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Cytoprotective effect of the small GTPase RhoB expressed upon treatment of fibroblasts with the Ras-glucosylating *Clostridium sordellii* lethal toxin

Johannes Huelsenbeck [a,1], Martin May [a], Florian Schulz [a], Ilona Schelle [a], Natalia Ronkina [b], Martin Hohenegger [c], Gerhard Fritz [d], Ingo Just [a], Ralf Gerhard [a], Harald Genth [a,*]

[a] *Institute of Toxicology, Hannover Medical School, D-30625 Hannover, Germany*
[b] *Institute of Biochemistry, Hannover Medical School, D-30625 Hannover, Germany*
[c] *Institute of Pharmacology, Medical University of Vienna, A-1090 Vienna, Austria*
[d] *Institute of Toxicology, Heinrich-Heine-University, D-40225 Düsseldorf, Germany*

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ABSTRACT

Mono-glucosylation of (H/K/N)Ras by *Clostridium sordellii* lethal toxin (TcsL) blocks critical survival signaling pathways, resulting in apoptosis. In this study, TcsL and K-Ras knock-down by siRNA are presented to result in expression of the cell death-regulating small GTPase RhoB. TcsL-induced RhoB expression is based on transcriptional activation involving $p38_{alpha}$ MAP kinase. Newly synthesized RhoB protein is rapidly degraded in a proteasome- and a caspase-dependent manner, providing first evidence for caspase-dependent degradation of a Rho family protein. Although often characterised as a pro-apoptotic protein, RhoB suppresses caspase-3 activation in TcsL-treated fibroblasts. The finding on the cytoprotective activity of RhoB in TcsL-treated cells re-enforces the concept that RhoB exhibits cytoprotective rather than pro-apoptotic activity in a cellular background of inactive Ras.

1. Introduction

The low molecular mass GTP binding proteins RhoA, RhoB and RhoC act as molecular switches, as they cycle between an active GTP-bound conformation and an inactive GDP-bound conformation. Rho-GTPases regulate the actin dynamics, cell adhesion, cell cycle progression, gene transcription, intracellular membrane trafficking, and cell survival [1]. These functions are important in tumorigenesis and tumor progression [2]. RhoA and RhoC are over-expressed in various types of tumors, and over-expression of RhoC protein has been shown to correlate with tumor metastasis [3,2]. The role of RhoB in human cancer development is controversial. RhoB has been found to be down-regulated in head and neck carcinoma and gastric and lung cancer but overexpressed in breast cancer [4,2]. Due to its pro-apoptotic activity RhoB is generally regarded as a tumor suppressor [5]. RhoB expression significantly inhibits proliferation, migration and invasion of cancer cells and also enhances chemosensitivity of malignant cells to anticancer drugs [6–9].

RhoB is an immediate-early gene product with a short half life period. RhoB expression has been reported in response to extracellular stimuli such as growth factors, cytotoxic and genotoxic agents [10–12]. The low intracellular level of RhoB is maintained by repression of *rhoB* promoter activity by oncogenes, including the epidermal growth factor receptor (EGFR), ErbB2, and Ras [8,9]. Farnesyltransferase inhibitors (FTIs) inhibits Ras through prevention of posttranslational farnesylation. FTI-induced Ras inhibition results in RhoB expression [13]. RhoB has been identified as a target in cancer therapy, based on the concept that RhoB expression is required for apoptosis of transformed cells [5,14].

