Inactivation of RhoA/B/C or H/K/N-Ras by large clostridial cytotoxins triggers up-regulation of the immediate early gene product RhoB

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

Dipl. Biochem. Johannes Hülsenbeck

geboren am 06.03.1978, in Münster (Westfalen)

-2007-

Die vorliegende Arbeit wurde in der Zeit von November 2003 bis März 2007 an den Instituten für Physiologische Chemie und Toxikologie der Medizinischen Hochschule Hannover unter der Betreuung von Prof. Dr. M. Gaestel und Prof. Dr. I. Just angefertigt.

Referent: **Prof. Dr. M. Gaestel**

Korreferent: **Prof. Dr. I. Just**

Prüfungstermin: **11.06.2007**

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel verfasst habe. Ich habe die Dissertation nicht als Diplomarbeit oder ähnliche Arbeit verwendet und abgesehen von den angegebenen Teilpublikationen nicht veröffentlicht.

Hannover, den 25.03.2007

(Johannes Hülsenbeck)

Danksagung

Mein Dank gilt all denen, die mich bei meiner Doktorarbeit direkt oder indirekt unterstützt haben.

Zunächst möchte ich mich bei Prof. Gaestel für die Übernahme des Referats dieser Arbeit bedanken.

Mein besonderer Dank gilt Prof. Just und Harald Genth für die Bereitstellung des Themas sowie eine Betreuung, die keine Wünsche offen ließ.

Den Arbeitsgruppenleitern in der Abteilung Toxikologie, Ralf Gerhard, Fred Hofmann und Andreas Pich danke ich für stete Diskussionsbereitschaft und viele Anregungen, ohne die die Qualität der Arbeit gelitten hätte. Besonderer Dank an Fred für die tatkräftige Unterstützung in allen molekularbiologischen Fragestellungen.

Für die Versorgung mit den alltäglichen Gebrauchsgütern im Labor und ein ständigen Zufluss an Toxinen danke ich Ilona Klose, die weit mehr leistet als „excellent technical assistance“. Weiterhin danke ich Helma Tatge, die jederzeit ansprechbar und äußerst hilfsbereit war. Obwohl sie nicht weniger hilfsbereit war, danke ich Sandra Hagemann vor allem für die gute Stimmung und eine Menge spaßige Unterhaltungen.

Mein Zuhause war die AG Genth. Verantwortlich waren hierfür nicht nur die ständigen Mitglieder Harald und Ilona, sondern auch etlichen Weggefährten, Doktoranden (Birgit Hartmann, Jan Markus Isermann, Ismael Halabi-Cabezon), Diplomanden (Florian Schulz), Praktikanten (Ina Bock, Valeria Cherkasov, Kathrin Gangl, Christina Knorr, Jakob Engel, Hermann-Josef Meyer). Hervorheben möchte ich Steffi Dreger, die an allen wichtigen Befunden dieser Dissertation Anteil hatte. Beruflich wie privat war und ist sie meine große Stütze. Dank Euch allen! Als beinahe zugehörig betrachte ich Hauke Suptuth („Genths and Friends“), dessen Humor manchmal für uns einfach zu trocken ist…

Dank gilt unseren Kooperationspartnern, Nils von Neuhoff aus der Molekularpathologie der MHH, für die Unterstützung bei der real time PCR sowie Gerhard Fritz aus der Toxikologie der Universität Mainz für fachmännischen Rat in Bezug auf RhoB.

Für den Ausgleich außerhalb des Labors danke ich meinen Kommilitonen, insbesondere Jens, Michi, Jazz, Julia, Karin und Klaus. Spezieller Dank an Jens für die Beratung bei der Erstellung dieser Arbeit.

Besonderen Dank an die Jungs vom TSV Kirchrode!

Last but not least danke ich meiner Familie, für Unterstützung, Rückzugsgebiet, Interesse und bedingungslose Hilfe in allen Lagen. Ohne Euch wäre ich nicht so weit gekommen.

Publikationen

Teile dieser Arbeit und themenverwandte Arbeiten wurden aus Prioritätsgründen bereits veröffentlicht:

Artikel

S.C. Dreger, **J. Huelsenbeck**, R. Gerhard, G. Fritz, I. Just, H. Genth: Inactivation of H-/K-/N-Ras is the basis of the cytotoxic effect of the *Clostridium sordelli* lethal toxin isoforms from strains 6018 and 9048. Manuscript in preparation.

J. Huelsenbeck, F. Schulz, S.C. Dreger, G. Fritz, I. Just, H. Genth: Ras dependent up-regulation of RhoB depends on p38 MAP kinase. Manuscript in preparation.

I. Halabi-Cabezon, **J. Huelsenbeck**, F. Hofmann, I. Just, H. Genth: Rac1 is the critical target of the clostridial glucosylating toxins. Manuscript in preparation.

J. Huelsenbeck, S.C. Dreger, R. Gerhard, G. Fritz, I. Just, H. Genth: Up-regulation of the immediate early gene product RhoB by exoenzyme C3 and toxin B from *Clostridium difficile*. Biochemistry, accepted Feb. 27th, 2007.

J. Huelsenbeck, S. Dreger, R. Gerhard, H. Barth, I. Just, H. Genth: Difference in the cytotoxic effects of toxin B from *Clostridium difficile* strain VPI10463 and toxin B from variant *Clostridium difficile* strain 1470. Infect Immun. 2007 Feb;75(2):801-9

H. Genth, **J. Huelsenbeck**, B. Hartmann, F. Hofmann, I. Just, R. Gerhard: Cellular stability of Rho-GTPases glucosylated by *Clostridium difficile* toxin B. FEBS Lett. 2006 Jun 12;580(14):3565-9.

Kurzartikel (Meeting Abstracts)

Vorträge

Mainz (Germany), 13th – 15th March 2007. 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
Title: Different cytotoxic effects of reference toxin B and variant toxin B from *Clostridium difficile*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 36, 141, Suppl. 1 MAR 2007

Dreger S, Isermann J, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Inactivation of H-/K-/N-Ras is the basis of the cytotoxic effect of *Clostridium sordellii* lethal toxin. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 36, 140, Suppl. 1 MAR 2007

Nottingham (United Kingdom), 21-25 June 2006, 5th International Meeting on the Molecular Biology and Pathogenesis of the Clostridia.
Title: Up-regulation of RhoB by clostridial cytotoxins

Mainz (Germany), 4th – 6th April 2006. 47th Spring Meeting of the German Society of Pharmacology and Toxicology.
Title: p38 MAPK-dependent modulation of RhoB up-regulation requires active Rac1
NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 53, 172, Suppl. 1 MAR 2006

Posterpräsentationen

Mainz (Germany), 13th – 15th March 2007. 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
Schulz F, **Huelsenbeck J**, Gerhard R, Kotlyarov A, Fritz G, Just I, Genth H. p38 MAP kinase and the cytotoxic effect of *Clostridium difficile* toxin B. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 38, 149, Suppl. 1 MAR 2007

Halabi-Cabezon I, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Glucosylation of RhoG contributes to the cytopathic effect of clostridial glucosylating toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 38, 150, Suppl. 1 MAR 2007

Nottingham (United Kingdom), 21-25 June 2006, 5th International Meeting on the Molecular Biology and Pathogenesis of the Clostridia.
Genth H, Dreger S, **Huelsenbeck J**, Just I. Distinct dynamics of the cytopathic and cytotoxic effect induced by *Clostridium difficile* toxin B.

Mainz (Germany), 4th – 6th April 2006. 47th Spring Meeting of the German Society of Pharmacology and Toxicology.
Dreger S, **Huelsenbeck J**, Just I, Genth H. The Cytopathic Activity of Clostridium difficile Toxin B Includes Up-Regulation of the Immediate Early-Gene RhoB and Caspase3-Activation. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 186, Suppl. 1 MAR 2006

Engel J, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Cross-Reactivity of the Anti-Toxin Against the Catalytic Domain of *C. sordellii* Lethal Toxin with *C. difficile* Toxin B. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 187, Suppl. 1 MAR 2006

Cherkasov V, **Huelsenbeck J**, Just I, Genth H. Glucosylation of Endogenous Rac1 as a Marker of the Cellular Activity of Clostridial Glucosylating Toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 188, Suppl. 1 MAR 2006

Berlin (Germany), 13th – 15th October 2005. 9th International Dahlem Symosium on Cellular Signal Recognition and Transduction.
Genth H, **Huelsenbeck J**, Dreger S, Hofmann F, Gerhard R. Clostridial factors associated to *Clostridium difficile* toxin A and toxin B responsible for their cytotoxic activity.

Huelsenbeck J, Gerhard R, Kotlyarov A, Just I, Genth H. p38 MAPK-dependent Modulation of RhoB Up-regulation Requires Active Rac1.

Canterbury (United Kingdom), 25th – 29th June 2005. 12th European Conference on Bacterial Protein Toxins.
Genth H, Isermann M, **Huelsenbeck J**, Hofmann F, and Just I. Difference in the protein substrate specifity between *Clostridium sordellii* lethal toxin and variant *Clostridium difficile* toxin B.

Huelsenbeck J, Gerhard R, Just I, and Genth H. Upregulation of RhoB by Clostridial Cytotoxins.

Mainz (Germany), 15th – 17th March 2005. 46th Spring Meeting of the German Society of Pharmacology and Toxicology.
Huelsenbeck J, Dreger S, Hartmann B, Gerhard R, Genth H. Up-regulation of RhoB protein by glucosylating toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R118-R118 493 Suppl. 1 FEB 2005

Isermann M, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Difference in the protein substrate specificity between *Clostridium sordellii* lethal toxin and variant *Clostridium difficile* toxin B 1470. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R117-R117 490 Suppl. 1 FEB 2005

Schulz F, **Huelsenbeck J**, Just I, Genth H. The catalytic domain of native large clostridial cytotoxins escapes antitoxin detection. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R117-R118 491 Suppl. 1 FEB 2005

Nice (France), 4th – 8th September, 2004. ELSO Meeting.
Genth H, Hartmann B, **Huelsenbeck J**, Just I, Gerhard R. Intracellular stability of Rho GTPases modified by ADP-ribosylation and glucosylation.

Mainz (Germany), 9th – 11th March 2004. 45th Spring Meeting of the German Society of Pharmacology and Toxicology.
Huelsenbeck J, Hartmann B, Gerhard R, Just I, Genth H. Difference in Rho-GTPase substrate specificity between wildtype and recombinant *Clostridium difficile* toxin B. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 369: R53-R53 211 Suppl. 1 MAR 2004

Toxin A (TcdA) und Toxin B (TcdB) aus *Clostridium difficile* sind Mono-Glucosyltransferasen, die RhoA, Rac1 und Cdc42 inaktivieren. Die Inaktivierung hat einen Zusammenbruch des Aktinzytoskeletts ("Zytopathischer Effekt") und die Apoptose von Zellen ("Zytotoxischer Effekt") zur Folge. Die Inaktivierung von RhoA wird dabei für den zytotoxischen Effekt verantwortlich gemacht. Bei Behandlung kultivierter Zellen mit TcdA wurde zudem eine Hochregulation von RhoB beobachtet. Die vorliegende Arbeit widmet sich den Mechanismen der Toxin-abhängigen RhoB Hochregulation und ihrer funktionellen Auswirkungen.

Nach Behandlung von Fibroblasten mit TcdB wurde eine Zeit- und Konzentrations-abhängige RhoB Hochregulation gemessen. Sowohl Actinomycin D als auch Cycloheximid blockierten die Hochregulation. Demnach waren mRNA und Protein *de novo* Synthese unbedingt erforderlich. Toxine, welche durch direkte Wirkung am Aktinzytoskelett dessen Zusammenbruch herbeiführen, wie das C2 Toxin aus *Clostridium botulinum* oder Latrunculin B, verursachten keine RhoB Hochregulation. Tatsächlich war die Inaktivierung von Rho die Ursache der Hochregulation, da sie ebenfalls nach Behandlung von Zellen mit dem RhoA/B/C inaktivierenden Exoenzym C3 aus *Clostridium limosum* auftrat. Das letale Toxin aus *Clostridium sordellii* (TcsL), welches Rac1 und H/K/N/R-Ras inaktiviert, verursachte ebenfalls eine RhoB Hochregulation. Im Gegensatz dazu wurde mit dem varianten Toxin B aus dem *Clostridium difficile* Stamm 1470 (TcdBF), welches Rac1 und R-Ras inaktiviert, keine RhoB Hochregulation beobachtet. Die Inaktivierung von H/K/N-Ras war somit ursächlich für die Hochregulation durch TcsL.

In p38 MAP Kinase defizienten Zellen wurde RhoB zwar nach Behandlung mit TcdB oder TcsL hochreguliert, jedoch in geringerem Ausmaß. Die p38 MAP Kinase scheint ein Verstärker der RhoB Hochregulation zu sein. Der RhoB Spiegel wurde durch proteasomale und Caspase-abhängige Degradation reguliert. Sowohl TcdB als auch TcsL verursachten somit eine Aktivierung der zellulären Caspasen. In mit TcsL behandelten Zellen konnte eine deutliche Steigerung der RhoB Aktivität festgestellt werden. Diese stieg zwar ebenfalls in mit TcdB behandelten, jedoch weniger deutlich. C3 war im Gegensatz dazu in der Lage, RhoB vollständig zu inaktivieren. Durch Inhibition der RhoB Hochregulation wurde der zytopathische Effekt der Toxine nicht beeinflusst. In synchronisierten S-Phase Fibroblasten verursachte TcdB einen Caspase-abhängigen zytotoxischen Effekt. Dieser Effekt konnte durch C3 blockiert werden. Somit wurde aktives RhoB für den zytotoxischen Effekt von TcdB benötigt.

Schlagworte: große clostridiäre Zytotoxine; Apoptose; Rho/Ras GTP bindende Proteine

Abstract

Clostridium difficile toxins A (TcdA) and B (TcdB) are mono-glucosyltransferases that inactivate RhoA, Rac1, and Cdc42. By these means, the toxins cause actin re-organisation ("cytopathic effect") and apoptosis ("cytotoxic effect"). The cytotoxic effect has generally been attributed to the inactivation of RhoA. Treatment of cultured cells with *Clostridium difficile* toxin A (TcdA) also causes RhoB up-regulation. This study focuses on the up-regulation of RhoB and its functional consequences in toxin-treated cells.

RhoB was up-regulated in response to treatment of fibroblasts with TcdB in a time- and concentration dependent manner. The up-regulation was abrogated by actinomycin D and cycloheximide, thus it was due to mRNA and protein *de novo* synthesis. When actin re-organisation was induced by toxins directly affecting the actin cytoskeleton, such as *Clostridium botulinum* C2 toxin or latrunculin B, no RhoB up-regulation was observed. Up-regulation of RhoB was due to inactivation of Rho, as it was also observed in cells treated with exoenzyme C3 from *Clostridium limosum* that specifically ADP-ribosylates RhoA/B/C but not Rac1 or Cdc42. RhoB was also up-regulated in cells treated with *Clostridium sordellii* lethal toxin (TcsL), which inactivates Rac1 and H/K/N/R-Ras. The up-regulation was based on the inactivation of H/K/N-Ras, as the variant *Clostridium difficile* toxin B from strain 1470 (TcdBF), which inactivates Rac1 and R-Ras, failed to do so.

In p38 MAP kinase knockout fibroblasts, RhoB was up-regulated to a minor extent compared to wild type cells after treatment with either TcdB or TcsL. This finding indicated that p38 MAP kinase was an enhancer of the RhoB response. The intracellular level of RhoB was regulated by proteasome- and caspase-dependent degradation in TcsL- as well as in TcdB-treated cells, indicating that both toxins caused an activation of caspases. RhoB was strongly activated in TcsL- and to a minor extent in TcdB-treated cells, most probably due to its glucosylation by TcdB. In contrast, RhoB was inactive in C3-treated cells, confirming the notion that C3 is an efficient inhibitor of RhoA/B/C. Inhibition of RhoB up-regulation did not affect actin re-organisation, indicating that RhoB did not regulate the cytopathic effect of the toxins. Synchronized fibroblasts were susceptible to the cytotoxic effect of TcdB as analysed in terms of annexin V staining. The cytotoxic effect was responsive to inhibition by either a pan-caspase inhibitor or exoenzyme C3, suggesting that active RhoB is required for the cytotoxic effect of TcdB.

Keywords: large clostridial Cytotoxins; apoptosis; Rho/Ras GTP-binding proteins

Content

1. Introduction

1.1 Large clostridial cytotoxins

Clostridium difficile causes antibiotic associated diarrhoea and pseudomembranous colitis (Just et al., 2001;Kelly and LaMont, 1998). After treatment with broad spectrum antibiotics, *C. difficile* overgrows the physiological flora of the gut. The bacteria then produce their main pathogenicity factors, toxin A (TcdA) and toxin B (TcdB). TcdA and TcdB belong to the family of large clostridial cytotoxins, which further comprises the lethal (TcsL) and hemorrhagic (TcsH) toxin from *C. sordellii* as well as the α-toxin (Tcnα) from *C. novyi*. The toxins are single chained proteins ranging from 250-308 kDa in size. They catalyze a glycosyltransferase reaction, by which means they inactivate their substrates, small GTP binding proteins of the Rho- and the Ras-family. These GTP binding proteins are the master regulators of the actin cytoskeleton (Boquet et al., 1998;Mackay and Hall, 1998). On cultured cell lines, the toxins cause an actin re-organisation resulting in rounding of cells ("cytopathic effect") (Chaves-Olarte et al., 1997). Furthermore, the toxins induce apoptosis ("cytotoxic effect").

The less common clostridial pathogen *Clostridium sordellii* causes endocarditis, arthritis, peritonitis, myonecrosis, and toxic shock syndrome (Gredlein et al., 2000;Lewis and Naylor, 1998;Sinave et al., 2002). *Clostridium sordellii* produces two main pathogenicity factors, the hemorrhagic toxin (TcsH) and the lethal toxin (TcsL), which are homologue to TcdA and TcdB, respectively (Just and Gerhard, 2004).

1.2 Structure of the toxins

TcdB and TcsL are single chain protein toxins (270 kDa). The toxins are homologous and have 76 % identical amino acids. Furthermore, they exhibit a three domain structure (Von Eichel-Streiber et al., 1994). The C-terminal harbours the receptor binding domain. Its binding to unknown receptors results in receptor-mediated endocytosis. A role for cellular surface sugars has been described (Frisch et al., 2003;Ho et al., 2005). The putative transmembrane domain is located in the central part of the toxins. This hydrophobous domain may be involved in the pore formation to enable the passage of the toxin from the endosome to the cytosol. The acidification of the endosomes during their processing is essential for the translocation into the cytosol (Qa'Dan et al., 2000). Only the N-terminal domain, which harbours the catalytic activity of the toxins, reaches the cytosol (Figure I.) (Pfeifer et al., 2003;Rupnik et al., 2005).

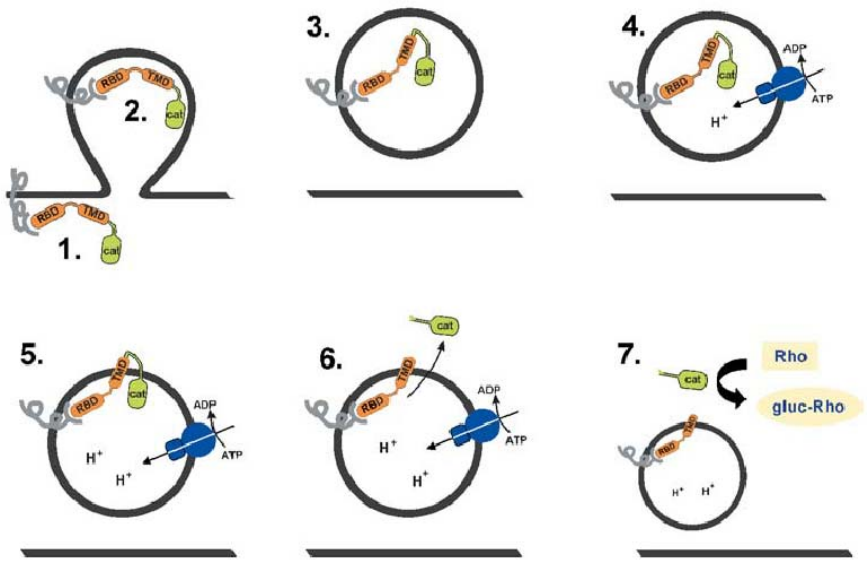

Figure I.: Proposed mechanism of toxin uptake

After binding of the receptor-binding domain (RBD) (1.), receptor mediated endocytosis takes place (2.), by which means an endosome is formed (3.). Subsequently, the endosome is acidified by an H^+-ATPase (4.). The acidification induces a conformational change of the toxin, allowing the transmembrane domain (TMD) to insert into the endosomal membrane and subsequently form a pore (5.) (Barth et al., 2001). The catalytical domain (cat) is cleaved from the toxin and released into the cytosol (6.), where it modifies its substrates, e.g. Rho GTP binding proteins (7.).

1.3 Enzymatic activity of TcdB and TcsL

TcdB and TcsL are glucosyltransferases that transfer a glucose moiety from their co-substrate, UDP-glucose, onto their protein substrates. A further factor required for the catalytic activity is Mn^{2+} or Mg^{2+}. Their substrates are small monomeric GTP binding proteins of the Rho- and the Ras-family (Figure II.).

