Yersinia Phosphatase Induces Mitochondrionally Dependent Apoptosis of T Cells*

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To evade the immune system, the etiologic agent of plague, Yersinia pestis, injects an exceptionally active tyrosine phosphatase called YopH into host cells using a type III secretion system. We recently reported that YopH acutely inhibits T cell antigen receptor signaling by dephosphorylating the Lck tyrosine kinase. Here, we show that prolonged presence of YopH in primary T cells or Jurkat T leukemia cells causes apoptosis, detected by annexin V binding, mitochondrial breakdown, caspase activation, and internucleosomal fragmentation. YopH also causes cell death when expressed in HeLa cells, and this cell death was inhibited by YopH-specific small molecule inhibitors. Cell death induced by YopH was also prevented by caspase inhibition or co-expression of Bcl-xL. We conclude that YopH not only paralyzes T cells acutely, but also ensures that the cells will not recover to induce a protective immune response but instead undergo mitochondrially regulated programmed cell death.

In historical times, the plague-causing bacterium Yersinia pestis has caused social devastation on a scale unmatched by any other infectious disease, with over 200 million human deaths (1, 2). Although this fatal bacterium is virtually eradicated and is effectively treated with antibiotic therapy, the rapidly fatal course of pneumonic plague, potential for secondary spread of infection, and developed capacity for massive aerosol distribution have made Y. pestis a feared biological weapon of terror (3). Thus, there exists a need for elucidation of aerosol distribution have made Y. pestis a feared biological weapon of terror (3). Thus, there exists a need for elucidation of aerosol distribution have made Y. pestis a feared biological weapon of terror (3). Thus, there exists a need for elucidation of aerosol distribution have made Y. pestis a feared biological weapon of terror (3). 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FIG. 1. YopH induces apoptosis in Jurkat T cells. 20 × 10⁶ Jurkat T cells were transiently transfected with 10 μg of the indicated expression plasmids. 24 h later, −10⁶ cells were washed in phosphate-buffered saline, stained with annexin V and propidium iodide for 5 min, and analyzed by FACS. A, representative dot plots of pEF5HA vector control, wild-type YopH, and YopH-D356A. The bottom right quadrant represents apoptotic cells positive for annexin V binding and negative for propidium iodide. The top two quadrants represent propidium iodide-positive cells that are either late apoptotic or necrotic cells. B, mean percentages of apoptotic cells from five independent experiments are shown. YopH-induced apoptosis is significantly different from vector control (p < 0.05, n = 5). C, mean percentage of total cell death is shown, i.e. apoptotic cells plus propidium iodide-positive cells. YopH induction of total cell death is significantly different from vector control (p < 0.05, n = 5). D, Jurkat cells were transiently transfected with vector control or YopH and then treated with 38 μM LTY9 or LTY11, as indicated. 48 h later, cell death was determined by trypan blue exclusion assay. E, similar experiments with HeLa cells transfected with YopH, or treated with 10 μM etoposide, in the presence of 48 μM LTY11 or LTY61. Induction of cell death was significantly different from vector control and inhibitor-treated (p < 0.0001, n = 2).
yukaryotic expression vector, which adds a 9-amino-acid hemagglutinin epitope to the N terminus of the insert, were as before (20). The cDNA encoding Bcl-xl and dominant negative caspase-9 were in the pcDNA3 expression vector.

**Cells and Cell Treatments—**Normal T lymphocytes were isolated from venous blood of healthy volunteers by Ficoll gradient centrifugation. Monocytes/macrophages were eliminated by adherence to plastic for 1 h at 37 °C. Jurkat T leukemia cells were kept at logarithmic growth in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, non-essential amino acids, and 100 units/ml each of penicillin G and streptomycin. Transfections were done by electroporation as described previously (21–23). HeLaS cells were maintained at logarithmic growth in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 100 units/ml each of penicillin G and streptomycin. HeLaS cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer’s protocol. ANT-YopH and ANT-YopH-D356A proteins were induced in transformed Echerichia coli and purified using Ni²⁺-nitrotriacetic acid. Both proteins were of equal high purity. They were added at 6 μg to cells in RPMI medium at 37 °C. BAF (capsase inhibitor III, Calbiochem) was added at a concentration of 10 μM immediately after transfection.

