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Phosphorylation of Rac1 at Ser-71 reduces *Clostridium difficile* toxin A–induced effects on the epithelial barrier function

Von der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover zur Erlangung des Grades einer Doktorin der Naturwissenschaften Dr. rer. nat. genehmigte Dissertation von

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig verfasst und die benutzten Hilfsmittel sowie Quellen vollständig angegeben habe.

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Hannover, den 19. Juni 2008
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Zusammenfassung

Die *Clostridium difficile* Toxine A (TcdA) und B (TcdB) verursachen die *Clostridium difficile*-assoziierte Diarrhoe und pseudomembranöse Colitis. Der epidermale Wachstumsfaktor (EGF) bewirkt eine Abschwächung der Toxin-induzierten Mukosaschädigung, wobei der zugrundeliegende Mechanismus bisher nicht bekannt war. In der vorliegenden Arbeit wurde mit Hilfe der intestinalen Epithelzellinie CaCo-2 ein Mechanismus aufgeklärt, über den ein Schutzeffekt von EGF gegenüber der Wirkung der Glucosyltransferase TcdA erfolgt: Die Phosphorylierung von Rac1, eines Substratproteins der clostridialen Glucosyltransferasen.

Die EGF-induzierte Phosphorylierung des Ser-71 von Rac1/Cdc42 korrelierte mit einer verminderten TcdA-katalysierten Rac1 Glucosylierung und mit einer Abschwächung der TcdA-induzierten Barrierestörung. Über einen spezifischen Inhibitor und im rekombinanten System wurde gezeigt, dass die Akt Kinase die Phosphorylierung von Rac1/Cdc42 bewirkte. Als wichtige Voraussetzung für einen schützenden Effekt dieser Ser-71 Phosphorylierung wurde der Aktivitätsstatus beider GTPasen bestimmt. Dabei konnte im Präzipitationsexperiment gezeigt werden, dass Phospho-Ser-71 Rac1/Cdc42 bevorzugt an das Effektorprotein Pak binden.

Zur weiteren Charakterisierung des phosphorylierten Rac1 und Cdc42 wurden beide phosphomimetische Mutanten erstellt, bei denen das Ser-71 zu einem phosphatgruppe-imitierenden Glutamat ausgetauscht wurde. Rac1 S71E und Cdc42 S71E zeigten gegenüber dem Wildtyp Rac1 bzw. Cdc42 identische Eigenschaften einer Nukleotidbindung und der nukleotidabhängigen Bindung an das Effektorprotein Pak und an das Inhibitorprotein Rho-GDI. Rac1 S71E war hingegen kein Substrat für TcdA oder die homologe Glucosyltransferase TcsL (lethale Toxin von *Clostridium sordellii*), wobei Cdc42 S71E ein schwaches Substrat für TcdA war. Die schützende Wirkung von Ser-71 phosphoryliertem Rac1 beruht demnach darauf, dass es kein Substrat für die Glucosyltransferasen mehr ist und eine Rac1-spezifische Signalübertragung aufrecht erhält.

Der kausale Zusammenhang einer Rac1 Phosphorylierung und der verminderten Wirkung von TcdA wurde im Transfektionsexperiment gezeigt. Rac1 S71E transfizierte Hep-2-Zellen waren partiell gegenüber den TcdA-induzierten morphologischen Änderungen geschützt. Noch deutlicher zeigte sich der Effekt bei einer Behandlung der Zellen mit TcsL, das nur Rac1, nicht aber RhoA als Substrat erkennt. Hier bewirkte Rac1 S71E einen vollständigen Schutz vor der Toxin-induzierten Zellabrundung. Neben der Aufklärung des Mechanismus, der einer schützenden Wirkung von EGF zugrunde liegt bzw. deutlich dazu beiträgt, wurde eine eigenständige Funktion von Rac1 S71E beschrieben. Die Färbung des Aktinzytoskeletts von Rac1 S71E-transfizierten Zellen weisen auf einen konstitutiv aktiven Zustand dieser Rac1 Mutante hin, die eine spezifische Änderung des Aktinzytoskeletts bewirkt. Diese Rac1 S71E-induzierten Filopodien ähneln den EGF-induzierten Zytoskelettänderungen und weisen auf eine Funktionsänderung von Rac1 durch Phosphorylierung hin.

Schlagworte: Glucosyltransferase, Rho GTPasen, Phosphorylierung

Abstract

Clostridium difficile toxin A (TcdA) and B (TcdB) are causative agents of *Clostridium difficile*-associated diarrhea and the pseudomembranous colitis. Epidermal growth factor (EGF) attenuates the toxin-induced damage of the mucosa, although the underlying mechanism was not yet known.

Using the intestinal epithelial model cell line CaCo-2, the present study describes the mechanism that contributes to the protective effect of EGF on the TcdA-induced alterations: The phosphorylation of Rac1, a substrate protein of the clostridial glucosyltransferases, is the critical step within the mechanism.

The EGF-induced phosphorylation of Ser-71 of Rac1/Cdc42 correlates with a reduced TcdA-catalyzed Rac1 glucosylation and a reduction of the TcdA-induced alteration of the epithelial barrier function. By using a specific inhibitor and in a recombinant system, it was shown that the Akt kinase induced the phosphorylation of Rac1/Cdc42. As a prerequisite for the protective effect, the activity status of both phosphorylated GTPases had to be shown. Further pull-down assays revealed that Phospho-Rac1/Cdc42 are preferentially bound to effector protein Pak and remain functional competent and active.

To characterize the phosphorylation of Rac1 and Cdc42 in more detail, both phosphomimetic mutants were generated by exchange of Ser-71 against the phosphomimetic amino acid glutamate. Compared to the wild type Rac1 and Cdc42, both phosphomimetic mutants showed an identical nucleotide binding and a nucleotide-dependent binding to the effector protein Pak and the inhibitor protein Rho-GDI. However, Rac1 S71E was neither substrate for TcdA nor for the homologue glucosyltransferase TcsL (lethal toxin from *Clostridium sordellii*), whereas Cdc42 S71E was a poor substrate for TcdA. Thus, the protective effect of the Ser-71 phosphorylated Rac1 is based on Rac1 not being substrate for the glucosyltransferases and the maintenance of the Rac1 specific signal pathways.

The causal relation between the Rac1 phosphorylation and the reduced TcdA effect was shown in transfection experiments. Rac1 S71E transfected Hep-2-cells were partially protected against TcdA-induced morphological changes. The effect became more obvious after treatment of the cells with TcsL, which only recognizes Rac1 as substrate but not RhoA. In this case, Rac1 S71E completely protected the cells against TcsL-induced cytopathic effect. In addition to revealing the mechanism of the protective effect of EGF, a distinct function of Rac1 S71E was described. Rac1 S71E-transfected cells showed specific alteration of the actin cytoskeleton. It can be assumed that Rac1 S71E primarily acts in the active state. The Rac1 S71E-induced filopodia resemble the EGF-induced alteration of the actin cytoskeleton and point out a change in Rac1 function by phosphorylation.

Key words: glucosyltransferase, Rho GTPases, phosphorylation

List of Contents

List of Figures

List of Tables

List of Abbreviations

ABTS	2,2'-azino-bis(3)-ethylbenzthiazoline-6-sulfonic acid
ADP	adenosine 5'-diphosphate
ARP	acidic repeat protein
ATP	adenosine 5'-triphosphate
BAD	pro-apoptotic Bcl-2 related protein
BSA	bovine serum albumin
CDAD	*Clostridium difficile*-associated diarrhea
Cdc42	cell division cycle 42
DAPI	4',6'-diamidino-2-phenylindole hydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
dNTPs	deoxynucleotide-triphosphates
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial cell-derived neutrophil-activating protein-78
FBS	fetal bovine serum
GAPs	GTPase-activating proteins
GDI	guanosine diphosphate-dissociation inhibitor
GDP	guanosine diphosphate
GEFs	guanine nucleotide exchange factors
Grb2	growth factor receptor-bound protein 2
GRO-α	growth-regulated oncogene-α
GST	Glutathione-S-transferase
GTP	guanosine triphosphate
GTPases	GTP-binding proteins
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN-γ	interferon-γ
IgG	immunoglobulin G
IL	interleukin
IPTG	isopropyl-D-thiogalatopyranosid
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
LIM	acronym of the three gene products Lin-11, Isl-1 and Mec-3
MEK	mitogen-activated protein kinase/extracellular signal to regulated kinase
MEM	Minimum Essential Medium
MLC	myosin light chain

NAD	nicotinamide adenine dinucleotide
NF-κB	nuclear factor-κB
non-gluc	non-glucosylated
NP40	Nonident P 40
MANT	N-methylanthraniloyl
MCP-1	monocyte chemoattractant protein-1
PAK	p21-activated kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	protein kinase-1
PI3-K	phosphatidylinosiol 3-kinase
PI(4,5)P2	phosphatidylinositol(4,5)-bisphosphate
PI-4-P5K	phosphatidylinositol-4-phosphate 5-kinase
PIP3	phosphatidylinositol(3,4,5)-trisphosphate
PKN	protein kinase N
PTB	phosphotyrosine binding domain
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family member A
ribos	ribosylated
ROK	Rho-associated kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH-2	Src homology 2
Ser	serine
Sos	son of sevenless
TBST	Tris buffered saline Tween-20
TcdA	*Clostridium difficile* toxin A
TcdB	*Clostridium difficile* toxin B
TcsH	*Clostridium sordellii* hemorrhagic toxin
TcsL	*Clostridium sordellii* lethal toxin
TEA	triethanolamine
TER	transepithelial electrical resistance
TNF-α	tumor necrosis factor-α
U	unit
UDP	uridine 5'-diphosphate
UDP-GlcNAc	uridine 5'-diphosphate-N-acetylglucosamine
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family Verprolin-homologous protein

1. Introduction

1.1. *Clostridium difficile*

Clostridium difficile is a gram-positive, spore-forming anaerobic bacterium that produces two pathogenic exotoxins: toxin A (TcdA) and B (TcdB) (Bongaerts and Lyerly, 1994; Lyerly and Wilkins, 1995; Taylor et al., 1981). The bacterium was first isolated from new born infants in the year 1935 (Hall and O'Toole, 1935). With introduction of the broad-spectrum antibiotics in the later half of the twentieth century, the antibiotic-associated diarrhea became more common. In the year 1977 the pseudomembranous colitis was first described as a complication of a *Clostridium difficile* infection (Kelly et al., 1994b; Larson and Proce, 1977).

Broad-spectrum antibiotics such as cephalosporins and clindamycin cause a disruption of the colonic microflora leading to a colonization of *Clostridium difficile* through the ingestion of heat-resistant spores, which convert to their active, vegetative form in the colon. There they proliferate and produce the major virulence factors TcdA and TcdB. The risk of enhanced colonization is increased by advanced age, a comprimized immune system or a severe underlying disease of the host (Frost et al., 1998). The clinical feature of a *Clostridium difficile* infection ranges from a mild to a moderate form of watery diarrhea with abdominal pain, fever and leucocytosis, sometimes development of the pseudomembranous colitis (PMC) with yellow-white plaques and a toxic megacolon (Rubin et al., 1995). The different types of clinical feature likely represent the outcome of cellular and molecular interactions between the host mucosal defense mechanisms and *Clostridium difficile* toxins A and B. TcdA and TcdB contribute to a release of mediators that govern the inflammatory process of the intestine by affecting epithelial cells, neurons of the enteric nervous system and mast cells. Both toxins cause an increase in the permeability of the intestinal epithelial barrier by disruption of the tight junctions (Hecht et al., 1988; Nusrat et al., 2001) and a recruitement of neutrophils to the site of infection (Kelly et al., 1994a). TcdA induces the production of substance P and its action involves the enteric nervous system (Mantyh et al., 1996; Pothoulakis et al., 1994). In response to TcdA, macrophages and mast cells produce factors such as TNF-α and IL1-β which further facilitate the inflammatory response (Calderón et al., 1998). TcdA also stimulates IL-8 secretion, other CXC or CC chemokines like GRO-α, ENA-78, MCP-1 and the production of reactive oxygen intermediates in human intestinal epithelial cells, and thus contributes to inflammation (Mahida et al., 1996; He et al., 2002; Kim et al., 2002).

The *Clostridium difficile*-associated diarrhea (CDAD) is usually treated orally with metronidazole or vancomycin. The problem with this approach is that antibiotics keep patients susceptible to a *Clostridium difficile* infection. Consequently, relapsing infection is a frequent complication occurring when antibiotics are discontinued. The use of the antibiotic rifaximin, vaccines

against *Clostridium difficile*, intravenous immunoglobulin therapy and human monoclonal antibodies to neutralize the toxins are being evaluated (Aslam et al., 2005; Aboudola et al., 2003; Giannasca and Warny, 2004; Kink and Williams, 1998; Salcedo et al., 1997).

1.2. Large clostridial cytotoxins

The family of large clostridial cytotoxins comprises *Clostridium difficile* toxin A and B (TcdA and TcdB), lethal toxin (TcsL), the hemorrhagic toxin (TcsH) from *Clostridium sordellii* and the α-toxin from *Clostridium novyii* (Bette et al., 1991; Aktories, 1997). Whereas *Clostridium difficile* toxins A and B are the major virulence factors in antibiotic-associated diarrhea and pseudomembranous colitis, the toxins from *Clostridium sordellii* and *Clostridium novyii* implicate gas gangrene syndrome (McGregor et al., 1989; Hatheway, 1990). The toxins are single chain proteins that exhibit glucosyltransferase activity towards their substrate proteins, the GTPases of the Rho family (Just et al., 1995a; Just et al., 1995b; Just et al., 1996; Popoff et al., 1996; Selzer et al., 1996; Genth et al., 1996). They differ in their protein substrate- and cosubstrate-specificity (see table 1.1). Whereas the α-toxin catalyzes the N-acetylglucosaminylation by using UDP-GlcNAc as cosubstrate, the other toxins use UDP-glucose to glucosylate their protein substrates (RhoA at Thr-35 or Rac1, Cdc42 and Ras at the homolog amino acid Thr-37).

toxin	*cosubstrate*	*protein substrate*
TcdA/TcdB	UDP-glucose	RhoA/B/C , Rac1 , Cdc42
TcsL	UDP-glucose	Rac1 , (Cdc42) , H/K/N-Ras , R-Ras
TcsH	UDP-glucose	RhoA/B/C , Rac1 , Cdc42
α-toxin	UDP-GlcNAc	RhoA/B/C , Rac1 , Cdc42

Tab. 1.1.: Cosubstrate- and protein substrate-specificity of large clostridial cytotoxins

Since Rho GTPases are master regulator of the actin cytoskeleton, their inactivation leads to reorganization of the actin cytoskeleton (Hall, 1998) and causes shrinking and rounding of eukaryontic cells (cytopathic effect) (Ottlinger and Lin, 1988). The breakdown of the actin cytoskeleton also affects migration, morphogenesis, cell devision (Jaffe and Hall, 2005) and membrane trafficking (Ridley, 2001). Additionally, several other cellular responses to the inactivation of Rho proteins by toxins are known, i.e. the inhibition of secretion and phospholipase D activity as well as the disregulation of apoptosis and phagocytosis. In addition to the cytopathic effect, the toxins induce apoptosis of cells (cytotoxic effect) (Brito et al., 2005; Brito et al., 2002; Carneiro et al., 2006).

1.3. Structure and uptake of the toxins

The large clostridial cytotoxins are single chain proteins. Until recently they were suggested to be structured in a tripartite manner (Just et al., 1995a; Von Eichel-Streiber et al., 1996). Further investigations expand this three-domain structure and propose a new model of a multi-domain structure. The structure is given on the basis of *Clostridium difficile* toxin B (see figure 1.1).

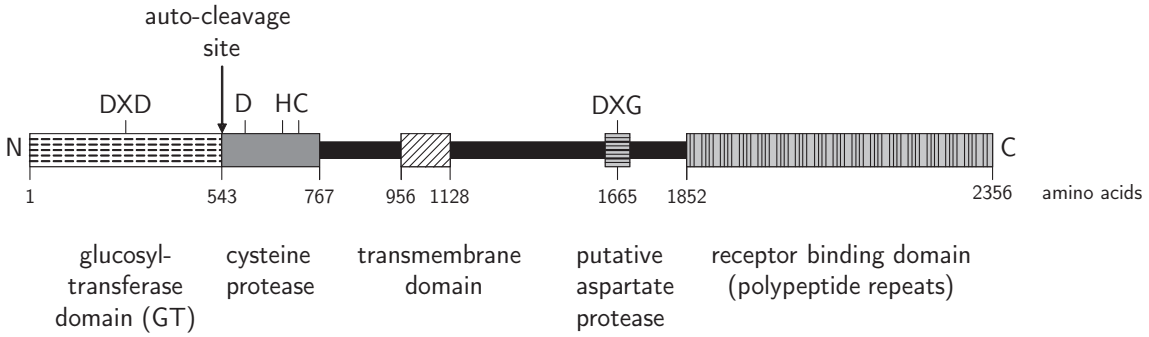

Fig. 1.1.: **Multi-domain structure of *Clostridium difficile* toxin B.** The N-terminus represents the glucosyltransferase domain showing the specific DXD motif (D286, D288). The cysteine protease domain with catalytic amino acids (D587, H653, C698) is placed downstream of the glucosyltransferase domain. The middle part of the protein harbors the transmembrane domain. The putative aspartate protease (D1663, S1664, G1665) is located downstream of the transmembrane domain. The C-terminus of the toxin harbors the receptor binding domain.

The N-terminus harbors the glucosyltransferase activity and therefore represents the domain that is crucial for inhibiton of Rho GTPases. The DXD motif (D286, D288), which is involved in manganese coordination, is typical for this domain (Reinert et al., 2005; Busch et al., 1998). A cysteine protease (D587, H653, C698), which is similar to *V. cholerae* RTX toxin (Sheahan et al., 2007; Egerer et al., 2007), is located downstream of the glucosyltransferase domain. It is essential for auto-cleavage of the toxin at amino acid L543 and the release of the catalytic domain in the cytosol (Pfeifer et al., 2003). The transmembrane domain in the middle part of the protein (residues 956-1128) is highly hydrophobic and is involved in translocation of the toxin into the cytosol. A DXG motif (D1663, S1664, G1665) is located more than 1000 amino acid residues distant from the proposed auto-cleavage site (L543). This motif as a putative aspartate protease is also proposed to be involved in processing of the toxin (Reineke et al., 2007). The C-terminus of the toxin consists of polypeptide repeats which are involved in receptor binding (Von Eichel-Streiber and Sauerborn, 1990; Dove et al., 1990; Von Eichel-Streiber et al., 1992).

After binding of the toxin to their specific but unknown receptor, the toxin-receptor-complex is endocytosed and the endosomes are formed. The acidification causes conformational changes of the toxin, allowing the insertion into the endosome membrane and subsequently formation of a pore (Barth et al., 2001; Giesemann et al., 2006). Then the toxin is translocated into the cytosol where the Rho GTPases are glucosylated and thereby inactivated.

1.4. Rho GTPases

Rho GTPases belong to the Ras superfamily of monomeric GTP-binding proteins. Their common feature is the molecular mass (18-28 kDa), the C-terminal posttranslational modification and their property to act as molecular switches cycling between an inactive GDP-bound state and an active GTP-bound state.

The best characterized members of the Rho family are RhoA, Rac1 and Cdc42 that regulate the assembly and organization of the actin cytoskeleton (Hall, 1998). They also play a role in a variety of cellular processes that depend on the actin cytoskeleton such as morphogenesis, endocytosis (Lamaze et al., 1996; Schmalzing et al., 1995), phagocytosis (Caron and Hall, 1998), cytokinesis (Drechsel et al., 1996; Prokopenko et al., 2000) and migration (Nobes and Hall, 1999; Jones and Blikslager, 2002). The formation of actin stress fibers is associated with RhoA (Ridley and Hall, 1992; Paterson et al., 1990), whereas Rac1 leads to membrane ruffles and lamellipodia (Ridley et al., 1992). Cdc42 induces the formation of filopodia and microspikes (Kozma et al., 1995; Nobes and Hall, 1995). The Rho GTPases also regulate a variety of signal pathways including the transcription factor NF-κB (Perona et al., 1997), the c-Jun N-terminal kinase (JNK), the p38 mitogen-activated protein kinase (Coso et al., 1995; Minden et al., 1995) as well as G1 cell cycle progression (Olson et al., 1995), apoptosis (Moorman et al., 1996) and cell transformation (Van Aelst and D'Souza-Schorey, 1997).