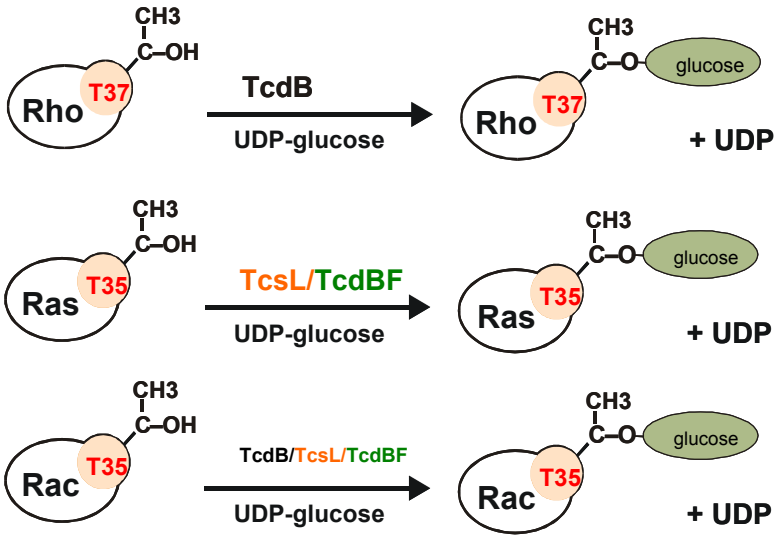

Figure II.: Substrate glucosylation of large clostridial cytotoxins

TcdB, TcdBF, and TcsL transfer a glucose moiety onto their substrates, small monomeric GTP binding proteins of the Rho- and the Ras-family. Rho is modified at threonine 37, whereas Rac and Ras are modified at threonine 35.

The glucose is covalently linked to a pivotal threonine residue, in the case of RhoA at threonine 37 (Just et al., 1995b), in the case of Rac1 and Ras at threonine 35 (Just et al., 1996;Popoff et al., 1996). This threonine residue is located within the effecter binding region of the Rho/Ras GTP binding proteins.

In contrast to their protein substrate specificity, TcdB and TcsL are highly specific regarding their co-substrate. The toxins exclusively recognize UDP-glucose as co-substrate. Supposedly, the toxins form a ternary complex with the UDP-glucose and their protein substrate to transfer the glucose moiety onto the threonine residue (Just and Boquet, 2000).

1.4 C3-like ADP-ribosyltransferases

The C3-like exoenzymes are single-chain proteins with a molecular mass of about 25 kDa. C3 specifically catalyzes the transfer of an ADP-ribose moiety from the co-substrate NAD^+ to asparagine 41 of RhoA/B/C (Sekine et al., 1989). This modification increases the affinity of the GTP binding proteins to their GDIs (Genth et al., 2003) and blocks the GEF mediated nucleotide exchange (Sehr et al., 1998). In consequence, C3 causes functional inactivation of the Rho GTP binding proteins. Further effects described for C3 treatment are alteration of e.g. epithelial and endothelial barrier functions (Nusrat et al., 1995;Stamatovic et al., 2003), the signaling of immune cells including phagocytosis (Caron and Hall, 1998), the production of cytokines (Chen et al., 2002), and adhesion (Laudanna et al., 1996). The family of C3-like transferases encompasses exoenzymes from *C. botulinum* (Popoff et al., 1990), *C. limosum* (Just et al., 1992), *B. cereus* (Just et al., 1995a), and *S. aureus* (Inoue et al., 1991). C3 was classified as exoenzyme because it lacks a membrane binding and translocation domain. To allow efficient cell entry of C3, chimera toxins have been constructed, exploiting e.g. the cell entry domains of other toxins such as the *C. botulinum* C2 toxin (receptor mediated endocytosis) (Barth et al., 1998). This chimera toxin has been applied in this study.

Table 1 lists the applied toxins as well as their respective catalyzed reactions and protein substrates (below).

Table 1: Substrate specificity and catalyzed reaction of the applied toxins

Toxin	catalyzed reaction	protein substrate				
TcdA/TcdB	glucosylation	RhoA/B/C	Rac1	Cdc42		
TcdBF	glucosylation		Rac1			R-Ras
TcsL	glucosylation		Rac1		H/K/N-Ras	R-Ras
C3	ADP-ribosylation	RhoA/B/C				

1.5 Small monomeric GTP binding proteins

The Rho- and the Ras-family are distinct subfamilies of GTP binding proteins within the Ras-superfamily as determined by homology (Hall, 1998). These small GTP binding proteins (molecular weight: 18-26 kDa) are nucleotide driven molecular switches, e.g. they are activated by binding to GTP. In their active, GTP-bound form, they interact with effecter proteins for signal transmission. Their state of activity is regulated by three groups of proteins (Figure III.):

I. Guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP and positively regulate the activity of the GTP binding protein.

II. GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity and negatively regulate their activity.

III. Guanine nucleotide dissociation inhibitors (GDIs), which stabilize the inactive GDP-bound form of the GTP binding protein in the cytosol, thereby inhibiting its translocation to the membranes.

The GTP binding proteins are thus regulated by the GDP/GTP-cycle (Fig. III.) and by a spatial cycle between membrane (active conformation) and cytosol (inactive conformation) (by GDIs).

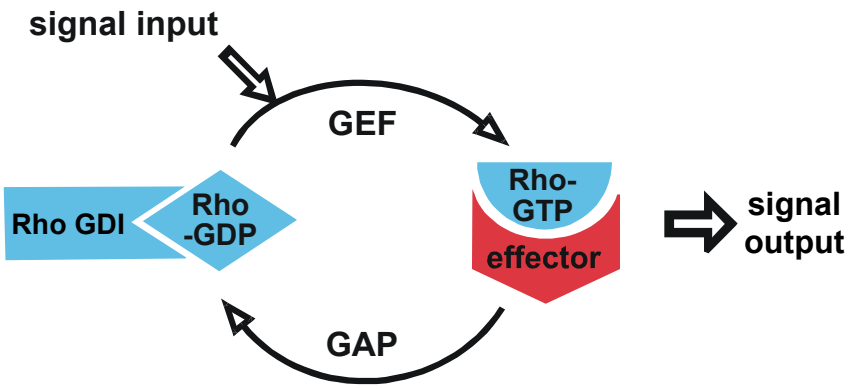

Figure III.: The activity of Rho/Ras-GTPases is regulated by GEFs, GAPs, and GDIs

The GTP binding proteins cycle between a GDP-bound, inactive, and a GTP-bound, active, conformation. Their activity is regulated by nucleotide exchange factors (GEF, activating), GTPase activating proteins (GAP, inactivating), and GDP-bound form stabilizing factors (GDI, inactivating).

A common feature of Rho- and Ras GTP binding proteins is their post-translational isoprenylation at the C-terminal, required for membrane binding. Furthermore, they feature two important functional domains, namely switch 1 and switch 2. These domains alter their three-dimensional structure upon exchange of GDP against GTP and vice versa (Wittinghofer and Pai, 1991). The GDP-bound inactive form does not interact with the respective effecter proteins. The conformational change upon nucleotide exchange to GTP enables this interaction, allowing signal transduction. Most effecter proteins are kinases that amplify and execute the Rho/Ras-signal.

The best characterized members of the Rho-family are RhoA, Rac1, and Cdc42 that regulate the actin cytoskeleton. They influence the morphology, motility, polarization, endocytosis, and vesicle trafficking (Etienne-Manneville and Hall, 2002). The formation of actin stress fibres is associated with RhoA (Ridley, 1997), whereas Rac1 induces membrane ruffling and Cdc42 the formation of filopodia (Nobes and Hall, 1995). The three GTP binding proteins further contribute to progression from G1 to S-phase, e.g. by up-regulation of cyclin D1 (Olson et al., 1995). For regulation of gene transcription of e.g. cyclin D1 and inflammatory proteins as interleukin 2, the GTP binding proteins activate distinct signaling pathways (especially those regulated by Jun-kinase, p38 MAP kinase, NFκB). Furthermore, they regulate apoptotic and inflammatory processes (Esteve et al., 1998; Jaffe and Hall, 2005; Van Aelst and D'Souza-Schorey, 1997).

Ras GTP binding proteins are associated with functions distinct from the Rho GTP binding proteins. Ras GTP binding proteins transmit signals from growth factor receptors via the Raf proto-oncogene to the nucleus that result in alterations of cell cycle proteins and transcription factors (Boguski and McCormick, 1993). Mutations of Ras GTP binding proteins are associated with ~30 % of all forms of cancer, making them potent proto-oncogenes themselves.

1.6 RhoB

RhoB is homologous to RhoA, both exhibit an identity of 86 % at the amino acid level and have therefore been suggested to have similar biological activities: They govern the dynamics of the actin cytoskeleton and both promote transactivation of the serum response element of the c-fos promoter and potentate the transforming activity of oncogenic Ras (Prendergast et al., 1995;Wennerberg and Der, 2004). Still, RhoB differs from RhoA in two important aspects. 1. While RhoA is constitutively expressed, RhoB is transcriptionally regulated in most cell lines, meaning that its basal level is low. It is up-regulated physiologically during S-phase (Zalcman et al., 1995). Due to its low half life period (mRNA: 20 minutes, protein: 2 hours), up-regulated RhoB is quickly degraded and its signaling terminated (Fritz et al., 1995;Lebowitz et al., 1995). Active RhoA and Ras GTP binding proteins regulate the RhoB level by suppression of *rhoB* promoter activity (Fritz and Kaina, 1997;Jiang et al., 2004b;Jiang et al., 2004a). 2. Due to differences in their respective C-terminals, RhoB can be either farnesylated or geranyl-geranylated, while RhoA is exclusively geranyl-geranylated (Adamson et al., 1992a). RhoA cycles between the plasma membrane and the cytosol, while RhoB has been found to be permanently localized to endosomes and lysosomes (Adamson et al., 1992b;Michaelson et al., 2001). Distinct functions of RhoB are partially based on this distinct localization (Wang and Sebti, 2005). RhoB regulates the trafficking of early endosomes (Rojas et al., 2004). Furthermore, alteration of RhoB signaling results in apoptosis induced by anti-cancer drugs (Fritz and Kaina, 2000;Liu et al., 2001). RhoB further suppresses NFκB and the survival kinase Akt in a PRK/PDK1-dependent manner, which may further contribute to the initiation of apoptosis (Fritz and Kaina, 2001;Jiang et al., 2004a). Finally, a direct interaction of RhoB with caspase-2 has been suggested (Kong and Rabkin, 2005).

1.7 Functional consequences GTP binding protein glucosylation

The receptor amino acid for glucosylation, threonine 37 (RhoA/RhoB) and threonine 35 (Rac/Cdc42/Ras) respectively, is located within the switch 1 region of the GTP binding proteins. The efficiency of glucosylation depends on the nucleotide bound. The threonine residue is easily accessible in the GDP-bound conformation. In the GTP-bound conformation, the threonine is hidden inside the molecule due to its interaction with a phosphate, and therefore it is hardly glucosylated (Just et al., 1995b).

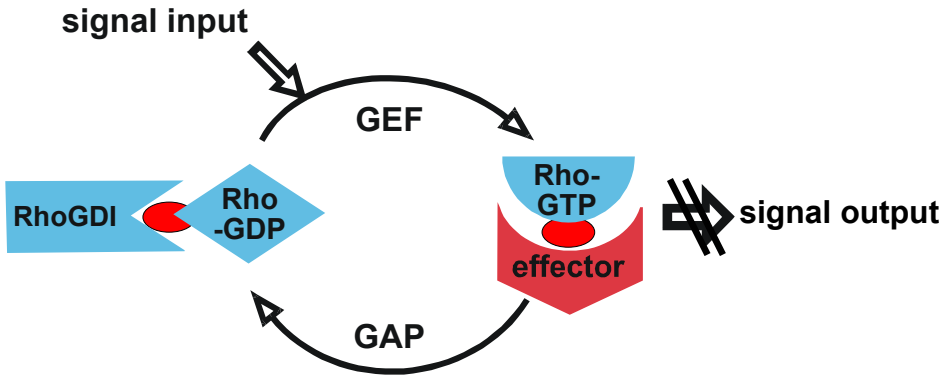

Figure IV.: Inhibition of Rho/Ras signal transduction by their glucosylation
Upon glucosylation, the Rho/Ras GTP binding proteins can no longer interact with GDI and their effecters.

The glucosylation freezes the Rho/Ras GTP binding proteins in their inactive state (Figure IV.). In consequence, the Rho/Ras-dependent signal transduction is blocked (Vetter et al., 2000). Except for this effect on the protein-effecter interaction, the glucosylation blocks the interaction with GDI. In consequence, glucosylated Rho accumulates at the plasma membrane (Genth et al., 1999). Inactivation of Rho causes actin re-organisation in cultured cells. The actin re-organisation may be the cause of the toxin-induced loss of barrier function of intestinal (TcdA/TcdB) and endothelial tissues (TcsL). The inactivation of the GTP binding proteins by glucosylating toxins further induces caspase-dependent apoptosis (Fiorentini et al.,

1998;Hippenstiel et al., 2002;Qa'Dan et al., 2002). Apoptosis of intestinal cells may play a role in the occurrence of the pseudomembranous colitis.

2. Aims of this project

Treatment of various cultured cell lines with TcdA causes an up-regulation of RhoB. This up-regulation was hypothesized to be based on the inactivation of Rac1 and the subsequent actin re-organisation (Gerhard et al., 2005). However, no function for RhoB has been described in cells exposed to the large clostridial cytotoxins.

The aim of this thesis is

1. to reveal molecular mechanisms governing the up-regulation of RhoB
    - → actin re-organisation by agents that manipulate actin dynamics
    - → inactivation of Rho/Ras GTP binding proteins by bacterial protein toxins with distinct substrate specificities

2. to analyse the posttranslational regulation of RhoB
    - → state of activity by effecter pulldown assay
    - → regulation of intracellular stability by inhibition of proteasomal and caspase-dependent degradation

3. to check on the function of up-regulated RhoB in the toxin-treated cell
    - → regulation of actin re-organisation by inhibition of RhoB up-regulation
    - → regulation of apoptosis by inhibition of RhoB

3. Materials and Methods

3.1 Materials

The GST-C21 vector construct was a kind gift of Dr. John Collard (Amsterdam). Commercially obtained reagents: latrunculin B, actinomycin D, caspase inhibitor I. (Z-VAD(OMe)-FMK), MG132, nocodazole (Calbiochem); cycloheximide (Sigma), antibodies: RhoB (BL927, Bethyl Laboratories); beta actin (AC-40, Sigma); horseradish peroxidase conjugated secondary antibodies mouse/rabbit (Rockland).

3.2 Toxin purification

TcdA, TcdB, TcdBF, and TcsL were purified from the respective *C. difficile* strains VPI 10463 and 1470 or the *C. sordellii* strain 6018 (Genth et al., 2000). A dialysis bag containing 900 ml of 0.9% NaCl in a total volume of 4 l brain heart infusion medium (Difco) was inoculated with 100 ml of an overnight culture of *C. difficile* (or *C. sordellii*) and grown under microaerophilic conditions at 37°C for 72 h. Proteins were precipitated from the culture supernatant by ammonium sulfate at 70% saturation. The precipitates were dialyzed against Tris-HCl buffer, pH 7.5, overnight, and loaded onto a MonoQ column (Amersham Biosciences). The toxins were subsequently eluted with Tris-HCl buffer, pH 7.5, containing 500 mM NaCl. *C. botulinum* C2 toxin, and C3 fusion-toxin were expressed in *E. coli* using the pGEX-2T vector system and purified with GSH Sepharose beads (AP Biotech) as described (Barth et al., 1998).

3.3 Cell culture

Fibroblast cell lines are widely used to study the function of Rho proteins. Furthermore, these cell lines are sensitive to the clostridial glucosylating toxins. Fibroblasts 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. Upon confluence (2 days), cells were washed twice with phosphate buffered saline. Cells were then treated with 2 ml of trypsin solution (2') and after addition of 5 ml full growth medium centrifuged (5', 800 rpm). Pellets were re-suspended in 10 ml full growth medium. The following volumes of the suspension were used:

Table 2: Volumes of cell suspension used for different formats of culture vessels

	Size of culture vessel (total volume)		
	75 cm^2 (25 ml)	3,5 cm^2 (2 ml)	24-well (24 ml)
NIH3T3	2 ml	0,3 ml	1 ml
MEF WT	3 ml	0,3 ml	1 ml
MEF $p38_{\alpha}$-/-	3 ml	0,3 ml	1 ml

3.4 Synchronization of cells

The thymidine double block technique was applied to synchronize NIH3T3 fibroblasts. Exponentially growing cells were exposed to 2 mM of 2'deoxy-thymidine in full growth medium at 37 °C and 5% CO_2 for 15 h. The medium was then removed and replaced by medium without thymidine. After 9 h, this medium was replaced by full growth medium containing 2 mM of 2'deoxy-thymidine for the second block. Cells were incubated for 15 h. Subsequently, the medium was replaced by thymidine free full growth medium for 1 h. Cells were then synchronized in S-phase as determined by FACS.

3.5 Treatment of cells

Fibroblasts were treated with TcdB, TcdBF, TcsL, C2 toxin, or cell permeable C3. The toxins were directly applied to sub-confluent cells to the growth medium. The following drugs were applied: MG132 (20 µM), a reversible and cell permeable

proteasome inhibitor; caspase inhibitor I (20 µM), a cell permeable pan-caspase inhibitor; cycloheximide (1 mM), a protein synthesis inhibitor; actinomycin D (5 µM), an inhibitor of transcription; latrunculin B, a toxin that disrupts the actin cytoskeleton by inhibition of actin polymerisation (2,5 µM); cytochalasin D, a toxin that disrupts the actin cytoskeleton (10 µM); nocodazole, a toxin that disrupts the microtubule system (20 µM).

3.6 Cytopathic effect of the toxins

Clostridial glucosylating toxins cause a re-organisation of the actin cytoskeleton due to the inactivation of Rho proteins. This re-organisation is reflected by rounding of the cultured cells. Sub-confluent fibroblasts were exposed to the toxins as indicated. Cells were then incubated for 4 h. Analysis was performed by a Zeiss Axiovert 200 M. The typical morphology (“cell rounding”) was recorded. Cell rounding was determined by counting and was given as the ratio of rounded per total cells in %.

3.7 Analysis of apoptosis

Membrane heterogeneity is a marker of viable cells. It is lost early during apoptotic processes. The membrane component phosphatidylserine is localized to the inner leaflet of the membrane in viable cells. When the heterogeneity gets lost, phosphatidylserine also distributes among the outer leaflet of the membrane and becomes accessible from the outside. Annexin V binds specifically to phosphatidylserine exposed at the outer leaflet of the membrane. Phosphatidylserine exposure is visualized by Annexin V labeled with Alexa Fluor 488 (Cambrex) added directly into the medium (1:50). Cells were analyzed by fluorescence microscopy using a Zeiss Axiovert 200 M (annexin V Alexa Fluor 488: excitation: 470 nm; emission: 515 nm).

3.8 Cell lysis

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' at 37 °C and 1400 rpm in a thermo shaker and subsequently sonified on ice. The lysate was then incubated for 10' at 95°C and submitted to SDS PAGE.

3.9 Western blot analysis

Lysate proteins were separated using SDS-PAGE and subsequently transferred onto nitrocellulose membranes (Schleicher and Schuell, Germany) by a tank blot system (120 V, 120'). The membranes were blocked with 5 % (w/v) non-fat dried milk in TRIS buffered saline supplemented with Tween 20 (50 mM TRIS, pH7,4, 150 mM NaCl, 0,05 % (w/v) Tween 20) for 60 minutes; incubation with the primary antibody was conducted over night at 4 °C, treatment with the secondary antibody for 2 h (22°C). For the chemoluminescence reaction, ECL Femto (Pierce) was used. All signals were analyzed densitometrically using the KODAK 1D software and normalized to beta actin signals.

3.10 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.

3.11 Semi-quantitative real-time PCR

Real time PCR is a specialized PCR protocol to detect changes of mRNA levels. It is based on a standard PCR protocol to amplify the sequence of interest. To detect the synthesized PCR product, a dsDNA specific, fluorescent dye (SYBR green) is added to the reaction mixture. By these means, an increase of synthesized product evokes an increase of fluorescence intensity which can be measured after each individual cycle. For quantification, a threshold value for the fluorescence intensity is set. The number of PCR cycles required to reach this threshold (crossing point) is used to compare treated samples to control samples, which are set to 1.0. 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'.

3.12 RhoB activity assay

Only GTP-bound and thus active RhoB interacts with its effecter proteins. Thus, a pulldown applying the Rho effecter Rhotekin is used to determine the activity state of RhoB (Gampel and Mellor, 2002). The Rho-binding domain of Rhotekin, C21, was expressed as GST-fusion protein in *E. 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 TcdB, or TcsL, or C3 as indicated were lysed in lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 5 mM MgCl2, 1 % NP-40, 1 mM PMSF, 5 mM DTT, Complete –EDTA). The soluble fraction was obtained by centrifugation (10.000 x 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.

3.13 Glucosylation reaction

The glucosylation reaction was performed in the presence of radio labelled UDP-[^{14}C]-glucose. Recombinant GTP binding proteins (1 µM) were incubated with TcdB or TcsL (20 nM) in glucosylation buffer (50 mM TRIS pH 7.2, 150 mM NaCl, 100 mM KCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 100 µg/ml BSA, 10 µM UDP-[^{14}C]glucose, 10 µM UDP-glucose) at 37 °C for the indicated periods. The reaction was terminated by addition of Laemmli sample buffer. Proteins were separated by SDS-PAGE and the gels dried onto Whatman paper. Radio-sensitive phosphorimaging films were exposed to the dry gels over night. The films were analyzed by phosphorImaging (Cyclone, Packard).

