Salmonella enterica SpvB ADP-Ribosylates Actin at Position Arginine-177—Characterization of the Catalytic Domain within the SpvB Protein and a Comparison to Binary Clostridial Actin-ADP-Ribosylating Toxins†

Henrike Hochmann,‡,§ Sascha Pust,‡,|| Guido von Figura,§ Klaus Aktories,§ and Holger Barth*,||

Department of Pharmacology and Toxicology, University of Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany, and Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Albertstrasse 25, D-79104 Freiburg, Germany

Received September 8, 2005; Revised Manuscript Received December 7, 2005

ABSTRACT: The SpvB protein from *Salmonella enterica* was recently discovered as an actin-ADP-ribosylating toxin. SpvB is most likely delivered via a type-III secretion system into eukaryotic cells and does not have a binding/translocation component. This is in contrast to the family of binary actin-ADP-ribosylating toxins from various *Bacillus* and *Clostridium* species. However, there are homologies in amino acid sequences between the C-terminal domain of SpvB and the catalytic domains of the actin-ADP-ribosylating toxins such as C2 toxin from *Clostridium botulinum* and iota toxin from *Clostridium perfringens*. We compared the biochemical properties of the catalytic C-terminal domain of SpvB (C/SpvB) with the enzyme components of C2 toxin and iota toxin. The specificity of C/SpvB concerning the modification of G- or F-actin was comparable to the C2 and iota toxins, although there were distinct differences regarding the recognition of actin isoforms. C/SpvB and iota toxin modify both muscle α-actin and nonmuscle β/γ-actin, whereas C2 toxin only modifies β/γ-actin. In contrast to the iota and C2 toxins, C/SpvB possessed no detectable glycohydrolase activity in the absence of a protein substrate. The maximal reaction rates were comparable for all toxins, whereas variable K_m values for NAD were evident. We identified arginine-177 as the modification site for C/SpvB with the actin homologue protein Act88F from *Drosophila*.

The intracellular acting pathogen *Salmonella enterica* is a Gram-negative bacterium that causes a variety of diseases, which range from gastroenteritis to typhoid fever in a broad host spectrum (*1*, *2*). *S. enterica* harbors an extra chromosomal plasmid with a highly conserved gene cluster called *spv* (*salmonella plasmid virulence*). This gene locus is associated with intracellular bacterial growth and systemic infection (*3*, *4*). It was shown that the infection efficiency is dramatically reduced when the *spv* genes are not expressed (*5*). The *spv* gene cluster comprises five genes. The transcriptional activator *spvR* controls an operon consisting of four genes *spvABCD* (*6*−*9*). Expression of the *spv* genes is upregulated after phagocytosis of *Salmonella* in response to the intracellular milieu (*10*, *11*). Recently, the *spvB* gene product was identified as a mono(ADP-ribosyl)transferase, which modifies actin and thereby leads to depolymerization of actin filaments (*12*, *13*).

It is still not completely clear and controversially discussed how the actin-ADP-ribosylating toxin from *S. enterica* (SpvB)[1] protein is transported into the cytosol of eukaryotic cells. The function of the N-terminal part of SpvB (Figure 1A), which has significant sequence similarity with the TcaC protein of *Photorhabdus luminescens* (*12*), is not known. Thus, no binding/translocation component was identified, which delivers SpvB into the cytosol of cells. This suggests that SpvB is not a binary toxin like all known actin-ADP-ribosylating toxins produced by *Bacillus* and *Clostridium* species. The *spv* locus is located on the virulence plasmid, which is connected with the *Salmonella* pathogenicity island 2 type-III secretion system (*14*, *15*). Therefore, transport of SpvB into the host cell via the type-III secretion system (TTSS) was deemed likely by investigators. On the other hand, it was reported that that mimicking of the intracellular ion concentrations leads to a secretion of SpvB protein in a TTSS independent manner *in vitro* and *in vivo* (*16*).

Thus far, less is known about the biochemical properties of SpvB. The existing biochemical data are restricted to the effect on actin polymerization following ADP-ribosylation. The C-terminal domain of SpvB (C/SpvB) contains the catalytic site and shares sequence similarity with other actin-ADP-ribosylating toxins such as C2 toxin from *Clostridium*

† This work was supported by the Deutsche Forschungsgemeinschaft (SPP 1150/BA 2087).

* To whom correspondence should be addressed: Department of Pharmacology and Toxicology, University of Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany. Telephone: 49-731-50023290. Fax: 49-731-50023872. E-mail: holger.barth@medizin.uni-ulm.de.

‡ These authors contributed equally to this work.

§ University of Freiburg.

|| University of Ulm.

[1] Abbreviations: Act88F, *Drosophila* indirect flight muscle protein; C2 toxin, *Clostridium botulinum* C2 toxin; C2I, enzyme component of C2; C/SpvB, C-terminal enzyme domain of SpvB; iota toxin, *Clostridium perfringens* iota toxin; iota a, enzyme component of iota toxin; PBS, phosphate-buffered saline; SpvB, actin-ADP-ribosylating toxin from *Salmonella enterica*.

