Real-time imaging of type III secretion: Salmonella SipA injection into host cells

Markus C. Schlumberger, Andreas J. Müller, Kristin Ehrbar, Brit Winnen, Iwan Duss, Bärbel Stecher, and Wolf-DietrichHardt*

Institute of Microbiology, Eidgenössische Technische Hochschule, Wolfgang-Pauli-Strasse 10, CH-8093 Zürich, Switzerland

Edited by John J. Mekalanos, Harvard Medical School, Boston, MA, and approved July 8, 2005 (received for review April 25, 2005)

Many pathogenic and symbiotic Gram-negative bacteria employ type III secretion systems to inject "effector" proteins into eukaryotic host cells. These effectors manipulate signaling pathways to initiate symbiosis or disease. By using time-lapse microscopy, we have imaged delivery of the Salmonella type III effector protein SipA/SppA into animal cells in real time. SipA delivery mostly began 10–90 sec after docking and proceeded for 100–600 sec until the bacterial SipA pool (6 ± 3 × 103 molecules) was exhausted. Similar observations were made for the effector protein SopE. This visualization of type III secretion in real time explains the efficiency of host cell manipulation by means of this virulence system.

type III effector protein | time-lapse microscopy

Type III secretion systems are of great importance in many pathogenic and symbiotic Gram-negative bacteria (1). These complex protein transport organelles are thought to function as molecular syringes and deliver bacterial "effector" proteins directly into eukaryotic host cells. Inside the host cell, effector proteins manipulate signaling pathways to establish a niche for symbiosis or disease (1). Efficient effector delivery and swift host cell invasion is crucial for the outcome of the bacteria–host cell interaction. However, the actual process of effector protein delivery is poorly characterized. To understand host cell manipulation, it is important to study type III effector protein injection in real time and to determine its kinetic parameters at the single-cell level.

The type III secretion system (TTSS) of Salmonella enterica serovar typhimurium (S. typhimurium) encoded in a chromosomal region called Salmonella pathogenicity island 1 (SPI-1 TTSS) serves as a paradigm for TTSS function (2). SPI-1 TTSS delivers a mixture of at least 12 effector proteins that induce responses like inflammation, membrane ruffling, and host cell invasion. For kinetic analysis of type III secretion, we have chosen the SPI-1 TTSS effector protein SipA, which plays a key role in mediating intestinal inflammation (3, 4) and is well characterized in its properties to bind and modulate the host actin cytoskeleton (5–7). SipA accumulates in the vicinity of the invading bacteria (5–10), which should allow sensitive detection of injected SipA by fluorescence microscopy techniques. In this study, we have analyzed the TTSS-mediated injection of SipA into host cells in real time by using time-lapse microscopy.

Materials and Methods

Bacterial Strains and Plasmids. pGFP–InvB was constructed by fusing via XhoI/BamH1 the 3′ end of the EGFP gene (pEGFP-actin, Clontech) to invB (PCR product; primers: 5′-CCGGTC-GAGCTATGCAACATTGGATATTACG-3′ and 5′-CGGGATCTCCTCTCATTAGGCGACCAGC-3′).

The mRFP1 expression plasmid pM1103 was constructed by PCR amplification of mRFP1 [template: mRFP1 in pRSETb (11); primers: 5′-CGCTAGCTGACAACAGGATATTACG-3′ and 5′-CGGGATCTCCTCTCATTAGGCGACCAGC-3′] and cloning via NheI/BamH1 mRFP1 into the backbone of plasmid EGFP-actin (Clontech).

S. typhimurium M1300 (SL1344, sipA45) was constructed by chromosomal integration of a suicide plasmid (tetR) encoding the C-terminal region of SipA fused to the M45 epitope (MDRSRDLRPFETETIRL) (12). M1301 (SL1344, sopE45) was constructed by chromosomal epitope tagging of sopE with the hemagglutinin (HA) epitope (YPDYDPDYA) as described in ref. 13. M1304 (SL1344, ΔsopABEE2, and sipAM45), M1301 (SL1344, ΔinvG, and sipAM45), and M1222 (SL1344, sipAM45, and sopE45) were obtained by phage transduction of sipAM45 from M1300 into M712 (ΔsopAΔsopABEE2) (12), SB161 (14), or M1001, respectively. Thus, M1300, M1304, M1301, and M1222 harbored a chromosomal M45-tagged version of SipA, and M1222 also carried a HA-tagged version of sopE. The strains were verified by Western blot and PCR. For infections, bacteria were grown in LB medium (0.3 M NaCl) for 12 h at 37°C and subcultured for 4 h in the same medium (SPI-1-inducing conditions).