4. Results

4.1 TcdB, TcdBF, and TcsL cause actin re-organisation in fibroblasts

TcdB, TcdBF, and TcsL are glucosyltransferases that modify distinct subsets of Rho and Ras proteins. Thereby, the toxins cause a re-organisation of the actin cytoskeleton, resulting in rounding of cells ("cytopathic effect"). NIH3T3 fibroblasts were exposed to increasing concentrations of the toxins. After 4 h, the cells were analyzed by phase contrast microscopy (Fig. 1B) and the cytopathic effect was quantified in terms of rounded per total cells. TcdB, TcdBF, and TcsL exhibited sigmoid toxin concentration – cytopathic effect curves (Fig. 1A). TcdB was one order of magnitude more potent than TcdBF and three orders of magnitude more potent than TcsL (Fig. 1A).

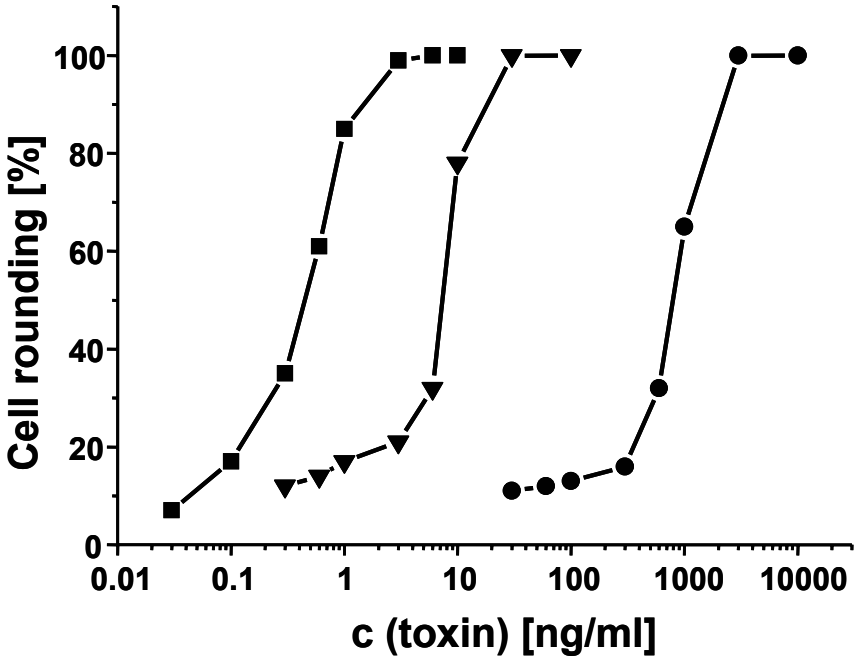

Fig. 1A: Cytopathic effect of TcdB, TcdBF, and TcsL

Fibroblasts were exposed to the indicated concentrations of TcdB (■), TcdBF (▼), or TcsL (●) for 4 h. The cells were analyzed by phase contrast microscopy and cell rounding determined by counting. Results displayed are the mean of three independent experiments.

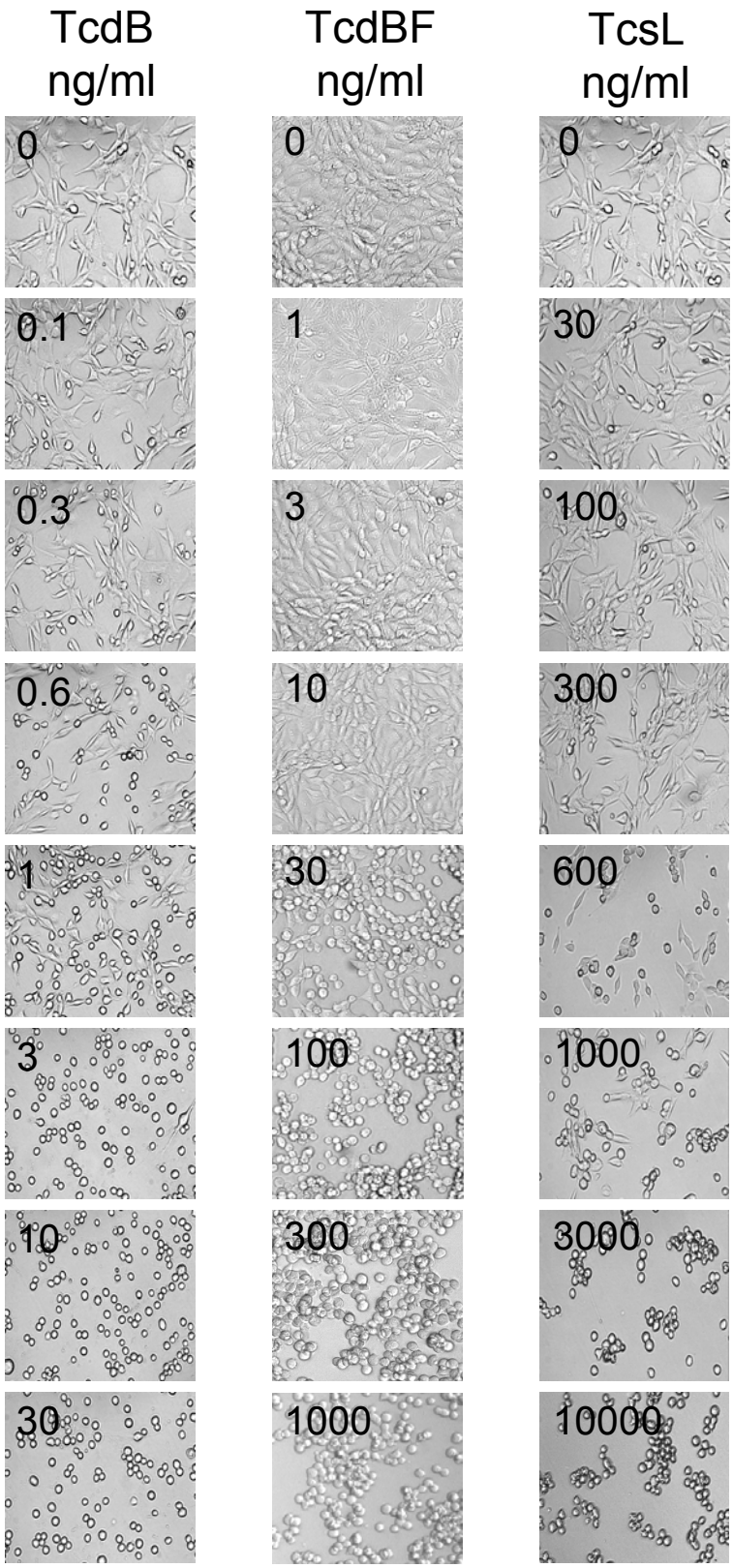

Fig. 1B: TcdB, TcdBF, and TcsL induce cell rounding in a concentration dependent manner

Fibroblasts were exposed to increasing concentration of the toxins as indicated for 4 h. Cells were then analyzed by phase contrast microscopy.

4.2 Up-regulation of RhoB by TcdB

Exposure of various cell lines to TcdA causes an increase of the RhoB level (Gerhard et al., 2005). This increase may be based on the inactivation of Rho GTP binding proteins. Both TcdA and TcdB glucosylate Rho, Rac, and Cdc42; therefore, TcdB likely causes an increase of RhoB, as well. To challenge this notion, fibroblasts were exposed to increasing concentrations of TcdB. The RhoB protein level increased in a concentration dependent manner up to 15-fold compared to untreated cells (Fig. 2A). To quantify the RhoB signals, beta actin was chosen as internal standard, as its level was not altered by TcdB-treatment (Fig. 2A).

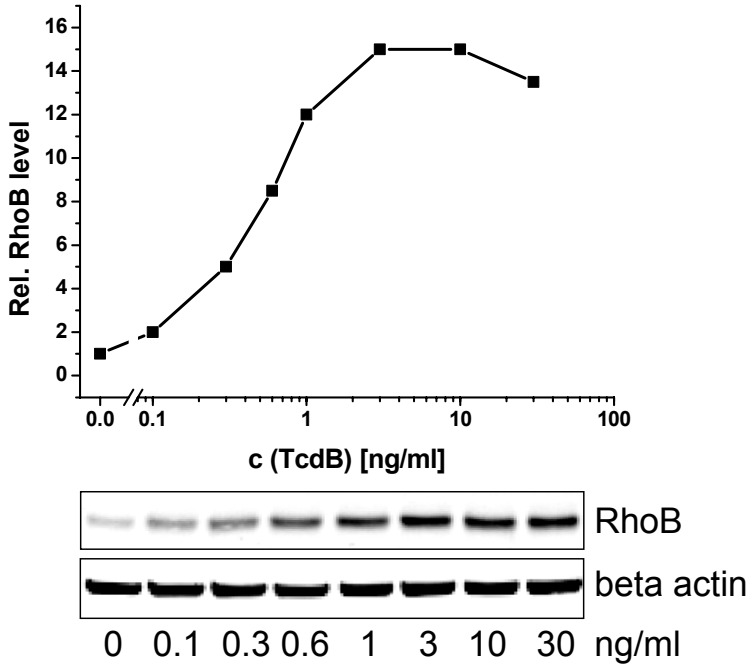

Fig. 2A: TcdB concentration dependent increase of the RhoB level

Fibroblasts were exposed to the indicated concentrations of TcdB for 4 h. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

Next, the increase of the RhoB level was assessed in a time-course experiment. After exposure to TcdB, the RhoB level increased in a time dependent manner (Fig. 2B). After 5 h, the level of RhoB did not increase further. The steady state level persisted for at least 20 h (Fig. 2B).

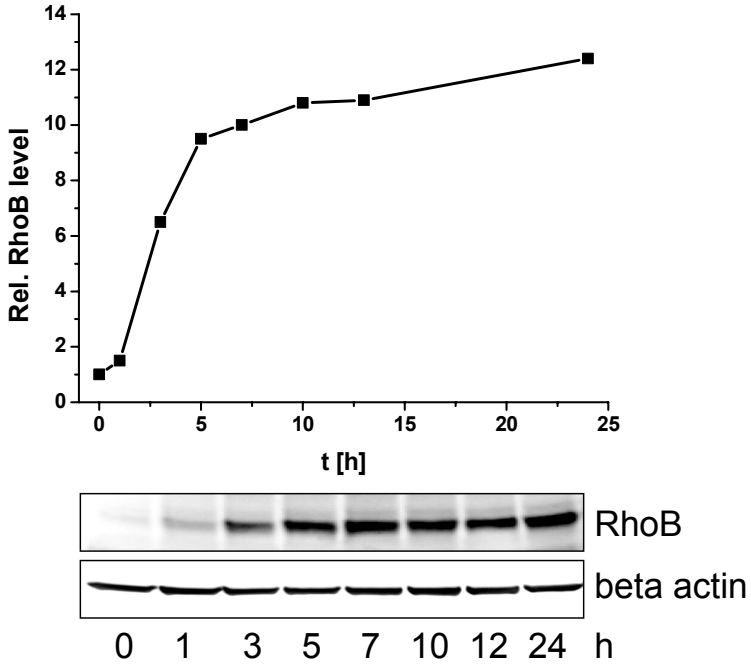

Fig. 2B: TcdB exposure time dependent increase of the RhoB level

Fibroblasts were exposed to TcdB (1 ng/ml) for the indicated periods. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

To test, if the observed increase of the RhoB level required protein *de novo* synthesis, fibroblasts were treated with cycloheximide (CHX) prior to toxin exposure. CHX pre-treatment completely inhibited up-regulation of RhoB indicating that the increase was due to protein *de novo* synthesis (Fig. 2C).

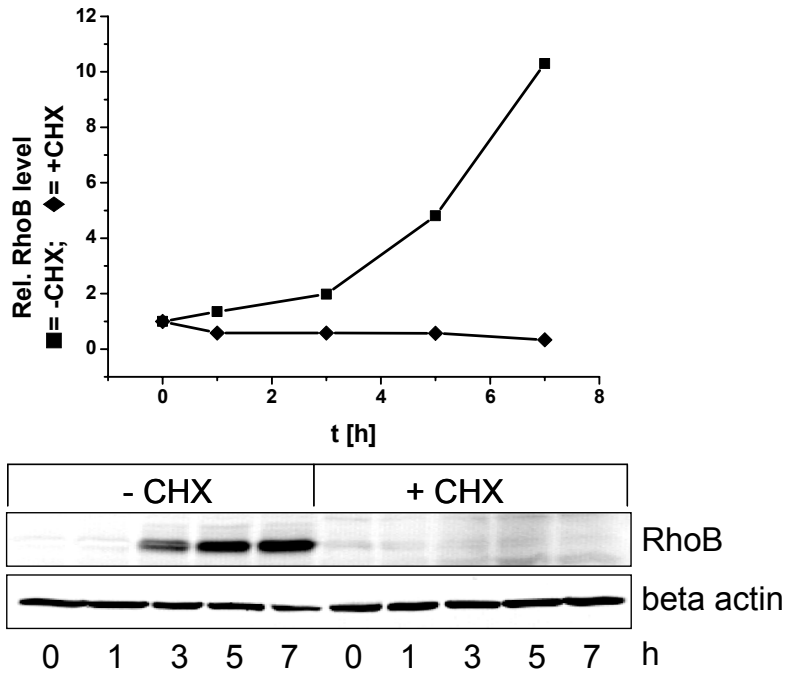

Fig. 2C: TcdB dependent increase of the RhoB level requires protein *de novo* synthesis
Fibroblasts were treated with cycloheximide (1 mM) (♦) or left untreated (■) for 1 h. TcdB (1 ng/ml) was then added and incubation continued for the indicated periods. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

To check, whether the up-regulation of RhoB protein was accompanied by an increase of *rhoB* mRNA, semi-quantitative real-time RT-PCR was applied. As internal standard, *beta actin* mRNA was used. RhoB mRNA levels increased in a concentration dependent manner up to 30-fold (Fig. 2D). The increase was blocked by actinomycin D. Pre-incubation of fibroblasts with actinomycin D accordingly inhibited the TcdB-induced RhoB up-regulation (Fig. 2E).

To summarize, RhoB protein was up-regulated by treatment of fibroblasts with TcdB. The up-regulation was due to transcriptional activation and required therefore mRNA and protein *de novo* synthesis.

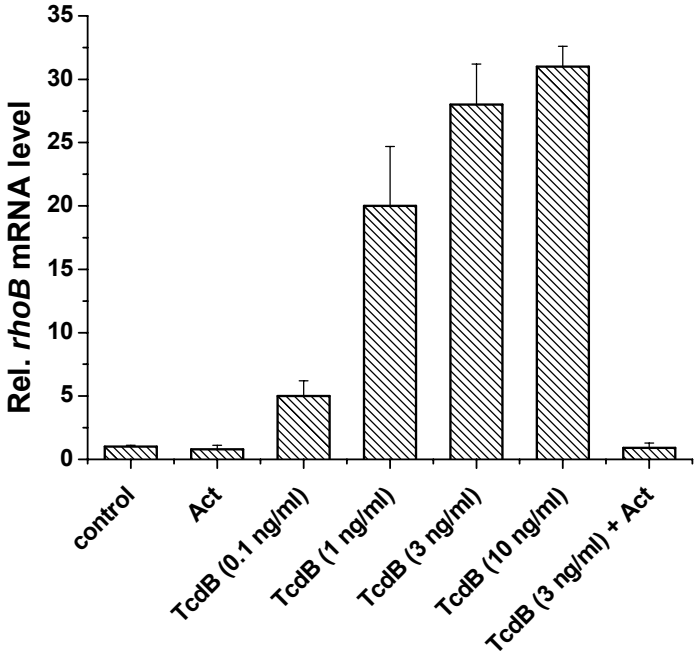

Fig. 2D: TcdB concentration dependent increase of the *rhoB* mRNA level

Fibroblasts were treated with actinomycin D (5 µM) or left untreated for 1 h. The indicated concentrations of TcdB were then added and incubation continued for 4 h. Total mRNA was prepared from treated cells and submitted to real-time RT-PCR. *rhoB* signals were normalized to *beta actin* signals. The control level was set 1.0. After every run, a melting curve was recorded to ensure the specificity of the reaction. Results displayed are the mean + S.D. of three independent experiments.

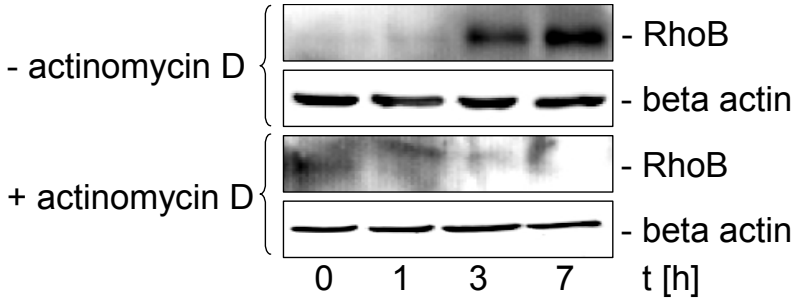

Fig. 2E: Inhibition of TcdB-induced RhoB up-regulation by actinomycin D

Fibroblasts were treated with actinomycin D (5 µM) or left untreated for 1 h. TcdB (1 ng/ml) was then added and incubation continued for the indicated periods. Western Blot analysis of the lysates was performed with the indicated antibodies.

4.3 Up-regulation of RhoB by C3 and TcdBF

To pinpoint Rho proteins responsible for RhoB up-regulation, the more specific cell permeable C3, inactivating exclusively Rho A/B/C, and the variant toxin B TcdBF, inactivating Rac1 and R-Ras, were applied. Fibroblasts were exposed to increasing concentrations of either C3 or TcdBF (equipotent regarding CPE). The RhoB level increased in a concentration dependent manner in both cases (Fig. 3A). C3 was at least as efficient as TcdB regarding RhoB up-regulation (> 13-fold). In contrast, TcdBF failed to induce strong RhoB up-regulation (< 5-fold).

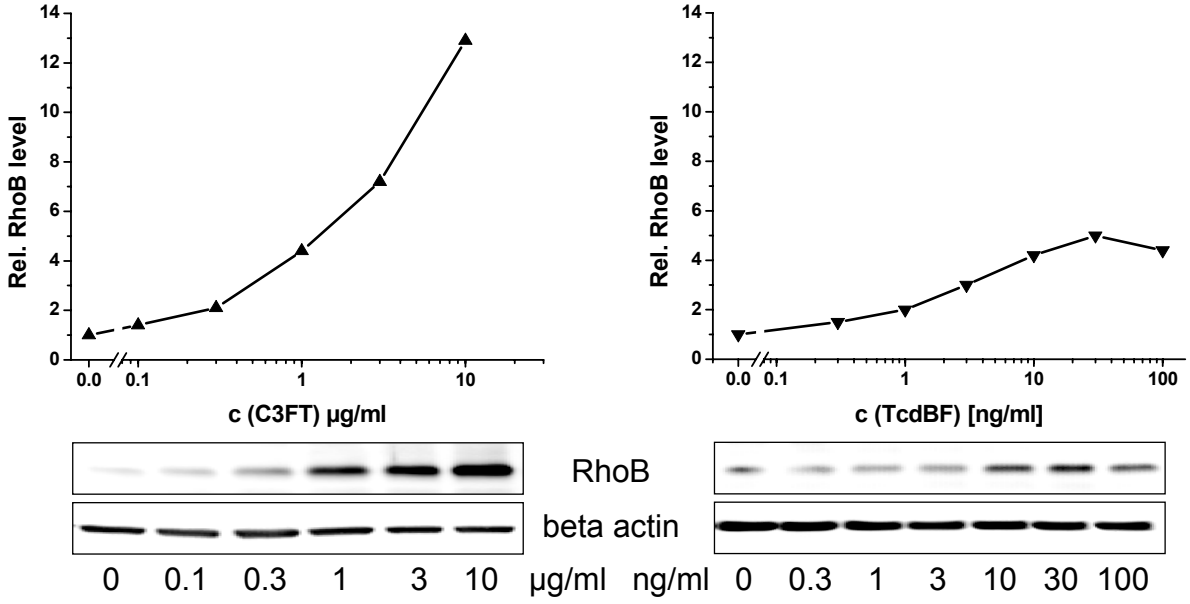

Fig. 3A: Up-regulation of RhoB by C3 is more efficient than by TcdBF

Fibroblasts were exposed to the indicated concentrations of C3 (1 µg/ml = 0,66 µg/ml C2I + 0,33 µg/ml C3FT) or TcdBF for 4 h. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

The lower efficiency of TcdBF was then confirmed in a time-course experiment. In fibroblasts exposed to TcdBF, the RhoB level slightly increased to about three fold (Fig. 3B). This elevated level persisted for at least 17 h. In contrast, C3 caused an up-regulation of the RhoB level by 10-fold, which lasted for at least 14 h (Fig. 3B).