Three groups of associated proteins regulate the activity state and localization of the Rho GTPases (see figure 1.2):

- the guanine nucleotide exchange factors (GEFs) catalyze the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) which is related with the membrane localization of the Rho GTPases (Zheng, 2001)
- the GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity and convert the GTP-bound form of Rho GTPases to the inactive GDP-bound form (Lamarche and Hall, 1994)
- the guanine nucleotide dissociation inhibitors (GDIs) stabilize the inactive GDP-bound form of the Rho GTPases in the cytosol (Rho-GDI complex) and thus prevent association with the membrane (Olofson, 1999)

The nucleotide exchange of GDP with GTP and vice versa induces conformational changes in two amino terminal regions of Rho GTPases, termed switch 1 and switch 2. The hydrolysis of GTP into GDP completes the cycle and terminates the signal transduction. In the GTP-bound form Rho GTPases undergo conformational changes in the switch 1 domain allowing interaction with effector proteins and thus initiating the downstream response. Different effector proteins are known. The effectors are often serine/threonine kinases which are activated by binding of GTP-bound GTPases (e.g., ROK, PAK, PKN) (Amano et al., 1999; Bagrodia et al., 1995; Tu and Wigler, 1999; Amano et al., 1996) to phosphorylate downstream targets. Additionally, Rho effectors also comprise proteins without enzymatic activity (e.g., DIA, rhotekin, rhophilin, WASP) (Watanabe et al., 1997; Miki et al., 1996; Symons et al., 1996) which may serve as nucleus for multi-protein complexes to connect different signaling pathways.

Rho GTPases exhibit sequences at their C-termini that are target structures for posttranslational modification with lipid moieties (i.e. farnesyl, geranyl-geranyl, palmitoyl and methyl).

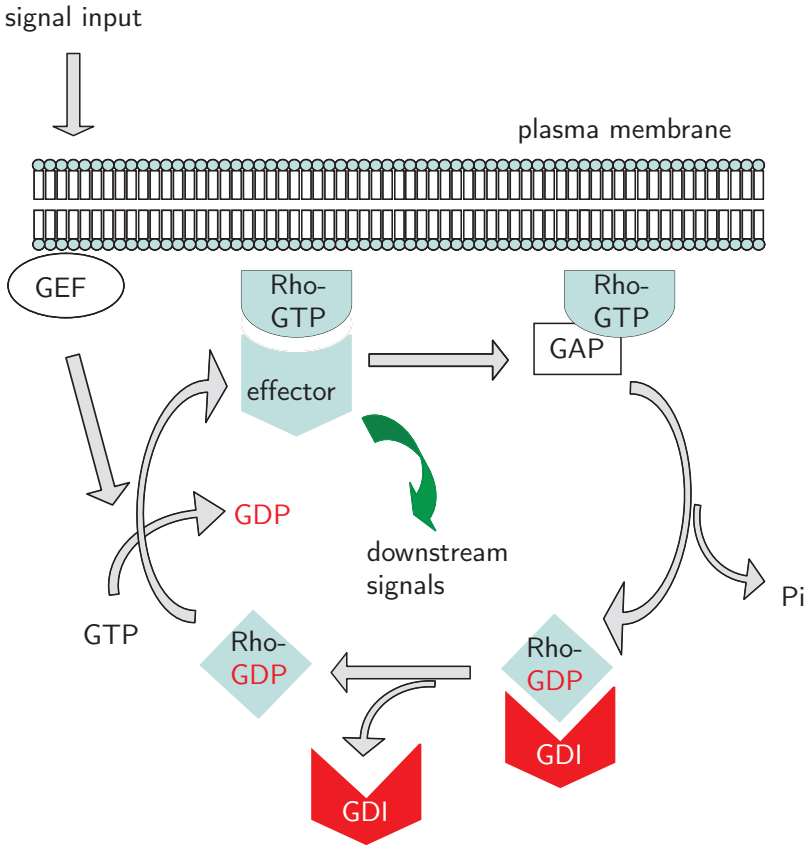

Fig. 1.2.: **Rho GTPase localization- and activity cycle.** Rho GTPase cycles between the inactive GDP-bound and the active GTP-bound form. Their activity is regulated by guanine nucleotide exchange factor (GEF), GTPase-activating protein (GAP) and guanine nucleotide dissociation inhibitor (GDI).

The majority of Rho GTPases are postranslationally modified by geranyl-geranyltransferases (e.g., RhoA, Rac1 and Cdc42). Others are processed solely by farnesyltransferases (e.g., RhoD, RhoE/Rnd3) or by both enzymes (such as RhoB) (Adamson et al., 1992; Cox et al., 1992; Lebowitz et al., 1997; Du et al., 1999). These modifications allow the activated Rho GTPases to associate with the cell membrane, where they exert their function. Furthermore the GTPases Rac1, Cdc42, RhoA, RhoB, RhoC and RhoG proteins harbor a specific sequence (^{64}`ydRIRplSYp`^{73}) that is recognized by Akt kinase (Kwon et al., 2000). The putative Akt kinase phosphorylation site (Ser-71) is located between the effector binding domain and the GTP-binding domain. The major effect of the Rac1 phosphorylation by Akt kinase is the inhibition of the GTP-binding without affecting the GTPase activity. In addition to Rac1 the Akt kinase modulates Rac1 GTPase regulatory proteins including BAD, 6-phosphofructo-2-kinase, glycogen synthase kinase-3, IRS-1, caspase-9 and human telomerase reverse transcriptase.

1.5. Functional consequence of the toxin-induced Rho GTPase glucosylation

The large clostridial cytoxins specifically glucosylate Rac1, Cdc42 or Ras at position Thr-35 or RhoA/B at the corresponding Thr-37 (Just et al., 1995a; Just et al., 1996; Just et al., 1995b). These target amino acid residues are highly conserved among the Rho GTPases and are located in the switch 1 region of these proteins which are involved in effector coupling. The threonine is preferentially modified in the GDP-bound form. In the GTP-bound form the threonine is participated in Mg^{2+} and nucleotide binding and is less accessible for the large clostridial cytoxins (Ihara et al., 1998).

The glucosylation inactivates the Rho GTPases (see figure 1.3). As a consequence, the effector coupling is inhibited and subsequently the signal transduction pathways are blocked. Furthermore, the nucleotide exchange by GEFs is prevented and the intrinsic and the GAP-stimulated GTPase activity is inhibited (Sehr et al., 1998; Herrmann et al., 1998). The glucosylation also blocks the interaction with GDI resulting in an accumulation of glucosylated GTPases at the plasma membrane (Genth et al., 1999).

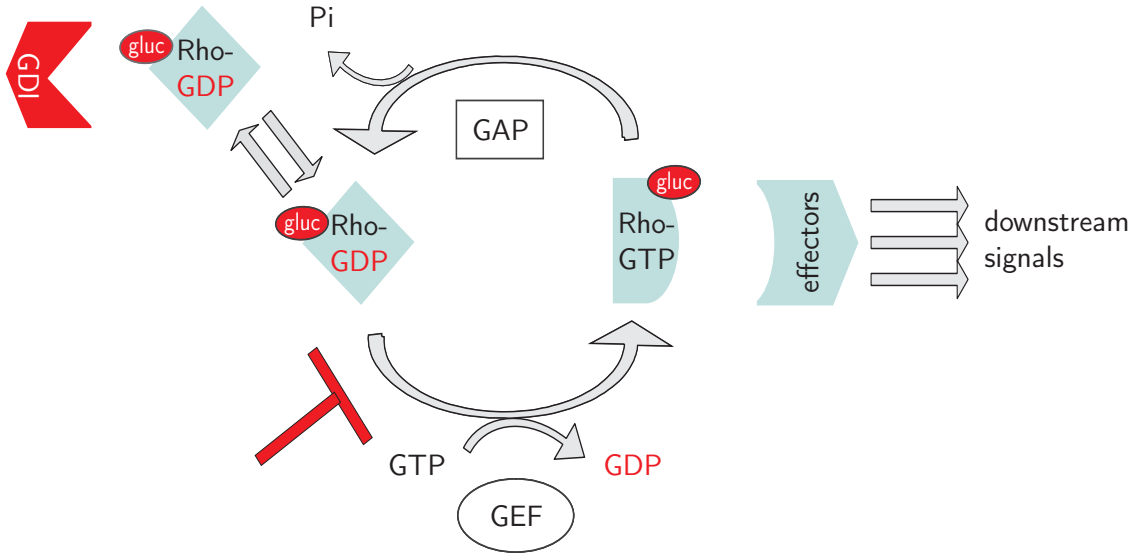

Fig. 1.3.: **Inactivation of Rho GTPases by glucosylation.** The most important consequence of glucosylation is the inhibition of effector coupling and subsequent blocking of signal transduction pathways.

Very recent investigations have shown that besides the amino acid residue Thr-37 also the Ser-73 of RhoA (Ser-71 of Rac1 and Cdc42) is important for interaction with the toxins (Jank et al., 2006; Jank et al., 2007). This amino acid is located at the end of the switch 2 region of the Rho GTPases. On the basis of the crystal structure of RhoA-GDP, Ser-73 is surface located in close vicinity to the acceptor Thr-37. Therefore, it is likely that this region is involved in the interaction with the glucosylating toxins. It is reasonable to assume that the polar Ser-73 in RhoA supports dipolar interactions with *Clostridium difficile* toxin B.

1.6. EGF signaling pathways

Binding of the epidermal growth factor (EGF) to its specific receptor (EGFR) induces dimerization of the receptor subunits and intermolecular transphosphorylation of several tyrosine residues within the cytoplasmic domains (Ullrich and Schlessinger, 1990). These tyrosines serve as binding sites for signaling proteins that contain Src homology 2 (SH-2) (such as Grb2) (Batzer et al., 1994) or phosphotyrosine-binding (PTB) domains and elicit the biological effects of the EGF receptor (EGFR) (Rozakis-Adcock et al., 1992; von Kriegsheim et al., 2006) by activation of different signal pathways (see figure 1.4).

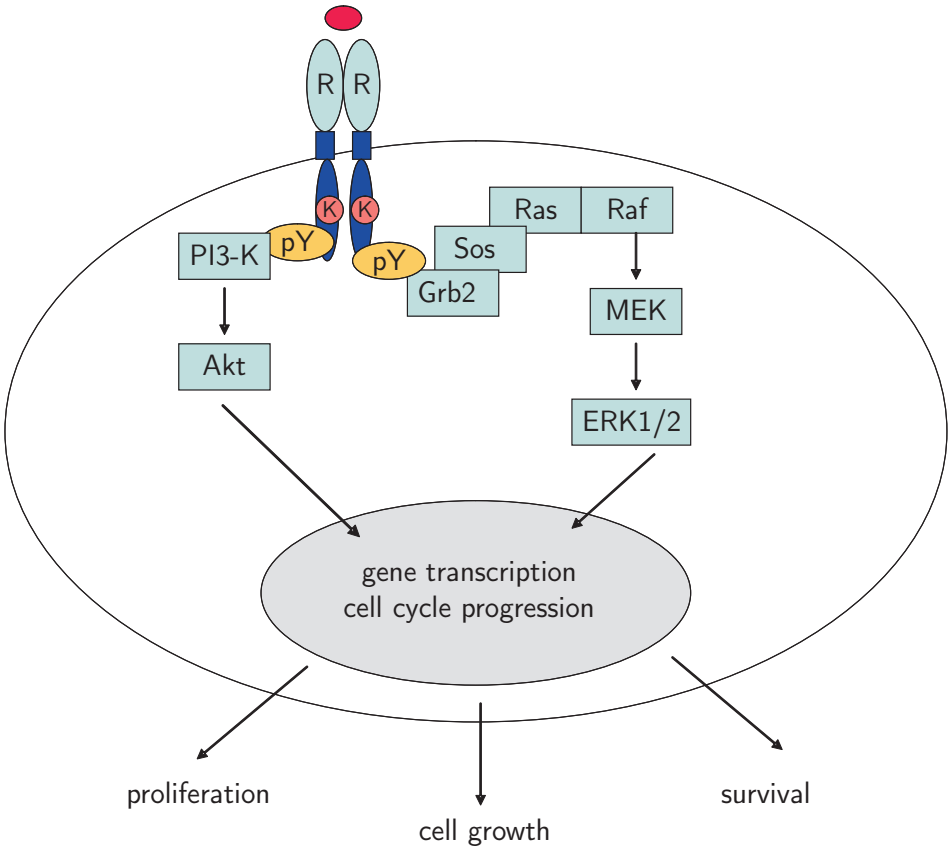

Fig. 1.4.: **Epidermal growth factor signaling pathways.** EGF exerts the biological effects by activating two signaling pathways: the Ras/Raf/MEK/ERK1/2 pathway and the PI3K/PDK 1/Akt/NF-κB pathway. These pathways are implicated in cell proliferation, cell growth and survival.

The Ras/Raf/MEK/ERK1/2 pathway is the best characterized EGF activated signal pathway: the interaction of the adaptor protein Grb2 with the phosphorylated receptor results in targeting of the guanylnucleotide exchange factor Sos to the membrane, where the Ras GTPases are located. Subsequently, activated GTP-bound Ras recruits the Ras activated factor (Raf) kinase to the plasma membrane resulting in Raf activation and phosphorylation of its downstream target MAPK/ERK kinase (MEK). MEK then induces the phosphorylation of extracellular regulated kinase (ERK) 1/2 (Kyriakis et al., 1992; Pearson et al., 2001). The activated ERKs are transported in the nucleus where they phosphorylate the transcription factors Elk1 and c-fos.

This pathway implicates cell proliferation, differentiation, cell growth and mitogenesis (LeVea et al., 2004; Kolch et al., 1991; Zhang and Liu, 2002; Mahimainathan et al., 2005).

Furtheron EGF activates the phosphatidylinosiol 3-kinase (PI3-K) to catalyze the formation of PIP3 via phosphorylation of PI(4,5)P2. The binding of PIP3 to the PH domain anchors Akt at the plasmamembrane and allows its phosphorylation and activation by 3-phosphoinositide-dependent protein kinase-1 (PDK1), another PH domain-containing protein. PI3-K suppresses apoptosis and promotes cell survival through activation of its downstream PDK1/Akt and NF-κB (Fresno Vara et al., 2004).

The activation or inactivation of Akt kinase is one of the critical regulatory points to deliver either a survival or an apoptotic signal. The Akt kinase has a direct effect on the apoptosis pathway, i.e. targeting the pro-apoptotic Bcl-2 related protein (BAD). It also affects the transcriptional response to apoptotic stimuli, for example by affecting the Forkhead factors and NF-κB as well as the activity of the p53 family. Additionally, Akt regulates the cell metabolism (Song et al., 2005).

2. Aim of this project

Treatment of the intestinal mucosa with the epidermal growth factor (EGF) has a protective effect against *Clostridium difficile* toxin-induced alteration of the intestinal barrier function. The aim of this study was to delineate the underlying mechanism by which EGF reduces the effect of *Clostridium difficile* toxin A (TcdA).

Three aspects have to be investigated:

The functional outcome of treatment with EGF. To investigate this, the intestinal epithelial model cell line CaCo-2 was used because of the advantage to measure transepithelial electrical resistance of the cell monolayer. Literature data strongly suggests phosphorylation of small GTP-binding proteins of the Rho family resulting from EGF treatment of cells. A correlation of Rac1 phosphorylation and reduction of TcdA-induced effects has to be shown.

Characterization of phosphorylated Rac1. The impact of Ser-71 phosphorylation on signal transduction of Rac1 has to be characterized with respect to nucleotide binding and effector coupling. A crucial point is to show whether phosphorylated Rac1 is capable of saving signal transduction. The generation of a phosphomimetic mutant, where Ser-71 is exchanged for glutamate, has to be performed to study the features of phosphorylated Rac1 in more detail.

Proof of protective effect of Rac1 S71E. The causal relationship of Rac1 Ser-71 phosphorylation and the protective effect against glucosylating toxins has to be shown in transfection experiments using the mutant Rac1 S71E. Only the evidence of Rac1 S71E being capable of reducing TcdA-induced morphological changes of cells will provide a conclusive mechanism by which phosphorylated Rac1 contributes to the protective effect of EGF.

3. Materials and Methods

3.1. Materials

Recombinant human epidermal growth factor (EGF) was purchased from R&D Systems (MN, USA). Glutathione Sepharose and Benzamidine Sepharose were from Amersham Biosciences (NJ, USA). Quickchange XL site-directed mutagenesis kit was from Stratagene (TX, USA) and TOPO-Cloning Kit from Invitrogen (CA, USA). Guanosine-5'-[γ-thio]triphosphate tetrasodium salt solution (GTP[γ]S), 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS), glutaraldehyde (25%) and 4',6'-Diamidin-2'-phenylindol-dihydrochlorid (DAPI) were from Sigma (MO, USA). Guanosine-diphosphate (GDP) was obtained from Boehringer Mannheim. UDP-[^{14}C]glucose (300 $^{\mathrm{mCi}}/_{\mathrm{mmol}}$) was from Biotrend (Germany) whereas [^{32}P]NAD (800 $^{\mathrm{Ci}}/_{\mathrm{mmol}}$) was purchased from Perkin Elmer (MA, USA). Akt-inhibitor II (SH-5) was from Calbiochem (NJ, USA). Active Akt1 was from Stressgen (BC, Canada). Fugene HD transfection reagent was from Roche (Germany). *Bacillus megaterium* protoplasts was from MoBITec (Germany). N-methylanthraniloyl (Mant)-GDP and Alexa Fluor 568 phalloidin were from Molecular Probes (OR, USA). All other chemicals were of the highest purity available. Oligonucleotides were purchased from Operon (Germany) and from MWG-Biotech AG (Germany). The lethal toxin from *Clostridium sordellii* (TcsL) was provided by Harald Genth (Hannover Medical School).

antigen	*mono-clonal*	*host*	*company*
Rac1 (clone 102)	yes	mouse	BD Biosciences Pharmingen, NJ, USA
Rac1 (clone 23A8)	yes	mouse	Upstate, NY, USA
RhoA (26C4)	yes	mouse	Santa Cruz Biotechnology, CA, USA
Phospho-Rac1/Cdc42 (Ser-71)	no	rabbit	Cell Signalling, MA, USA
Phospho-Akt (Ser-473)	no	rabbit	Cell Signalling, MA, USA
Tubulin-α (clone YL1/2)	yes	rat	Linaris, Germany
HA (clone HA-7)	yes	mouse	Sigma, MO, USA

Tab. 3.1.: Primary antibodies used

host	*animal*	*conjugated*	*company*
goat	rabbit	peroxidase	Rockland, PA, USA
goat	mouse	peroxidase	Rockland, PA, USA
goat	mouse	Alexa Fluor 594	Molecular Probes, OR, USA
goat	mouse	Alexa Fluor 488	Molecular Probes, OR, USA
goat	rat	Fluoprobes S 488	Interchim, France

Tab. 3.2.: Secondary antibodies used

3.2. Methods

3.2.1. Cell biology methods

3.2.1.1. Cell culture

CaCo-2 cells were cultured in DMEM medium, supplemented with 10% $^{v}/_{v}$ fetal bovine serum (FBS), 100 $^{U}/_{ml}$ penicillin, 100 $^{\mu g}/_{ml}$ streptomycin and 1% $^{v}/_{v}$ non-essential amino acids at 37 °C and in a humidified atmosphere with a CO_2 content of 5%.

Hep-2 cells were grown in MEM Eagle's medium, supplemented with 10% $^{v}/_{v}$ FBS, 100 $^{U}/_{ml}$ penicilline, 0.1 $^{mg}/_{ml}$ streptomycine at 37 °C and in a humidified atmosphere with a CO_2 content of 5%.

Both cell lines were subcultured twice a week: they were washed once with PBS and then incubated with Trypsin-EDTA solution for 5-10 min at 37 °C. Afterwards, they were resuspended in their cell culture medium and seeded in flasks or appropriate multiwell plates.

3.2.1.2. Transepithelial electrical resistance (TER)

TER was determined by epithelial Voltohmmeter (EVOM) equipped with Endom 24-chamber for 12-well filter inserts. An AC square wave (20 µA) was applied across the confluent and 7 days old CaCo-2 cell monolayers, which were grown on transwell filters (pore size 0.4 µM, Becton Dickinson). Only monolayers with an initial resistance of >150 $\Omega \cdot cm^2$ were used for experiments. TcdA was added only to the apical compartment, whereas EGF was added to both the apical and basolateral compartment. The voltage was measured and the electrical resistance was calculated.