**YopH Inhibitors—**For Jurkat and HeLa cell treatments, LTY11, LTY12, and LTY61 were added at 36 or 48 μM after transfection of YopH was completed.

**Cell Death Assays—**The percentage of cell death was determined by using trypan blue (Invitrogen) exclusion. 48 h after transfection, cells were diluted 50:50 (v/v) in 0.4% trypan blue in phosphate-buffered saline and viewed under a microscope, and total cell number as well as the number of trypan blue-positive (dead) cells were counted in four fields of view using a hemacytometer. The annexin V-FITC apoptosis detection kit (BioVision Research Products, Mountain View, CA) was used according to the manufacturer’s protocol, and cells were analyzed on a BD Biosciences FACSort. Internucleosomal DNA fragmentation was determined using the nucleosome enzyme-linked immunosorbent assay (Oncogene Research Products, San Diego, CA) according to the manufacturer’s protocol.

Mitochondrial integrity assays were performed by staining 10⁶ cells with the tetramethylrhodamine, 5(6)-acetoxymethyl ester-tetramethylrhodamine-bocyanine iodide (JC-1) for 15 min at room temperature in the dark. The cells were washed in phosphate-buffered saline and subsequently analyzed on a BD Biosciences FACSort.

**Caspase Assays—**Caspase activity was measured as described previously (24, 25). Briefly, 10⁶ cells were lysed in 50 μl of modified radioimmune precipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate) and left on ice for 15 min, and centrifugation was done at 14,000 rpm for 10 min. Protein concentration was determined using the Pierce BCA protein assay according to the manufacturer’s protocol with bovine serum albumin as standard. Total caspase activity was measured using 100 μM Ac-DEVD-AFC (Enzyme Systems Products, Aurora, OH) in caspase buffer (10 mM Pipes, pH 7.2, 100 mM NaCl, 10% sucrose, 0.1% Chaps, 1 mM EDTA, and 10 mM dithiothreitol). Amidolytic activity was measured on an f-max Molecular Device spectrofluorometer at 37 °C (excitation, λ = 405 nm; emission, λ = 510 nm). A CaspACE™ FITC-VAD-fmk in situ marker (Promega, Madison, WI) was used to stain active caspases at a concentration of 10 μM for 10 min in the dark, and the cells were then washed in phosphate-buffered saline and analyzed on a BD Biosciences FACSort.

**Data Analysis and Statistics—**Data collected by FACS were analyzed with FlowJo for Windows v5.0 (Tree Star Inc., Ashland, OR). Statistical analyses were performed with GraphPad Prism v4.0 for Windows (GraphPad Software Inc., San Diego, CA). All experiments were analyzed by two-way analysis of variance followed by Bonferroni posttests.

## RESULTS

**YopH Induces Apoptosis in Jurkat T Cells—**In preliminary transfection experiments with Jurkat T cells and normal human T lymphocytes, we observed that the number of YopH-positive cells in many experiments was much lower than the number of cells expressing the catalytically inactive YopH-D356A or a control protein (e.g. GFP). This phenomenon was particularly evident at 48 or 72 h after transfection and was more prominent with normal human T lymphocytes when compared with Jurkat. The inequality of YopH- versus YopH-D356A-positive cell numbers between samples was abolished by the inclusion of Z-VAD-fmk, a polycaspase inhibitor, in the culture medium (data not shown). This observation led us to ask whether YopH expression results in caspase activation and subsequent apoptosis in T cells. To test this notion, we transfected Jurkat with the empty pEF5HA vector, wild-type YopH, or the catalytically inactive mutant YopH-D356A and analyzed the cells at 24 h after transfection by staining with FITC-annexin V and propidium iodide. This method of analysis reveals two important aspects of cell death: (i) phosphatidylserine exposure on the extracellular face of the plasma membrane by FITC-annexin V binding and (ii) loss of plasma membrane integrity, resulting in entry of propidium iodide into the cells. YopH caused a significant increase in the percentage of cells double-positive for annexin V and propidium iodide (Fig. 1, A and C). This population of cells represents either late stage apoptosis or necrosis. YopH also increased single-positive cells for annexin V, which corresponds to apoptotic cells that have not yet lost outer membrane integrity but have lost phosphatidylserine asymmetry (Fig. 1, A and B). The cells expressing YopH first go through the annexin V single-positive stage and then proceed to the late stages of apoptosis and become positive for propidium iodide as well, thus accumulating in the double-positive population. Also, cells co-transfected with YopH and GFP and then analyzed by flow cytometry at 24 h (Table I) showed that fewer cells were alive in the YopH-transfected population, fewer GFP-expressing cells remained, and many more of the green fluorescent protein-positive cells were also annexin V-positive. The catalytically inactive (but substrate-binding) YopH-D356A mutant had a small (and not significant) effect when compared with vector controls in this and some other experiments. Thus, the catalytic activity of YopH is essential for its ability to induce apoptosis.