Lethal Toxin from *Clostridium sordellii* (TcsL) is the major virulence factor of *C. sordellii*-associated disease in animals and humans, which includes myonecrosis, sepsis and shock [15,16]. TcsL enters target cells by receptor-mediated endocytosis and efficaciously mono-glucosylates (and thereby inactivates) the Ras subtype proteins (H/K/N)Ras and Rap(1/2). TcsL less efficaciously glucosylates Rac1 and Cdc42, while Rho(A/B/C) are not glucosylated [17–19]. Treatment of cells with TcsL results in inhibition of PI3K/

Abbreviations: FTI, farnesyltransferase inhibitor; MEF, mouse embryonic fibroblasts; TcdBF, toxin B from the *Clostridium difficile* serotype F strain 1470; TcsL, lethal Toxin from *Clostridium sordellii*; TUDCA, tauroursodesoxycholic acid

* Corresponding author. Fax: +49 511 532 2879.
E-mail address: genth.harald@mh-hannover.de (H. Genth).
[1] Present address: Institut für Toxikologie, Universität Mainz, D-55131 Mainz, Germany.

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Akt-mediated survival signaling and activation of p38 MAP kinase, with both effects contributing to the initiation of apoptosis of cultured myeloid and epithelial cells [20–23].

In this study, TcsL-induced RhoB expression is characterised in detail. TcsL treatment is presented to result in pronounced RhoB expression based on transcriptional activation involving $p38_{alpha}$ MAP kinase. RhoB, that is not glucosylated by TcsL, becomes activated in TcsL-treated cells and protects cells from TcsL-induced apoptosis. RhoB is caspase-dependently degraded, which is interpreted in terms of an amplification of pro-apoptotic signaling process initiated by the toxin. This is the first report showing that a Rho subtype protein is degraded in a caspase-dependent manner.

2. Materials and methods

2.1. Materials

Toxin B (TcdBF) and TcsL were purified from the *Clostridium difficile* serotype F strain 1470 and the *Clostridium sordellii* strain 6018, respectively [24]. The GST-C21 vector construct was a kind gift of Dr. John Collard (Amsterdam). Commercially obtained reagents: Actinomycin D (Calbiochem), cycloheximide (Calbiochem), caspase inhibitor I. (Z-VAD(OMe)-fmk) (Calbiochem), LY294002 (Calbiochem), MG132 (Calbiochem), SB203580 (Calbiochem), Tauroursodeoxycholic acid (Sigma); antibodies: K-Ras(Mab F234, SantaCruz); Ras(Mab27H5, Cell Signaling); Rac1(Mab 102; Transduction Lab); Rac1(Mab 23A8, Upstate); RhoA(Mab 26C4, SantaCruz); RhoB (Mab C-5, SantaCruz); beta-actin(Mab AC-40, Sigma), and horseradish peroxidase conjugated secondary antibodies rabbit/mouse (Rockland).

2.2. Methods

2.2.1. Cell culture

NIH3T3 fibroblasts, E1A/Ras-transformed RhoB +/− and RhoB −/− mouse embryonic fibroblasts (MEFs) (kindly provided by Dr. George Prendergast, Lankenau Institute for Medical Research, Wynnewood, Pennsylvania) [7,25], and $p38_{alpha}$−/− and the corresponding $p38_{alpha}$ wt MEFs (kindly provided by Dr. Angel Nebreda, Institute for Research in Biomedicine, Barcelona, Spain) [26] were cultivated in Dulbecco's modified essential medium (Biochrom + 10% FCS, 100 μg/ml penicillin, 100 U/ml streptomycin and 1 mM sodium pyruvate) at 37 °C and 5% CO_2 according to standard protocols. For all experiments, cells were seeded subconfluently into 3.5 cm-diameter dishes. Cells were washed once with phosphate buffered saline. They were then scraped into Laemmli sample buffer (200 μl). The obtained suspension was incubated for 10 min at 37 °C and 1400 rpm in a thermo shaker and subsequently sonified on ice. The lysate was then incubated for 10 min at 95 °C and submitted to Western blot analysis.