3.2.2. Biochemical methods

3.2.2.1. Expression of recombinant *Clostridium difficile* toxin A (TcdA)

Recombinant TcdA was expressed as His-tagged protein using the *Bacillus megaterium* expression system (Burger et al., 2003). His-tagged toxin A was purified from the soluble fraction by affinity chromatography using HiTrap chelating HP column loaded with Ni^{2+}. Purified TcdA was stored in 20 mM Tris pH 7.2, 50 mM NaCl, 20% $^{v}/_{v}$ glycerol. For experiments only the recombinant *Clostridium difficile* toxin A was used.

3.2.2.2. Expression of recombinant proteins

GTPases, Pak-Crib domain and Rho-GDI were expressed as GST-fusion proteins in *E. coli* after induction with 100 µM isopropyl-D-thiogalatopyranosid (IPTG) for 3 hours at 37 °C. Affinity purification was performed using Glutathione Sepharose. The GST-tagged GTPases were cleaved by thrombin (1.5 U) in 0.5 ml thrombin buffer (50 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$) overnight at 4 °C. Thrombin was removed from supernatants by precipitation with benzamidine Sepharose at 4 °C.

3.2.2.3. Phosphorylation of recombinant Rac1

Recombinant Rac1 (600 ng) was incubated with the activated Akt kinase (1 µg) in 50 µl kinase buffer (0.5 mM ATP, 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.2 mM EGTA, 100 $\mu g/ml$ BSA, 0.1 M KCl, 1 mM sodium orthovanadate, 20 mM Hepes pH 7.2) for 90 min at 30 °C. Samples were submitted to SDS-PAGE and Western blotting. Ser-71 phosphorylation of Rac1 was checked using a specific Phospho-Rac1 antibody.

3.2.2.4. Pull-down assays and nucleotide exchange

After EGF-stimulation and toxin-treatment, the culture medium was removed and CaCo-2 cells were washed with ice-cold PBS. Cells were lysed by adding 500 µl Fish-buffer (50 mM Tris pH 7.4, 2 mM $MgCl_2$, 10% v/v glycerol, 100 mM NaCl, 1% v/v NP40, 0.5 mg/ml BSA). After 5 min incubation on ice the lysates were centrifugated at 16.000 g for 5 min. The supernatant was used for pull-down experiments. 20 µl of bead slurry consisting of approximately 15 µg GST-Pak-Crib domain or GST-Rho-GDI were added to each sample and rotated at 4 °C for 45 min. The beads were collected by centrifugation at 10.000 g and washed twice with Fish-buffer. GTPases were eluted by incubation with Laemmli buffer at 95°C for 5 min. Samples were submitted to SDS-PAGE and Western blotting.

Pull-down experiments with recombinant Rac1, Rac1 S71E, Cdc42 and Cdc42 S71E (300 ng each) were performed in 500 µl Fish-buffer under identical conditions.

The exchange of nucleotides bound to the forementioned GTPases was done using 10 mM EDTA to extract the bound nucleotide. After loading with 0.1 mM GDP or GTP[γ]S at 30 °C for 15 min, 50 mM $MgCl_2$ was added to stabilize the GTPase nucleotide complex.

3.2.2.5. Glucosyltransferase assay

Recombinant Rac1, Rac1 S71E, Cdc42 or Cdc42 S71E (2 µg each) were incubated in 20 µl glucosylation buffer (50 mM Hepes pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 µM UDP-[^{14}C]glucose (300 $mCi/mmol$)) at 37 °C. The glucosylation reaction was started by addition of 30 ng recombinant TcdA. Samples of 20 µl were taken at indicated time points and the reaction was stopped by adding Laemmli buffer followed by 2 min boiling at 95 °C. SDS-PAGE and subsequent filmless autoradiography (Cyclon, Canberra Packard) was performed for documentation of [^{14}C]glucosylated GTPases. OptiQuant software (Canberra Packard) was used for quantification of detected signals.

The competition study of glucosylation reaction was performed with either 500 ng recombinant Rac1 alone or in combination with Rac1 S71E in a molar ratio of 1:1 and 1:10 in 30 µl glucosylation buffer. The reaction was started by addition of 20 ng of recombinant TcdA and 8 µM UDP-[^{14}C]glucose (300 $^{mCi}/_{mmol}$) and stopped after 15 min.

In the time-dependent competition study either 500 ng recombinant Rac1 alone or in combination with 2 µg Rac1 S71E was incubated in 25 µl glucosylation buffer with 20 ng of recombinant TcdA and 10 µM UDP-[^{14}C]glucose (300 $^{mCi}/_{mmol}$). Samples of 20 µl were taken at indicated time points. SDS-PAGE and documentation of [^{14}C]glucosylated GTPases were performed as described previously.

3.2.2.6. [^{32}P]ADP-ribosylation

Untreated Caco-2 cells and cells treated with EGF (100 $^{ng}/_{ml}$, 120 min), TcdA(300 $^{ng}/_{ml}$, 120 min) or a combination of EGF followed by TcdA were harvested in PBS. After cell lysis with sonication, 20 µg of protein was used for reaction. The ADP-ribosylation was performed using 0.75 µCi [^{32}P]NAD (800 $^{Ci}/_{mmol}$) and 1 µg of exoenzyme C3 in 20 µl ribosylation buffer (50 mM Hepes pH 7.4, 5 mM $MgCl_2$, 2.5 mM DTT, 2.5 µM NAD, 10 mM thymidine) for 30 min at 37 °C. SDS-PAGE and filmless autoradiography (Cyclon, Canberra Packard) was performed for documentation of [^{32}P]ADP-ribosylated RhoA. OptiQuant software (Canberra Packard) was used for quantification of detected signals.

3.2.2.7. Mant-GDP binding

To study the nucleotide binding of the GTPases the binding of the fluorophore-coupled GDP (MANT-GDP) was detected. Recombinant Rac1 or Rac1 S71E (each 20 µg) were equilibrated in a quartz glass cuvette in 500 µl triethanolamine buffer (150 mM NaCl, 2.5 mM $MgCl_2$, 10 mM Triethanolamine (TEA) pH 7.5) for 2 min at 30 °C. The photometrical detection was started by adding Mant-GDP to a final concentration of 100 µM. The fluorescence was measured every 0.5 s (excitation 357 nm, emission 444 nm) in a Jasco FP-6500 spectrofluorometer over a period of 300 s.

3.2.2.8. Glutaraldehyde cross-linking

Appropriate GTPases Rac1, Rac1 S71E, RhoA, Rac1ΔC-8 and Rac1 S71EΔC-8 (each 5 µg) were first loaded with 0.1 mM GDP or GTP[γ]S in 15 µl glucosylation buffer (50 mM Hepes pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $MnCl_2$) for 15 min at 30 °C, and then incubated with Rac1 S71E (5 µg) or Pak-Crib domain for 30 min at 4 °C. Cross-linking of associated proteins was performed by adding glutaraldehyde to a final concentration of 2 mM. Samples were incubated for 30 min at 37 °C. Glycine was added to a final concentration of 200 mM to stop the cross-linking reaction and to remove unreacted glutaraldehyde. The reaction mixture was further incubated for 60 min at RT. The proteins were separated by SDS-PAGE and visualized by Coomassie staining.

3.2.2.9. Gel filtration chromatography by Superdex 75 column

A Superdex 75 column (HR 10/30) gel filtration column (Amersham Biosciences) coupled to a Bio-Rad biologic liquid chromatography system was used to analyze the homo- and heterophilic interactions of the small GTPases Rac1, RhoA (Genbank Acc. No. L25080) and Rac1 S71E. For analysis 60 µg of Rac1, RhoA, Rac1 S71E or a combination of these GTPases were incubated in 100 µl glucosylation buffer (50 mM Hepes pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $MnCl_2$) in the presence of 100 µM GDP for 30 min on ice. The mixture was then loaded onto the column and eluted with buffer containing 50 mM Hepes pH 7.6, 5 mM $MgCl_2$, 150 mM NaCl and 1 mM DTT at a flow rate of 0.4 ml/min. The elution profiles were monitored by UV absorption at 280 nm.

3.2.3. Immunological methods

3.2.3.1. Western blot analysis

Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membrane using "semi-dry" techniques (17 V for 72 min). After blocking with 5% w/v nonfat dry milk in TBST (50 mM Tris HCl pH 7.2, 150 mM NaCl, v/v 0.05% Tween 20) the membrane was incubated overnight with the primary antibody at 4 °C. Then it was washed thoroughly with TBST and incubated for 1 hour with horseradish peroxidase conjugated goat anti-rabbit or anti-mouse IgG at RT. The detection was performed by chemiluminescence (Super Signal West Femto, Pierce). The signals were analyzed densitometrically using the KODAK 1D software.

3.2.3.2. Transfection and immunofluorescence staining

Hep-2 cells were seeded onto coverslips in 24-well plates 24 hours prior to transfection. Transfection was performed according to manufacturer's protocol using Fugene HD (Roche). After 20 hours untreated cells or those treated for 4 hours with TcdA (150 ng/ml) or for 5 hours with TcsL (600 ng/ml) were fixed in 4% v/v formaldehyde/4% w/v sucrose in PBS, permeabilized for 5 min in 0.2% v/v Triton X-100 in PBS and then blocked for 1 hour with 5% w/v BSA in PBS at RT. Cells were stained with HA antibody (1:500 diluted in 1% w/v BSA in PBS) for 45 min at RT followed by oregon green conjugated goat anti-mouse secondary antibody (1:500 in 1% w/v BSA in PBS) for 45 min. Filamentous actin was labeled with Alexa Fluor 568 conjugated to phalloidin (3 U/ml in PBS). Microtubules were stained with Tubulin-α antibody (1:500 diluted in 1% w/v BSA in PBS) for 45 min at RT followed by goat anti-rat Fluoprobes S 488 secondary antibody (1:500 in 1% w/v BSA in PBS) for 45 min. Subsequently, the nuclei was counterstained with DAPI. The coverslips were mounted onto glass slides with Mowiol or Antifade mounting medium (Molecular Probes) and subjected to fluorescence microscopy (Zeiss Axiovert 200).

3.2.3.3. TcdA binding assay

The binding of the GTPases to the recombinant TcdA was investigated in the following assay. The microtiter plates were first coated with recombinant TcdA (30 ng per well in 100 µl PBS) at RT for 1 hour, and afterwards blocked for 1 hour with TBST (50 mM Tris HCl pH 7.2, 150 mM NaCl, 0.05% $^{v}/_{v}$ Tween 20). Binding of Rac1 and Rac1 S71E to the coated plates was performed in 100 µl PBS for 1 hour using indicated amounts of the GTPases. Then the microtiter plates were washed three times with TBST and bound Rac1 or Rac1 S71E was detected by anti-Rac1 ((clone 23A8) 1:1000 in TBST) for 1 hour. The plate was washed three times, probed with horseradish peroxidase conjugated secondary anti-mouse IgG (1:1000 in TBST) followed by three washing cycles. 30 min after addition of 100 µl 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate the absorption was determined at 405 nm by Anthos 2020 ELISA reader.

3.2.4. Molecular biology methods

3.2.4.1. Generation of phospomimetic Rac1 and Cdc42 mutants

Mutation of serine 71 of Rac1 (Genbank Acc. No. M29870) and Cdc42 (Genbank Acc. No. M35543) to glutamate was performed by Quickchange XL site-directed mutagenesis kit using the following primers for amplification:

Rac1 S71E sense: `5'-GATTACGCCCCCTAGAATATCCGCAAACAGATGTG-3'`
Rac1 S71E antisense: `5'-CACATCTGTTTGCGGATATTCTAGGGGGCGTAATC-3'`
Cdc42 S71E sense: `5'-GATTACGACCGCTGGAATATCCACAAACAGATGTA-3'`
Cdc42 S71E antisense: `5'-TACATCTGTTTGTGGATATTCCAGCGGTCGTAATC-3'`

Phosphomimetic mutants of pGEX-2T Rac1, pcDNA3.1+ Rac1 (3× HA), pGEX-2T Cdc42 and pcDNA3.1+ Cdc42 (3× HA) were generated.

The PCR reactions contained: 2.5 mM dNTPs, 10 ng of the respective expression vector, 125 ng sense primer, 125 ng antisense primer, 5 µl 10× reaction buffer, 3 µl QuickSolution and 2.5 U Pfu Turbo DNA-polymerase. Water was added to obtain the final volume of 50 µl. The following PCR conditions were used: one cycle of 95 °C for 1 min, 18 cycles of 95 °C for 50 sec, 60 °C for 50 sec and 68 °C for 8 min followed by one cycle of 68 °C for 10 min. After digest of the parental, methylated DNA with DpnI (20 U) at 37 °C the transformation in XL-Gold ultracompetent cells was performed according to standard protocol by using 5 µl of DpnI digested PCR product.

3.2.4.2. Generation of C-terminal truncated GST-fusion Rac1 and Rac1 S71E

The carboxyl-terminal truncated Rac1ΔC-8 and Rac1 S71EΔC-8 were generated by PCR-based amplification technique using Taq-polymerase and the following primers:

Rac1ΔC-8 sense: `5'-GGATCCATGCAGGCCATCAAGTGTGTGGTGG-3'`
Rac1ΔC-8 antisense: `5'-GAATTCCTACTTCTTCACGGGAGGCGGGCAGAGG-3'`

The PCR reactions contained: 2.5 mM dNTPs, 25 ng DNA, 100 ng sense primer, 100 ng antisense primer, 5 µl 10× S (Taq-polymerase)-buffer and 5 U Taq-polymerase. Water was added to obtain the final volume of 50 µl. The following PCR program was used: one cycle of 94 °C for 1 min, 20 cycles of 94 °C for 30 sec, 68 °C for 30 sec and 68 °C for 1 min and followed by one cycle at 68 °C for 1 min.

The PCR product was cloned into the TOPO-vector (TOPO-Cloning Kit). The coding sequence for truncated Rac1 and Rac1 S71E was mobilized from TOPO-vector by BamH I and EcoR I restriction enzyme digest and ligated into an appropriately cleaved pGEX-2T vector. Truncated GST-fusion Rac1 and Rac1 S71E were expressed as described in chapter 3.2.2.2.

4. Results

4.1. Effect of treatment with EGF on Rho GTPases

4.1.1. EGF reduces the effect of *Clostridium difficile* toxin A on epithelial barrier function

A sensitive method to determine alteration of the barrier function is the measurement of the transepithelial electrical resistance (TER). Each measurement was standardized by setting the TER to 100% at the time point of addition of the substances (see figure 4.1).

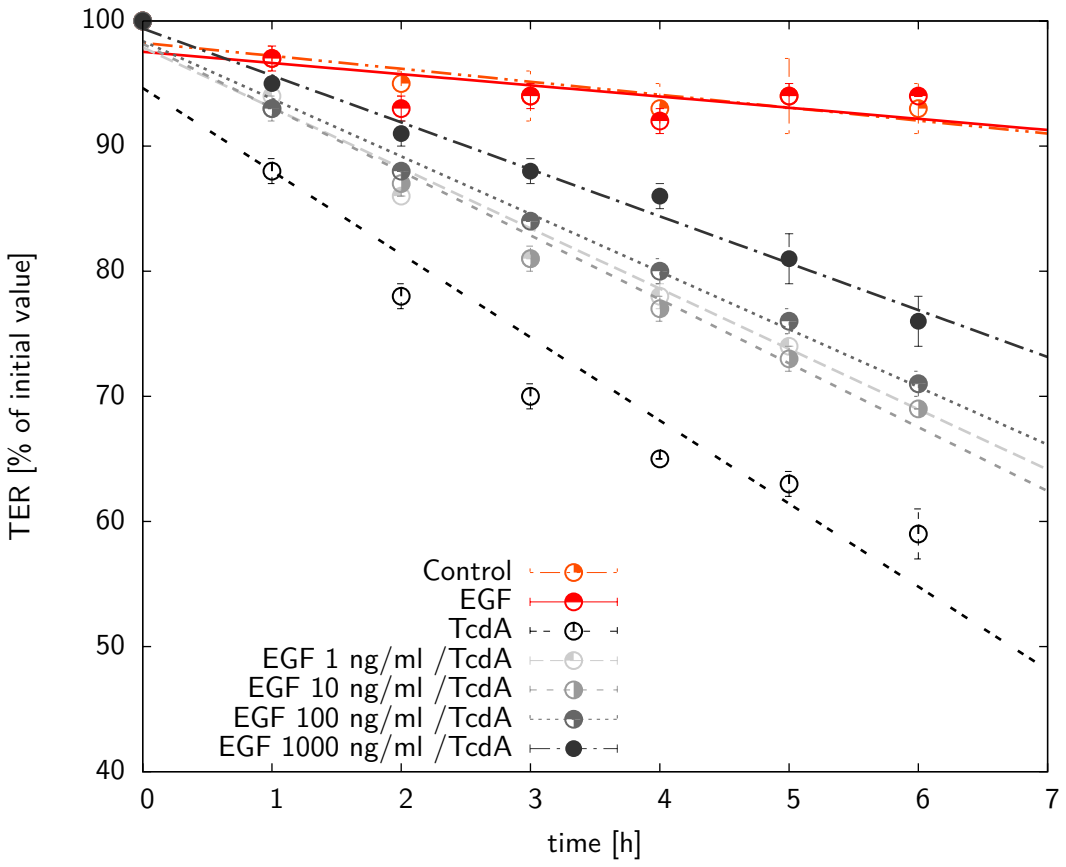

Fig. 4.1.: **EGF attenuated TcdA-induced cytopathic effect.** Measurement of the transepithelial electrical resistance (TER) was performed to elucidate the concentration-dependent effect of EGF on the *Clostridium difficile* toxin A-induced changes on the CaCo-2 monolayer. The initial level was set to 100%. All values are expressed as means ± standard deviation (n=3).

EGF alone did not affect the TER. TcdA (80 ng/ml) caused a decrease in TER to 60±2% within 6 hours. Preincubation of CaCo-2 cells with 10, 100 and 1000 ng/ml of EGF reduced concentration-dependently the effect of TcdA to 70±1%, 71±1% and 77±2%, respectively.

Thus, EGF attenuates the TcdA-induced alteration on epithelial barrier function.

4.1.2. EGF induces phosphorylation of Rac1 and Cdc42 via Akt kinase

EGF activates the PI3-kinase/Akt pathway. By Western blot analysis we examined whether Rac1 and Cdc42 are downstream targets of the EGF-receptor signal pathway. EGF treatment of CaCo-2 cells induced phosphorylation of Akt as well as of Rac1 and Cdc42 in a time-dependent manner. The level of phosphorylated Akt and Rac1/Cdc42 were increased by the factor four after 120 min (see figure 4.2). The phospho-specific antibody cannot differentiate between phosphorylated Rac1 and Cdc42. Therefore, it is assumed that phosphorylation of both, Rac1 and Cdc42 occurs if not shown otherwise in a recombinant system.

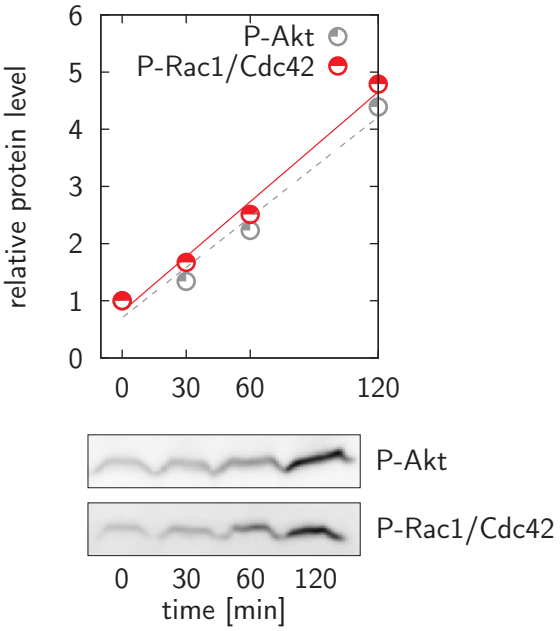

Fig. 4.2.: **EGF induced phosphorylation of Akt and Rac1/Cdc42.** CaCo-2 cells were treated with EGF (100 ng/ml) for 120 min. Western blot analysis of cell lysates was performed with anti-P-Akt (Ser-473) and anti-P-Rac1/Cdc42 (Ser-71). Western blot signals were analyzed densitometrically and displayed in the graph. The initial level of phosphorylated Akt or Rac1/Cdc42 was set to 1.0.

To determine whether Rac1/Cdc42 are phosphorylated by Akt kinase, the specific inhibitor SH-5 (10 µM) was applied to cells before EGF treatment. The inhibitor alone had no effect on the level of phosphorylated Rac1 and Cdc42 whereas the EGF-induced Rac1/Cdc42 phosphorylation was reduced to control level (see figure 4.3).

