B-ind1, a Novel Mediator of Rac1 Signaling Cloned from Sodium Butyrate-treated Fibroblasts

Delphine Courilleau‡, Eric Chastre, Michèle Sabbah, Gérard Redeuilh, Azeddine Atti, and Jan Mester§
From the INSERM U482, Hôpital Saint-Antoine, 184 Rue du Faubourg Saint-Antoine, 75571 Paris cedex 12, France

Sodium butyrate is a multifunctional agent known to inhibit cell proliferation and to induce differentiation by modulating transcription. We have performed differential display analysis to identify transcriptional targets of sodium butyrate in Balb/c BP-A31 mouse fibroblasts. A novel butyrate-induced transcript B-ind1 has been cloned by this approach. The human homologue of this transcript contains an open reading frame that codes for a protein of 370 amino acids without known functional motifs. In transfected cells, the B-ind1 protein has been found to potentiate different effects of the small GTPase Rac1, such as c-Jun N-terminal kinase activation and transcriptional activity of nuclear factor κB (NF-κB). In addition, we have demonstrated that B-ind1 forms complexes with the constitutively activated Rac1 protein. To investigate the role of B-ind1 in Rac1 signaling, we have constructed several deletion mutants of B-ind1 and tested their ability to affect the activation of NF-κB by Rac1. Interestingly, the fragment encoding the median region of human B-ind1 acted as a dominant-negative variant to block Rac1-mediated NF-κB activity. These data define B-ind1 as a novel component of Rac1-signaling pathways leading to the modulation of gene expression.

Sodium butyrate is a multifunctional agent known as an efficient inhibitor of cell proliferation, an inducer of differentiation in numerous cell lines and apoptosis in certain cell types (1, 2). Sodium butyrate exerts most of its biological effects through a reversible inhibition of histone deacetylases (HDAC),† resulting in histone hyperacetylation and in selective changes in gene expression. Effects similar to those of sodium butyrate are also induced by structurally unrelated inhibitors of HDAC such as trichostatin A (3). The acetylation state of core histones plays a critical role in the regulation of transcription. In addition, a DNA butyrate-response element conferring the ability of sodium butyrate to induce gene expression has been identified (4, 5). However, sodium butyrate also inhibits the expression of several genes including c-myc (6) and cyclin D1 (7), p21WAF1, a cellular inhibitor of cyclin-dependent kinase and of the progression in G1 phase of the cell cycle, is induced by sodium butyrate as well (8, 9). We have previously reported that in the mouse fibroblast cell line BP-A31 sodium butyrate inhibits the hyperphosphorylation of the tumor suppressor retinoblastoma protein via a transcription-dependent mechanism (10). However, sodium butyrate induces only a small increase in the level of p21WAF1 transcript in this cell line, suggesting that other butyrate-induced gene products may be involved in its antiproliferative action.

Van Lint et al. (11) showed that only a small subset of gene promoters is sensitive to inhibition of histone deacetylation by trichostatin A. Thus, cloning of transcripts induced by inhibitors of HDAC may facilitate the identification of effectors responsible for the antiproliferative and/or differentiating actions of this class of drugs. Using the differential display technique (12), we cloned the cDNA of a new gene transcript induced by sodium butyrate in the mouse fibroblast cell line BP-A31. The protein encoded by this transcript (designated B-ind1 for butyrate-induced 1) does not affect cell proliferation and apoptosis but does appear to participate in the control of the transcriptional activity of NF-κB that is stimulated by sodium butyrate. In this context, we show that B-ind1 acts downstream of the small GTPase Rac1 in the biochemical pathway leading to NF-κB activation. B-ind1 also collaborates with the activated form of Rac1 to induce c-Jun N-terminal kinase (JNK) activity. Taken together, these data implicate B-ind1 as a novel component of the sodium butyrate and Rac1-signaling pathways that influence the regulation of gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The chemically transformed mouse fibroblast BP-A31 cell line was maintained in minimal essential medium α supplemented with 6% fetal calf serum. For synchronization in early G1 phase, the cells were cultured for 1 or 2 days in serum-free minimal essential medium α supplemented with 2.5 μM FeSO4, Simian COS-7 fibroblast-like cells were cultured in Dulbecco modified Eagle’s medium with 10% fetal calf serum.