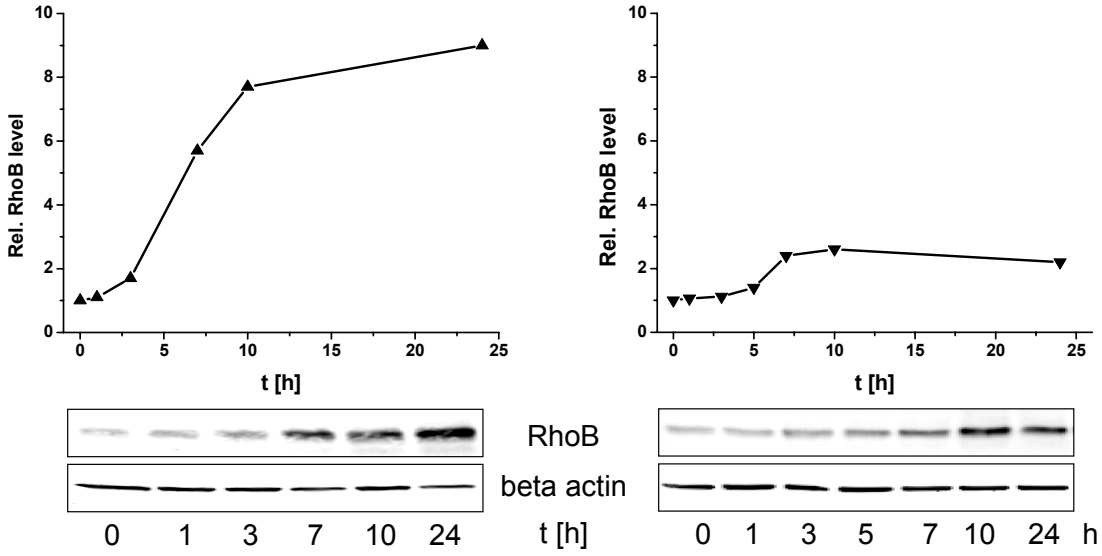

Fig. 3B: Time dependent increase of the RhoB level after C3- or TcdBF-treatment

Fibroblasts were exposed to C3 (1 µg/ml) or TcdBF (10 ng/ml) for the indicated periods. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

The requirement of protein *de novo* synthesis for C3-induced RhoB up-regulation was confirmed by pre-treating fibroblasts with cycloheximide. The C3 dependent RhoB up-regulation was completely inhibited (Fig. 3C).

TcdB and C3 were both strong inducers of RhoB up-regulation, while TcdBF proved to be a poor inducer. RhoB up-regulation was mainly governed by inactivation of RhoA/B/C. A direct comparison of TcdB- and TcdBF-induced RhoB up-regulation emphasizes this notion (Fig. 3D).

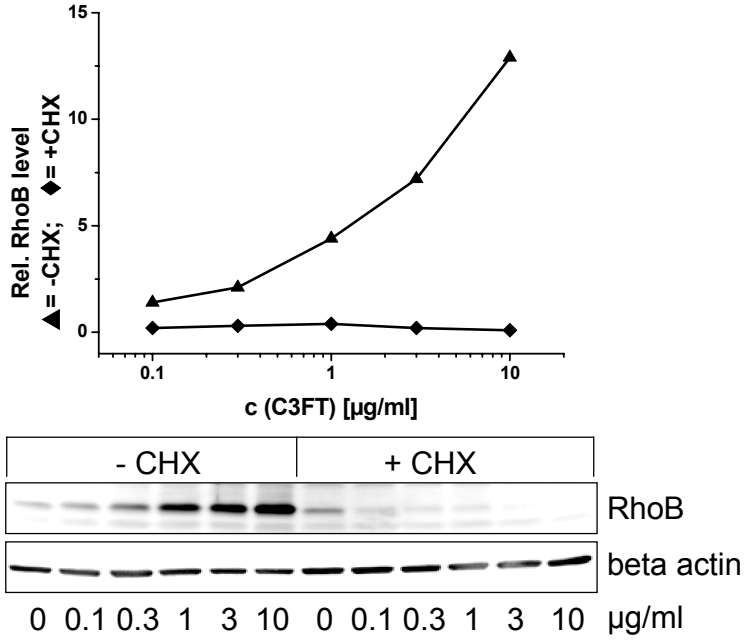

Fig. 3C: C3 dependent increase of the RhoB level requires protein *de novo* synthesis

Fibroblasts were treated with cycloheximide (1 mM) (♦) or left untreated (▲) for 1 h. The indicated concentrations of C3 were then added and incubation continued for 4 h. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

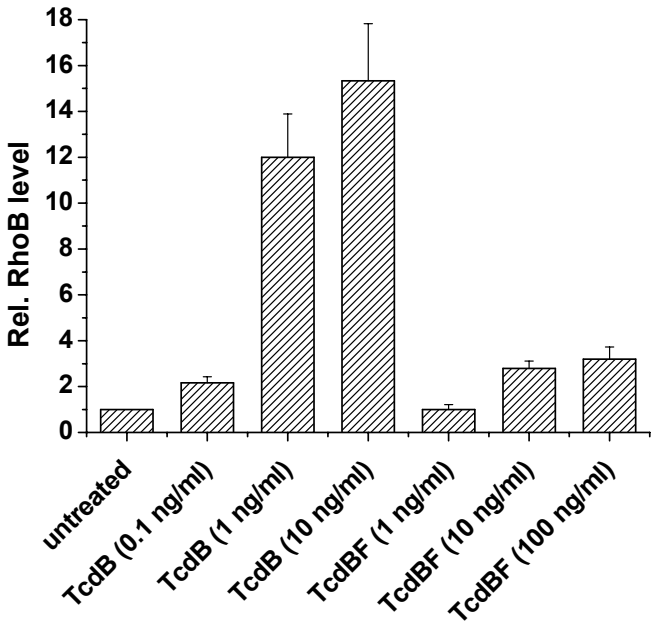

Fig. 3D: TcdB is a more powerful inducer of RhoB protein than TcdBF

Fibroblasts were exposed to the indicated toxin concentrations for 4 h. Western Blot analysis of the lysates were performed with RhoB and beta actin antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0. Results displayed are the mean + S.D. of three independent experiments.

4.4 Up-regulation of RhoB by TcsL

H/K/N-Ras suppresses *rhoB* promoter activity (Jiang et al., 2004a). Inactivation of H/K/N-Ras may therefore result in “de-suppression” and activation of the *rhoB* promoter. To check this notion, *Clostridium sordellii* lethal toxin (TcsL) which glucosylates Rac1, R-Ras, and H/K/N-Ras, was applied. Fibroblasts were exposed to increasing concentrations of TcsL. The RhoB level increased in a concentration dependent manner to the 12-fold level compared to untreated cells (Fig. 4A). The observed increase was more pronounced than in TcdBF-treated fibroblasts (Fig. 4A).

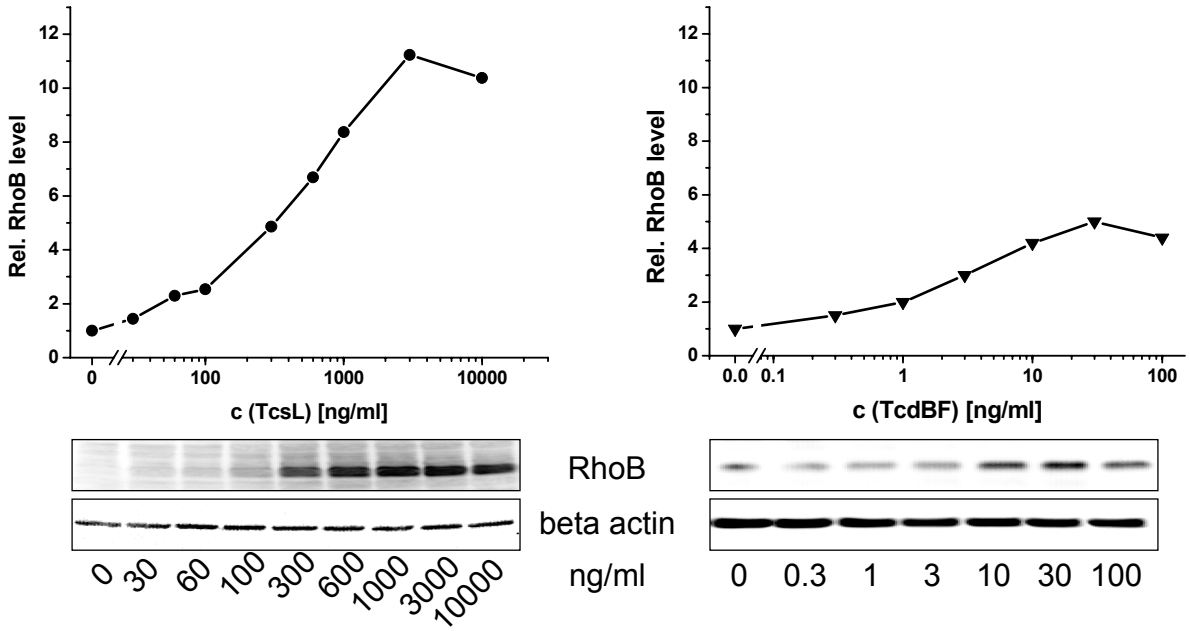

Fig. 4A: TcsL and TcdBF concentration dependent increase of the RhoB level

Fibroblasts were exposed to the indicated concentrations of TcsL or TcdBF for 4 h. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

The increase of the RhoB level was also observed in time-course experiments. The RhoB level in TcsL-treated fibroblasts increased in a time dependent manner for about 12 h (Fig. 4B). The steady state level of RhoB persisted for another 12 h.

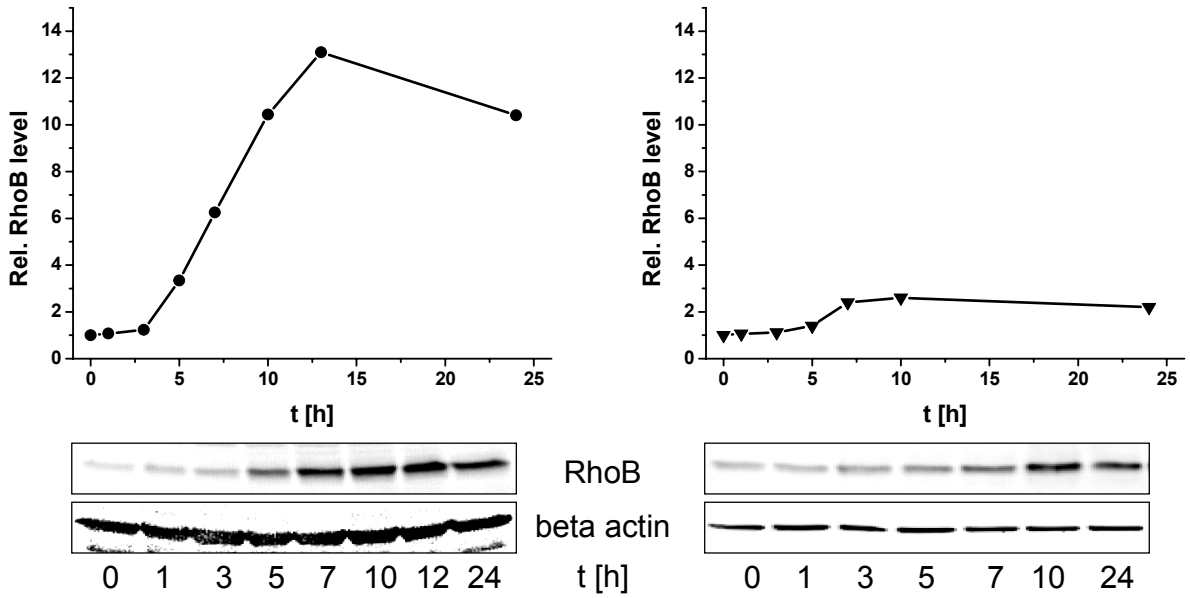

Fig. 4B: Up-regulation of RhoB in a time dependent manner by TcsL- or TcdBF-treatment
Fibroblasts were exposed to TcsL (1 µg/ml) or TcdBF (10 ng/ml) for the indicated periods. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

TcsL-induced RhoB up-regulation required protein *de novo* synthesis, as pre-treatment of fibroblasts with cycloheximide abolished it (Fig. 4C). Furthermore, *rhoB* mRNA levels after TcsL-treatment were determined by real-time RT-PCR. The *rhoB* mRNA level increased in a concentration dependent manner. Pre-treatment with actinomycin D inhibited the increase of *rhoB* mRNA as well as RhoB protein, indicating that RhoB was transcriptionally regulated (Fig. 4D+E). The minor RhoB up-regulation by TcdBF may also be based on the inactivation of H/K/N-Ras. At high concentrations, TcdBF glucosylated N-Ras (exemplarily) in a recombinant system, however to a lower extent compared to TcsL (Fig. 4F).

Application of TcsL thus proved the existence of a second, Ras-dependent pathway to induce RhoB up-regulation by transcriptional activation.

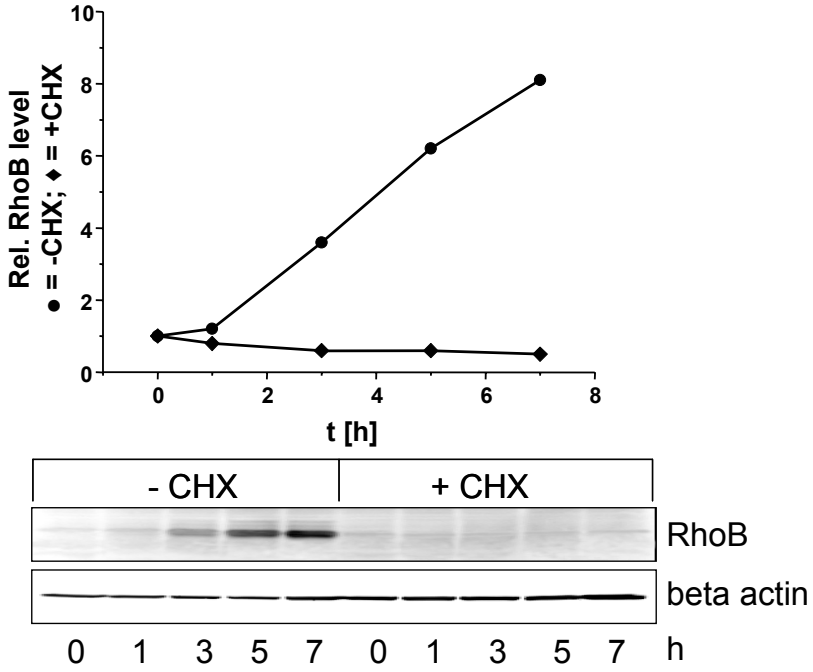

Fig. 4C: TcsL dependent increase of the RhoB level requires protein *de novo* synthesis
Fibroblasts were treated with cycloheximide (1 mM) (♦) or left untreated (●) for 1 h. TcsL (1 µg/ml) was then added and incubation continued for the indicated periods. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. The control level was set 1.0.

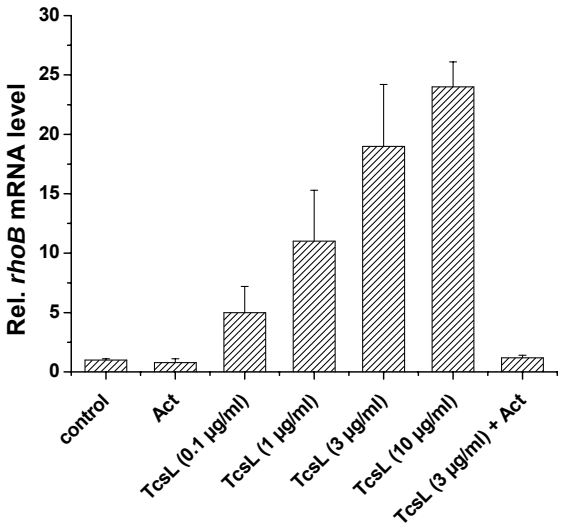

Fig. 4D: TcsL concentration dependent increase of the RhoB mRNA level
Fibroblasts were treated with actinomycin D (5 µM) or left untreated for 1 h. The indicated concentrations of TcdB were then added and incubation continued for 4 h. Subsequently, total mRNA was prepared from treated cells and submitted to real-time RT-PCR. *rhoB* signals were normalized to *beta actin* signals. The control level was set 1.0. After every run, a melting curve was recorded to ensure the specificity of the reaction. Results displayed are the mean + S.D. of three independent experiments.

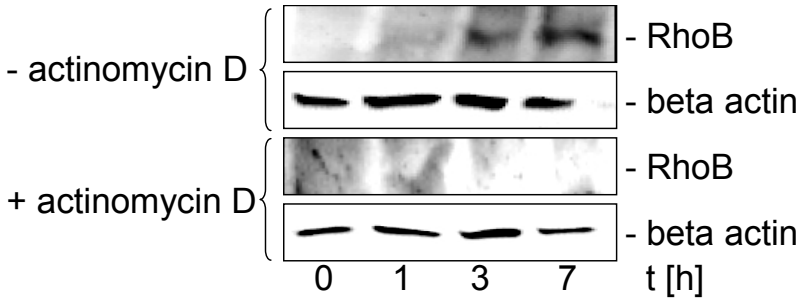

Fig. 4E: Inhibition of TcdB-induced RhoB up-regulation by actinomycin D

Fibroblasts were treated with actinomycin D (5 µM) or left untreated for 1 h. TcsL (1 µg/ml) was then added and incubation continued for the indicated periods. Western Blot analysis of the lysates was performed with the indicated antibodies.

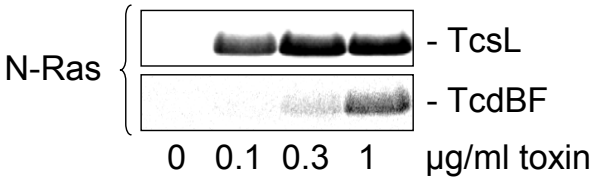

Fig. 4F: TcdBF glucosylates N-Ras at high concentrations of the toxin

Recombinant N-Ras (1 µM) was incubated with TcdBF or TcsL as indicated in the presence of UDP-[^{14}C]-glucose at 37 °C for 30 minutes. Protein glucosylation was detected by phosphorImager analysis.

4.5 p38 MAPK is an enhancer of RhoB up-regulation

In TcdA-treated cells, RhoB up-regulation is p38 MAP kinase-dependent (Gerhard et al., 2005). Therefore, a regulating role of the p38 MAP kinase was checked in TcdB- and TcsL-treated fibroblasts. To this end, a $p38_{\alpha}$-/- mouse embryological fibroblasts (MEF) cell line was compared with wild type (WT) MEFs. WT and $p38_{\alpha}$-/- fibroblasts were exposed to increasing concentrations of TcdB or TcsL. The cytopathic effect of TcdB and TcsL on WT and $p38_{\alpha}$-/- MEFs was determined (Fig. 5A). Both WT and $p38_{\alpha}$-/- fibroblasts were equally sensitive. Furthermore, the cytopathic effects of TcdB and TcsL on ME fibroblasts were comparable to their respective CPEs on NIH3T3 fibroblasts.

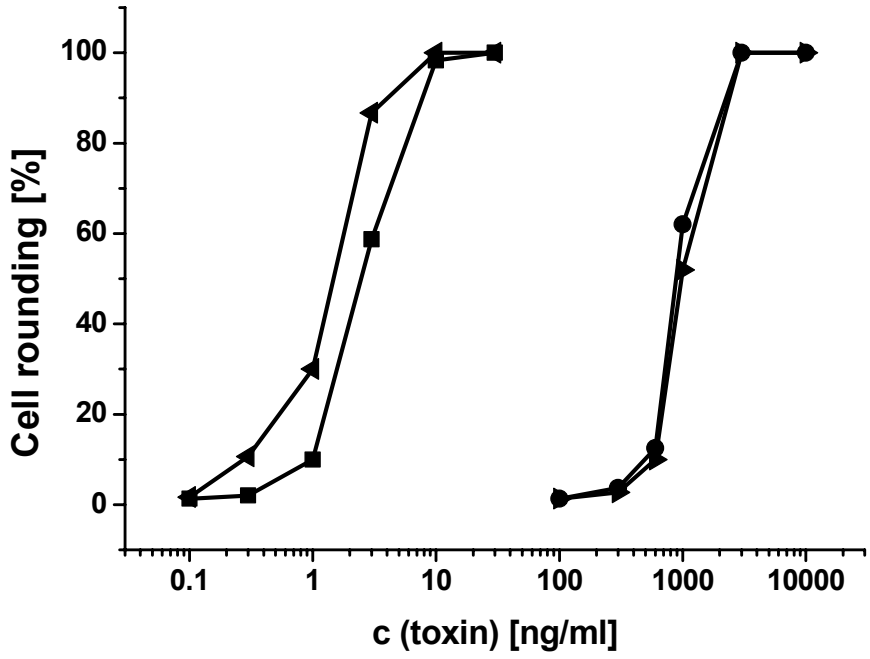

Fig. 5A: Cytopathic effect of TcdB and TcsL on mouse embryological fibroblasts

WT or $p38_{\alpha}$-/- fibroblasts were exposed to the indicated concentrations of either TcdB (WT = ■; $p38_{\alpha}$-/- = ◀), or TcsL (WT = ●; $p38_{\alpha}$-/- = ▶) for 4 h. The cells were analyzed by phase contrast microscopy and cell rounding determined by counting. Results displayed are the mean of three independent experiments.

Next, the RhoB level in WT and p38$_\alpha$-/- fibroblasts after treatment with TcdB was assessed. RhoB was up-regulated in WT as well as in p38$_\alpha$-/- MEFs. The maximal relative RhoB level, however, was lower in p38$_\alpha$-/- cells; thus, the efficacy of TcdB to up-regulate RhoB was reduced. The same results were found in TcsL-treated fibroblasts (Fig. 5B). These findings showed that the p38 MAP kinase pathway was not required for RhoB up-regulation. Instead of an essential regulator, the p38 MAP kinase pathway appeared to be a non-essential enhancer of RhoB up-regulation, whose absence resulted in a reduced RhoB up-regulation.