This data confirms that Akt is the upstream kinase, eventually leading to Rac1 and Cdc42 phosphorylation.

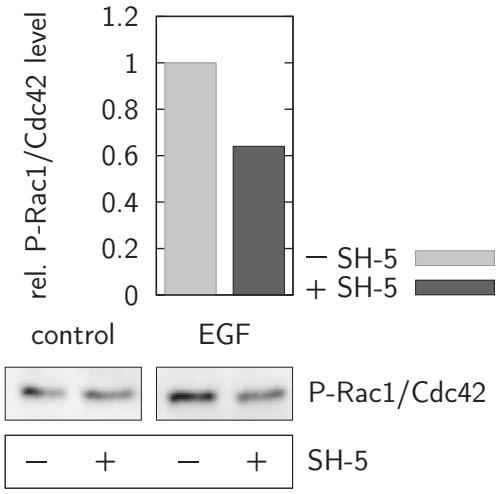

Fig. 4.3.: **EGF induced phosphorylation of Rac1 and Cdc42 via Akt kinase.** CaCo-2 cells were treated with the Akt kinase inhibitor SH-5 (10 µM) 15 min before EGF (100 $^{ng}/_{ml}$) was applied for further 120 min. Western blot analysis of cell lysates was performed with anti-P-Rac1/Cdc42 (Ser-71). Western blot signals were analyzed densitometrically and displayed in the graph. The initial level was set to 1.0.

Additionally, the direct phosphorylation of Rac1 by Akt kinase was verified in a recombinant system. The phosphorylation of Rac1 was shown by Western blot analysis using the phospho-specific Rac1 antibody (see figure 4.4).

The Western blot analysis confirms that the activated Akt kinase directly phosphorylate Rac1 at Ser-71.

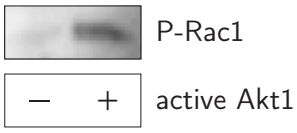

Fig. 4.4.: **Akt phosphorylated Rac1.** The phosphorylation of Rac1 by Akt1 kinase was investigated in a recombinant system. Recombinant Rac1 (12 $^{µg}/_{ml}$) was incubated with the active Akt1 kinase (20 $^{µg}/_{ml}$) for 90 min at 30 °C. Phosphorylation of Rac1 was checked by Western blot analysis with P-Rac1/Cdc42 (Ser-71) antibody.

These findings show that an EGF-induced increase in Phospho-Rac1 is due to phosphorylation by Akt1.

4.1.3. Ser-71 Phospho-Rac1/Cdc42 are associated with the effector protein Pak

The interaction of Rac1/Cdc42 with both the Crib-domain of the effector protein Pak as well as its inhibitor Rho-GDI was examined by pull-down assays. Western blot analysis of lysates from untreated and EGF treated cells revealed that phosphorylated Rac1/Cdc42 is bound to the effector protein Pak whereas no binding to its inhibitor Rho-GDI was observed (see figure 4.5, upper panel). The total amount of either P-Rac1/Cdc42 or RhoA is shown in figure 4.5 (lower panel). The functionality of Rho-GDI was analyzed by detection of RhoA, which was found to be associated with Rho-GDI.

This data indicates that Rac1 and Cdc42 are not negatively regulated by phosphorylation at Ser-71 but remain functional competent and active.

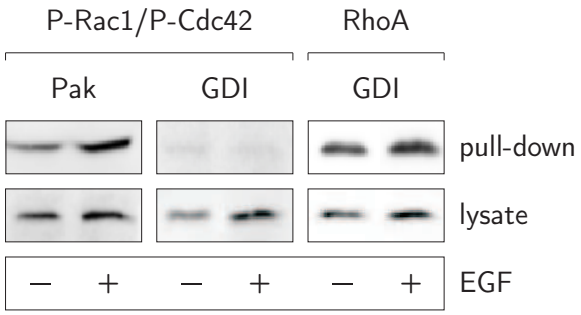

Fig. 4.5.: **Ser-71 phosphorylated Rac1/Cdc42 bound to effector protein Pak.** The lysates of untreated or EGF treated CaCo-2 cells (100 ng/ml, 120 min) were used for pull down assays using Pak-Crib domain and Rho-GDI. Total (lower panel) and precipitated (upper panel) phosphorylated Rac1/Cdc42 was detected by Western blot analysis. The detection of RhoA in Rho-GDI pull down was used as positiv control for functional Rho-GDI.

4.1.4. Treatment with EGF diminishes TcdA-glucosylation of Rac1 but not of RhoA

To study whether the protective effect of EGF correlates with a reduced TcdA-induced Rac1 glucosylation we performed Western blot analysis with specific antibodies recognizing only non-glucosylated or total Rac1. TcdA treatment of CaCo-2 cells decreased the level of non-glucosylated Rac1 over the observed time period of 4 hours to 40%. Preincubation with EGF (100 ng/ml) reduced the TcdA-induced Rac1 glucosylation, resulting in 70% of non-glucosylated Rac1 (see figure 4.6 - upper panel). The level of total Rac1 is shown in the lower panel, as it was not altered by TcdA treatment.

To test whether EGF also affects Rho glucosylation we performed a [^{32}P]ADP-ribosylation assay. Glucosylated RhoA is not substrate for exoenzyme C3. Thus, the level of ADP-ribosylated RhoA reflects the non-glucosylated pool. For comparison of RhoA- and Rac1 glucosylation in differentiated CaCo-2 cells 300 ng/ml TcdA was applied. This was the minimum concentration where a significant RhoA glucosylation was achieved and Rac1 glucosylation was not at its maximum. Lysates of untreated CaCo-2 cells, or cells treated with EGF (100 ng/ml, 120 min), TcdA (300 ng/ml, 120 min) or the combination of both were analyzed by Western blot analysis

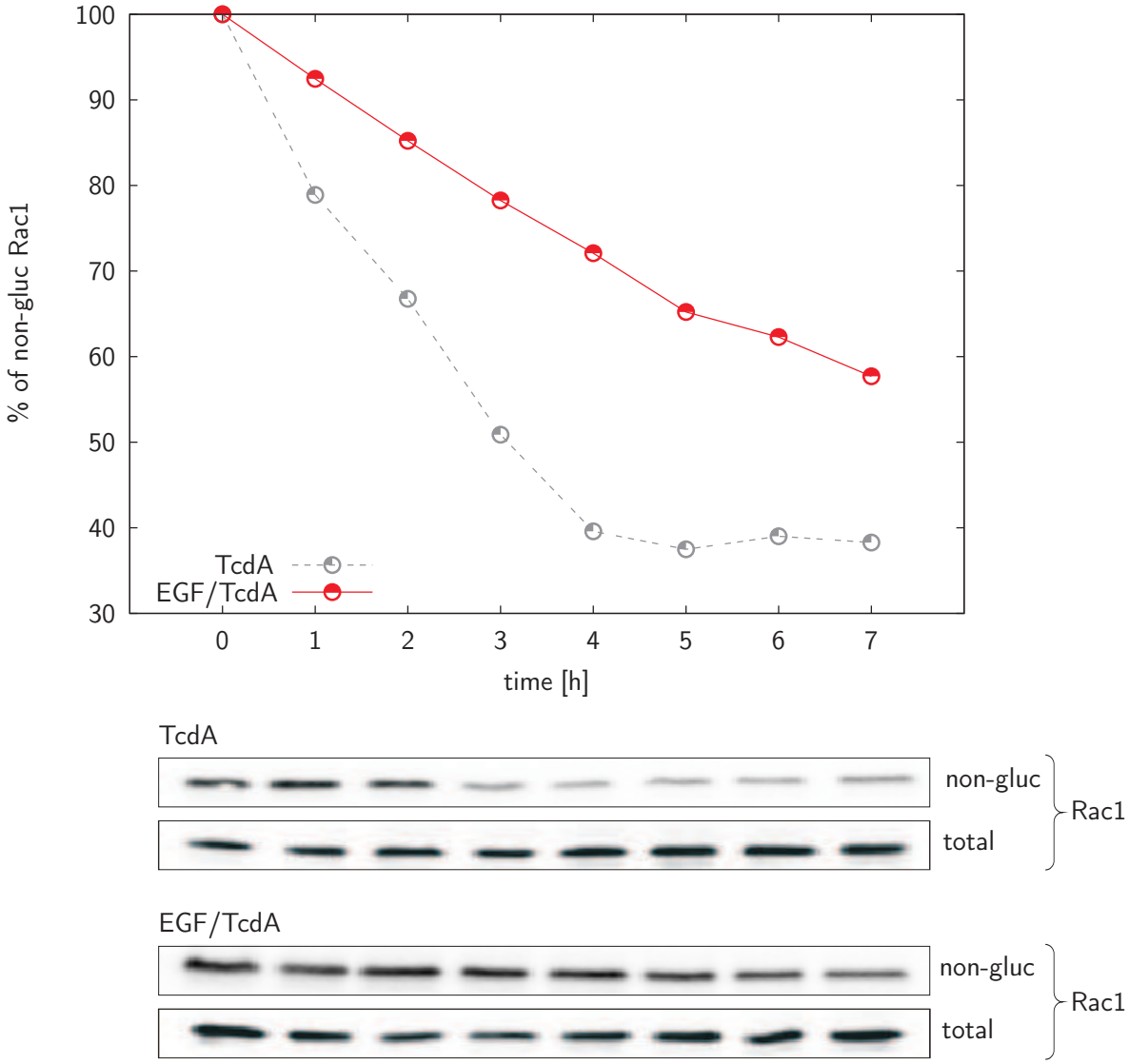

Fig. 4.6.: **Treatment with EGF diminished the TcdA-catalyzed Rac1 glucosylation.** Caco-2 cells were preincubated with EGF (100 ng/ml) for 120 min followed by TcdA treatment (80 ng/ml) for indicated time periods. Western blot analysis of lysates was performed with specific Rac1 antibodies recognizing the non-glucosylated form or the total Rac1. Signals were analyzed densitometrically and displayed in a graph.

using a Rac1 antibody, which only detects the non-glucosylated form. Protein levels were normalized to the initial value (see figure 4.7, dark bars). EGF alone had no effect on the Rac1 level. TcdA treatment alone led to a decrease to 16±1.5%, whereas preincubation of cells with EGF reduced the TcdA-induced Rac1 glucosylation to 28±3%.

The [^{32}P]ADP-ribosylation assay revealed that EGF treatment alone did not influence the level of ribosylated RhoA. No differences in the radioactive signals (60%±2%) of TcdA treated cells and cells pretreated with EGF (see figure 4.7, light bars) were observed. The TcdA-catalyzed glucosylation of RhoA was not affected by EGF.

The finding that glucosylation of RhoA was unaffected indicated that uptake of TcdA was not altered by EGF treatment.

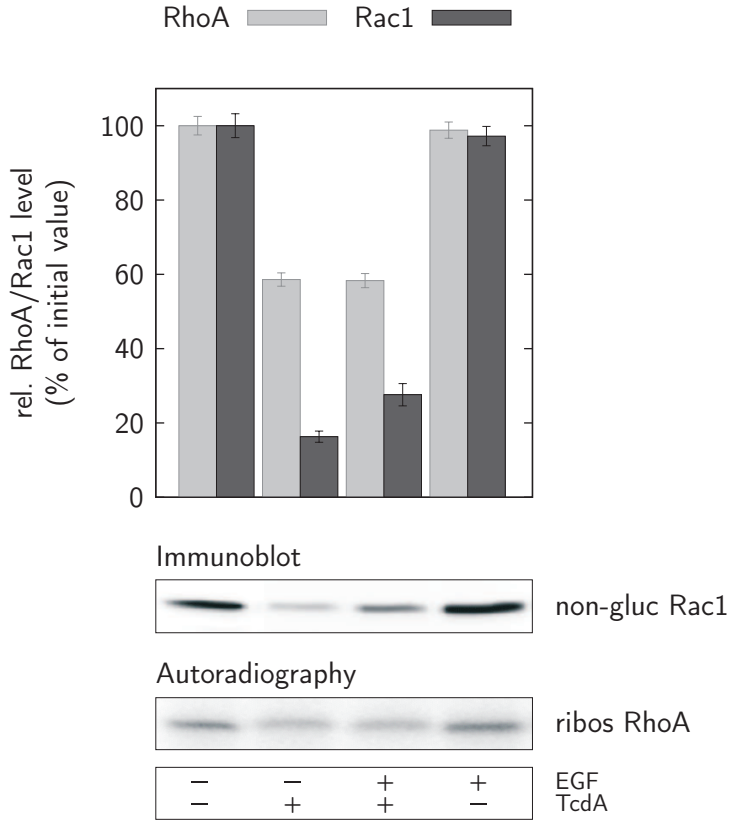

Fig. 4.7.: **Treatment of cells with EGF did not affect RhoA glucosylation.** Lysates of untreated CaCo-2 cells or cells treated with EGF (100 ng/ml, 120 min), TcdA (300 ng/ml, 120 min) or the combination of both were analyzed by Western blot using the Rac1 antibody which detects only the non-glucosylated form. Additionally, ADP-ribosylation of the lysates using [^{32}P]NAD and the exoenzyme C3 from *Clostridium botulinum* was performed to detect the non-glucosylated RhoA. The signals of non-glucosylated Rac1 and RhoA were analyzed densitometrically. The control level was set 100%. All values are expressed as means ± standard deviation (n=3).

Therefore, the protective effect of EGF is specific for Rac1 but not for RhoA.

4.2. Phosphomimetic Rac1 and Cdc42 mutant

4.2.1. Rac1 S71E and Cdc42 S71E bind to Pak or Rho-GDI in a nucleotide-dependent manner

To study the outcome of Ser-71 phosphorylation of Rac1 and Cdc42 in more detail we generated their phosphomimetic mutants (Rac1 S71E and Cdc42 S71E) by exchange of Ser-71 to the phosphomimetic amino acid glutamate (S71E). This amino acid exchange via mutagenesis is an established method to study the function of protein phosphorylation.

As the GTPases act as molecular switches between the GTP-bound active state and the GDP-bound inactive state, we first investigated the nucleotide binding using the fluorophore-coupled GDP (MANT-GDP). The change of the fluorescence emission of MANT-GDP reflects the nucleotide binding to the GTPases. The addition of MANT-GDP to GDP-loaded-Rac1 and Rac1 S71E resulted in an increase in MANT fluorescence emission within 150 s before reaching a plateau.

Both GTPases showed identical binding of this nucleotide (see figure 4.8), indicating that phosphorylation of Rac1 at Ser-71 does not interfere with the GDP-binding.

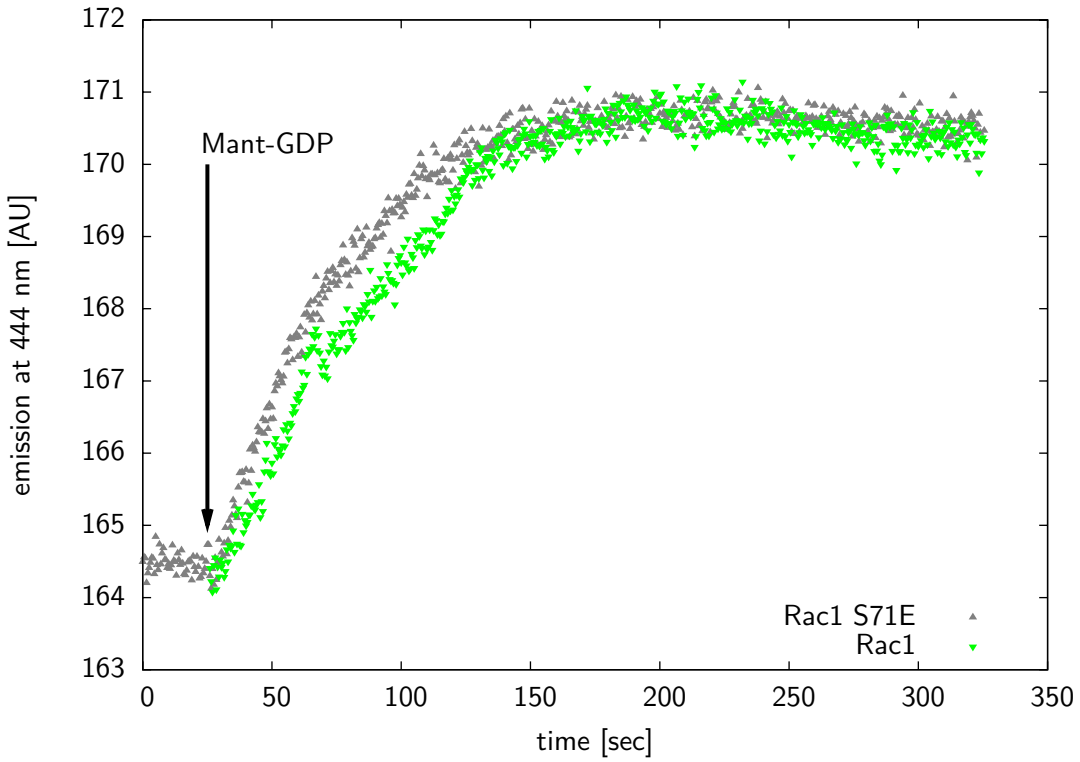

Fig. 4.8.: **Binding of MANT-GDP to Rac1 and Rac1 S71E was identical.** The nucleotide binding to Rac1 and Rac1 S71E was monitored by using the MANT-fluorophore-coupled GDP. Fluorescence emission was measured every 0.5 s at 444 nm in a Jasco FP-6500 spectrofluorometer over a period of 300 s. The arrow indicates the addition of MANT-GDP.

As previously shown in figure 4.5, phosphorylated Rac1 and Cdc42 bind to the effector protein Pak. To exclude unspecific binding to Pak we performed pull-down assays using the phosphomimetic mutants. The active GTP-bound Rac1 and Rac1 S71E bound to Pak, whereas the GDP-bound form associated with Rho-GDI (see figure 4.9, left panel). Identical nucleotide-dependent binding was also observed for Cdc42 and Cdc42 S71E (right panel).

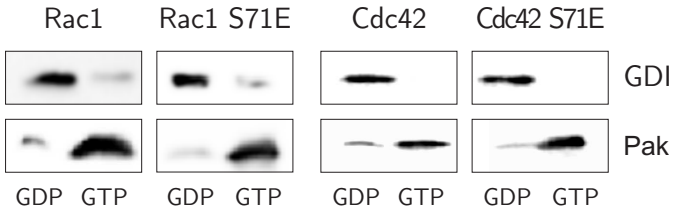

Fig. 4.9.: **Binding of Rac1 S71E and Cdc42 S71E to effector protein Pak and inhibitor Rho-GDI was nucleotide-dependent.** The GTPases Rac1, Rac1 S71E, Cdc42 and Cdc42 S71E (0.6 µg/ml) were first loaded with either 0.1 mM GDP or 0.1 mM GTP[γ]S and then their interaction with either Pak-Crib domain (lower panel) or Rho-GDI (upper panel) was analyzed by Western blot with specific Rac1 and Cdc42 antibodies.

The data suggests that the effector coupling was not affected by phosphorylation of Rac1 at Ser-71.

4.2.2. Mutation of serine 71 to glutamate changes the substrate modification and the binding to TcdA

Rac1 and Cdc42 are glucosylated by TcdA and *Clostridium sordellii* lethal toxin (TcsL). Thus, the question arose whether the amino acid exchange of serine to glutamate of recombinant Rac1 and Cdc42 affects the substrate modification by both toxins.

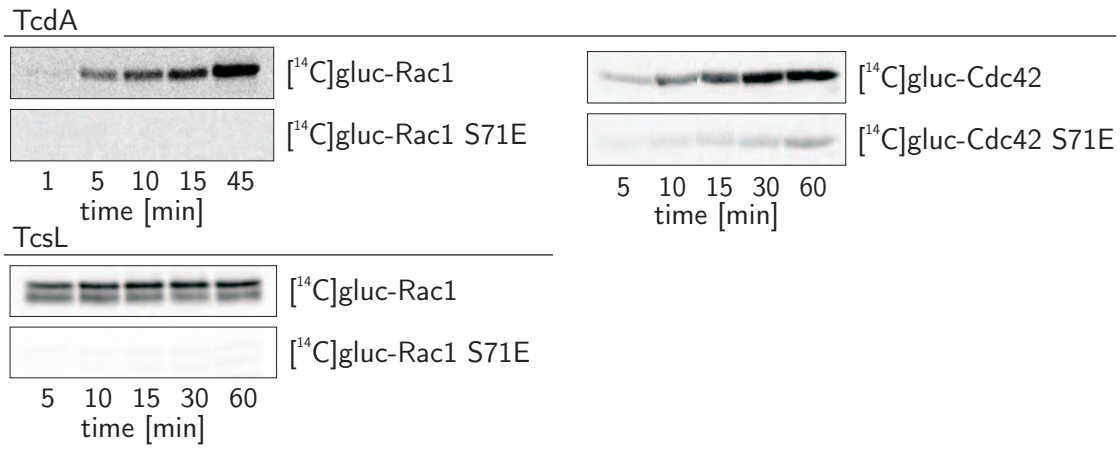

Fig. 4.10.: **Rac1 S71E was neither substrate for TcdA nor TcsL.** Recombinant Rac1, Cdc42, Rac1 S71E or Cdc42 S71E (80 µg/ml each) were incubated with TcdA (1.3 µg/ml). Rac1 or Rac1 S71E were also incubated with TcsL (1.6 µg/ml). Protein [^{14}C]glucosylation was analyzed by filmless autoradiography.