For transient transfection, COS-7 cells (5 × 105 cells/dish) were seeded in 60-mm Petri dishes and transfected the following day with 5 μg of DNA using the LipofectAMINE plus kit (Life Technologies, Inc.). The transfection was stopped after 3 h by switching to standard growth medium. Cell extracts were collected 36 h later for kinase assays, Western blot analysis, or immunoprecipitation.

For the luciferase assay, 105 cells/dish were seeded in 35-mm Petri dishes and transfected the following day with 1.5 μg of DNA. The transfection was stopped after 3 h by switching to standard growth medium. Cell extracts were collected 36 h later. Luciferase activities were measured using the dual luciferase kit (Promega), and firefly luciferase activity in each sample was normalized with Renilla luciferase activity. Light emission was measured for 12-s intervals using a luminometer (Berthold).

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‡ To whom correspondence should be addressed. Tel.: 33-1-49-28-46-14; Fax: 33-1-44-74-93-18; E-mail: mester@adr.st-antoine.insERM.fr.

§ The abbreviations used are: HDAC, histone deacetylase; NF-κB, nuclear factor κB; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; hB-ind1, human B-ind1.
Cloning of B-ind1—Total RNA extracted from BP-A31 cells treated with or without 6 mM sodium butyrate in the presence or absence of serum was reverse transcribed in reaction mixtures containing the one-base-anchored oligo(dT) primer (H-T11 A). PCR was performed on the reverse transcription reaction products with the oligo(dT)-anchored primer and the arbitrary primer H-AP3 (5′-AAGCTTGTGTCAG-3′) (GenHunter Corp.) in the presence of [α-32P]dATP. The PCR products were resolved on a 6% acrylamide/urea gel and subjected to autoradiography. The cDNA corresponding to autoradiographic bands overexpressed in butyrate-treated cells but not in serum-treated cells were eluted from the gel, amplified, and subcloned in pCR-TRAP cloning vector (GenHunter Corp.). The murine sequence of B-ind1 was cloned and expressed in Escherichia coli as described previously (15).

RNA Analysis—Total cellular RNA was extracted by the LiCl/urea method, fractionated by formaldehyde/agarose gel electrophoresis, and analyzed by Northern blotting. The blots were hybridized with the murine B-ind1 cDNA fragment obtained as described below and labeled with the multiprime kit (Amersham Pharmacia Biotech).

Cloning of hB-ind1 Protein—Differential display PCR was used to identify proteins induced by sodium butyrate (12). Serum-starved quiescent BP-A31 murine fibroblasts were incubated for 6 h in the absence or presence of sodium butyrate, and differential display PCR was performed with different primer combinations. With 3 one-base oligo dT-anchored primers and 4 arbitrary primers (16) we detected cDNA in which expression was up-regulated by sodium butyrate in the presence or absence of serum (data not shown). To confirm these results, bands corresponding to differentially expressed cDNA were excised from the gel, and the cDNA was amplified by PCR using the combination primers described above for differential display and then used as probes in Northern blot analysis. This report focuses on the functional characterization of the cDNA designated as B-ind1.

The PCR-derived probe corresponding to B-ind1 hybridized with a single band of 3.5 kilobases. This RNA was weakly expressed in quiescent BP-A31 fibroblasts and was induced by sodium butyrate in the absence or presence of cycloheximide (Fig. 1A) indicating that the induction of B-ind1 expression does not require de novo protein synthesis. Analysis of the expression pattern of B-ind1 revealed that the mRNA was expressed in all mouse tissues examined (Fig. 1B). A strong signal was detected in the testis, kidney, brain, and liver whereas much weaker signals were observed in the skeletal muscle, spleen, and heart.

To obtain the entire coding sequence, we screened a human brain cDNA library with a partial cDNA clone. Analysis of the complete cDNA clone (hB-ind1) revealed an open reading frame that encoded a predicted protein of 370 amino acids (GenBank/EBI accession number AJ271081). A search of the data bases using PROSITE revealed no known functional motifs although significant homologies outside of the active site with the putative tyrosine phosphatase PTPLA cloned from mouse and human (17) and with protein coding sequences from organisms as distant as Caenorhabditis elegans and yeast were identified. In addition, hB-ind1 exhibits homology with the human p23 (18) as well as related proteins from other species including chicken, yeast, and C. elegans.

