A Cell-permeable Fusion Toxin as a Tool to Study the Consequences of Actin-ADP-ribosylation Caused by the Salmonella enterica Virulence Factor SpvB in Intact Cells

Received for publication, November 2, 2006, and in revised form, January 24, 2007 Published, JBC Papers in Press, February 5, 2007, DOI 10.1074/jbc.M610254200

Sascha Pust 1, Henrike Hochmann 1, Eva Kaiser 1, Guido von Figura 1, Karin Heine 1, Klaus Aktories 1, and Holger Barth 1,2

From the 1Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Albert-Einstein-Allee 11, D-89081 Ulm, Germany and 2Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Albertstrasse 25, D-79104 Freiburg, Germany

The virulence factor SpvB is a crucial component for the intracellular growth and infection process of Salmonella enterica. The Spv protein mediates the ADP-ribosylation of actin in infected cells and is assumed to be delivered directly from the engulfed bacteria into the host cell cytosol. Here we used the binary Clostridium botulinum C2 toxin as a transport system for the catalytic domain of SpvB (C/SpvB) into the host cell cytosol. A recombinant fusion toxin composed of the enzymatically inactive N-terminal domain of C. botulinum C2 toxin (C2IN) and C/SpvB was cloned, expressed, and characterized in vitro and in intact cells. When added together with C2II, the C2IN-C/SpvB fusion toxin was efficiently delivered into the host cell cytosol and ADP-ribosylated actin in various cell lines. The cellular uptake of the fusion toxin requires translocation from acidic endosomes into the cytosol and is facilitated by Hsp90. The N- and C-terminal domains of SpvB are linked by 7 proline residues. To elucidate the function of this proline region, fusion toxins containing none, 5, 7, and 9 proline residues were constructed and analyzed. The existence of the proline residues was essential for the translocation of the fusion toxins into host cell cytosol and thereby determined their cytopathic efficiency. No differences concerning the mode of action of the C2IN-C/SpvB fusion toxin and the C2 toxin were obvious as both toxins induced depolymerization of actin filaments, resulting in cell rounding. The acute cellular responses following ADP-ribosylation of actin did not immediately induce cell death of J774.A1 macrophage-like cells.

Several pathogenic bacteria produce toxins and effector proteins, which attack the cytoskeleton of eukaryotic cells by mono-ADP-ribosylation of actin. In past years, we focused on the mode of action of the Clostridium botulinum C2 toxin as the prototype of binary actin-ADP-ribosylating toxins (1). The enzyme component of the C2 toxin (C2I)3 ADP-ribosylates G-actin at arginine 177 (2). This leads to depolymerization of actin filaments and finally to cell rounding. The proteolytically activated binding/translocation component (C2IIa) forms heptamers, which assemble with C2I and bind to the cellular receptor (3). Following receptor-mediated endocytosis, C2IIa forms pores in the membrane of acidic endosomes. For translocation of the C2I protein through the lumen of these pores, a partial unfolding of C2I is required (4). The subsequent refolding of C2I in the cytosol is facilitated by the host cell chaperone Hsp90 (5). The interaction of C2I with C2IIa is mediated by the N-terminal domain of the C2I protein (C2IN, amino acid residues 1–225). C2IN, which is enzymatically inactive and does not induce cell rounding when applied in combination with C2IIa to cells, was successfully used as an adaptor for the C2IIa-mediated delivery of different proteins into the cytosol of eukaryotic cells (6).

The SpvB protein from Salmonella enterica was identified as a new member of bacterial actin-ADP-ribosylating enzymes (7). We identified arginine 177 as the modification site for C/SpvB within the actin homolog Act88F (Drosophila indirect flight muscle actin) (8). Our finding was confirmed by a recent publication (9) reporting that arginine 177 is the modification side in mammalian actin for SpvB. S. enterica is a Gram-negative facultative intracellular pathogen, which causes diseases ranging from mild gastroenteritis to severe systemic infections in humans (10, 11). During infection Salmonella grows and replicates inside macrophages in a special membrane compartment, called the Salmonella-containing vacuole (SCV) (12).

As the final step in the infection process, Salmonella induces cell death of infected macrophages. Intracellular pathogenesis depends on the presence of the Salmonella pathogenicity island 2 (SPI2), which encodes for several effectors and for a type III secretion system (SPI2-encoded TTSS, for review see 13). A plasmid, which contains the Salmonella plasmid virulence (spv)

* This work was supported by Deutsche Forschungsgemeinschaft Grant SPP 1150/BA 2087. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Both authors contributed equally to this work.

1 To whom correspondence should be addressed. Tel: 49-731-50065503; Fax: 49-731-50065502; E-mail: holger.barth@uni-ulm.de.

3 The abbreviations used are: C2I, enzyme component of C. botulinum C2 toxin; C2IN, N-terminal domain of C2I; C2IN-C/SpvB, fusion toxin consisting of the N-terminal domain of C2I and the C-terminal domain of S. enterica SpvB; SCV, Salmonella-containing vesicles; spv, Salmonella plasmid virulence; C/SpvB, enzyme domain of S. enterica SpvB; PBS, phosphate-buffered saline; TTSS, type III secretion system; DMEM, Dulbecco’s modified Eagle’s medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; GST, glutathione S-transferase.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 14, pp. 10272–10282, April 6, 2007 © 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
gene cluster, is also essential for intracellular growth of Salmonella and therefore for the virulence (14, 15). This plasmid comprises the four-gene operon spvABCD (16–18), in which spvB encodes for the ADP-ribosyltransferase (7, 19). It is supposed that SpvB is delivered into the cytoplasm via the SPI2-encoded TTSS (13, 20). However, the mechanism underlying the translocation of the SpvB protein across the membrane of the Salmonella-containing vacuole is not known. The N-terminal domain of SpvB might be involved in translocation of SpvB, and the C-terminal catalytic domain (C/SpvB, amino acid residues 375–591) contains the ADP-ribosyltransferase activity (7, 8). It is supposed that SpvB is delivered into the cytoplasm via the SPI2-encoded