**YopH-induced Cell Death Is Blocked by a Specific Small Molecule Inhibitor—**To directly test whether the catalytic activity of YopH is essential for its ability to induce cell death, we transfected Jurkat cells with YopH and then treated the cells with the specific YopH inhibitors, LTY11 and LTY9, generated in our laboratory.2 Both are membrane-permeable competitive small molecule inhibitors that specifically block the enzymatic activity of YopH with Ki values of 143 and 208 nM, respectively, using the general tyrosine phosphatase substrate p-nitrophenyl phosphate in an in vitro assay. Jurkat cells were transfected with YopH and then kept in culture in the presence of LTY11 or LTY9, which largely (but not completely) abolished the ability of YopH to cause cell death (Fig. 1D). In the absence of YopH, these inhibitors did not affect cell death. We also used HeLa cells, which typically have higher transfection efficiencies and lower levels of background cell death when compared with Jurkat cells. Expression of YopH caused a highly significant increase in the amount of cell death when compared with empty vector (Fig. 1D), and this cell death was completely

| Table I | Death of Jurkat T cells co-transfected with YopH and GFP |
|---------|-------------------------------------------------------|
|         | Live cells† | GFP †, ‡ | GFP †, annexin †, ‡ |
| GFP     | 88          | 54.7     | 25.5                  |
| YopH + GFP | 36.2      | 23.4     | 51.9                  |
| YopH-DA + GFP | 76.6  | 39.1     | 43.2                  |

† The percentage of live cells was determined by flow cytometry using forward and side scatter gating for live versus dead cells.
‡ The percentage of GFP-positive cells was determined by flow cytometry using untransfected cells as a negative control.
†, ‡ The percentage of Annexin-positive cells was determined by flow cytometry using Alexa Fluor594-conjugated annexin V.

2 Tautz, L., Bruckner, S., Sareth, S., Alonso, A., Bogetz, J., Bottini, N., Pellechia, M., and Mustelin, T. (2005) *J. Biol. Chem.* 280, 9400–9408.
YopH Causes Apoptosis of T Cells

YopH Activates Caspases—To directly measure whether YopH expression resulted in caspase activation, Jurkat cells were transiently transfected with empty vector, wild-type YopH, YopH-D356A, or PTEN (as positive control) and subjected to *in vitro* caspase activity assays 48 h later, which showed that YopH caused a significant 3-fold increase in caspase activity when compared with vector control cells (Fig. 2A). Importantly, caspase activity in cells transfected with the catalytically inactive mutant of YopH was indistinguishable from vector-transfected control cells, whereas PTEN induced a 5-fold activation of caspases. We conclude that YopH activates caspases in a manner that is dependent upon its catalytic activity.

We have previously shown that the tyrosine kinase Lck is a target of YopH that accounts for its ability to inhibit T cell receptor signal transduction (20). To test whether Lck was important for the ability of YopH to induce caspase activation, we transfected the Lck-deficient Jurkat cell line JCaM1 with empty vector, wild-type YopH, or YopH-D356A and assayed *in vitro* caspase activity 48 h later. YopH did not cause caspase activation in this Lck-deficient cell line (Fig. 2B), whereas PTEN did. We conclude that YopH may well reduce T cell survival because it reduces basal Lck phosphorylation at Tyr-394 (20). If so, JCaM1 cells survive in the absence of Lck because additional mutations have uncoupled Lck from caspase activation or because of other alterations in the machinery for apoptosis. The induction of cell death in HeLa cells by YopH may be related to the dephosphorylation of other Src family kinases and perhaps other tyrosine kinases involved in survival of cells.