10.1021/bi051810w CCC: $33.50
Published on Web 01/06/2006

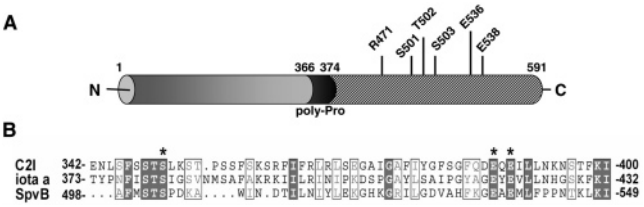


FIGURE 1: Amino acid alignment of the actin-ADP-ribosyltransferases *C. botulinum* C2I, *C. perfringens* iota a, and *S. enterica* SpvB. (A) SpvB protein (65 kDa) from *S. enterica* consists of 591 amino acid residues and represents an actin-ADP-ribosylating toxin. The C-terminal domain (amino acid residues 375−591, 24.5 kDa) shares homology to other known ADP-ribosyltransferases and harbors the conserved amino acid residues, typical for ADP-ribosyltransferases (R471, S501, T502, S503, E536, and E538). Between the N-terminal domain and the C-terminal domain of the SpvB protein, a polyproline region (amino acid residues 366−374) is located. (B) Catalytic domains of C2I, iota a, and SpvB were aligned by CLUSTALW. Amino acid identities within the proteins are marked in gray frames, and similarities are marked in white frames. The conserved amino acids, which are essential for ADP-ribosyltransferase activity, are indicated by asterisks.

botulinum and iota toxin from *Clostridium perfringens* (*17*). An amino-acid-based alignment of the ADP-ribosyltransferases C/SpvB, C2 toxin, and iota toxin is shown in Figure 1B. In particular, motifs that are essential for ADP-ribosyltransferase activity remain conserved within these toxins. The binary actin-ADP-ribosylating toxins have been studied in detail during the past years in our and other laboratories. A separated binding/translocation component mediates delivery of the enzyme component into the cytosol by pore formation in acidic endosomes (*18*). Cellular chaperones facilitate the translocation of the enzyme components across endosomal membranes (*19*).

In this work, we performed a detailed biochemical analysis of the C/SpvB protein in comparison to the C2 and iota toxins. We show that there are differences in the enzymatic activities of the C/SpvB and clostridial toxins during the initial phase. Concerning the maximum ADP-ribosylation of actin and pH dependency, there are no significant differences between these proteins. We demonstrate that C/SpvB ADP-ribosylates β/γ-actin (*13*) as well as α-actin, which is comparable to iota toxin, but is in contrast to the C2 toxin that only modifies β/γ-actin. Furthermore, C2I has a 5-fold higher K_m value for NAD than C/SpvB and iota a. Surprisingly, SpvB showed no NAD-glycohydrolase activity, although a poor activity for C2 toxin and a high activity for iota toxin is evident. Moreover, we identified arginine-177 in an actin homologue of a *Drosophila* indirect flight muscle protein as the modification site for C/SpvB.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were obtained from Hermann GbR (Freiburg, Germany). Polymerase chain reactions were performed with a T1 thermocycler from Biometra (Göttingen, Germany), and DNA sequencing was done with an ABI PRISM 310 genetic analyzer from Perkin−Elmer (Langen, Germany). Turbo Pfu polymerase was purchased from Stratagene (La Jolla, CA); glutathione-Sepharose 4B was from Amersham Bioscience Europe (Freiburg, Germany); and thrombin was from Sigma (Deisenhofen, Germany). The α-skeletal muscle actin (rabbit) and the β/γ-cytoplasmic nonmuscle actin (human platelets) were purchased from Cytoskeleton (Denver, CO), and [adenylate-^{32}P]NAD was from Perkin−Elmer (Boston, MA). The complete protease inhibitor was from Roche (Basel, Switzerland).

Expression and Purification of Recombinant Proteins. The proteins *C. botulinum* C2I and *C. perfringens* iota a were expressed as GST fusion proteins in *E. coli* BL21. Proteins were purified as described previously (*20*, *21*) and incubated with thrombin (3.25 NIH units/mL of bead suspension) for cleavage of the GST domain. The C/SpvB protein represents the catalytic domain (amino acid residues 375−591) of the full-length protein (see Figure 1A) and was purified following the protocol of Otto et al. (*12*); however, after IPTG induction, *E. coli* cells were grown at 37 °C and sterile filtration was not done.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed according to the method of Laemmli (*22*). The proteins were stained with Coomassie Brilliant Blue R-250.

ADP-Ribosylation Assay. The ADP-ribosylation assay was performed (30 min, 37 °C) with 6 μM β/γ-actin in HEPES buffer (50 mM, pH 7.4), containing 2 mM $MgCl_2$, complete protease inhibitor (according to manual of the manufacturer), 200 μM [adenylate-^{32}P]NAD, and 1 μM of each toxin C2I, iota a, or C/SpvB. The reaction was stopped by the addition of Laemmli buffer and heated for 5 min at 95 °C. Radiolabeled proteins were detected by SDS−PAGE and analyzed by phosphorimaging. For ADP-ribosylation of G-/F-actin, the reaction was done with 60 nM toxins in G buffer (5 mM Tris-HCl at pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM DTT added with 6 mM KCl and 60 mM $MgCl_2$) for G-actin or in F buffer (G buffer plus 1 mM ATP) for F-actin. For further stabilization of F-actin, additionally, 120 μM phalloidin was supplemented to the reaction mixture.

NAD-Glycohydrolase Assay. For detection of glycohydrolase activity, the recombinant toxins (1 μM each) were incubated with 200 μM [*adenylate*-^{32}P]NAD in 50 mM HEPES (pH 7.5) for 7 h at 37 °C. Aliquots of the reaction mixture were separated by thin-layer chromatography on TCL aluminum sheets (Silica Gel 60 F_{254}) with 66% 2-propanol and 0.33% ammonium sulfate and analyzed by phosphorimaging.