Between both domains a region of seven proline residues with a yet unknown function is present. Biochemical studies on the mode of action of SpvB in intact cells are limited by the fact that SpvB is not taken up when applied to cultured cells. To characterize the cellular consequences of actin-ADP-ribosylation by C/SpvB, we used the C. botulinum C2 toxin as a delivery system for the catalytic domain of SpvB into the cytosol. We constructed a recombinant fusion toxin, consisting of the N-terminal adaptor domain of the C2I component (C2IN) and the catalytic domain of SpvB (C/SpvB), and analyzed the cellular effects caused by the fusion toxin. Finally, we identified the functional role of the polyproline stretch, located between the domains C2IN and C/SpvB in the fusion toxin.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from Hermann GB R (Freiburg, Germany). PCRs were performed with a T1 thermocycler from Biometra (Göttingen, Germany). DNA sequencing was done with an ABI PRISM 310 genetic analyzer from PerkinElmer Life Sciences. Taq polymerase was purchased from Roche Diagnostics, and Turbo Pfu polymerase was purchased from Stratagene (La Jolla, CA). Glutathione-Sepharose 4B was from Amersham Biosciences; cell culture medium was from Biochrom (Berlin, Germany); fetal calf serum was from PAN Systems (Aidenbach, Germany); and thrombin was from Sigma. ECL, Complete protease inhibitor mixture, annexin-V-FLUOS, trypsin, and trypsin inhibitor were from Roche Diagnostics. Alexa594/phalloidin, and ProLong Gold antifade reagent were obtained from Molecular Probes (Eugene, OR); β/γ-actin was from Cytoskeleton (Denver, CO). The 6-biotin-17-NAD was purchased from R&D Systems. The horseradish peroxidase-coupled anti-rabbit antibody was from Santa Cruz Biotechnology. Alexa594/phalloidin, 4,6-diamidino-2-phenylindole, and ProLong Gold antifade reagent were obtained from PerkinElmer Life Sciences.

Cloning of C2IN-C/SpvB Fusion Toxins—The pET32a plasmid encoding the thioredoxin-S-tag-SpvB protein, encompassing residues 374–591, was kindly provided from Dr. F. Koch-Nolte (Hamburg, Germany). For cloning of an SpvB construct containing the residues 374–591, the pET encoding SpvB was amplified by PCR with 100 ng of plasmid DNA in a total volume of 50 μl with 2.5 units of TaqDNA polymerase in a reaction mixture (10 mM Tris, 15 mM MgCl2, 50 mM KCl (pH 8.3)), including deoxynucleoside triphosphates (2.5 mM each) and 12.5 pmol of the primers 5′CSpvB-BglII (5′-GGAGAGATCTCTATCTGATCTGATCCTATG3′) and 3′CSpvB-BglII (5′-GGAGATCTCTATGATCTGATCCTATGATCCTATG3′). The amplification was done by 25 cycles of denaturing at 94 °C for 30 s, primer annealing at 50 °C for 1 min, and extension at 72 °C for 90 s. The received PCR product was cloned into TOPO-TA vector (Invitrogen), according to the manufacturer’s instructions. For fusion to the C2IN fragment, the SpvB gene was excised with BglII and cloned into the BamHI-digested pGEX-C2IN vector (6). The resulting C2IN-C/SpvB Pro fragment was ligated and transformed into competent E. coli XL cells from Stratagene (La Jolla, CA). For insertion of a proline linker (according to residues 367–373 in full-length SpvB), linking C2IN to C/SpvB, a QuikChange site-directed mutagenesis, according to the QuiKChange manual, was performed by using 5′C2IN(pro5)/SpvB primer (5′-G CGT GAT TT TTT TAT AAT AAA GGA TGT CCG CCA CCT CCT CTT TAT ATG GGA GGT AAT TCA TCT CG-3′) and 3′C2IN(pro5)/C/SpvB (5′-CG AGA TGA ATT ACC TCC CAT CAT CGG AGG AGG TGG AGA TCC TTT ATT ATA AAA ATC-3′) for C2IN-C/SpvB(pro5), 5′C2IN(pro7)/C/SpvB primer (5′-G CGT GAT TT TTT TAT AAT AAA GGA TGT CCG CCA CCT CCT CTT CCG ATG ATG GGA GGT AAT TCA TCT CG-3′) and 3′C2IN(pro7)/C/SpvB primer (5′-CG AGA TGA ATT ACC TCC CAT CAT CGG AGG AGG CGGTGG CCG AGG TGA TCC TTT ATT ATA AAA ATC AAG C-3′) for C2IN-C/SpvB(pro7). For the linker with 9 proline residues, a QuiKChange mutagenesis of the C2IN(pro7)/C/SpvB was performed by using 5′C2IN(pro9)/C/SpvB (5′-G CGT GAT TT TTT TAT AAT AAA GGA TGT CCG CCA CCT CCT CTT TAT ATG GGA GGT AAT TCA TCT CG-3′) and 3′C2IN(pro9)/C/SpvB primer (5′-CG AGA TGA ATT ACC TCC CAT CAT CGG AGG AGG CGGTGG CCG AGG TGA TCC TTT ATT ATA AAA ATC AAG C-3′) for C2IN-C/SpvB(pro9). The resulting plasmids were transformed into Escherichia coli TG1 cells.

