Universitätsklinikum Hamburg-Eppendorf
Institut für Immunologie
Direktor: Prof. Dr. med. Marcus Altfeld

Molecular characterization of single-domain antibodies against *Clostridium difficile* toxins

Dissertation

zur Erlangung des Grades eines Doktors der Medizin

an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von

Oliver Moritz Strysio

aus Goslar

Hamburg, 2020

Angenommen von der Medizinischen Fakultät am: 27.10.2020
Veröffentlicht mit Genehmigung der Medizinischen Fakultät der Universität Hamburg
Prüfungsausschuss, der Vorsitzende: Prof. Dr. Friedrich Koch-Nolte
Prüfungsausschuss, zweiter Gutachter: Prof. Dr. Holger Rohde

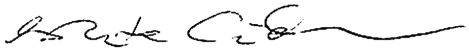
Date: 01/31/2017

Marta Ciechonska
60B Gowlett Read
London SE154HY
UK

Post-doctoral Associate, Department of Life Sciences, Sir Alexander Fleming Building (SAF), Imperial College London.

I, Marta Ciechonska, testify that I have fully read over the doctoral thesis of Oliver Moritz Strysio and declare that the English is clear and free of grammatical errors.

Full name MARTA CIECHONSKA

Signature 

Contents

Publications

Unger, Mandy, Anna Marei Eichhoff, Lucas Schumacher, **Moritz Strysio**, Stephan Menzel, Carsten Schwan, Vanina Alzogaray, et al. 2014. “Selection of Nanobodies That Block the Enzymatic and Cytotoxic Activities of the Binary Clostridium Difficile Toxin CDT..” Scientific Reports 5 (December): 7850–50. doi:10.1038/srep07850.

List of abbreviations

AA	Amino acid
ADP	adenosine diphosphate
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride
AF647	Alexa Fluor 647
AP	Alkaline Phosphatase
ART2	ADP-ribosyl transferase 2
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BCR	B cell receptor
BisTris	2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol
BSA	bovine serum albumin
CCCNA	cell culture cytotoxicity neutralization assay
CD	cluster of differentiation
CDAD	*Clostridium difficile* associated disease
CDI	*Clostridium difficile* infection
cDNA	complementary DNA
CDR	complementarity determining region
CDT	*Clostridium difficile* transferase
CH	constant domain of the antibody heavy chain
CL	constant domain of the light chain
CPD	cysteine protease domain
CPE	cytopathic effect
CROPs	c-terminally-located combined repetitive oligopeptides

DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylendiamintetraacetate
ELISA	enzyme-linked immunosorbent assay
et al.	*et altera*
Fab	fragment antigen binding
FACS	flourescence-activated cell sorting
Fc	fragment of crystalization
FCS	foetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
FR	framework regions
GDH	glutamate dehydrogenase
GTD	glucosyl transferase domain
HCl	hydrochloric acid
HCAbs	llama heavy chain only antibodies
HEK	human embryonal kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HSP90	heat shock protein 90
IDSA	Infectious Diseases Society of America
Ig	immunglobulin
IgNAR	immunoglobulin new antigen receptor

IL-8, 1β	interleukin-8, 1β
IMAC	immobilized metal ion affinity chromatography
$InsP_6$	inositol hexakisphosphate
IPTG	isopropylthio-β-galactoside
kDa	kilodalton
LacZ	ß-galactosidase coding gene
LAMP	loop-mediated isothermal amplification
LB	Luria broth
LCT	large clostridial toxin
LSR	lipolysis-stimulated lipoprotein receptor
mAb	monoclonal antibody
MCS	multiple cloning site
MES	2-(N-morpholino)ethanesulfonic acid
MST	microscale thermophoresis
MW	molecular weight
NAATs	nucleic acid amplification tests
NaCl	sodium chloride
NAD^+	nicotinamide adeninine dinucleotide
NALP3	NACHT, LRR and PYD domains-containing protein 3
Nb	nanobody
N-AP	nanobody alkamline phosphatase (fusion protein)
Ni-NTA	nickel-nitriloacetic acid
OD	optical density
PBS	phosphate buffer saline
PaLoc	pathogenicity locus

PBL	peripheral blood lymphocyte
PCR	polymerase chain reaction
PE	R-Phycoerythrin
PelB	pectate lyase B
PhoA	alkaline phosphatase gene
pNPP	para-nitrophenylphosphate
PMC	pseudomembranous colitis
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institue - cell culture medium
sdAb	single domain antibody
SDS-PAGE	sodium dodecyl sulfate polyacrylamide
SHEA	The Society for Healthcare Epidemiology of America
SOC	super optimal broth with catabolite repression
TAE	tris-acetate-EDTA
TcdA	toxin A (of *Clostridium difficile*)
TcdB	toxin B (of *Clostridium difficile*)
TMB	3,3′,5,5′-tetramethylbenzidine
U	unit
UV	ultraviolet
V	volt
VH	variable domain of the heavy chain
V_HH	variable domain of the heavy chain only antibody
VL	variable domain of the light chain

Abstract

Clostridium difficile is a gram-positive gut bacterium that provokes severe and relapsing diarrhea. It is the primary causative agent of antibiotic-associated diarrhea and pseudomembranous colitis and one of the leading causes of hospital-associated infections. The major virulence factors are the two high molecular cytotoxins of *C. difficile*, toxin A (TcdA) and toxin B (TcdB). Additionally, hypervirulent strains (e.g. ribotypes 027 and 078) produce a binary toxin, *C. difficile* transferase (CDT). The emergence of hypervirulent strains has led to a dramatic increase in the prevalence of *C. difficile* infections, as well as in associated mortality rates. These developments require new diagnostic and therapeutic tools to control the spread and the severity of *C. difficile* infections. A clinical study demonstrated that a mixture of systemically administered monoclonal antibodies against TcdA and TcdB significantly diminished the frequency of relapsing infections. Single-domain antibodies (sdAb) or nanobodies (Nbs) derived from llama heavy chain only antibodies (hcAbs) provide similar beneficial properties as conventional antibodies such as target specificity and binding strength. At the same time, they are more cost-effective, more robust and have a greater tissue penetration capacity. Prior to this study, toxin-specific Nbs had been generated in the Nolte lab from llamas immunized with the ADP-ribosyltransferase domain of CDTa, the cysteine protease domain of TcdA, and the glucosyltransferase domain of TcdB. The aim of this study was to further characterize these Nbs. The Nbs were expressed in *E. coli* periplasm at yields of 0.1–5 mg per liter of culture medium and the target specificities were verified by ELISA. The binding affinities of two Nbs were determined by microscale thermophoresis to lie in the lower nM range, i.e. comparable to those of mAbs. Nb-alkaline phosphatase fusion proteins were generated as diagnostic tools to directly detect clostridial toxins. Functional assays identified Nbs capable to block the enzymatic activities of the toxins. Five of ten CDTa-specific Nb families contained members that effectively blocked CDTa-mediated ADP-ribosylation of actin. Two TcdB-specific Nbs inhibited TcdB-mediated glucosylation of Rac1, and two TcdA-specific Nb families blocked CPD-mediated autoproteolysis of TcdA. The results of this study have identified a set of Nbs that provide a basis for the development of new diagnostic and therapeutic tools to better identify and treat *C. difficile* infections.

Zusammenfassung

Clostridium difficile ist ein gram-positives Bakterium, welches schwere und rezidivierende Diarrhoen verursacht. Es ist der häufigste Erreger Antibiotika-assoziierter Diarrhoe und Pseudomembranöser Enterocolitis und einer der bedeutendsten Erreger nosokomialer Infektionen. Seine wichtigsten Virulenzfaktoren sind die Zytotoxine, Toxin A (TcdA) und (TcdB) Toxin B. Hypervirulente Stämme vom Ribotyp 027 und 078 besitzen zusätzlich ein drittes Toxin, die *C. difficile* Transferase (CDT). Da die Ausbreitung dieser Stämme in den letzen Jahren zu einem dramatischen Anstieg der Infektions- und Sterblichkeitsraten geführt hat, sind neue Diagnostika und Therapeutika erforderlich, um die Ausbreitung und Schwere der Infektionen eindämmen zu können. In einer klinischen Phase-2-Studie wurde gezeigt, dass der Einsatz von systemisch verabreichten monoklonalen Antikörpern gegen die Toxine A und B die Rezidivrate der durch *C. difficile* verursachten Diarrhoen signifikant mindert. Die Produktion monoklonaler Antikörper ist jedoch aufwändig und kostenintensiv. Einzeldomänen-Antikörper, auch Nanobodies (Nbs) genannt, welche Derivate sogenannter Schwere-Ketten-Antikörper sind, stellen eine wertvolle Alternative zu herkömmlichen Antikörpern dar. Ebenso wie konventionelle Antikörper besitzen sie eine hohe Spezifität und Bindungsaffinität. Gleichwohl lassen sie sich kostengünstig produzieren, weisen eine hohe chemische und thermische Stabilität und, aufgrund ihrer geringen Größe, eine gute Gewebegängigkeit auf. Die Nbs für diese Studie waren im Nolte Labor zuvor aus immunisierten Lamas isoliert worden. Die Nbs wurden in dieser Studie in *E. coli* produziert und deren Toxin-Spezifität mittels ELISA verifiziert. Die Bindungsaffinitäten lagen, vergleichbar mit denen von monoklonalen Antikörpern, im niedrigen nanomolaren Bereich. Durch molekulare Klonierung wurden Nb-Alkalische Phosphatase Fusionsproteine hergestellt, welche als Agens zur Detektion von *C. difficile* in direkten Nachweisverfahren eingesetzt werden können. Schließlich wurde der Einfluss der Nbs auf die enzymatischen Funktionen der Clostridientoxine untersucht. Drei CDTa-spezifische Nb Familien inhibierten die CDT vermittelte ADP-Ribosylierung von Aktin. Zwei TcdB-spezifische Nb Familien verhinderten die TcdB-vermittelte Glucosylierung von Rac1 und zwei TcdA-spezifische Nb Familien blockierten die TcdA-vermittelte Autoproteolyse von TcdA. Die in dieser Studie untersuchten Nbs stellen eine Basis für die Entwicklung neuer Diagnostika und Therapeutika dar, um *C. difficile* Infektionen zukünftig besser identifizieren und behandeln zu können.

1 Introduction

1.1 *Clostridium difficile* infection

1.1.1 Epidemiology

Clostridium difficile is considered the most important cause of hospital-associated infections (Rupnik, Wilcox, and Gerding 2009). Fecal colonization with *C. difficile* is seen in >20% of adults that are hospitalized for more than one week (Clabots et al. 1992; Riggs et al. 2007). Incidence rates and severity of C. difficile infections (CDIs) have markedly increased in the U.S.A., Canada, and Europe since the year 2000 (Fauci et al. 2008). In the US, hospital discharges with CDI increased from 3.8 per 1000 discharges in 2000 to more than 10 per 1000 discharges in 2012. Particularly among patients ≥65 years of age, infection rates have increased dramatically (Lucado, Gould, and Elixhauser 2012; Lessa, Gould, and McDonald 2012; Leffler and Lamont 2015) (**Fig. 1.1**). A study of 28 community hospitals in the southern United States suggests that *C. difficile* has replaced MRSA (methicillin-resistant Staphylococcus aureus) as the most common cause of healthcare-associated infections (Miller et al. 2011). In Montreal and Quebec, hospitals reported a fourfold increase of CDIs from 1997 to 2009 with a directly attributable mortality of 1.5% to 6.9%, respectively (Fauci et al. 2008).

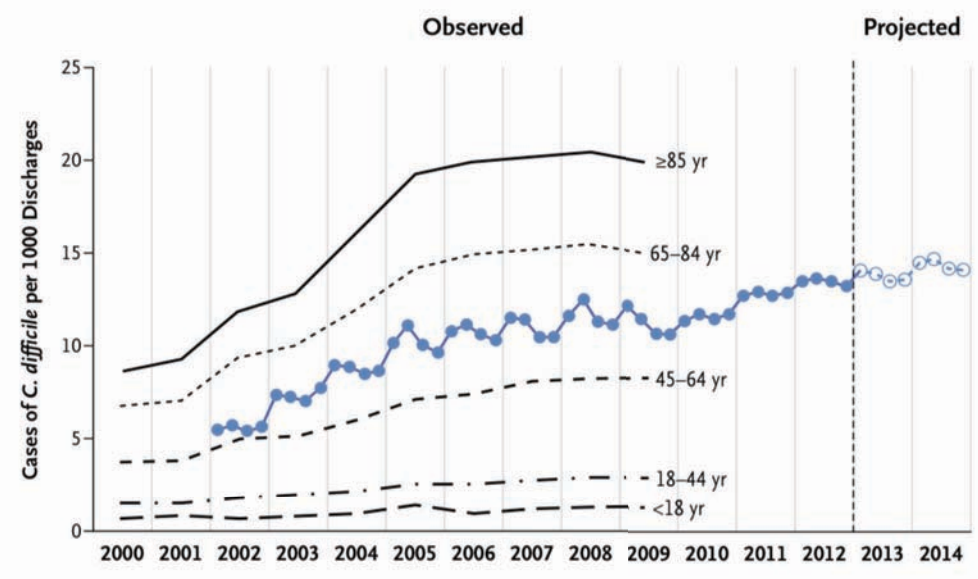


Fig 1.1: Incidence of nosocomial Clostridium difficile Infection. The overall incidence of C. difficile infections by year is shown in blue. The incidence according to patient age is depicted in black. This Figure was published by Daniel A. Leffler in the New England Journal of Medicine in 2015 . The Data are from Steiner et al. and Lessa et al..

In England, the number of fatal (CDI as primary cause of death) infections rose from 499 patients in 1999 to 3393 in 2006 (Kelly and LaMont 2008). Notably, the reported incidence of *C. difficile* during the mid- and late 1990s was rather stable (McDonald, Owings, and Jernigan 2006). In Saxony, Germany, the incidence of CDIs is relatively low, but a substantial increase was seen from 2002 to 2006 (1.7–3.8 to 14.8 per 100,000) (Burckhardt et al. 2008). CDI most often afflicts the elderly. In the aforementioned study from Quebec, the incidence of *C. difficile* infections in patients >65 was 867 per 100,000, compared to ~58 per 100,000 in the group of patients of 17–64 years of age (Pepin et al. 2004).

The drastic change in CDI rates and particularly the increased mortality, is largely attributed to a newly-recognized hypervirulent strain called BI/NAP1/027 (Rupnik, Wilcox, and Gerding 2009). This strain was first identified in 2005, and produces not only two large clostridial cytotoxins (LCTs), but also a third toxin, *Clostridium difficile* transferase (McDonald et al. 2005; Perelle et al. 1997). CDT was found in all isolates of the BI/NAP1/027 strain, and the prevalence of CDT producing strains among all other *C. difficile* strains has recently increased in several countries in Europe (Goorhuis et al. 2008; Rupnik et al. 2008).

1.1.2 Pathogenesis and clinical manifestation

Clostridium difficile is an obligatory anaerobic, gram-positive, spore-forming bacillus. The spores can be found in a variety of natural environments, such as soil, water, animals (including pets and meats) and vegetables (Saif and Brazier 1996). Of particular importance for the infection with *C. difficile* is the high occurrence of spores in hospitals and other health care facilities. Besides hospitalization, risk factors for the *C. difficile* infection include age, underlying chronic disease, gastrointestinal surgery, tube feeds, and most importantly, antibiotic treatment (Fauci et al. 2008). Virtually all antibiotics have been associated with CDI. Notably, some of them carry higher risks of inducing CDI. These are clindamycin, cephalosporins and, more recently, fluoroquinolones (Loo et al. 2005; Muto et al. 2005; Johnson et al. 1999; Gaynes et al. 2004; Pepin et al. 2005).

Spores that are ingested can survive the acidic environment of the stomach, germinate in the small bowel, and colonize the lower intestinal tract. The resulting bacteria cross the mucus layer and adhere to intestinal epithelial cells. Toxigenic strains of *C. difficile* secrete at two large cytotoxins (toxins A and B), and in case of BI/NAP1/027 a third binary toxin (CDT). CDT was shown to induce the formation of microtubule-based protrusions, which

facilitate adherence of *C. difficile* bacteria to epithelial cells. TcdA and TcdB compromise the epithelial barrier function by impairing the actin cytoskeleton (Schwan, Stecher, Tzivelekidis, van Ham, Rohde, Hardt, Wehland, and Aktories 2009b).

Furthermore, these toxins are capable of arousing an immune response which leads to the damage of epithelial cells, infiltration of neutrophils, and cytokine production (Kelly et al. 1994; Voth and Ballard 2005; Madan and Jr 2012). The clinical manifestation may vary from mild diarrhea to more severe outcomes including fever, abdominal pain and leukocytosis. The fulminant CDI may be accompanied by severe complications, including pseudomembranous colitis (PMC), colon perforations or toxic megacolon, sepsis, shock and death(Rupnik, Wilcox, and Gerding 2009).

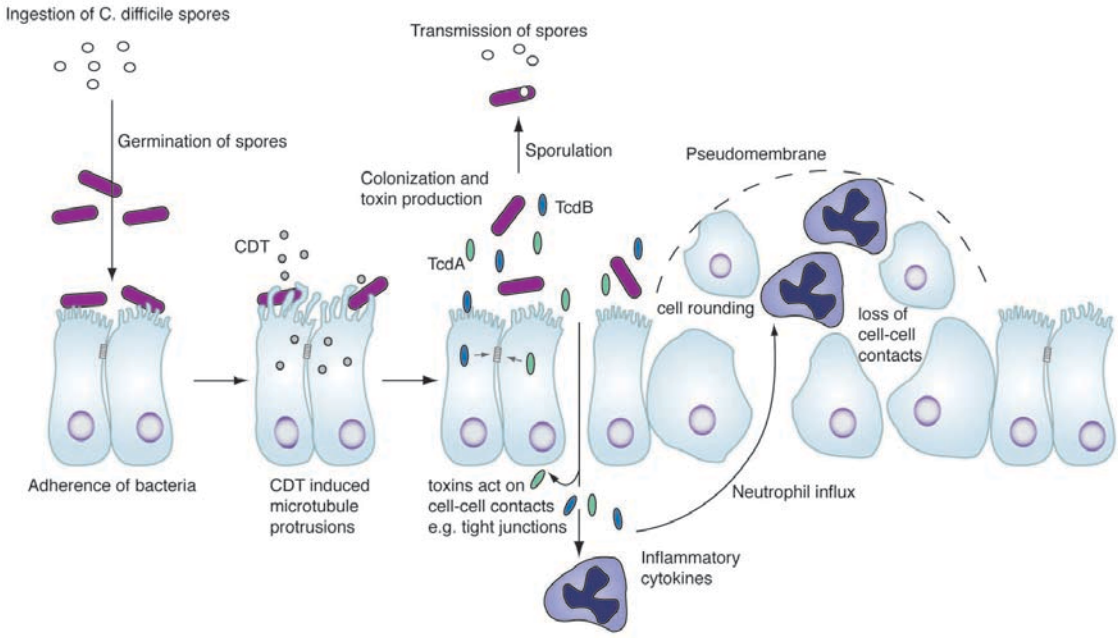


Fig. 1.2: Pathogenesis of *Clostridium difficile* infection. After ingestion, *C. difficile* spores germinate into their vegetative form. Several factors predispose the host to bacterial colonization, most importantly, the disruption of the intestinal flora upon antibiotic treatment. Toxigenic strains of *C. difficile* produce up to three different toxins (TcdA, TcdB and CDT). CDT is known to be produced by the hypervirulent strain BI/ NAP1/027. Recent research indicates that this binary toxin induces the formation of microtubule-based protrusions that increase bacterial adherence to epithelial cells. TcdA and TcdB compromise the epithelial cell barrier by disturbing the actin cytoskeleton, which leads to the loss of cell-cell contacts and to the rounding up of cells. This ‘cytopathic effect‘ is mediated by the glucosylation of small Rho GTPases. These proteins play an important role in the organization and function of the actin cytoskeleton and the cellular structures it builds (i.e. tight junctions). Furthermore, TcdA and TcdB are immunogenic and induce epithelial cells, mast cells and phagocytes to produce proinflammatory cytokines. The release of cytokines leads to inflammation and recruitment of neutrophils. Pseudomembranous colitis (PMC) is a severe form of CDI and is characterized by eroded and necrotic areas of the epithelium that are interspersed with inflammatory cells. Cell debris covered with fibrin appear like a membrane (pseudomembrane). The figure was modified from Shen et al. and Rupnik et al. (Shen 2012; Rupnik, Wilcox, and Gerding 2009).

In conclusion, three conditions are crucial for the development of CDI: (i) the exposure to antibiotics; (ii) the infection with toxigenic strains of *C. difficile*; and (iii) an insufficient immune response of the host to *C. difficile* toxins. Of particular note is the propensity of CDI to recur in certain individuals. As *C. difficile* spores are not directly affected by the antibiotic treatment, recurrent infections are reported in 15–25% of CDIs. Those individuals who have experienced a recurrent infection, have an increased propensity for a subsequent second (40%) and third (60%) infection (McCollum and Rodriguez 2012).

1.1.3 Diagnosis and Diagnostics

Clinical diagnosis

CDI is the cause for 15–25% of antibiotic-associated diarrhea (Katz 2006). The clinical manifestation of CDI has a wide range, varying from mild diarrhea to severe and fulminant colitis. Mild forms of *C. difficile* associated disease often resolve with discontinuation of antibiotic treatment, whereas severe forms can be complicated by the appearance of ileus and toxic megacolon, bowel perforation, sepsis, and shock. These complicated infections are associated with a high mortality of up to ~7% (Dubberke et al. 2008). Diarrhea is the most common symptom of patients that carry *C. difficile*. The stool of patients with CDI has a characteristic odor and ranges from soft and formed to unformed watery and bloody stools. Further clinical and laboratory findings include abdominal pain (22%), fever (28%), and leukocytosis (50%) (Fauci et al. 2008).

Laboratory testing

Testing for *C. difficile* is performed when there are clinical symptoms that indicate CDI. It is not useful to test asymptomatic patients (Cohen et al. 2010). Several diagnostic assays are available to test for *C. difficile* (**Table 1)**. The gold standard is an anaerobic bacterial culture in a selective culture medium with a subsequent cell culture cytotoxicity neutralization assay (CCCNAs) with a monolayer of a sensitive human cell line. The toxins, if present, induce a cytopathic effect (CPE) that leads to cell rounding detectable by light microscopy. If a CPE is observed, neutralization of the effect by *C. difficile* antiserum confers specificity to the test. Although this combinational method provides high sensitivity as well as a high specificity, it is too time consuming and too costly to serve as a method for clinical practice. The most frequently used diagnostic methods are based on enzyme immunoassays (EIAs), including solid phase and rapid immunochromatographic

card tests. The immunoassays available can detect either a metabolic enzyme of *C. difficile* (glutamate dehydrogenase, GDH), which is expressed by toxigenic and non-toxigenic strains, or the clostridial toxins themselves. The GDH assay is a very sensitive test but if positive, further testing for toxigenic strains is required. Initially, toxin EIAs detected only TcdA, and subsequently, kits that detect both TcdA and TcdB have become available. Toxin EIAs are fast and easy to perform, relatively inexpensive, and provide a high degree of specificity. A disadvantage is that the performance of commercially available kits varies in sensitivity, and not a single kit meets the criteria for an acceptable test (sensitivity >90%, false positive rate of ≤3%) (Planche et al. 2008). As a result, official test regimes recommend combining both tests. Firstly, the GDH assay and in case of a positive result, subsequent examination for the presence of toxins in the sample using a toxin EIA. The C. DIFF Quik Chek complete™, which works on the basis of an immunochromatogenic membrane, combines both tests in a single device. However the toxin EIA component of the test has a poor sensitivity of merely 61–78% (Quinn et al. 2010; Swindells et al. 2010). More recent technologies include nucleic acid amplification methods (NAATs) based on either PCR (polymerase chain reaction)- or LAMP (loop-mediated isothermal amplification)-mediated amplification of toxin-encoding DNA. The PCR-based methods target a conserved region of tcdB, whereas the LAMP-based method amplifies a conserved region of tcdA, which is also present in tcdA–, tcdB+ strains. By providing sufficient sensitivity as well as specificity, these methods are superior to the EIA-based methods but not to toxigenic culture-based CCCNAs (Carroll and Bartlett 2011). The American society for Microbiology proposes NAAT as a one-step diagnosis method, making it particularly attractive for clinicians in comparison to the two- or three-step EIA-based techniques. The limited use of these methods is mainly explained by their high cost. For the detection of the binary toxin CDT, PCR-based commercial methods are available.