In Vitro Translation and Separation of ADP-Ribosylated Act88F from Endogenous Actin. In this study, we used the *Drosophila* indirect flight muscle actin (Act88F) as a highly conserved homologue for full-length mammalian actin as described in other studies (*23*). The phosphate-buffered saline (PBS) plasmid encoding the *Drosophila* indirect flight muscle Act88F protein (wild type) was kindly provided by Dr. John C. Sparrow (York, U.K.). For *in vitro* translation, the insert was cloned into a pET21a vector. The Act88F(wt) insert was amplified by PCR with Turbo *Pfu* DNA Polymerase (Stratagene, La Jolla, CA) as described in the manual of the instructor with the primers 5′-*Bam*HI-Act88F (CATGCCGGATCCACCATGTGTGACGATGATGCGGG) and 3′-*Hin*dIII-Act88F (CAGCGGAAGCTTTTAAAAGCATTTGCGGTGAACG). The amplification was done with 25 cycles of denaturing at 94 °C for 30 s, primer annealing at 52 °C for 1 min, and extension at 72 °C for 2 min. The PCR product was then digested with *Bam*HI and *Hin*dIII, ligated with *Bam*HI/*Hin*dIII digested pET21a vector, and transformed into Epicurian XL cells from Stratagene (La Jolla, CA). To prepare the Act(R177Q) mutant, a Quickchange mutagenesis

of the Act88F(wt) plasmid was performed as described in the manual of the instructor, by using the primers 5′-Act88Fwt/R117Q (CCCACGCCATTCTGCAGCTGGATCTGGCTGG) and 3′-Act88Fwt/R177Q (CCAGCCAGATCCAGCTGCAGAATGGCGTGGG). The resulting plasmid was transformed into Epicurian XL cells from Stratagene. The *in vitro* translation was performed at 30 °C for 90 min with the TNT T7 Coupled Reticulocyte Lysate System (Promega, Mannheim, Germany) as described in the manual of the instructor. For control of the translation efficiency, 1 μL of [^{35}S]-L-methionine (Hartmann Analytic, Braunschweig, Germany) was added and the translated protein was analyzed via SDS−PAGE and phosphorimaging. In parallel, the *in vitro* translation was performed without [^{35}S]-L-methionine. Subsequently, an ADP-ribosylation assay was performed in HEPES buffer (50 mM, pH 7.4) containing 2 mM $MgCl_2$, complete protease inhibitor, 100 μM [adenylate-^{32}P]NAD, and 1 μM C2I or C/SpvB in a final volume of 500 μL for 60 min at 37 °C. A 400 μL solution of actin G buffer (pH 7.0) was added, and the samples were incubated with 40 μL pre-equilibrated talon beads (Metal Affinity Resin, Clontech, Palo Alto, CA) for 15 h at 4 °C to bind His-tagged Act88F to the beads. The beads were centrifuged (8000*g* for 3 min at 4 °C) and washed 3 times with 800 μL of equilibration/washing buffer (50 mM Na_3PO_4, 300 mM NaCl, and 0.05% Triton X-100 at pH 7.0). After further centrifugation, 40 μL of Laemmli buffer with DTT was added to the beads and the mixture was heated for 10 min at 95 °C. The supernatant was subjected to SDS−PAGE, and radiolabeled proteins were detected by phosphorimaging.

All experiments were performed at least 3 times, and the results from representative experiments are shown in the figures. Values are given as mean ± standard deviation (SD) ($n \geq 3$).

RESULTS

Substrate Specificity of the Actin-ADP-Ribosylating Toxins C2I, Iota a, and C/SpvB. The recombinant proteins C/SpvB, C2I, and iota a were purified, and their correct molecular weights were confirmed by Coomassie staining following SDS−PAGE (Figure 2A). The proteins were tested for their enzyme activities by *in vitro* ADP-ribosylation of actin with the radioactive cosubstrate [adenylate-^{32}P]NAD. The radioactive ADP-ribosylated actin was detected by SDS−PAGE and subsequent autoradiography (Figure 2B). C2I, iota a, and C/SpvB all harbored ADP-ribosyltransferase activity, and the three enzymes comparably modified actin. Next, the substrate specificities of C/SpvB, C2 toxin, and iota toxin were compared according to the actin isoforms. Therefore, we used highly purified rabbit muscle α-actin and a nonmuscle β/γ-actin mixture from human platelets as substrates for ADP-ribosylation. Each of the three toxins (100 nM final concentration in the assay) was incubated with 1 μM of either α-actin or β/γ-actin and with 200 μM [adenylate-^{32}P]NAD. The amount of ADP-ribosylated actin differed dependent upon the actin isoform and toxin used as an enzyme. C2I, iota a, and C/SpvB all modified nonmuscle β/γ-actin to a comparable extent (Figure 3A). In contrast, there were differences when muscle α-actin was used as a substrate (Figure 3A). Muscle α-actin was not a substrate for C2I, whereas C/SpvB and iota a both modified α-actin to a comparable level of about 20% in comparison to β/γ-actin.

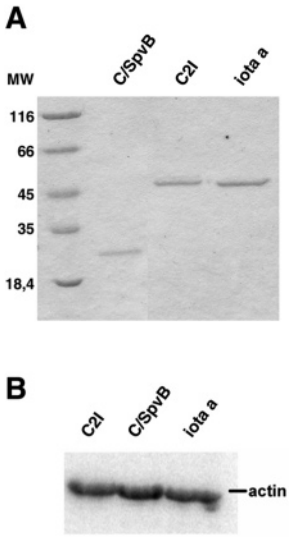


FIGURE 2: Characterization of the recombinant proteins C/SpvB, C2I, and iota a. The purified recombinant toxins (C/SpvB, 24.5 kDa; C2I, 49.4 kDa; iota a, 48 kDa) were analyzed by SDS−PAGE and subsequent Coomassie staining (A). The enzyme activities of the proteins were analyzed by *in vitro* ADP-ribosylation of actin (B). Actin (6 μM), [^{32}P]NAD (200 μM), and the respective toxin (1 μM of each toxin) were incubated, and the radioactively labeled actin was detected by SDS−PAGE and subsequent autoradiography.

It was next tested whether the toxins could ADP-ribosylate G-actin and/or F-actin. For the enzyme assay, G or F buffer was used and, to stabilize the F-actin, phalloidin was added to the F buffer. C/SpvB weakly modified F-actin (Figure 3B), whereas G-actin was a good substrate for ADP-ribosylation. Although the data demonstrate that C/SpvB, C2 toxin, and iota toxin prefer monomeric G-actin as a substrate, these toxins also marginally ADP-ribosylate F-actin.