Expression and Purification of Recombinant Proteins—The components of C. botulinum C2 toxin, C2I and C2II, were expressed as GST fusion proteins in E. coli BL21 cells. Proteins were purified as described previously (3, 6, 21, 22) and incubated with thrombin (3.25 NIH units/ml of bead suspension) for cleavage of the GST domain. C2II was activated with trypsin for 30 min at 37 °C (3). The C/SpvB protein was purified following the protocol of Otto et al. (7). However, after isopropyl 1-thio-β-D-galactopyranoside induction the E. coli cells grew at 37 °C, and no sterile filtration was done. The proteins C2IN-C/SpvB (pro5), C2IN-C/SpvB (pro7), C2IN-C/SpvB (pro9), and C2IN-C/SpvBΔ pro were expressed as recombinant glutathione S-transferase fusion proteins in E. coli. Bacteria were grown at 37 °C in 2 liters of LB medium, containing 100 μg/ml ampicillin to an absorbance (600 nm) of 0.8. After addition of isopropyl 1-thio-β-d-galactopyranoside the cultures were grown for 18 h at 29 °C. The bacteria were sedimented at 5,000 × g (10 min, 4 °C) and resuspended in ice-cold lysis buffer (10 mM NaCl, 20 mM Tris·HCl (pH 7.4), 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Following sonication, the bacterial debris was sedimented at 12,000 × g (10 min, 4 °C), resuspended, and sedimented again. The supernatant was added to a 50% slurry of glutathione-Sepharose 4B in PBS (400 μl/liter) and incubated for 60 min at room temperature. After sedimentation at 3350 × g (3 min at 4 °C), the pellet was washed.
twice with ice-cold wash buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4)) and two times with ice-cold PBS (8000 × g, 3 min at 4 °C). Thereafter, the beads were incubated with 500 µl of PBS, containing thrombin (3.25 NIH units/ml) for 60 min at room temperature. For the elimination of thrombin, the beads were sedimented (8000 g) and sedimented at 8000 × g for 1 min at 4 °C. Afterward, an aliquot of the supernatant was subjected to a 12.5% SDS-PAGE, and the protein concentration was determined by densitometry using Photoshop 7.0 software.

**Cell Culture and Cytotoxicity Assays**—Vero cells were cultured in DMEM (Invitrogen) containing 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamate, and 0.1 mM nonessential amino acids. HeLa cells were cultured in minimum Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-glutamate. NIH 3T3 cells and J774.A1 macrophage-like cells were cultured in DMEM (Invitrogen) containing 10% fetal calf serum, 4 mM L-glutamate, 1.5 g/liter sodium pyruvate, and 4.5 g/liter glucose. All cell lines were cultured at 37 °C and 5% CO₂. Cells were trypsinized and reseeded for at most 15–20 times. For cytotoxicity experiments, cells were seeded in culture dishes and incubated with the respective toxin in complete medium.

**Cell Vitality After Intoxication**—Cell viability was determined by using the CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay from Promega, referred to as the MTS assay according to the method of Laemmli (23). The proteins were stained with Coomassie Brilliant Blue R-250. For immunoblot analysis, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked for 30 min with 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBS-T), and the proteins were probed with anti-C2IN antiserum (rabbit, 1:5000). After washing with PBS-T, the blot was incubated for 1 h with an anti-rabbit antibody coupled to horseradish peroxidase (1:2000 in PBS-T). The membrane was washed again, and proteins were visualized using the ECL system according to the manufacturer’s instructions.

**Assay for Toxin Binding to the Cell Surface**—Confluently grown Vero cells were incubated in serum-free DMEM at 4 °C with the toxins (C2IIa + C2I; C2IIa + fusion toxins) to allow the binding of the proteins to the cell surface. Before adding to the cells, the toxin components were preincubated on ice for 15 min. As a control, the single components of the binary toxins (C2IIa, C2I, fusion toxins) were taken. Following a washing step with serum-free DMEM, the cells were lysed in RIPA buffer, and equal amounts of protein (50 µg of each sample) were subjected to SDS-PAGE. The proteins were transferred from the gel onto a nitrocellulose membrane using the semi-dry system. The membranes were blocked overnight with 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBS-T) followed by a 1.5-h incubation with the antiserum (anti-C2IN, rabbit, 1:5000). After washing with PBS-T, the membrane was incubated with a donkey anti-rabbit antibody coupled to horseradish peroxidase (1:2000 in PBS-T) for 1 h and washed, and the C2IN-containing proteins were detected using the ECL system.

**Assay for Translocation of Toxins across the Cell Membrane**—Confluently grown HeLa and Vero cells were treated in serum-free DMEM for 30 min at 37 °C with 100 mM bafilomycin.
A1 to inhibit the physiological uptake pathway for C2 toxin. Subsequently, the cells were incubated for 20 min at 4 °C with the toxins (C2I + C2IIa; C2IN/C/SpvB + C2IIa; preincubated for 15 min at 4 °C to allow complex formation) and for 15 min at 4 °C in the presence of bafilomycin to allow the binding of the toxins to the cell surface. The medium was removed, and warm acidic medium (37 °C, pH 4.6, containing bafilomycin A1) was added to the cells for 5 min at 37 °C. This acidic pulse mimics the endosomal conditions. Finally, the acidic medium was replaced by neutral medium (pH 7.5) containing bafilomycin A1, and cells were incubated at 37 °C. The cytopathic effect of the toxin, i.e., cell rounding, was detected by taking pictures of the cells after various incubation periods.

Reproducibility of the Experiments and Statistics—All experiments were performed independently at least three times. Results from representative experiments are shown in the figures. Values (n ≥ 3) are calculated as mean ± S.D. using the SigmaPlot software.

RESULTS

Cloning, Expression, and Biochemical Characterization of the C2IN-C/SpvB Fusion Toxin—To deliver the catalytic domain of the ADP-ribosyltransferase SpvB (C/SpvB) into the cytosol of eukaryotic cells, we used a C2 toxin-based transport mechanism. To this end, a fusion toxin was cloned, consisting of amino acid residues 1–225 of the C2I enzyme component (C2IN, adaptor for the interaction with the C2IIa transport component) and the C/SpvB domain, encompassing residues 374–591 of the SpvB protein (Fig. 1A). The wild type S. enterica SpvB protein harbors a region of seven-proline residues between the N- and C-terminal domains, according to the amino acid residues 367–373 in the SpvB protein. We included this seven-proline region into the C2IN-C/SpvB fusion toxin between the two domains C2IN and C/SpvB (C2IN-C/SpvB (pro7) in the following referred to as C2IN-C/SpvB). To compare the fusion toxin with the C/SpvB domain and the C. botulinum C2I ADP-ribosyltransferase, the proteins C/SpvB, C2I, and C2IN-C/SpvB were expressed as recombinant glutathione S-transferase (GST)-fused proteins with a C2I-specific antiserum. In the C2IN-C/SpvB lane of the SDS-PAGE and subsequent Coomassie staining (Fig. 1B), the lower band represents part of the C2IN domain because of degradation. D, ADP-ribosylation of actin by C/SpvB, C2I, and C2IN-C/SpvB. The toxins (1μM of each protein) were incubated with 200μM [32P]NAD as co-substrate and 6μM actin for 30 min at 37 °C. The radiolabeled, i.e., ADP-ribosylated, actin was detected by SDS-PAGE and subsequent autoradiography. E, cytopathic effects induced by the microinjected proteins C/SpvB and C2IN-C/SpvB. Vero cells were subconfluently seeded on CELLlocate coverslips (Eppendorf), and microinjection of the fusion toxin C2IN-C/SpvB (50 nM, right panel) and the catalytic domain of SpvB (C/SpvB, 50 nM, mid panel) was performed. In the left panel, the injection buffer without any protein was injected into cells as a control. The pictures of the cells were taken immediately and after the indicated incubation periods following microinjection.