The German AWMF (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften e.V.) are yet to release a diagnostic procedure for *C. difficile* infections. The Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA) recommend the aforementioned two-step method that combines the GDH-screening assay with a subsequent toxin EIA.

Table 1: Performance characteristics of diagnostic tests for C. difficile. The table is modified from Carroll et al., Badger et al. and McCollum et al..

Assay	Advantages	Limitations	Sens./Spec.	Reference
CCCNA	high specificity	time consuming (2 days)	67–87/97–100	(Eastwood et al. 2009; Peterson et al. 2007; Stamper, Babiker, et al. 2009; Barbut et al. 2009)
Toxigenic culture + CCCNA	higher sensitivity than CCCNA alone	time consuming (7 days)	N/A	N/A
EIA for GDH	sensitive, simple, low costs, good screening test	Requires a second test that is specific for toxigenic bacteria	71–100/76–98	(Crobach et al. 2009; Fenner et al. 2008; Snell et al. 2004; Ticehurst et al. 2006; Zheng et al. 2004)
EIA for LCTs	low cost, simple, specific	variable sensitivity	31–99/84–100	(Crobach et al. 2009; Eastwood et al. 2009; Planche et al. 2008)
NAATs	rapid and sensitive	potential for false positives, costly	89–97/95–99	(Babady et al. 2010; Barbut et al. 2009; Eastwood et al. 2009; Goldenberg, Dieringer, and French 2010; Huang et al. 2009; Karre et al. 2011; Knetsch et al. 2010; Kvach et al. 2010; Norén et al. 2011; Novak-Weekley et al. 2010; Stamper, Babiker, et al. 2009; Stamper, Alcabasa, et al. 2009; Swindells et al. 2010; Terhes et al. 2009)

1.1.4 Therapy

1.1.4.1 Antibiotic treatment of *C. difficile* associated disease

The increasing incidence of health-care associated infections demands effective prevention strategies to combat transmission of *C. difficile*. The SHEA/IDSA guidelines from 2010 provide recommendations on CDI prevention. Compliance with hand hygiene is indispensable. Single-use disposable equipment should be used and non-disposable materials should be dedicated to the patients room. Other materials should be thoroughly cleaned and disinfected after use. Contact precautions are to be maintained at least until the resolution of diarrhea. For environmental surfaces disinfection with chlorine-containing cleaning agents is essential to inactivate *C. difficile* spores. A hospital-based infection control program and antibiotic stewardship are recommended. Patients with CDI or suspected CDI should be isolated in a single room.

The discontinuation of antibiotic treatment alone often resolves CDI associated symptoms. However, it is difficult to predict which patients are at risk of developing severe symptoms.

Therefore, *C. difficile* specific antibiotic treatment is indicated for all patients diagnosed with CDI. In an initial episode of mild to moderate CDAD, metronidazole in a dosage of 500 mg, three times each day, is the medication of choice. Orally administered metronidazole is almost completely absorbed and has to be secreted into the colon to reach the bacteria (Aktories 2009). Secretion of metronidazole into the gut lumen is highest in the state of inflammation, it decreases with disappearing symptoms, and is lowest in asymptomatic patients (McCollum and Rodriguez 2012). Therefore, metronidazole is inefficient in preventing CDI recurrences.

According to the American College of Gastroenterology, severe CDI presents with hypoalbuminemia (< 3 g/dl) and either a white blood cell count of more than 15,000 cells/ µl or abdominal tenderness without criteria of complicated disease (Surawicz et al. 2013). In a randomized controlled trial published in 2007, oral treatment of vancomycin (97% cured) was superior to metronidazole (76% cured) (Zar et al. 2007). However, both antibiotics performed equally well in patients with mild to moderate CDI. Vancomycin (125 mg) is recommended to be administered four times a day, for ten to fourteen days. For patients suffering from complicated CDI (ileus, shock, or hypotension), the guidelines recommend higher doses of vancomycin (500 mg, every 8 hours) and/or rectally administered vancomycin (500 mg in 100 ml saline solution, every 6 hours) (Cohen et al. 2010; Apisarnthanarak, Razavi, and Mundy 2002). Patients with symptoms of fulminant CDI (colon perforations, toxic megacolon, sepsis, or shock) show the highest mortality rate. In this case, subtotal colectomy can reduce the risk of death.

Recurrent *C. difficile* infections are accompanied by substantial morbidity, frustration, and cost. The recommended treatment includes a tapering course of oral vancomycin followed by pulsed dosing. This treatment allows *C. difficile* spores to geminate to their vegetative form, which is then susceptible to the subsequent antibiotic dosage. Thereby the ‘gut-load‘ of *C. difficile* spores can be diminished.

1.1.4.2 Novel Therapies

Fidaxomicin and Rifaximin

Fidaxomicin is a macrocyclic antibiotic drug that was approved by the United States Food and Drug Administration for CDI in may 2011. In a randomized controlled clinical trial, the efficacy of fidaxomicin was similar to that of orally administered vancomycin (88.2% cure vs 85.8%). However, the recurrence rates of CDI were significantly lower among

patients treated with fidaxomicin (Louie et al. 2011). The rifamycin derivative rifaximin was also shown to effectively reduce recurrence of CDI, when combined with standard therapy (Garey et al. 2011).

Monoclonal Antibodies

Two humanized monoclonal antibodies against TcdA and TcdB were tested in a phase 2 clinical trial to treat severe and recurrent CDI (Lowy et al. 2010). The antibodies were administered together as a single infusion, each at a dose of 10 mg per kg body weight in addition to either metronidazole or vancomycin. Antibody treated patients had significantly lower recurrence rates than placebo treated controls (7% vs. 25%; $P<0.001$).

1.1.4.3 Fecal Transplant

Fecal transplantation (intestinal microbiota transplantation, fecal bacteriotherapy) is a method that employs a healthy donor's feces to re-establish the normal colonic flora of a patient with CDI. Since disruption of the colonic microbiota is a key event in the pathogenesis of CDI, it appears plausible that patients with refractory CDIs may benefit from fecal transplantation. The method entails screening donors for infectious agents such as viruses (HIV, hepatitis) and then transplantation of stool via various delivery methods including naso-gastric tube, upper endoscopy, colonoscopy, and retention enema. Although fecal transplantation was first described in 1958 by Eisemann et al. it is still poorly used in clinical practice. However, from the few studies that exist, it appears to be a very effective method to cure recurrent CDIs. Meta-analysis of studies revealed an overall cure rate of 92%, regardless of the transplantation method used (Gough, Shaikh, and Manges 2011). In addition, fecal transplantation is a simple, cheap, effective method with rare adverse effects. Considering the astonishing cure rates, it may well be the 'yuck' factor that restrains clinicians and patients from using this method to treat recurrent CDIs (Palmer 2011).

1.2 Structure and function of the clostridial toxins

1.2.1 The large clostridial (cyto)toxins (LCTs)

TcdA and TcdB from *Clostridium difficile* belong to the family of LCTs. This family also includes the lethal (TcsL) and hemorrhagic (TcsH) toxins of *Clostridium sordellii*, the α-

toxin (TcnA) from *Clostridium novyi*, and the TpeL toxin of *Clostridium perfringens* (Alouf 2005). These family members share sequence identity ranging from 42 to 96%, glucosyltransferase activity, cytotoxicity, and a high molecular weight of 250–308 kDa (Pruitt and Lacy 2012). TcdA and TcdB have an overall sequence identiy of 63% and share a similar multi-modular domain structure (Lyras et al. 2009). They are huge proteins with molecular weights of 308 (TcdA) and 269.6 kDa (TcdB). The corresponding genes, termed tcdA and tcdB, are located on a 19.6 kb pathogenicity locus (PaLoc). Other genes located on the PaLoc encode negative (tcdC) and positive (tcdR) regulators of toxin production (Rupnik, Dupuy, et al. 2005), and a gene (tcdE) encoding a holin-like protein required for efficient secretion of TcdA and TcdB (Govind and Dupuy 2012).

TcdA and TcdB compromise the intestinal epithelial cell barrier by at least two pathophysiologic pathways. One appears to involve the production of proinflammatory cytokines and the other involves the transfer of glucosyl residues onto small Rho GTPases, which induces rounding and the eventual death of target cells. The exact mechanisms by which clostridial toxins induce cytokine production and thereby inflammation are not entirely understood, but several hints have become apparent in the last two decades. In a rodent model it was shown that TcdA and TcdB alone, can induce characteristic symptoms of CDI, including diarrhea, hemorrhage, and intestinal fluid accumulation (Lyerly et al. 1985; Gilbert et al. 1989; Kelly and Kyne 2011). Notably, the toxins were shown to trigger the secretion of proinflammatory cytokines (e.g. IL-1ß, TNF-α, IL-8) by epithelial cells and mucosal immune cells (Pothoulakis 2000; Savidge et al. 2003). The activation of an ASC-containing inflammasome and the activation of the p38 kinase by TcdA and TcdB were uncovered as pathophysiologic pathways leading to the secretion of IL-1ß and IL-8, respectively (Ng et al. 2010; Warny et al. 2000). Adaptive immunity plays an important role in alleviating the disease symptoms, and defective humoral responses are associated with more severe CDI (Warny et al. 1994). Consistently, systemic administration of toxin-neutralizing antibodies diminished disease symptoms and lowered recurrence rates (Babcock et al. 2006).

1.2.1.1 The ABCD model of toxin uptake

The toxin structure and mode of action is described as the ABCD model, an extension of the AB-toxin model (Jank and Aktories 2008). This model describes the structure-function relationship of the clostridial glucosylating toxins. Each step of the toxin‘s uptake and processing is mediated by one of their protein domains. Both, TcdA and TcdB, carry the same multi-modular structure that consists of an enzymatically **a**ctive domain (**A; GTD**), a **b**inding domain (**B; CROP; RBD**), a self-**c**utting protease domain (**C; CPD**), and a **d**elivery domain (**D; DD**).

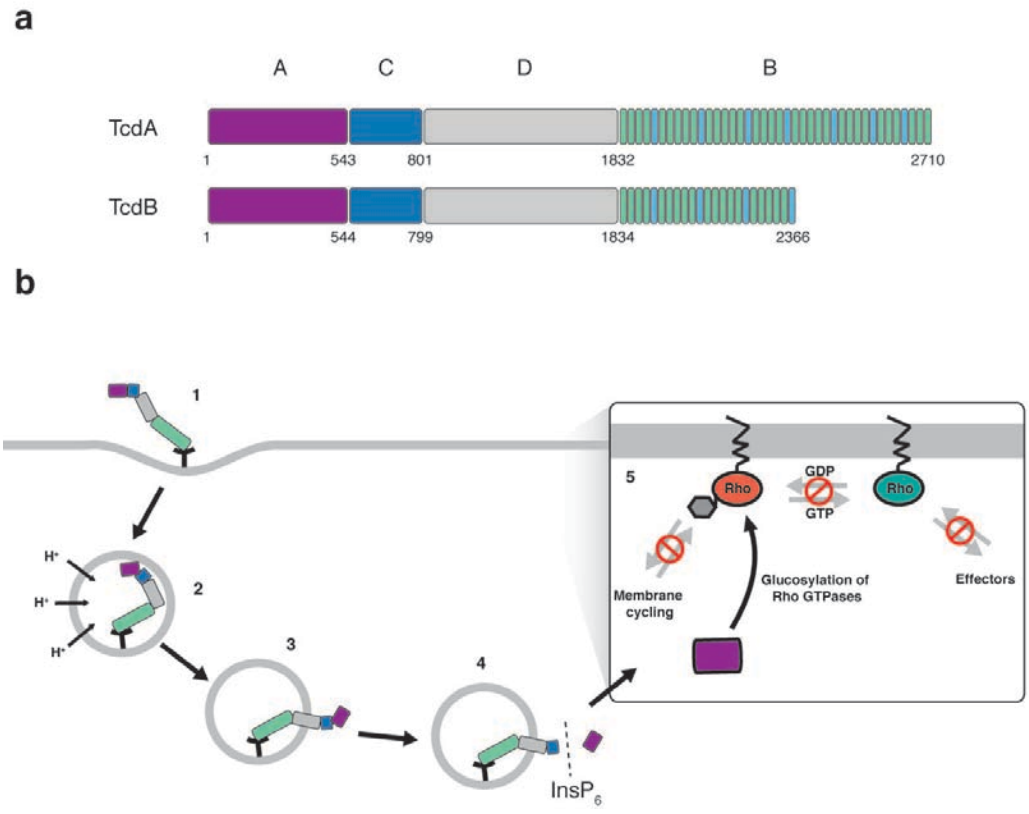


Fig. 1.3: The ABCD model of clostridial glucosylating toxins and the mechanism of cellular uptake.
(a) TcdA and TcdB share a similar domain structure. The biologically active (A) glucosyltransferase domain (GTD) is located N-terminally (violet). The B component (green, light blue) is responsible for binding of the toxin to the cell surface. It consists of repetitive elements termed combined repetitive oligopeptides (CROP). The D (delivery) domain (grey) is thought to insert into the endosomal membrane building a pore to translocate the A and C domain into the cell cytosol. The Cysteine protease domain (blue) is the C (cutting) component, which cleaves off the A domain to release it into the cytosol. b) The cellular uptake of clostridial glucosylating toxins is divided into four main steps, which are facilitated by each individual domain. (1) The toxin binds to a yet unknown cell surface receptor resulting in receptor mediated endocytosis. (2-3) Acidification of the endosomal milieu triggers conformational changes of the toxin that lead to D (delivery)-mediated pore formation trough which the GTD is translocated. (4) The A domain is released into the cytosol by $InsP_6$ mediated autoproteolysis. (5) The GTD domain glucosylates small Rho GTPases at the threonine residue 37. The glucosylation of small GTPases inhibits them from binding to effector molecules, as well as membrane cycling and switching from GDP to GTP bound state.

Binding to the target cell's surface

Nearly one third of TcdA is highly repetitive (Davies et al. 2011). The repeating units are located at the C-terminus and are termed C-terminally combined repetitive oligopeptides (CROPs). CROPs are acknowledged to function as binding domains of TcdA. CROPs consist of multiple 19–24 amino acid short repeats (SRs) and 31 amino acid long repeats (LRs) (Eichel-Streiber and Sauerborn 1990; Jank, Giesemann, and Aktories 2007a). TcdA and TcdB differ in the length of their CROPs. TcdA contains 32 SRs and 7 LRs, whereas the CROPs of TcdB are considerably shorter, consisting of 19 SRs and 4 LRs. Each repeat encodes a single hairpin structure, resulting in a corkscrew-like superfold, termed solenoid. Such structures increase the binding surface and are often found among bacterial cell-surface binding proteins (Fernández-Tornero et al. 2001). Although carbohydrates are presumed to be involved in binding of TcdA, the precise cell surface receptors are still unknown. Initially, the CROPs of TcdA were shown to bind Gal-α-(1,3)-Gal-ß-(1,4)-GlcNAc (Pruitt and Lacy 2012; Krivan et al. 1986). However, this sugar compound does not exist in humans (Jank and Aktories 2008). Since then, other receptors for TcdA have been reported, including human I, X and Y blood antigens as well as a human glycosphingolipid, which all contain a ß-Gal-(1,4)-ß-GlcNAc core structure (Pruitt and Lacy 2012; Teneberg et al. 1996; Tucker and Wilkins 1991). It is not known whether these putative receptors are relevant for toxin uptake by human intestinal epithelial cells. No receptor has been identified for TcdB to date. Furthermore, a recent study has identified CROP-independent binding using truncated LCTs ($TcdA_{1–1851}$; $TcdB_{1–1874}$) devoid of the CROP region. This additional binding activity outside the carbohydrate-binding repeats is suspected to be within the amino acid residues 1101–1874 (TcdA) and 1529–1874 (TcdB) (Olling et al. 2011; Genisyuerek et al. 2011; Pruitt et al. 2012).

Toxin uptake and pore formation

Cell surface binding of the B domain induces clathrin- and dynamin-dependent endocytosis. Alternative clathrin-independent uptake of toxins is suspected, although the molecular mechanisms are as yet unsolved (Gerhard et al. 2013; Olling et al. 2011; Papatheodorou et al. 2010). Within the endosomal compartment, the toxins undergo a pH-dependent conformational change. As the endosomal pH decreases, hydrophobic residues within the delivery domain insert into the endosomal membrane building a pore to translocate the GTD domain into the cytosol (Barth et al. 2001; Giesemann et al. 2006).

Bafilomycin, an endosomal H^+-ATPase inhibitor, blocks the translocation of LCTs, indicating that acidification is essential for toxin processing (Qa'Dan, Spyres, and Ballard 2000). The amino acid residues 956–1128 are particularly hydrophobic and have been suggested to build at least a part of the pore (Eichel-Streiber et al. 1992; Eichel-Streiber and Sauerborn 1990). For TcdB, residues 830–990 constitute the minimal pore forming region. Within this region, residues Glu970 and Glu976 were shown to act as pH-sensors mediating pore formation (Genisyuerek et al. 2011).

Autoproteolysis

When the N-terminal glucosyltransferase domain reaches the host‘s cytoplasm, it is cleaved off by the adjacent cysteine protease domain. This autoproteolytic processing of LCTs was initially shown for TcdB (Pfeifer et al. 2003; Egerer et al. 2007). Further analyses revealed the cleavage site to be localized between the residues Leu543 and Gly544 (Rupnik, Pabst, et al. 2005). The initiation of the autoproteolytic cleavage is controlled by the cytosolic molecule inositol hexakisphosphate ($InsP_6$) (Reineke et al. 2007). $InsP_6$ binds to CPD distantly from the active center, and allosterically induces cleavage. Three conserved residues (TcdA: Cys700, His655, Asp589; TcdB: Cys698, His653, Asp587) have been shown to be essential for the auto-proteolytic processing of TcdA and TcdB.

Glucosyltransfer

Following autoproteolytic cleavage, the N-terminal glucosyltransferase domain (A; GTD) is released into the cytosol. Using UDP-Glucose as substrate, GTD transfers the glucosyl-moiety onto Thr37 of RhoA and the corresponding threonine residue of other small GTPases (Thr35 of Rac and Cdc42), leading to the inactivation of these proteins. Rho GTPases serve as molecular switches to regulate a range of cellular processes, most importantly, organization of the actin cytoskeleton (Bishop and Hall 2000). In the active, GTP-bound state, the protein conformation allows Rho proteins to interact with their effector molecules (Aktories and Barbieri 2005). Binding of effector molecules to Thr37 is particularly important (Genth, Aktories, and Just 1999; Jank, Giesemann, and Aktories 2007a). Hydrolysis of Rho-bound GTP to GDP confers Rho proteins into their inactive conformation in which they are not able to bind to effector molecules. The modification of Thr37 by TcdA or TcdB is mainly carried out during the inactive, GDP-bound state (Just et

al. 1995; Genth, Aktories, and Just 1999). In vitro experiments revealed that the Rho family members RhoA, RhoB, RhoC, Rac1, Cdc42 and TC10 are targets of the LCTs (Jank, Giesemann, and Aktories 2007b; Genth et al. 2008). TcdA was shown to also modify Rap1 and Rap2, two proteins which are more closely related to the family of Ras proteins. Ras is involved in the regulation of cell-cell junctions, cell proliferation and survival. A distinct variant of TcdB also glucosylates Ras proteins. It carries the tail of the conventional TcdB, which is linked to the GTD domain of TcsL and is produced by the *C. difficile* strains 1470, 8864 and C34 (Huelsenbeck et al. 2007; Mehlig et al. 2001; Chaves-Olarte et al. 1999). All of the $TcdA^{-}/TcdB^{+}$ strains that have been characterized produce these functional hybrids (Pruitt and Lacy 2012). The crystal structures of the GTDs of TcdA and TcdB were solved and are accessible via the Protein Data Bank (GTD-TcdA: pdb 3SS1/3SRZ (Pruitt et al. 2012); GTD-TcdB: pdb 2BVM (Reinert et al. 2005)).

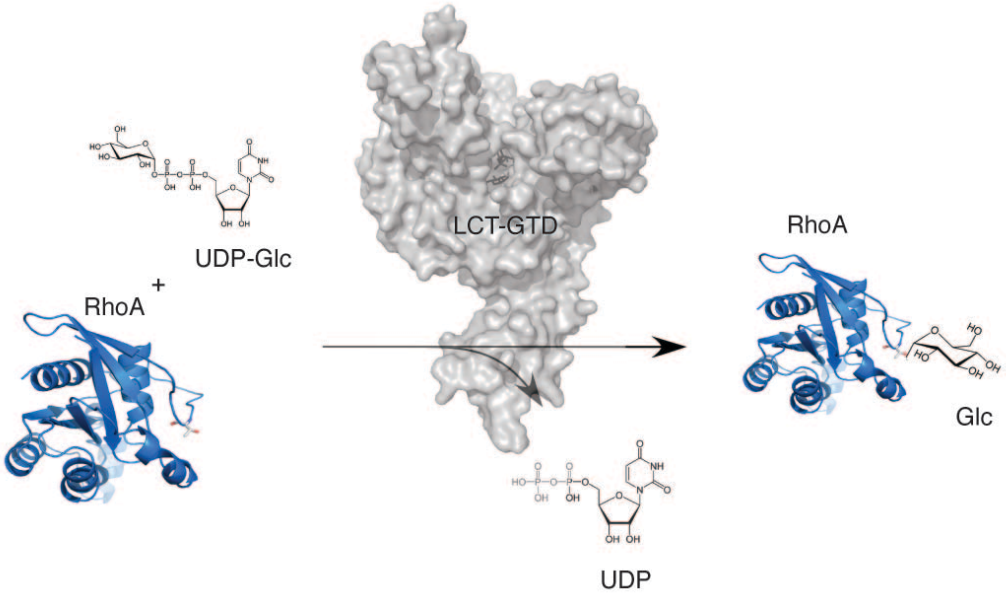


Fig. 1.4: Mechanism of GTD-mediated glucosylation of small Rho GTPases. GTD is the N-terminal enzyme domain, which is released into the cytosol upon autoproteolytic cleavage of TcdA and TcdB. GTD catalyses mono-O-glucosylation of small Rho GTPases (i.e. RhoA), thereby inactivating these proteins. The crystal structures were modeled with PyMol using the coordinates from the pdb files GTD-TcdB: 2BVM and RhoA: 1CC0.