Kinetics of Toxin-Induced ADP-Ribosylation of Actin. To characterize the maximal modification of actin, the ADP-ribosylation of β/γ-actin was studied with an individual toxin (50 nM) and 150 μM [adenylate-P^{32}PP]NAD for 1−15 min. No significant differences between the toxins were observed for the maximal ADP-ribosylation of actin (Figure 4A). However, at lower concentrations (1 nM iota a, 2.5 nM C2I, and 10 nM C/SpvB), it was obvious that kinetics during the linear phase of the reaction varied for each toxin. When the maximal velocities during the initial phase of ADP-ribosylation were compared, iota a exhibited the highest enzyme activity, followed by C2I and then the C/SpvB fragment. These results were confirmed by the actin modification level of each toxin (Figure 4B).

To study the cosubstrate dependency, we incubated increasing amounts of [adenylate-^{32}P]NAD (0−20 μM) with 6 μM actin and 10 nM C2I or C/SpvB or 5 nM iota a for 8 min at 37 °C. To determine the K_m value for NAD, at least three independent experiments for each toxin were analyzed by using Lineweaver−Burk plots. Comparable K_m values were detected for iota a (3.8 ± 0.2 μM) and C/SpvB (4.4 ± 0.6 μM), while the C2I value was 21.3 ± 1.2 μM (Table 1).

NAD-Glycohydrolase Activity of C2I, Iota a, and C/SpvB. The NAD-glycohydrolase activity of the individual toxins was clearly different. Iota a possessed a 36-fold increase in activity relative to the auto-hydrolysis of NAD after 7 h at 37 °C, while there was also a pronounced 6-fold increase in

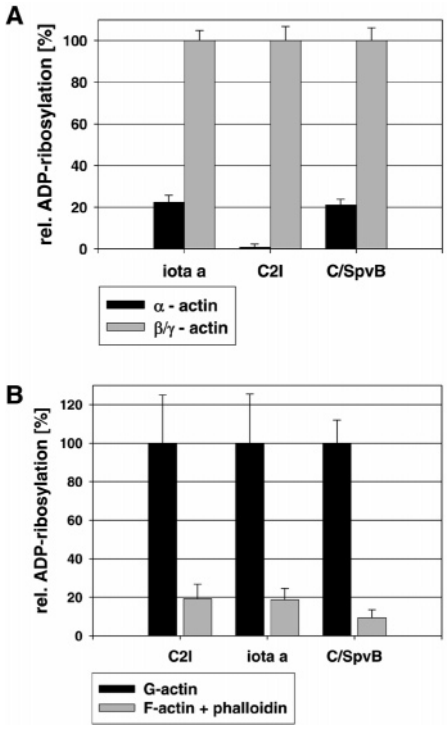
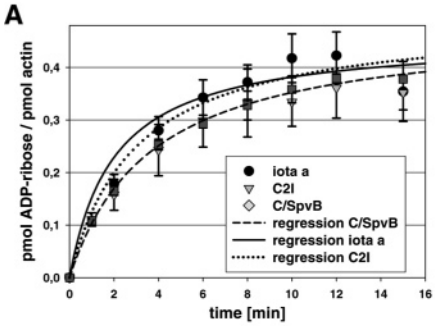


FIGURE 3: Substrate specificity of the actin-ADP-ribosyltransferases iota a, C2I, and C/SpvB. The substrate specificity concerning different actin isoforms for iota a, C2I, and C/SpvB is shown in A. For detection of actin isoform specific ADP-ribosylation, 1 μM of either α-actin or β/γ-actin was incubated with 100 nM of each toxin and 200 μM [^{32}P]NAD for 30 min at 37 °C. For ADP-ribosylation of G- or F-actin, the reaction was performed in G or F buffer containing 200 μM [^{32}P]NAD for 15 min at 37 °C (B). For stabilization of F-actin, phalloidin was added to the F buffer. The final toxin concentrations in the assay were 60 nM. For each toxin, the rate of ADP-ribosylation for β/γ-actin (A) and G-actin (B) was set to 100%. The values in A and B represent means ± SD of three independent measurements.



B

toxin	mol ADP-ribose/ mol actin	actin modification level [min^{-1}]
iota a	0.26 ± 0.05	77.1 ± 4.6
C2I	0.09 ± 0.01	27.8 ± 1.6
C/SpvB	0.04 ± 0.01	13.0 ± 2.2

FIGURE 4: Kinetics of actin-ADP-ribosylation by iota a, C2I, and C/SpvB. Under saturated conditions (50 nM of the respective toxin, 150 μM [^{32}P]NAD, and 6 μM β/γ-actin), no significant difference ($n = 3$) in the time-dependent ADP-ribosylation of actin was detected (A). Results are means ± SD for three independent measurements. With reduced toxin concentrations (iota a, 1 nM; C2I, 2.5 nM; C/SpvB, 10 nM), different kinetics in the linear range were detected after extrapolation to a value of 5 nM ($n = 5$, $t = 4$ min, shown in B). The *actin modification levels* were calculated at the actual toxin concentrations as mentioned above. The calculation was done at all time points of the linear phase: *actin modification level* = amount of modified actin/amount of toxin × min^{-1}. The values presented in A and B were means ± SD of three independent measurements.

NAD-glycohydrolase activity for C2I (parts A and B of Figure 5). In contrast, no significant NAD-glycohydrolase activity was detected for the C/SpvB protein. In parallel, we also tested the toxins for ADP-ribosylation of actin to confirm that the toxins, which had been included in the NAD-glycohydrolase assay, all harbored enzyme activity. Therefore, aliquots from the NAD-glycohydrolase assay solutions were removed after the 7 h incubation period and analyzed for ADP-ribosylation of actin as described above. Taken together, C/SpvB, C2I, and iota a ADP-ribosylated actin in a comparable manner (data not shown).