Lysozyme Treatment. For immunostaining of effector proteins in the bacterial cytosol (see Figs. 3D and 4), S. typhimurium immobilized on gelatin-coated cover slips (no host cell contact) or infection assays were fixed for 20 min at 22°C in 4% paraformaldehyde/4% sucrose/PBS, incubated in 20% sucrose in PBS for 10 min at 22°C, permeabilized with buffer A (50 mM EDTA/20 mM Tris-HCl/1.8 g/liter glucose/0.1% Triton X-100, pH 8) for 5 min at 22°C, washed three times in buffer B (10 mM EDTA/25 mM Tris-HCl/1.8 g/liter glucose, pH 8) at 22°C, and incubated for 1 h in buffer B plus 5 g/liter lysozyme at 4°C. Afterward, immunostaining was performed as described below.

Immunofluorescence Microscopy. Cos7 cells were seeded onto glass coverslips in DMEM plus 5% FCS (PAA, Linz, Austria), transfected with PolyFect (Qiagen, Valencia, CA), and grown to 70–80% confluency. Cells were infected for the indicated time with S. typhimurium at a multiplicity of infection of 25 bacteria per cell, fixed with 4% paraformaldehyde in PBS/4% sucrose for 20 min at 22°C, and permeabilized in 0.1% Triton X-100 for 5 min (see Figs. 1C and D, 2, and 3A). S. typhimurium was stained with a rabbit anti-Salmonella O5,4,5,12 (8) antiserum (Difco) and goat anti-rabbit-FITC (Fig. 1), anti-rabbit-7-amino-4-methylcoumarin-3-acetic acid (see Fig. 2) or anti-rabbit-horseradish (see Figs. 3D and 4B and C) conjugate. SipA45 and/or SopE45 were stained with monoclonal mouse anti-M45 [kindly provided by P. Hearing (State University of New York, Stony Brook)] or rabbit anti-HA (Santa Cruz Biotechnology) and goat anti-mouse-Cy3 (see Figs. 1, 3A, and 4) or anti-mouse-Cy5 (see Figs. 2 and 3D) and/or goat e-rabbit-FITC (see Fig. 4D and E) conjugates (Jackson ImmunoResearch). F-actin filaments were stained with tetramethylrhodamine B isothiocyanate–phallolidin (Sigma). To determine bacterial intracellular/
extracellular localization (see Fig. 1C), bacteria were stained with rabbit anti-Salmonella O-1,4,5,12 (8) antiserum and goat anti-rabbit-FITC before permeabilization. Subsequently, all bacteria were stained again but with a goat anti-rabbit-7-amino-4-methylcoumarin-3-acetic acid conjugate.

Images were taken with a confocal system (PerkinElmer/Zeiss; see below). To localize SipA at high resolution, confocal image stacks (0.2-μm focal plane distance) were acquired in the green (bacteria) and red (SipAM45) channel with a Plan Neofluar ×100 oil objective, an aperture setting of 1.3, and a ×1.6 Optivar (Zeiss). Three-dimensional reconstructions were built by using Volocity 2.6.1 software (Improvision, Conventry, U.K.).

**Time-Lapse Microscopy.** pGFP–InvB-transfected Cos7 cells on glass-bottom dishes (MatTek, Ashland, MA) were transferred into Hank’s balanced salt solution (GIBCO) at 37°C, mounted onto a heated specimen holder (37°C) on a Zeiss Axiovert 200m inverted microscope, and infected (multiplicity of infection = 25). Time series were recorded with an Ultraview confocal head (PerkinElmer), a krypton argon laser (643-RYB-A01, Melles Griot, Didam, The Netherlands) and a Plan Neofluar ×63 oil Ph3 objective with an aperture setting of 1.25 (Zeiss). At each time point, a stack of five fluorescent images (0.5-μm focal plane distance) and a single phase contrast image at the z position of the third image slice (focused on the edge of a transfected cell) was recorded. An additive projection of the GFP fluorescence signals was used for evaluation. An 8-sec delay was inserted after recording each image stack, leading to intervals of 16–25 sec between imaged time points, depending on the exact settings of image acquisition. After imaging, cells were fixed and stained as described above.