1.2.2 *Clostridium difficile* transferase (CDT)

The *Clostridium difficile* transferase (CDT) is a binary toxin. As in case of other AB-toxins, CDT consists of two independent proteins encoded by two genes that are unrelated to the LCT-encoding PaLoc (Barth et al. 2004). CDT**a** (48 kDa) is the **a**ctive component, carrying the enzymatic activity, whereas CDT**b** (74 kDa) is the cell surface **b**inding component. CDTb is produced as a precursor molecule of 99 kDa. It is thought to be cleaved to its active compound (of 75 kDa) by various bacterial and mammalian serine-type proteases. This was shown for its homolog iota toxin (Ib) from *Clostridium perfringens*, and is also presumed for CDTb, however, the definite enzymes which are involved are unknown. Loss of the N-terminal 24 amino acids induces a conformational change and facilitates homoheptamerization. The Lipolysis-stimulated lipoprotein receptor (LSR) was identified as the cellular receptor of CDTb (Papatheodorou et al. 2011). Upon binding, CDTb induces clustering of LSR in lipid rafts (Papatheodorou et al. 2013). The A and B components are then internalized via endocytosis (Gibert et al. 2010; Pust, Barth, and Sandvig 2010). Ultimately, CDTa is released from an acidified endosome into the host cell‘s cytosol via transmembrane pores, formed by CDTb heptamers (Barth et al. 2004; Kaiser et al. 2011; Blocker et al. 2001; Barth et al. 2000). Acidification seems to be crucial for the insertion of CDTb into the membrane, as the translocation and release of CDTa into the cytosol is blocked by the addition of Bafilomycin A (an inhibitor of endosomal acidification). These findings are underlined by in vitro studies that demonstrate toxin uptake of the *C. botulinum* toxin C2 into NIH3T3 cells, by simply lowering the pH of the cell culture medium (Blocker et al. 2001; Barth et al. 2000). Partial unfolding of the active A compound during translocation was shown for C2 and is also assumed for CDTa. This hypothesis is supported by the finding that chaperoning proteins, such as heat-shock protein 90 (HSP90) and peptidyl-prolyl cis/trans Isomerases (i.e. cyclophilin A) facilitate translocation of CDTa (Kaiser et al. 2009; Haug et al. 2003; Kaiser et al. 2011). Thus, treatment with HSP90 inhibitors delays the CDTa induced cytopathic effect. Furthermore, direct binding of CDTa to HSP90 and cyclophilin A was shown in an *in vitro* assay (Kaiser et al. 2011).

The ADP-ribosylation of actin

CDTa consists of two structurally related domains, most likely generated by gene duplication (Han et al. 1999). The C-terminal domain carries a functional ADP-ribosyl

transferase (ART) domain, whereas the N-terminal (pseudo) ART domain underwent an evolutionary loss of amino acids that are crucial for the ART activity. Instead of catalyzing the transfer of ADP-Ribose onto G-actin, the N-terminal component is thought to interact with the binding component CDTb (Sundriyal et al. 2009). The target of CDTa is G-actin which is mono-ADP-ribosylated at Arg177. Thereby, the polymerization of G-actin to its filamentous form F-actin is sterically inhibited (Aktories et al. 1986; Aktories et al. 2011). ADP-ribosylation of actin also affects the interaction of G-actin with ATP. Polymerization of actin requires hydrolysis of ATP. In ADP-ribosylated actin, the affinity to ATP is decreased and ATP hydrolysis is inhibited (Perieteanu et al. 2010; Geipel et al. 1989; Geipel, Just, and Aktories 1990).

ADP-ribosylation of actin also affects the microtubule system. Microtubules are long intracellular filaments consisting of α-/β-tubulin heterodimers. Their orientation is polarized: a slowly growing minus-end, which is stabilized at the microtubule organizing centre and a faster growing plus end, which is directed to the cellular cortex. Physiologically, the growth of the plus end side is stopped when the microtubules reach the cortical actin network. The stabilization of microtubules here is mediated by capture proteins (Gerding et al. 2014; Lansbergen and Akhmanova 2006; Siegrist and Doe 2007). However, upon treatment with CDT, microtubule growth does not stop at the cell membrane.

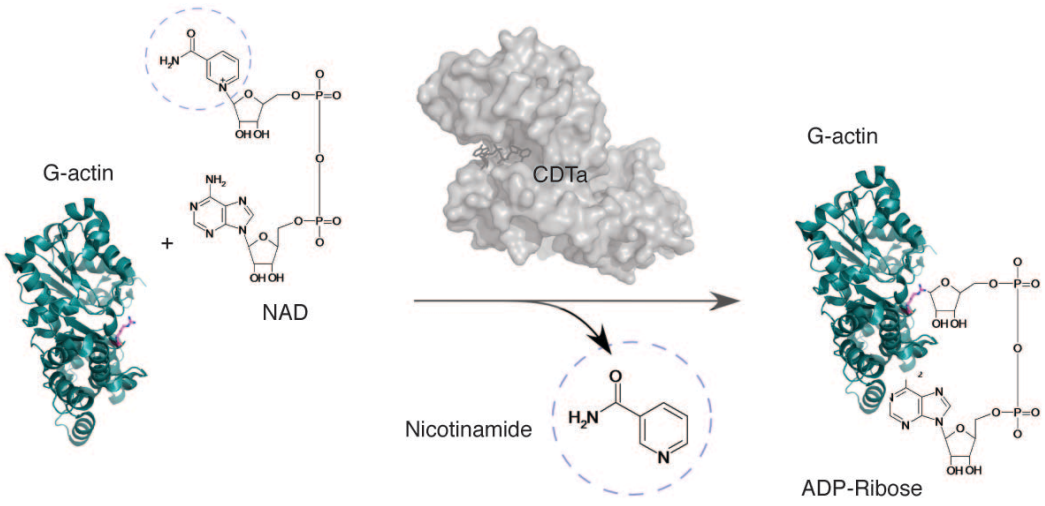


Fig. 1.5: Mechanism of CDTa mediated ADP-ribosylation of G-actin. The enzymatically active domain of CDTa has ADP-ribosyltransferase activity, which transfers ADP-ribose from NAD onto the side chain of an arginine residue of G-actin. Thereby, the polymerisation of G-actin to F-actin is sterically inhibited and the cellular actin cytoskeleton collapses. The crystal structures were modeled with PyMol using the coordinates from the pdb files CDTa: 2WN7; G-actin: 2GWJ.

Epithelial cells build microtubule-based protrusions of 0.05–0.5 µm in diameter and lengths of more than 150 µm that may facilitate adherence and colonization of the bacteria to the cell surface (Schwan, Stecher, Tzivelekidis, van Ham, Rohde, Hardt, Wehland, and Aktories 2009a).

1.3 Antibodies

1.3.1 Conventional antibodies

Antibodies are *the* effector molecules of the immune system in all vertebrates. Paul Ehrlich introduced the term 'antibodies' in his article 'Experimental studies on Immunity', published in 1891. In his side chain theory, for which he became the Nobel prize winner in 1908, Ehrlich proposed the fundamental concept on how these molecules act: specifically binding 'toxins' on cell surface receptors, followed by the amplification and secretion of these receptors to build soluble molecules that neutralize 'toxins'. In principle, this concept is still valid.

Antibodies are composed of two copies each of two distinct polypeptide chains forming a heterotetrameric molecule. One chain, termed heavy chain, is of 50 kDa molecular mass, and the other, termed light chain, has an average molecular mass of 25 kDa. All proteins of the immunoglobulin superfamily, which the antibodies/immunoglobulins belong to, have a common structure motif, the immunoglobulin domain (Ig-domain). It is constructed from 100–110 amino acids that are arranged in two ß-sheets. Each of these sheets is comprised of three to five antiparallel ß-strands. The strands are connected by loops and by a highly conserved disulfide bond that connects and stabilizes the two ß-sheets. The heavy chain consists of four to five (V_H, C_H1, C_H2, C_H3, (C_H4)) such domains, whereas the light chain is composed of two (C_L, V_L) Ig-domains. The N-terminal domain of both the heavy and the light chain has the highest sequence variability and is therefore called variable domain (light chain:V_L; heavy chain: V_H). The antigen binding surface consists of three loops of each of the variable domains. These six regions are more termed complementarity determining region (V_H CDR1–3, V_L CDR1–3). The C-terminal domains of the heavy chain (C_H2–C_H3 or C_H2–C_H4) comprise the Fc (fragment crystallizable) region. Antibodies are subdivided into isotypes by the amino acid sequence of their heavy chain: IgM (µ), IgD (δ), IgG (γ), IgA (α) and IgE (ε). The heavy chain of IgG, IgD and IgA isotypes has a proline-rich portion between the C_H2 and the C_H1 that confers mobility on the two arms of

the immunoglobulin. This region is referred to as the hinge region. This hinge region is replaced by an additional constant region in IgM and IgE antibodies. In humans, four subclasses of IgG ($IgG_{1–4}$) and two subclasses of IgA ($IgA_{1–2}$) are known.

Effector functions of immunoglobulins are mediated via their Fc tail. Four main effector functions of the Fc region are known: i) phagocytosis of IgG-coated antigens by macrophages and neutrophils mediated via the Fcγ receptor. ii) the binding of IgE‘s Fc portion to their corresponding receptor (Fcε receptor) on mast cells, basophils and activated eosinophils facilitates the release of inflammatory mediators. iii) the binding the Fc tail of IgM and IgG to factors of the complementarity system activates the classical complement cascade. iv) Fc receptors mediate the transfer of immunoglobulin across mucosal membranes (IgA) and across the blood-placenta barrier (IgG). By binding to the Fc receptor, which is expressed on immune cells (i.e. monocytes and macrophages), immunoglobulins carry out their effector functions, such as opsonization and neutralization of antigens, sensitization of mast cells and NK cells, and activation of the complement system (Murphy 2012). IgM is the the first antigen receptor on immature and mature naïve B cells. It is an immunoglobulin of the initial immune response and it is secreted as a pentameric molecule. It efficiently agglutinates antigens, although its repertoire is more limited than for that of the others (e.g. IgG). IgD is expressed on mature B cells. Its role in the immune response is still largely unknown. IgG is the predominant class of antibodies in human sera and extracellular fluids. It is effective in opsonization (e.g. pathogens) and activation of the complement system. IgA is found on the external barrier of the body, such as in the mucosal membranes of the gut, lung, or the urogenital tract. IgAs are transferred via breast milk, so that infants gain passive immunity prior to the development of their own mature immune system. The IgE isotype of immunoglobulins activates mast cells and eosinophil granulocytes. These types of antibodies are important for protection against worm infections and are known to play a key role in allergic reactions.

1.3.2 Heavy-chain antibodies (HCAbs) and their derivative nanobodies

Heterotetrameric y-shaped antibodies are a common feature of immune systems in vertebrates. Homodimeric derivates of these conventional antibodies devoid of the light chain occur naturally in camelids. These antibodies are composed of heavy chains only and are therefore called HCAbs. They (Hamers-Casterman et al. 1993). The family of camelidae, which belongs to the order of Artiodactyla, comprises camels (*Camelus*

dromedarius, Camelus bactrianus), llama (Lama glama, Lama guanicoe), and vicugna (Vicugna vicugna, Vicugna pacos). Interestingly, these peculiar HCAbs are not found in other suborders of Artiodactyla, such as Tylopoda and Ruminantia. In humans, production of (defective) HCAbs has been observed in rare lymphoplasmatic malignancies termed heavy chain disease (Fauci et al. 2008; Alexander et al. 1982). In the sera of camels, up to 50% of the circulating IgGs are HCAbs (Maass et al. 2007). This high amount of HCAbs reflects the importance of the HCAbs in the camelid immune response. Three IgG subisotypes are known; the IgG1 subclass, which displays a conventional antibody structure, and the IgG2 and IgG3 subclasses, which consists of HCAbs (Nguyen et al. 2002). All IgG isotypes are able to bind to the surface of monocytes and macrophages, indicating that they are able to recruit these cells in the course of an immune response (Daley et al. 2010). In contrast to conventional antibodies, HCAbs lack the C_H1 domain and are devoid of the light chain. Consequently, the hinge region directly links the variable domain to the C_H2 domain.

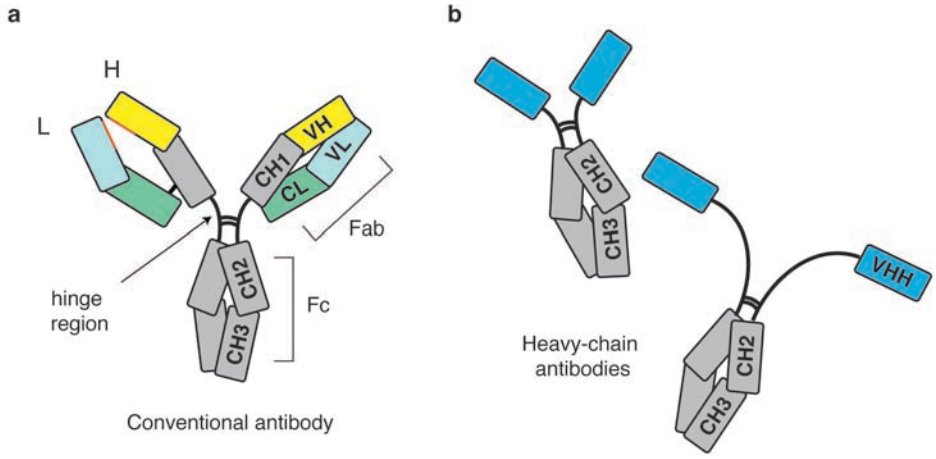


Fig. 1.6: Structural comparison of conventional antibodies and heavy chain antibodies. Llamas and other camelids possess two types of IgG antibodies. The IgG1 subclass has the conventional heterotetrameric antibody structure containing two heavy and two light chains. In contrast, the IgG2 and IgG3 of llamas solely consist of two heavy chains, and are therefore termed heavy chain only antibodies (HCAbs). a) The heavy chain of a conventional IgG antibody contains three constant domains (C_H1–C_H3) and one variable domain (V_H). The light chain has two domains, a constant- (C_L) and a variable (V_L) domain. The C_H1 and C_H2 domains are linked by a proline rich amino acid sequence, which confers flexibility to the arms of the Y-shaped molecule. The quaternary structure is stabilized by at least four disulfide bonds, two link the hinge regions of both heavy chains, and a further disulfide links each C_H1 and C_L domains. The variable domains of heavy and light chain form the antigen binding paratope. The interactions of these domains is stabilized by hydrophobic residues within their FR2 (marked orange). The C_H2 and C_H3 domains of the heavy chains together form the Fc-region, and the combination of C_L–V_L and C_H1–V_H is referred to as Fab fragment. b) HCAbs of camelids are devoid of light chains and the heavy chains lack the C_H1 domain. The hydrophobic residues within the FR2 of the variable domains are replaced by hydrophilic ones. The paratope of HCAbs is formed only by the variable domain of the heavy chain, which is is designated V_HH. The hinge region directly links the V_HH and the C_H2 domain, and is either of a short (IgG3) or of a long type (IgG2).

The length of the hinge region differs among IgG subtypes. The IgG2 subtype contains a long hinge region of 35 amino acids whereas the IgG3 isotype contains a shorter hinge of 12 amino acids (Conrath et al. 2003). The molecular weight of a HCAb is approximately half of that of a conventional antibody (~75 kDa *vs.* 150 kDa). The N-terminal variable domain of the HCAbs is termed V_HH (variable domain of heavy chain antibody). When expressed as a recombinant protein, it is called a single domain antibody (sdAb) or nanobody (Nb). The V_HH contains the antigen binding paratope formed by the hypervariable loops (CDR) which are flanked by a more conserved framework, similar to that seen in conventional VH domains. The V_HH domain consists of a ß-sheet sandwich with a four-stranded (A, B, E, D) and a five-stranded (G, F, C, C‘, C‘‘) ß-sheet connected by a conserved disulfide bond between the cysteine residues 23 (in strand B) and 94 (in strand F). The hypervariable loops are located between the B – C (CDR1), C‘ – C‘‘ (CDR2) and the F – G (CDR3) strands. Collectively, the CDR loops create a surface, which interacts with the antigen. In conventional antibodies, this platform contains the CDR loops of the heavy and the light chain, which together make up an area of 600–900 Å. The surface of the V_HH paratope is just slightly smaller (600–800 Å). This may seem counter-intuitive, since the loss of half of the interacting CDR loops (CDRs of the light chain) does not halve the interacting surface. This paradox observation can be attributed to the enlargement of the CDR1 and particularly the CDR3 loops in HCAbs, by which the antigen binding surface of the variable domain is increased. The enlargement of the CDR1 loop is due to an incremental increase on the germline level. To explain the enlarged CDR3 loop, several mechanisms have been proposed, including a higher activity of the deoxynucleotidyl transferase during the V-D-J rearrangement, positive selection at the pre-B-cell receptor stage of V_HHs containing long CDR3 loops, and negative selection during affinity maturation of short CDR3 loops (Muyldermans 2013). In conventional antibodies, the interface of the VH and the VL domains is stabilized by the interaction of hydrophobic residues (Val47, Gly49, Leu50, Trp52) that are highly conserved and stabilize the interaction of both domains. In contrast, the corresponding residues of HCAbs are replaced by hydrophilic residues (e.g., Glu49, Arg50) that increase the solubility of the domain (Arbabi Ghahroudi et al. 1997). In addition to the canonical disulfide bond, a subset of camelid-HCAbs contains an additional disulfide bond connecting the CDR3 with the CDR1 (camels) or with the CDR2 (llama). Several deductions can be made on the architecture of the CDR loops from their amino acid sequences. V_HH with short CDR3

loops that jut out of the antigen interaction surface often contain a tyrosine at position 42 (e.g. anti-GFP V_HH). A specific CDR3 formation termed 'stretched twisted turn', was observed for V_HHs containing a long CDR3 loop and a phenylalanine at position 42 (Sircar et al. 2011). In general, long CDR3 loops are stabilized by an additional disulfide bond, and/or an aromatic core formation (Trp118 (FR4), Tyr93 (FR3), and Phe117 or Tyr117).

Properties of sdAbs

Nanobodies are easily expressed in bacterial (*E. coli*) or eukaryotic cells (*P. pastoris,* HEK-cells), yielding high protein amounts at relatively low costs (Wesolowski et al. 2009; Holliger and Hudson 2005). Moreover, they show high thermal stability, high stability under variable pH conditions, high stability against unfolding, and protease resistance (Dumoulin et al. 2002; Harmsen et al. 2006).

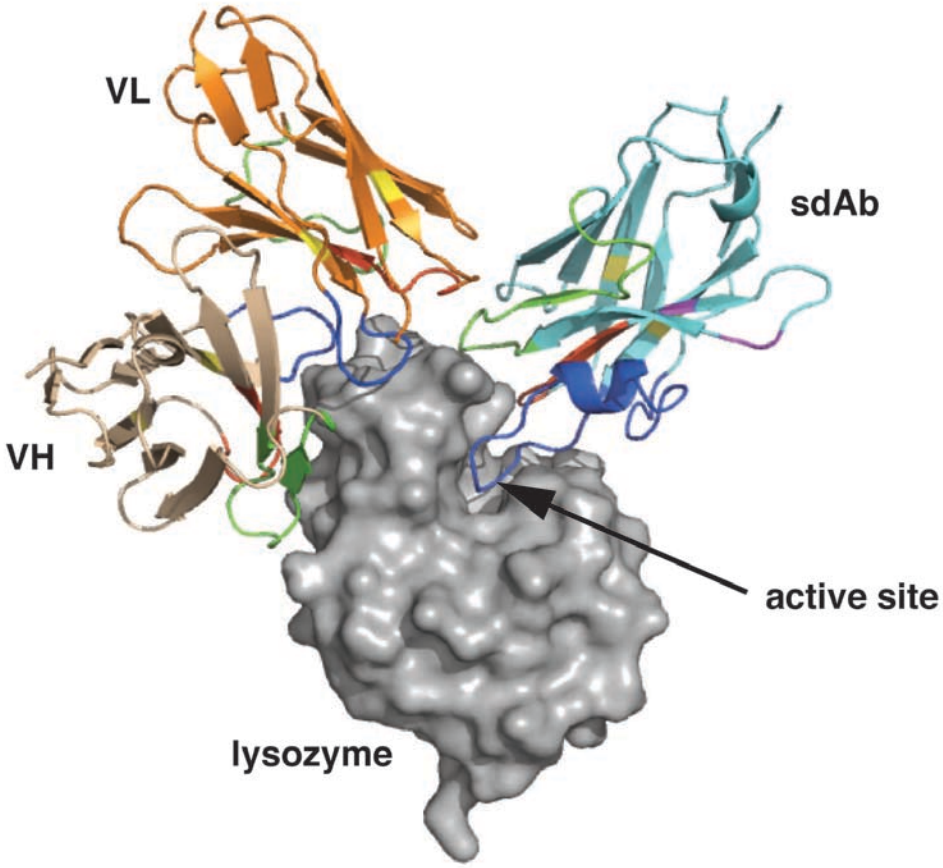


Fig. 1.7: Binding of a scFv (single chain variable fragment (VH/VL)) and a sdAb (V_HH) to their common target (lysosyme). Conventional antibodies tend to from planar interactions with their antigen, whereas the long fingerlike CDR3 region of V_HHs can access cryptic sites on their target, such as the active site crevice of lysozyme. CDR1, CDR2 and CDR3 are color-coded red, green and blue respectively. Canonical cysteines forming a conserved disulfide bridge between ß strands of FR1 and FR3 are shown in yellow. Hydrophilic amino acid substitutions in FR2, which render V_HHs with a high water solubility are colored pink. The figure was taken from Danquah et al. (Danquah 2013). The pdb files 1MEL (sdAb) and 1IC4 (scFv) were used for modeling.

The small size of nanobodies allows for optimal tissue penetration properties as well as quick systemic clearance. As nanobodies are easily reformatted, they can be used as modules in a variety of applications. These include homo- or heteropolymeric molecules with increased avidity; Nb-Fc fusion proteins with restore effector functions and increased serum half-life (Scheuplein et al. 2010; Tijink et al. 2008); and alkaline phosphatase fusion proteins as tools in histology or ELISA (Swain et al. 2011). With longer CDR1/3 loops, V_HHs possess a prolate domain structure that is ideally suited for gripping clefts or cavities in proteins. Active sites of enzymes are often located in such indents; and complementary V_HHs can impede enzymatic activity (De Genst et al. 2006). This inherent capacity to block enzymatic activity is a key feature of V_HHs and has been observed for several other target enzymes, such as bacterial toxins or leukocyte ecto-enzymes (e.g. SpvB from *Salmonella*; ART2.2) (Alzogaray et al. 2011; Koch-Nolte et al. 2007).

1.3.3 The antibody response to *Clostridium difficile*

C. difficile activates the host‘s adaptive immune system. In patients with a CDI, systemic antibodies can be detected against toxins and non-toxin antigens (Kyne et al. 2001; Kyne et al. 2000). Within the general population, 24% are seropositive for IgG antibodies against TcdA and/or TcdB (Bacon and Fekety 1994). It is thought that antibody production is initially stimulated in infancy and is maintained throughout adult life by constant environmental exposure to *C. difficile* (Sánchez-Hurtado et al. 2008). Experimental animal models (mice, hamsters) have shown that passive transfer of antiboides against *C. difficile* toxins can be protective (Giannasca et al. 1999; Babcock et al. 2006). Furthermore, it has been shown that particular the IgG response to TcdA is associated with asymptomatic carriage of *C. difficile*, and an adequate IgM response to TcdA during acute episodes of infection correlates with lower recurrence rates (Kyne et al. 2001; Kyne et al. 2000). This was also shown to be the case for anti-TcdB antibodies (Leav et al. 2010). In a recent passive immunization study, monoclonal antibodies against TcdA and B reduced the recurrence rates of CDI (Lowy et al. 2010).

2 Aims of the study

The goal of this study was to clone and produce recombinant C. difficile toxin-specific nanobodies in monomeric and dimeric formats and to verify their binding specificities. These nanobodies were to be reformatted further into dimeric Nb-alkaline phosphatase fusion proteins and evaluated as diagnostic tools in direct binding ELISAs. Biotinylatable Nbs were to be produced in order to further multimerize Nbs onto streptavidin. Finally, monomeric Nbs were to be tested for their capacity to block the enzymatic activities of the two large clostridial TcdA and TcdB cytotoxins as well as the binary CDT toxin.