Influence of pH on ADP-Ribosylation of Actin by C2I, Iota a, and C/SpvB. The pH dependency of the enzyme activity was studied by *in vitro* ADP-ribosylation of 20 nM toxin and 4 μM β/γ-actin. The enzyme activities for all toxins were tested at pH values of 4−10 (Figure 5C). The pH optimum for C2I and C/SpvB was between pH 7−8. A comparable enzyme activity for iota a was detected in a pH range from 6 to 10. In contrast, decreased enzyme activities were measured for C2I and C/SpvB at pH values of 9 and 10. When compared to C2I and iota a, the C/SpvB enzyme activity was more sensitive to low (≤6) and high (≥9) pH values.

Arginine-177 Is the Modification Site for C/SpvB in the Recombinant Drosophila Actin Homologue Act88F. To identify the acceptor amino acid for ADP-ribosylation by SpvB, we used the actin homologue Act88F, which is a *Drosophila* indirect flight muscle protein, as a substrate. The *in vitro* translated proteins Act88F wt and Act88F R177Q were subjected to *in vitro* ADP-ribosylation by C2I or C/SpvB. To verify the amount of the translated proteins Act88F wt and Act88F R177Q, we performed in parallel an *in vitro* translation containing [^{35}S]-L-methionine and detected the translated radiolabeled proteins by autoradiography. Both proteins were translated in comparable amounts (Figure 6A) and subsequently used for ADP-ribosylation experiments. The nonradioactive proteins Act88F wt and Act88F R177Q were incubated with either C2I or C/SpvB in the presence of radioactive cosubstrate [adenylate-^{32}P]NAD. The ADP-ribosylated Act88F proteins were detected by autoradiography (Figure 6B). Both C2I and C/SpvB strongly ADP-ribosylated the Act88F wt protein, but only a marginal signal was detectable when the Act88F R177Q protein was used as a substrate for the toxins, indicating that arginine-177

Table 1: ADP-Ribosylation of Actin with *C. botulinum* C2I, *C. perfringens* Iota a, or *S. enterica* C/SpvB: Determination of the K_m Values for NAD[a]

toxin	K_m of NAD (μM)
iota a	3.8 ± 0.2
C2I	21.3 ± 1.2
C/SpvB	4.4 ± 0.6

[a] For determination of the K_m values, actin (6 μM) was ADP-ribosylated in the presence of 10 nM C/SpvB or C2I and 5 nM Iota a with increasing amounts of NAD (0−20 μM) for 8 min at 37 °C. The data were analyzed by using Lineweaver−Burk plots ($n = 3$).

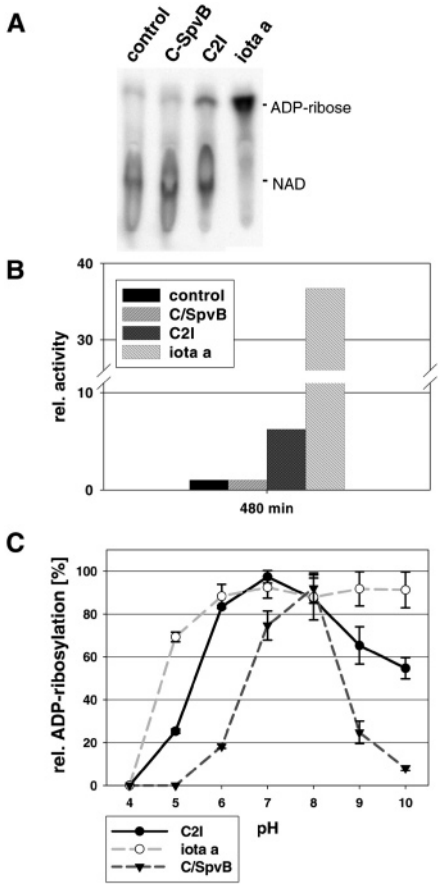


FIGURE 5: NAD-glycohydrolase activity and pH-dependent ADP-ribosylation of actin by C2I, iota a, and C/SpvB. For detection of glycohydrolase activity, the recombinant toxins were incubated with [^{32}P]NAD in 50 mM HEPES buffer at 37 °C for 7 h. The cleaved ADP-ribose moiety was separated form NAD by thin-layer chromatography (A). The control lane without any toxin shows the low basal auto-hydrolysis of NAD. Quantification of the radiolabeled ADP-ribose is shown in B. The pH-dependent ADP-ribosylation (pH values of 4−10) was measured with 4 μM β/γ-actin, 150 μM [^{32}P]NAD, and 20 nM of each individual toxin (C). The maximal enzyme activity (pH optimum) for each toxin was set to 100%. Values are means ± SD of three independent measurements.

represents the modification site for ADP-ribosylation by C2I and C/SpvB.

DISCUSSION

Various bacteria produce and secrete toxins, which attack eukaryotic cells by ADP-ribosylation of actin. This leads to a depolymerization of actin filaments and finally to a complete destruction of the cytoskeleton. Until recently, all known bacterial actin-ADP-ribosyltransferases revealed a binary form of organization consisting of two separate proteins, an enzyme component and a binding/translocation component. The family of clostridial actin-ADP-ribosylating toxins comprises the *C. botulinum* C2 toxin (*24*) and the iota-like toxins consisting of the *C. perfringens* iota toxin (*25*), *C. difficile* CDT (*26*, *27*), and *C. spiroforme* toxin (*28*). Besides the clostridial toxins, there is the binary ADP-ribosyltransferase VIP (vegetative insecticidal protein) from *Bacillus cereus*. Recently, a new actin-ADP-ribosylating protein was identified in *S. enterica*. The SpvB protein is essential for the intracellular growth of *Salmonella* in macrophages and therefore for the pathogenic effects of this bacterium (*14*, *29*, *30*). The catalytic site of SpvB shares