For comparison of independent experiments, the background-corrected fluorescence signal was normalized as described in ref. 15 by using rotavirus-like particles containing 120 copies of a chimerical GFP-VP2 capsid protein (16) for calibration (for details, see Supporting Materials and Methods).

To assay depletion of intrabacterial effectors during the infection, time-lapse microscopy was modified as follows: The infection (multiplicity of infection = 25) of Cos7 cells infected for 30 min with wild-type S. typhimurium (M1300) or with the noninvasive strain M1304. Bacteria were stained before (green) and after (blue) permeabilization of the Cos7 cells to distinguish intracellular (blue) and extracellular (cyan in merge) bacteria. (D) Three-dimensional reconstruction of confocal image stacks showing SipA (red) delivered by individual bacteria (green; anti-LPS) of S. typhimurium strain M1300 or M1304 into a Cos7 cell 10 min after infection.

**Results and Discussion.**

SipA is highly expressed by S. typhimurium population (6 ± 3 × 10^7 SipA molecules per expressing bacterium) (Fig. 1 A and B; see also Materials and Methods, Fig. 5, which is published as supporting information on the PNAS web site, and Supporting Materials and Methods).

When tissue culture cells were infected with wild-type S. typhimurium (M1300) (see Materials and Methods), SipA formed 1–15 small foci (median = 5) next to the internalized bacteria (Figs. 1 C and D and 5F). SipA deposition required a functional SPI-1 TTSS and host cell contact (M1301) (Fig. 1C and Fig. 6, which is published as supporting information on the PNAS web site). Additional experiments were performed with the S. typhimurium strain M1304 (ΔsopABEE2) (see Materials and Methods), which injects SipA but lacks the key effector proteins mediating membrane ruffling and host cell invasion. M1304 docked to host cells, remained extracellular and did not induce membrane ruffling. SipA injected by M1304 formed often one (sometimes several) focus of larger size (Figs. 1 C and D and 5F). We reasoned that the formation of one larger SipA focus and the...
lack of membrane ruffling might help to set up sensitive fluorescence microscopy assays for measuring SipA arrival in the host cell.

We used two complementary assays to analyze SipA injection into host cells by time-lapse video microscopy: One assay detects the arrival of SipA in the host cell cytosol in real time, the second assay measures SipA depletion from the bacterial cytosol.

For live imaging of SipA injection, we required a probe highlighting SipA upon arrival in the host cell cytosol. This probe was derived from the *S. typhimurium* protein InvB. InvB is a 6-kDa type III secretion chaperone for SipA (17) that binds to SipA in the bacterial cytosol. InvB is required for type III secretion of SipA, but it is not delivered into the host cell. The equilibrium binding constant of the SipA GST–InvB complex was in the range of 10 nM ($K_D = k_{off}/k_{on}$; $k_{on} = 2 \pm 1 \times 10^7$ M$^{-1}$sec$^{-1}$ and $k_{off} = 2 \times 10^{-5}$ sec$^{-1}$) (Fig. 7, which is published as supporting information on the PNAS web site). This binding matches the affinity of many antibody–antigen interactions and indicated that a GFP–InvB probe could stably bind SipA in the host cell cytosol (Fig. 2A). Thus, we constructed a plasmid for a fusion between EGFP and the N terminus of InvB (pGFP–InvB). GFP–InvB was evenly distributed in the cytoplasm of transfected Cos7 cells (Fig. 2B). Nuclear staining was also observed. The distribution of GFP–InvB did not change upon infection with a *S. typhimurium* strain lacking SipA or a functional SPI-1 TTSS [M712, ∆sipAasopABEE2 (12); M1301, ∆invG] (Fig. 2C and F). In contrast, infection with *S. typhimurium* M1304 resulted in recruitment of GFP–InvB to the sites of bacteria–host cell contact (Fig. 2D). Immunostaining confirmed that GFP–InvB was recruited to the SipA foci (Fig. 2D), and control experiments with Cos7 cells coexpressing GFP–InvB and mRFP-1 confirmed that GFP–InvB recruitment was solely attributable to SipA injection (Fig. 2E).

SipA and the recruited GFP–InvB colocalized with F-actin (Fig. 2D), which is in line with the actin binding and polymerizing properties of SipA (5, 18–20). SipA focus formation and GFP–InvB recruitment were cytochalasin D–insensitive (data not shown), and actin remodeling per se did not redistribute GFP–InvB (RhoAV14/F2SN or Rac1V12 expressing cells) (Fig. 8, which is published as supporting information on the PNAS web site). Thus, binding of the GFP–InvB reporter to SipA did not interfere with SipA function and provides a useful assay for live imaging of SipA injection into host cells.

