Yersinia enterocolitica induces apoptosis in macrophages by injecting the plasmid-encoded YopP (YopJ in other Yersinia species). Recently it was reported that YopP/J is a member of an ubiquitin-like protein cysteine protease family and that the catalytic core of YopP/J is required for its inhibition of the MAPK and NF-κB pathways. Here we analyzed the YopP/J-induced apoptotic signaling pathway. YopP-mediated cell death could be inhibited by addition of the zVAD caspase inhibitor, but not by DEVD or YVAD. Generation of truncated Bid (tBid) was the first apoptosis-related event that we observed. The subsequent translocation of tBid to the mitochondria induced the release of cytochrome c, leading to the activation of procaspase-9 and the executioner caspases-3 and -7. Inhibition of the postmitochondrial executioner caspases-3 and -7 did not affect Bid cleavage. Bid cleavage could not be observed in a yopP-deficient Y. enterocolitica strain, showing that this event requires YopP. Disruption of the catalytic core of YopP abolished the rapid generation of tBid, thereby hampering induction of apoptosis by Y. enterocolitica. This finding supports the idea that YopP/J induces apoptosis by directly acting on cell death pathways, rather than being the mere consequence of gene induction inhibition in combination with microbial stimulation of the macrophage.

A number of bacterial pathogens resist the defense mechanisms of their host by a recently discovered virulence mechanism called type III secretion. By this mechanism, bacteria adhering at the surface of eukaryotic cells inject proteins in the cytosol of these cells. In the archetypal Yersinia, Yersinia pestis, the agent of bubonic plague, Yersinia pseudotuberculosis, and Yersinia enterocolitica, the injected proteins are called Yops. Yops are encoded by a large virulence plasmid, and their main target seems to be the macrophage (for review see Ref. 1). One of the Yops, called YopP in Y. enterocolitica and YopJ in Y. pseudotuberculosis and Y. pestis, causes a variety of effects, such as suppression of tumor necrosis factor α (TNF-α)1 and interleukin-8 production, as the result of blockade of the activation of mitogen-activated protein kinase (MAPK) kinases (MKKs), MAPK, and nuclear factor κB (NF-κB) (2–6) and finally induction of apoptosis in macrophages (7, 8). YopP-induced apoptosis by Y. pseudotuberculosis has also been observed during an experimental mouse infection thereby showing that apoptosis plays a role in the establishment of a systemic infection (9). YopP/J interacts with IKKβ and MKKs, and recently it has been suggested that YopP/J belongs to a family of cysteine proteases related to the ubiquitin-like protein proteases (6, 10). Ubiquitin-like protein proteases cleave the C terminus of an 11-kDa small ubiquitin-related modifier SUMO-1. The protease YopJ was shown to reduce the cellular concentration of SUMO-1-conjugated proteins in an overexpression experiment; however, no direct substrate of YopJ was yet identified. Mutation of the YopJ catalytic cysteine results in the loss of its protease activity and hampers its capacity to inhibit NF-κB and MKK activation. It has been suggested that YopP/J-induced apoptosis is due to its capability of inhibiting the activation of NF-κB (5). However, the exact mechanism by which YopP/J induces apoptosis is not yet known.

One of the earliest and most consistently observed features of apoptosis is the activation of procaspases, a family of cysteine proteases that cleave their substrates after an aspartic acid residue (11). Caspases are synthesized as zymogens (procaspases) that become activated via proximity-induced auto-