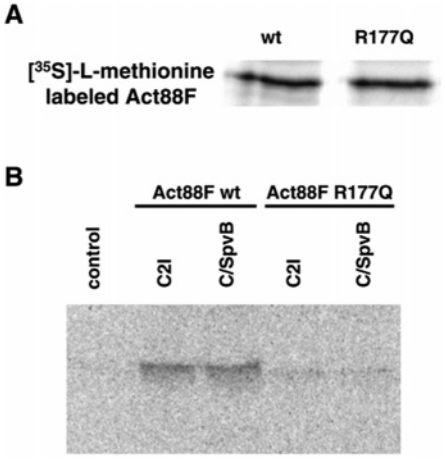


FIGURE 6: ADP-ribosylation of Act88F wt and Act88F R177Q by C2I and C/SpvB. Recombinant His-tagged Act88F proteins (wt and R177Q) were expressed using the *in vitro* reticulocyte translation system. To control the translation efficiency for both Act88F proteins, the translation reaction was done in parallel with [^{35}S]-L-methionine. (A) Radiolabeled Act88F proteins were detected by autoradiography. (B) To test Act88F proteins as substrates for C2I and C/SpvB, *in vitro* translated nonradioactive Act88F proteins (wt and R177Q) were used for ADP-ribosylation with radioactive [^{32}P]-NAD and the enzymes C2I and C/SpvB. Subsequently, His-tagged Act88F was purified from reticulocyte actin by affinity chromatography (talon beads). The [^{32}P]-ADP-ribosylated Act88F wt and Act88F R177Q proteins were detected by SDS−PAGE and subsequent autoradiography, which is shown in B.

homology to the clostridial actin-ADP-ribosylating toxins and contains amino acid residues that are conserved within all arginine-specific ADP-ribosyltransferases (*12*). Interestingly, the N-terminal domain of SpvB does not share any homology to the enzymatic components of the known binary toxins. Moreover, no binding component was identified, thus leading to the conclusion that SpvB is not a binary toxin. It was supposed that SpvB is translocated by a type-III secretion system directly from the bacteria, which are located within cellular vesicles, into the cytosol of host cells (*13*).

To study whether SpvB is related to the C2 or iota-like toxins, with respect to ADP-ribosylation of actin, we analyzed the biochemical properties of the catalytic domain of SpvB (C/SpvB) and compared this enzyme with others like C2I from *C. botulinum* and iota a from *C. perfringens*. Moreover, we identified the modification site for SpvB as arginine-177 in the actin homologue Act88F protein from *Drosophila*. Until this study, the acceptor amino acid for ADP-ribosylation by SpvB was not identified by any group. Arginine-177 is the modification site for all actin-ADP-ribosylating toxins, which have been characterized by date. The C/SpvB protein consists of 217 amino acids and represents the catalytic domain of the full-length SpvB protein (Figure 1A). It harbors a conserved EXE motif in its catalytic domain (Figure 1B), which is conserved within the family of actin-ADP-ribosyltransferases and determines the acceptor amino acid specificity for arginine in other ADP-ribosyltransferases (*12*). We used the actin homologue of *Drosophila* indirect flight muscle protein Act88F wt and the R177Q mutant to identify the modification site for C/SpvB. These proteins can be recombinantly expressed, and exchanges of amino acid residues are readily performed. To exclude the possibility that the exchange of R177Q in the Act88F protein leads to conformational changes, we per-

Table 2: Overview of the Biochemical Properties of the Actin-ADP-Ribosyltransferases *S. enterica* C/SpvB, *C. botulinum* C2 Toxin, and *C. perfringens* Iota Toxin

	C/SpvB	C2 toxin	iota toxin
bacteria	*Salmonella enterica*	*Clostridium botulinum*	*Clostridium perfringens*
toxin type	type-III-secreted toxin (?), no binding component identified	binary toxin; enzyme component C2I and binding/translocation component C2IIa	binary toxin; enzyme component Ia and binding/translocation component Ib
cellular uptake	via type-III secretion system (?)	via binding/translocation component C2II (receptor-mediated endocytosis) translocation from acidic endosomes into cytosol	via binding/translocation component lota B (receptor-mediated endocytosis), translocation from acidic endosomes into cytosol
substrate specificity	nonmuscle (β/γ) and muscle (α) G-actin	only nonmuscle (β/γ) G-actin	nonmuscle (β/γ) and muscle (α) G-actin
glycohydrolase activity	−	+	++
acceptor amino acid on Aet88F	Arg 177	Arg 177	Arg 177
actin modification level (min^{-1})	13.0 ± 2.2	27.8 ± 1.6	77.1 ± 4.6
K_m value for NAD	4.4 ± 0.6	21.3 ± 1.2	3.8 ± 0.2

formed an *in vitro* protease digest assay with Act88F wt and Act88F R177Q. The proteins were incubated with either trypsin or V8 protease, and the resulting digest patterns were analyzed by SDS−PAGE and subsequent Coomassie staining (data not shown). No differences were observed between Act88F wt and Act88F R177Q, thus suggesting that significant conformational changes were not induced by residue exchange at arginine-177. Moreover, Act88F wt and Act88F R177Q polymerized similarly under *in vitro* conditions. This confirms a correct conformation for the Act88F R177Q protein (data not shown). Either C/SpvB or C2I (*23*) modified Act88F. In contrast, only a marginal signal was detected for either toxin when the Act88F R177Q protein was used as a substrate. This result strongly suggests that Act88F is ADP-ribosylated at arginine-177 by SpvB. This specific arginine residue is the major modification site for SpvB, as it is for other binary actin-ADP-ribosylating toxins. The weak radioactive labeling of Act88F R177Q most likely results from remaining endogenous actin that was bound nonspecifically to the talon beads during the purification step of the Act88F R177Q protein from cell lysate.