2.2.2. RhoB activity assay

The Rho-binding domain of Rhotekin, C21, was expressed as GST-fusion protein in *Escherichia coli*. After their lysis using French Press, the soluble fraction was obtained by centrifugation (20.000 rpm, 20′). It was incubated with glutathione–Sepharose for 30′ at 4 °C and subsequently washed. 3T3 fibroblasts treated with either TcdBF or TcsL as indicated were lysed in lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM PMSF, 5 mM DTT, Complete –EDTA). The soluble fraction was obtained by centrifugation (10000×*g*, 5′). It was then added to the glutathione-bound GST-C21 for 1 h (4 °C). After washing of the beads, RhoB was eluted by incubation with Laemmli sample buffer at 95 °C (10′). Samples were submitted to SDS–PAGE and Western blotting.

2.2.3. Knockdown of gene expression using siRNAs

The following Ras-specific siRNAs were used in this study: siRNA to H-Ras, GAGUGGAGGAUGCCUUCUA; siRNA to K-Ras, GCUC AGGACUUAGCAAGAA; and siRNA to N-Ras, AAAGCGCACUGA CAAUCCA. These siRNAs (20 nM) were transfected into Hela cells (n = 50,000) by using JetPRIME transfection reagent (PeqLab, Erlangen, Germany) according to the manufacturer's protocol. After 48 h, the effects of knockdown on RhoB expression were assessed. The level of protein knockdown by siRNA was determined by Western-blot analysis.

2.2.4. RNA purification and RT-reaction

Total RNA was purified from fibroblasts using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Shortly, cells were lysed in lysis buffer. The RNA was extracted and bound to silica membranes after addition of ethanol. Contaminating DNA was cleaved by DNase I digestion and bound RNA washed with the supplied buffer. Total cellular RNA was then eluted with RNase free water. 2 μg of RNA were then used as template in the RT-reaction, which was performed with the Omniscript RT-Kit (Qiagen) according to the manufacturer's instructions.

2.2.5. Semi-quantitative real-time PCR

The real-time PCR was conducted using the QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler (Roche). The cDNA obtained from the RT-reaction was diluted (1:1000) to avoid overloading. Primers (3 μM) used were: β-actin: 5′-CCT GCT TGC TGA TCC ACA TC-3′ and 5′-GCA TTG CTG ACA GGA TGC AG-3′, RhoB: 5′-CCG AGG TAA AGC ACT TCT GC-3′ and 5′-CCG AGC ACT CGA GGT AGT CA-3′. After every run, a melting curve was recorded to ensure the specificity of the reaction.

2.2.6. Analysis of rhoB promoter activity

Subconfluent NIH3T3 fibroblasts were transiently transfected with 2 μg of the 3.5 kb *rhoB* promoter CAT construct [10] for 14 h, applying FuGENE 6 Transfection Reagent (Roche). Cells were then treated as indicated and harvested. The protein concentration of the lysates was normalized using Bradford test. The level of CAT expression was analyzed using an enzyme-linked immunosorbent assay (CAT-Elisa kit, Roche) according to the manufacturer's instructions.

2.2.7. Caspase-3 colorimetric assay

Caspase-3 activity was analyzed using the Caspase-3 colorimetric assay (R&D Systems), according to the manufacturer's instructions. Cells were exposed to the toxins and drugs as indicated. Cells were lysed and subsequently incubated with the colorimetric substrate DEVD-pNA at 37 °C for 3 h. The caspase-3-dependent release of the reporter molecule pNA was quantified by photometry using a scanning multi-well spectrophotometer at 405 nm.

3. Results

3.1. TcsL-induced expression and activation of RhoB

Pronounced RhoB expression was observed upon treatment of NIH3T3 fibroblasts with increasing concentrations of TcsL (Fig. 1A). TcsL-catalysed glucosylation of (H/K/N)Ras and Rac1 was evidenced using the glucosylation-sensitive Ras(27H5) and Rac1(102) antibodies, respectively [27,17]. Either antibody recognizes glucosylation in terms of apparently decreasing levels of either (H/K/N)Ras or Rac1 protein (Fig. 1A). (H/K/N)Ras was more efficaciously glucosylated than Rac1 (Fig. 1A), consistent with former observations [17]. The levels of K-Ras or Rac1 were not changed upon TcsL treatment, as analyzed using alternative