The glucosylation assay showed that Rac1 and Cdc42 were glucosylated in a time-dependent manner whereas Rac1 S71E was not modified by TcdA. Cdc42 S71E however was a poor substrate. In accordance, Rac1 but not Rac1 S71E was modified by TcsL (see figure 4.10). The data indicates that Ser-71 of the GTPases plays an important role in substrate modification.

The fact that Rac1 S71E is correctly folded was shown by nucleotide binding in figure 4.11 and by pull-down assays in figure 4.9. To check whether the missing substrate modification is related to reduced binding to TcdA, a binding assay was performed. Increasing concentrations of Rac1 and Rac1 S71E were applied to TcdA-coated microtiter plates. The level of bound protein was measured in an ELISA-reader. Both GTPases bound to TcdA in a concentration dependent manner, but in low concentrations the binding of Rac1 S71E was about 2 to 3 times weaker compared to Rac1 (see figure 4.11). In higher concentrations the binding was alike as they come close to TcdA saturation. This data shows that Ser-71 of Rac1 plays an important role in the interaction with TcdA.

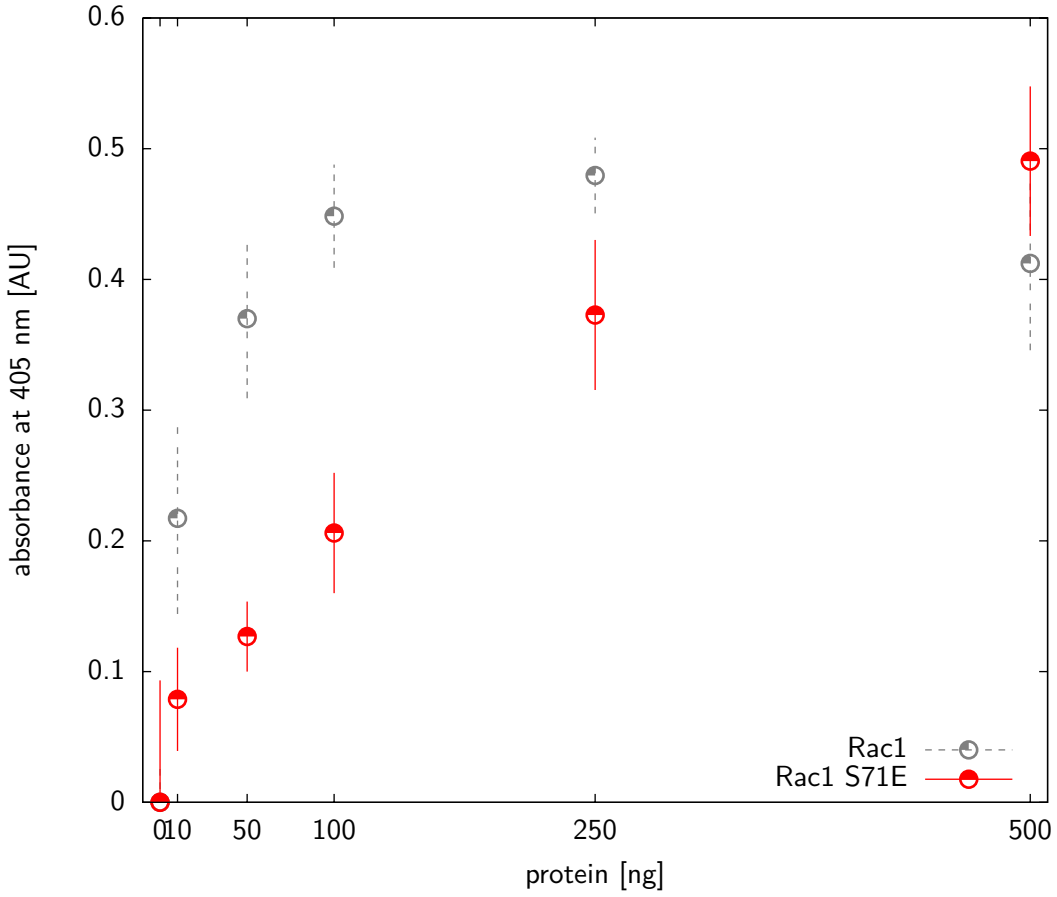

Fig. 4.11.: **Rac1 S71E bound weaker to TcdA than Rac1.** The interaction of Rac1 as well as Rac1 S71E with TcdA was assessed in an ELISA binding assay. Increasing concentrations of either Rac1 or Rac1 S71E were applied to TcdA-coated microtiter plates. The level of bound protein was determined at a wavelength of 405 nm in an Anthos 2020 ELISA reader.

From the previous data, we conclude that the exchange of serine to glutamate at position 71 of Rac1 and Cdc42 affects the substrate modification as well as its binding to TcdA.

4.2.3. Rac1 S71E prevents glucosylation of Rac1 but not of RhoA and Cdc42

Since Rac1 S71E binds to TcdA but is not substrate for glucosylation, sequesteration of TcdA by Rac1 S71E was assayed in a competition assay with Rac1. First the concentration-dependent effect of Rac1 S71E on Rac1 glucosylation was analyzed. The glucosylation of Rac1 alone was set to 100%. The presence of an equimolar amount of Rac1 S71E reduced the glucosylation of Rac1 to 60%. However, a 10 fold excess reduced the Rac1 glucosylation to 20% (see figure 4.12).

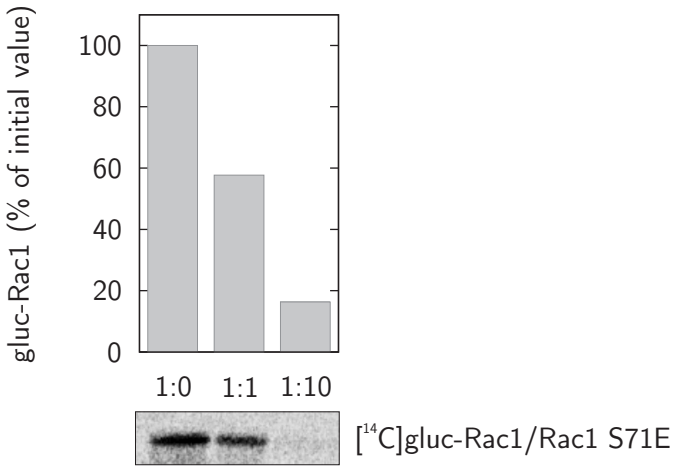

Fig. 4.12.: **Rac1 S71E competed with Rac1 in a glucosylation assay.** Glucosylation of Rac1 (17 µg/ml) and Rac1 S71E in a molar ratio of 1:1 or 1:10 was determined after adding TcdA (2.7 µg/ml). The reaction was stopped after 15 min and the protein glucosylation was analyzed by filmless autoradiography.

The reduced glucosylation of Rac1 might be due to sequesteration of either TcdA or Rac1 by Rac1 S71E. To differentiate between sequestering of TcdA and homodimerization of Rac1 S71E, competition studies with RhoA and Cdc42 were performed. Figure 4.13 shows time-dependent glucosylation of Rac1, RhoA and Cdc42 in the absence or presence of Rac1 S71E in a four fold excess. In the absence of Rac1 S71E the GTPases were glucosylated in a time-dependent manner. The presence of Rac1 S71E clearly reduced the Rac1 glucosylation by a factor of four. No changes in the glucosylation of RhoA and Cdc42 in the presence of Rac1 S71E were observed. Competition studies showed sequestering of Rac1 and not of TcdA by Rac1 S71E.

This suggests homodimerization as a reason for reduced Rac1 glucosylation in the presence of Rac1 S71E.

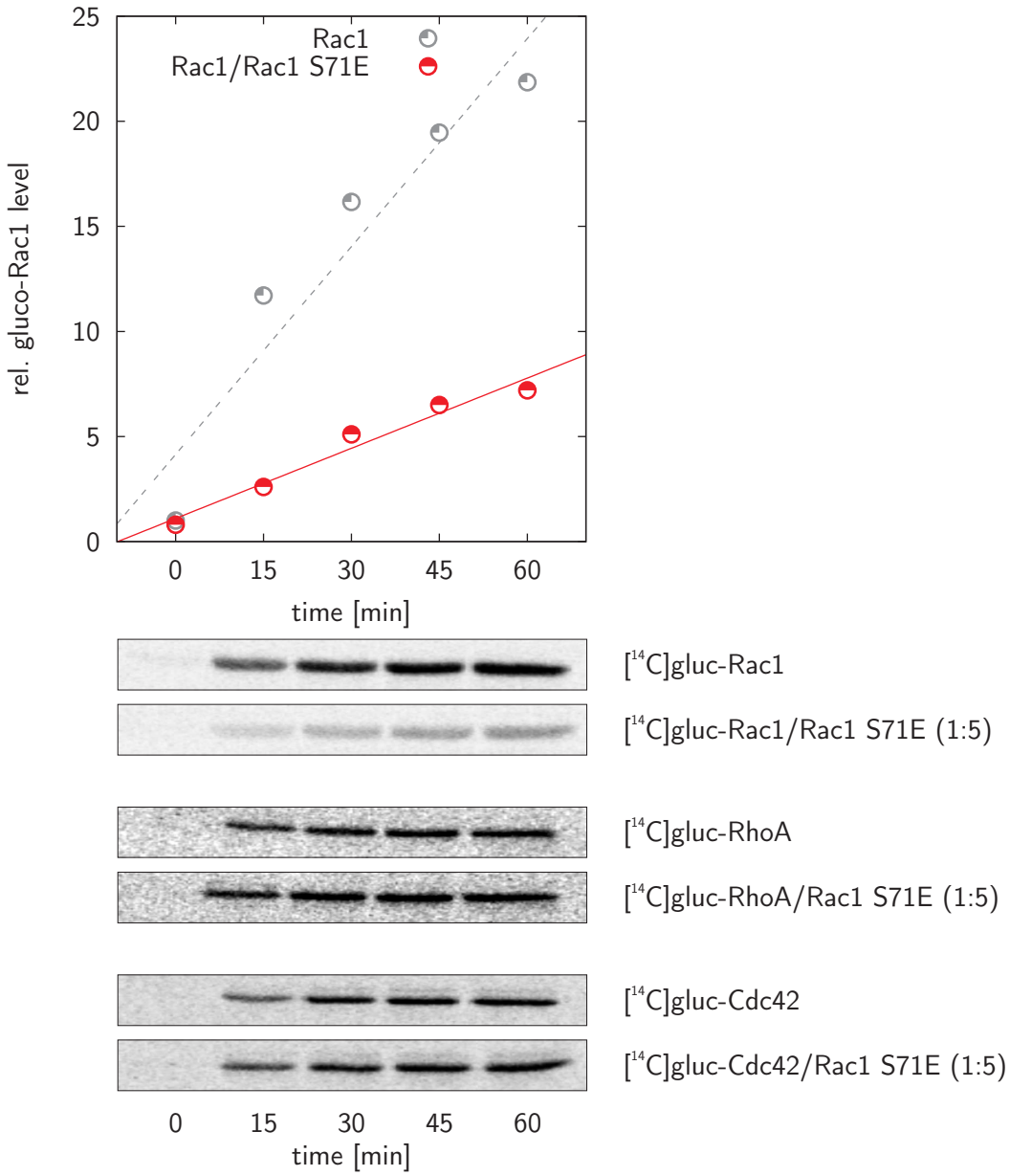

Fig. 4.13.: **Rac1 S71E specifically competed with Rac1 but not with RhoA and Cdc42.** The time-dependent glucosylation of the GTPases Rac1, RhoA or Cdc42 (20 µg/ml each) alone or in presence of Rac1 S71E (80 µg/ml) was performed by adding TcdA (1.6 µg/ml). The samples were taken at indicated time points. Protein glucosylation was analyzed by filmless autoradiography. The signals of the Rac1 and Rac1 S71E were analyzed densitometrically and displayed in the graph.

4.2.4. The C-terminus contributes to substrate recognition

By using the C-terminal deleted (last eight residues) forms Rac1ΔC-8 and Rac1 S71EΔC-8 we investigated the relevance of the C-terminus in substrate recognition by TcdA. The glucosylation assay showed that Rac1ΔC-8 is still substrate for TcdA (see figure 4.14). Rac1 S71EΔC-8 was a weak substrate for TcdA whereas full length Rac1 S71E was not modified by TcdA (compare figure 4.10). The data suggested that the C-terminus of the GTPases is important for the substrate recognition by TcdA.

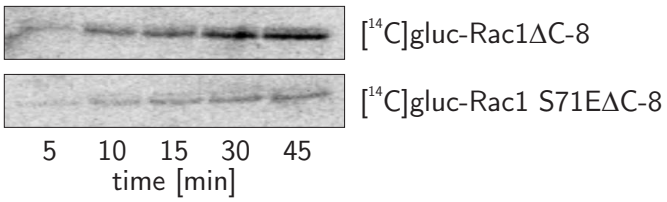

Fig. 4.14.: **Rac1 S71EΔC-8 was glucosylated by TcdA.** Recombinant Rac1ΔC-8 or Rac1 S71EΔC-8 (each (80 µg/ml) were incubated with TcdA (1.3 µg/ml) in presence of UDP-[^{14}C]glucose. The protein glucosylation was analyzed by filmless autoradiography at indicated time points.

The competition experiments, as shown in figure 4.13, were repeated using Rac1ΔC-8 (see figure 4.15). In contrast to full length, Rac1 S71EΔC-8 in 4 fold excess did not compete with Rac1ΔC-8.

In combination with competition experiments using full length GTPases, these findings indicate that Rac1 and Rac1 S71E form complexes and that the eight C-terminal amino acids are essential for mutual binding.

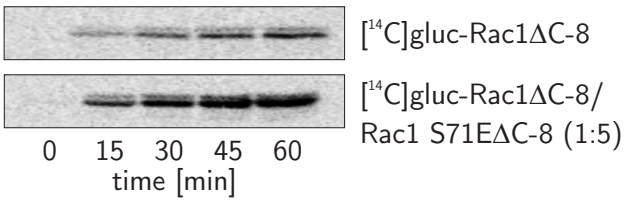

Fig. 4.15.: **Rac1 S71EΔC-8 did not compete with Rac1ΔC-8.** The time-dependent glucosylation of Rac1ΔC-8 (20 µg/ml) alone or in combination with Rac1 S71EΔC-8 (80 µg/ml) was performed by addition of TcdA (1.6 µg/ml). The samples were taken at indicated time points. Protein glucosylation was analyzed by filmless autoradiography.

4.2.5. The C-terminus contributes to the interaction of Rac1 and Rac1 S71E

Cdc42 and Rac2 have been reported to form homodimers (Zhang and Zheng, 1998). To examine the possible interaction between Rac1 and Rac1 S71E, the proteins were subjected to gel filtration chromatography column Superdex 75.

First the chromatography profiles of the molecular weight standards exoenzyme C3 (24 kDa), Ovalbumin (45 kDa), BSA (66 kDa) and IgG (anti-TcdA 542) (150 kDa) were used to determine the retention times (see figure 4.16).

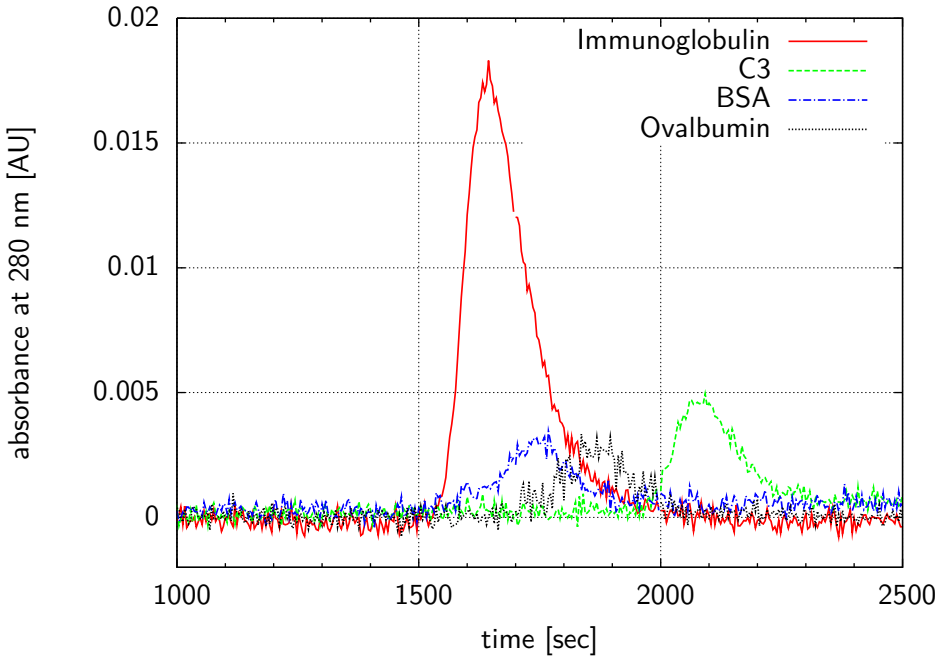

Fig. 4.16.: **S75 column chromatograms of standard proteins.** To calibrate the S75 column, (25 µg) of proteins with known molecular weights (see table 4.1) were separated by S75 column.

Table 4.1 summarizes the observed data.

standard	*molecular weight [kDa]*	*retention time [s]*
exoenzyme C3	24	2100
Ovalbumin	45	1900
BSA	66	1760
IgG (Anti-TcdA 542)	150	1650

Tab. 4.1.: Retention times of molecular weight standards

Rac1, Rac1 S71E or RhoA (60 µg each) alone or in combination with Rac1 S71E (30 µg each) were analyzed by gel filtration chromatography. In figure 4.17, Rac1 eluted in two peaks with maxima at 1550 sec (peak 1) and at 2100 sec (peak 2) (upper panel). The average molecular weight was estimated as >150 kDa for peak 1 and 21 kDa for peak 2. The mass distribution of each peak was 50% of total mass as calculated from the area under the curve. The Rac1 S71E peaks were detected at 1550 sec (equivalent to >150 kDa; 25% of total mass) and at 2100 sec which is equivalent to 21 kDa (75% of total mass).