labeled, i.e. ADP-ribosylated actin, is shown in Fig. 1D. This result demonstrates that the C2IN-C/SpvB fusion toxin was active in vitro. To test the activity of C2IN-C/SpvB in intact cells, the protein was microinjected into Vero cells. Directly and
SpvB Fusion Toxin Induces Actin-ADP-ribo-ylation in Cells

after the indicated incubation periods, pictures from identical cells were taken (Fig. 1E). Injection of control buffer as a control for the microinjection procedure did not influence the morphology of cells (Fig. 1E, left panel). When the C/SpvB protein as a positive control was injected, cells rounded up and finally detached from the coverslip (Fig. 1E, middle panel). Similarly, cells microinjected with C2IN-C/SpvB rounded up within a period of 60 min (Fig. 1E, right panel), indicating that the C2IN-C/SpvB fusion toxin destroyed the structure of the actin cytoskeleton in infected cells.

Cellular Uptake Mechanism of the C2IN-C/SpvB Fusion Toxin—Next, we tested whether the fusion toxin C2IN-C/SpvB was delivered into cells by the transport component C2IIa. To this end, subconfluently growing Vero cells were incubated with the C2IN-C/SpvB fusion toxin (1 μg/ml) together with C2IIa (2 μg/ml). As a positive control, the C. botulinum C2 toxin (C2I + C2IIa) was used. As further controls, cells were incubated with the single components C2I (200 ng/ml), C2IN-C/SpvB (1 μg/ml), and C2IIa (1 μg/ml) and without any protein. Cells were incubated for 3 h at 37 °C; pictures were taken, and the morphology of the cells was determined as an end point for the cytopathic activity of the individual protein combinations (Fig. 2A). Only such cells, which have been treated with the combination of C2IIa plus C2IN-C/SpvB or C2IIa plus C2I, showed a round morphology. As expected, treatment of the cells with the single components C2IIa, C2I, or C2IN-C/SpvB did not induce any morphological alterations, indicating that for transport of the C2IN-C/SpvB fusion toxin into cells C2IIa is essentially needed. After a 3-h incubation period, the majority of the cells were rounded up (Fig. 2A). This change in morphology was comparable with the rounding of cells, which have been treated with C2I and C2IIa.

To confirm that the morphological effects caused by the toxins were because of the ADP-ribosylation of actin within the cytosol of intact cells, we analyzed the state of actin-ADP-ribo-ylation from cells, which have been incubated with C2IIa plus C2IN-C/SpvB. Again, the combination C2IIa plus C2I was used as a control. Following incubation with the toxins, cells were lysed, and the cellular protein was subsequently incubated with biotin-labeled NAD⁺ and C2I for ADP-ribosylation of actin. Proteins were separated by SDS-PAGE, and ADP-ribosylated actin was detected by the use of streptavidin coupled to peroxidase and a subsequent chemiluminescence detection of the labeled proteins (Fig. 2B). The more actin already ADP-ribosylated in the intact cells by the action of the intracellular toxin, the less was labeled during the subsequent in vitro reaction, i.e. no actin signals were detectable from intoxicated cells. The ADP-ribosylation of actin from cell lysates confirmed that actin was already ADP-ribosylated in the intact cells by the C2IN-C/SpvB protein during incubation of the cells with C2IN-C/SpvB plus C2IIa (Fig. 2B). Incubation of cells with the single components C2IIa and C2IN-C/SpvB resulted in a strong signal of biotin-labeled actin, indicating that actin was not ADP-ribosylated in intact cells. This result clearly demonstrates that cell rounding was because of the activity of the fusion toxin within the cytosol of treated cells.

The C2IIa-mediated translocation of C2I from acidic endosomes into the cytosol was blocked by the drug bafilomycin A1, which prevents acidification of the endosomal lumen. Cells, which have been pretreated with bafilomycin A1, were protected from the cytopathic effects and did not round up. As shown in Fig. 3A, bafilomycin A1 protected the cells from cytopathic effects of the C2IN-C/SpvB fusion toxin, indicating that the fusion toxin takes the same route into the cytosol as C2 toxin does.

For C2 toxin and C2 toxin-derived fusion toxins, it is known that inhibition of the host cell chaperone Hsp90 prevents the C2IIa-facilitated translocation of C2I from acidic endosomes into the cytosol. Again, the cytopathic effect of the C2IN-C/SpvB fusion toxin, i.e. the number of rounded cells, was reduced when cells were pretreated with the Hsp90 inhibitor radicicol prior to application of C2IIa plus C2IN-C/SpvB (Fig. 3B). This observation corroborates that the C2IN-C/SpvB fusion toxin is taken up into the cytosol of
target cells via the same Hsp90-dependent mechanism as the C2 toxin. Most likely, the translocation of the fusion toxin through the C2IIa pore requires at least a partial unfolding of the protein and a subsequent Hsp90-dependent refolding of the enzyme in the cytosol.