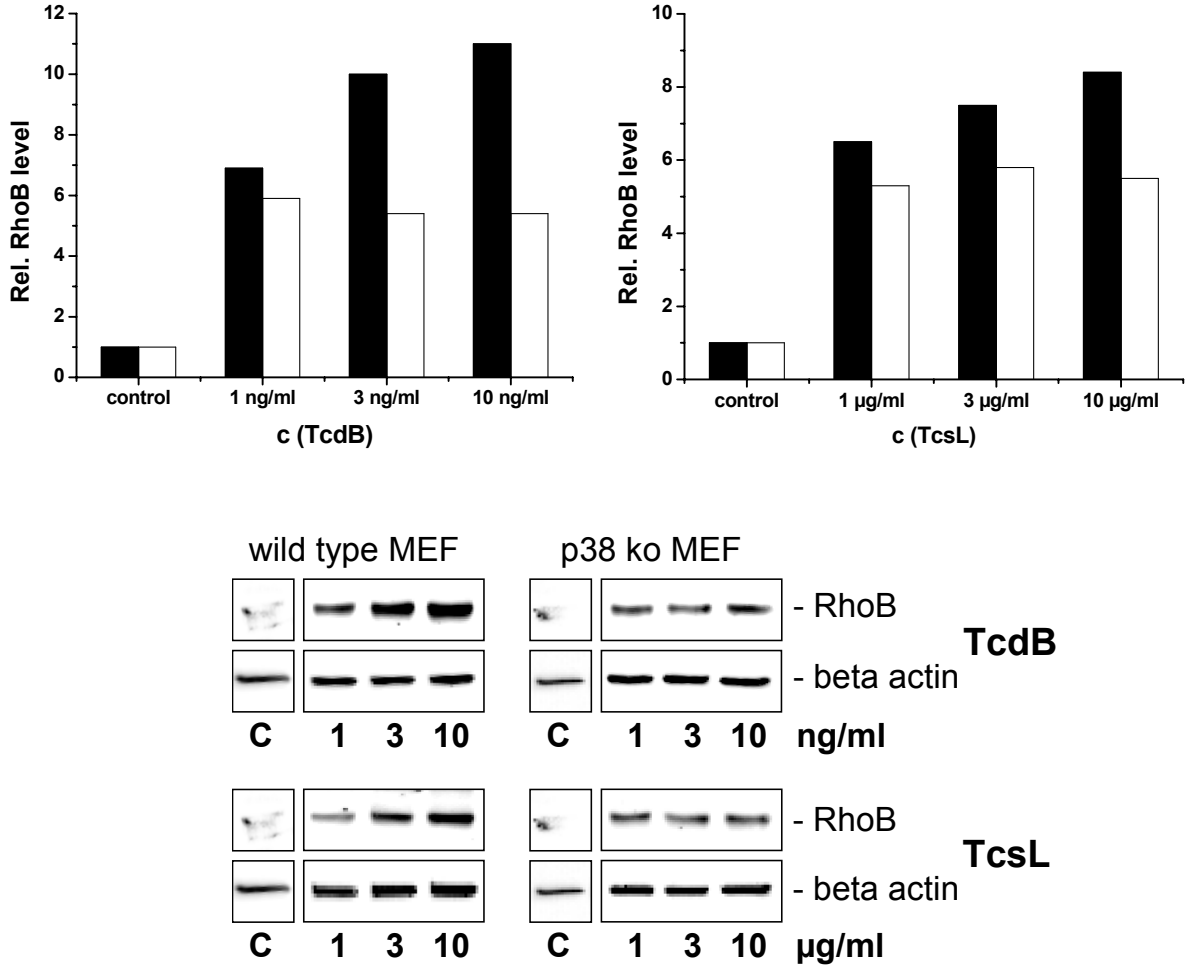

Fig. 5B: Reduced efficacy of TcdB and TcsL to up-regulate RhoB in p38$_\alpha$ MAPK -/- fibroblasts
WT (filled bars) or p38$_\alpha$ MAP kinase -/- (blank bars) fibroblasts were exposed to the indicated concentrations of TcdB or TcsL for 4 h. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals.

4.6 RhoB is degraded by caspases and the proteasome

The RhoB protein level is not only governed by transcriptional activation, but also by degradation. RhoB exhibits a short half life period of 2 h (Lebowitz et al., 1995). The more stable homologue RhoA is degraded in a proteasome-dependent manner (Lanning et al., 2004). To determine the degradation of up-regulated RhoB, fibroblasts were treated with TcsL for 4 h (Fig. 6A). Cycloheximide was then applied and the RhoB level monitored during the next 4 h. After addition of CHX, up-regulated RhoB was rapidly degraded. The half life period was estimated to ~ 1 h. To check, if RhoB was proteasomally degraded, fibroblasts were treated with the proteasome inhibitor MG132 prior to toxin exposure (Fig. 6A). After addition of CHX, the RhoB protein level still dropped but slower, indicating that RhoB was indeed proteasomally degraded.

Caspases are activated by treatment of cells with TcsL (Petit et al., 2003). To find out, if RhoB was degraded by caspases, fibroblasts were treated with a pan-caspase inhibitor (Zhu et al., 1995) prior to toxin treatment. Note that the inhibitor did not prevent RhoB up-regulation. The pan-caspase inhibitor stabilized RhoB to a similar extent as the proteasome inhibitor, indicating that RhoB was degraded in a caspase-dependent manner. Next, fibroblasts were treated with proteasome plus pan-caspase inhibitor prior to toxin exposure (Fig. 6A). After addition of CHX, the RhoB level remained constant, showing that the RhoB level was regulated by proteasome- and caspase-dependent degradation. Furthermore, this finding indicated that TcsL-treatment caused an activation of caspases in fibroblasts.

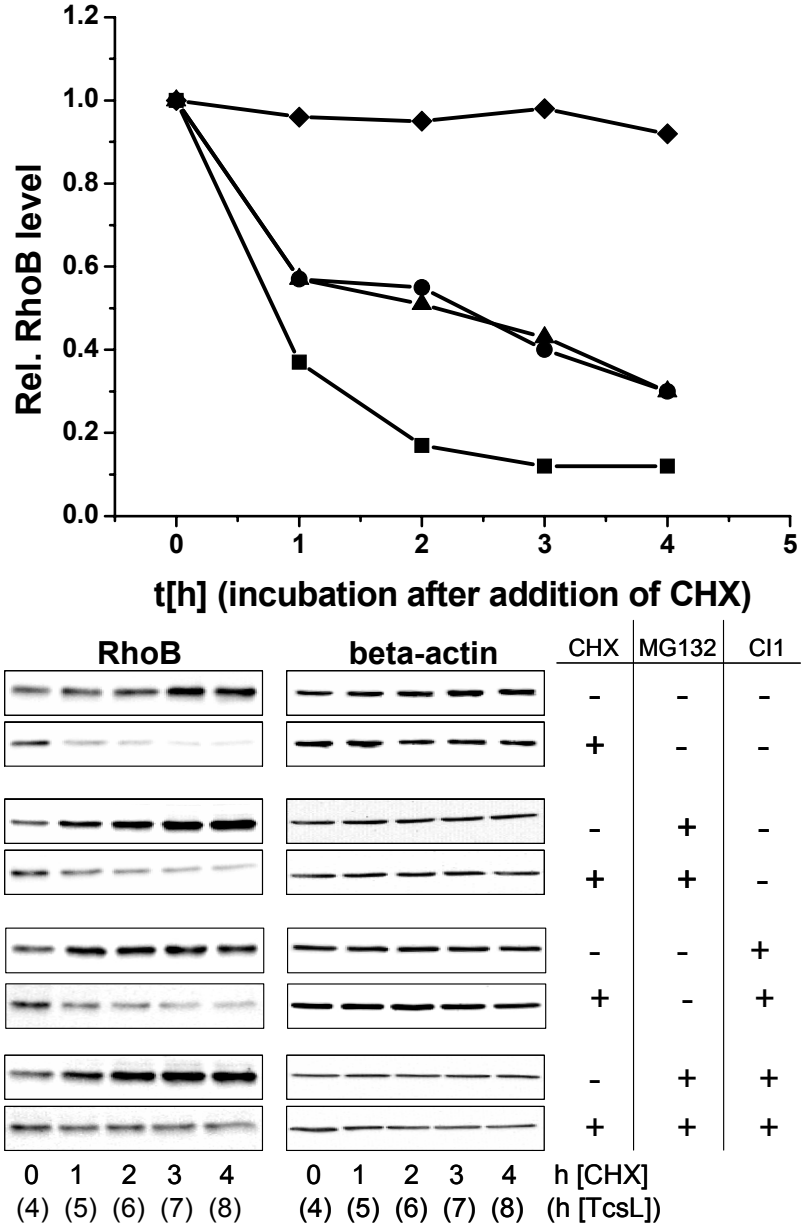

Fig. 6A: RhoB is degraded by caspases and the proteasome in fibroblasts exposed to TcsL
Fibroblasts were treated with either MG132 (20 µM), caspase inhibitor I (20 µM) or MG132 plus caspase inhibitor I (20 µM each) or left untreated for 1 h. TcsL (1 µg/ml) was then added and incubation continued for 4 h. Subsequently, cycloheximide (1 mM) was added or not and incubation continued for the periods indicated. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. RhoB signal intensity at t = 0 was set 1.0.

The same set of experiments was performed applying TcdB instead of TcsL. The results were alike; RhoB was partially stabilized by treatment with either the proteasome (Fig. 6B) or the pan-caspase inhibitor (data not shown). Treatment with proteasome plus pan-caspase inhibitor completely stabilized RhoB (Fig. 6B).

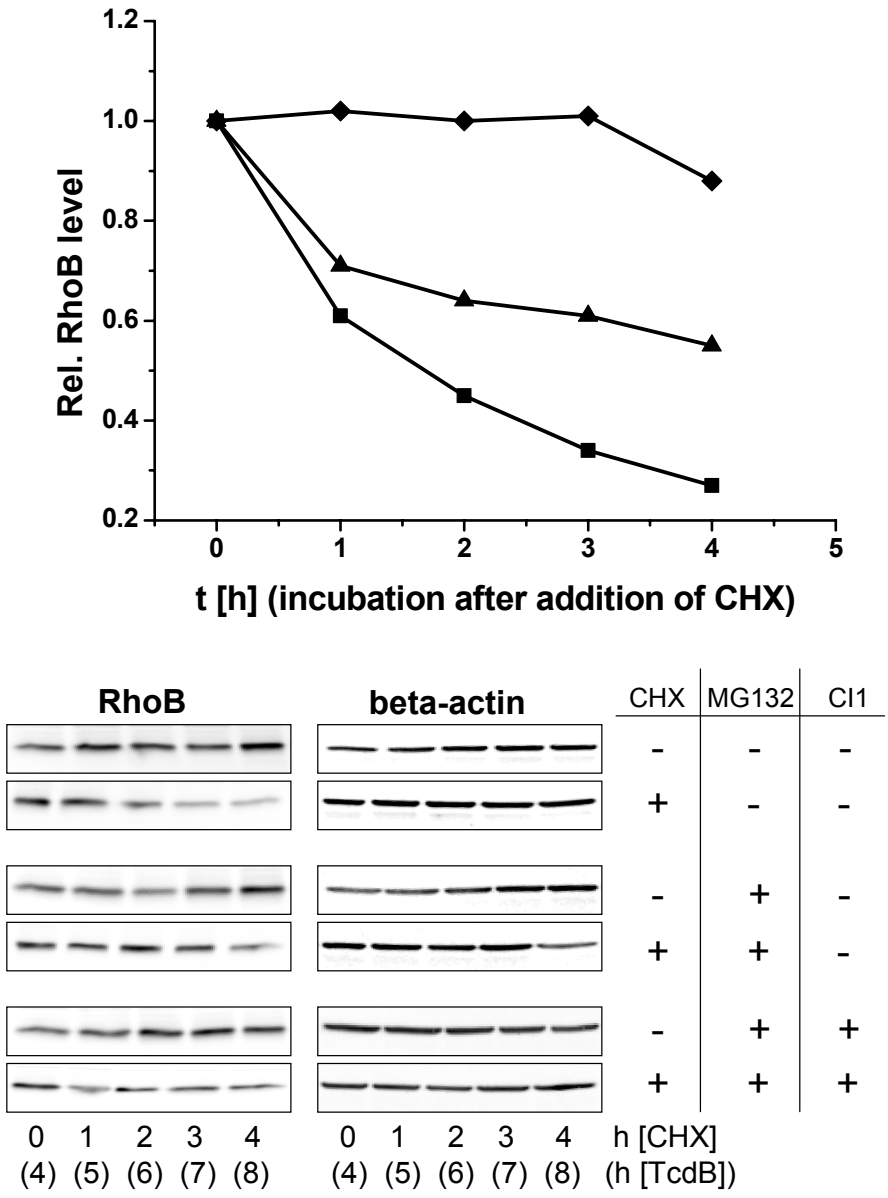

Fig. 6B: RhoB is degraded by caspases and the proteasome in fibroblasts exposed to TcdB Fibroblasts were treated with either MG132 (20 µM) or MG132 plus caspase inhibitor I (20 µM each) or left untreated for 1 h. TcdB (1 ng/ml) was then added and incubation continued for 4 h. Subsequently, cycloheximide (1 mM) was added or not and incubation continued for the periods indicated. Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals. RhoB signal intensity at t = 0 was set 1.0.

In conclusion, the RhoB level was regulated by proteasome- and caspase-dependent degradation in fibroblasts treated with either TcdB or TcsL. The observed caspase activity is in line with reports on TcdB- and TcsL-induced caspase-dependent apoptosis (Fiorentini et al., 1998;Hippenstiel et al., 2002;Petit et al., 2003).

4.7 Activation state of RhoB in toxin-treated fibroblasts

RhoB signaling does not only require an increase of the protein itself, but its active, GTP-bound form. Therefore, the Rhotekin pulldown was performed. The pulldown exploits that only GTP-bound RhoB binds to its effecter Rhotekin. To this end, fibroblasts were treated with increasing concentrations of either TcdB or TcsL to cause RhoB up-regulation. The lysates were used for the pulldown.

TcdB induced an up-regulation as well as an activation of RhoB compared to untreated cells (Fig. 7A). The activation was about 7-fold. Interestingly, the increase of RhoB activity (7-fold) was less than the increase of RhoB protein (15-fold; Fig. 2A) induced by TcdB.

TcsL also induced RhoB protein and activity (Fig. 7A). Regarding RhoB activity, however, it was a more potent inducer than TcdB, activating RhoB by > 20-fold.

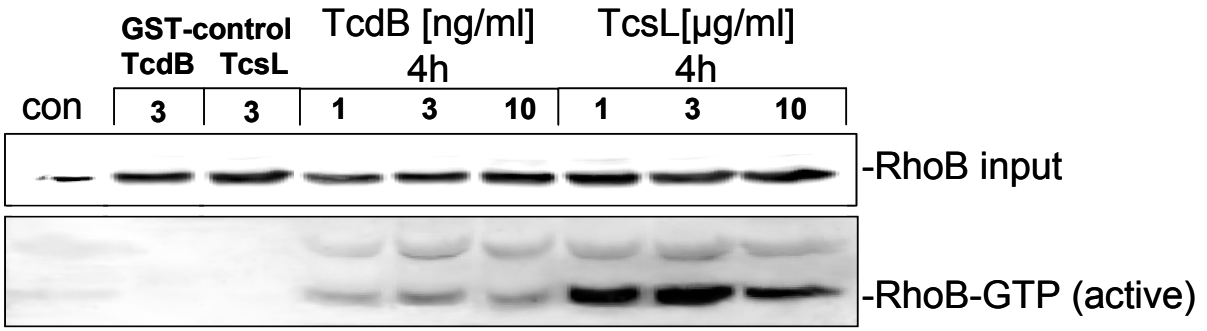

Fig. 7A: Increase of RhoB activity in Fibroblasts exposed to either TcdB or TcsL

Fibroblasts were exposed to the indicated concentrations of either TcdB or TcsL for 4 h. Cells were then lysed and the lysates used for the pulldown assay using GSH-sepharose bound GST-Rhotekin-C21 fusion protein. Total and precipitated RhoB was detected by Western Blot with the RhoB antibody. As negative reference, GST bound to GSH-sepharose was applied.

RhoB is a substrate of TcdA (Gerhard et al., 2005). Therefore, it was likely to be also glucosylated by TcdB. This notion would explain the lower activity of RhoB in TcdB-treated cells. To address this matter, recombinant RhoA, RhoB, and Rac1 were submitted to [^{14}C]-glucosylation by TcdB and TcsL (Fig. 7B). RhoA and RhoB were substrates of TcdB but not of TcsL. Glucosylation of Rac1 by either toxin proved comparable catalytic activity. This finding indicated that the lower activity of RhoB in cells exposed to TcdB was probably due to its partial modification.

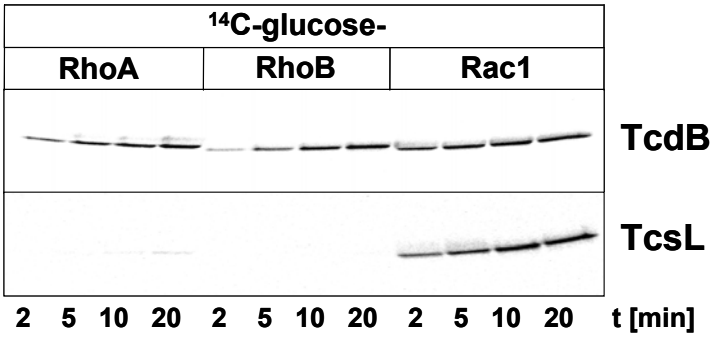

Fig. 7B: RhoB is a substrate of TcdB but not of TcsL

Recombinant RhoA, RhoB, or Rac1 (1 µM) was incubated with TcdB or TcsL (20 nM) in the presence of UDP-[^{14}C]-glucose at 37 °C for the indicated periods. Protein glucosylation was detected by phosphorImager analysis.

RhoB is also a substrate of C3. To check, whether RhoB was active in C3-treated fibroblasts, the Rhotekin pulldown was applied. Even though RhoB was up-regulated by C3-treatment of fibroblasts, no active RhoB could be precipitated (Fig. 7C, lower panel). In the Western blot from the lysates, a shift of the RhoB band to an apparent higher molecular weight indicated its ADP-ribosylation (Fig. 7C, upper panel). Thus, up-regulated RhoB was completely modified and inactive.

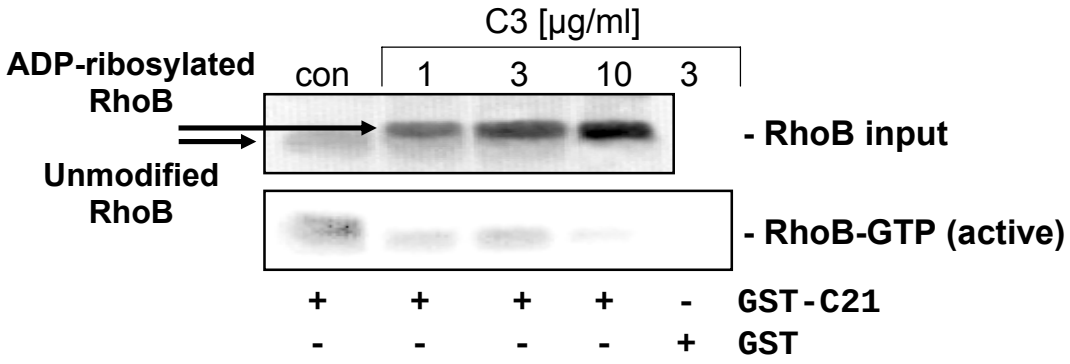

Fig. 7C: RhoB is inactive in fibroblasts exposed to C3

Fibroblasts were exposed to the indicated concentrations of C3 for 4 h. Cells were then lysed and the lysates submitted to the pulldown assay using GSH-sepharose bound GST-Rhotekin-C21 fusion protein. Total and precipitated RhoB was detected by Western blot with the RhoB antibody. As negative reference, GST bound to GSH-sepharose was applied. A shift to an apparent higher molecular weight of RhoB is indicated by the arrows.

4.8 Up-regulated RhoB does not influence the cytopathic effect

RhoB was up-regulated and its activity increased in response to treatment of cells with TcdB or TcsL. A distinct effect of TcdB and TcsL is the cytopathic effect. A comparison between RhoB up-regulation and actin re-organisation revealed similar kinetics of both effects (Fig. 8A), indicating that both were governed by the inactivation of Rho GTP binding proteins.

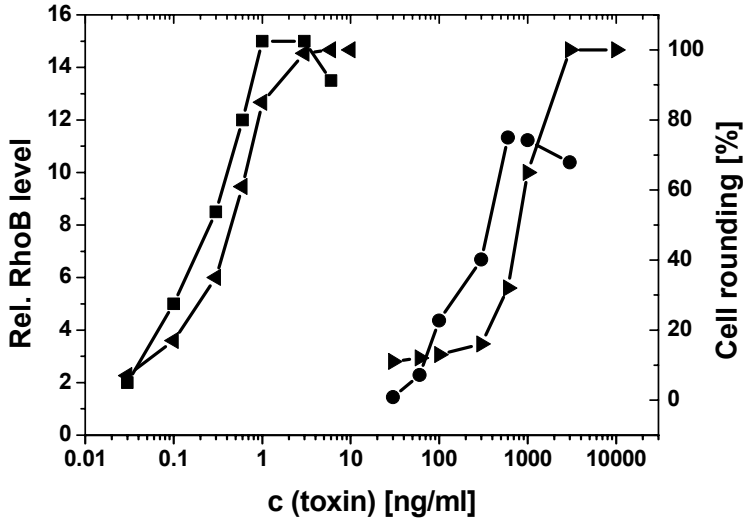

Fig. 8A: Combination of Fig. 1A, 2A, and 4A

Displayed are RhoB up-regulation by TcdB (■) and TcsL (●) as well as cell rounding induced by TcdB (◄) and TcsL (►).