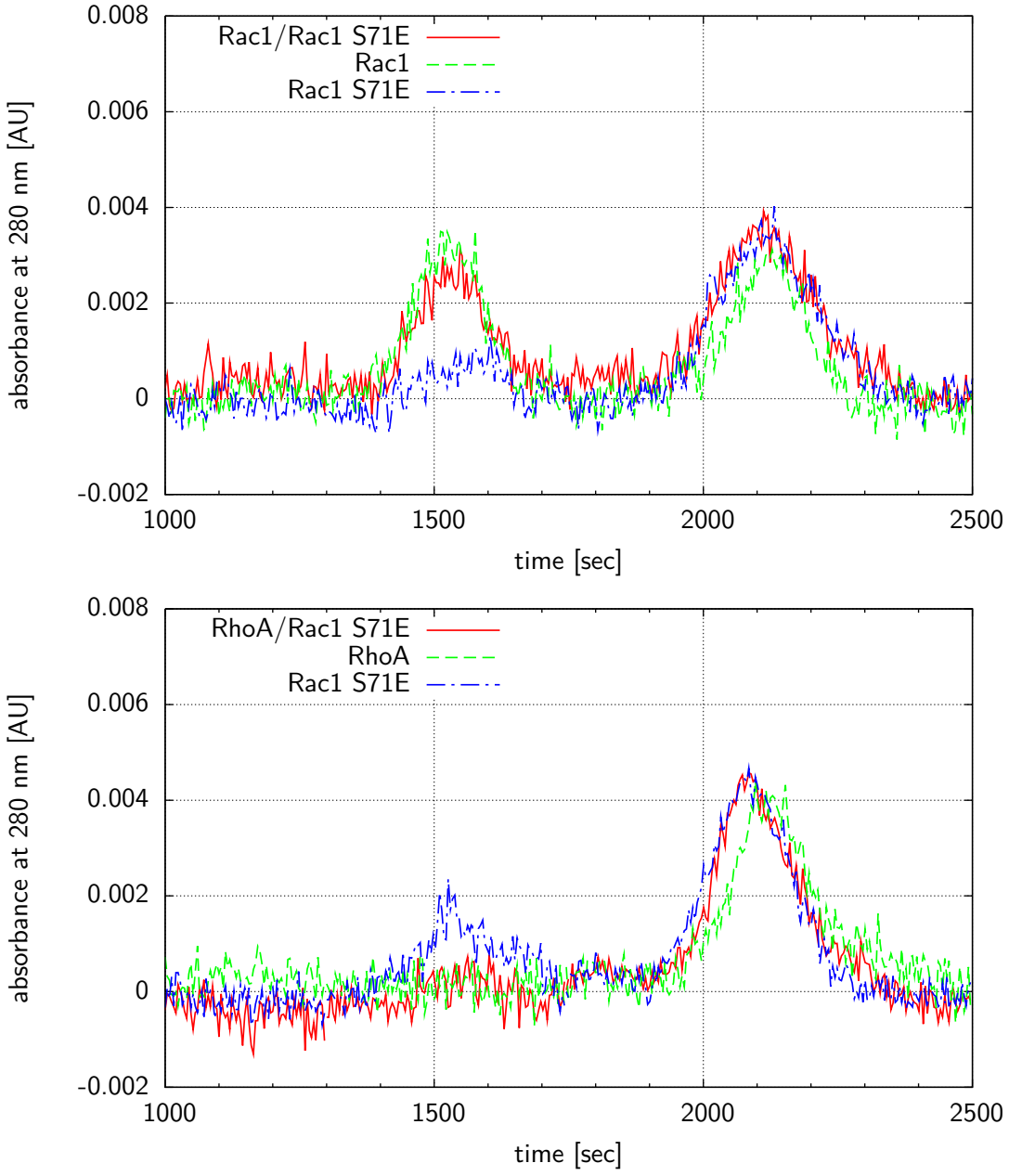

Fig. 4.17.: **Rac1 S71E specifically interacted with Rac1 but not with RhoA.** The diagram showed the complex formation of Rac1, Rac1 S71E, RhoA (60 µg in total) or combinations of Rac1 S71E with Rac1 or RhoA (30 µg each) respectively, using gel filtration chromatography (S75 column).

The combination of Rac1 and Rac1 S71E also showed two peaks with equal mass distribution (50% each). The chromatogram showed that Rac1 alone or in combination with Rac1 S71E forms protein complexes of >150 kDa. This complex formation was less pronounced with Rac1 S71E alone.

As the peak of >150 kDa is above the exclusion limit of the S75 column with 100 kDa no statement about the correct mass of the complexes was possible.

The same analysis was performed in the presence of RhoA. Separation of RhoA alone showed only one peak at 21 kDa (lower panel in figure 4.17). The chromatogram of the RhoA and Rac1 S71E combination revealed only one detectable peak at 21 kDa. From this data we conclude that Rac1 S71E specifically interacts with Rac1 but not with RhoA.

The carboxyl-terminal domain of the Rho family proteins, spanning the last eight amino acid residues including the CAAX isoprenylation sequence was suggested to have a role in homodimer formation. Therefore, we used the mutants of Rac1 and Rac1 S71E (ΔC-8) as described in section 4.2.4. Both truncated forms were found to be present only in the monomeric form (21 kDa) (see figure 4.18) and did not form complexes.

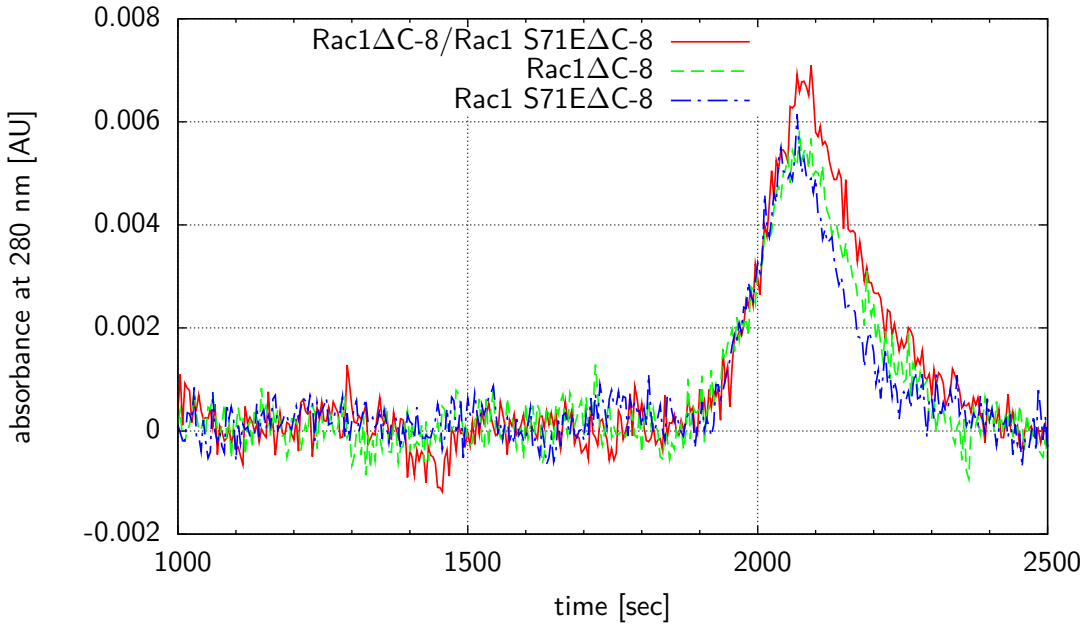

Fig. 4.18.: **Interaction of Rac1 and Rac1 S71E occurred via C-terminus.** The effect of the C-terminus on protein interaction was analyzed by gel filtration chromatography (S75 column) using 60 µg in total of Rac1ΔC-8, Rac1 S71EΔC-8 or a combination of both.

Thus, the Rac1 specific interaction is mediated by the C-terminus of the proteins.

4.2.6. Rac1 and Rac1 S71E are able to form higher molecular weight complexes

The gel filtration chromatography of Rac1 and Rac1 S71E indicated protein-protein interactions which were further investigated by chemical cross-linking. This technique involves the formation of covalent bonds between two proteins by using bifunctional reagents such as glutaraldehyde that react with surface amino acid residues of the protein. Only if two proteins physically interact with each other they can be covalently cross-linked.

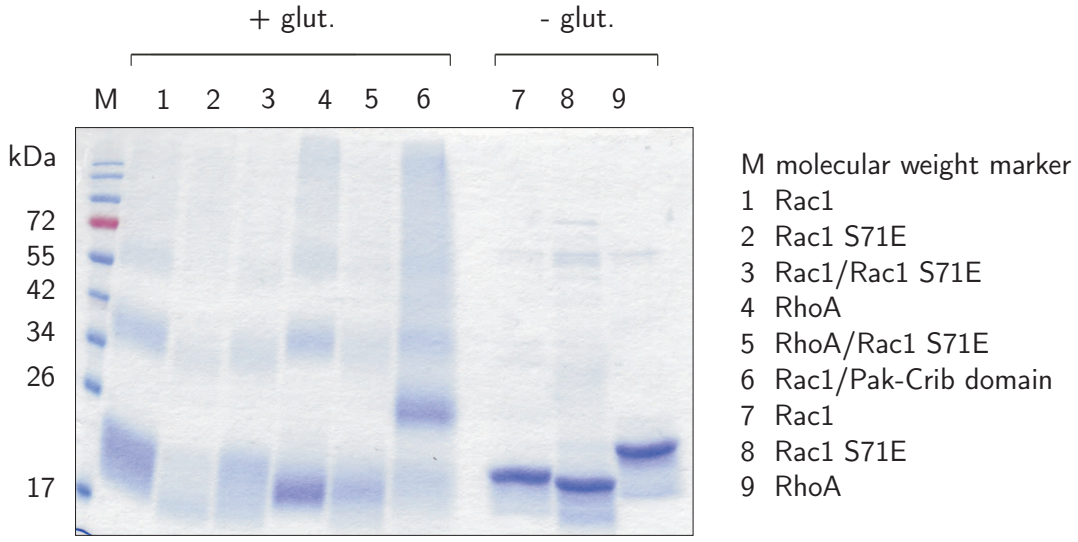

Fig. 4.19.: **Rac1, Rac1 S71E and RhoA formed higher complexes.** GDP-loaded Rac1, -Rac1 S71E and -RhoA were cross-linked with glutaraldehyde (glut.) for 30 min at 37 °C. The reaction was stopped by addition of glycine. Cross-linked proteins were analyzed by 12.5 % SDS-PAGE. As positive control the interaction of GTP-loaded Rac1 with the Pak-Crib domain (lane 4) was analyzed.

The cross-linking with glutaraldehyde revealed that Rac1 or Rac1 S71E alone form complexes of differing masses of about 40 kDa, 60 kDa and 80 kDa representing dimers, trimers and tetramers, respectively (see figure 4.19). The 40 kDa band was most prominent. The monomeric form with a mass of 21 kDa was also present.

Since all studied GTPases formed homodimers or -trimers in cross-linking experiments, formation of heterodimers or -trimers could not be distinguished. It is noteworthy that cross-linking of Rac1 S71E interfered with Coomassie staining and immunodetection (data not shown).

The cross-linking experiments of truncated Rac1 (Rac1ΔC-8) and Rac1 S71E (Rac1 S71EΔC-8) with glutaraldyhyde (see figure 4.20) also showed the formation of complexes of about 40 kDa, 60 kDa and 80 kDa, which represent dimers, trimers and tetramers. The most prominent band was the 21 kDa monomer. Since Rac1ΔC-8 and Rac1 S71EΔC-8 formed homodimers or -trimers in cross-linking experiments, formation of heterodimers or -trimers could not be distinguished.

The cross-linking experiments of truncated Rac1 and Rac1 S71E showed that these proteins also form homodimers, a finding which is in contrast to gel filtration chromatography experiments (see figure 4.18).

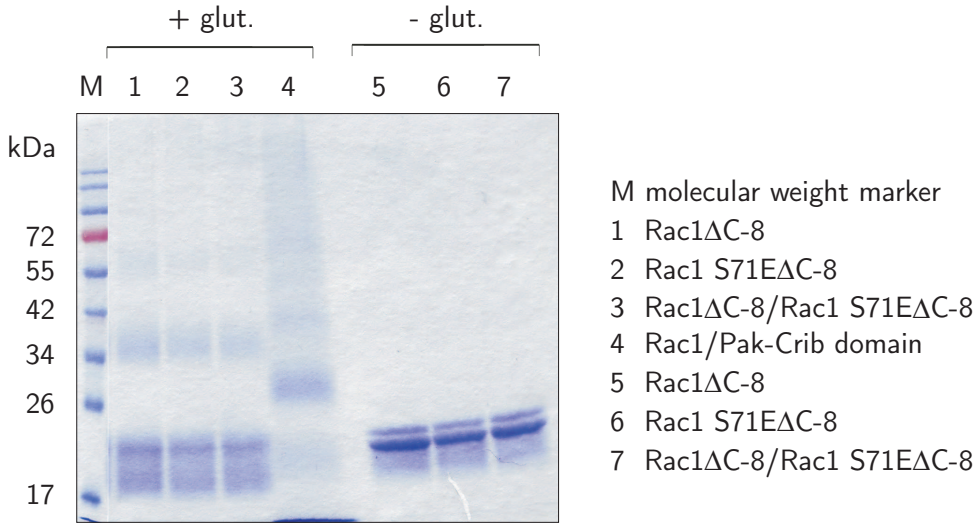

Fig. 4.20.: **Rac1ΔC-8 and Rac1 S71EΔC-8 also formed higher complexes.** GDP-loaded Rac1ΔC-8 and -Rac1 S71EΔC-8 were cross-linked with glutaraldehyde (glut.) for 30 min at 37 °C. The reaction was stopped by adding glycine. The proteins were analyzed by 12.5 % SDS-PAGE.

Therefore, cross-linking under the used conditions is not an appropriate method for analysis of heterodimer or -trimer formation.

4.3. Changes in actin cytoskeleton and cell morphology of transfected Hep-2 cells

4.3.1. The overexpression of constitutive active Rac1 (Rac1 G12V) and dominant negative Rac1 (Rac1 T17N) altered cell morphology of Hep-2 cells

One major function of the Rho GTPases (Cdc42, Rac1, RhoA) is the regulation of the actin cytoskeleton which determines the cell morphology. Active RhoA induces formation of actin stress fibers. Activation of Rac1 leads to formation of lamellipodia and membrane ruffles whereas Cdc42 induces formation of microspikes and filopodia. The effect of constitutive active Rac1 (c.a., Rac1 G12V) and dominant negative Rac1 (d.n., Rac1 T17N) on actin filaments and the resulting cell morphology of Hep-2 cells was examined. Overexpression of Rac1 G12V induced the typical formation of prominent membrane ruffles. Cells transfected with Rac1 T17N rounded up and showed a clear condensation of chromatin in the nucleus as shown by DAPI-staining (see figure 4.21). The overexpression of the wild type forms of Rac1 and Cdc42 did not affect the Hep-2 cell morphology. Both wild type GTPases were also located in the nucleus. Transfection with the empty vector (pcDNA) had no effect on actin filaments and the cell morphology.

4.3.2. The overexpression of Rac1 S71E in Hep-2 cells induced the formation of membrane ruffles and filopodia-like structures

First the effect of EGF, which induced phosphorylation of Rac1 and Cdc42, on cell morphology and the actin filament was investigated. EGF treatment (100 $^{ng}/_{ml}$) of Hep-2 cells for 2 hours led to formation of actin bundles and filopodia-like structures (see figure 4.22). The phosphomimetic Rac1 mutant (Rac1 S71E) induced the formation of lamellipodia- and filopodia-like structures as well as of membrane ruffles compared to wild type Rac1. In contrast to dominant negative Rac1, Rac1 S71E did not induce cell rounding indicating rather an active role of the phosphorylated Rac1. Cells transfected with the phosphomimetic Cdc42 mutant (Cdc42 S71E) showed an increased formation of microspikes and actin bundles compared to wild type Cdc42. The phosphorylation of Rac1 and Cdc42 also affected their distribution within the cell. In contrast to the wild type, the phosphomimetic mutants are located in the perinuclear region.

The phosphomimetic forms of Rac1 and Cdc42 induced effects on the actin cytoskeleton that resembled in part the effects induced by EGF. Furthermore, phosphorylation seems to affect intracellular localization of Rac1 and Cdc42.

Effects of the Rac1 S71E and Cdc42 S71E on the microtubular system were investigated by tubulin staining (see figure 4.23). The microtubular system was affected neither by overexpression of Rac1, Rac1 G12V and Cdc42 nor by the phosphomimetic mutants Rac1 S71E and Cdc42 S71E.

The phosphomimetic Rac1 mutant (lamellipodia- and filopodia-like structures and membrane ruffles) and phosphomimetic Cdc42 mutant (microspikes and actin bundles) induced morphological changes were based only on alteration of the actin cytoskeleton.

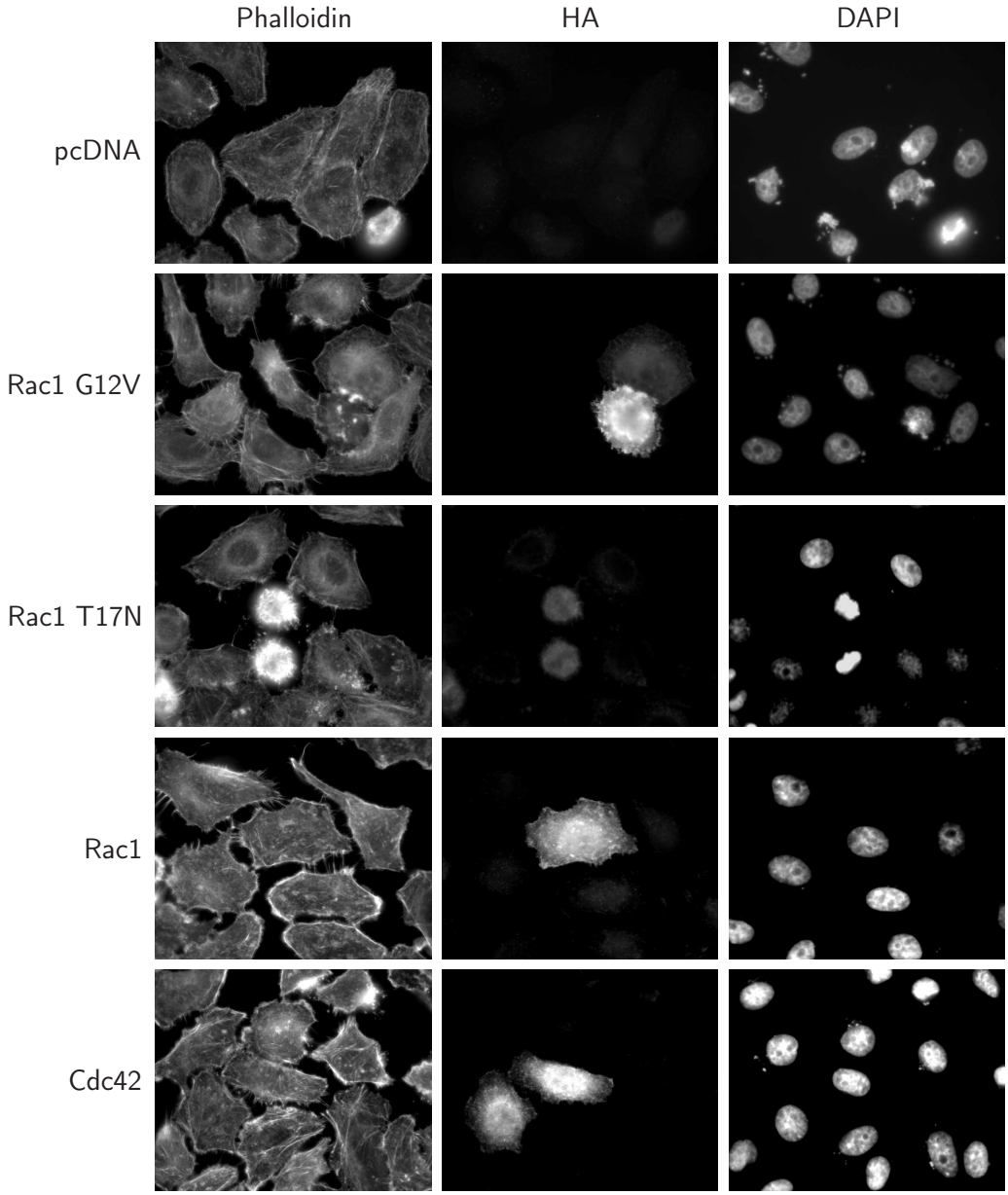

Fig. 4.21.: **Rac1 G12V and Rac1 T17N affected the cell morphology.** Hep-2 cells grown on coverslips were transfected with HA-tagged c.a Rac1 (Rac1 G12V), HA-tagged d.n. Rac1 (Rac1 T17N), HA-tagged Rac1, HA-tagged Cdc42 and the vector (pcDNA) alone. After 20 hours immunofluorescence staining with anti-HA was performed. Additionally the nuclei were stained with DAPI and the actin filaments with Alexa Fluor 568 phalloidin.

