis (Fig. 8B). Thus, overall, the previously reported data (28) were confirmed by our own results.

To summarize, the data available strongly indicate that CCAAT-binding factors, putatively NF-YA, act in concert with the transcription factor ATF-2 in stimulating rhoB gene expression upon genotoxic stress. A possible function of ATF-2 might be to increase the frequency of transcription initiation after a particular type of stimulus. Notably, ATF-2 is a substrate for JNKs/SAPKs and p38 MAP kinase and is believed to play a central role in the stimulation of c-jun expression by UV irradiation (21). With respect to rhoB regulation, it is therefore tempting to speculate that, in analogy to the regulation of c-jun (with c-Jun/ATF-2 being of particular relevance), genotoxic stress leads to activation of ATF-2/NF-Y, which finally leads to an increase in rhoB gene expression. Bearing in mind that MAP kinases (i.e. JNK and p38 kinase) are important for the stimulation of c-jun expression by UV light (21), this hypothesis at the same time implies that MAP kinases are involved in rhoB regulation. Our initial hypothesis that MAP kinase-related pathways do not participate in the regulation of rhoB was based on the observation that pharmacological inhibition of MAP kinases does not prevent activation of rhoB expression by UV irradiation (18). However, recently we showed that even induction of c-jun by UV light is not blocked by pharmacological inhibition of JNK1 activity (3). It appears that JNK1 is not an essential requirement for expression of c-jun, possibly because under conditions of JNK1 inhibition other JNK isoenzymes come into play and compensate for loss of JNK1 activity. Therefore, with respect to a possible involvement of MAP kinase-related pathways in rhoB regulation, conclusions drawn from experiments using pharmacological MAP kinase inhibitors might be misleading. Nevertheless, the essential requirement of a CCAAT box for transcriptional activation of rhoB by DNA-damaging treatments represents a novel mechanism of regulation of an early responsive gene in cells exposed to genotoxic agents.

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