Actin re-organisation, however, might also be a regulator of RhoB up-regulation. To challenge the notion that actin re-organisation was a trigger of RhoB up-regulation, the cell permeable C2 toxin was applied; it causes actin re-organisation by ADP-ribosylation of actin monomers. Fibroblasts were exposed to increasing concentrations of C2 and actin re-organisation and the RhoB level were analyzed (Fig. 8B). Furthermore, cells were incubated with agents affecting actin (latrunculin B, cytochalasin D) or microtubule (nocodazole) dynamics (Fig. 8C). Neither C2 nor any of these agents caused an up-regulation of RhoB, indicating that mere actin re-organisation was not sufficient.

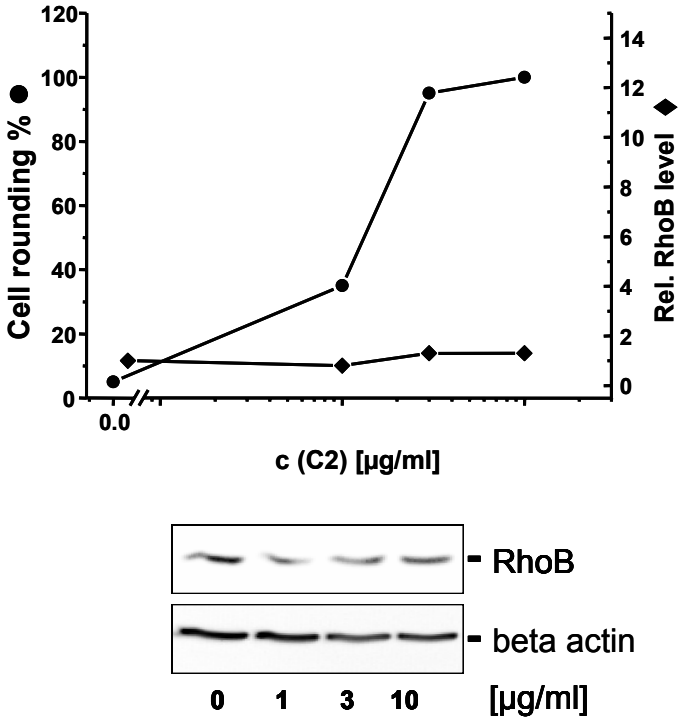

Fig. 8B: RhoB is not up-regulated after GTP binding protein independent actin re-organisation
Fibroblasts were exposed to the indicated concentrations of C2 toxin (1 µg = 0,66 µg C2I + 0,33 µg C2II) for 4 h. Cells were then analyzed by microscopy (●). Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals (♦) were normalized to beta actin signals.

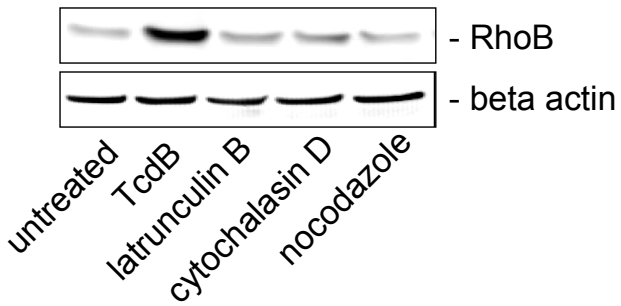

Fig. 8C: RhoB is not up-regulated in response to changes of actin or microtubule dynamics
Fibroblasts were exposed to TcdB (1 ng/ml), latrunculin B (2,5 mM), cytochalasin D (2 mM), or nocodazole (20 µM) for 4 h. Western Blot analysis of the lysates was performed with the indicated antibodies.

RhoB is a regulator of the actin cytoskeleton as shown before (Aspenström et al., 2004); therefore, it was likely to regulate actin re-organisation. To check, if active RhoB was a regulator of actin re-organisation, cell rounding was analyzed in fibroblasts treated with cycloheximide prior to exposure to TcdB. RhoB protein up-regulation was abolished (Fig. 8D+2C); cell rounding, however, occurred similar to cells exposed to TcdB only and was thus not influenced by cycloheximide (Fig. 8D).

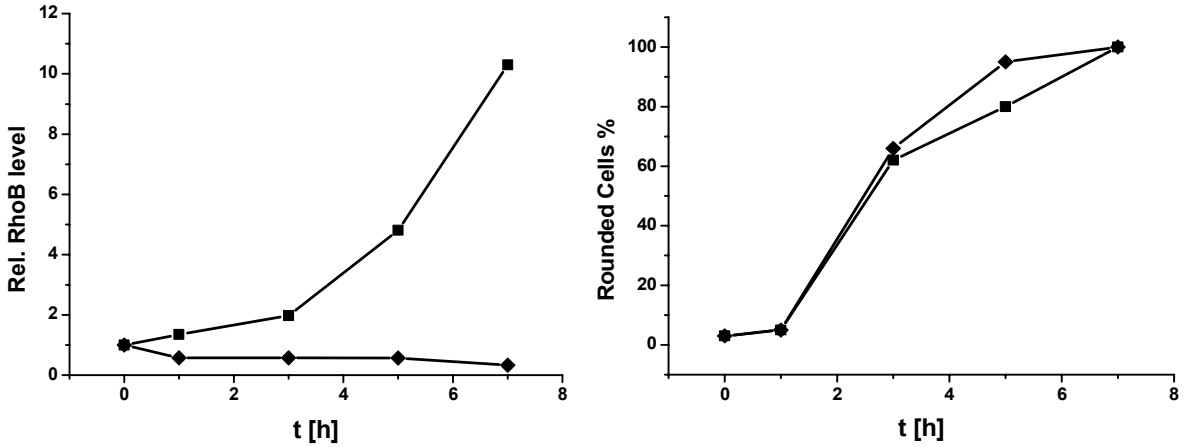

Fig. 8D: Inhibition of RhoB up-regulation by cycloheximide does not alter actin re-organisation
Fibroblasts were incubated with cycloheximide (1 mM) or left untreated for 1 h. Cells were then exposed to TcdB (1 ng/ml) for the indicated time periods. Cells were anaylzed microscopically and cell rounding determined by counting (no CHX = ■; CHX added = ♦). Western Blot analysis of the lysates was performed with the indicated antibodies. Signals were analyzed densitometrically. RhoB signals were normalized to beta actin signals.

These findings showed that RhoB up-regulation was not a consequence of actin re-organisation and cell rounding. Up-regulated RhoB appeared not to be involved in cell rounding. The similar kinetics of cell rounding and RhoB up-regulation indicated that both effects were regulated by the inactivation of Rho or Ras GTP binding proteins.

4.9 RhoB regulates the cytotoxic effect of TcdB

TcdB and TcsL have been reported to induce apoptosis in cultured cell lines (Fiorentini et al., 1998;Hippenstiel et al., 2002;Petit et al., 2003). This “cytotoxic effect” of TcdB and TcsL has been attributed to the inactivation of Rho- or Ras GTP binding proteins. A role of RhoB in the cytotoxic effect has not been evaluated, yet.

To assess the cytotoxic effect of TcdB, synchronized S-phase fibroblasts were exposed to increasing concentrations of the toxin for 12 h. TcdB-treatment induced complete rounding of the cells. The ratio of annexin V positive cells versus total cells increased in a concentration dependent manner to ~ 70 % (Fig. 9A). This increase was completely blocked by application of Z-VAD(OMe)-FMK, a pan-caspase inhibitor (Fig. 9A). These results indicated that TcdB was a potent inducer of caspase-dependent apoptosis in fibroblasts. This finding, however, did not provide evidence on a possible role of RhoB in the cytotoxic effect of TcdB.

TcdB and C3 both inactivate RhoA. In contrast, RhoB is active in TcdB- but not in C3-treated cells. To check, if active RhoB was required for the cytotoxic effect, synchronized S-phase fibroblasts were exposed to C3 and its cytotoxic effect determined by annexin V staining. C3 did not cause an increase in annexin V positive cells (Fig. 9B).

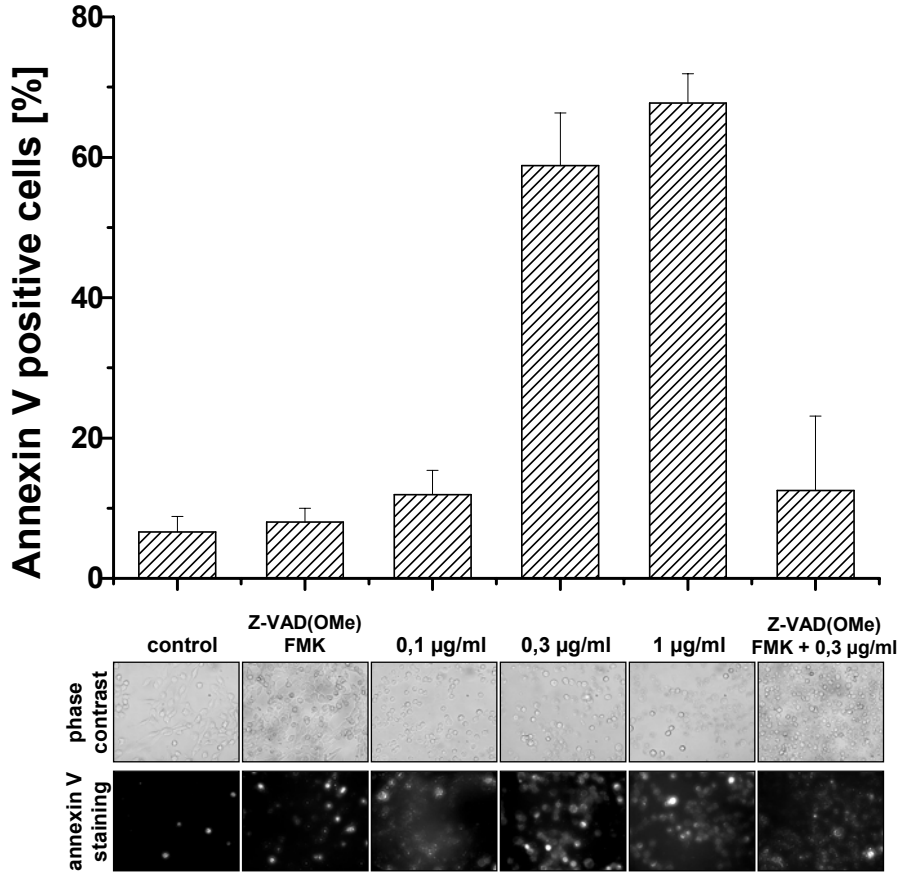

Fig. 9A: TcdB concentration dependent increase of annexin V positive fibroblasts

Synchronized S-phase fibroblasts were treated with Z-VAD(OMe)-FMK for 1 h or left untreated. They were then exposed to the concentrations of TcdB as indicated for 12 h. Then, Annexin V conjugated with Alexa Fluor 488 (1:50) was added directly to the medium. Cells were analyzed by phase contrast and fluorescence microscopy.

C3 was then used in a co-treatment experiment, applying both C3 and TcdB. Cells that were co-treated with both toxins did not exhibit an increase of the ratio of annexin V positive cells, indicating that C3 inhibited the cytotoxic effect of TcdB (Fig. 9C). This result was independent of the order of application of the toxins, indicating that it was not due to interference with the respective up-take mechanisms.

To summarize, TcdB induced caspase-dependent apoptosis in fibroblasts. This effect was inhibited when fibroblasts were co-treated to C3. C3 in turn was shown to completely inhibit RhoB activity. Its impact on the cytotoxic effect of TcdB is most likely based on the inactivation of RhoB.

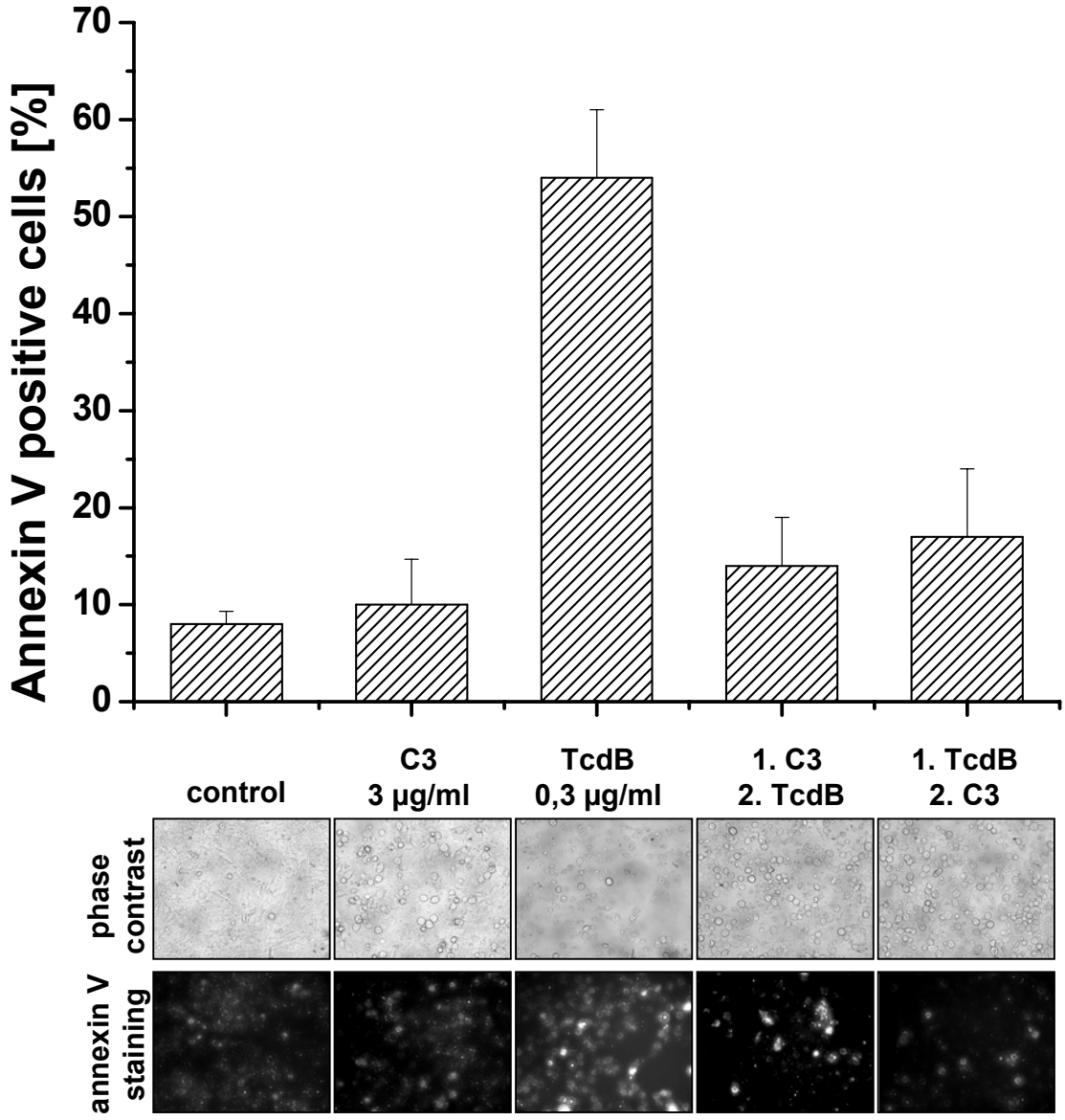

Fig. 9B: TcdB-induced apoptosis is inhibited by C3

Synchronized S-phase fibroblasts were treated with C3 (3 µg/ml) or TcdB (0,3 µg/ml) for 1 h. Cells exposed to TcdB were then exposed to C3 (3 µg/ml) and vice versa. Further samples were left with one toxin. Incubation was continued at 37°C and 5 % CO_2 for 11 h. Then, Annexin V conjugated with Alexa Fluor 488 (1:50) was added directly to the medium. Cells were analyzed by phase contrast and fluorescence microscopy.

5. Discussion

5.1 Up-regulation of RhoB

RhoB is a small GTP binding protein of the Rho family, exhibiting 86 % identity at the amino acid level with RhoA (Chardin et al., 1988). The immediate early gene product RhoB is short-lived and transcriptionally regulated, while RhoA is constitutively expressed (Jahner and Hunter, 1991). RhoB is up-regulated in response to distinct triggers: 1. cellular stress, e.g. by growth factors (Jahner and Hunter, 1991); 2. genotoxic stress, e.g. by DNA alkylating agents (Fritz et al., 1995); 3. inhibition of Rho/Ras signaling, e.g. by statins (Holstein et al., 2002), farnesyltransferase- and geranylgeranyl-transferase inhibitors (Delarue et al., 2007), *Clostridium difficile* toxin A (Gerhard et al., 2005). Up-regulation caused by TcdA has been attributed to actin re-organisation caused by the toxin, as latrunculin B has been suggested to induce up-regulation of RhoB.

rhoB belongs to the family of immediate early genes, some of which (e.g. connective tissue growth factor, CTGF) are up-regulated in response to changes in actin dynamics (Chowdhury and Chaqour, 2004;Ott et al., 2003). Our findings showed that RhoB up-regulation and actin re-organisation exhibited comparable kinetics in TcdB- and C3-treated cells. *rhoB*, however, was not responsive to changes of actin dynamics. Neither actin de-polymerisation by the C2 toxin (Fig. 7B), nor latrunculin B, cytochalasin D, nor disruption of microtubules by nocodazole (Fig. 7C) caused an up-regulation of RhoB. Thus, RhoB up-regulation is only temporally correlated but not a consequence of actin re-organisation.

RhoB up-regulation might, like actin re-organisation, be a consequence of the inactivation of Rho proteins. TcdA and TcdB both glucosylate the same Rho proteins, namely Rho, Rac, and Cdc42 (Table 1) (Boquet and Lemichez, 2003;Just et al., 1995b; Just and Gerhard, 2005). We found that RhoB was up-regulated in a time-

and concentration dependent manner by TcdB-treatment (Fig. 2A+B), indicating that RhoB up-regulation was a consequence of the inactivation of Rho proteins by TcdB (Fig. V). The up-regulation of RhoB was also observed at the mRNA level (Fig. 2D). Inhibition of *rhoB* transcription did not only abolish the increase of the mRNA but also the protein level (Fig. 2D/E). Furthermore, protein *de novo* synthesis was required for RhoB up-regulation (Fig. 2C). Taken together, these results indicate that RhoB was up-regulated due to transcriptional activation by treatment of fibroblasts with TcdB.

Gerhard et al. further suggested that the inactivation of Rac1 by TcdA triggers the up-regulation of RhoB. This suggestion was based on the notion that Rac1 is a part of the PI3 kinase/Akt signalosome (Fukuhara et al., 1999). Interrupting this pathway has been shown to increase RhoB expression (Jiang et al., 2004b). To challenge this notion, the variant toxin B, named TcdBF, which glucosylates Rac1 and R-Ras but not RhoA/B/C (Table 1) (Chaves-Olarte et al., 1999), was applied. The up-regulation of RhoB caused by TcdBF was marginal compared to TcdB (Fig. 3D). Previously, a report from Fritz and Kaina stated that Rac1 was indeed an activator of the *rhoB* promoter, as its activity increased in cells that ectopically expressed Rac1 (Fritz and Kaina, 1997). Thus, activation rather than inactivation of Rac1 causes RhoB up-regulation. In this line, the Rac activating cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* causes RhoB up-regulation (S. Dreger, unpublished observation).

We found that C3-treatment of cells caused pronounced RhoB up-regulation in a time- and concentration dependent manner (Fig. 3A+B). The up-regulation was as efficient as TcdB-induced RhoB up-regulation, indicating that inactivation of RhoA/B/C was sufficient. This view is supported by Fritz et al. who found that ectopic expression of GDI1, a negative regulator of the activity of RhoA, Rac1, and Cdc42, activates the *rhoB* promoter (Fritz and Kaina, 1997). Jiang et al. suggested that active RhoA suppressed the *rhoB* promoter activity (Jiang et al., 2004a). Thus, we

suggest that inactivation of RhoA lifts the suppression of the *rhoB* promoter, which results in up-regulation of RhoB.

TcsL that does not inactivate RhoA/B/C also induced up-regulation of RhoB in a time- and concentration-dependent manner (Fig. 4A+B). mRNA and protein *de novo* synthesis were required for the up-regulation (Fig. 4C/E); accordingly, an increase of *rhoB* mRNA was observed, as well (Fig. 4D). In comparison to TcdBF, TcsL was more efficient regarding RhoB up-regulation, equalling TcdB. This effect is most likely based on the inactivation of H/K/N-Ras by TcsL (Just et al., 1996:Popoff, 1996). In this line, ectopic expression of constitutively active Ras GTP binding proteins suppresses the *rhoB* promoter activity (Jiang et al., 2004b). Thus, RhoB is up-regulated by distinct pathways, dependent on the inactivation of either RhoA or H/K/N-Ras, but independent of changes in actin dynamics (Fig. V). The marginal RhoB up-regulation by TcdBF may be based on the finding that TcdBF glucosylates other Ras GTP binding proteins than R-Ras at high concentrations of the toxin.