3 Materials

3.1 Laboratory equipment

Equipment	Type	Manufacturer
Adjustable volume pipette	Pipetman	Gilson, Middleton
	Type ‘research’	Eppendorf, Hamburg
Agarose gel electrophoresis	Model 40-0708	PEQLAB, Erlangen
Autoclave	2540 EK Varioklav	Tuttnauer H&P Labortechnik, Oberschleißheim
Blood counting chamber	various	Labor Optik, Lancing
Centrifuge	Rotanta 460R	Hettich, Tuttlingen
	Type 5417	Eppendorf, Hamburg
	Type 5424	Eppendorf, Hamburg
	Biofuge pico	Heraeus, Hanau
CO_2 incubator	Thermicon T	Heraeus, Hanau
Cooler	Stratacooler	Stratagene, Waldbronn
Contamination monitor	Contamat FHT 111 M	Thermo Scientific, Erlangen
Electrophoresis System	XCell SureLock Mini-Cell	Invitrogen, Groningen
ELISA reader	Victor^3	Perkin-Elmer, Waltham
Freezer	–80 °C HFC 586 Basic	Eberline, Erlangen
Laboratory Scale	Analytical plus	Ohaus, Greifensee
	Type 1412	Sartorius, Göttingen
Microbiological incubator	B6060	Heraeus, Hanau
	Incubat® 80/85	Melag, Berlin
Microscale Thermophoresis	Monolith NT.115	NanoTemper, Munich
Microwave	M 637 EC	Miele, Gütersloh
Pipet-Aid	‘Express’	BD Biosciences, Heidelberg
pH meter	InLab Routine pro	Mettler, Toledo
Photometer	Ultraspec 2000	Pharmacia Biotech, Freiburg
	Smartspec 3000	BioRad, Munich
	Nanodrop 2000c	PEQLAB, Erlangen
Table-top processor	Curix60	AGFA, Köln

Gel documentation	Edas290 + Camera DC290	Kodak, Stuttgart
Thermocycler	Biometra T3 / T Gradient	Biometra, Göttingen
Thermomixer	Thermomixer compact	Eppendorf, Hamburg
Incubator shaker	Ecotron/Unitron	inforsHT, Bottmingen
Gel system	Perfect Blue Gel System Mini S	PEQLAB Ltd., Erlangen
Laminar flow	Gelaire BSB 4	Gelman, Singapur
Nitrogen tank	K series	Taylor-Wharton, Husum
Power supply for Agarose gel	Standard power pack P25	Biometra, Göttingen
Power supply for SDS PAGE	Power Pac 200	BioRAD, Munich
Roller	SRT 6	Stuart, Staffordshire
Table centrifuge	5415D	Eppendorf, Hamburg
Scanner	Canonscan 9800 F	Canon, Tokyo
Ultracentrifuge	RC 26 PLUS	Sorvall, Buckinghamshire
Ultracentrifuge rotors	SA-300, SS 34	Sorvall, Buckinghamshire
UV transilluminator	Type TI	Biometra, Göttingen
Vortex mixer	Genie 2T	Neolab, Heidelberg

3.2 Consumables

Consumable	**Type/Size**	**Manufacturer**
Culture plates	10 cm	Greiner, Solingen
Destaining bags	Type E732	AMRESCO, Solon
Dispenser tips	Combitips	Eppendorf, Hamburg
Disposable pipettes	diverse	BD Biosciences, Heidelberg
Disposable pipette tips	diverse	Sarstedt, Nümbrecht
Disposable syringe	diverse	Braun, Melsungen
Electroporation cuvette	MicroPulser Cuvettes	BioRad, Munich
Erlenmeyer flask		Coming, Inc., Acton
Filter paper	Whatman	Schleicher & Schuell, Einbeck
Microtiter plates, 96-well	MaxiSorp	Nunc, Langenselbold
Nitrocellulose membrane	Hybond-N+	Amersham, Piscataway

Parafilm		VWR, Darmstadt
Photographic processing	Fixer	AGFA, Köln
	Developer	AGFA, Köln
Sterile filtration units	Steriflip / Stericup	Millipore, Schwalbach/Ts.
Gloves	Reha-Soft	Hartmann, Heidenheim

3.3 Chemicals

Product	**Manufacturer**
Acetone	Merck, Darmstadt
Agarose	Invitrogen, Karlsruhe
Aqua ad iniectabila	Baxter, Lessines
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	Sigma-Aldrich, Munich
Carbenicillin	Serva, Munich
Dinatriumhydrogenphosphat-Dihydrat	Merck, Darmstadt
DNA-loading buffer (6x)	Fermentas, St. Leon-Rot
DNA-loading buffer (6x) with SDS	Fermentas, St. Leon-Rot
dNTPs	Toyobo, Osaka
Ethanol	Merck, Darmstadt
Ethanol, denatured	Walter CMP GmbH, Kiel
Ethidium bromide	Molecular Probes, Eugene
Ethylenediaminetetraacetic acid, sodium salt (EDTA)	Sigma-Aldrich, Munich
Glycerol	Omni Lifescience, Bremen
Imidazole	Merck, Darmstadt
IPTG (Isopropyl-1-thio-"-D-galactopyranoside)	Fermentas, St. Leon-Rot
Kanamycin	Sigma-Aldrich, Munich
Magnesium chloride hexahydrate	Fluka, Neu-Ulm
Magnesium sulfate heptahydrate	Merck, Darmstadt
Sodium chloride	J. T. Baker, Deventer
Sodium citrate	Merck, Darmstadt
Sodium sodecyl sulfate (SDS)	Sigma-Aldrich, Munich

Sodium hydroxide	Merck, Darmstadt
Sodium sulfate	Merck, Darmstadt
Hydrochloric acid	Merck, Darmstadt
Tetracyclin	Sigma-Aldrich, Munich
tris(hydroxymethyl)aminomethane (TRIS)	Merck, Darmstadt
tris(hydroxymethyl)aminomethane, chloride salt (TRIS-HCl)	Sigma-Aldrich, Munich
UDP-Glucose	Ascent Scientific, Bristol
X-gal (5-Bromo-4-chloro-3-inodlyl-"-D-Galactopyranoside)	Sigma-Aldrich, Munich

3.4 Reagents

3.4.1 Media buffers and solutions

3.4.1.1 Solutions for protein biochemistry

Solution	**Ingredients**
BCIP stock solution	0.5% (w/v) 5-Brom-4-chlor-3 indolylphosphate in DMF
Coating buffer (ELISA)	0,1 M NaHCO3, pH 8,8
Blot buffer (WB)	10% methanol, 0.1% antioxidant, 1 x transfer buffer
Blocking solution	5% milk powder in PBS
Detection buffer AP	13 mM pNPP in TBS, 0.1 M MgCl2, 0,1 M NaCl, pH 9,5
Detection buffer HRP	TMB 0.4 g/l
Dulbecco‘s PBS	2.67 mM KCl, 1.47 mM KH_2PO_4, 2.67 mM KCl, 1.47 mM KH_2PO_4,
LDS loading buffer (4x)	200 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol
MES buffer (20x)	50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3
MST buffer	25 mM Tris/HCl pH 8, 100 mM NaCl, 0,1% BSA, 0.1% TWEEN-20, 0.5 mM DTT
Stop solution HRP	0.5 M H_2SO_4
TBS	0.025 M Tris-HCl, pH 7.4, 0.15 M

Transfer buffer (20x)	0.5 M Bicin, 0.5 M Bis-Tris, 20,5 mM
Washing buffer	0.05% TWEEN-20 in TBS

3.4.1.2 Media for bacteria cultures

Media	Manufacturer	Ingredients
LB	Invitrogen, Karlsruhe	10 g/l tryptone, 10 g/l NaCl, 5 g/l
$LB_{(Carb)}$		10 g/l tryptone, 10 g/l NaCl, 5 g/l (+ 100 µg/ml Carbenicilin)
$LB_{(Carb)}$ agar	BD Difco, Heidelberg	10 g/l Trypton, 10 g/l NaCL, 5 g/l Hefeextrakt, 15 g/l Agar (+ 100 µg / ml Carbenicilin)
$2xYT_{(Carb/Kana)}$	BD Difco, Heidelberg	16 g/l Trypton, 5 g/l NaCl, 10 g/l, Hefeextrakt, 15 g/l Agar (+ 100 µg / ml Carbenicilin / 100 µg / ml Kanamycin)
SOC		2 % Trypton, 0,5 % Hefeextrakt, 8,6 mM NaCl, 2,5 mM KCl, 20 mM MgSO4, 20 mM Glucose

3.4.1.3 Solutions for protein production and purification

Solution	Manufacturer	Ingredients
AEBSF stock solution	MP Biomedicals, Irvine	100 mM
Elution buffer Ni-NTA		50 mM Na_2PO_4 pH 8.0, 0.3 M NaCl, 100 mM imidazole
IPTG stock solution	MBI Fermentas, St. Leon-Rot	1 M
Lysozyme stock solution	Roche, Basel	10 mg/ml
TS lysis buffer		30 mM Tris-HCl, pH 8, 20% Saccharose, 500 mM AEBSF, 1% lysozyme
Washing buffer Ni-NTA		50 mM Natriumphosphat pH 8, 0,3 M NaCl, 4 mM Imidazol

3.4.2 DNA and protein standards

Type of standard	Manufacturer
DNA:	
GeneRuler 1 kb DNA ladder	MBI Fermentas, St. Leon-Rot

Proteins:	
Supermark	customized
Novex Sharp Pre-stained	Invitrogen, Karlsruhe

3.4.3 Affinity cromatography matrices

Matrix	**Manufacturer**
Ni-NTA agarose	QIAGEN, Hilden
Ni-TED agarose	Macherey & Nagel, Düren

3.4.4 Antibiotics

Antibiotic	**Concentration**
Carbenicilin (stock solution)	100 mg/ml
Kanamycin (stock solution)	100 mg/ml

3.4.5 Antibodies

Specificity	**Organism**	**Clone**	**Manufacturer**
anti-c-Myc	mouse monoclonal	9E10, IgG1κ	BD Biochiences, Franklin Lakes
anti-Rac1	mouse monoclonal	23A8, IgG2b	Abcam, Cambridge
anti-Rac1	mouse monoclonal	102/Rac1 IgG2b	BD Biochiences, Franklin Lakes
anti-c-Myc-HRP	rabbit polyclonal		Santa Cruz Biotechnology, Dallas

3.4.6 Kits

Kit	**Manufacturer**
Alexa Fluor 680/647 Protein Labeling Kit	Molecular Probes, Eugene
BCA Protein Assay Reagent	Pierce, Rockford
Biotin Protein Ligase Kit	Source BioScience, Nottingham
Colloidal blue staining kit	Invitrogen, Karlsruhe
Gel extraction NucleoSpin Extract II	Macherey-Nagel, Düren
PhoA Color System	QBiogene, Quebec
Plasmid preparation QIAprep Spin Miniprep Kit	QIAGEN, Hilden

3.5.7 Oligonucleotides

DNA oligonucleotides were used for PCR amplification and the site directed mutagenesis. All oligonucleotides were commercially synthesized by Metabion. Sequence data are given in the appendix **(**see **section 8.1)**.

3.4.8 Plasmids

Plasmid	**Size**	**Manufacturer**
pHEN2	4.5 kbp	Institut Leloir, Buenos Aires
pQUANTagen	6.5 kbp	QBiogene, Quebec
pMA_RQ	2.8 kbp	Invitrogen, Karlsruhe

3.4.9 Enzymes

3.4.9.1 Recombinant toxins from *C. difficile*

Recombinant *C. difficile* toxins were kindly provided by the group of Prof. Dr. Klaus Aktories, University Hospital, Freiburg. Full length and truncated proteins were produced, using the Bacillus megaterium expression system (Burger et al. 2003).

Toxin	**Amino acids**	**[kDa]**	**Domains**
Toxin A:			
$TcdA_{full\ length}$	1–2710	308	GTD, CPD, DD, RBD
$TcdA_{1–809}$	1–809	93	GTD, CPD
$TcdA_{574–778}$	574–778	23	CPD
Toxin B:			
$TcdB_{full\ length}$	1–2366	270	GTD, CPD, DD, RBD
$TcdB_{1–807}$	1–807	93	GTD, CPD
$TcdB_{1–548}$	1–548	64	GTD
CDTa:			
$CDTa_{full\ lenght}$	1–463	53	ART, pseudoART

3.4.10 *E. coli* strains

Strain / Manufacturer	Genotype
One Shot® BL21 Star™ / Invitrogen Karlsruhe	F-ompT hsdSB (rB-mB-) gal dcm rne131 (DE3) pLysS (CamR)
TG1 / Stratagene, Waldbronn	F' [traD36 proAB+ lacIq lacZΔM15]supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, (rK-mK-)
HB2151 / Amersham	K12 D(lac-pro), ara, nalr, thi/F'[proAB, lacIq, lacZDM15]
XL2 blue / Stratagene, Waldbronn	endA1 supE44 thi-1 hsdR17 recA1 gyrA96 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr].
XL10-Gold / Stratagene, Waldbronn	endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tetR F'[proAB lacIqZΔM15 Tn10(TetR Amy CmR)]

3.4.11 Cell lines

Eucaryotic cell lines were purchased from the American Type Culture Collection (ATCC).

Name	Origin	Species
HEK-293T	embryonic kidney cells	Homo sapiens

4 Methods

4.1 Methods in molecular biology

4.1.2 Heat shock transformation of chemically competent bacteria

Bacteria were thawed on ice and incubated with 10 ng DNA on ice for 30 minutes. Subsequently, bacteria were heat-pulsed in a water bath (42 °C) for 30 seconds. After another two minutes on ice, pre-warmed SOC media was added and the tubes were incubated for 1 hour at 37 °C while shaking at 230 rpm. The transformation mixture was plated out on agar plates containing antibiotics and incubated overnight at 37 °C.

4.1.3 Preparation of plasmid DNA

Small scale plasmid DNA preparations were carried out using the QIAprep® Spin Miniprep Kit (QIAGEN). *E. coli* bacteria were cultured in 3 ml cell culture media (TB, LB or 2xYT) for 16 hours, which contained antibiotics. Plasmid DNA was extracted following the manufacturer‘s protocol.

4.1.4 Quantification of DNA

The concentration of double-stranded DNA (e.g. plasmids, restriction digestion fragments) were determined with a micro-volume spectrophotometer (NanoDrop 2000c, Thermo scientific). The absorbance was measured at 260 nm wavelength, using an extinction coefficient of 0.020 (µg/ml)-1 cm-1. Consequently, an absorbance of ‘1’ corresponds to a concentration of 50 µg/ml double-stranded DNA. The ratio of the absorbances at 260 and 280 nm was used as a marker of purity. Samples with a 260/280 nm ration of 1.8–2.0 were considered as pure. For some applications, especially for the ligation of DNA fragments, the amount of DNA was estimated by agarose gel analysis. A standard DNA marker (GeneRuler 1 kb DNA ladder, Fermentas) was used to estimate the amount of loaded DNA.

4.1.5 Restriction digestion of plasmid DNA

For cloning and for fragment size analysis, double-stranded plasmid DNA was digested with restriction enzymes and buffer solutions from New England Biolabs (specified in table below). The appropriate buffer solutions were determined using the ‘double-digest’ online tool provided by NEB (http://www.neb.com). One µg DNA was digested with 1–1,5

U of endonuclease in a PCR cycler at a volume of 20–50 µl. The incubation time recommended by the manufacturers was adjusted to account for inadequate digest (e.g. incomplete digest). Subsequent to digestion, restriction enzymes were heat inactivated.

4.1.5.1 Restriction digest of pMA_RQ and pHEN2_s+16Apa

Component	Volume	Time	Temperature
Apa1 digest:			
Template DNA	1,5–3 µg		
BSA (10x)	2 µl		
Buffer 4 (10x)	2 µl	2 h	25 °C
Apa1	1 µl	20‘	65 °C
H_2O	ad		
	Σ = 20 µl		
Sfi1 digest:			
Template DNA	1,5–3 µg		
BSA (10x)	2 µl		
Buffer 4 (10x)	2 µl	2 h	50 °C
Sfi1	1 µl		
H_2O	ad		
	Σ = 20 µl		

4.1.5.2 Restriction digest of pHEN2 and pQ

Component	Volume	Time	Temperature
Sfi1 digest:			
Template DNA	3 µg		
BSA (10x)	2 µl		
Buffer 4 (10x)	2 µl	2 h	50 °C
Sfi1	1 µl		
H2O	ad		
	Σ = 20 µl		

Not1 HF:			
Template DNA	1.5–3 µg		
BSA (10x)	2 µl		
Buffer 4 (10x)	2 µl	2.5 h	37 °C
Not1 HF	1 µl		
H2O	ad		
	Σ = 20 µl		

4.1.6 Dephosphorylation of DNA fragments

After restriction digestion, Antarctic Phosphatase (NEB) was used to remove the 5‘ phosphate groups of the target plasmid. Since DNA ligases requires 5‘ phosphoryl groups for ligation, dephosphorylation of 5‘ groups decreases the amount of vector backbone self ligation. Two µl of Antarctic Phosphatase buffer and 1µl of Antarctic Phosphatase (5000 U/ml) were added to 20 µl of digestion reaction. Dephosphorylation was carried out at 37 °C for 2 hours, followed by heat inactivation of the Antarctic Phosphatase at 65 °C for 20 minutes. All reactions were run in a PCR cycler.

4.1.7 Agarose gel electrophoresis and documentation

Agarose (1%) was boiled in tris-acetate-EDTA (TAE) buffer. Gel casting trays containing a sample comb were filled and ethidium bromide (0.1 µg/ml) was added. After solidifying, the gel was transferred to an electrophoresis chamber and run in TAE-buffer. Samples were mixed 1:6 with loading buffer, loaded into the gel, and run at ~5 V/cm field intensity. Analysis and documentation of the agarose gel were performed under UV-light, using a BioVision 3000 WL documentation chamber from Vilber Lourmat, Eberhardzell.

4.1.8 Extraction of double-stranded DNA from an agarose gel

DNA extractions from agarose gels were carried out using the QIAquick® Gel Extraction Kit (QIAGEN), following the manufacturer‘s protocol. Depending on the expected amount of DNA, 15–30 µl de-ionized water or PBS were used for the elution.

4.1.9 Ligation of DNA fragments

For all cohesive end ligations, T4 ligase was used. Ligations were carried out in T4 buffer following the manufacturer‘s protocol. For standard ligation, linearized vector backbone

and insert were mixed using a 1:3 molar ratio. 50 ng of linearized vector backbone were mixed with the corresponding amount of insert. Calculations were verified using the LIGation.CALCulator tool provided by the University of Duesseldorf.

4.1.10 Cloning

The V_HH-encoding DNA-fragment was cloned from pHEN2 V_HH phagemids into pQUANTagen plasmids, to produce Nbs-AP fusion proteins, and into pHEN2Avi plasmids, to produce biotinylatable Nbs. pHEN2 was digested with Sfi1 and Not1 (NEB) restriction enzymes. V_HH cDNA was separated from linearized vector, purified, and ligated into either pQ or pHEN2 Avi **(**see **section 8.3).** The pHEN2 Avi vector was generated by cloning the s14RBD cDNA followed by an Avi- and a His_6-tag into the pHEN2 vector backbone via Sfi1 and Apa1 restriction sites.

4.1.11 Polymerase Chain Reaction (PCR)

PCR was used to amplify a V_HH encoding fragment out of the phagemid vector pHEN2 and to perform site directed mutagenesis. The process of amplification includes three basic steps:

1. Denaturation of double-stranded DNA: the reaction mix is heated to 94–96 °C. At this temperature, hydrogen bonds, which connect the DNA‘s nucleotides, break down. Thus the DNA is mostly present as single stranded DNA.
2. Annealing of primer: a fast cool down to 55–65 °C allows the primers to bind. The drastic decrease of temperature is important, since that prevents the refolding from single-stranded to double-stranded DNA.
3. Elongation of DNA strands: once the primers have bound, the DNA polymerase elongates the initial DNA oligomer, beginning at the 3‘ end. The temperature during the elongation period depends on the type of polymerase used. Typically, temperatures between 68–72 °C are optimal for polymerization. For detailed protocols see tables below.

4.1.11.1 Standard PCR protocol for the amplification of VHH cDNA

Component	Volume	Temperature	Time	Cycles
template DNA	100 ng	95°C	1‘	
Primer 1	1 µl	95°C	20“	
Primer 2	1 µl	55°C	105“	x 40
dNTPs (10 µM)	1 µl	70°C	5‘	
Buffer (10x)	5 µl	70°C	30“	
KOD Hot Start PM	0.5 µl	4°C	pause	
H2O	ad			
	Σ = 50 µl			

4.1.12 Site directed DNA Mutagenesis of pQ_s+16

Primers containing Sfi1 and Not1 restriction sites were designed *in silico* using DNA Lasergene® software and oligonucleotides were purchased from Metabion. The 35–37mer primers carry overlapping ends that allow specific binding to the vector DNA. The intermediate part of the primers differs from the vector sequence and contains the Sfi1 recognition sequence. This sequence of 13 nucleotides (five more than the Not1 restriction site) was inserted sequentially by site directed DNA mutagenesis, using the SfiA primer first, followed by tthe SfiB primer secondly. PCR reactions were run, using the KOD Hot Start DNA Polymerase Kit (Novagen®).

4.1.12.1 PCR protocol for the site directed mutagenesis of pQ_s+16

Component	Volume	Temperature	Time	Cycles
template DNA	50 ng	95°C	2‘	
Primer 1	1 µl	95°C	30“	
Primer 2	1 µl	50°C	60“	20
dNTPs (10 µM)	1 µl	70°C	4‘	
Buffer (10x)	5 µl	70°C	10‘	
KOD Hot Start PM	0.5 µl	4°C	pause	
H2O	ad			
	Σ = 50 µl			

4.1.13 DNA sequencing

All DNA samples were sequenced at Seqlab, Goettingen. Therefore, 700 ng of template DNA together with 20 pmol of the respective primer were submitted in a total volume of 7 µl. De-ionized water was used to resuspend the DNA.

4.2 Methods in cell biology

4.2.1 Cell culture of human embryonic kidney cells (HEK-293 cells)

Cell cultures were grown in a steam-saturated incubator at 37 °C and 5% CO_2. Adherent HEK cells were cultured in petri dishes in complete DMEM medium. Every 2–3 days the cells were subcultured (1:10 – 1:20) to new petri dishes, in which cells were washed with PBS and detached with Trypsin.

4.2.2 Cell count determination using a Neubauer chamber

The Neubauer chamber is a glass microscope slide with a rectangular indentation that creates a chamber. The cells from four major squares were counted and an average calculated. To distinguish living cells from dead cells, the cell solution was diluted (1:10) with trypan blue (0.1%), a membrane impermeable dye. Therefore, cells that have lost their membrane integrity are stained blue. These cells were not taken into account. Cell number per ml were calculated as: mean cell number per major quadrant x dilution factor x 10^4

4.2.3 Preparation of HEK-293 cell lysates

HEK cell lysates were used for ADP-ribosylation assays. T75 cell culture flasks were washed with $PBS^{+/+}$ and the cells were detached using 1 ml trypsin. After 2–3 minutes of incubation, 30 ml DMEM + 10 % FCS were added to inactivate trypsin and cells were counted. 1.5x107 cells were washed with $PBS^{+/+}$, taken up in 500 µl lysis buffer ($PBS^{+/+}$, 1% TX 100, 1 mM AEBSF), and incubated on ice for 20 minutes. The cell suspension was centrifuged at 800 x g at 4°C for 10 minutes and supernatants were transferred into fresh Eppendorf tubes. Lysates were stored at 4 °C or directly used for ADP-ribosylation assays.