To demonstrate that the effect of C2IN-C/SpvB was not restricted to one specific cell type, several eukaryotic cell types were tested for their sensitivity toward the new fusion toxin. The cell lines HeLa, Vero, NIH 3T3, and Caco-2 were incubated at 37 °C in the presence of C2IN-C/SpvB plus C2IIa, and following a 3-h incubation period, the cytopathic effect of the toxin was analyzed by monitoring alterations in the cell morphology. All the tested cell lines, which have been treated with C2IN-C/SpvB (1/250 µg/ml) plus C2IIa (2 µg/ml), showed cell rounding (Fig. 4A). In contrast, an incubation of cells with the single components C2I, C2IN-C/SpvB, and C2IIa did not change the morphology of the cells (data not shown). The cells were lysed, and a subsequent in vitro ADP-ribosylation of actin was performed with C2I and biotin-labeled NAD, as shown in the lower panel. Therefore, a weak signal indicates a strong ADP-ribosylation of actin in the intact cells by the toxin. B, time course of the cytopathic effects of the toxins on Vero and HeLa cells. Vero and HeLa cells were incubated with C2IN-C/SpvB(pro7) (1 µg/ml) plus C2IIa (2 µg/ml) at 37 °C. Pictures were taken after the indicated times, and the percentages of rounded cells were determined. Values are means ± S.D. of three independent measurements.

FIGURE 4. Cytopathic effect of the C2IN-C/SpvB fusion toxin on various eukaryotic cell lines. A, subconfluently growing HeLa, Vero, NIH 3T3, and Caco-2 cells were incubated with C2IN-C/SpvB(pro7) (1 µg/ml) plus C2IIa (2 µg/ml) for 4 h at 37 °C. For control, cells were left untreated. Pictures were taken, and the percentage of rounded cells was determined. Subsequently, the cells were lysed and an ADP-ribosylation of cellular actin was performed with C2I and biotin-labeled NAD, as shown in the lower panel. Therefore, a weak signal indicates a strong ADP-ribosylation of actin in the intact cells by the toxin.
cells to about 20%, no significant decrease of viable cells was detected after treatment of cells with each of the actin-ADP-ribosylating toxins (data not shown). This result indicates that within the time frame, in which the total amount of cellular actin was ADP-ribosylated by the toxins, cell death is not essentially and directly a consequence of actin-ADP-ribosylation in J774.A1 cells.

Role of the Proline Linker Region between the Domains C2IN and C/SpvB in the C2IN-C/SpvB Fusion Toxin—Finally, we studied the role of the seven proline residues located between the domains C2IN and C/SpvB in the C2IN-C/SpvB fusion toxin (see Fig. 1A). The catalytic domain of SpvB (C/SpvB), containing no polyproline region, is capable to ADP-ribosylate actin in vitro (see Fig. 1D). Moreover, the C/SpvB protein induced morphological alterations, i.e. cell rounding of Vero cells following microinjection (Fig. 1E, middle panel). Therefore, the presence of the polyproline sequence is not absolutely essential for the ADP-ribosyltransferase activity in vitro and in the cytosol of intact cells.

We constructed the following C2IN-C/SpvB fusion toxins with variations in the length of the proline region: C2IN-C/SpvB(Δpro) without any proline region; C2IN-C/SpvB(pro5) containing five proline residues; the C2IN-C/SpvB(pro7), which corresponds to the above characterized C2IN-C/SpvB and reflects the seven proline residues in the S. enterica SpvB protein; and C2IN-C/SpvB(pro9), which contains nine proline residues. The constructs were confirmed by DNA sequence analysis. The recombinant GST fusion proteins were purified as described above, and the resulting proteins were analyzed by SDS-PAGE and subsequent Coomassie staining (Fig. 6A). Moreover, all fusion toxins were detected by the specific antiserum raised against C2IN (Fig. 6B). The enzyme activities of the proteins were tested by ADP-ribosylation of non-muscle (β/γ) actin with [32P]NAD as a co-substrate. All tested fusion toxins showed a detectable enzyme activity (Fig. 6C). However, in presence of 5 nM of each fusion toxin, the kinetics of actin-ADP-ribosylation revealed that depending on the length of the proline linker the enzyme activity of the toxins decreased because C2IN-C/SpvB(pro9) exhibited the highest enzyme activity followed by C2IN-C/SpvB(pro7), C2IN-C/SpvB(pro5), and C2IN-C/SpvB(Δpro). If high concentrations of each fusion toxin (i.e. 1 μM) were tested in the actin-ADP-ribosylation assay, no differences in the in vitro enzyme activities were detected in our assay.

Next, the various fusion toxins were tested for their cytotoxic activity toward intact cells. To this end, the toxins (400 ng/ml and 1.5 μg/ml of each protein) were applied with C2IIa (800 ng/ml and 2 μg/ml) to Vero cells. Pictures from the cells were taken after the indicated incubation periods, and the percentages of rounded cells were determined. In the presence of C2IIa, the effect of the fusion toxins in cells correlated with the existence and the length of the proline linker region between

---

4 K. Heine, unpublished observations.
Within 6 h of incubation, the fusion toxin C2IN-C/SpvB(pro9) plus C2IIa induced only a marginal number of rounded cells, comparable with untreated cells. Interestingly, the fusion toxin C2IN-C/SpvB(pro9) showed an advanced cytopathic effect compared with the C2IN-C/SpvB(pro7) protein and with the C2IN-C/SpvB(pro5) protein, when the proteins were applied to cells together with C2IIa.

We tested whether the proline linker region had any influence on the transport of the fusion toxin by C2IIa, and we compared the two proteins C2IN-C/SpvB(pro9) and C2IN-C/SpvB(pro7) for their ability to interact with C2IIa and to translocate into the host cell cytosol. The first step in the cellular uptake mechanism was the C2IIa-mediated binding of the fusion toxins to the cell surface receptor. This step was analyzed by detecting cell-bound toxin with a specific antiserum against the C2I protein. To prevent any endocytosis, cells were incubated for 20 min at 4 °C with C2IIa (800 ng/ml) plus 400 ng of one of each of the fusion toxins C2IN-C/SpvB(Dpro), C2IN-C/SpvB(pro5), C2IN-C/SpvB(pro7), and C2IN-C/SpvB(pro9) (upper part). In a second experiment, 2 μg/ml C2IIa was used in combination with 1.5 μg/ml of each of the fusion toxins (lower part). At the indicated time points pictures were taken, and the percentage of rounded cells was determined. Values are means ± S.D. of three independent measurements.