Table 1: Substrate specificity and catalyzed reaction of the applied toxins

Toxin	catalyzed reaction	protein substrate				
TcdA/TcdB	glucosylation	RhoA/B/C	Rac1	Cdc42		
TcdBF	glucosylation		Rac1			R-Ras
TcsL	glucosylation		Rac1		H/K/N-Ras	R-Ras
C3	ADP-ribosylation	RhoA/B/C				

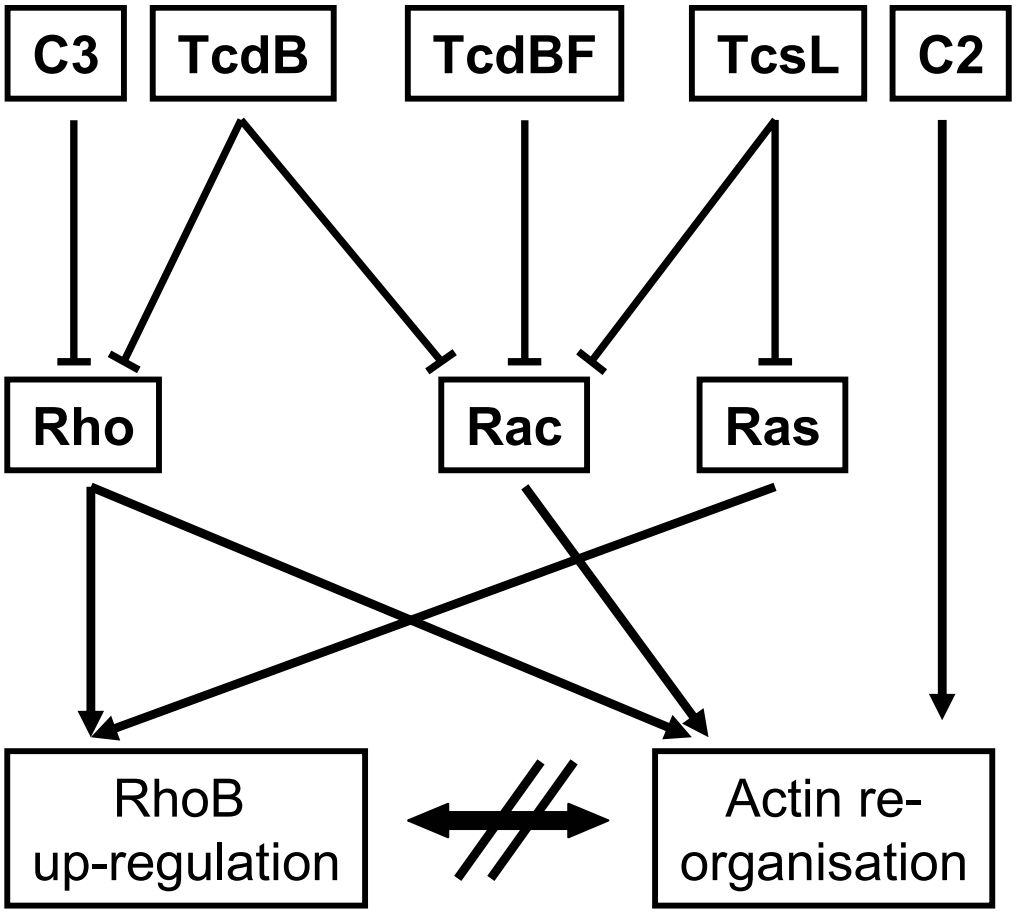

Fig. V: Model of RhoB up-regulation

RhoB is up-regulated after inactivation of either Rho or Ras GTP binding proteins. Both RhoB up-regulation and actin re-organisation are parallel downstream effects the inactivation of Rho proteins.

5.2 Regulation of RhoB by the p38 MAP kinase

The p38 MAP kinase is positively regulated by Rac1 and Cdc42 (Kyriakis and Avruch, 2001). One must therefore expect that p38 MAPK is inactivated in TcdB-treated cells. The opposite is true. p38 MAP kinase phosphorylation increased in TcdA-treated cells (Gerhard et al., 2005). This increase was probably based on changes in actin dynamics (Nemeth et al., 2004), as it correlated with the toxin-induced actin re-organization. We found that the RhoB level was reduced by ~30 % in TcdB- and TcsL-treated p38 -/- MEF compared to WT MEF. Thus, p38 MAP kinase is an enhancer of – but not essential for – RhoB up-regulation. Most probably, p38 MAP kinase increases either *rhoB* promoter activity or *rhoB* mRNA stability. The mechanism of this regulation is the topic of further investigation currently conducted in our lab.

5.3 Posttranslational regulation of RhoB

The level of RhoA and RhoB is suggested to be regulated by degradation by the 26S proteasome (Engel et al., 1998;Lanning et al., 2004). The RhoB protein half life period was re-analyzed in toxin-treated cells applying cycloheximide and $t_{1/2}$ was determined. It was 1-2 h and ~1 h in TcdB- and TcsL-treated cells, respectively (Fig. 5A/B). These findings are in line with former reports on the RhoB half life period of 2 h (Lebowitz et al., 1995).The half life period increased when cells were incubated with an inhibitor of the 26S proteasome prior to toxin treatment. Surprisingly, the inhibitor failed to completely block degradation of RhoB, indicating the existence of proteasome-independent degradation. TcdB and TcsL both cause an activation of caspases in cultured cell lines (Hippenstiel et al., 2002;Petit et al., 2003). Caspases are proteases (Black et al., 1989) whose activation mediates apoptotic processes (Jacobson and Evan, 1994). Application of a pan caspase inhibitor increased the

RhoB half-life period to a similar extent as the proteasome inhibitor (Fig. 5A). Co-application of both proteasome and caspase inhibitor completely blocked degradation of RhoB in TcsL- as well as in TcdB-treated cells (Fig. 5A/B). This is the first evidence that RhoB is a substrate of caspases.

5.4 Activation state of RhoB

RhoB up-regulation was associated with an increase of RhoB activity in TcdB- and TcsL treated cells (Fig. 6A). Even though both toxins caused comparable RhoB up-regulation, the increase of RhoB activity was more pronounced in TcsL-treated fibroblasts. This difference originated from the distinct substrate specificities of the toxins. TcdB was found to modify RhoB as described before (Wilde et al., 2003), while TcsL did not (Fig. 6B). The observed increase of RhoB activity in TcdB-treated cells, however, indicated that RhoB was only partially modified. Even though TcdB has been characterized as Rho inactivating, it caused an activation of RhoB. Molecular effects of TcdB so far attributed to the inactivation of Rho may thus be based on the activation of RhoB instead. In line with these findings, an increase of RhoB activity has been reported on TcdA-treated cells (Gerhard et al., 2005). In contrast, no active RhoB was found in C3-treated cells in spite of the profound RhoB up-regulation. This finding further supported former notions that C3 is an efficient inhibitor of RhoB (Just et al., 1992). The difference between TcdB and C3 regarding the activity state of RhoB may originate from their distinct intracellular localization. TcdB most likely localizes to the cytoplasma membrane, as it has been described for TcsL (Mesmin et al., 2004). In contrast, C3 imported as chimeric protein localizes to endosomes (C. Mühlenstädt, unpublished observation). RhoB is permanently localized to endosomes. Therefore, RhoB may be accessible to C3, while it escapes

its modification by TcdB. Furthermore, RhoB has recently been shown to be a poor substrate of TcdB compared to e.g. RhoA (Huelsenbeck et al., 2007b).

Thus, the cellular RhoB protein level was regulated at three distinct stages: 1. by transcriptional activation, allowing a rapid increase of the protein level; 2. by degradation, either proteasome- or caspase-dependent, allowing a rapid decrease of the protein level; 3. by nucleotide exchange, allowing regulation of the activity of the present protein. This very subtle regulation of RhoB control suggests that its correct function is crucial for cellular survival. A comparable set of regulation is found e.g. for cyclins that are regulated by transcriptional activation, degradation, localization, and phosphorylation (Diehl, 2002). Cyclins are essential for cell cycle progression. Even though RhoB is reportedly up-regulated during S-phase, no role for RhoB has been described in cell cycle progression. Instead, it appears to be important for regulating cellular sensitivity to cellular damage during replication and to induce apoptotic cell death, when necessary. Obviously, a protein that comprises the activity to regulate apoptosis needs to be closely regulated. An excess of its activity could contribute to unnecessary cell death, whereas a lack of activity may result in unchallenged growth of cells, as observed in the cancer field (Mazieres et al., 2004).

5.5 Involvement of RhoB in the cytotoxic effect of TcdB

Apoptotic stimuli are classified into two groups. Perturbations of homeostasis, e.g. by growth factor withdrawal or DNA damage, cause apoptosis on a large time scale within the range of days rather than hours. In contrast, direct stimulators of apoptotic pathways such as the Fas ligand or tumor necrosis factor induce apoptosis within minutes or hours (Ashkenazi and Dixit, 1998)(Evan and Littlewood, 1998). We observed the cytotoxic effect of TcdB as early as three hours after toxin-treatment in RBL cells (Huelsenbeck et al., 2007), suggesting that it was due to direct stimulation of an apoptotic pathway. We applied S-phase synchronized cells, as RhoB is physiologically up-regulated during S-phase (Zalcman et al., 1995), indicating its importance during this phase. TcdB exhibited a cytotoxic effect in a concentration dependent manner in synchronized fibroblasts (Fig. 9A), which was responsive to a pan caspase inhbitor (Z-VAD(OMe)-FMK), a finding in line with the reported caspase activation by TcdB (Hippenstiel et al., 2002;Qa'Dan et al., 2002). In contrast, C3 failed to cause apoptosis in synchronized fibroblasts (Fig. 9B). The cytotoxic effect of TcdB has been attributed to the inactivation of RhoA. RhoA, however, was inactive in C3-treated cells. In contrast, RhoB was active in TcdB- but inactive in C3-treated cells (Fig. 7A+C). Inactivation of RhoB by C3 abrogated the cytotoxic effect of TcdB (Fig. 9B), suggesting that active RhoB is required for the cytotoxic effect of TcdB. TcsL causes detachment of fibroblasts from the surface, blocking the microscopic approach and making the differentiation between apoptosis and anoikis impossible (Gilmore, 2005).

The cytotoxic effect of TcdB was characterized by an early onset of apoptosis and the requirement of active RhoB and caspases. Findings from RhoB knockout cells indicate that RhoB may play a role in the physiological arrest- and repair-mechanism after genotoxic stimuli such as UV-light or chemotherapeutic agents. RhoB is

required to induce apoptosis after DNA damage in Ras transformed cells (Prendergast, 2001). In contrast, RhoB protects fibroblasts from radiation induced mitotic cell death. In irradiated cells, RhoB aggravates the G2 arrest and inhibits centrosome overduplication (Milia et al., 2005). These reports suggest a regulatory role of RhoB in apoptosis. RhoB appears to be involved in the decision of the cell to undergo DNA-repair or apoptosis upon DNA damage (Fritz and Kaina, 2000). Alterations of RhoB signaling are therefore fatal in two ways: A loss of RhoB signaling leads to uncontrolled growth (Liu et al. 2001), excess activation of RhoB causes profound apoptosis across a population of cells (this study). Our finding that fibroblasts as well as RBL cells (Huelsenbeck et al., 2007a) are most sensitive to TcdB during S-phase implicate that RhoB may also be a sensor for cellular damage during replication. RhoB may further influence susceptibility to proapoptotic stimuli by regulating how signaling molecules are trafficked under stressful conditions (Kamasani et al., 2004). RhoB knockout cells or RhoB siRNA are useful tools to identify those RhoB-dependent pathways leading either to cellular survival or apoptosis.

6. Non-standard abbreviations

C21	Rho-binding domain of Rhotekin
cat	catalytic domain
CHX	cycloheximide
CNF1	*Escherichia coli* cytotoxic necrotizing factor 1
CPE	cytopathic effect (round cells/total cells)
FT	GST-fusion toxin
GAP	GTP binding protein activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
MEF	mouse embryonic fibroblast
RBD	receptor binding domain
TcdA	*Clostridium difficile* toxin A
TcdB	*Clostridium difficile* toxin B
TcdBF	variant *Clostridium difficile* toxin B
TcsH	*Clostridium sordellii* hemorrhagic toxin
TcsL	*Clostridium sordellii* lethal toxin
TMD	trans membrane domain

7. Reference List

Adamson,P., Marshall,C.J., Hall,A., and Tilbrook,P.A. 1992a. Post-translational modifications of $p21^{rho}$ proteins. *J. Biol. Chem.* 267:20033-20038.

Adamson,P., Paterson,H.F., and Hall,A. 1992b. Intracellular localization of the $p21^{rho}$ proteins. *J. Cell Biol.* 119:617-627.

Ashkenazi,A., and Dixit,V.M., 1998. Death receptors: signaling and modulation. *Science*. 281(5381):1305-8.

Aspenström,P., Fransson,A., and Saras,J. 2004. Rho GTPases have diverse effects on the organization of the actin filament system. *Biochem. J.* 377:327-337.

Barth,H., Hofmann,F., Olenik,C., Just,I., and Aktories,K. 1998. The N-terminal part of the enzyme component (C2I) of the binary *Clostridium botulinum* C2 toxin interacts with the binding component C2II and functions as a carrier system for a Rho ADP-ribosylating C3-like fusion toxin. *Infect. Immun.* 66:1364-1369.

Barth,H., Pfeifer,G., Hofmann,F., Maier,E., Benz,R., and Aktories,K. 2001. Low pH-induced formation of ion channels by *Clostridium difficile* toxin B in target cells. *J. Biol. Chem.* 276:10670-10676.

Black,R.A., Kronheim,S.R., Merriam,J.E., March,C.J., and Hopp,T.P., 1989. A pre-aspartate-specific protease from human leukocytes that cleaves pro-interleukin-1 beta. *J. Biol. Chem.* 264: 5323 – 5326.

Boguski,M.S., and McCormick,F. 1993. Proteins regulating Ras and its relatives. *Nature* 366:643-654.

Boquet,P., and Lemichez,E. 2003. Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis ? *Trends Cell Biol.* 13:238-246.

Boquet,P., Munro,P., Fiorentini,C., and Just,I. 1998. Toxins from anaerobic bacteria: specificity and molecular mechanisms of action. *Curr. Opin. Microbiol.* 1:66-74.

Caron,E., and Hall,A. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* 282:1717-1721.

Chardin,P., Madaule,P., and Tavitian,A. 1988. Coding sequence of human rho cDNAs clone 6 and clone 9. *Nucl. Acids Res.* 16:2717.

Chaves-Olarte,E., Löw,P., Freer,E., Norlin,T., Weidmann,M., Von Eichel-Streiber,C., and Thelestam,M. 1999. A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. *J. Biol. Chem.* 274:11046-11052.

Chaves-Olarte,E., Weidmann,M., Von Eichel-Streiber,C., and Thelestam,M. 1997. Toxins A and B from *Clostridium difficile* differ with respect to enzymatic potencies, cellular substrate specificities, and surface binding to cultured cells. *J. Clin. Invest.* 100:1734-1741.

Chen,L.Y., Zuraw,B.L., Liu,F.T., Huang,S., and Pan,Z.K. 2002. IL-1 receptor-associated kinase and low molecular weight GTPase RhoA signal molecules are required for bacterial lipopolysaccharide-induced cytokine gene transcription. *J. Immunol.* 169:3934-3939.

Chowdhury,I., and Chaqour,B. 2004. Regulation of connective tissue growth factor (CTGF/CCN2) gene transcription and mRNA stability in smooth muscle cells. Involvement of RhoA GTPase and p38 MAP kinase and sensitivity to actin dynamics. *Eur. J. Biochem.* 271:4436-4450.

Clark,E.A., and Brugge,J.S. 1995. Integrins and signal transduction pathways: The road taken. *Science* 268:233-239.

7. Reference list

Delarue,F.L., Adnane,J., Joshi,B., Blaskovich,M.A., Wang,D.A., Hawker,J., Bizouarn, F., Ohkanda,J., Zhu,K., Hamilton,A.D., Chellappan,S., Sebti,S.M, 2007. Farnesyltransferase and geranylgeranyltransferase I inhibitors upregulate RhoB expression by HDAC1 dissociation, HAT association and histone acetylation of the RhoB promoter. *Oncogene.* 26(5):633-40.

Diehl, J.A. 2002. Cycling to cancer with cyclin D1. *Cancer Biol. Ther*. 1(3):226-31.

Engel,M.E., Datta,P.K., and Moses,H.L. 1998. RhoB is stabilized by transforming growth factor β and antagonizes transcriptional activation. *J. Biol. Chem.* 273:9921-9926.

Esteve, P., Embade, N., Perona, R., Jimenez, B., del Peso, L., Leon, J., Arends, M., Miki, T., Lacal J.C. 1998. Rho-regulated signals induce apoptosis in vitro and in vivo by a p53-independent, but Bcl2 dependent pathway. *Oncogene.* 17(14):1855-69.

Etienne-Manneville, S., Hall, A. 2002. Rho GTPases in cell biology. *Nature*. 420(6916):629-35.

Evan,G. and Littlewood,T., 1998. A matter of life and cell death. *Science*. 281(5381):1317-22.

Fiorentini,C., Fabbri,A., Falzano,L., Fattorossi,A., Matarrese,P., Rivabene,R., and Donelli,G. 1998. *Clostridium difficile* toxin B induces apoptosis in intestinal cultured cells. *Infect. Immun.* 66:2660-2665.

Frisch,C., Gerhard,R., Aktories,K., Hofmann,F., and Just,I. 2003. The complete receptor-binding domain of Clostridium difficile toxin A is required for endocytosis. *Biochem. Biophys. Res. Commun.* 300:706-711.

Fritz,G., and Kaina,B. 2001. Ras-related GTPase Rhob represses NF-kappaB signaling. *J. Bio. Chem.* 276(5):3115-22.

Fritz,G., and Kaina,B. 2000. Ras-related GTPase RhoB forces alkylation-induced apoptotic cell death. *Biochem. Biophys. Res. Commun.* 268:784-789.

Fritz,G., and Kaina,B. 1997. 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. *J. Biol. Chem.* 272:30637-30644.

Fritz,G., Kaina,B., and Aktories,K. 1995. The Ras-related small GTP-binding protein RhoB is immediate- early inducible by DNA damaging treatments. *J. Biol. Chem.* 270:25172-25177.

Fukuhara,S., Murga,C., Zohar,M., Igishi,T., and Gutkind,J.S. 1999. A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. *J. Biol. Chem.* 274:5868-5879.

Gampel,A., Mellor,H. 2002. Small interfering RNAs as a tool to assign Rho GTPase exchange-factor function in vivo. *Biochem. J.* 366(Pt 2):393-8.

Genth,H., Gerhard,R., Maeda,A., Amano,M., Kaibuchi,K., Aktories,K., and Just,I. 2003. Entrapment of Rho ADP-ribosylated by Clostridium botulinum C3 exoenzyme in the Rho-guanine nucleotide dissociation inhibitor-1 complex. *J. Biol. Chem.* 278:28523-28527.

Genth,H., Selzer,J., Busch,C., Dumbach,J., Hofmann,F., Aktories,K., and Just,I. 2000. New method to generate enzymatically deficient *Clostridium difficile* toxin B as an antigen for immunization. *Infect. Immun.* 68:1094-1101.

Genth,H., Aktories,K., Just,I. 1999. Monoglucosylation of RhoA at threonine 37 blocks cytosol-membrane cycling. *J. Biol. Chem.* 274(41):29050-6.

Gerhard,R., Tatge,H., Genth,H., Thum,T., Borlak,J., Fritz,G., and Just,I. 2005. Clostridium difficile toxin A induces expression of the stress-induced early gene product RhoB. *J. Biol. Chem.* 280:1499-1505.

Gilmore,A.P. 2005. Anoikis. *Cell Death Differ.* 12 Suppl 2:1473-7.

Gredlein,C.M., Silverman,M.L., and Downey,M.S., 2000. Polymicrobial Septic Arthritis Due to *Clostridium* Species: Case Report and Review. *Clin. Inf. Diseases.* 30:590-594.

Hall,A. 1998. G proteins and small GTPases: distant relatives keep in touch. *Science* 280:2074-2075.

Hippenstiel,S., Schmeck,B., N´Guessan,P.D., Seybold,J., Krüll,M., Preissner,K., Von Eichel-Streiber,C., and Suttorp,N. 2002. Rho protein inactivation induced apoptosis of cultured human endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 283:L830-L838.

Ho,J.G., Greco,A., Rupnik,M., and Ng,K.K. 2005. Crystal structure of receptor-binding C-terminal repeats from Clostridium difficile toxin A. *Proc. Natl. Acad. Sci. U. S. A* 102:18373-18378.

Holstein,S.A., Wohlford-Lenane,C.L., and Hohl,R.J. 2002. Consequences of mevalonate depletion. Differential transcriptional, translational, and post-translational up-regulation of Ras, Rap1a, RhoA, AND RhoB. *J. Biol. Chem.* 277:10678-10682.

Huelsenbeck,J., Dreger,S., Gerhard,R., Barth,H., Just,I., and Genth,H. 2007a. Difference in the Cytotoxic Effects of Toxin B from Clostridium difficile Strain VPI 10463 and Toxin B from Variant Clostridium difficile Strain 1470. *Infect. Immun.* 75:801-809.