4.3 Methods in protein biochemistry

4.3.1 Cultivation of *E. coli*

A single bacterial colony was picked from an agar plate to inoculate 2–5 ml of preculture media (TB, LB or 2xYT). After incubation overnight at 37 °C, the cell suspension was centrifuged, the supernatant was discarded and the cell pellet resuspended in fresh culture medium of the required volume. For plasmid preparation purposes, the cell pellet was resuspended in lysis buffer (buffer P1) from the QIAprep® Spin Miniprep Kit (QIAGEN) **(**see **preparation of plasmid DNA)**.

4.3.2 Expression of recombinant nanobodies in different formats

4.3.2.1 Expression of monomeric V_HHs

Two plasmids (pHEN2 and pHEN2Avi) carrying V_HH cDNA were used to express monomeric Nbs in *E. coli* HB2151. Cells were cultured in TB, LB, or 2xYT media containing carbenicillin (100 µg/ml). Bacterial cultures were induced using IPTG (0.5 mM final concentration), upon reaching an optical density at 600 nm (OD_{600}) of 0.5. The cells were harvested after 3.5 h of cultivation at 37 °C. The periplasmic fraction was removed by osmotic shock and monomeric Nbs were purified via Ni-NTA or Ni-TED immobilized metal affinity chromatography (IMAC) (see **section 4.3.3.**).

4.3.2.2 Expression of Nb-AP fusion proteins

The pQUANTagen (pQ) expression system was used to produce nanobody-alkaline phosphatase (Nb-AP) fusion proteins. pQ contains the bacterial alkaline phosphatase gene (PhoA) as well as a multiple cloning site (MCS) within the PhoA gene. Inframe isertion of a V_HH encoding DNA fragment results allows expression of a Nb-AP fusion protein, carrying a leader peptide N-terminally and a functional alkaline phosphatase enzyme at the C-terminus. The leader peptide controls the export of the protein into *E. coli* periplasmic space, where it is removed proteolytically and alkaline phosphatase spontaneously dimerizes. Bacteria (*E. coli* XL2 blue, Stratagene) were cultured in TB, LB, or 2xYT media containing carbenicillin (100 µg/ml). Protein expression was induced at OD_{600} = 0.5 using IPTG (0.5 mM final concentration). The cells were harvested after 3.5 h of cultivation at 37 °C and the periplasmic fraction was removed by osmotic shock.

4.3.3 Purification of His_6-tagged nanobodies

All His_6-tagged Nbs were purified via immobilized metal affinity chromatography (IMAC). Ni^{2+}-complexes of immobilized TED or NTA served as stationary phase for the separation of His_6-tagged proteins by ligand exchange. For small scale purification, His_6-tagged proteins were separated in a batch procedure. Eppendorf tubes were loaded with 50 µl of Ni^{2+}-NTA matrix and washed with $PBS^{-/-}$. Periplasmic lysates from 40–200 ml *E. coli* cell cultures were added and incubated for one hour while rolling at 25 °C. The matrix was washed with PBS, containing low concentration of imidazole (3.3 mM). His_6-tagged proteins were eluted with 200 µl of a higher concentrated (100 mM) imidazole buffer. For large scale purification, Ni^{2+}-TED (Protino® Ni-TED 1000 Packed Columns, Macherey-Nagel) matrices were loaded with periplasmic lysates from 150–500 ml *E. coli* cultures. Purification was carried out following the manufacturer‘s protocol. The imidazole containing elution buffer was replaced by PBS, using PD-10 desalting columns (GE). Pure protein fractions were concentrated to 0.1–1 µg/ml, using Amicon Ultra centrifugal filter units (Millipore), and stored at 4 °C.

4.3.4 Sodium dodecylacetate gel electrophoresis (SDS-PAGE)

HEK cell lysates and purified proteins were analysed on precast gels (NuPAGE® Bis-Tris, Invitrogen), containing 10–12 % Bis-Tris and 4–20 % Tris-glycine. Electrophoresis was carried out in XCell SureLock® Mini-Cell chambers (Invitrogen**)** at 200 V for 45 minutes in MES buffer. Proteins were stained with Coomassie Brilliant Blue (Invitrogen).

4.3.5 Quantification of proteins

The amount of purified Nbs was determined with a UV/Vis spectrophotometer (NanoDrop 2000c). The protein concentration was calculated using absorption at 280 nm, molecular weight, and the molar extinction coefficient. The extinction coefficient was determined, using the ProtParam tool from the EXPASY server (http://web.expasy.org/protparam/).

$$c = A_{280} \times \frac{M_W}{\varepsilon}$$

c = concentration

A_{280} = absorption at 280 nm wavelenght

M_W = molecular weight

ε = molar extinction coefficient

To verify the results from NanoDrop measurements, the protein amount of purified Nbs was determined via the Bicinchoninic acid assay (BCA™ protein assay kit, Pierce). In this colorimetric assay, the protein bond of every protein reduces $CuSO_4$ to Cu^{2+}, which in turn builds chelates with BCA. The chelate appears purple in solution, and the amount of chelate formation is proportional to the amount of protein in the sample.

4.3.6 Western blot analysis

Western blotting was performed after SDS-PAGE Electrophoresis as described in **section 4.3.4**. Proteins were blotted onto PVDF membrane, and sequentially probed with primary antibodies against rac1 (23A8, Sigma-Aldrich; Rac1/102, BD Pharmingen) and f-actin (clone 9E10, BD Biosciences). Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG (source: sheep) and enhanced chemiluminescence (ECL reagent, Amersham Biosciences).

4.3.7 ADP-Ribosylation assay

For ADP-ribosylation of actin, CDTa or SpvB (0.2 µM) were incubated with HEK cell lysates in the presence of ^{32}P-NAD for 10 minutes at 37 °C. For blocking experiments, CDTa or SpvB (0.2 µM) were incubated with purified Nbs (2 µM) at room temperature for 20 minutes prior to the addition of ^{32}P-NAD and HEK cell lysates. The reaction was stopped using NuPAGE® sample buffer, and proteins were analyzed by SDS-PAGE. Radiolabeled proteins were detected by exposure to an X-ray film (GE Healthcare) for 15 h at –80 °C.

4.3.8 Rac1 glucosylation assay

300 ng $TcdB_{1–546}$ were preincubated with 4.5 µg Nbs l-7.II or s+12 in PBS (Gibco) for 20 minutes at room temperature in the presence of a bovine serum albumin (BSA) or a monoclonal antibody Nika102 (anti-ART2) as carrier protein. Recombinant Rac1 (600 ng) and UDP-glucose (10 µM, Ascent scientific) were added and incubated at 37 °C for 30 minutes. Western Blot analyses wer performed using two anti-Rac1 mAbs, recognizing total Rac1 (clone 23A8, Sigma-Aldrich) or non-modified Rac1 (clone Rac1/102, BD Pharmingen) as described in in **section 4.3.6**.

4.3.9 $InsP_6$-mediated autoproteolysis of TcdA

Cleavage assays were performed in 100 mM Tris-HCl buffer, pH 7.5 at a final volume of 20 µl. One µg of $TcdA_{1–809}$ was incubated with titrated concentrations of Nbs (1.25 µg – 0.08 µg) at room temperature for 1 hour. Reactions were initiated by addition of 100 µM inositol hexakisphosphate ($InsP_6$) and incubated at 37 °C for 1 hour. The reaction was stopped by heating samples at 75 °C in SDS-PAGE sample buffer. Reaction products were analyzed by SDS-PAGE and Coomassie staining as described in **section 4.3.4**.

4.4 Immunological Methods

4.4.1 Enzyme-linked immunosorbent assay (ELISA)

Antigens (100 ng / well) were adsorbed to wells of 96-well Nunc Maxisorp plates (Rochester, NY) at 4 °C overnight. The wells were washed twice with PBS containing 0.05% Tween-20 (PBST) and blocked with PBS containing 5% nonfat powdered milk at room temperature for 2 hours. Subsequently, wells were washed with PBST. Prior to their use as detection reagents, Nbs were dimerized to improve their avidity. C-Myc-tagged anti-TcdA Nbs were dimerized by incubating at a 2:1 molar ratio with anti-c-Myc mAb (clone 9E10, BD Pharmingen) at room temperature for 30 minutes. After incubation with dimerized detection reagents at room temperature for 60 minutes, wells were washed three times with PBST. HRP-conjugated anti-mouse-IgG secondary antibodies (GE Healthcare) were added to detect bound c-myc-dimerized Nbs. Unbound secondary antibodies were washed off and HRP‘s substrate (3,3′′,5,5′′-Tetramethylbenzidine = TMB) was added. Anti-CDTa Nbs were genetically fused to alkaline phosphatase (AP), generating bivalent Nb-AP fusion proteins, (via spontaenous dimerization of the phosphatase domain in solution). Since Nb-AP fusion proteins exhibit inherent enzymatic activity, these antibodies were detected directly (i.e. without secondary Ab) after washing using para-Nitrophenylphosphate (pNPP). Both substrates were purchased from Sigma-Aldrich. The absorbance was measured using a Victor3 ELISA-reader (Perkin-Elmer Waltham USA).

4.4.2 Microscale Thermophoresis (MST)

$TcdA_{1–809}$ was conjugated to fluorochrome Alexa647 according to the manufacturer's instructions (Molecular Probes, Eugene, (OR), USA). MST assays were carried out as

described (Wienken et al, 2010) using serial dilutions of unlabelled monomeric Nbs mixed with Alexa647-labeled $TcdA_{1–809}$ (12,5 nM) in MST buffer and incubated for 10 minutes. MST assays were measured in a NanoTemper Monolith NT.115. Nb concentrations were plotted againist percent changes of normalized fluorescence (ΔFnorm [%]). The curve fitting and determination of K_D-values were carried out using GraphPad Prism software.

5 Results

5.1 Previous work

Four llamas were immunized to induce toxin-specific heavy-chain antibodies. Llama 5 was immunized with the cysteine protease domain (CPD) of TcdA, llama 5026 with the glucosyltransferase domain (GTD) of TcdB, llama 6 with the enzymatically active domain of CDTa and llama 5037 with a 'cocktail' of the three toxins. The llamas were immunized and bled as indicated in **Fig. 8.1** (see **appendix**). Peripheral blood lymphocytes (PBLs) were extracted and prepared on day ten and eleven after the final boost immunization. RNA was isolated from PBLs and transcribed into cDNA. To amplify the V_HH coding regions, partially degenerate forward and reverse primers (either for long or short hinge regions) were used. The amplified cDNA was digested using Sfi1 and Not1 and cloned into the pHEN2 phagemid vector to generate a phage library. Sequencing of V_HHs from the primary libraries revealed a broad repertoire of V_HH clones. Toxin-specific VHHs were selected by panning on immobilized toxins.

5.2 Multiple V_HH protein sequence alignment

The selected V_HH sequences had CDR3 coding regions of 6–23 amino acids. In addition, all clones possess the canonical disulfide bond, which connects a cysteine residue from the FR1 with a cysteine residue from the FR3, and the characteristic hydrophilic amino acid residues in the FR2 (Muyldermans et al. 1994). An additional disulfide bond between the adjacent CDR1 and CDR3 loops, was present in 75% (llama 5), 10% (llama 5026) and 5% (llama 5037) of sequenced V_HH clones. By the length and the sequence identity of their CDR3 regions, 5 (TcdA), 4 (TcdB), and 10 (CDTa) distinct V_HH families were defined. **Table 2** shows a multiple sequence alignment of the CDR regions of toxin-specific V_HHs. The first character of the family name indicates the presence of a long (l) or short (s) hinge region. The existence of an additional disulfide bond is emblematized by a 'plus', whereas sequence families having only the canonical disulfide bond are identified by a minus sign. The digit indicates the number of amino acids comprising the CDR3 region. Sequences within one family are ordered according to the frequency (fq) of their appearance upon panning on immobilized toxins. Sequence variations within one family are highlighted in dark grey.

Table 2: Multiple sequence alignment of clostridial toxin-specific V_HHs (CDR regions). Family names indicate the presence of a short (s) or long hinge (l), the absence (-) or presence (+) of a disulfide bond, and the length of the CDR3 in numbers of amino acid residues. The sequences within one family are ordered according to the frequency (fq) of their appearance upon panning on immobilized toxins. Sequence variations within one family are marked grey.

Anti-TcdA-CPD

Llama 5

Name	Fq	CDR1	CDR2	CDR3
l-10a	5	LTFSLYRMG	TITSNGA	GFTTTSGYNY
l-10b	3	LTFSLYRMG	TITSGGD	GFTTTSGYNY
l-10c	2	LTFSLHRMG	TITSSGD	GPTTTSGYQY
l-10d	2	LTFSLYRMG	TITSSGG	GVTTTSGYEY
l-10e	2	LTFSLYKMG	TITTSGG	GAPTTSGYKY
l-11a	3	PTFNSNPMG	TITRNGR	RSDASSPTYDY
l-11b	1	GTFSSYAMG	TITRNGR	RSDASSPTYDY
l-14	2	STFSTYHMA	GISWTGA	YPSDSRQVAPNFKY
s-12a	1	SIFSIIALR	GLTTDDT	DVADSVTTYTDL
s-12b	1	SGYSIIALR	GLTTDDT	DVQDSSTMYTDL
s-20		FSFGNYDMS	AIDSGGG	STAGLPVQSIVAITTREYNY

Anti-TcdB-GTD

Llama 5037

Name	Fq	CDR1	CDR2	CDR3
l-7Id	5	SIYSIKPMA	LISSTGT	WVDGKNY
l-7Ia	4	SITGVKPMA	SIYSGDG	WVDGKNY
l-7Ib	4	SIYSIKPMA	LVTSTGT	WVAGKNY
l-7Ic	4	SISSLKPMA	VIFSTGT	WVDGKNY
l-7Ie	1	SINSLKPMA	LIFSTGT	WVDGKNY

Llama 5026

Name	Fq	CDR1	CDR2	CDR3
l-14	4	RTFSSYTMG	GISRSGG	SPSSTWYRSGEFDY
s+12	2	STFS--IMG	AINRGGT	RPYGCNRPECDD
l-7II	2	STFSTSPMG	VIYSAGS	WLGGNEY

Anti-CDTa

Llama 6

Name	Fq	CDR1	CDR2	CDR3
l+8	2	LTFDKYAIG	SCINTSD	ECGGYGAH
l-14.1a	1	RTFTTYAMA	AITRADN	GDWGAYTLNTAYAN
l-14.1b	1	RTFTSYAMA	AITRADN	GDWGAYSLNTAYAN
l-14.2	2	HSFSSLDMA	QISWSGS	TYRPNTFTPAEYDY
l-15.1a	3	SLRSIAVMG	RITSGGY	GGFTEAYSGTYYPDS
l-15.1b	1	SLRSLAVMG	RITSGGY	GGFTEAYSGTYYPDS
l+18a	2	FTFDEFAIG	CIRSSDG	LGSGYGCSLFTTTVGMDY
l+18b	2	FTFDDGAIG	CISSADH	LGSGYGCYAFTPAYGMDY
l+18c	2	FTFDEGAIG	CIASSHG	LGSGYGCYLFTTTVGMDY
l+18d	1	FTFDEGAIG	CISDSDG	LGSGYGCSAFTTTYDMDY
l+18e	1	FTFNDGAIG	CISGADG	LGSGYGCYLWTTTVDMDY
l+18f	1	FTLDEGAIG	CMSGADG	LGSGYGCYLFTTTVDMDY

Llama 5037

Name	Fq	CDR1	CDR2	CDR3
s-14	5	GTISNYVMG	AITSSGG	DRGTYGFDLDDFGS
s-15	2	DTDRDYRMA	AINWSGG	DKFMWRMVLPGDYDY
s+16	2	FALTWHAIG	CISTSDG	DRGAYLTTGCLKRYDY
s-17a	5	RTFSNYAMA	GISRISS	MGAIFAVPGTGVNEYDR
s-17b	1	RTFSNYSFA	GISRTGR	MGALFALPGTGINEYDI
s-21a	13	LTFSSYAMG	AISWANG	ADQRVGYIEYYSGSSGKEYDY
s-21b	3	LTFSNYAMG	AISWANG	AAQRIGYIEYYSGSSGKEYDY
s-21c	1	LTFSNYAMG	AISWTNG	ANQRIGYIEYYSGNSGKEYDY
s-21d	1	RTFSSYAMG	AISWANG	ADQRIGYIEYYSGSSGKEYDY

5.3 Mutagenesis and DNA sequencing of nanobody-AP fusion proteins

To allow for an easy transfer of V_HH cDNA from the pHEN2 library into the pQUANTagen expression system, the pQ vector was modified. Since the V_HH cDNA in the pHEN2 vector is flanked by Sfi1 and Not1 restriction sites, Sal1 and Nde1 restriction sites surrounding the V_HH cDNA in the pQ vector were changed into Sfi1 and Not1 restriction sites, respectively by site directed PCR mutagenesis. DNA sequence analysis confirmed the introduction of the two new restriction sites (**Fig. 5.1**).

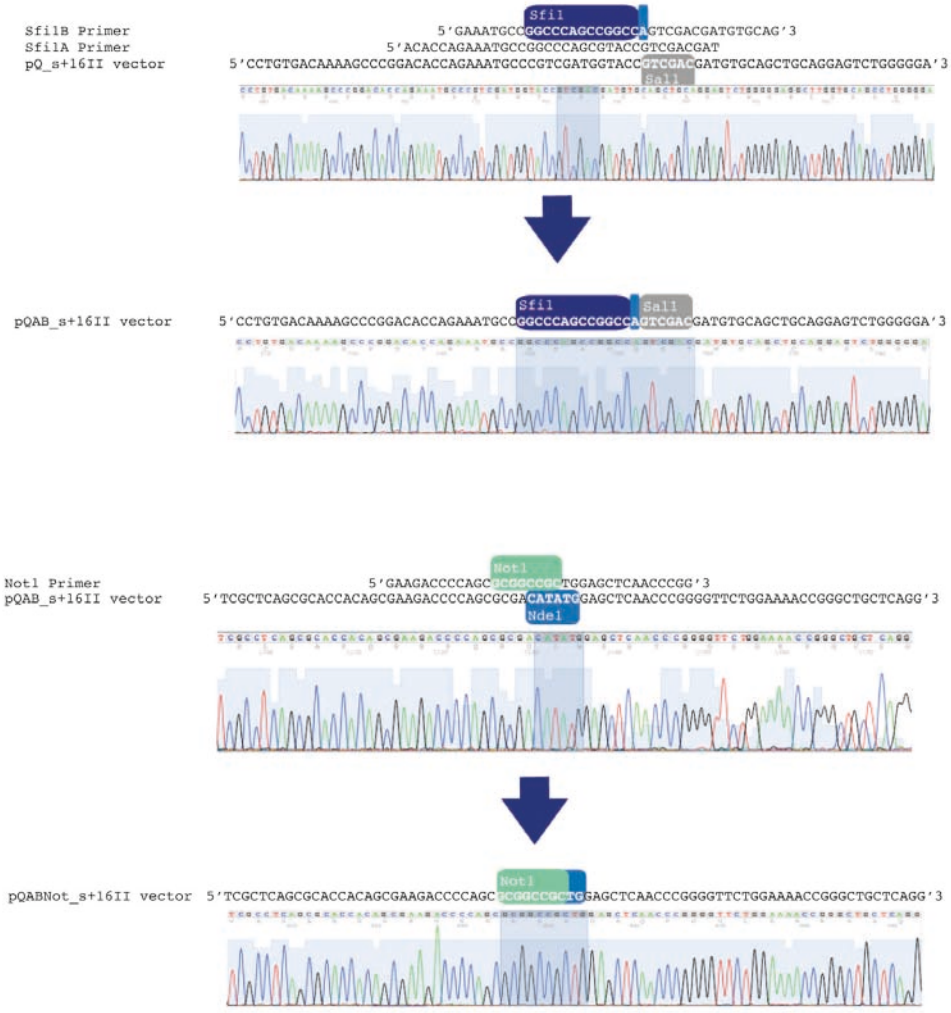


Fig. 5.1: Introduction of Sfi1 and Not1 restriction sites into the pQUANTagen expression vector using site directed mutagenesis. Alignment of primer and vector DNA sequences with corresponding chromatograms of the vector DNA. Primers containing either Sfi1 or Not1 restriction sites were used for PCR amplification. As a template, the pQ_s+16 expression vector was used. The Sfi1 restriction site was introduced sequentially in two steps using the SfiA Primer in the first and the SfiB Primer in the second round of PCR amplification. After successful insertion of the Sfi1 site, pQ_s+16AB was used as a template for the insertion of the Not1 restriction site.

5.4 Recombinant production of nanobodies in different formats

5.4.1 Expression and purification of monomeric nanobodies

Monomeric nanobodies were produced to analyze their target specificity and capacity to block the enzymatic activities of *C. difficile* toxins. Monomeric Nbs were expressed in *E. coli* HB 2151 using the prokaryotic expression vector pHEN2. Bacteria were cultured and protein expression was induced at OD_{600} 0.5 using IPTG. Cells were harvested after 3.5 hours of cultivation at 37 °C. Subsequently, the periplasmic fraction was recovered by osmotic shock and monomeric Nbs were purified via immobilized metal affinity chromatography, yielding protein concentrations of up to 5 mg/l culture. According to the length of their CDR3 regions, Nbs have molecular masses in the range of 14–18 kDa (arrow). **Fig. 5.2** shows the SDS-PAGE analysis of crude periplasmic lysates (PPLs), obtained from 15 ml *E. coli* cultures. Purified Nbs from respective PPLs are shown below. The appearance of double bands, e.g. in PPLs containing Nbs l-11a or l-11b, may be due to proteolytic cleavage of the Nb tail region, which consists of a c-Myc- and a His_6-tag.

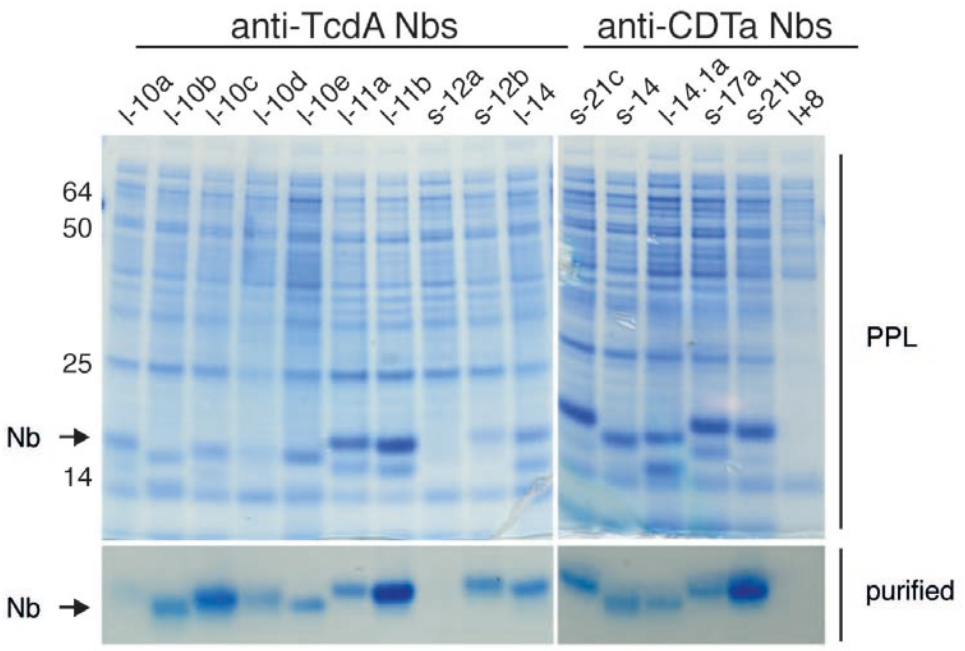


Fig. 5.2: Protein production and purification of Nbs directed against TcdA and CDTa of *C. difficile.* SDS PAGE size fractionation of *E. coli* HB 2151 periplasmic lysates (upper panel) and IMAC purified His_6-tagged monomeric Nbs (lower panel). For the SDS-PAGE analysis of periplasmic lysates, lanes were loaded with 10 µl PPL obtained from 15 ml *E. coli* cell cultures. The lower panel shows the SDS-PAGE analysis after IMAC purification chromatography in a batch format, using a 50 µl Ni-NTA matrix. 10 µl of the elution fraction (200 µl) were applied to the gel .