FIGURE 6. Influence of the proline region located in C2IN-C/SpvB on the activity of the fusion toxin. A, following expression, affinity purification, and removal of the GST domain by thrombin, the recombinant fusion toxins C2IN-C/SpvB(Dpro), C2IN-C/SpvB(pro5), C2IN-C/SpvB(pro7), and C2IN-C/SpvB(pro9) were analyzed by SDS-PAGE and subsequent Coomassie staining. B, fusion proteins (250 ng of protein each) were analyzed by SDS-PAGE and subsequently detected with an antibody raised against C2IN. The upper bands represent the fusion toxins, whereas the lower signals are the degraded C2IN fragments. C, ADP-ribosylation of actin by the fusion toxins. Each fusion toxin (5 nM final concentration) was incubated with 6 μM actin and with 150 μM [32P]NAD for the indicated times. The ADP-ribosylated actin was detected by SDS-PAGE and subsequent autoradiography, and the modification level of actin was calculated as mol of ADP-ribose/mol of actin. D, cytopathic effects of the fusion toxins on Vero cells. Vero cells were incubated for up to 6 h at 37 °C with C2IIa (800 ng/ml) plus 400 ng of one of each of the fusion toxins C2IN-C/SpvB(Dpro), C2IN-C/SpvB(pro5), C2IN-C/SpvB(pro7), and C2IN-C/SpvB(pro9) (upper part). In a second experiment, 2 μg/ml C2IIa was used in combination with 1.5 μg/ml of each of the fusion toxins (lower part). At the indicated time points pictures were taken, and the percentage of rounded cells was determined. Values are means ± S.D. of three independent measurements.
pretreated with bafilomycin A1 to block endosomal acidification and thereby the physiological uptake of C2 toxin and the C2 toxin-derived fusion toxins (see Fig. 3A). Subsequently, cells were incubated at 4 °C with C2IIa plus C2IN-C/SpvB(pro7) and C2IN-C/SpvB(pro7), respectively. As a positive control, C2I plus C2IIa was used. As further controls, the single components or no protein was applied to the cells. Following binding of the toxins to the cell surface, cells were subjected for 5 min to pre-warmed medium (37 °C), containing bafilomycin A1 at pH 4.6 versus pH 7.5. The acidic conditions trigger membrane insertion and pore formation of C2IIa, which enables C2I or the respective fusion toxin to translocate through the C2IIa pore into the cytosol. After the short acidification, cells were incubated at 37 °C at pH 7.5 in the presence of bafilomycin A1, and the numbers of rounded cells were determined. As shown in Fig. 7B, only cells that have been treated with either C2IIa plus C2I or C2IIa plus C2IN-C/SpvB(pro7) rounded up, even in the presence of bafilomycin A1 and after exposition to the acidic shift, although no cell rounding was observed for cells that have not been incubated in acidic medium or for cells treated with the single components C2I, C2IIa, C2IN-C/SpvB(pro7), or C2IN-C/SpvB(Apro), respectively (data not shown). Only few cells were rounded when C2IIa plus C2IN-C/SpvB(Apro) was applied. This finding indicates that the proline linker region is essential for the C2IIa-facilitated translocation of the fusion toxins across cell membranes under acidic conditions.

**DISCUSSION**

The pathogenesis of systemic *Salmonella* infections is characterized by survival and proliferation of bacteria inside macrophages, leading to cell death and to intercellular spread of the bacteria. To promote cellular uptake and intracellular survival, *Salmonella* has evolved sophisticated strategies to directly modify the host cell cytoskeleton. To this end, a variety of virulence factors is delivered into the cytosol of infected cells in a precisely spatiotemporal coordinated manner by two different type III secretion systems encoded on the pathogenicity islands SPI1 and SPI2. The intracellular survival and the replication of *Salmonella* in macrophages are promoted by the SPI2-mediated translocation of bacterial effectors into the host cell cytosol. The SPI2 effectors induce local actin polymerization, resulting in focal actin meshwork around the SCV (24), which is important for intracellular replication of bacteria in the SCV. Some strains of *Salmonella* harbor the *spv* virulence cluster, which encodes for the ADP-ribosyltransferase SpvB and enhances the ability of *Salmonella* to induce systemic disease (15). SpvB ADP-ribosylates G-actin at arginine 177, resulting in depolymerization of actin filaments in host cells. In *Salmonella*-infected cells, SpvB modulates the vesicle-associated actin polymerization to achieve release of bacteria from SCV, and it was reported that SpvB contributes to cell death of infected macrophages (25). However, the precise consequence of actin-ADP-ribosylation by SpvB in intact cells and its contribution to cell death are not completely understood.

To study the effects of actin-ADP-ribosylation by SpvB in intact cells apart from other SPI2 effector proteins, the cell-permeable fusion toxin C2IN-C/SpvB was used. In microscopic time lapse experiments with GFP-effector transfected cells, the depolymerization of actin filaments because of C2 toxin- and C2IN-C/SpvB-mediated ADP-ribosylation was analyzed. Interestingly, many filaments started to disrupt from their central region. Based on previous reports, it was expected that depolymerization starts at the end of the filaments because of the capping function of ADP-ribosylated actin (26). The disruption of actin filaments following treatment with actin-ADP-ribosylating toxins might be because of interference of ADP-ribosylated actin monomers in the treadmilling process of actin polymerization and depolymerization (for review see Ref. 27). A deranged actin polymerization cycle might be leading to an increased tension in the actin filaments resulting in their disruption and subsequent depolymerization. Recently, Stebbins and co-workers (9) reported on a steric antagonism of actin polymerization by SpvB. By crystallizing ADP-ribosylated and thereby polymerization-deficient actin, no dramatic conformational changes in actin were found. Based on their study, they draw the conclusion that polymerization of ADP-ribosylated actin is inhibited most likely through a steric disruption of intrafilament contact sites. It requires further investigation to