Effect of hB-ind1 on Sodium Butyrate-induced Activation of NF-κB—Because sodium butyrate is involved in the control of cell growth and apoptosis, we first examined whether any of these processes might be affected by hB-ind1. In COS-7 cells, using stably expressed hB-ind1 as well as hB-ind1 expressed under transient transfection conditions, we were unable to detect any effect of hB-ind1 on cell proliferation and apoptosis (data not shown).

Previous studies have shown that sodium butyrate induces
the activity of NF-κB (19), a transcription factor implicated in the control of many cellular processes including inflammatory response, differentiation, cell proliferation, and apoptosis (20). To investigate the potential role of hB-ind1 in sodium butyrate-dependent activation of NF-κB activity, we studied the expression of a luciferase reporter plasmid placed downstream of three tandem NF-κB binding sites of the IgGκ gene and a conalbumin basal promoter, (Igκ3-conaluc. In these experiments, we used COS-7 cells, which are characterized by high expression of transgenic genes and a low, butyrate-insensitive content of endogenous B-ind1 mRNA (data not shown). Transient transfection of the promoter (Igκ3-conaluc) into COS-7 cells resulted in low basal levels of transcription in untreated cells and an activation of 800-fold with sodium butyrate treatment. In cells transfected with hB-ind1, we observed an additional increase in sodium butyrate induction of (Igκ3-conaluc) promoter activity (Fig. 2), raising the possibility that B-ind1 may play a role in sodium butyrate-mediated NF-κB activation.

**hB-ind1 Potentiates Rac1-induced NF-κB Activation**—NF-κB activity is induced by members of the Rho family of small GTPases (Rho, Rac1, and Cdc42) (21). These proteins function as binary switches by cycling between the active GTP-bound state and the inactive GDP-bound state. Constitutive functional activation is achieved by mutations in amino acid positions 12 or 61 analogous to those of p21<sup>ras</sup> (22). We investigated whether hB-ind1 may participate in the pathway initiated by small GTPases and leading to NF-κB activation. To test this possibility, COS-7 cells were transfected with (Igκ3-conaluc together with an expression vector coding for the activated form of Rac1 (Rac Q61L) in the presence or absence of hB-ind1. As expected, expression of the activated form of Rac1 increased NF-κB activity. Interestingly, the effect of Rac1 on the transcriptional activity of NF-κB was potentiated in cells cotransfected with hB-ind1 (Fig. 3, A and B). Similar results were obtained with the HeLa cell line (data not shown).

To further investigate the functional importance of B-ind1 in Rac1 signaling and to identify the domains involved, we constructed several deletion mutants of hB-ind1 and tested their ability to affect the activation of NF-κB by Rac1. Two truncation mutants, fragments N (amino acids 1–199) and C (amino acids 197–370), failed to potentiate Rac1-induced NF-κB activation. Moreover, cotransfection of the median fragment M (amino acids 101–277) inhibited the Rac1-induced NF-κB activation (Fig. 3B). When increasing levels of the M mutant expression vector were cotransfected, we observed a dose-dependent inhibition of Rac1-induced activation of NF-κB (Fig. 3C). These results strongly suggest that the M fragment acts as a dominant-negative variant to block Rac1-mediated NF-κB activity.

**hB-ind1 Potentiates Rac1-induced JNK Activation**—In addition to NF-κB, the Rho family of GTPases controls at least two other transduction pathways, one regulating the mitogen-activated protein kinase/JNK pathway (23) and the second controlling the actin cytoskeleton organization (24). We searched to establish whether expression of hB-ind1 could also potentiate
the stimulation of JNK activity by Rac1. COS-7 cells were cotransfected with Flag-JNK and Rac Q61L expression vectors either in the presence or absence of hB-ind1, and JNK activity was determined in Flag immunoprecipitates using GST-c-Jun-(1–79) as substrate. As described previously, coexpression of Rac Q61L led to a significant increase in JNK activity of 3-fold. Expression of hB-ind1 failed to activate JNK but potentiated the Rac Q61L-mediated JNK activation by 5.6-fold. Similar results were obtained with another constitutively active form of Rac, Rac V12 (Fig. 4).