1 The abbreviations used are: TNF-α, tumor necrosis factor-α; cmk, chloromethylketone; fmk, fluoromethylketone; Ac-YVAD, acetyl-Tyr-Val-Ala-Asp; B-D, benzyloxycarbonyl-Asp(OMe); zDEVD, benzyloxycarbonyl-Ala-Ala-Asp; zVAD, benzoyloxycarbonyl-Val-Ala-Asp(OMe); zDEVD, benzoyloxycarbonyl-Ala-Ala-Asp(OMe)-Gluf(OMe)-Val-Ala-Asp(OMe); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling; SUMO-1, small ubiquitin-related modifier-1.
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proteolysis by interaction with adaptor proteins or by cleavage via upstream proteases in an intracellular cascade (12–14). Two main procaspase activation pathways during apoptosis have been proposed: (i) the extrinsic activation of initiator procaspases, triggered by the formation of a receptosome complex; and (ii) the intrinsic activation of initiator procaspases, initiated by the formation of an apoptosome complex. The extrinsic activation of initiator procaspases is initiated by the death domain-containing receptors of the TNF receptor superfamily (14, 15). Binding of the ligand to the cell surface receptor leads to trimerization (16) of the receptors and subsequently oligomerization of their cytosolic death domains eventually leading to the recruitment of initiator procaspases, such as procaspase-8. This complex is called the death-inducing signaling complex (17). During this process caspase-8 can initiate cell death by directly cleaving the downstream executioner procaspases-3, -6, and -7 (13, 18). In some cases, however, the death-inducing signaling complex does not generate sufficient caspase-8 levels to allow efficient proteolytic activation of the downstream executioner procaspases, and amplification of the caspase cascade by mitochondrial-derived factors is required to kill the cell (19). A molecular link connecting the death-inducing signaling complex activation and mitochondria is the caspase-8-mediated cleavage of Bid, a pro-apoptotic member of the Bcl-2 family. The C-terminal part of Bid (tBid) translocates to the mitochondria, where it induces the release of cytochrome c (20, 21). Cytochrome c, together with dATP/ATP, binds the apoptotic protease activating factor-1 resulting in the formation of the apoptosome complex (22), which leads to recruitment and autoactivation of procaspase-9. In turn, caspase-9 activates procaspases-3, -6, and -7, thus initiating the caspase cascade (23). The consecutive activation of both initiator procaspases, procaspase-8 at the level of the death receptor complex and procaspase-9 at the postmitochondrial level, then leads to sufficient activation of the executioner procaspases resulting in cell demise. Intrinsic activation of procaspases, triggered by environmental insults, senescence, and developmental programs, involves the release of cytochrome c from the mitochondrial intermembrane space to the cytosol leading to assembly and activation of the apoptosome complex igniting the caspase cascade. The mechanism by which this cytochrome c release is induced is not yet clear.

In this report we describe that activation of procaspases is essential in Y. enterocolitica YopP-mediated cell death. Moreover, YopP-mediated apoptosis initiates the cell death pathway upstream of the cytosolic protein, Bid. Bid cleavage results in the subsequent release of cytochrome c from the mitochondria eventually leading to the activation of procaspase-9 and the executioner procaspases-3 and -7. The cleavage of Bid and the release of cytochrome c can both be inhibited by broad-spectrum caspase inhibitors, suggesting that YopP induces activation of upstream caspases, most likely caspase-8. Finally, we provide evidence that the protease activity of YopP is crucial for the induction of apoptosis in macrophages by Yersinia.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Bacterial Strains, and Growth Conditions—**Escherichia coli DH5α was used for standard manipulations. E. coli SM10 pir+ (24) was used to deliver mobile plasmids in Y. enterocolitica. Y. enterocolitica E40(pYV40) (7) is a wild-type, low virulence strain from serotype O:9 (25). Y. enterocolitica E40(pMSK41) is a YopP knockout (yopP53 allele) of E40(pYV40) (7). E40(pAB409) is a multiknockout mutant of E40(pYV40) unable to produce YopH, -O, -P, -E, -M, and -B (2). Y. enterocolitica A127(pYV127) is a wild-type, high virulence strain from serotype O:8 (2, 26).