**FIGURE 7. Role of the proline residues in the fusion toxin for the C2IIa-mediated receptor binding and translocation into the cytosol.** A, detection of the cell-bound fusion toxins C2IN-C/SpvB(Apro) and C2IN-C/SpvB(pro7). Vero cells were incubated at 4 °C for 20 min with C2IN-C/SpvB(Apro) or with C2IN-C/SpvB(pro7) (each 1 μg/ml) together with C2IIa (2 μg/ml). For control, cells were left untreated. To demonstrate the specific C2IIa-mediated binding of the fusion toxins to cells, cells were incubated with C2IN-C/SpvB(Apro) and C2IN-C/SpvB(pro7) in the absence of C2IIa. Cells were washed and lysed, and following SDS-PAGE, the proteins were blotted onto nitrocellulose. The fusion toxins were detected with an anti-C2IN antibody that encodes for the ADP-ribosyltransferase SpvB and a secondary anti-rabbit antibody coupled to horseradish peroxidase. Note that binding of the fusion toxins to cells is specific and dependent on C2IIa (left panel). Right panel, detection of the amount of cell-bound fusion toxins C2IN-C/SpvB(Apro) and C2IN-C/SpvB(pro7) after 3 and 20 min. The conditions of the experiment were precisely as described above. The fusion toxins were applied to cells only in combination with C2IIa. B, C2IIa-mediated translocation of the toxins into the cytosol. HeLa cells were pretreated for 1 h at 37 °C with bafilomycin A1 (100 nm final concentration) to block endosomal acidification. Subsequently, cells were incubated at 4 °C with 2 μg/ml C2IN-C/SpvB(Apro) plus 1 μg/ml C2IIa or with 2 μg/ml C2IN-C/SpvB(pro7) plus 1 μg/ml C2IIa, respectively. As a positive control, cells were treated with C2IIa (1 μg/ml) plus C2I (0.5 μg/ml). As a negative control, cells were treated without any toxin. The cells were subjected for 5 min to an acidic pulse (medium at 37 °C, pH 4.6, containing bafilomycin A1). The cells were further incubated at 37 °C at pH 7.5 in the presence of bafilomycin A1, and pictures were taken after 90 min.
test whether this model is the underlying mechanism for the toxin-induced effects on actin filaments observed in intact cells.

We used the C2IN-C/SpvB fusion toxin and the C. botulinum C2 toxin to separate the consequences of actin-ADP-ribosylation, e.g., the depolymerization of actin filaments from the induction of cell death. According to the postulated model of Salmonella infection, the intracellular secretion of effectors by the SPI2-TTSS induces a delayed type of apoptosis in infected macrophages (25), and SpvB was proposed to contribute to this phenomenon. Interestingly, it was reported earlier that C2 toxin did not immediately induce cell death following ADP-ribosylation of actin in various cell types (28–32). When macrophages were challenged with the C2IN-C/SpvB fusion toxin, ADP-ribosylation of actin did not trigger cell death, even when the total amount of cellular actin was ADP-ribosylated by the toxin. However, this observation does not exclude that actin-ADP-ribosylation might result either directly or indirectly in delayed macrophage cell death. In our experiments the fusion toxin harbors only the catalytic domain of SpvB; therefore, we cannot exclude that the N-terminal domain of SpvB may contribute either directly or indirectly to cell death by modulating C/SpvB to exhibit any cytopathic activity. To further investigate the role of SpvB in cell death of macrophages, we will construct fusion toxins containing C2IN and either the N-terminal domain of SpvB or the full-length SpvB protein.

As an advantage of the fusion toxin, the consequences of actin-ADP-ribosylation can be studied in intact cells without overlapping effects from intracellular growing Salmonella. On the other hand, the artificial delivery of an isolated domain of a virulence factor into the cytosol most likely does not reflect the spatiotemporal distribution of the ADP-ribosyltransferase in Salmonella-infected cells. In infected cells, SpvB is delivered from SCV-located Salmonella into the cytosol, most likely via the SPI2-TTSS. On the amount of translocated SpvB, a more local distribution of SpvB around the SCV might be possible, initially resulting in an actin-ADP-ribosylation around the vacuoles, accompanied by a vacuole-associated actin depolymerization. On the other hand, it was shown that the complete amount of actin was ADP-ribosylated in Salmonella-infected cells.

The translocation mechanism of proteins through the C2IIa pore and through a Salmonella type III needle complex might be comparable. For translocation through the C2IIa pore, C2I and C2I-derived fusion toxins require at least a partial unfolding (4). Refolding of the proteins in the cytosol is facilitated by the host cell chaperone Hsp90 (5). Here we report that the cytopathic mode of action of the C2IN-C/SpvB fusion toxin also requires Hsp90 activity, suggesting that this protein becomes unfolded during C2IIa-mediated translocation and is refolded into its active conformation in the cytosol. If SpvB translocates through a type III needle complex from intracellularly growing Salmonella into the host cell cytosol and as the inner diameter of the C2IIa pore (2.7 nm) and of the type III needle complex are similar (33–35), unfolding for passage through the needle and subsequent refolding might be also important during the physiological translocation of SpvB.

Finally, we analyzed the role of the polyproline region located in the fusion toxin for its functional role. The full-length SpvB protein also harbors a seven-proline residue region between the N- and C-terminal domains. We demonstrated recently that the proline region has no significant effects on the ADP-ribosyltransferase activity of the catalytic domain of SpvB (8). Moreover, we can exclude that the proline region is compulsive for the ADP-ribosylation of actin by C/SpvB in intact cells, because microinjection of C/SpvB without the proline residues also led to depolymerization of actin filaments. When the conditions of C2IIa-mediated toxin translocation from acidic endosomes were experimentally mimicked on the surface of intact cells, the cytopathic effects of the C2IN-C/SpvB fusion toxins revealed a crucial influence of the presence and the length of the proline region for translocation of the fusion toxins through the C2IIa pore. The proline stretch between the two domains may enhance the flexibility of the fusion toxin and facilitate its unfolding and subsequent refolding, as the presence of proline residues results in special type helices (36). If the SpvB protein actually translocates via the SPI2-TTSS in Salmonella-infected macrophages, it would be interesting to study whether the seven proline residues between the two domains of SpvB are essentially involved in the intracellular secretion of SpvB into the host cell cytosol.