5.4.2 Cloning and expression of dimeric nanobody-AP fusion proteins

In order to generate genetically fuse individual nanobodies to dimeric E. coli alkaline phosphatase (PhoA), seven anti-CDTa Nbs were cloned into the pQUANTagen vector via Sfi1 and Not1 (see **section 4.1.10**). These pQ-anti-CDTa vector constructs and a mock construct (IgG_{3000}) were expressed in *E. coli* and the periplasmic lysates were subjected to analysis by SDS-PAGE and Coomassie staining (**Fig. 5.3a**). After size fractionation, one discrete band at 65 kDa is seen in every PPL (**Fig. 5.3a arrow, lane 1–7**), except in the PPL from the mock transformed *E. coli* (**Fig. 5.3a lane 8**). Since the molecular weight of a Nb-AP fusion protein is expected to be ~65 kDa (Nb=15 kDa + AP=50kDa), the additional bands in lanes 1–7 may display the successful expression of Nb-AP fusion proteins. To verify the presence of a functional alkaline phosphatase in these PPLs, the substrate para-nitrophenylphosphate (pNPP) was added and conversion to NPP was determined spectrophotometrically (**Fig. 5.3b**). Due to their higher avidity (compared to monomeric Nbs) and their inherent enzymatic acitivity, Nb-APs allow direct-binding ELISA assays (see **section 5.5.1**).

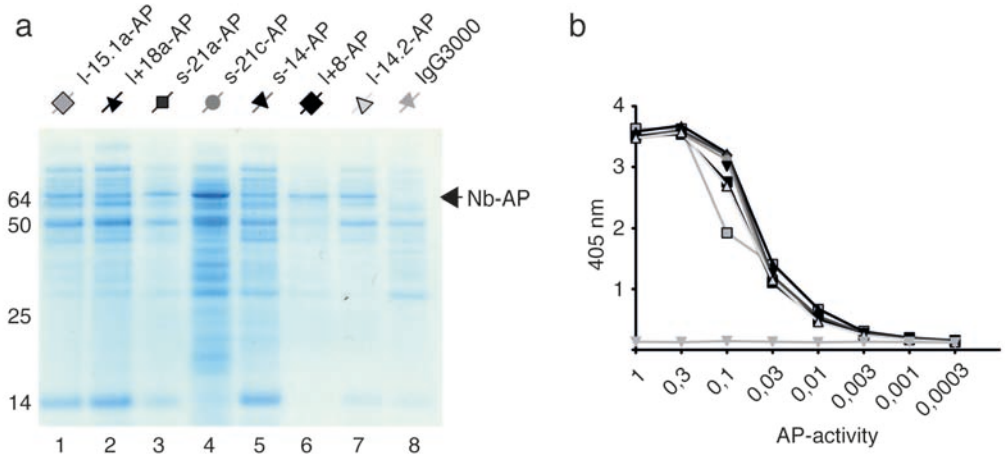


Fig. 5.3: Protein production and enzymatic activity of anti-CDTa Nb-AP fusion proteins. a) SDS-PAGE analysis of Nb-AP containing PPLs. To equal the protein amounts of Nb-APs, distinct volumes of PPLs were used (1: 5 µl; 2: 7,5 µl; 3: 2,5 µl; 4: 0,5 µl; 5: 9 µl; 6: 0,5µl; 7: 2,5 µl; 8: 2,5 µl). b) To detect AP activity, these volumes of PPLs were diluted with PBS (ad 100 µl PBS = AP activity set to 1) and titration was carried out in 1:√10 dilution steps. 50µl of para-nitrophenophosphate (pNPP) was added and the amount of reaction product (NPP) was determined spectrophotometrically at 405 nm wavelength after 3 hours.

5.4.2 Generation of biotinylatable nanobodies

Nanobodies that carry a specific amino acid sequence of 12 residues (GLNDIFEAQ**K**IE = Avi tag) can be biotinylated at the lysine residue within this sequence. The biotinylation is catalyzed by the bacterial enzyme BirA which recognizes the Avi-tag and attaches biotin to the nitrogen of lysine‘s epsilon amino group. Biotinylation allows attachment of nanobodies to streptavidin, a tetramer in which every subunit can bind a biotin molecule. To produce biotinylatable Nbs, a cDNA sequence containing the Nb s14RBD linked to the Avi/His_6-tag was designed *in silico* and purchased commercially in a plasmid (pMA-RQ, see **appendix 8.3**). For the expression in *E. coli*, V_HH cDNA, as well as both tags, were cloned into the pHEN2 vector via Sfi1 and Apa1 restriction sites **(Fig. 5.4a)**. Insertion was verified by DNA sequencing, using the pHEN2 sequencing primer LMB3 and fdseq (see **appendix 8.1**). Via Sfi1 and Not1, two Nbs (anti-CDTa s-21a and anti TcdA l-11a) were transferred into the pHEN2Avi vector. The pHEN2Avi vector provides a template for the production of nanobodies in a biotinylatable format. After transformation of *E. coli* HB2151 with pHEN2Avi, s14RBD (anti-TcdA) and s-21a (anti-CDTa) carrying an Avi-tag, the proteins were expressed and purified from the periplasmic space of E. coli, yielding protein amounts of 0,5 mg/l cell culture (**Fig. 5.4b**).

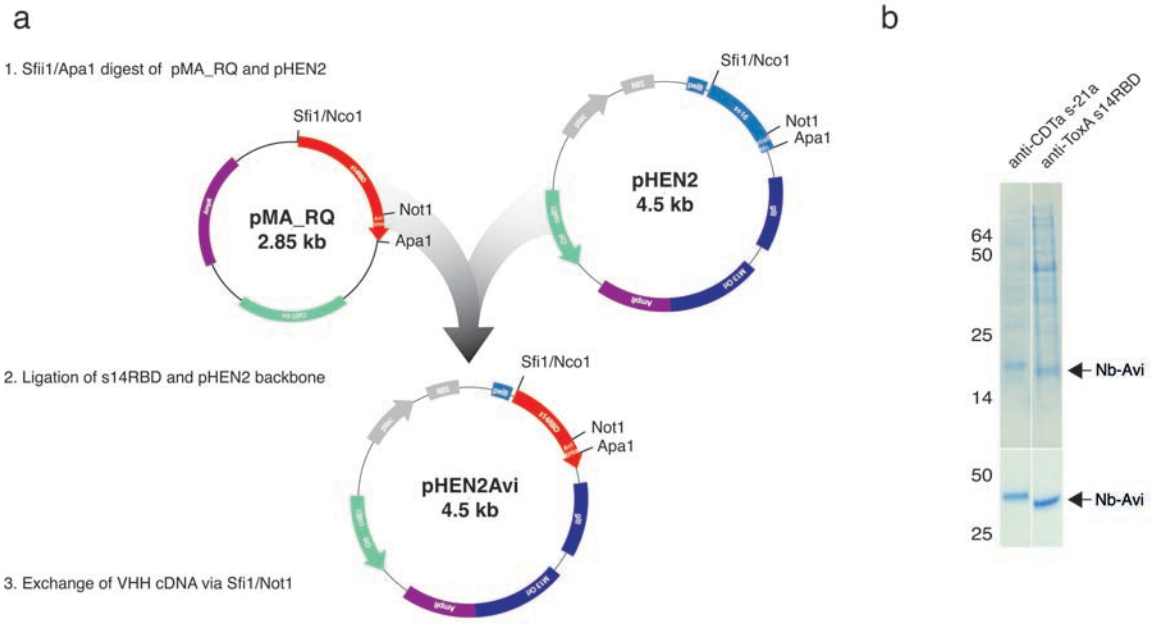


Fig. 5.4: Generation of biotinylatable Nbs. a) The pMA_RQ_s14RBD and the pHEN2_s+16 were digested using Sfi1 and Apa1 endonucleases. The s14RBD cDNA was ligated into the pHEN2 vector backbone. Exchange of V_HH cDNA was done via Sfi1 and Not1 sites. b) SDS-PAGE analysis of crude PPLs containing indicated Avi/His_6-tagged Nbs (upper panel) and IMAC purified Avi/His_6-tagged Nbs (lower panel). Each lane was loaded with 10 µl of a 15 ml bacterial culture (top) or 10 µl of 200 µl elution fraction (bottom).

5.5 Binding specificity and strength of nanobodies to their target toxin

5.5.1 Nanobodies specifically bind to clostridial toxins

The selection of Nbs was done via phage display by panning on immobilized toxins. Clones of four anti-TcdA and six anti-CDTa sequence families were selected and tested for their target specificity. After recombinant production in *E. coli*, an ELISA was performed to verify their binding specificity. ELISA plates were coated with 100 ng of indicated toxins. Prior to incubation with the toxins, nanobodies were dimerized. Anti-TcdA nanobodies were dimerized using an anti-c-Myc mAb, and anti-CDTa nanobodies were expressed as Nb-alkaline phosphatase fusion proteins, which dimerize spontaneously in solution. Bound anti-c-Myc antibodies were detected indirectly via peroxidas-conjugated anti-mouse IgG secondary antibody and the substrate TMB (anti-TcdA), bound Nb-AP fusion proteins were detected directly by adding AP‘s substrate pNPP (anti-CDT). Seven Nbs from three Nb sequence families bound the CPD protease domain of TcdA as well as the TcdA holotoxin **(Fig. 5.5a)**. One clone (s-20) did not show any detectable binding to either CPD or the TcdA holotoxin, indicating that this clone was falsely selected via phage display. None of the Nbs showed any detectable cross-binding to the TcdB holotoxin. The weaker detection signal for TcdA holotoxin than for TcdA-CPD, may be explained by a ten-fold higher molar coating concentration of TcdA-CPD than for the holotoxin. The results confirm that three of four Nb families selected by panning on immobilized TcdA-CPD indeed are TcdA-specific. Similarly, five of ten Nb families selected by panning on CDTa were identified as specific binders by ELISA using anti-c-Myc antibodies for dimerization (Unger 2012). The specificity of these Nb-families was confirmed with respective Nb-AP fusion proteins (results not shown).

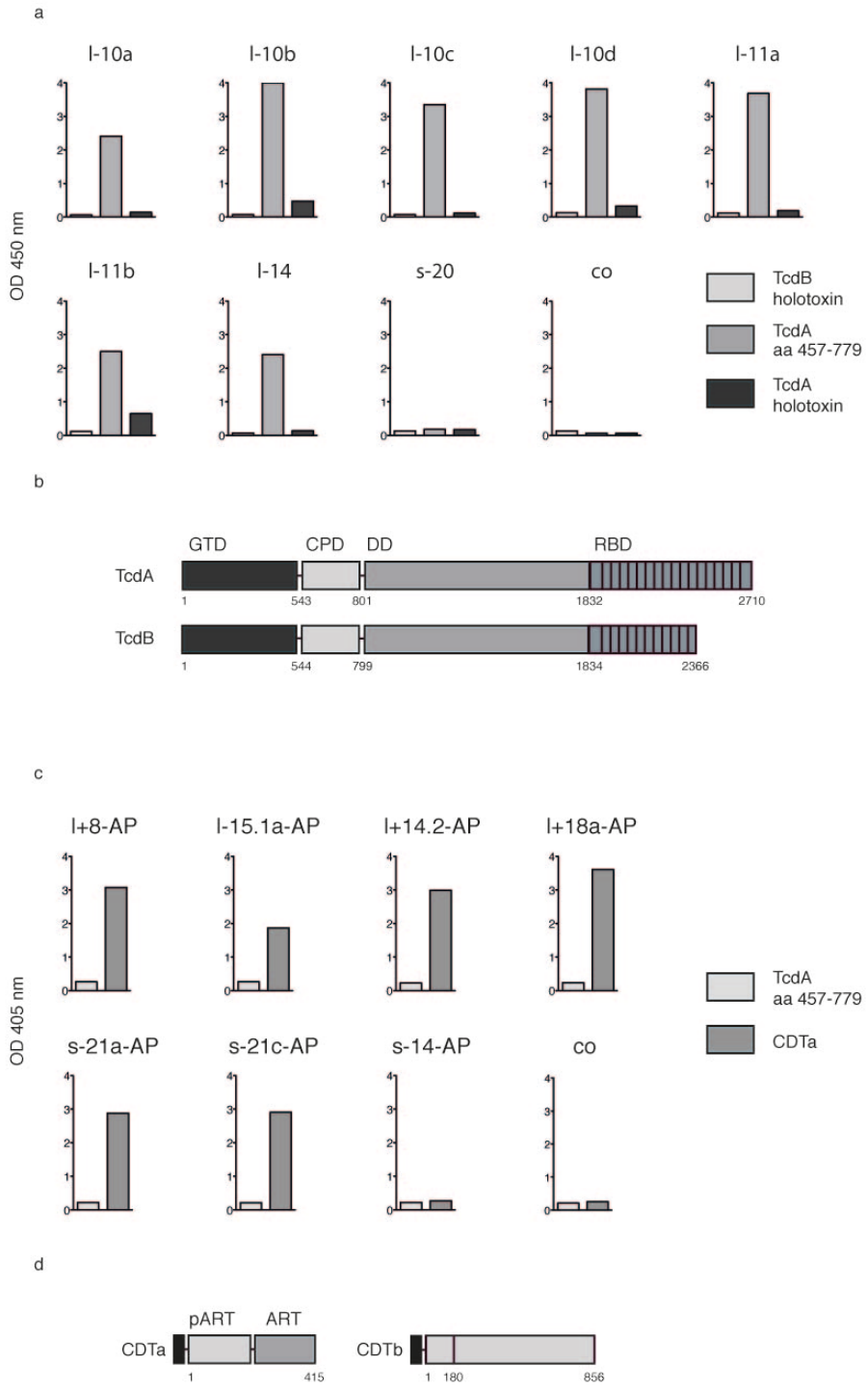


Fig. 5.5: Binding of selected anti-TcdA and anti-CDTa Nbs to their specific target. a) ELISA plates were coated with 100 ng of the indicated toxins. Eight selected Nbs from four different anti-TcdA Nb families were tested for binding, anti-ART2 Nb s+16 was used as negative control (co). Prior to incubation with the toxins, Nbs were dimerized using an anti-c-Myc mAb (100 ng Nb, 700 ng anti-c-Myc). Bound antibodies were detected using a horse radish peroxidase-coupled anti-mouse IgG secondary antibody and TMB as substrate. c) ELISA plates were coated with CDTa (200 ng) or TcdA-CPD (100 ng). Toxins were incubated with PPLs containing ~ 250 ng Nb-AP fusion protein (control gel is shown in **Fig. 5.3a**) and incubated for 1 hour. PPL containing a nonfunctional AP-fusion protein (AP-IgG300) was used as control (co). After washing, bound fusion proteins were detected directly by adding pNPP. b, d) Schematic diagrams of the domain structures of the analyzed *C. difficile* toxins.

5.5.2 The dissociation constants (K_D) of anti-TcdA nanobodies

The affinities of monomeric Nbs to their target often lie in the nanomolar range, similar to the binding affinities of monoclonal antibodies. The K_D values for two selected anti-TcdA Nbs were estimated using Microscale Thermophoresis (MST). Binding anti-TcdA Nbs to Alexa647-labeled $TcdA_{1–809}$ influences the thermal migration behavior of the labeled binding parter. Changes in fluorescence values at the infra-red heated spot are shown as percent change of normalized fluorescence values. Y-values at high Nb concentration reflect fluorescence values of bound toxins. The fluorescence of the unbound toxin decreases at low Nb concentrations, reflecting alterated thermophoresis of Alexa647 $TcdA_{1–809}$. K_D values were derived from the fitted curves (l-11a: 86 nM; l-10a: 2 nM). An anti-ART2 Nb (s+16) was used as negative control (**Fig. 5.6**).

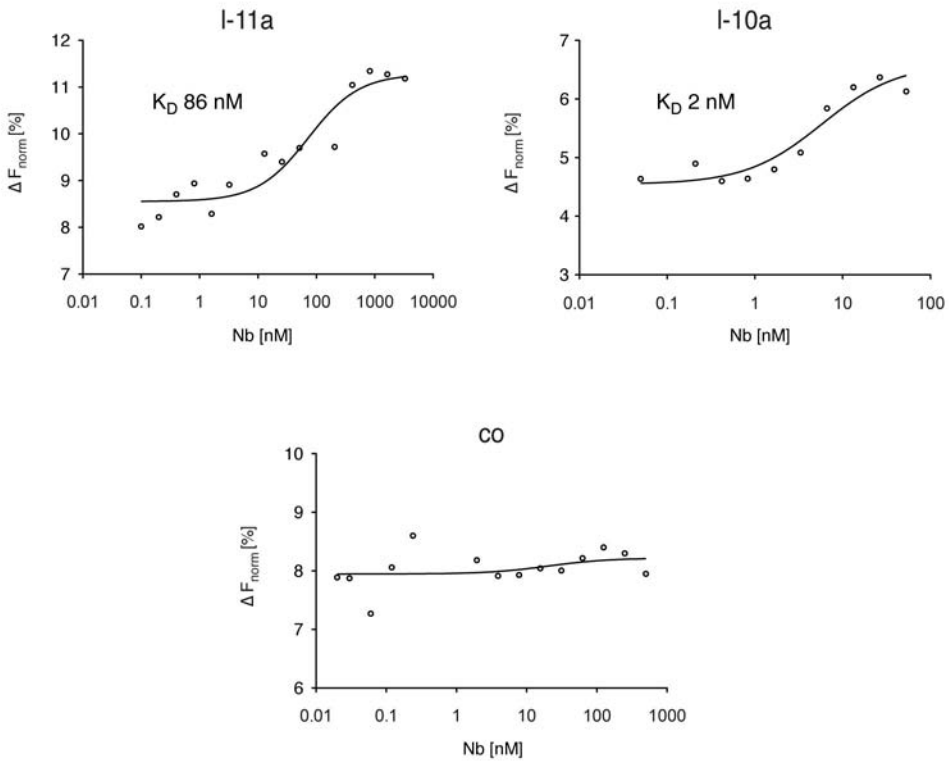


Fig. 5.6: K_D estimation of two anti-TcdA Nbs. To determine the binding affinity of anti-TcdA Nbs and their specific toxin, Nbs were titrated 1:2 starting at 3,3 µM. The concentration of the fluorochrome coupled $TcdA_{1-809}$ was constant (12,5 nM). Anti-ART2 Nb s+16 was used as negative control (co). Percent change of normalized fluorescence values is shown on y-axes.

5.6 Capacity of nanobodies to inhibit the activity of their target‘s enzymatic subunits

5.6.1 Nb l+8 inhibits CDTa-mediated ADP-ribosylation of actin

Nb l+8 was shown to bind CDTa in ELISA assays and was able to diminish CDTa‘s cytotoxic effect on cultured MDCK-II cells (**Fig. 5.5; Unger et al.** (Unger et al. 2014)). A radioactive ADP-ribosylation assay was used in order to determine whether l+8 can inhibit the actin-ADP-ribosyltransferase activity of CDTa. CDTa was preincubated in the absecence or presence of Nb l+8 before addition to HEK cell lysates that contained endogenous actin and exogenously added ^{32}P-NAD. SpvB, an ADP-ribosylating toxin of *Salmonella enterica,* was used as control. Both toxins target the arginine residue 177 on actin. After SDS-PAGE size fractionation, ^{32}P-ADP-ribosylation of actin was visualized by autoradiography. A band corresponding to ^{32}P-ADP-ribosylated actin was seen after incubation with CDTa or SpvB in the absence of Nb l+8, but ADP-ribosylation of actin by SpvB was less efficient than by CDTa. Addition of Nb l+8 completely blocked the CDTa mediated ADP-ribosylation of actin, but had no influence on the ADP-ribosylation catalysed by SpvB (**Fig. 5.7**).

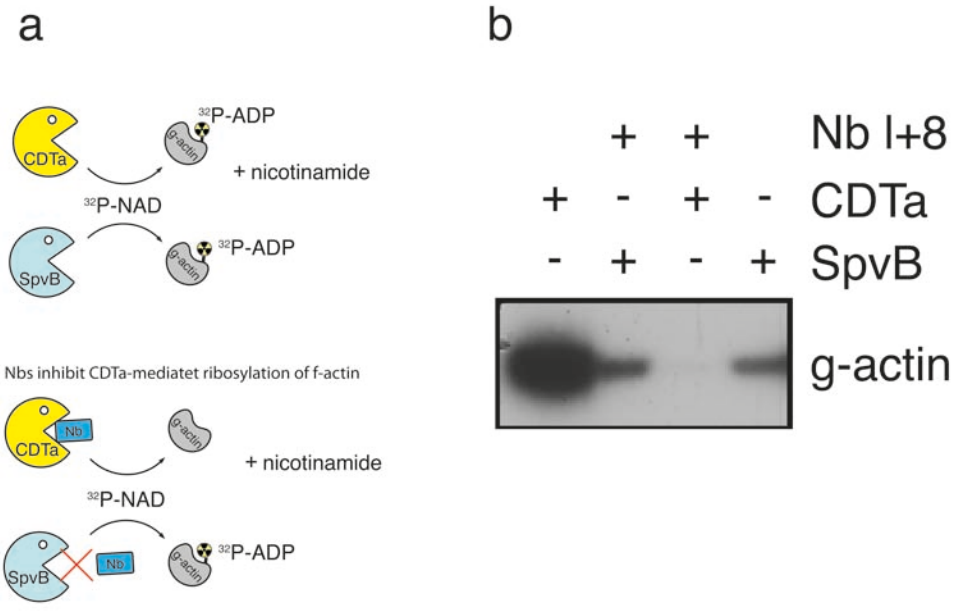


Fig. 5.7: The CDTa-specific nanobody l+8 blocks the-ribosylation of g-actin monomers by CDTa. a) Schematic representation of the ADP-ribosylation of actin by CDTa and SpvB and the specific inhibition of CDTa by Nb l+8. CDTa as well as Spvb both catalzye NAD-dependent ADP ribosylation of g-actin in the absence of Nb l+b. In the presence of Nb l+8, CDTa is blocked, whereas SpvB maintains its ADP-ribosyltransferase activity. b) HEK cell lysates were incubated with either CDTa (100 ng) or SpvB (50 ng) in the presence of and ^{32}P-NAD. Toxins were preincubated with Nbs (300 ng) for 20 minutes at RT. The ADP-ribosylation of actin was visualized by autoradiography after size fractionation by SDS-PAGE.