Plasmid pMSK13 is a mobilizable plasmid containing yopF4ewmt (from the pYV plasmid of Y. enterocolitica E40). Plasmid pRB16 is a derivative of pMSK13 encoding yopF6ewmt in which the catalytic cysteine is replaced by a threonine. The mutant was engineered by site-directed mutagenesis as described by Kunkel et al. (27) using the mutation primer 5′-ACTAAAAATACGGTTTTACAGGTACT-3′, which induces a FinA restriction site (underlined). Plasmids pMSK13 and pRB16 were used to complement the yopP knockout mutant. For reasons of clarity, strains E40(pMSK41)(pMSK13) and E40(pMSK41)(pRB16) will be referred to as YopPE40wt. YopP strains. Plasmid pGD2 was obtained by cloning yopF2ewmt, the wild type yopF gene from strain A127, into the eukaryotic expression vector pEFB6(TK) expressing vector (Invitrogen, Carlsbad, CA) in frame with the Myc and His tags, using EcoRI and NotI sites. pcDNA1-TRADD was described elsewhere (28). pcDNA1-FADD was constructed in a similar way as pcDNA1-TRADD by fusing the human FADDp. pEF1-Bid was made by cloning the reverse transcription polymerase chain reaction fragment of full-length Bid (from mouse lung mRNA) in the pEF1TK expressing vector (Invitrogen, Carlsbad, CA). pIKK2(Flag) an expression vector for IKK2/IKKβ, was a generous gift from Drs. J. Schmitt and R. de Martin (University of Vienna, Vienna, Austria).

Bacteria were pregrown overnight in brain-heart infusion (Difco) before infection bacteria were diluted 1:20 in fresh brain-heart infusion and cultured under continuous shaking (110 rpm) for 120 min at room temperature; subsequently the bacteria were induced for Yop secretion by incubation for 30 min in a shaking water bath (110 rpm) at 37 °C. Prior to infection bacteria were washed with RPMI 1640 (Life Technologies, Inc.).

**Cell Culture and Macrophage Infection—**Murine monocye-macrophage J774A.1 cells (ATCC TIB67) were cultured at 37 °C under 6% CO2 in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), sodium pyruvate (1 mM), and β-mercaptoethanol (2 × 10–5 M). Unless otherwise indicated, macrophages were seeded in medium without antibiotics at a density of 105/cm2 15 h before infection. Macrophages were infected with Y. enterocolitica grown under conditions for moderate Yop induction at 37 °C (see above) with a multiplicity of infection (m.o.i.) of 50.

**Caspase Inhibitor Studies—**J774A.1 macrophages were seeded at 5 × 104 cells in 24-well tissue culture plates on glass coverslips, 15 h in advance of infection. 100 μM of the caspase peptide inhibitors (Calbiochem) benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD.fmk), benzyloxycarbonyl-Ala-Asp(OMe)-fluoromethylketone (zDEVD.fmk), and benzoylcarbonyl-Ala-Asp(OMe)-Ala-Asp-fluoromethylketone (zDEVD.fmk), and benzoylcarbonyl-Ala-Asp-OMe-Ala-Asp-fluoromethylketone (zAD.fmk) were added 2 h before infection. Infection of the macrophages was done as described above. Immunofluorescence detection of cytochrome c-ment-gemion DNA was performed using a terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay, essentially as described by Mills et al. (7). Cell morphology was analyzed by light microscopy.

**Measurement of Caspase Activity—**Cell extracts were prepared by lysing the cells in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10% glycerol, 10 μg/ml leupeptin, 0.3 mM phenylmethylsulfonyl fluoride, and 0.1 mM phenylmethylsulfonyl fluoride. Caspase activity was determined by incubation of cell lysates (containing 40 μg of total protein) with 50 μM of the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-Asp-Glu-Ala-Asp-fluoromethylketone (Ac-DEVD-amc) (Peptide Institute Inc., Osaka, Japan) in 150 μl of cell-free system buffer, comprising 10 mM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM MgCl2, 0.5 mM EGTA, 2 mM MgCl2, 0.5 mM pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM dithiothreitol (13). The release of fluorescent aminomethylcoumarin was measured by fluorometry and expressed as fluorescence increase per min (ΔF/min). (Cytoflor; PerSeptive Biosystems, Cambridge, MA).