**hB-ind1 Forms Complexes with Constitutively Activated Rac1**—Our data on the regulation of gene expression by Rac1 are consistent with the possibility that hB-ind1 and the constitutively activated form of Rac1 may interact to produce transactivation of NF-κB-dependent promoters. To test this possibility, COS-7 cells were transfected with Flag-Rac Q61L and X-press hB-ind1. Complexes were precipitated with anti-Flag antibody directed toward tagged Rac Q61L followed by immunoblotting with anti-X-press to monitor the presence of hB-ind1 wild-type (WT) or mutant (M) alone or together with Flag-Rac Q61L as indicated. After 36 h, cells were harvested. Left panel, lysates were immunoprecipitated with Flag monoclonal antibody M2 and assayed for JNK activity using GST-Jun as substrate. Bottom, anti-M2 immunoblotting of whole extracts confirmed that similar amounts of Flag-JNK were transected in each sample. The results were quantified by PhosphorImager, and the ratio between 32P-labeling (GST-Jun) and ECL+ luminescence (Flag-JNK) is shown (Quantitation).

One important finding of our study is the demonstration that hB-ind1 is involved in the signaling pathway mediated by the small GTPase Rac1. In particular, hB-ind1 potentiated the Rac1-induced transcription and JNK activation. Moreover, the median region comprising amino acids 101–277 acted as a dominant-negative variant to inhibit the induction of the NF-κB-dependent promoter by Rac1. The mechanism of action of hB-ind1 may rely on the formation of complexes between Rac1 and its targets. Although the sequence of hB-ind1 reveals no Cdc42/Rac-interactive binding site, a distinctive domain contained in several previously identified Rac1 targets (25), the existence of protein complexes containing Rac1 and hB-ind1 was evidenced by coimmunoprecipitation experiments. Because other proteins without the Cdc42/Rac-interactive binding site associate with Rac1 (26), it is possible that hB-ind1 favors the maintenance of Rac1 complexes with its effector systems. This hypothesis is substantiated by the inhibition of the Rac signaling pathway by the median fragment M of hB-ind1, although this fragment did not form stable complexes with Rac1. The M fragment may act as a dominant-negative variant by competing with endogenous wild-type B-ind1 to sequester downstream targets required in Rac1 signaling.

In addition, the hB-ind1 protein displays homologies with p23 and related proteins from other species including chicken, yeast, and *C. elegans*. This suggests that hB-ind1 may be a member of a large family of proteins. Human p23 was first identified as a protein associated with the progesterone receptor (18) and was later shown to be in fact a heat shock (Hsp90)-binding protein (27, 28). Both p23 and its yeast homolog Sba1 (29) appear to act as Hsp90 co-chaperones, but each possesses a chaperone activity of its own (30). p23 has been postulated to crystallize as a homodimer.2 These observations lead us to speculate that hB-ind1 may form homodimers with a chaperone type activity toward Rac1. In that case, the dominant-negative action of the M fragment would result from the formation of M/wild-type B-ind1 dimers, depleting Rac1 of its chaperone.

The role of B-ind1 in the cellular effects of sodium butyrate is not clear. The B-ind1 transcript was present in all tissues examined; its induction by sodium butyrate in cultured cells is not an universal process, and its overexpression in transfected COS-7 cells did not induce cell growth arrest or apoptosis. In the transformed mouse embryo fibroblasts, the cell in which this transcript has been cloned, B-ind1 may participate in the initial differentiation program.

In conclusion, the strategy of cloning transcripts induced by HDAC inhibitors has enabled us to identify a novel protein that...
is conserved over a wide range of eukaryotic organisms with tissue-specific expression related to specific functions of differentiated cells. The participation of this protein in signaling by the small GTPase Rac1 is an important although perhaps not unique aspect of its activity; B-ind1 and related proteins may have other cellular functions that remain to be discovered.

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