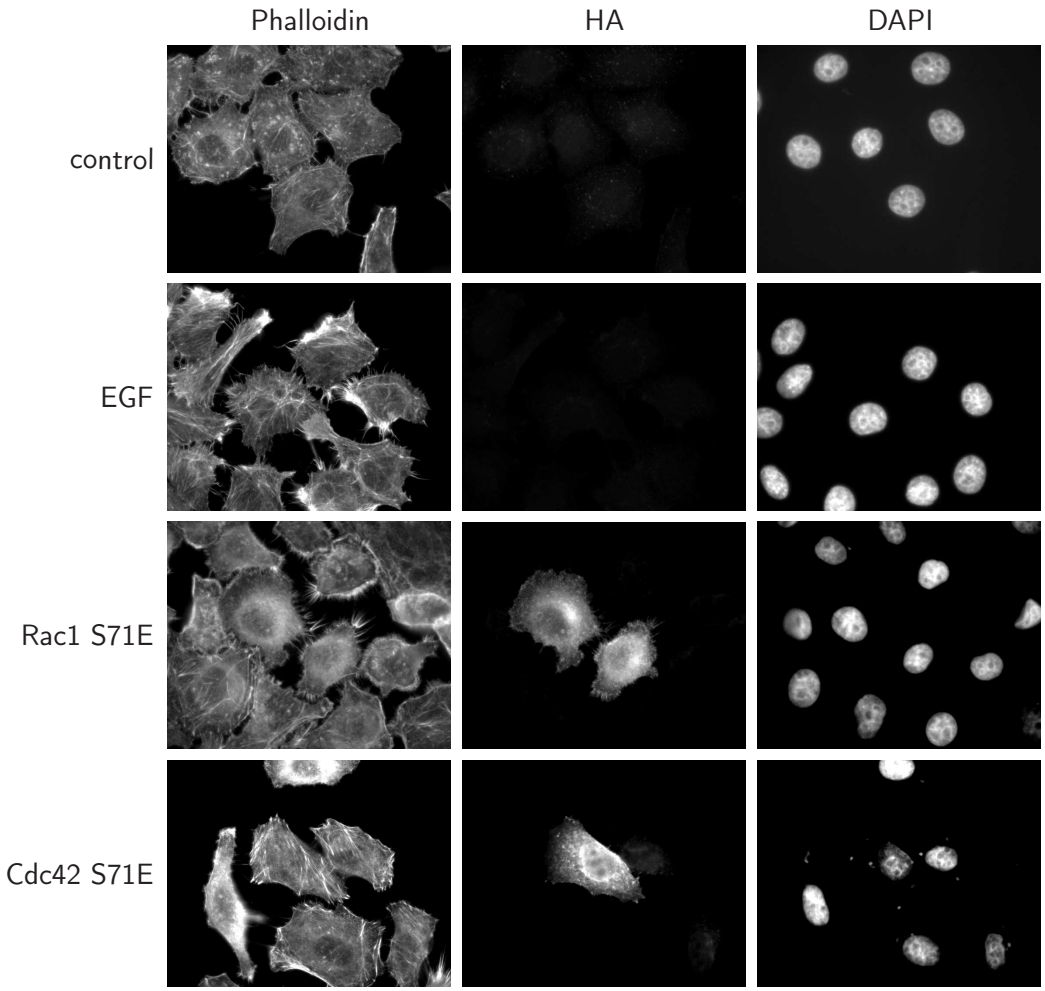

Fig. 4.22.: **Morphological effects induced by EGF, Rac1 S71E and Cdc42 S71E.** Hep-2 cells grown on coverslips were transfected with HA-tagged Rac1 S71E and Cdc42 S71E. After 20 hours the immunofluorescence staining with anti-HA was performed. Additionally, the nuclei were stained with DAPI and the actin filaments with Alexa Fluor 568 phalloidin. For comparison of morphological effects, Hep-2 cells were incubated with EGF (100 $^{ng}/_{ml}$) for 2 hours.

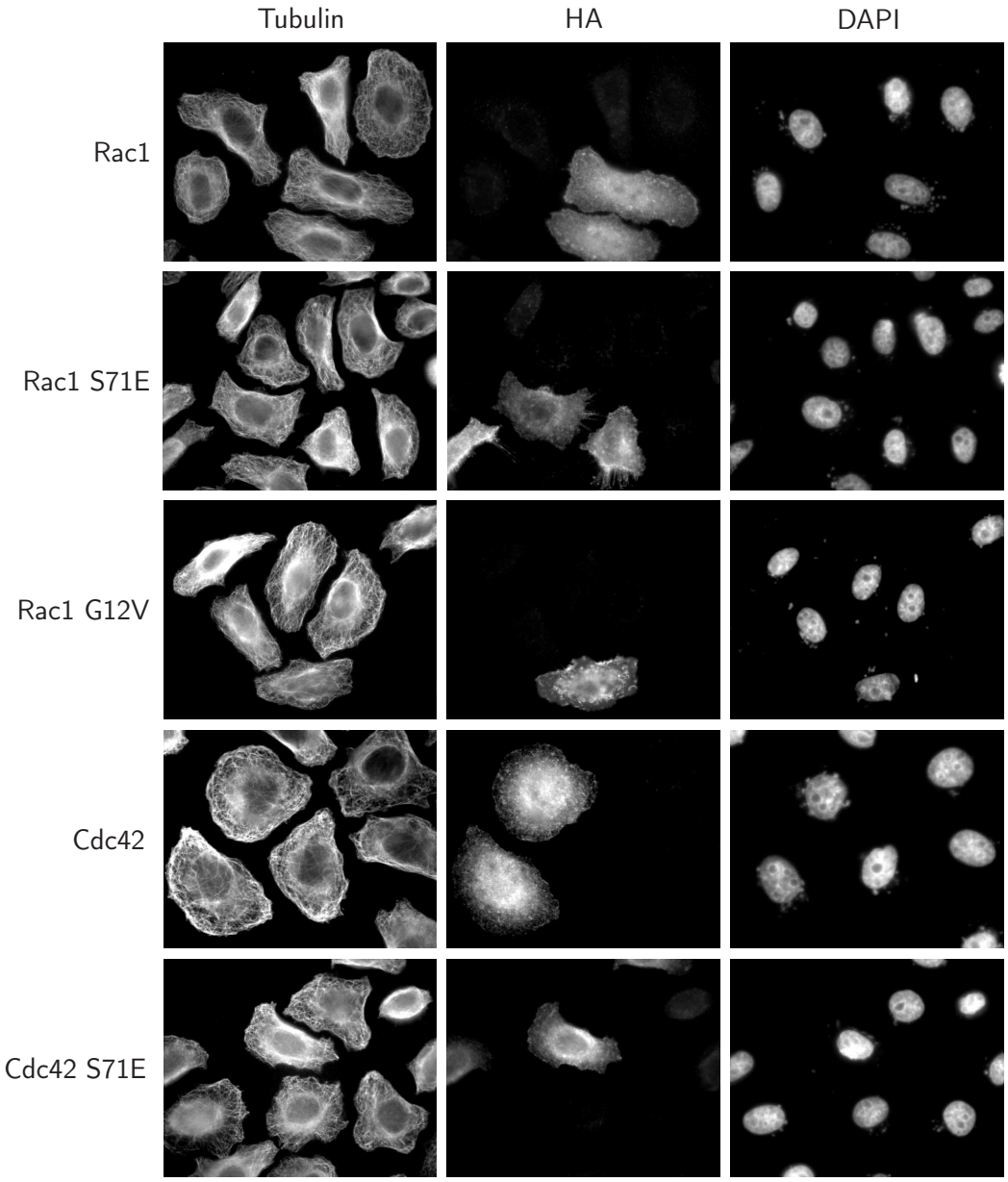

Fig. 4.23.: **Overexpression of Rac1 S71E and Cdc42 S71E did not affect the microtubular system.** Hep-2 cells grown on coverslips were transfected with HA-tagged Rac1, Rac1 S71E, Rac1 G12V, Cdc42 and Cdc42 S71E. After 20 hours the immunofluorescence staining with anti-HA was performed. Additionally the nuclei were stained with DAPI and the microtubular system with anti-Tubulin-α.

4.3.3. Overexpression of the Rac1 S71E mutant protects against toxin-induced cytopathic effect

EGF has been reported to attenuate the *Clostridium difficile* toxin A- and B-induced damage of human colonic mucosa (Riegler et al., 1997). We showed that the EGF treatment of cells resulted in phosphorylation of the GTPases Rac1 and Cdc42. The Ser-71 phosphorylated GTPases are not (Rac1) or restricted (Cdc42) substrates for the toxins. To proof a protective role, Hep-2 cells were transfected with Rac1, Rac1 S71E and Rac1 G12V before toxin treatment. The phosphorylated Rac1 was neither substrate for *Clostridium difficile* toxin A (TcdA) nor for *Clostridium sordellii* lethal toxin (TcsL) compared to phosphorylated Cdc42 which was poor substrate. Therefore, we focussed our studies on the effect of the phosphorylated Rac1 (see figure 4.24). Hep-2 cells transfected with the Rac1 S71E showed partial reduction of the TcdA-induced cytopathic effect compared to Rac1 transfected cells. As positive control Rac1 G12V was used, as it is known not to be substrate for TcdA. Rac1 G12V transfected cells were also partially protected against the TcdA-induced cytopathic effect.

Rac1 S71E and Rac1 G12V transfected cells were not completely protected against the TcdA-induced cytopathic effect. As TcdA modifies RhoA/B/C, Rac1 and Cdc42 and only Rac1 is protected against the TcdA-induced glucosylation, Hep-2 cells showed alteration of cell morphology based on modification of Rho and Cdc42.

To further highlight the protective effect of phosphorylated Rac1 we used TcsL, as this toxin only modifies Rac1 and Ras. Rac1 S71E and Rac1 G12V transfected Hep-2 cells were completely protected against the TcsL-induced cytopathic effect and showed no alteration in cell morphology compared to Hep-2 cells expressing wild type Rac1. Furthermore, TcsL treatment resulted in a strong condensation of chromatin in the nucleus in un- and Rac1-transfected Hep-2 cells, which was blocked by Rac1 S71E and Rac1 G12V.

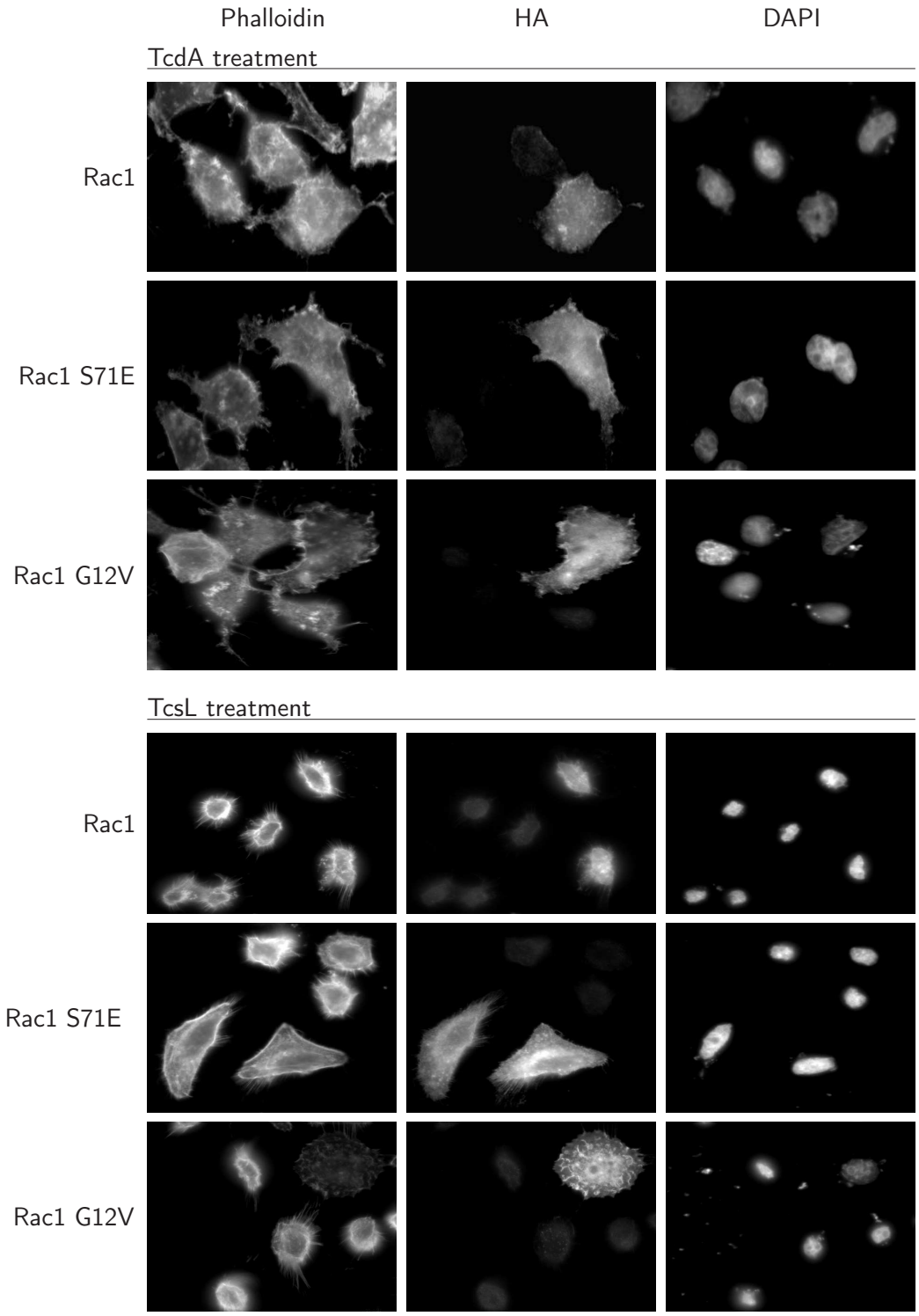

Fig. 4.24.: **Rac1 S71E attenuated the TcdA- and TcsL-induced cytopathic effect.** Hep-2 cells grown on coverslips were transfected with HA-tagged Rac1, Rac1 S71E and Rac1 G12V. After 20 hours the cells were treated with TcdA (150 $^{ng}/_{ml}$) for 4 hours or TcsL (600 $^{ng}/_{ml}$) for 5 hours, immunofluorescence staining was performed with anti-HA, DAPI (for nuclei) and Alexa Fluor 568 phalloidin (for actin cytoskeleton).

5. Discussion

EGF reduced the effect of *Clostridium difficile* toxin A on the epithelial barrier function. Corresponding findings were previously observed, showing the protective effect of EGF on toxin-induced damage of the intestine (Riegler et al., 1997; Lawrence et al., 1997). The present study describes one mechanism that contributes to the protective EGF effect on the TcdA-induced alteration of the epithelial barrier function. EGF treatment activated the Akt kinase and led to phosphorylation of Rac1 and Cdc42 at Ser-71 as shown here in CaCo-2 cells. The inhibition of the Akt-kinase by the specific inhibitor clearly reduced EGF-induced Rac1 and Cdc42 phosphorylation to control level. Thus, Rac1 and Cdc42 were substrates of the Akt kinase (see figure 5.1), as it was described by Kwon et al. (2000). In our study EGF functions as a reference substance. Any other growth factor or substance that induces activation of Akt is suggested to display the same effects towards Rac1/Cdc42.

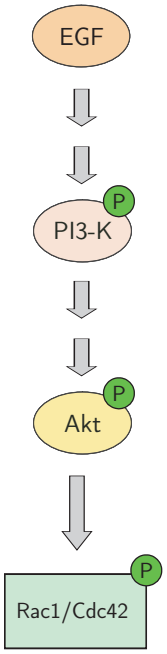

Fig. 5.1.: **EGF-AKT-Rac1/Cdc42 pathway.** EGF induced phosphorylation of Rac1/Cdc42 via Akt kinase.

5.1. Ser-71 phosphorylation does not inhibit Rac1 function

Despite the fact that Rac1 and Cdc42 are phosphorylated by the Akt kinase, almost nothing is known about the function of Ser-71 phosphorylation of Rac1. Rac1 and Cdc42 are not negatively regulated by phosphorylation at Ser-71 and remain functional competent and active as shown by pull-down assays. To study the effect of Rac1 and Cdc42 phosphorylation in more detail Ser-71 was exchanged to the phosphomimetic amino acid glutamate (S71E). This amino acid exchange via mutagenesis is an established method to study the function of protein phosphorylation. We demonstrated that the phosphomimetic Rac1 mutant (Rac1 S71E) exhibited no differences in binding to the nucleotide GDP, shown by MANT-GDP binding, compared to wild type Rac1. Furthermore, pull-down experiments indicated that the GTPase effector coupling of both phosphomimetic mutants (Rac1 S71E and Cdc42 S71E) was also not altered and depended on the bound nucleotide. Data published by Kwon et al. regarding the phosphorylation of Rac1 by Akt kinase showed that the GTPase activity is unchanged whereas GTP-binding was reduced to some extent (Kwon et al., 2000). The same authors reported that mutation of Ser-71 to alanine of Rac1 completely abolished GTPase activity and GTP-binding.

We assume that this effect is caused by a change in polarity as the Ser-71 is polar whereas the alanine is not polar.

5.2. Ser-71 phosphorylated Rac1 is not substrate for TcdA

As Rac1 and Cdc42 are substrates of *Clostridium difficile* toxin A (TcdA) and *Clostridium sordellii* lethal toxin (TcsL), the effect of phosphorylation on substrate recognition by both toxins was examined. The exchange of Ser-71 to glutamate in Rac1 (and Cdc42) alters the substrate recognition by TcdA and TcsL. Rac1 and Cdc42 were glucosylated whereas Rac1 S71E was not modified by TcdA. Cdc42 S71E however was a poor substrate. Single amino acid residues within and adjacent to the switch II domain are described to be important for the substrate properties of the Rho GTPases and the interaction with the toxins. The Ser-73 of RhoA (corresponding to Ser-71 in Rac1 and Cdc42) is located at the surface in close vicinity to the acceptor Thr-37 and is therefore involved in the interaction with the glucosylating toxins. The role of Ser-73 in RhoA in substrate recognition by glucosylating toxins was also described by Jank and coworkers (2006; 2007). The authors showed that Ser-73 is decisive for being substrate for TcdB or TcsL.

Thus, the amino acid residue at position 73 in RhoA (position 71 in Rac1/Cdc42) plays a crucial and determining role for the interaction of the Rho protein family members with large clostridial cytotoxins. This was also shown in our TcdA binding assay.

Interestingly, differences in glucosylation of Rac1 S71E and Cdc42 S71E are based on the C-terminus as the amino acids around the phosphorylation site are highly homologous. C-terminal deleted GTPase Rac1ΔC-8 was still substrate for TcdA. Rac1 S71EΔC-8 was a poor substrate, whereas Rac1 S71E full length was not modified. Therefore, the C-terminus of Rac1 contributes to substrate recognition by the toxins.

A different mechanism also contributes to the prevention of the Rac1 S71E glucosylation. In a competition assay the sequestration of TcdA by Rac1 S71E was excluded as Rac1 S71E only

reduced the glucosylation of Rac1 but not of RhoA and Cdc42. The competition of Rac1 and Rac1 S71E seemed to be based on the homodimerization of both GTPases. The phosphomimetic Rac1 mutant did not compete with RhoA or Cdc42. It is in favor of our hypothesis that these GTPases showed divergent sequences at their carboxyl-terminal region. The carboxyl-terminal region represents the most divergent sequence in the family of the Rho GTPases. Most members contain a stretch of two to six poly-lysine and/or arginine residues directly before the isoprenylation CAAX-sequence. Because of its positive charge on the surface of the molecules the carboxyl-terminal region was assumed to play a role in homodimer formation and membrane targeting.

To examine the suggested dimerization of Rac1 and Rac1 S71E we performed gel filtration chromatography using Superdex 75 column and cross-linking with glutaraldehyde. The gel filtration chromatography revealed the formation of higher molecular weight complexes (>150 kDa) suggesting a specific interaction between Rac1 and Rac1 S71E. The chromatography of the RhoA and Rac1 S71E combination did not exhibit such complexes. The cross-linking experiment of Rac1, Rac1 S71E and RhoA alone with glutaraldehyde resulted in formation of dimers, trimers and tetramers of all GTPases. Therefore, no evidence of dimerization of Rac1 and Rac1 S71E or RhoA and Rac1 S71E was given. However, both techniques give us a hint of potential complex formation. Data that is not exactly in line with our results was published by Zhang and Zheng (1998) regarding the formation of reversible dimers of the Rho GTPases RhoA, Cdc42 and Rac2. Further on, we showed that the C-terminus deleted GTPases Rac1ΔC-8 and Rac1 S71EΔC-8 were not able to form higher complexes. Corresponding data was also observed by Zhang and Zheng (1998) showing that the Cdc42ΔC-7 and the RhoAΔC-8 are present only in monomeric form. On the basis of not published data by Zhang and Zheng they described that GTPases of the Rho family containing the polybasic sequences at the carboxyl termini are not only able to form homodimers but can also form higher complexes. GTPases which lack the polybasic residues in this region, e.g. TC10 and RhoB, are only present in the monomeric form. Based on their data Zhang and Zheng proposed a non-symmetric configuration of the Rho GTPases in the homodimer state. This configuration explains the competition of Rac1ΔC-8 with full length Rac1 S71E and full length Rac1 with Rac1 S71EΔC-8 (data not shown).

Presumably, the observed homodimerization of Rac1 and Rac1 S71E has no physiological significance. The cellular GTPases are additionally isoprenylated at their C-terminus which possibly interferes with homodimerization. Most likely, GDI acts as an additional regulator to prevent formation of higher complexes. The investigation of this phenomenon is, however, beyond the aim of this study.