5.6.2 Nbs. l-7.II and s+12 inhibit TcdB-mediated glucosylation of Rac1

Two anti-TcdB Nb families (l-7.II and s+12) were previously identified as efficient toxin binders (Unger 2012). These were tested for their capacity to block the TcdB-GTD mediated glucosylation of Rac1. After preincubation of Nbs and TcdB, the reaction was started by the addition of UDP-glucose and recombinant Rac1. After SDS-PAGE size fractionation, the glucosylation status of Rac1 was analysed by WB using Rac1-specific monoclonal antibodies (**Fig. 5.8**). While mAb 23A8 recognizes both, native and glucosylated Rac1 (total Rac1), binding of mAb Rac1/102 is blocked by glucosylation, i.e. Rac1/102 recognizes non-glucosylated Rac1 only (non-Glc Rac1). The results show a constant band for mAb 23A8, confirming that equal amounts of Rac1 were loaded. In contrast, Rac1/102 can only detect a band in the absence of the substrate UDP-glucose or when Rac1 is incubated with the actin ADP-ribosylating toxin SpvB as negative control. In presence of anti-TcdB Nbs l-7.II and s-12, a band is detected by Rac1/102, indicating that these Nbs (partially) inhibit GTD-mediated glucosylation of Rac1 in a concentration dependent manner (**Fig. 5.8**).

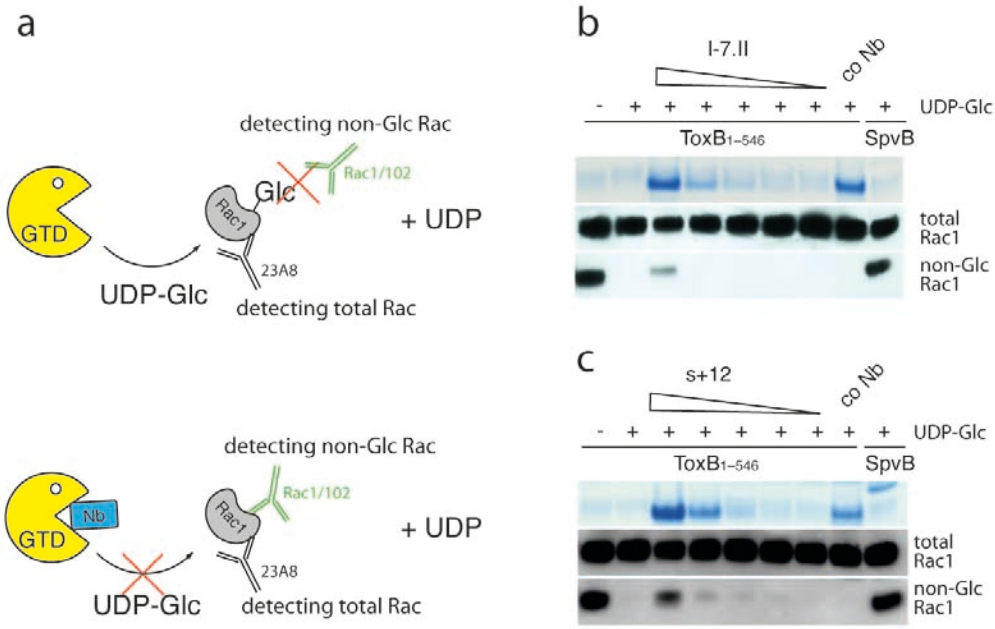


Fig. 5.8: Nbs l-7.II and 2+12 inhibit TcdB-mediated glucosylation of Rac1. a) Schematic representation of the posttranslational modification of Rac1 catalyzed by the glucosyltransferase domain (GTD) of toxin B and its inhibition by Nbs l-7.II and s-12. Glucosylated Rac1 is recognised by mAb 23A8, but not by mAb Rac1/102. b) and c) $TcdB_{1–546}$ (300 ng) was preincubated with 4,5 µg of Nbs 2971 L-7.II or 2967 S+12, respectively, for 20 min at RT before addition of of recombinant Rac1 (600 ng) and UDP-Glucose (10 µM) and further incubation for 30 min at 37° C. After SDS-PAGE size fractionation, WBs were carried out, using two different primary antibodies, recognizing either total (23A8) or non-glucosylated Rac1 (Rac1/102).

5.6.3 Inhibition of the autoproteolytic cleavage of $TcdA_{1–809}$

Selected TcdA-specific Nbs were tested for their capacity to block Ins6P-induced autoproteolysis of TcdA mediated by its cysteine-protease domain (CPD). Cleavage of $TcdA_{1–809}$ was monitored using SDS-PAGE by the appearance of bands with sizes of 63 kDa and 30 kDa, corresponding to the GTD and CPD domains, respectively (**Fig. 5.9).** The results show that the autoproteolysis of $TcdA_{1–809}$ and $TcdB_{1–807}$ is induced by the addition of $InsP_6$. Addition of Nbs l-10a and l-11a results in a dose-dependent inhibition of CPD-mediated autoproteolysis of $TcdA_{1–809,}$ with a complete blockade at a seven-fold molar excess. Other members of these Nb families (l-10d, l-10c, l-11b) exhibited similar enzyme-blocking activity (not shown). In contrast, Nb l-14 did not inhibit the proteolytic cleavage of $TcdA_{1–809}$. Moreover, none of the anti-TcdA Nbs inhibited the autoproteolysis of $TcdB_{1–807}$, confirming their high target specificity.

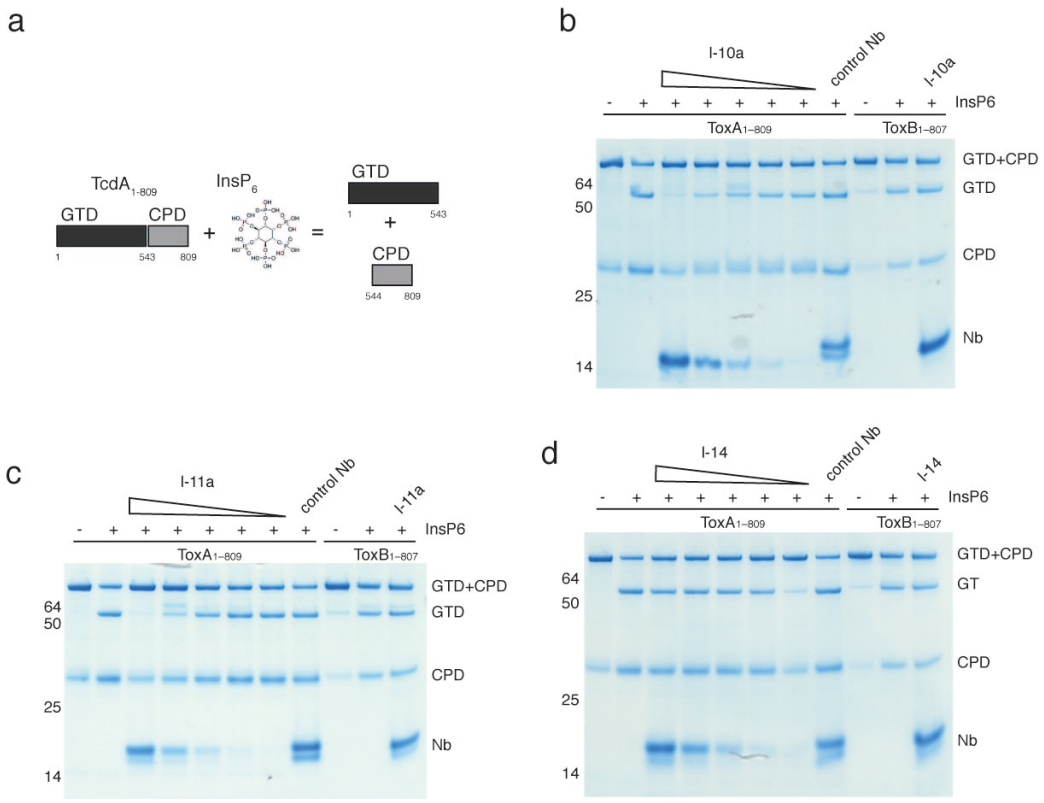


Fig. 5.9: Nbs l-10a and l-11a block CPD mediated autoproteolysis of TcdA. a) Schematic depiction of the autoproteolysis of $TcdA_{1–809}$. In the presence of $InsP_6$, $TcdA_{1–809}$ is cleaved into its two constituent domains, GTD (63 kD) and CPD (30 kD). b-c) One µg of $TcdA_{1–809}$ was incubated with titrated amounts of anti-TcdA Nbs l-10a, l-11a or l-14 (1,25–0,08 µg) for 1 hour at RT. The CPD mediated autoproteolysis of $TcdA_{1–809}$ was induced by addition of $InsP_6$ (100 µM). The appearance of 30 kDa and 63 kDa cleavage fragments indicates successfull cleavage. A saturating dose of a control Nb (1,25 µg) was used as a negative control. The effect of a saturating dose of TcdA-specific Nbs on autoproteolysis of TcdB was analyzed in parallel.

6 Discussion

This study identified nanobodies that effectively bind and block the enzymatic activities of the three clostridial enzyme toxins. As discussed in the following section, these nanobodies hold promise as diagnostics to improve the detection of *C. difficile* toxins in patient stool samples and as new potential therapeutics to reduce toxin-mediated damage to colonic epithelial cells during CDI.

6.1 The specificity and functional activity of anti-toxin nanobodies

Toxin-specific anobodies were generated by immunizing llamas with the recombinant enzymatic domains of the clostridial toxins (CDTa-ART; TcdA-CPD; TcdB-GTD) (**Fig. 8.1, appendix**). Five TcdA, ten CDTa and four TcdB nanobody families were selected from immune phage display libraries by binding to immobilized toxins. When expressed in a monovalent (anti-TcdA and anti-TcdB) or bivalent (anti-CDTa-AP) format, the target specificity was verified by ELISA for three TcdA, five CDTa, and three TcdB Nb families (**Fig. 5.5; and data not shown for TcdB**). Using microscale thermophoresis, the binding affinity for two anti-TcdA nanobodies was determined to be in the nanomolar range. The K_D of Nb l-10a (2 nM) is comparable or even lower than the K_D of other anti-TcdA nanobodies such as A20.1 (2 nM) and AA.6 (19.7 nM) (Hussack, Arbabi-Ghahroudi, et al. 2011). The lower K_D-values of conventional antibodies may explained by their higher valency (dimeric mAbs vs. monomeric sdAb). Dimerization of Nbs (Nb-AP fusion proteins) was shown to increase the binding affinity (avidity) more than tenfold (Swain et al. 2011).

Selected nanobodies that had been verified as toxin binders by ELISA were functionally examined for their capacity to inhibit the enzymatic domains against which they were raised. The CDTa-specific nanobody l+8 effectively blocked the ADP-ribosylation of actin (**Fig 5.7**). This nanobody also neutralized the cytotoxicity of CDTa in a cell culture assay (**Unger et al.** (Unger et al. 2014)). Nanobodies l-7.II and s+12 from two of the three TcdB-GTD-specific Nb families were shown to partially inhibit the TcdB-mediated glucosylation of Rac1 (**Fig 5.8**). Among three TcdA-CPD-specific Nb families (l-10, l-11, l-14), two families (l-10 and l-11) blocked the CPD-mediated autoproteolysis of TcdA (**Fig. 5.9**). However, these anti-TcdA and anti-TcdB Nbs did not display any effective toxin

neutralization in cell culture assays. Interestingly, the majority of the selected Nb clones seem to recognize active sites on their target antigen. This observation supports the conclusions reached by a study that compared the epitopes of Nbs and mAbs to hen egg lysozyme): Nbs show a much higher propensity to bind to clefts or cavities on proteins (e.g. the active site crevice of lysozyme) than conventional antibodies (De Genst et al. 2006).

The binding epitopes of the nanobodies described in this thesis on the respective toxins are not known. However, it is tempting to speculate that GTD-TcdB- and CDTa-antagonistic nanobodies directly block the UDP-binding and NAD-binding active site of the toxin, respectively (**Fig. 6.1**). For the CPD domain of toxin A, two distinct mechanisms of inhibition are conceivable: Inhibitory nanobodies might bind to either the active site of the protease or to the allosteric, Ins6P-binding site. In contrast nanobodies that bind CPD-TcdA but do not block its enzymatic activity (e.g. Nb l-14) likely bind to a region that does not interfere with the enzymatic activity.

Knowledge of the epitope specificity is important for assembling distinct nanobodies into multimers. Therefore, it might be useful to investigate wether inhibitory Nbs bind to overlapping or distinct epitopes. Cross-blockade epitope mapping analyses, e.g. the sequential incubation of the toxin with unconjugated and Nb-AP fusion proteins, is a suitable method to address this question.

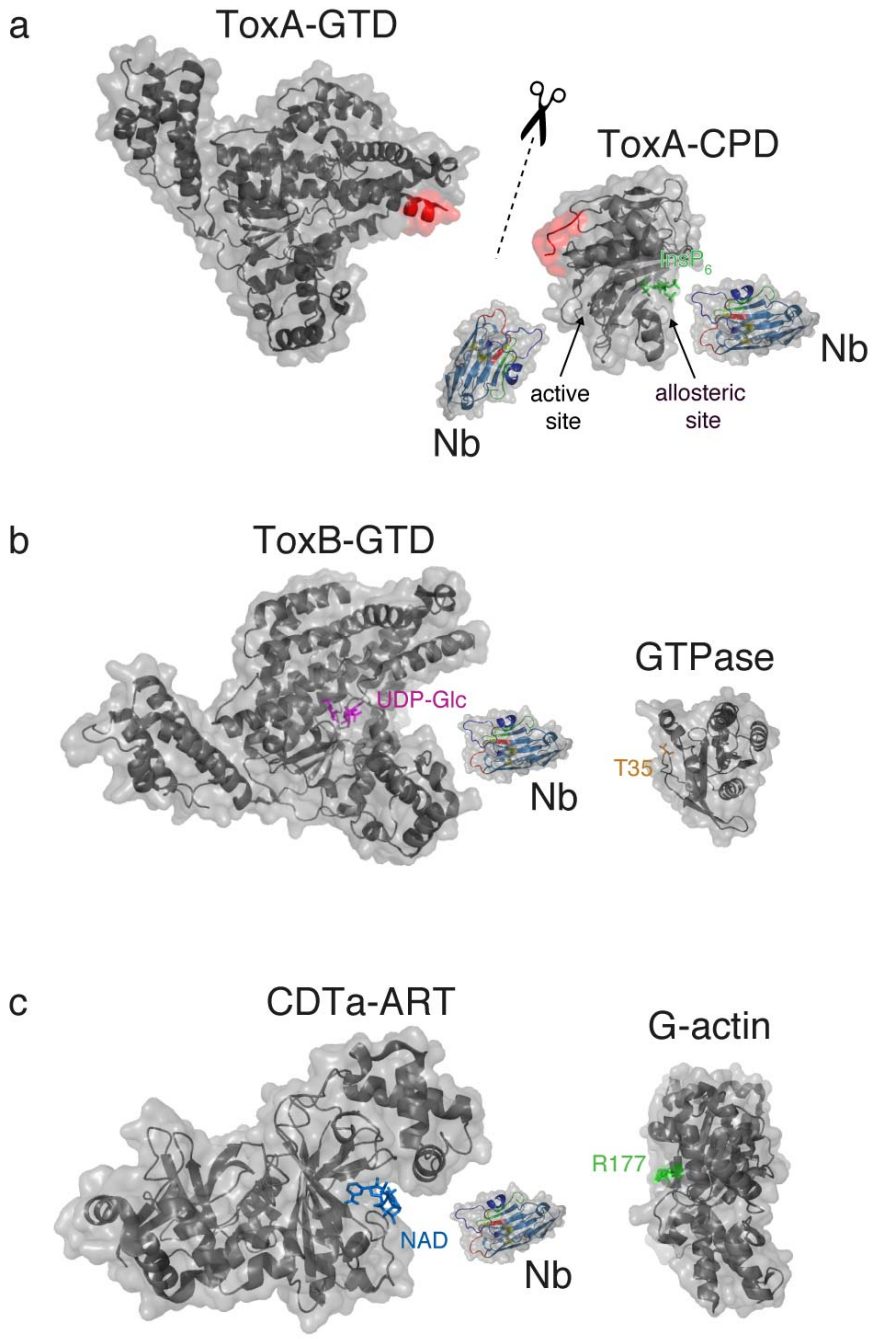


Fig. 6.1: Hypothetical binding sites and mode of action of toxin-specific nanobodies. a) The glucosyltransferase domain (TcdA-GTD; PDB: 3ss1) and the cysteine protease domain (TcdA-CPD; PDB: 3HO6) of TcdA are shown. Residues adjacent to the cleavage site (Leu543 and Gly544) of TcdA are marked red. The cysteine protease domain contains the allosteric Ins6P-binding site and the protease active site. Autoproteolytic processing of TcdA may be blocked by nanobody binding to either of these two regions. b) The glucosyltransferase domain of TcdB catalyzes the glucosylation of small GTPases at threonine T35 in Rac1, Cdc42 or T37 in RhoA (shown here; PDB:1CC0) (Just et al. 1995). c) CDTa (PDB: 2WN7) ADP-ribosylates G-actin at arginine R177 (green) using NAD^+ (blue) as substrate. The CDTa blocking Nbs presumably have overlapping epitopes in the region of its active site. The model crystal structure of the indicated nanobodies (Nbs) is taken from a lysozyme blocking Nb (PDB: 1MEL).

Due to their good solubility and small size, nanobodies can be easily reformatted. Molecular cloning allows their utility to be adapted to certain diagnostic and/or therapeutic applications (**Fig. 6.2**). Dimeric or multimeric nanobodies permit a potential increase in binding avidity compared to their monomeric derivates (Danquah 2013; Zhu et al. 2010). They are generated by cloning V_HH coding sequences interspersed with glycine/serine rich ($[G_3S]_4$) flexible linker regions into a bacterial or eukaryotic expression systems. One such example of a multimeric nanobody derivate is 'ABA'(Yang et al. 2014). It is a tetravalent molecule consisting of four V_HH domains that have distinct epitope specificities. The domains displayed in pink (**Fig. 6.2c**) are two identical V_HH domains (Nb E3) directed against the GT domain of TcdB, whereas the blue and green domains (Nbs AH3, AA6) are directed against two distinct epitopes of TcdA. Compared to their monomeric derivatives, this tetrameric construct was shown to have a 300-(heterotetrameric) fold higher capacity to neutralize TcdA in cytotoxicity assays (Shoemaker and Feng 2013; Yang et al. 2014). By cloning the ABA construct into an adenoviral vector (e.g. pAdEasy-1), functional replication-deficient adenovirus particles expressing ABA were generated (**Fig. 6.2d**) (Yang et al. 2016).

The fusion of nanobodies to bacterial alkaline phosphatase provides a strategy to directly detect clostridial toxins (**Fig. 6.2e**). Nb-AP fusion proteins were generated using the pQUANTagen cloning protocol, whereby the V_HH cDNA is genetically linked to the PhoA gene (**Fig. 5.3**). The PhoA signal peptide, which is located N-terminally of the V_HH gene, directs the fusion protein into the periplasmic space, where it spontaneously dimerizes (Inouye et al. 1981; Ducancel et al. 1993). Dimeric Nbs impart a higher affinity (avidity) than their monomeric equivalents (Swain et al. 2011). In addition, the signal intensity can be augmented using AP-coupled mAbs against AP (Hohmann et al. 1988). Epitope mapping using Nb-AP fusion proteins also provides a method to identify nanobodies recognizing non-overlapping epitopes.

In order to create tetrameric binding molecules, nanobodies can be biotinylated at a specific site and then coupled to streptavidin (**Fig. 6.2f**). This process is carried out using the Avi-tag™ technology. Nanobodies are genetically fused to the Avi-tag (GLNDIFEAQ**K**IE) and can then be biotinylated at the lysine residue within the Avi-tag (indicated by bold letter) using the bacterial enzyme BirA. Subsequent incubation of biotinylated Nbs with streptavidin in a 4:1 molar ratio creates Nb tetramers, which may perform better in absorbing bacterial toxins than their monomeric derivatives.

The reformatted nanobodies reported in this study may contribute to a more sensitive detection of clostridial toxins from stool samples. The gold standard of testing for *C. difficile* and their toxins is a combination of a bacterial culture and a subsequently performed cytotoxicity assay. This testing procedure is highly sensitive as well as highly specific. However, with a turnaround time of 72 hours, it is too time-consuming for modern clinical test regimes (see **section 1.1.3.**). This is why these methods were, for the

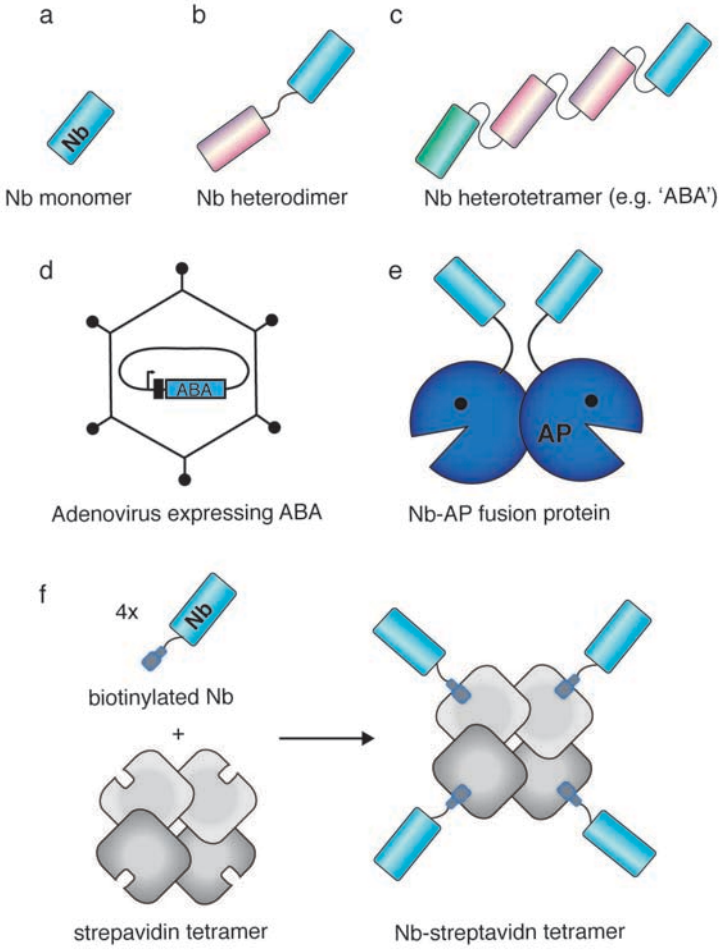


Fig. 6.2: Various Nb formats for improved absorption and detection of clostridial toxins. Monomeric and multimeric nanobodies can be produced in pro- or eucaryotic expression systems. a) Monomeric nanobodies contain only a single binding unit for their target. b) Bivalent Nbs are produced by genetically linking two V_HH domains via a flexible peptide linker, e.g. a glycine/serine rich sequence (Shoemaker and Feng 2013). c) Analogously, tetrameric derivates are produced by cloning four V_HH coding sequences containing interspersed linker sequences(Yang et al. 2014). d) By subcloning the aforementioned fragment into an adenoviral expression system (pAdEasy-1), ABA expressing virions can be produced (Yang et al. 2016). e) The pQuantagen cloning protocol provides a means for genetic linkage of Nbs to the *E. coli* alkaline phosphatase (AP) to produce Nb-AP fusion proteins. f) Nbs containing an Avi-tag can be biotinylated using the bacterial enzyme BirA. These biotinylated Nbs can be multimerized onto streptavidin forming a tetravalent molecule.

most part, replaced by rapid and cheap EIA-based methods, which detect toxins A and/or B from stool samples. Solid phase ELISAs and immunochromatographic lateral flow tests are currently the most frequently used toxin tests (Carroll and Bartlett 2011). Although these methods provide high specificity, on average, they lack a sufficient sensitivity (60% to 81%) (Eastwood et al. 2009).