**Western Analysis of YopP, Cytochrome c, Caspases, and Bid—**For analysis of YopP, cytochrome c, caspase, and Bid cleavage, macrophages were infected with Y. enterocolitica or were left untreated as a control. After 3 h, cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), resuspended in 0.002% digitonin (RBI, Natick, MA) PBS, and incubated for 3 min on ice. Cell membranes and organelles were pelleted by centrifugation (15,000 × g for 5 min at 4 °C). Subsequently, 50 μg of cytosolic protein or an equivalent of 2.5 × 105 cells were loaded per lane on 15% polyacrylamide gels. After electrophoresis, the gels were stained, and the bands were visualized (BA 83; Schleicher & Schuell, Dassel, Germany), which was then probed with a rabbit polyclonal anti-YopP antisemur, mouse monoclonal antibody to cytochrome c (Pharmining, San Diego, CA), polyclonal antiserum raised against recombinant murine caspases-3 or -7 (29), and polyclonal rabbit anti-murine caspase-9 antisemur (cell sig- naling technology; New England Biolabs Inc., Beverly, MA) or probed at
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4 °C with a polyclonal goat serum raised against Bid (R & D Systems, Abingdon, UK). The anti-YopP antisera was raised against purified YopP produced in strain E40(pAB409(pMSK13)), affinity purified on nitrocellulose-bound YopP, and concentrated by passing through a protein G column (Mab kit; Amersham Pharmacia Biotech). Primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, or rabbit anti-goat IgG (DAKO A/S, Glostrup, Denmark) for cytochrome c, caspases and YopP, and Bid, respectively, and visualized by enhanced chemiluminescence as described in the manufacturer’s instructions (Roche Molecular Biochemicals).

RESULTS

Caspases Mediate Yersinia YopP-induced Cell Death—Previous results indicated that YopP/J is required for Yersinia to induce apoptosis in macrophages (7, 8). To determine whether procaspase activation was involved in YopP/J-mediated apoptosis, J774A.1 macrophages were pretreated with various irreversable caspase inhibitors prior to infection with E40(pMSK41)(pMSK13), a Y. enterocolitica yopP knockout strain overproducing YopPE40WT (YopPE40WT-). Ac-YVAD.cmk and zDEVD.fmk are specific inhibitors that bind to the active site of caspase-1 and caspases-3/7, respectively, whereas B-D.fmk is a broad-spectrum caspase inhibitor, and zVAD.fmk is a rather specific inhibitor of the apoptosis-related initiator caspases, caspases-8 and -9 (30). zAAD, a granzyme B inhibitor, was used as a control. As shown in Fig. 1A, YopP-mediated apoptosis is characterized by the induction of membrane blebbing and nuclear condensation and fragmentation. Treatment of macrophages with the caspase inhibitors zDEV.D.fmk or Ac-YVAD.cmk or the granzyme B inhibitor zAAD.fmk prior to Y. enterocolitica infection could not prevent YopP-mediated apoptotic cell death. Nevertheless, incubation with zDEVD.fmk resulted in the complete inhibition of intracellular DEVDase activity, as determined by a fluorogenic substrate assay (data not shown). In contrast, pretreatment of the cells with the caspase inhibitors zVAD.fmk and B-D.fmk completely prevented YopP-mediated cell death. Similar results were obtained when analyzing DNA fragmentation in these cultures by means of TUNEL staining (Fig. 1B). Incubation of the cells with the inhibitor alone did not alter their survival rate (data not shown). These results suggest that YopP/J kills macrophages by activating procaspases, but inhibition of caspase-1, -3, or -7 activity cannot prevent the execution of apoptosis.

Yersinia-induced Apoptosis Results in Early Bid Cleavage and Cytochrome c Release—To determine the molecular mechanism of Yersinia-mediated apoptosis we examined the intracellular events taking place by means of a time kinetics experiment evaluating procaspase activation, Bid cleavage, and cytochrome c release. J774A.1 macrophages were infected with the wild type Y. enterocolitica A127 (pYV127) strain or with the virulence plasmid-cured isogenic strain (pYV127−), and at different time intervals cytosolic fractions of these cultures were prepared. Non-infected cells served as a control. YopP could be detected in the macrophage cytosol in substantial amounts as soon as 40 min after co-incubation of cells with bacteria (Fig. 2A). The first apoptosis-related intracellular event that we were able to monitor was Bid cleavage (Fig. 2B). Bid does not contain a transmembrane domain and is located in the cytosol. Cytosolic Bid was cleaved to a 13-kDa fragment (tBid), and the concentration of tBid gradually increased from 60 min on to 80 min after infection of the macrophages. Bid protein is a specific proximal substrate of caspase-8 in the signaling pathway to apoptosis (20, 21). We were not able to analyze endogenous procaspase-8 expression and/or activation in J774A.1 cells because of the lack of anti-murine caspase-8 antibodies with sufficient detection efficiency. It has been described that the C-terminal fragment of Bid translocates from the cytosol to the mitochondrial membrane, a process that finally leads to the release of cytochrome c (20, 21). Cytochrome c release became evident 20 min after the generation of tBid in the cell (Fig. 2B). The release of cytochrome c results in a rapid assembly of the apoptosome complex as witnessed by the processing of procaspase-9 to its effectorically active p35/p37 subunits, eventually leading to the activation of the downstream executioner procaspases-3 and -7 (Fig. 2C). This activation of the apoptotic cascade finally resulted in the total demise of the macrophage resulting in the loss of the cell membrane integrity (Fig. 2D). None of the apoptotic events took place in non-infected cells or cells treated with the plasmid-cured strain (pYV127−). Taken together, our results indicate that Yersinia infection results in a rapid generation of tBid that will translocate to the mitochon-
dria and induce the release of cytochrome c leading to the assembly of the apoptosome resulting in the activation of the executioner procaspases.

**YopP Is Required for Bid Cleavage, Cytochrome c Release, and Procaspase Activation**—To investigate which molecular events observed during *Yersinia*-induced apoptosis, viz. Bid cleavage, cytochrome c release, and procaspase activation, were YopP-dependent we infected J774A.1 macrophages with the plasmid-cured A127 strain (pYV127) or with the wild type A127 strain (pYV127) at a m.o.i. of 50. Non-infected (NI) J774A.1 cells were used as a control. At different time intervals cytosolic fractions of J774A.1 cells were prepared by digitonin lysis, and analyzed by polyacrylamide gel electrophoresis and Western blotting, making use of anti-YopP (A), anti-Bid (B), anti-cytochrome c (C), or anti-caspase-9, -3, or -7 (C) antibodies. As a control for cell death, the percentage of cells that lost plasma membrane integrity (D), determined by propidium iodide (PI) uptake, was analyzed.

**Fig. 2.** Time kinetic analysis of YopP translocation, Bid cleavage, cytochrome c release, and procaspase activation during *Y. enterocolitica*-induced apoptosis of macrophages. J774A.1 macrophages were infected with a plasmid-cured A127 strain (pYV127) or with the wild type A127 strain (pYV127) at a m.o.i. of 50. Non-infected (NI) J774A.1 cells were used as a control. At different time intervals cytosolic fractions of J774A.1 cells were prepared by digitonin lysis, and analyzed by polyacrylamide gel electrophoresis and Western blotting, making use of anti-YopP, anti-Bid, anti-cytochrome c, or anti-caspase-9, -3, or -7 antibodies. As a control for cell death, the percentage of cells that lost plasma membrane integrity, determined by propidium iodide uptake, was analyzed.

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FIG. 3. YopP is necessary for the induction of Bid cleavage, cytochrome c release, and procaspase-3 and -7 activation during Y. enterocolitica-induced apoptosis of macrophages. J774A.1 were infected for 3 h with Y. enterocolitica YopP+ and YopP40C172T, strains at a m.o.i. of 50 with or without pretreatment of the caspase inhibitor zVAD.fmk (100 μM). Equivalent amounts of proteins were analyzed by Western blot making use of anti-Bid (A), anti-cytochrome c antibodies (A), or anti-caspases-3 or -7 antibodies (B). Open and closed arrows indicate cleaved and uncleaved proteins, respectively. Caspase activity in cell extracts was measured making use of the Ac-DEVD aminomethyl coumarin fluorogenic substrate, as described under "Experimental Procedures." (C). NI, non-infected.

FIG. 4. YopP-induced Bid cleavage is not the result of a caspase-3-dependent feedback loop. Control and zDEVD.fmk (100 μM) pretreated J774A.1 macrophages were infected with the YopP40C172T strain, and 2.5 h after infection cells were lysed. A, cell lysates were analyzed by polyacrylamide gel electrophoresis and Western blotting, making use of an anti-Bid antiserum. B, activity was determined by incubation of cell lysates with the fluorogenic substrate Ac-DEVD.ame, as described under "Experimental Procedures." NI, non-infected.

These data indicate that YopP/J protease activity is required both for the inhibition of NF-κB signaling and the induction of apoptosis by Yersinia.

YopP Does Not Interact with TRADD, FADD, or Bid—In cells in which clustering of death receptors causes weak procaspase-8 activation, the mammalian Bid protein is a specific proximal substrate of caspase-8 in the signaling pathway (19). YopP/J has been shown to bind to members of the MAPK family and to IKKβ and to interfere with these signaling pathways. The mechanism by which YopP/J induces apoptosis is not yet completely clear, but our data suggest an implication of caspase-dependent cleavage of Bid. A possible hypothesis could be that YopP/J clusters pro-apoptotic signaling molecules thereby starting the cell death machinery. For this reason we analyzed whether YopP could bind directly to pro-apoptotic signal transduction molecules such as TRADD, FADD, or Bid. Therefore, we overexpressed these molecules in HEK293T cells, together with YopP40C172T and performed an immunoprecipitation experiment. FADD, TRADD, and Bid could not be co-immunoprecipitated with YopP40C172T (Fig. 6), indicating that YopP is not able to cluster these pro-apoptotic mediators thereby initiating an apoptotic cascade. As a positive control, we could co-immunoprecipitate IKKβ with YopP40C172T (6). Additionally, YopP40C172T overexpression did not lead to Bid processing (data not shown), indicating that Bid is not a direct substrate for the YopP protease.
DISCUSSION

Previously it was shown that YopP/J is required for Yersinia-induced apoptosis in macrophages, but the exact mechanism of action remained unknown (7, 8). Although the signals that generally lead to apoptosis are quite diverse, the activation of procaspases plays a central role in the initiation and execution phase of apoptosis. The caspase family comprises three groups, the inflammatory caspases (-1, -4, -5, and -11), the initiator caspases (2, -8, -9, and -10), and the executioner caspases (-3, -6, and -7). In this study we wanted to determine which signaling pathways are activated in YopP-mediated cell death. We demonstrated an early Bid cleavage after YopP was injected into the cells by the infecting bacteria. Bid cleavage clearly occurred before cytochrome c release, suggesting that the truncated Bid relocates to the outer mitochondrial membrane and initially causes the release of cytochrome c (20, 21). Subsequent assembly of the cytochrome c-apoptotic protease activating factor-1-ATP-procaspase-9 complex then initiated the activation of procaspase-9 leading to postmitochondrial procaspase-3 and -7 activation (30, 35). This seems to be a very rapid event, because cytochrome c release, procaspase-9 activation, and procaspase-3 and -7 activation all occur simultaneously. Because tBid generation becomes evident clearly before the release of cytochrome c, our data indicate that Bid cleavage is not the result of postmitochondrial caspase activity as described in other apoptotic systems (31). Inhibition of the executioner caspases-3 and -7 was not able to block YopP-mediated apoptosis. Nevertheless, pretreatment of cells with this inhibitor completely blocked the YopP-induced DEVDase activity, thus suggesting that the caspases-3 and -7 are dispensable for the onset of apoptosis and merely play an executing role in the late stage of apoptosis (36, 37). Furthermore, Ac-YVAD.cmK, an
inhibitor of caspase-1, also failed to block YopP-induced apoptosis despite the fact that procaspase-1 becomes activated upon *Y. enterocolitica* infection, indicating that caspase-1 activity is not required for Yersinia-mediated macrophase apoptosis. This finding indicates that *Yersinia* activates a different cell death pathway in macrophages than *Shigella* or *Salmonella*, two pathogens that make use of a type III protein secretion mechanism similar to that of *Yersinia* sp. *Shigella* and *Salmonella* both produce similar apoptotic invasin proteins, named IpaB and SipB, respectively (38). During infection these proteins are translocated to the cell and bind to procaspase-1, resulting in its activation. In contrast to *Yersinia*-mediated apoptosis, pretreatment of macrophages with the Ac-YVAD.cmk caspase-1 inhibitor prevented the induction of apoptosis by *Shigella* or *Salmonella* (39, 40). Furthermore, caspase-1-deficient macrophages are protected against *Shigella*- or *Salmonella*-induced cell death (41). When cells were pretreated with the caspase inhibitors zVAD.fmk and B-D.fmk, YopP-mediated apoptosis could be completely blocked. In addition, YopP-induced Bid cleavage and the subsequent release of cytochrome c were completely blocked in the presence of zVAD.fmk. Therefore it seems that YopP-mediated cell death is mainly caused by the activation of initiator procaspases acting upstream of caspases-3 and -7. Bid can be cleaved by different proteases such as caspase-3 (31) or -8 (20, 21) or granzyme B (42). Although processing of endogenous procaspase-8 could not be analyzed because of the lack of anti-mouse caspase-8 antiserum with sufficient detection efficiency, procaspase-8 activation may play a key role in YopP-mediated apoptosis because (a) YopP-induced procaspase-3 activation occurs only after Bid had been cleaved, and Ac-DEVD.fmk (a potent caspase-3 inhibitor) treatment could not prevent Bid cleavage; and (b) the granzyme B inhibitor zAAD.fmk did not affect the YopP-dependent apoptosis; in addition, granzyme B is normally not expressed in macrophages. The presently known activation mechanisms of pro-apoptotic initiator procaspases include two main pathways, the death receptor-receptor-initiated activation of procaspase-8 in the death-inducing signaling complex (43) and the apoptosisome-mediated mitochondrial activation of procaspase-8 by caspase-3 (32). Because the executioner procaspases became activated after Bid had been cleaved, and caspase-3/7 inhibition could not prevent the onset of the apoptotic program or the cleavage of Bid, it is less likely that procaspase-8 would be activated by the generation of postmitochondrial caspase activity. IpaB or SipB can bind directly to procaspase-1, thereby inducing procaspase-1 activation that is crucial for the induction of apoptosis by *Shigella* or *Salmonella* infections. Could YopP/J make use of a similar mechanism by binding to procaspase-8? We were not able to show a clear interaction between YopP and procaspase-8 in a yeast two hybrid experiment. Another possibility might be that YopP/J would cluster intracellular pro-apoptotic adapter molecules involved in the activation of procaspase-8 such as TRADD or FADD, thereby mimicking the death-inducing signaling complex as formed by death receptors of the TNF-receptor superfamily. Upon receptor activation these receptors can recruit the adaptor molecules TRADD and/or FADD. Binding of FADD subsequently can recruit procaspase-8 or -10, and this event leads to proximity-induced autocleavage of these procaspases (44). Immunoprecipitation experiments with YopP could confirm that YopP is able to interact with IKKβ (6) but did not provide evidence for binding of YopP with TRADD or FADD. Hence, the trigger that leads to YopP/J-induced procaspase activation required for Bid cleavage remains elusive. Given the observation that YopP is a protease we also considered whether Bid could be a direct substrate for YopP. Overexpression of YopP in HEK293T cells did not lead to processing of Bid or apoptosis. Overexpression of wild type YopP, but not of YopPΔ127C172T, resulted in the inhibition of NF-κB activation indicating that YopP has enzymatic activity upon overexpression in HEK293 cells. YopP/J is not only responsible for the induction of apoptosis, but it also strongly interferes with macrophase signal transduction resulting in a blockade of two major cell signaling cascades; the MAPK and the NF-κB pathway are both involved in transcriptional gene control (2–5). Indeed, the production of proinflammatory cytokines would be disadvantageous for the extracellular lifestyle of *Yersinia*. It has been shown that YopP/J inhibits these pathways by interacting directly with IKKβ and several MKKs, thereby preventing their activation (6). Recently it was suggested that the YopP/J-induced apoptosis of macrophages could merely result from its inhibition of NF-κB activation in combination with lipopolysaccharide (LPS) (5, 45). In contrast to macrophages, other cell types (fibroblasts and HeLa cells) do undergo apoptotic cell death upon *Yersinia* infection. Nevertheless, YopP/J is translocated efficiently in HeLa cells (4), resulting in inhibition of NF-κB activation and interleukin-8 cytokine production (4, 5). YopP/J exerts its effect on HeLa cells, but this does not lead to the induction of programmed cell death, suggesting the absence of a signaling pathway in this cell type when compared with macrophages. As shown for *Yersinia* and *E. coli*, LPS can trigger apoptosis when NF-κB activation is inhibited (5, 46). LPS binds the CD14 receptor, a receptor only present on monocytes and macrophages. The LPS signal is transduced across the plasma membrane by the toll-like receptor 4 (TLR-4), which associates with CD14 to form the LPS receptor complex (47). Recently it was shown that another class of microbial surface molecules, the bacterial lipoproteins (BLPs), induced apoptosis in macrophages by binding TLR-2 (48). Furthermore, epithelial cell lines transfected with TLR-2 underwent bacterial lipoprotein-mediated apoptosis. Altogether these studies suggest that bacteria-mediated apoptosis requires the presence of Toll-like receptors at the cell surface. During *Yersinia* infection, LPS and/or BLPs and YopP might both be required to fulfill the process of apoptosis. In addition it was suggested that the BLP-stimulated TLR-2-triggered apoptosis occurs via a path-