All known bacterial, actin-ADP-ribosylating toxins block the polymerization of actin, ultimately leading to a fatal collapse of the microfilament network. Thereby, the toxins turn G-actin into a capping protein that binds to the barbed ends of actin filaments and inhibit further polymerization (*31*). For both C2I (*24*) and iota a (*32*), G-actin is the major substrate, while polymerized F-actin is only poorly ADP-ribosylated. This agrees with our findings that C/SpvB also modifies G-actin with minimal alterations made upon F-actin.

Most likely the remaining ADP-ribosylation of F-actin (10−20%) is due to the fact that nonpolymerized G-actin is still available under these assay conditions, thus resulting in a background signal for ADP-ribosylation of F-actin. Nonmuscle actin isoforms (β and γ) are the preferred substrates for the C2 and iota toxins (*24*, *33*). However, only iota toxin has been described to modify also α-actin (*32*). Concerning the modification of the actin isoforms, our data show that C/SpvB behaves like iota toxin.

As described previously by Otto et al. (*12*), we were not able to detect any NAD-glycohydrolase activity for C/SpvB. In contrast, iota a and C2I both possess significant NADase activities. As reported earlier by Nagahama et al. (*34*), we also identified a similar K_m value of 3.8 ± 0.2 μM for iota a, and in this current study, we discovered similar results for C/SpvB (4.4 ± 0.6 μM). These findings however are in contrast to earlier reports for C2I by Aktories et al. (*35*), with a determined K_m value of 21.3 ± 1.2 μM. This disagreement might be due to the fact that for the present work highly purified actin was used instead of cell lysate protein.

In our hands, iota a and C2I exhibited ADP-ribosyltransferase activity in a pH range from 5 to 10. Iota a was active at higher pH values than C2I, and this was most likely linked to protein stability. C/SpvB exhibited significant activities at pH values from 6 to 9. The enhanced sensitivity of C/SpvB to pH could be due to the fact that the recombinant C/SpvB protein represents only one domain of the full-length SpvB protein, and therefore, the C/SpvB protein could be more sensitive to unfolding by acidic pH when compared to the full-length versions of SpvB, C2I, and iota a.

Taken altogether, C2I, iota a, and C/SpvB ADP-ribosylate actin that ultimately cause comparable ill effects upon the cytoskeleton of eukaryotic cells. C/SpvB shares arginine-177 as the modification site on the actin molecule with all known family members of the binary actin-ADP-ribosylating toxins various *Bacillus* and *Clostridium* species. However, the toxins differ concerning specific biochemical properties (for an overview, see Table 2).

ACKNOWLEDGMENT

We thank Dr. Friedrich Koch-Nolte (Hamburg, Germany) and Dr. John Sparrow (York, U.K.) for providing *Salmonella* C/SpvB cDNA and *Drosophila* indirect flight muscle protein Act88F cDNA, respectively. We also thank Peter Gebhardt and Brigitte Neufang for technical assistance. We thank Dr. Bradley G. Stiles (USAMRIID, Fort Detrick, MD) for semantic help with the English.

REFERENCES

1. Rodriguez, M., de, D., I, and Mendoza, M. C. (1998) Extraintestinal salmonellosis in a general hospital (1991 to 1996): Relationships between *Salmonella* genomic groups and clinical presentations, *J. Clin. Microbiol. 36*, 3291−3296.
2. Fierer, J., and Guiney, D. G. (2001) Diverse virulence traits underlying different clinical outcomes of *Salmonella* infection, *J. Clin. Invest 107*, 775−780.
3. Libby, S. J., Adams, L. G., Ficht, T. A., Allen, C., Whitford, H. A., Buchmeier, N. A., Bossie, S., and Guiney, D. G. (1997) The spv genes on the *Salmonella* dublin virulence plasmid are required for severe enteritis and systemic infection in the natural host, *Infect. Immun. 65*, 1786−1792.