To better mimic the scenario in a lymph node of an infected patient, where the bacteria inject YopH protein directly into the cytoplasm of T lymphocytes, we incubated freshly isolated peripheral blood leukocytes (>80% T lymphocytes) with negative control buffer, the membrane-permeable fusion protein ANT-YopH (20), or etoposide as positive control for 24 h and then analyzed *in vitro* caspase activity. In normal T lymphocytes, ANT-YopH induced a very high level of caspase activity when compared with mock control (Fig. 2C). When compared with etoposide-treated cells, which typically have very high levels of caspase activity, ANT-YopH induced a higher increase in caspase activity. These data show that YopH is a very potent inducer of caspase activation in normal human T lymphocytes.

**YopH Causes Loss of Mitochondrial Membrane Potential**—An important event associated with the transduction of many apoptotic signals is the loss of mitochondrial membrane potential caused by translocation of pro-apoptotic Bcl-2 family members to the outer mitochondrial membrane. These pro-

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**FIG. 2. Caspase activation by YopH.** A, Jurkat T cells were transiently transfected with 10 μg of the indicated expression plasmids. Caspase activity was measured 48 h after transfection by analyzing the relative fluorescence units generated as a result of Ac-DEVD-AFC cleavage and then normalized for protein concentration. YopH significantly increased the *in vitro* caspase activity of Jurkat cells (*p* < 0.001, *n* = 3). B, JCaM1 cells were transiently transfected with 10 μg of the indicated expression plasmids. Caspase activity was measured 48 h after transfection by analyzing the relative fluorescence units generated as a result of Ac-DEVD-AFC cleavage normalized for protein concentration. Normal human T lymphocytes were treated with 6 μM of the indicated fusion protein, an equal volume of buffer, or 10 μM etoposide. Caspase activity was measured 24 h after treatment. YopH significantly increased the *in vitro* caspase activity of Jurkat cells (*p* < 0.01, *n* = 3). PBL, peripheral blood leukocytes.
apoptotic molecules, such as Bax, form pores in the membrane, causing breakdown of the membrane potential and release of other proapoptotic molecules from the intermembrane space (26). Therefore, to test the hypothesis that YopH expression would result in loss of mitochondrial membrane potential, we transfected Jurkat cells with empty vector, YopH, or YopH-D356A. B, representative dot plots of vector-transfected control, wild-type YopH, and YopH-D356A. Cells with intact mitochondria are positive for both FL-1 and FL-2 channels, whereas cells with no remaining intact mitochondria will lose FL-2. C, results from four independent transfections are represented graphically. YopH significantly reduces the number of cells with intact mitochondria ($p < 0.05, n = 4$).

**Fig. 3. YopH causes mitochondrial breakdown.** Jurkat T cells were transiently transfected with 10 μg of the indicated expression plasmids; 24 h later, the cells were stained with JC-1 and analyzed by FACS. A, representative digital images of vector-transfected control, wild-type YopH, and YopH-D356A. B, representative dot plots of vector-transfected control, wild-type YopH, and YopH-D356A. Cells with intact mitochondria are positive for both FL-1 and FL-2 channels, whereas cells with no remaining intact mitochondria will lose FL-2. C, results from four independent transfections are represented graphically. YopH significantly reduces the number of cells with intact mitochondria ($p < 0.05, n = 4$).

YopH-induced Cell Death Is Mitochondrially Mediated—Members of the Bcl-2 family of proteins are critical regulators of apoptosis via control of mitochondrial outer membrane characteristics. Bcl-xL, an anti-apoptotic member of this family, is able to counteract the effects of the pro-apoptotic members that
are responsible for the mitochondrial membrane permeabilization and eventual cell death (27). To test whether YopH induction of caspase activity was mitochondrially mediated, we co-transfected Bcl-xL with YopH and determined the percentage of cells with activated caspases as measured by FACS analysis of FITC-VAD-fmk-stained cells. We found that YopH caused an increase in cells positive for active caspases (Fig. 4A). More importantly, expression of Bcl-xL blocked this increase. These data implicate mitochondria as a key regulator involved in the activation of caspases in response to YopH expression.