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3 G. D. and G. C., unpublished results.

4 M. V. G. and P. V., unpublished results.

5 G. D. and G. C., unpublished results.

6 M.-P. S. and G. C., unpublished results.
way involving MyD88, FADD, and caspase-8 (49). These data indicate that the early Bid cleavage observed in our experiments could well be because of activation of the initiator procaspase-8 by TLR-2 signaling. In this context, YopP/J would only be required to inhibit gene activation thereby preventing the up-regulation of anti-apoptotic proteins in the macrophage. This hypothesis would imply that infection of macrophages with the YopPE40C172T+ mutant strain would not affect the early Bid processing, already significant 20 min after YopP injection in the macrophage cytosol upon infection. Because we show here that Bid cleavage is impaired upon YopPE40C172T+ infection, we speculate that YopP/J induces apoptosis by a direct action on cell death signaling pathways.

Previous studies indicated that YopP/J interacts with MKKs and IKKβ and prevents their phosphorylation. However, MKK SUMOylation was not observed, and data on I KKβ SUMOylation are not available; hence the targets for YopP/J de-SUMOylation remain to be identified. MKKs could serve as shuttles to escort YopP/J to the signaling complex where it would affect critical SUMO-1-conjugated proteins. It has been observed that the NF-κB signaling molecules such as I KKβ can be recruited to the TNF receptor signaling complex (50); a similar recruitment event could be valid for the Toll-like receptors. Hence, it should be considered that YopP/J may directly affect Toll-like receptor signaling in this way causing apoptotic signaling in macrophages.

Together, our data support a model in which the YopP/J protease activity targets both anti- and pro-apoptotic pathways (Fig. 7). In our model YopP/J-mediated de-SUMOylation alters the Toll-like receptor signaling in a way allowing procaspase-8 activation leading to Bid processing. Truncated Bid then translocates to the mitochondria inducing the release of cytochrome c, resulting in the assembly of the apoptosome and the generation of activated caspase-9 and the executioner caspases-3 and -7. Identification of the YopP/J targets will be important to further elucidate the YopP/J signaling pathways.

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