Acknowledgments—We thank Dr. Friedrich Koch-Nolte (Hamburg, Germany) for providing Salmonella C/SpvB cDNA. We thank Ulrike Binder (University of Ulm Medical Center) and Peter Gebhardt (University of Freiburg) for expert technical assistance.

REFERENCES

1. Aktories, K., and Barth, H. (2004) Int. J. Med. Microbiol. 293, 557–564
2. Aktories, K., Bärmann, M., Ohishi, L., Tsuyama, S., Jakobs, K. H., and Habermann, E. (1986) Nature 322, 390–392
3. Barth, H., Blöcker, D., Behlke, I., Bergsma-Schutter, W., Brisson, A., Benz, R., and Aktories, K. (2000) J. Biol. Chem. 275, 18704–18711
4. Haug, G., Wilde, C., Leemhuis, J., Meyer, D. K., Aktories, K., and Barth, H. (2003) Biochemistry 42, 15284–15291
5. Haug, G., Leemhuis, J., Tiemann, D., Meyer, D. K., Aktories, K., and Barth, H. (2003) J. Biol. Chem. 278, 32266–32274
6. Barth, H., Hofmann, F., Olenik, C., Just, I., and Aktories, K. (1998) Infect. Immun. 66, 1364–1369
7. Otto, H., Tezcan-Merdol, D., Girisch, R., Haag, F., Rhen, M., and Koch-Nolte, F. (2000) Mol. Microbiol. 37, 1106–1115
8. Hochmann, H., Pust, S., von FIGuera, G., Aktories, K., and Barth, H. (2006) Biochemistry 45, 1271–1277
9. Margarit, S. M., Davidson, W., Frego, L., and Stebbins, C. E. (2006) Structure (Lond.) 14, 1219–1229
10. Fierer, J., and Guiney, D. G. (2001) J. Clin. Investig. 107, 775–780
11. Rodriguez, M., de Diego, I., and Mendoza, M. C. (1998) J. Clin. Microbiol. 36, 3291–3296
12. Guiney, D. G., and Lesnick, M. (2005) Clin. Immunol. 114, 248–255
13. Hensel, M., Shear, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F. C., and Holden, D. W. (1998) Mol. Microbiol. 30, 163–174
14. Gulig, P. A., Doyle, T. J., Hughes, J. A., and Matsui, H. (1998) Infect. Immum. 66, 2471–2485
15. Libby, S. J., Adams, L. G., Ficht, T. A., Allen, C., Whitford, H. A., Buchmeier, N. A., Bossie, S., and Guiney, D. G. (1997) Infect. Immun. 65, 1786–1792
16. Fang, F. C., Krause, M., Roudier, C., Fierer, J., and Guiney, D. G. (1991) J. Bacteriol. 173, 6783–6789
17. Grob, P., and Guiney, D. G. (1996) J. Bacteriol. 178, 1813–1820
18. Guiney, D. G., Libby, S., Fang, F. C., Krause, M., and Fierer, J. (1995) Trends Microbiol. 3, 275–279
SpvB Fusion Toxin Induces Actin-ADP-ribosylation in Cells

19. Tezcan-Merdol, D., Nyman, T., Lindberg, U., Haag, F., Koch-Nolte, F., and Rhen, M. (2001) *Mol. Microbiol.* 39, 606–619
20. Ochman, H., Soncini, F. C., Solomon, F., and Groisman, E. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 7800–7804
21. Barth, H., Roebling, R., Fritz, M., and Aktories, K. (2002) *J. Biol. Chem.* 277, 5074–5081
22. Blöcker, D., Behlke, J., Aktories, K., and Barth, H. (2001) *Infect. Immun.* 69, 2980–2987
23. Laemmli, U. K. (1970) *Nature* 227, 680–685
24. Steele-Mortimer, O., Brumell, J. H., Knodler, L. A., Meresse, S., Lopez, A., and Finlay, B. B. (2002) *Cell Microbiol.* 4, 43–54
25. Libby, S. J., Lesnick, M., Hasegawa, P., Weidenhammer, E., and Guiney, D. G. (2000) *Cell Microbiol.* 2, 49–58
26. Wegner, A., and Aktories, K. (1988) *J. Biol. Chem.* 263, 13739–13742
27. Pollard, T. D. (2002) *Harvey Lect.* 98, 1–17
28. Barth, H., Klingler, M., Aktories, K., and Kinzel, V. (1999) *Infect. Immun.* 67, 5083–5090
29. Hippenstiel, S., Tannert-Otto, S., Vollrath, N., Krüll, M., Just, I., Aktories, K., Von Eichel-Streiber, C., and Suttrop, N. (1997) *Am. J. Physiol.* 272, L38–L43
30. Hippenstiel, S., Schmeck, B., Seybold, I., Krull, M., Eichel-Streiber, C., and Suttrop, N. (2002) *Biochem. Pharmacol.* 64, 971–977
31. Linseman, D. A., Laessig, T., Meintzer, M. K., McClure, M., Barth, H., Aktories, K., and Heidenreich, K. A. (2001) *J. Biol. Chem.* 276, 39123–39131
32. Zepeda, H., Considine, R. V., Smith, H. L., Sherwin, J., Ohishi, I., and Simpson, L. L. (1988) *J. Pharmacol. Exp. Ther.* 246, 1183–1189
33. Chakravortty, D., Rohde, M., Jager, L., Deiwick, J., and Hensel, M. (2005) *EMBO J.* 24, 2043–2052
34. Marlovits, T. C., Kubori, T., Sukhan, A., Thomas, D. R., Galan, J. E., and Unger, V. M. (2004) *Science* 306, 1040–1042
35. Schleberger, C., Hochmann, H., Barth, H., Aktories, K., and Schulz, G. E. (2006) *J. Mol. Biol.* 364, 705–715
36. Williamson, M. P. (1994) *Biochem. J.* 297, 249–260