Next, we tested whether Bcl-xL could also prevent cell death induced by YopH. Although catalytically active YopH expression induced about a 2-fold increase in the percentage of dead cells, as measured by trypan blue exclusion (Fig. 4B), co-expression of Bcl-xL blocked this effect of YopH. We also demonstrate that caspase activity is required for YopH-induced cell death as the broad spectrum caspase inhibitor BAF completely blocked the effect of YopH. We conclude from these data that the cell death induced by catalytically active YopH involves an obligatory mitochondrial step and requires caspase activity.

YopH Induces Internucleosomal Fragmentation—A typical late stage characteristic of apoptosis is internucleosomal genomic DNA degradation (28). To evaluate the ability of YopH to cause this genomic DNA fragmentation pattern, we used a nucleosome enzyme-linked immunosorbent assay kit. We found that YopH causes internucleosomal fragmentation and that this effect again is dependent on its tyrosine phosphatase activity. BAF blocked the increase in DNA fragmentation due to YopH expression (Fig. 4C).

**DISCUSSION**

In T cells, apoptosis is most commonly initiated in response to death receptor ligation resulting in activation of caspase 8 (extrinsic pathway) or cellular stress followed by mitochondrial release of cytochrome c and subsequent activation of caspase 9 (intrinsic pathway) (26, 29). However, activation of programmed cell death can be much more complex, and it is difficult to specifically implicate a single pathway responsible for the death of cells. Because of the multiple possible entry points...
points into the apoptotic execution phase, it is important to delineate the pathway of apoptotic signaling events triggered by any specific stimulus. Here, we have demonstrated that the Yersinia phosphatase YopH causes annexin V binding, caspase activation, mitochondrial membrane potential loss, and internucleosomal DNA degradation in Jurkat cells. In addition, the Lck-deficient JCaM1 Jurkat cell line is not susceptible to YopH-induced caspase activation. Bel-2 co-expression blocks the YopH-induced caspase activation, DNA degradation, and cell death. Caspase inhibition also inhibits the DNA degradation and cell death induced by YopH. Moreover, YopH expression in HeLa cells also causes cell death, and YopH-specific inhibitors block the cell death induced by YopH. These results demonstrate that YopH activates mitochondrially regulated apoptosis of Jurkat T cells.

We have previously shown that YopH targets Lck and thus reduces basal tyrosine phosphorylation as well as antigen-stimulated tyrosine phosphorylation, efficiently inhibiting antigen-induced stimulation of T cells (20). Inactivation of Lck by YopH results in loss of phosphorylation of all downstream targets, a situation that is virtually indistinguishable from a broad and indiscriminate dephosphorylation of proteins by YopH. Since most phosphotyrosine-containing proteins are continuously dephosphorylated by efficient endogenous protein tyrosine phosphatases, a sharp decline in their rate of phosphorylation following Lck inactivation will result in a rapid clearance of total tyrosine phosphorylation. The global loss of tyrosine phosphorylation may lead to either the loss of inhibition of apoptotic mechanisms or the stimulation of a proapoptotic pathway. Here, we have shown that the induction of caspase activity by YopH is abolished in the Lck-deficient Jurkat cell line JCaM1. Lck activity may be needed for survival of normal Jurkat, and inhibition of Lck by YopH may result in the stimulation of apoptotic signal transduction. In this case, the transduction of the apoptotic signal is undoubtedly mediated by signals transmitted to mitochondria, integrated by Bcl-2 family members, and carried out by caspases.

Pathogenic bacteria have evolved numerous ways of evading the innate and adaptive immune systems (16). Y. pestis has adopted one of the most successful strategies, namely a type III secretion system that injects a set of tranquilizing proteins directly into the cytoplasm of macrophages and lymphocytes (4, 8). As a result, the cells are unable to respond, and the bacteria can multiply unopposed by the normal mechanisms of host defense (6). We have previously implicated YopH as a key mediator of Y. pestis evasion of the immune response acting acutely to inhibit T cell activation (20). Here, we show that YopH induces programmed cell death in T cells. A similar induction apoptosis by another Yop virulen protein, YopJ, was reported in macrophages (30). Thus, it seems that several effectors YopS may act to kill host cells, perhaps in a manner that involves synergistic actions, e.g. by YopH and YopJ. This may be critical in vivo, where the amounts of Yop proteins injected by bacteria into host cells must be relatively small. Nevertheless, the outcome is striking: acute inhibition of signal transduction in cells of both the innate and the adaptive immune systems followed by programmed cell death of these cells before they can recover to initiate host defense.

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