4. Gulig, P. A., Danbara, H., Guiney, D. G., Lax, A. J., Norel, F., and Rhen, M. (1993) Molecular analysis of spv virulence genes of the *Salmonella* virulence plasmids, *Mol. Microbiol. 7*, 825−830.
5. Gulig, P. A., and Curtiss, R., III (1988) Cloning and transposon insertion mutagenesis of virulence genes of the 100-kb plasmid of *Salmonella typhimurium*, *Infect. Immun. 56*, 3262−3271.
6. Fang, F. C., Krause, M., Roudier, C., Fierer, J., and Guiney, D. G. (1991) Growth regulation of a *Salmonella* plasmid gene essential for virulence, *J. Bacteriol. 173*, 6783−6789.
7. Grob, P., and Guiney, D. G. (1996) *In vitro* binding of the *Salmonella* dublin virulence plasmid regulatory protein SpvR to the promoter regions of spvA and spvR, *J. Bacteriol. 178*, 1813−1820.
8. Grob, P., Kahn, D., and Guiney, D. G. (1997) Mutational characterization of promoter regions recognized by the *Salmonella* dublin virulence plasmid regulatory protein SpvR, *J. Bacteriol. 179*, 5398−5406.
9. Guiney, D. G., Libby, S., Fang, F. C., Krause, M., and Fierer, J. (1995) Growth-phase regulation of plasmid virulence genes in *Salmonella*, *Trends Microbiol. 3*, 275−279.
10. Fierer, J., Eckmann, L., Fang, F., Pfeifer, C., Finlay, B. B., and Guiney, D. (1993) Expression of the *Salmonella* virulence plasmid gene spvB in cultured macrophages and nonphagocytic cells, *Infect. Immun. 61*, 5231−5236.
11. Rhen, M., Riikonen, P., and Taira, S. (1993) Transcriptional regulation of *Salmonella enterica* virulence plasmid genes in cultured macrophages, *Mol. Microbiol. 10*, 45−56.
12. Otto, H., Tezcan-Merdol, D., Girisch, R., Haag, F., Rhen, M., and Koch-Nolte, F. (2000) The spvB gene-product of the *Salmonella enterica* virulence plasmid is a mono(ADP-ribosyl)transferase, *Mol. Microbiol. 37*, 1106−1115.
13. Tezcan-Merdol, D., Nyman, T., Lindberg, U., Haag, F., Koch-Nolte, F., and Rhen, M. (2001) Actin is ADP-ribosylated by the *Salmonella enterica* virulence-associated protein SpvB, *Mol. Microbiol. 39*, 606−619.
14. Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F. C., and Holden, D. W. (1998) Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages, *Mol. Microbiol. 30*, 163−174.
15. Ochman, H., Soncini, F. C., Solomon, F., and Groisman, E. A. (1996) Identification of a pathogenicity island required for *Salmonella* survival in host cells, *Proc. Natl. Acad. Sci. U.S.A. 93*, 7800−7804.
16. Gotoh, H., Okada, N., Kim, Y. G., Shiraishi, K., Hirami, N., Haneda, T., Kurita, A., Kikuchi, Y., and Danbara, H. (2003) Extracellular secretion of the virulence plasmid-encoded ADP-ribosyltransferase SpvB in *Salmonella*, *Microb. Pathog. 34*, 227−238.
17. Pizza, M., Masignani, M., and Rappuoli, R. (1999) Molecular, functional and evolutionary aspects of ADP-ribosylating toxins, in *The Comprehensive Sourcebook of Bacterial Protein Toxins* (Freer, J. H., and Alouf, J. E.) pp 45−72, London, U.K.
18. Barth, H., Blöcker, D., Behlke, J., Bergsma-Schutter, W., Brisson, A., Benz, R., and Aktories, K. (2000) Cellular uptake of *Clostridium botulinum* C2 toxin requires oligomerization and acidification, *J. Biol. Chem. 275*, 18704−18711.
19. Haug, G., Leemhuis, J., Tiemann, D., Meyer, D. K., Aktories, K., and Barth, H. (2003) The host cell chaperone Hsp90 is essential for translocation of the binary *Clostridium botulinum* C2 toxin into the cytosol, *J. Biol. Chem. 278*, 32266−32274.
20. Barth, H., Roebling, R., Fritz, M., and Aktories, K. (2002) The binary *Clostridium botulinum* C2 toxin as a protein delivery system: Identification of the minimal protein region necessary for interaction of toxin components, *J. Biol. Chem. 277*, 5074−5081.
21. Blöcker, D., Behlke, J., Aktories, K., and Barth, H. (2001) Cellular uptake of the binary *Clostridium perfringens* iota-toxin, *Infect. Immun. 69*, 2980−2987.
22. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
23. Just, I., Hennessey, E. S., Drummond, D. R., Aktories, K., and Sparrow, J. C. (1993) ADP-ribosylation of *Drosophila* indirect flight muscle actin and arthrin by *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin, *Biochem. J. 291*, 409−412.
24. Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K. H., and Habermann, E. (1986) Botulinum C2 toxin ADP-ribosylates actin, *Nature 322*, 390−392.
25. Stiles, B. G., and Wilkens, T. D. (1986) Purification and characterization of *Clostridium perfringens* iota toxin: Dependence on two nonlinked proteins for biological activity, *Infect. Immun. 54*, 683−688.
26. Gülke, I., Pfeifer, G., Liese, J., Fritz, M., Hofmann, F., Aktories, K., and Barth, H. (2001) Characterization of the enzymatic component of the ADP-ribosyltransferase toxin CDTa from *Clostridium difficile*, *Infect. Immun. 69*, 6004−6011.
27. Popoff, M. R., Rubin, E. J., Gill, D. M., and Boquet, P. (1988) Actin-specific ADP-ribosyltransferase produced by a clostridium difficile strain, *Infect. Immun. 56*, 2299−2306.
28. Popoff, M. R., and Boquet, P. (1988) Clostridium spiroforme toxin is a binary toxin which ADP-ribosylates cellular actin, *Biochem. Biophys. Res. Commun. 152*, 1361−1368.
29. Uchiya, K., Barbieri, M. A., Funato, K., Shah, A. H., Stahl, P. D., and Groisman, E. A. (1999) A *Salmonella* virulence protein that inhibits cellular trafficking, *EMBO J. 18*, 3924−3933.
30. Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., Mastroeni, P., and Fang, F. C. (2000) *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase, *Science 287*, 1655−1658.
31. Aktories, K., and Wegner, A. (1989) ADP-ribosylation of actin by clostridial toxins, *J. Cell Biol. 109*, 1385−1387.
32. Schering, B., Bärmann, M., Chhatwal, G. S., Geipel, U., and Aktories, K. (1988) ADP-ribosylation of skeletal muscle and non-muscle actin by *Clostridium perfringens* iota toxin, *Eur. J. Biochem. 171*, 225−229.
33. Vandekerckhove, J., Schering, B., Bärmann, M., and Aktories, K. (1988) Botulinum C2 toxin ADP-ribosylates cytoplasmic β/g-actin in arginine 177, *J. Biol. Chem. 263*, 696−700.
34. Nagahama, M., Sakaguchi, Y., Kobayashi, K., Ochi, S., and Sakurai, J. (2000) Characterization of the enzymatic component of *Clostridium perfringens* iota-toxin, *J. Bacteriol. 182*, 2096−2103.
35. Aktories, K., Ankenbauer, T., Schering, B., and Jakobs, K. H. (1986) ADP-ribosylation of platelet actin by botulinum C2 toxin, *Eur. J. Biochem. 161*, 155−162.

BI051810W