5.3. Overexpression of Rac1 S71E induced Cdc42-like morphological changes in Hep-2 cells

The existence of a common effector binding motif of Rac1 and Cdc42 (referred to as a CRIB motif) corresponds to the fact that Rac1 and Cdc42 exhibit a sequence homology of about 70%. Both GTPases bind to certain common target proteins involved in actin reorganization, thus leading to induction of lamellipodia and filopodia. Both GTPases bind to the isoform 1, 2 and 3 of Ser/Thr kinases Pak which regulate the actin cytoskeleton in cooperation with signals of MLC-kinase and LIM-kinase. Cdc42 induces microspikes and filopodia by acting via WASP/Arp2/3/Profilin pathway. The binding of Rac1 to PI-4-P5K or to the WAVE/Profilin/Arp2/3 complex causes the formation of lamellipodia and membrane ruffles (Bishop and Hall, 2000). However, the mechanisms of lamellipodia and filopodia formation are less understood at the biochemical level, partly because of their highly dynamic nature.

Treatment of Hep-2 cells with EGF for 2 hours induced the phosphorylation of Rac1 and Cd42, resulting in formation of actin bundles and filopodia-like structures. In comparison with EGF cells were transfected either with phosphomimetic Rac1 or the phosphomimetic Cdc42 mutant. The phosphomimetic Rac1 mutant induced the formation of filopodia-like structures, lamellipodia and membrane ruffles which was not observed in Rac1 transfected cells. Therefore, Rac1 S71E primarily acts in the active state. The formation of filopodia suggests an activation of Cdc42. The Rac1 S71E transfected cells clearly differed in morphology and actin cytoskeleton from cells overexpressing the dominant negative Rac1 (Rac1 T17N). Rac1 T17N cells rounded up and exhibited strong condensation of the chromatin in the nucleus. This data indicated that Rac1 S71E is still active and is in line with pull-down experiments. However, the overexpression of the phosphomimetic Cdc42 mutant did not induce membrane ruffles but formation of microspikes and actin bundles. The alteration due to both phosphomimetic mutants considerably resembled the morphological effects induced by EGF. The fact that Rac1 is capable of inducing filopodia is against common notion that Cdc42 is in charge of microspike and filopodia formation. Corresponding data showed that Cdc42 can activate Rac1 to transform filopodial protrusions to lamellipodia (Nobes and Hall, 1995).

Since the phosphorylation of Rac1 completely blocked the glucosylation by the toxins compared to phosphorylated Cdc42, which was a poor substrate, we focused our studies on the effect of phosphorylated Rac1 on the toxin-induced cytopathic effect (cell rounding). In TcdA-treated cells transfected with Rac1 S71E or Rac1 G12V, only Rac1-dependent signal pathways are maintained in an active state. The other GTPases Rho and Cdc42 were still inactivated by glucosylation. Cells overexpressing Rac1 were not protected and rounded up. To circumvent the effect of glucosylated Cdc42 and RhoA we used TcsL which specifically modifies Rac1 and the GTPases of the Ras family (H/K/N-Ras and R-Ras). Cells transfected with Rac1 S71E as well as Rac1 G12V were completely protected against the TcsL-induced cytopathic effect while the overexpression of Rac1 had no protective effect. Rac1 S71E seems to act in dominant positive way by maintaining the Rac1 signaling pathways. Further, Rac1 S71E acts stronger than wild type Rac1 to functionally compensate for glucosylated Rac1. An alternative explanation could be the sequestration of TcsL by Rac1 S71E, thereby inactivating the toxin. The sequestration of TcsL was not analyzed and has to be proven in TcsL binding assay. This data underlines the hypothesis that the cytopathic effect induced by clostridial glucosylating toxins is not exclusively mediated by inactivation of only one Rho GTPase.

In summary, the present study describes a mechanism by which EGF affects the toxin-induced alteration of the epithelial barrier function for the first time. The Akt mediated phosphorylation and activation of Rac1 and Cdc42 is the critical step within this mechanism (see figure 5.2). A further interesting outcome of this study is the characterization of Ser-71 phosphorylated Rac1 and its related distinct effects on actin cytoskeleton and cell morphology.

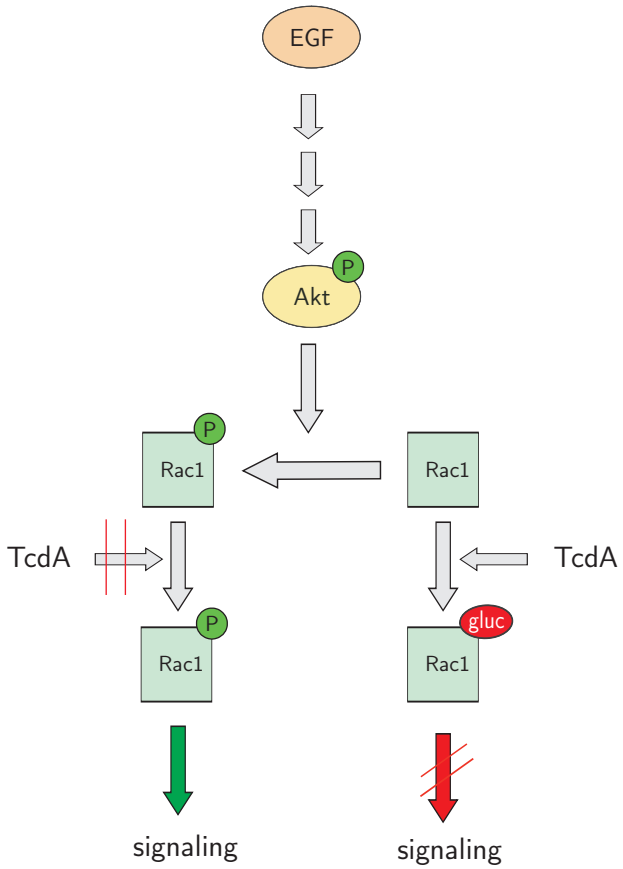

Fig. 5.2.: **EGF-Akt-Rac1 signal pathway.** EGF induces phosphorylation of Rac1. Phosphorylated Rac1 is not substrate for glucosylating toxins and remains signaling competent.

An increase in Rac1 phosphorylation might be a therapeutical approach to prevent or reduce symptoms of *Clostridium difficile*-associated diarrhea (CDAD). As TcdA and TcdB from *Clostridium difficile* are the causative agents of diarrhea and inflammation, EGF could have a supportive role in tissue repair by resolution of CDAD or by prevention of relapses. Furthermore, EGF might be the reason for protection of the developing intestine of babies (up to 6 months) which are resistant to the toxins (Bolton et al., 1984). Probiotics stimulate the gut immune cells to release inflammatory (TNF-α, IFN-γ) and regulatory (IL-4, IL-10, IL-12) cytokines which were also described to modulate the toxin effects by activating the Akt kinase (Osawa et al., 2002; Bommhardt et al., 2004). A contribution of phosphorylated Rac1 to the protective effect of probiotics has to be shown in continuative studies.

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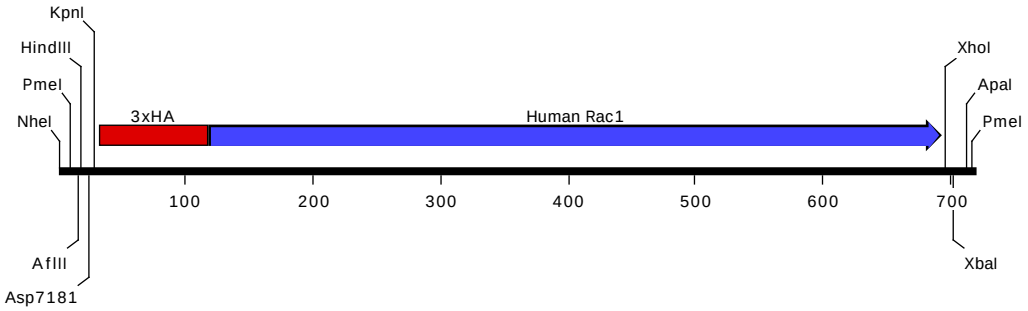
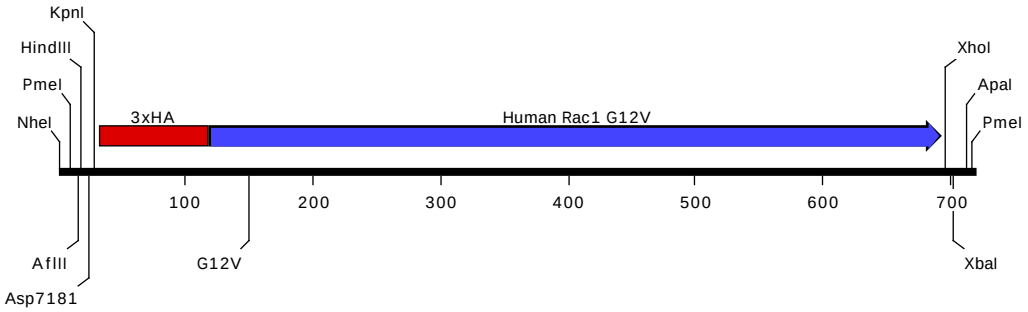
A. Supplements

A.1. Nucleotide sequences of obtained GTPases

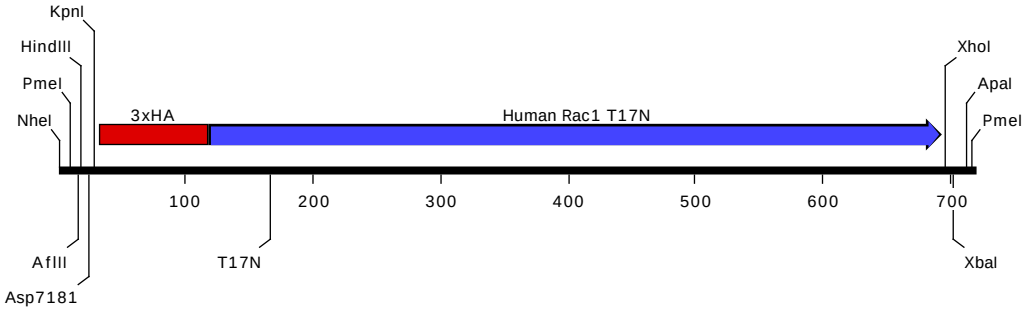
Human Rac1 (3×HA), GB Acc. No. M29870, Clone ID RAC010TN00



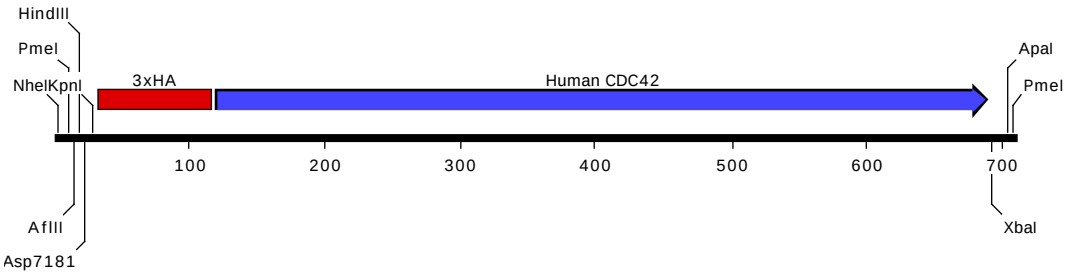
Human Rac1 G12V (3×HA), GB Acc. No. M29870, Clone ID RAC010TNC0



Human Rac1 T17N (3×HA), GB Acc. No. M29870, Clone ID RAC010TND0



Human Cdc42 (3×HA), GB Acc. No. M35543, Clone ID CDC420TN00



A.2. Nucleotide sequences of generated GTPases

The multiple cloning sites contain the following ligated GTPases. Highlighted in blue are the recognition site of restriction endonucleases: BamH I (5'end) and EcoR I (3'end). The mutations (S71E) are highlighted in red.

Rac1 in pGEX-2T vector

CTGGTTCCGCGTGGATCTCCGGGAATTTCCGGTGGTGGTGGTGGATCCATGCAGGCCATCAAGTGTGTGGTGGTGGGA
GACGGAGCTGTAGGTAAAACTTGCCTACTGATCAGTTACACAACCAATGCATTTCCTGGAGAATATATCCCTACTGTC
TTTGACAATTATTCTGCCAATGTTATGGTAGATGGAAAACCGGTGAATCTGGGCTTATGGGATACAGCTGGACAAGAA
GATTATGACAGATTACGCCCCCTATCCTATCCGCAAACAGATGTGTTCTTAATTTGCTTTTCCCTTGTGAGTCCTGCA
TCATTTGAAAATGTCCGTGCAAAGTGGTATCCTGAGGTGCGGCACCACTGTCCCAACACTCCCATCATCCTAGTGGGA
ACTAAACTTGATCTTAGGGATGATAAAGACACGATCGAGAAACTGAAGGAGAAGAAGCTGACTCCCATCACCTATCCG
CAGGGTCTAGCCATGGCTAAGGAGATTGGTGCTGTAAAATACCTGGAGTGCTCGGCGCTCACACAGCGAGGCCTCAAG
ACAGTGTTTGACGAAGCGATCCGAGCAGTCCTCTGCCCGCCTCCCGTGAAGAAGAGGAAGAGAAAATGCCTGCTGTTG
TAAGAATTCATCGTGACTGACTGACG

Rac1 S71E in pGEX-2T vector

CTGGTTCCGCGTGGATCTCCGGGAATTTCCGGTGGTGGTGGTGGATCCATGCAGGCCATCAAGTGTGTGGTGGTGGGA
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TTTGACAATTATTCTGCCAATGTTATGGTAGATGGAAAACCGGTGAATCTGGGCTTATGGGATACAGCTGGACAAGAA
GATTATGACAGATTACGCCCCCTAGAATATCCGCAAACAGATGTGTTCTTAATTTGCTTTTCCCTTGTGAGTCCTGCA
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ACAGTGTTTGACGAAGCGATCCGAGCAGTCCTCTGCCCGCCTCCCGTGAAGAAGAGGAAGAGAAAATGCCTGCTGTTG
TAAGAATTCATCGTGACTGACTGACG

Rac1ΔC-8 in pGEX-2T vector

CTGGTTCCGCGTGGATCTCCGGGAATTTCCGGTGGTGGTGGTGGATCCATGCAGGCCATCAAGTGTGTGGTGGTGGGA
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ACAGTGTTTGACGAAGCGATCCGAGCAGTCCTCTGCCCGCCTCCCGTGAAGAAGTAGGAATTCATCGTGACTGACTGA
CG

Rac1 S71EΔC-8 in pGEX-2T vector

CTGGTTCCGCGTGGATCTCCGGGAATTTCCGGTGGTGGTGGTGGATCCATGCAGGCCATCAAGTGTGTGGTGGTGGGA
GACGGAGCTGTAGGTAAAACTTGCCTACTGATCAGTTACACAACCAATGCATTTCCTGGAGAATATATCCCTACTGTC
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ACAGTGTTTGACGAAGCGATCCGAGCAGTCCTCTGCCCGCCTCCCGTGAAGAAGTAGGAATTCATCGTGACTGACTGA
CG

Cdc42 in pGEX-2T vector

CTGGTTCCGCGTGGATCTCCGGGAATTTCCGGTGGTGGTGGTGGATCCATGCAGACAATTAAGTGTGTTGTTGTGGGC
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TTTGACAACTATGCAGTCACAGTTATGATTGGTGGAGAACCATATACTCTTGGACTTTTTGATACTGCAGGGCAAGAG
GATTATGACAGATTACGACCGCTGAGTTATCCACAAACAGATGTATTTCTAGTCTGTTTTTCAGTGGTCTCTCCATCT
TCATTTGAAAACGTGAAAGAAAAGTGGGTGCCTGAGATAACTCACCACTGTCCAAAGACTCCTTTCTTGCTTGTTGGG
ACTCAAATTGATCTCAGAGATGACCCCTCTACTATTGAGAAACTTGCCAAGAACAAACAGAAGCCTATCACTCCAGAG
ACTGCTGAAAAGCTGGCCCGTGACCTGAAGGCTGTCAAGTATGTGGAGTGTTCTGCACTTACACAGAAAGGCCTAAAG
AATGTATTTGACGAAGCAATATTGGCTGCCCTGGAGCCTCCAGAACCGAAGAAGAGCCGCAGGTGTGTGCTGCTATGA
GAATTCATCGTGACTGACTGACG

Cdc42 S71E in pGEX-2T vector

CTGGTTCCGCGTGGATCTCCGGGAATTTCCGGTGGTGGTGGTGGATCCATGCAGACAATTAAGTGTGTTGTTGTGGGC
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ACTGCTGAAAAGCTGGCCCGTGACCTGAAGGCTGTCAAGTATGTGGAGTGTTCTGCACTTACACAGAAAGGCCTAAAG
AATGTATTTGACGAAGCAATATTGGCTGCCCTGGAGCCTCCAGAACCGAAGAAGAGCCGCAGGTGTGTGCTGCTATGA
GAATTCATCGTGACTGACTGACG

B. Curriculum Vitae

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Nationality:	German

Education

09/1987 – 06/1991	Primary School, Magdeburg
09/1991 – 07/1999	"Wilhelm-Raabe-Gymnasium", Magdeburg
07/1999	Abitur (Grade: "gut")
10/1999	Started Studies at Leibniz University Hannover, Biochemistry
07/2002	Vordiplom (Grade: "befriedigend")
09/2004	Diplomarbeit / Diploma thesis "Charakterisierung des Endosomen-lokalisierten RhoB im Verlauf der Behandlung eukaryontischer Zellen mit *Clostridium difficile* Toxin A" at the Institute of Toxicology at the Hannover Medical School, supervised by PD Dr. Ralf Gerhard (Grade: "sehr gut")
05/2005 – 06/2008	Dissertation at the Institute of Toxicology at the Hannover Medical School, supervised by PD Dr. Ralf Gerhard

Academic Publications

Schoentaube, J., Olling, A., Tatge, H., Just, I., and Gerhard, R. 2008. Serine-71 phosphorylation of Rac1 diminishes the effect of *Clostridium difficile* toxin A on epithelial barrier function. *Manuscript submitted for publication.*

Gerhard, R., Nottrott, S., **Schoentaube, J.**, Tatge, H., Olling, A., and Just, I. Jun 2008. Glucosylation of Rho GTPases by *Clostridium difficile* toxin A triggers apoptosis in intestinal epithelial cells. *J. Med. Microbiol.* 57:765-770.

Nottrott, S., **Schoentaube, J.**, Genth, H., Just, I., and Gerhard, R. Aug 2007. *Clostridium difficile* toxin A-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. *Apoptosis.* 12(8):1443–1453.

Teichert, M., Tatge, H., **Schoentaube, J.**, Just, I., and Gerhard, R. Oct 2006. Application of Mutated *Clostridium difficile* toxin A for Determination of Glucosyltransferase-Dependent Effects. *Infect.Immun.* 74(10):6006–6010.

Speeches

Mainz (Germany), 11—13 March 2008. 49th Spring Meeting of the German Society of Pharmacology and Toxicology.
Schoentaube, J., Just, I., and Gerhard, R. 2008. Rac1 phosphorylation attenuates the effect of *Clostridium difficile* toxin A on the epithelium barrier function. Naunyn-Schmiedeberg's Archives of Pharmacology, 377:27, 95. Suppl. 1.

Mainz (Germany), 13—15 March 2007. 48th Spring Meeting of the German Society of Pharmacology and Toxicology.
Schoentaube, J., Just, I., and Gerhard, R. 2007. Rac1 phosphorylation protects from *Clostridium difficile* toxin A-induced cytotocity. Naunyn-Schmiedeberg's Archives of Pharmacology, 375:36, 139. Suppl. 1.

Poster Presentations

Maribor (Slovenium), 6—9 June 2007. Second international Clostridium difficile Symposium.
Schoentaube, J., Just, I., and Gerhard, R. 2007. Rac1 phosphorylation protects against *Clostridium difficile* toxin A-induced cytophathic effect. In *Clostridium difficile: Organism, Disease, Control and Prevention – Abstract Book*, 93, P43.

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Nottrott, S., **Schoentaube, J.**, Genth, H., Just, I., and Gerhard, R. 2007. *Clostridium difficile* toxin A-induced apoptosis. Naunyn-Schmiedeberg's Archives of Pharmacology, 375:81, 398. Suppl. 1.

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Languages

German	Mother tongue
English	Speaking and writing fluent
French / Spanish	Basic