Huelsenbeck,J., Dreger,S.C., Gerhard,R., Fritz,G., Just,I., Genth,H. 2007b. Upregulation of the immediate early gene product RhoB by exoenzyme C3 from *Clostridium limosum* and toxin B from *Clostridium difficile*. *Biochemistry*. accepted Feb. 27th

Inoue,S., Sugai,M., Murooka,Y., Paik,S.-Y., Hong,Y.-M., Ohgai,H., and Suginaka,H. 1991. Molecular cloning and sequencing of the epidermal cell differentiation inhibitor gene from Staphylococcus aureus. *Biochem. Biophys. Res. Commun.* 174:459-464.

Jacobson,M.D., and Evan,G.I., 1994. Apoptosis. Breaking the ICE. *Curr. Biol.* 4(4):337-40.

Jaffe, A.B., Hall, A. 2005. Rho GTPases: biochemistry and biology. Annu. Rev. Cell Dev. Biol. 21:247-69.

Jahner,D., and Hunter,T., 1991. The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. *Mol. Cell. Biol*. 11(7):3682-90.

Jiang,K., Delarue,F.L., and Sebti,S.M. 2004a. EGFR, ErbB2 and Ras but not Src suppress RhoB expression while ectopic expression of RhoB antagonizes oncogene-mediated transformation. *Oncogene* 23:1136-1145.

Jiang,K., Sun,J., Cheng,J., Djeu,J.Y., Wei,S., and Sebti,S. 2004b. Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis. *Mol. Cell Biol.* 24:5565-5576.

Just,I., and Boquet,P. 2000. Large clostridial cytotoxins as tools in cell biology. *Curr. Top. Microbiol. Immunol.* 250:97-107.

Just,I., and Gerhard,R. 2004. Large clostridial cytotoxins. *Rev Physiol Biochem Pharmacol* 152:23-47.

Just,I., Hofmann,F., Genth,H., and Gerhard,R. 2001. Bacterial protein toxins inhibiting low-molecular-mass GTP-binding proteins. *Int. J. Med. Microbiol.* 291:243-250.

7. Reference list

Just,I., Mohr,C., Schallehn,G., Menard,L., Didsbury,J.R., Vandekerckhove,J., van Damme,J., and Aktories,K. 1992. Purification and characterization of an ADP-ribosyltransferase produced by *Clostridium limosum*. *J. Biol. Chem.* 267:10274-10280.

Just,I., Sehr,P., Jung,M., van Damme,J., Puype,M., Vandekerckhove,J., Moss,J., and Aktories,K. 1995a. ADP-ribosyltransferase type A from turkey erythrocytes modifies actin at Arg-95 and Arg-372. *Biochemistry* 34:326-333.

Just,I., Selzer,J., Hofmann,F., Green,G.A., and Aktories,K. 1996. Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J. Biol. Chem.* 271:10149-10153.

Just,I., Selzer,J., Wilm,M., Von Eichel-Streiber,C., Mann,M., and Aktories,K. 1995b. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* 375:500-503.

Kamasani, U., Huang, M., DuHadaway, J.B., Prochownik, E.V., Donover, P.S., and Prendergast, G.C. 2004. Cyclin B1 Is a Critical Target of RhoB in the Cell Suicide Program Triggered by Farnesyl Transferase Inhibition. *Cancer Res*. 64, 8389–8396.

Kamasani, U., Prendergast, G.C. 2005. Genetic response to DNA damage: proapoptotic targets of RhoB include modules for p53 response and susceptibility to Alzheimer's disease. *Cancer Biol. Ther*. 4(3):282-8.

Kelly,C.P., and LaMont,J.T. 1998. *Clostridium difficile* infection. *Annu. Rev. Med.* 49:375-390.

Kong,J.Y., and Rabkin,S.W. 2005. The association between RhoB and caspase-2: changes with lovastatin-induced apoptosis. *Biochem. Cell Biol.* 83:608-619.

Kyriakis,J.M., and Avruch,J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* 81:807-869.

Lanning,C.C., Daddona,J.L., Ruiz-Velasco,R., Shafer,S.H., and Williams,C.L. 2004. The Rac1 C-terminal polybasic region regulates the nuclear localization and protein degradation of Rac1. *J. Biol. Chem.* 279:44197-44210.

Laudanna,C., Campbell,J.J., and Butcher,E.C. 1996. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science* 271:981-983.

Lebowitz,P.F., Davide,J.P., and Prendergast,G.C. 1995. Evidence that farnesyltransferase inhibitors supress ras transformation by interferingwith rho activity. *Mol. Cell. Biol.* 15:6613-6622.

Lewis,C.J., and Naylor,R.D., 1998. Sudden death in sheep associated with *Clostridium sordellii*. *Vet. Rec.* 142(16):417-21.

Liu,A., Cerniglia,G.J., Bernhard,E.J., and Prendergast,G.C. 2001. RhoB is required to mediate apoptosis in neoplastically transformed cells after DNA damage. *Proc. Natl. Acad. Sci. U. S. A* 98:6192-6197.

Mackay, D.J., Hall, A. 1998. Rho GTPases. *J. Biol. Chem.* 273(33):20685-8.

Mazieres J, Antonia T, Daste G, Muro-Cacho C, Berchery D, Tillement V, Pradines A, Sebti S, Favre G. 2004. Loss of RhoB expression in human lung cancer progression. *Clin. Cancer Res*. 10(8):2742-50.

Michaelson,D., Silletti,J., Murphy,G., D'Eustachio,P., Rush,M., and Philips,M.R. 2001. Differential Localization of Rho GTPases in Live Cells. Regulation by hypervariable regions and rhogdi binding. *J. Cell Biol.* 152:111-126.

7. Reference list

Milia,J., Teyssier,F., Dalenc,F., Ader,I., Delmas,C., Pradines,A., Lajoie-Mazenc,I., Baron,R., Bonnet,J., Cohen-Jonathan,E., Favre,G., Toulas,C. 2005. Farnesylated RhoB inhibits radiation-induced mitotic cell death and controls radiation-induced centrosome overduplication. *Cell Death Differ.* 12(5):492-501.

Nemeth,Z.H., Deitch,E.A., Davidson,M.T., Szabo,C., Vizi,E.S., and Hasko,G. 2004. Disruption of the actin cytoskeleton results in nuclear factor-kappaB activation and inflammatory mediator production in cultured human intestinal epithelial cells. *J. Cell Physiol* 200:71-81.

Nobes,C.D., and Hall,A. 1995. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81:53-62.

Nusrat,A., Giry,M., Turner,J.R., Colgan,S.P., Parkos,C.A., Carnes,D., Lemichez,E., Boquet,P., and Madara,J.L. 1995. Rho protein regulates tight junctions and perijunctional actin organization in polarized epithelia. *Proc. Natl. Acad. Sci. USA* 92:10629-10633.

Olson, M.F., Ashworth, A. Hall, A. 1995. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science*. 269(5228):1270-2.

Ott,C., Iwanciw,D., Graness,A., Giehl,K., and Goppelt-Struebe,M. 2003. Modulation of the expression of connective tissue growth factor by alterations of the cytoskeleton. *J. Biol. Chem.* 278:44305-44311.

Petit,P., Bréard,J., Montalescot,V., El Hadj,N.B., Levade,T., Popoff,M., and Geny,B. 2003. Lethal toxin from Clostridium sordellii induces apoptotic cell death by disruption of mitochondrial homeostasis in HL-60 cells. *Cellular Microbiology* 5:761-771.

Pfeifer,G., Schirmer,J., Leemhuis,J., Busch,C., Meyer,D.K., Aktories,K., and Barth,H. 2003. Cellular uptake of Clostridium difficile toxin B. Translocation of the N-terminal catalytic domain into the cytosol of eucaryotic cells. *J. Biol. Chem.* 278:44535-44541.

Popoff,M.R., Boquet,P., Gill,D.M., and Eklund,M.W. 1990. DNA sequence of exoenzyme C3, an ADP-ribosyltransferase encoded by Clostridium botulinum C and D phages. *Nucl. Acids Res.* 18:1291.

Popoff,M.R., Chaves,O.E., Lemichez,E., Von Eichel-Streiber,C., Thelestam,M., Chardin,P., Cussac,D., Chavrier,P., Flatau,G., Giry,M., Gunzburg,J., and Boquet,P. 1996. Ras, Rap, and Rac small GTP-binding proteins are targets for Clostridium sordellii lethal toxin glucosylation. *J. Biol. Chem.* 271:10217-10224.

Prendergast,G.C. 2001. Actin' up: RhoB in cancer and apoptosis. *Nat. Rev. Cancer* 1:162-168.

Prendergast,G.C., Khosravi-Far,R., Solski,P.A., Kurzawa,H., Lebowitz,P.F., and Der,C.J. 1995. Critical role of Rho in cell transformation by oncogenic Ras. *Oncogene* 10:2289-2296.

Qa'Dan,M., Spyres,L.M., and Ballard,J.D. 2000. pH-induced conformational changes in *Clostridium difficile* toxin B. *Infect. Immun.* 68:2470-2474.

Qa'Dan,M., ´Ramsey,M., Daniel,J., Spyres,L.M., Safiejko-Mroczka,B., Ortiz-Leduc,W., and Ballard,J.D. 2002. Clostridium difficile toxin B activates dual caspase-dependent and caspase-independent apoptosis in intoxicated cells. *Cell. Microbiol.* 4:425-434.

Ridley,A.J., 1997. The GTP-binding protein Rho. *Int. J. Biochem. Cell. Biol.* 29(11):1225-9.

Rojas,R., Ruiz,W.G., Wang,E., Kinlough,C.L., Poland,P.A., Hughey,R.P., Dunn,K.W., and Apodaca,G. 2004. RhoB-dependent modulation of early endocytic traffic in Madin-Darby canine kidney cells. *J. Biol. Chem.*

7. Reference list

Rupnik,M., Pabst,S., Rupnik,M., Von Eichel-Streiber,C., Urlaub,H., and Soling,H.D. 2005. Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of Clostridium difficile toxin B (TcdB) by host cells. *Microbiology* 151:199-208.

Sehr,P., Joseph,G., Genth,H., Just,I., Pick,E., and Aktories,K. 1998. Glucosylation and ADP-ribosylation of Rho proteins - Effects on nucleotide binding, GTPase activity, and effecter-coupling. *Biochemistry* 37:5296-5304.

Sekine,A., Fujiwara,M., and Narumiya,S. 1989. Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. *J. Biol. Chem.* 264:8602-8605.

Sinave,C., Le,T.G., Blouin,D., Leveille,F., and Deland,E. 2002. Toxic shock syndrome due to Clostridium sordellii: a dramatic postpartum and postabortion disease. *Clin. Infect. Dis.* 35:1441-1443.

Stamatovic,S.M., Keep,R.F., Kunkel,S.L., and Andjelkovic,A.V. 2003. Potential role of MCP-1 in endothelial cell tight junction "opening": signaling via Rho and Rho kinase. *J. Cell. Sci.* 116:-4615.

Takai,Y., Kaibuchi,K., Kikuchi,A., and Kawata,M. 1992. Small GTP-binding proteins. *Int. Rev. Cytol.* 133:187-230.

Van Aelst,L., and D'Souza-Schorey,C. 1997. Rho GTPases and signaling networks. *Genes & Development* 11:2295-2322.

Vetter,I.R., Hofmann,F., Wohlgemuth,S., Herrmann,C., and Just,I. 2000. Structural consequences of mono-glucosylation of Ha-Ras by Clostridium sordellii lethal toxin. *J. Mol. Biol.* 301:1091-1095.

Von Eichel-Streiber,C., Habermann,E., Weidmann,M., and Hofmann,F. 1994. Genetical analysis of the large Clostridial cytotoxins - a comparison of *Clostridium novyi*, C. *sordellii* and C. *difficile* toxins. *In* Bacterial protein toxins, Zbl.Bakt.Suppl.24. Freer, editor. Fischer,G., Stuttgart, Jena, New York. 51-59.

Wang,D.A., and Sebti,S.M. 2005. Palmitoylated cysteine 192 is required for RhoB tumor-suppressive and apoptotic activities. *J. Biol. Chem.* 280:19243-19249.

Wennerberg,K., and Der,C.J. 2004. Rho-family GTPases: it´s not only Rac and Rho (and I like it). *J. Cell Sci.* 117:1301-1312.

Wilde,C., Vogelsang,M., and Aktories,K. 2003. Rho-specific Bacillus cereus ADP-ribosyltransferase C3cer cloning and characterization. *Biochemistry* 42:9694-9702.

Wittinghofer,A., and Pai,E.F. 1991. The structure of *ras* protein: a model for a universial molecular switch. *Trends Biochem Sci.* 16:382-387.

Zalcman,G., Closson,V., Linarès-Cruz,G., Lerebours,F., Honoré,N., Tavitian,A., and Olofsson,B. 1995. Regulation of Ras-related RhoB protein expression during the cell cycle. *Oncogene* 10:1935-1945.

Zhu,H., Fearnhead,H.O., Cohen,G.M., 1995. An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. *FEBS Lett*. 374(2):303-8.

8. Curriculum vitae

Name: Johannes Hülsenbeck
Geburtstag: 06.03.1978
Geburtsort: Münster

Eltern: Martin Hülsenbeck
Superintendent i.R.

Claudia Hülsenbeck, geb. Nell
Referentin der Erwachsenenbildung im ev. Kirchenkreis Bielefeld

Akademischer Werdegang

1984: Einschulung, Haardschule, Marl

1988: Gymnasium Petrinum, Recklinghausen

1992: Ratsgymnasium, Bielefeld

1994: Eintritt in die gymnasiale Oberstufe

1994/1995: Auslandsaufenthalt in Syracuse, U.S.A, Besuch der East-Syracuse Minoa High School

1997: Abitur am Ratsgymnasium Bielefeld, Gesamtnote 1.6

1997/1998: Zivildienst an den von Bodelschwinghschen Anstalten, Bethel

10/1998: Beginn des Studiums der Biochemie mit Studienziel Diplom an der Ruhr-Universität Bochum

9/2000: Vordiplom an der Ruhr-Universität Bochum in Biochemie, Gesamtnote „gut“

10/2000: Aufnahme des Hauptstudiums in Biochemie an der Universität Hannover, Studienziel Diplom

9/2003: Diplom in Biochemie an der Universität Hannover, Gesamtnote „sehr gut“
Titel der Diplomarbeit: „Darstellung und Charakterisierung von rekombinantem *Clostridium difficile* Toxin B“, angefertigt in der Abteilung Toxikologie der Medizinischen Hochschule Hannover

11/2003-06/2007: Anfertigung der Dissertation in der Abteilung Toxikologie der Medizinischen Hochschule Hannover, Thema: „Zelluläre Antworten auf clostridiale Zytotoxine“
Betreuer: Prof. Dr. Ingo Just, Dr. Harald Genth

8. Curriculum vitae

Wissenschaftliche Publikationen

S.C. Dreger, **J. Huelsenbeck**, R. Gerhard, G. Fritz, I. Just, H. Genth: Inactivation of H-/K-/N-Ras is the basis of the cytotoxic effect of the *Clostridium sordelli* lethal toxin isoforms from strains 6018 and 9048. Manuscript in preparation.

J. Huelsenbeck, S.C. Dreger, R. Gerhard, G. Fritz, I. Just, H. Genth: *Clostridium sordellii* lethal toxin causes up-regulation of the immediate early gene product RhoB by inactivation of H-/K-/N-Ras. Manuscript in preparation.

I. Halabi-Cabezon, **J. Huelsenbeck**, F. Hofmann, I. Just, H. Genth: Glucosylation of Rac1 governs the cytopathic effect of the clostridial glucosylating toxins. Manuscript in preparation

J. Huelsenbeck, S. Dreger, R. Gerhard, G. Fritz, I. Just, H. Genth: Up-regulation of the immediate early gene product RhoB by exoenzyme C3 and toxin B from *Clostridium difficile*. Biochemistry, accepted Feb. 27th, 2007.

J. Huelsenbeck, S.Dreger, R. Gerhard, H. Barth, I. Just, H. Genth: Difference in the cytotoxic effects of toxin B from *Clostridium difficile* strain VPI10463 and toxin B from variant *Clostridium difficile* strain 1470. Infect Immun. 2007 Feb;75(2):801-9

H. Genth, **J. Huelsenbeck**, B. Hartmann, F. Hofmann, I. Just, R. Gerhard: Cellular stability of Rho-GTPases glucosylated by *Clostridium difficile* toxin B. FEBS Lett. 2006 Jun 12;580(14):3565-9.

Kongressbeiträge

Vorträge

Mainz (Germany), 13th – 15th March 2007. 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
Title: Different cytotoxic effects of reference toxin B and variant toxin B from *Clostridium difficile*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 36, 141, Suppl. 1 MAR 2007

Nottingham (United Kingdom), 21-25 June 2006, 5th International Meeting on the Molecular Biology and Pathogenesis of the Clostridia.
Title: Up-regulation of RhoB by clostridial cytotoxins

Mainz (Germany), 4th – 6th April 2006. 47th Spring Meeting of the German Society of Pharmacology and Toxicology.
Title: p38 MAPK-dependent modulation of RhoB up-regulation requires active Rac1
NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 53, 172, Suppl. 1 MAR 2006

Posterpräsentationen

Mainz (Germany), 13th – 15th March 2007. 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
Huelsenbeck J, Dreger S, Hofmann F, Just I, Genth H. Different cytotoxic effects of reference toxin B and variant toxin B from *Clostridium difficile*. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 36, 141, Suppl. 1 MAR 2007
Dreger S, Isermann J, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Inactivation of H-/K-/N-Ras is the basis of the cytotoxic effect of *Clostridium sordellii* lethal toxin. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 36, 140, Suppl. 1 MAR 2007

Schulz F, **Huelsenbeck J**, Gerhard R, Kotlyarov A, Fritz G, Just I, Genth H. p38 MAP kinase and the cytotoxic effect of *Clostridium difficile* toxin B. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 38, 149, Suppl. 1 MAR 2007
Halabi-Cabezon I, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Glucosylation of RhoG contributes to the cytopathic effect of clostridial glucosylating toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 375: p. 38, 150, Suppl. 1 MAR 2007

Nottingham (United Kingdom), 21-25 June 2006, 5th International Meeting on the Molecular Biology and Pathogenesis of the Clostridia.
Genth H, Dreger S, **Huelsenbeck J**, Just I. Distinct dynamics of the cytopathic and cytotoxic effect induced by *Clostridium difficile* toxin B.

Mainz (Germany), 4th – 6th April 2006. 47th Spring Meeting of the German Society of Pharmacology and Toxicology.
Dreger S, **Huelsenbeck J**, Just I, Genth H. The Cytopathic Activity of Clostridium difficile Toxin B Includes Up-Regulation of the Immediate Early-Gene RhoB and Caspase3-Activation. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 186, Suppl. 1 MAR 2006

Engel J, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Cross-Reactivity of the Anti-Toxin Against the Catalytic Domain of *C. sordellii* Lethal Toxin with *C. difficile* Toxin B. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 187, Suppl. 1 MAR 2006

Cherkasov V, **Huelsenbeck J**, Just I, Genth H. Glucosylation of Endogenous Rac1 as a Marker of the Cellular Activity of Clostridial Glucosylating Toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 372: p. 57, 188, Suppl. 1 MAR 2006

Berlin (Germany), 13th – 15th October 2005. 9th International Dahlem Symosium on Cellular Signal Recognition and Transduction.
Genth H, **Huelsenbeck J**, Dreger S, Hofmann F, Gerhard R. Clostridial factors associated to *Clostridium difficile* toxin A and toxin B responsible for their cytotoxic activity.

Huelsenbeck J, Gerhard R, Kotlyarov A, Just I, Genth H. p38 MAPK-dependent Modulation of RhoB Up-regulation Requires Active Rac1.

Canterbury (United Kingdom), 25th – 29th June 2005. 12th European Conference on Bacterial Protein Toxins.
Genth H, Isermann M, **Huelsenbeck J**, Hofmann F, and Just I. Difference in the protein substrate specifity between *Clostridium sordellii* lethal toxin and variant *Clostridium difficile* toxin B.

Huelsenbeck J, Gerhard R, Just I, and Genth H. Upregulation of RhoB by Clostridial Cytotoxins.

Mainz (Germany), 15th – 17th March 2005. 46th Spring Meeting of the German Society of Pharmacology and Toxicology.
Huelsenbeck J, Dreger S, Hartmann B, Gerhard R, Genth H. Up-regulation of RhoB protein by glucosylating toxins. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R118-R118 493 Suppl. 1 FEB 2005

Isermann M, **Huelsenbeck J**, Hofmann F, Just I, Genth H. Difference in the protein substrate specificity between *Clostridium sordellii* lethal toxin and variant *Clostridium difficile* toxin B 1470. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R117-R117 490 Suppl. 1 FEB 2005

Schulz F, **Hulsenbeck J**, Just I, Genth H. The catalytic domain of native large clostridial cytotoxins escapes antitoxin detection. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 371: R117-R118 491 Suppl. 1 FEB 2005

Nice (France), 4th – 8th September, 2004. ELSO Meeting.
Genth H, Hartmann B, **Huelsenbeck J**, Just I, Gerhard R. Intracellular stability of Rho GTPases modified by ADP-ribosylation and glucosylation.

Mainz (Germany), 9th – 11th March 2004. 45th Spring Meeting of the German Society of Pharmacology and Toxicology.
Huelsenbeck J, Hartmann B, Gerhard R, Just I, Genth H. Difference in Rho-GTPase substrate specificity between wildtype and recombinant *Clostridium difficile* toxin B. NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY 369: R53-R53 211 Suppl. 1 MAR 2004