To study SipA injection into host cells we have imaged GFP–InvB recruitment by time-lapse confocal microscopy. Cos7 cells expressing GFP–InvB were infected with *S. typhimurium* M1304. To monitor bacterial docking and GFP–InvB recruitment, we recorded one phase-contrast image and a stack of confocal GFP fluorescence images at each time point in 16- to 25-sec intervals (Fig. 3A Center; see Movie 1, which is published as supporting information on the PNAS web site). The fluorescence images were analyzed to quantify GFP–InvB recruitment (Fig. 3A; see Materials and Methods and Fig. 9, which is published as supporting information on the PNAS web site).

Fig. 3A shows a typical time course. Once bacteria had docked to the cells they stayed on for the rest of the experiment (Fig. 3A, arrows). In these cases GFP–InvB recruitment was observed shortly upon docking (Fig. 3A, arrow in GFP channel). After imaging, the cells were fixed immediately, and SipA was immunostained, verifying the specificity of GFP–InvB recruitment to SipA foci (Fig. 3A Right).

Beginning of GFP–InvB recruitment could frequently be detected 16–90 sec (median = 54 sec) after bacterial docking (Fig. 3B). Generally, the recruitment followed a linear time course. From the slopes of this linear phase of GFP–InvB recruitment we estimated the individual rates of SipA injection by single bacteria (Fig. 3C, right scale). Later on, GFP–InvB recruitment leveled off in a plateau (Fig. 3C). In most cases, 100–600 sec were sufficient to reach the plateau of GFP–InvB recruitment (Fig. 3C, curves 1–7 and 10). At this stage, SipA was no longer detected inside the bacterial cytosol (Fig. 3D). Thus, the plateau was attributable to exhaustion of the intrabacterial SipA pool, and the levels of the individual plateaus may reflect
In conclusion, M1304 bacteria harbor \( \approx 1 \times 10^3 \) SipA molecules before host cell contact (Fig. 1A and B) and require \( \approx 100–600 \) sec to deliver this cargo into the host cell, which roughly corresponds to translocation rates between 7 and 60 molecules per sec (see Supporting Materials and Methods). Although variable, SipA transport rates seem to be in the same order of magnitude as the flagellin transport rates during assembly of the phylogenetically related flagella system [20–40 flagellin per sec (21, 22)]. Future work will have to determine whether one or more of the 10–100 SPI-1 TTSS present on each bacterium (23) contribute to injection and whether the number of TTSS participating in transport dictates the overall injection rate.
Transport by the SPI-1 TTSS depletes intrabacterial SipA and SopE pools. (A) Strategy for detecting effector protein injection into host cells by monitoring the presence of SipA in the bacterial cytosol. (B) Representative images of S. typhimurium M1304 in the early, intermediate, or late phase of SipA injection into Cos7 cells. Cells were fixed, permeabilized with lysozyme, and LPS (blue) and SipA (red) were immunostained (see Materials and Methods). (C) Time course of SipA depletion during the infection of Cos7 cells with S. typhimurium M1300 (wild type; black circles), M1304 (noninvasive; white circles), and M1301 (SPI-1 TTSS-disrupted; crosses). (D) Parallel detection of SopE and SipA depletion from the bacterial cytosol. Cos7 cells were infected for 30 min with wild-type S. typhimurium M1222, fixed, permeabilized with lysozyme, and immunostained for SopE (green) and SipA (red). The arrows point to a group of bacteria that had completed the SipA and SopE injection. Arrowheads indicate bacterium still harboring SopE and SipA in the cytosol. (E) Depletion of SopE and SipA from the bacterial cytosol. Infection was monitored by phase-contrast time-lapse microscopy. Cells were fixed and stained as in D. For each bacterium, the graph shows the time between docking and fixation, the location of SipA (as in B), and the presence/absence of SopE in the bacterial cytosol. Lines connect SipA and SopE data from the same bacterium.

SipA transport kinetics were verified in a second type of assay that monitors depletion of the intrabacterial SipA pool as a function of time (Fig. 4A). Cos7 cells were infected with S. typhimurium M1304, and bacterial docking was monitored by phase-contrast time-lapse microscopy. After 10, 15, or 25 min, the cells were fixed. The time-lapse movie revealed how long each bacterium had docked to the host cell before fixation. The SipA pools inside the bacterial and the host cell cytosol were immunostained. By confocal microscopy we assigned each SipA-expressing bacterium to one of the following three categories: (i) SipA in the bacterial but not the host cell cytosol (little or no injection), (ii) SipA in the bacterial and the host cell cytosol (injection ongoing), or (iii) SipA in the host cell but not the bacterial cytosol (injection advanced/complete) (Fig. 4B). Partial depletion of intrabacterial SipA was observed as early as 80 sec after docking of S. typhimurium M1304 to Cos7 cells (Fig. 4C). SipA was no longer detected in the bacterial cytosol 300–500 sec after docking. This timeframe agrees well with the results from live cell imaging (Fig. 3). Injection required a functional SPI-1 TTSS because M1301 (ΔinvG; see Materials and Methods) retained the SipA within the bacterial cytosol even after extended periods of host cell contact (Fig. 4C and Fig. 10, which is published as supporting information on the PNAS web site).

Infections with wild-type S. typhimurium M1300 yielded results similar to those for the noninvasive strain M1304 (Fig. 4C). Advanced/complete SipA injection was generally observed 80–200 sec after docking. This time was somewhat faster compared with M1304 and might be attributable to the rapid engulfment by the host cell. Engulfment increases the bacterial surface area engaged and may allow employing additional TTSS to accelerate completion of SipA injection (Fig. 5F).

SipA is just one of the SPI-1 TTSS effector proteins. It was of interest whether other effector proteins [i.e., SopE (24)] were injected with similar kinetics. SopE did not accumulate in defined foci inside the host cell. Therefore, we used a modified version of the intrabacterial depletion assay (see Fig. 4C) and the S. typhimurium wild-type strain M1222 (see Materials and Methods) in this experiment. As expected from earlier studies (25), all sipA expressing bacteria also expressed sopE (1,000 ± 500 molecules of SopE per cell) (Fig. 11, which is published as supporting information on the PNAS web site). Cos7 cells were infected with M1222, and bacterial docking was monitored by phase-contrast time-lapse microscopy. Cells were fixed. SipA and SopE were immunostained, and effector protein injection was evaluated by fluorescence microscopy (Fig. 4D). Each SipA-expressing bacterium was scored with respect to the time of docking before the fixation, the distribution of SipA (see Fig. 4B), and the presence/absence of SopE in the bacterial cytosol (Fig. 4E, pairs of green or red circles). Advanced or complete SipA and SopE injection was observed when wild-type S. typhimurium M1222 had docked for 100–200 sec, which indicated that SipA and SopE were injected with similar kinetics.
In conclusion, assays monitoring SipA depletion from the bacterial cytosol and SipA arrival in the host cell cytosol yielded equivalent results: M1304 could transport 6–3 × 10^3 molecules of SipA within 100–600 sec. With this strain, live cell imaging allowed sensitive detection of SipA injection as early as 16–25 sec after docking.

The threshold for detection of GFP–InvB recruitment (~100 GFP–InvB molecules) indicated that the fastest bacteria start SipA injection ~10 sec after docking. Considering that two InvB can bind per SipA (26, 27), we estimated that SipA can reach concentrations sufficient for remodeling the host cell actin cytoskeleton in vitro and in vivo within 16–25 sec after docking (5, 7, 18, 28). SopE is injected with kinetics similar to those of SipA but it does not accumulate in foci (Fig. 4E). Future work will address how such differences in effector localization and concentration affect the primary host cell response.

This study presents a real-time visualization of the type III secretion process and explains why *S. typhimurium* can trigger host cell responses within 1 min after docking (29–31). However, the analysis of the kinetics of effector protein delivery did vary somewhat between individual bacteria. Therefore, host cell responses may also vary. Real-time analysis of effector protein transport and localization at a single-cell level by assays like those described here are a key step toward understanding the critical early phase of the bacteria–host interaction that initializes symbiosis or disease.

We thank Jean Cohen (Centre National de la Recherche Scientifique/L’Institut National de la Recherche Agronomique, Gil-sur-Yvette, France) for the gift of GFP virus-like particles and Uwe Schlattner for help with surface plasmon resonance experiments. We also thank Yves Barral, Kurt Dittmar, Jan Ellenberg, Matthias Peter, and the members of the W.-D.H. laboratory for helpful discussions.