The nanobodies from this study are promising agents to improve the current diagnostic methods for *C. difficile* toxins. Streptavidin-coupled nanobodies can be used to enrich clostridial toxins from stool samples or culture supernatants; Nb-AP fusion proteins can be used for direct toxin detection in EIA-based methods. Particularly in the detection of hypervirulent strains such as BI/ NAP1/027, anti-CDTa Nbs represent a valuable tool to absorb and detect CDTa from stool samples. Immunoassays that detect CDT are not currently commercially available. Instead, cost-intensive PCR-based methods are used to detect the presence of the CDT encoding genes (Carman et al. 2011; Zhou 2014). The I+8 anti-CDTa Nb might also be of value in experimental settings, for example, to assess the differential role of CDTa compared to the LCTs in *C. difficile* animal models (Cowardin et al. 2016).

Besides conventional solid state ELISA assays, the characterized nanobodies might also be applied to multiplex bead assays. This technique offers a 10–100 fold lower detection limit than conventional ELISAs. Magnetic bead-conjugated Nbs can be incubated with, and extracted from a numerous sample volume than that used for solid-phase ELISAs.

6.3 Nanobodies as a therapeutic option for severe and relapsing CDI

Despite their limitations antibiotics remain the mainstay of CDI treatment. Insufficient protection from recurrent infections and the emergence of hypervirulent strains, that are accompanied by high mortality rates are compelling reasons to develop new CDI therapeutics. In this section, therapeutic concepts, beyond the scope of conventional antibiotic treatment, are discussed.

Nitazoxanide is an antiparasitic drug that is also effective against *C. difficile*. Two clinical trials have shown nitazoxanide to be as effective as metronidazole or vancomycin, though the study excluded patients with fulminant CDI. The use of Nitazoxanide is seen as a salvage therapy for patients who do not respond to metronidazole (Musher et al. 2007). Rifaximin has been shown to be of value as an adjunct in combination with standard

therapy; however, this substance can not be considered a therapeutic breakthrough (Gerding and Johnson 2010). Toxin binding agents, such as anion-exchange resins (cholestyramine and colestipol) and oligosaccharide sequences attached to an inert support (SYNSORB) did not show satisfying results in placebo controlled clinical trials (Gerding and Johnson 2010; Mogg et al. 1982; Kurtz et al. 2001; Heerze et al. 1994). Fecal transplantation has been a viable therapeutic option since the 1960s and its high efficiency in abolishing recurrent CDIs (92% cure rate) was recently verified in clinical trials (Bauer and van Dissel 2009; Bakken 2009).

Preliminary trials of parenteral vaccination containing toxoids A and B were shown to induce substantial antibody responses in healthy adults (Aboudola et al. 2003; Kotloff, Wasserman, and Losonsky 2001). An ongoing phase III study (Cdiffence), is currently evaluating the efficacy, immunogenicity and safety of a *C. difficile* toxoid vaccine. Although antibody responses to TcdA and B correlate with asymptomatic carriage of toxigenic *C. difficile*, the extent of the vaccines protective effect is still unclear. Additionally, it remains to be seen wether toxoid vaccines induce a sufficient and durable immune response in the elderly population. Furthermore, an appropriate at-risk population for vaccination has to be defined (Gerding and Johnson 2010).

In 2010, a systemically administered combination of monoclonal antibodies against TcdA and TcdB was shown to significantly decrease the recurrence rates of CDI in a phase 2 study. Seven percent of patients who received mAbs in addition to the standard treatment (metronidazole or vancomycin) had a recurrent infection ($P < 0.001$), compared to 25% in the placebo group. Seven percent of patients of the antibody treated group who had more than one prior CDI episode had further recurrences; this figure rose to 38% in the placebo group ($P = 0.006$). Of patients carrying the BI/NAP1/027 strain, 8% of the antibody treated group and 32% of the placebo group had recurrent infections ($P = 0.06$) (Lowy et al. 2010). These results clearly demonstrate that mAbs are a valuable therapeutic option to treat severe and relapsing CDI. However, the cost of treatment with monoclonal antibodies is very high.

Nanobodies are promising agents that provide the same outstanding features of conventional antibodies, such as target specificity and a low level of adverse effects. At present, several nanobody constructs are being tested in preclinical studies. The bispecific tetrameric Nb construct ABA, which is directed against TcdA and TcdB, was tested in an *in vivo* study using mice that were orally infected with *C. difficile* spores. 24h after infection,

ABA (40, 200 and 1000µg/kg) were injected peritoneally. ABA treated mice were significantly protected from severe weight loss and accompanying mortality. None of the mice treated with 200 or 1000µg/kg ABA died in the course of CDI, whereas one out of ten mice died in the group of 40 µg/kg ABA treated mice compared to 6 out of ten in the untreated group (Yang et al. 2014). The same authors recently published an *in vivo* study, where they systemically administered replication-deficient adenovirus expressing ABA. Mice that were transduced with one dose of the adenovirus displayed high serum levels of ABA for more than one month, and were fully protected against systemic challenge with either orally administered spores or intraperitoneally administered toxins (Yang et al. 2016). Similar promising results were reported for an altered version of ABA designated VNA2-Tcd (Schmidt et al. 2016). This construct carries two distinct TcdB-specific nanobodies (5D, E3, both directed against the GT domain of TcdB) instead of two copies of Nb-E3.

In principle, nanobodies could also be administered to the gastrointestinal tract. For oral administration nanobodies would need to be protected against the low pH of the stomach and proteolytic enzymes secreted by the pancreas. Colonic administration is also conceivable, e.g. as a complement to stool transplantation. Some nanobodies are stable at low pH-conditions and can be stabilized further by the introduction of an additional disulfide bond (Ala/Gly54Cys and Ile78Cys) (Hussack, Hirama, et al. 2011). In addition, nanobodies can also be protected from degradation by encapsulation using chitosan-alginate microcapsules (Li et al. 2009). To better absorb the toxins, biotinylated Nbs could be multimerized onto streptavidin (see **section 5.4.2**) and to higher molecular structures, such as magnetic or sepharose beads, to further increase the rate of toxin absorption. Finally, Nbs could be produced locally in the lumen of the gut, e.g. by engineered commensal lactobacilli. This strategy was used to express two TcdB specific nanobodies (VHH-B2, VHH-G3) either as secretory proteins or as cell-wall anchored proteins (Andersen et al. 2016). In a prophylactic setting, these engineered lactobacilli shoed therapeutic efficacy in a hamster model of *C. difficile* associated disease.

7 Perspective

The Nbs characterized in this study contribute to the development of improved diagnostics and therapy for *Clostridium difficile* associated disease.

8 Appendix

8.1 Oligonucleotides (Primer)

Name	Sequence	Description
PhoA Seq for	5‘GCA CTG GCA CTC TTA CCG TTA C 3‘	Sequencing pQUANTagen
PhoA Seq rev	5‘CAG TCT GAT CAC CCG TTA AAC 3‘	
Fdseq1 (pHEN2, for)	5‘GAA TTT TCT GTA TGA 3‘	Sequencing pHEN2
Lmb3 (pHEN2, rev)	5‘CAG GAA ACA GCT ATG AC 3‘	
Sfi1A	5’ACA CCA GAA ATG CCG GCC CAG CGT ACC GTC GAC GAT 3‘	Mutagenesis (Sfi1/Not1) pQUANTagen
Sfi1B	5‘GAA ATG CCG GCC CAG CCG GCC A GTC GAC GAT GTG CAG 3‘	
Not1	5‘GAA GAC CCC AGC GCG GCC GCT GGA GCT CAA CCC GG 3‘	
SHF (IgG2c, for)	5‘ TCG CGG CCC AGC CGG CCA TGG CGC AGG TSM ARC TGC AGG AGT CWG G 3‘	Cloning of immune libraries from llama cDNA
SHF (IgG2c, rev)	5‘ ATG GTG ATG ATG ATT GTG CGG CCG CGC TGG GGT CTT CGC TGT GGT GCG 3‘	
LHF (IgG2b, for)	5‘ TCG CGG CCC AGC CGG CCA TGG CCG ATG TGC AGC TGC AGG MGT CWG GRG GAG G 3‘	
LHR (IgG2b, rev)	5‘ ATG GTG ATG ATG ATG TGC GGC CGC TGG TTG TGG TTT TGG TGT CTT GGG 3‘	

8.2 Immunization scheme of llamas

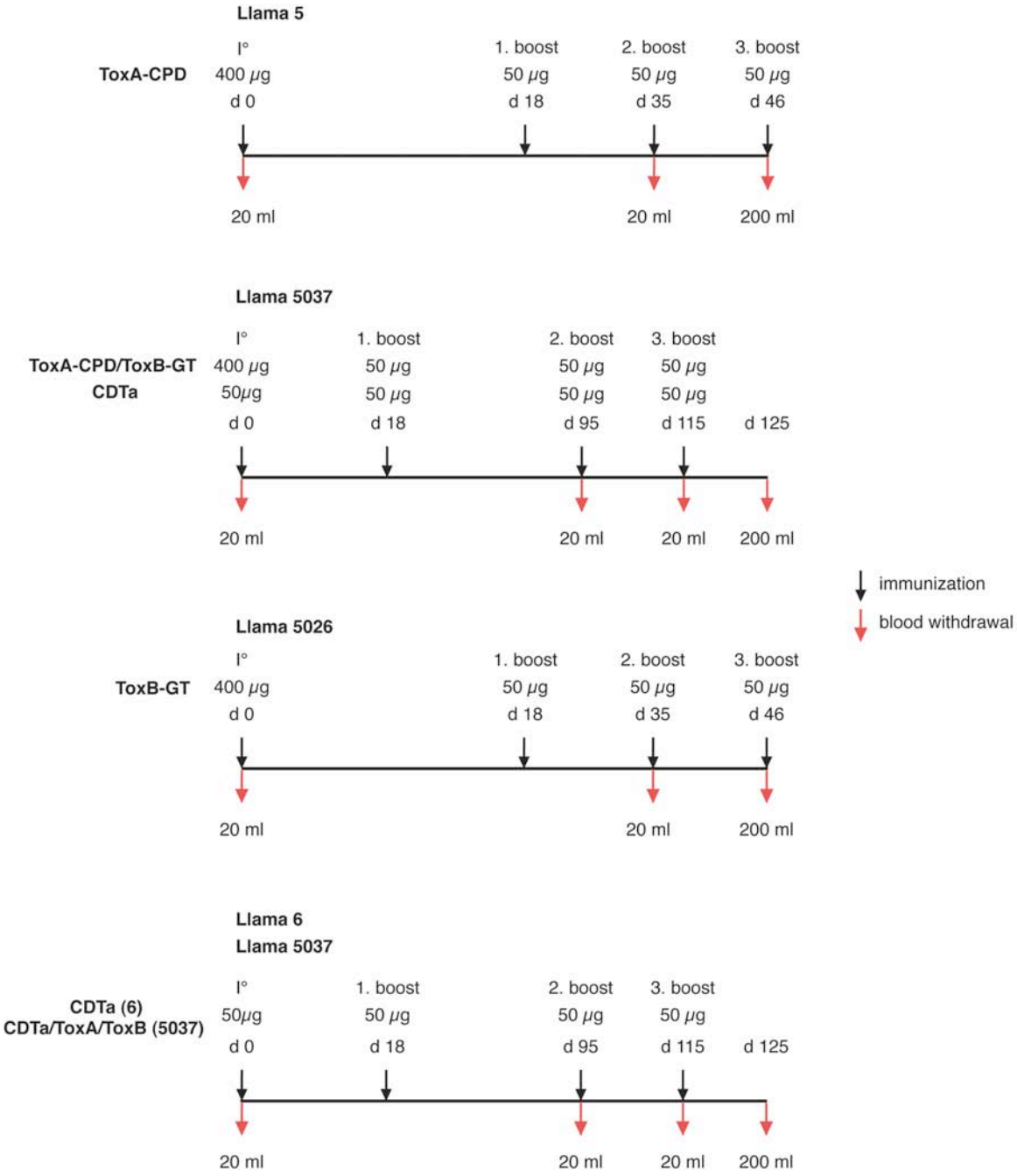
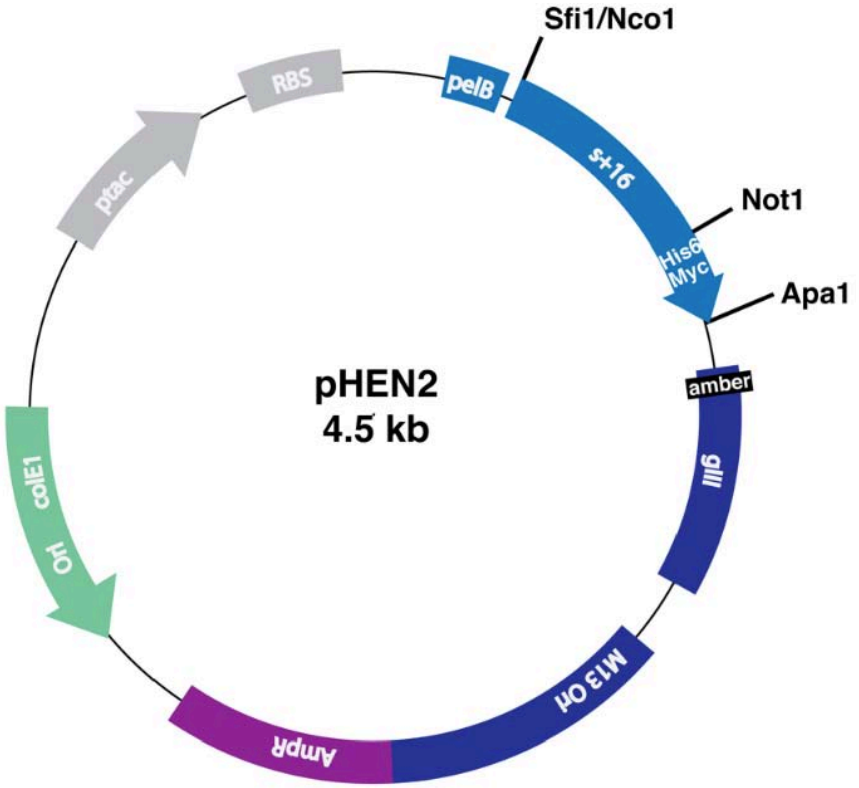
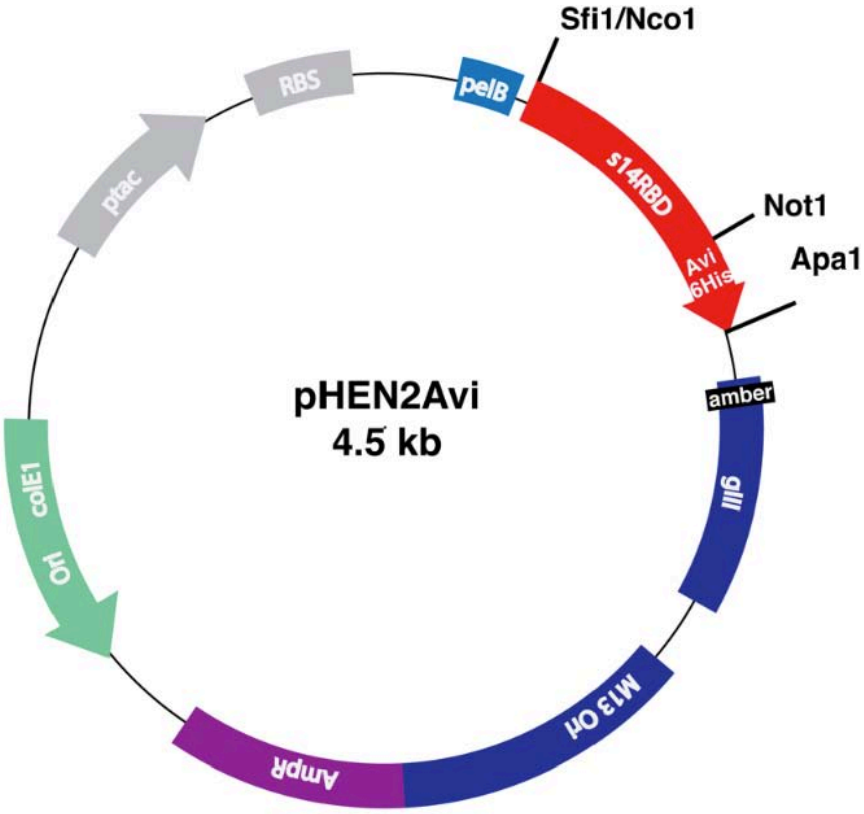
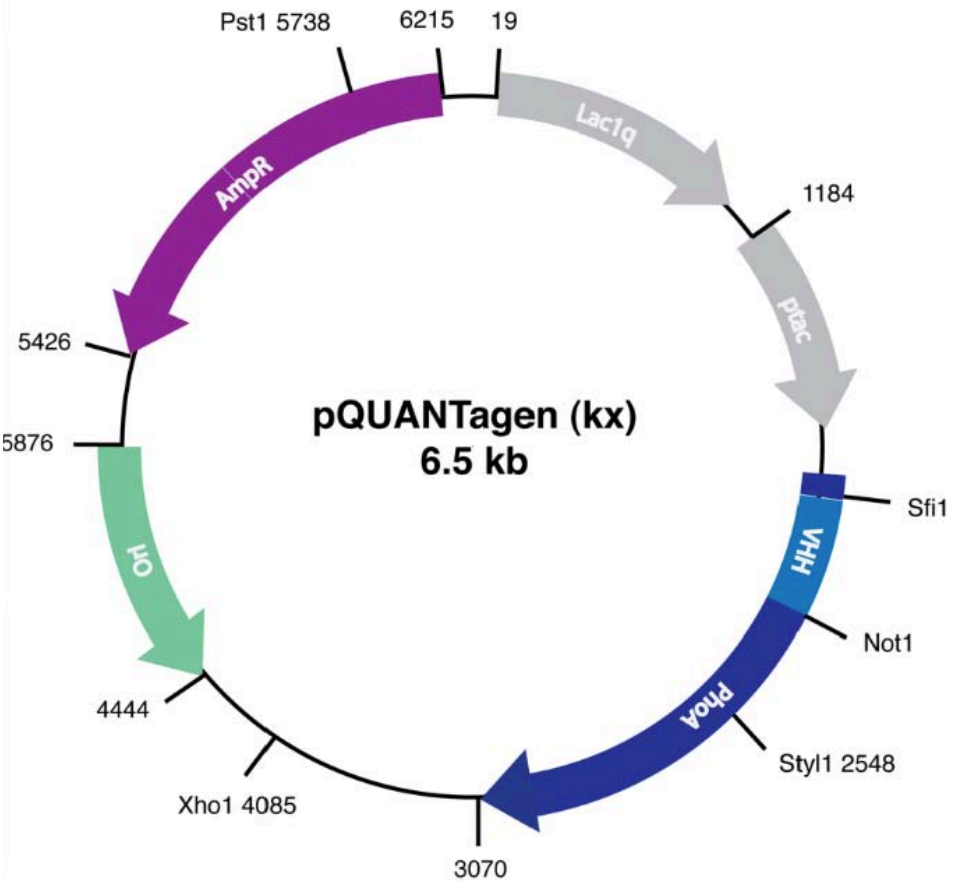


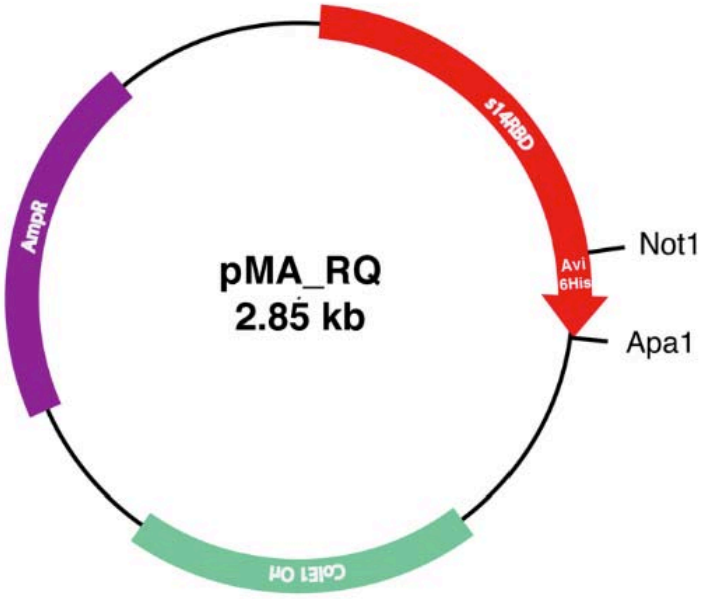
Fig. 8.1 Immunization scheme of llamas with recombinant toxins. Four llamas (5, 6, 5026, 5037) were immunized with the indicated toxin fragments to induce the production of HCAbs. For the primary immunization, 400 µg (toxA-CPD, TcdB-GTD) or 50 µg (CDTa) were dissolved in 400 µl of PBS, emulsified in 500 µl Specol adjuvant and injected subcutaneously into the llamas neck. The llamas 5 and 5026 were boosted two times and the llamas 6 and 5037 three times. For each boost immunization 50 µg of toxin fragments were used. The llama 5037 were immunized with a mixture of TcdA-CPD/TcdB-GTD and CDTa. Blood samples (red arrows) were taken after indicated days.

8.3 Plasmid maps






Pst1 5738
6215
19
Lac1q
1184
ptac
Sfi1
VHH
Not1
PhoA
StyI1 2548
3070
Xho1 4085
4444
Ori
5876
5426
AmpR
pQUANTagen (kx)
6.5 kb


S14RBD
Not1
Avi
6His
Apa1
ColE1 Ori
AmpR
pMA_RQ
2.85 kb

8.4 V_HH protein sequence alignments

9 References

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10 Danksagung

Mein ganz besonderer Dank gilt meinem Doktorvater Prof. Friedrich Koch-Nolte für die Überlassung des spannenden Themas, die hervorragende wissenschaftliche und methodische Betreuung und die Anleitung zum selbstständigen wissenschaftlichen Arbeiten. Die zahlreichen Gespräche während der Doktorarbeit und im Rahmen des studienbegleitenden Mentoringprogramms haben mir viele Denkanstöße und Inspiration für meine wissenschaftliche Arbeit gegeben und mich auch persönlich sehr bereichert.

Mein Dank gilt auch Herrn PD Dr. Thomas Jacobs und Herrn Prof. Dr. Ansgar W. Lohse, die als Betreuungskommission meine Arbeit mit Diskussionen und Anregungen unterstützt haben.

Ganz herzlich möchte ich mich bei allen Mitarbeitern des Instituts für Immunologie für die schöne Zeit und die ausgesprochen angenehme Arbeitsatmosphäre im Labor bedanken. Ganz besonders danke ich Dr. Welbeck Danquah und Dr. Mandy Unger für die Einführung in die Arbeit mit den Einzeldomänenantiköpern sowie für die vielen wertvollen Tipps bei praktischen Problemen.

Anna-Marei Eichhoff danke ich für die praktische Unterstützung bei der Durchführung der Experimente zur Untersuchung der Hemmung der Autoproteolyse von Toxin A und für das Korrekturlesen dieser Arbeit.

Ebenfalls für das Korrekturlesen danke ich Alex Fusco und Marta Ciechonska.

Ich danke den Mitgliedern des SFB 841 „Leberentzündung: Infektion, Immunregulation und Konsequenzen“ der DFG für die finanzielle Unterstützung meiner Arbeit und für die Möglichkeit an vielfältigen Seminaren und Fortbildungsveranstaltungen teilzunehmen.

Ich danke ich meinen Eltern für ihre immerwährende Unterstützung.

Meiner Freundin Charlotte danke ich für all die Energie, die sie mir verleiht.

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt

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Unterschrift: