Apoptosis 2003; **8**: 179–190

Potential role of the EPEC translocated intimin receptor (Tir) in host apoptotic events

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Apoptosis, or programmed cell death, is a well-ordered process that allows damaged or diseased cells to be removed from an organism without severe inflammatory reactions. Multiple factors, including microbial infection, can induce programmed death and trigger reactions in both host and microbial cellular pathways. Whereas an ultimate outcome is host cell death, these apoptotic triggering mechanisms may also facilitate microbial spread and prolong infection. To gain a better understanding of the complex events of host cell response to microbial infection, we investigated the molecular role of the microorganism Enteropathogenic *Escherichia coli* (EPEC) in programmed cell death. We report that wild type strain of EPEC, E2348/69, induced apoptosis in cultured PtK2 and Caco-2 cells, and in contrast, infections by the intracellularly localized *Listeria monocytogenes* did not. Fractionation and concentration of EPEC-secreted proteins demonstrated that soluble protein factors expressed by the bacteria were capable of inducing the apoptotic events in the absence of organism attachment, suggesting adherence is not required to induce host cell death. Among the known EPEC proteins secreted via the Type III secretion (TTS) system, we identified the translocated intimin receptor (Tir) in the apoptosis-inducing protein sample. In addition, host cell ectopic expression of an EPEC GFP-Tir showed mitochondrial localization of the protein and produced apoptotic effects in transfected cells. Taken together, these results suggest a potential EPEC Tir-mediated role in the apoptotic signaling cascade of infected host cells.

Keywords: apoptosis; EPEC; mitochondria; pedestals; stress fibers; Tir.

Introduction

The utilization of host machinery to establish and spread bacterial infection involves numerous signaling pathways, and there is growing evidence that many bacteria proceed to induce the eventual death of the infected cells via an apoptotic mechanism.[1–4] Whereas many of the early steps in pathogen interactions with host cells are known, the molecular events leading to subsequent host-cell death are poorly understood. Both Gram-positive and Gram-negative microbial pathogens have been found to trigger apoptotic events, using different pathways with possible selective survival strategies custom designed for each organism.[5,6] Some organisms seem to induce apoptotic events, whereas others activate preventive apoptotic signals.[7,8] The Gram-positive organism *Staphylococcus aureus*, a nosocomial pathogen, secretes the protein alpha-toxin thereby inducing release of cytochrome C and activating the caspase signaling pathways in host cell mitochondria that lead to apoptosis.[9] Organisms of the Gram-negative *Enterobacteriaceae* such as, *Yersinia*, translocate soluble YOP proteins that promote activation of pro-apoptotic caspase activated pathways.[10,11] Other Gram-negative organisms, such as *Helicobacter pylori, Neisseria* and *Salmonella*, also have been shown to induce apoptotic effects, whereas *Listeria* induction of apoptotic events depends on the type of host cell infected.[12–14]

Enteropathogenic *Escherichia coli* (EPEC), is a Gram-negative pathogen that causes diarrhea in a variety of animals and humans.[15–17] These organisms invade and attach to the intestinal epithelium, inducing rearrangements in the actin cytoskeleton of the host cells that lead to the eventual destruction of the epithelial microvilli.[18] Insertion of the EPEC protein, translocated intimin receptor (Tir), into the host cell membrane allows the bacteria to adhere to the host cell through the binding interaction between the host membrane anchored Tir and the EPEC membrane protein, intimin.[19] This interaction results as Tir, via a type III secretion system, is inserted in an orientation that creates three distinct protein domains. The first and third domains are intracellular to the host cytoplasm and the second domain is extracellular and binds to the EPEC intimin.[20] Beneath each attachment site, actin-rich pedestals form in the host cell with the EPEC

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riding externally atop.[21] Tir insertion into the host membrane results in interactions between its intracellular first and third domains and host cell proteins. We and others, have identified several interacting host cytoskeletal proteins.[22–25] Whereas the significance of pedestal formation to infection is poorly understood, mutants lacking the ability to induce pedestals have greatly reduced virulence.[18] Mutants lacking Tir and intimin are unable to attach efficiently and do not induce pedestals, emphasizing the importance of these bacterial proteins.

There are many potential ways that insertion of a bacterially expressed protein and subsequent alteration to the host cell membrane could induce a response leading to apoptotic events. The physical insertion alone could potentially compromise the integrity of the host cell membrane triggering multiple host warning pathways. Several reports support EPEC-induced apoptosis, but the mechanisms remain unclear.[26–28] There has also been a conflicting report indicating that apoptosis is reduced in the intestines of REPEC-infected rabbits.[8] To understand the molecular connections between EPEC and apoptotic events, we explored the potential role of EPEC Tir and apoptosis in several different cultured cell lines. Using hallmark apoptotic phenotypes of DNA fragmentation and cytoskeletal disorganization, we found EPEC-induced apoptotic effects 8–12 hours post-infection. Parallel infection of sister cultures with *Listeria monocytogenes* did not induce apoptotic effects. Whereas *L. monocytogenes* and EPEC differ in their mode of interaction with host cells, they are similar in their ability to harness and utilize host cytoskeletal molecules to achieve locomotion. Incubation of host cells' growth medium exposed to EPEC and subsequently cleared of organisms, induced apoptosis suggesting that bacterial adherence was not required for programmed cell death. Among the proteins in the medium was Tir. Finally, ectopic expression of Tir in the host cells leads to localization of Tir to the mitochondria, and apoptosis in the transfected cells. These results suggest a potential Tir-mediated role of EPEC induced apoptosis.

Material and methods

Bacterial stains, cultured cells, growth media and chemicals

Infectious bacterial strains used in this study were wild type *Escherichia coli* E2348/69, and *Listeria monocytogenes* SLCC5764. Non-infectious *E. coli* strain DH5alpha was used for cloning purposes (Invitrogen/Life technologies, Carlsbad, CA). Unless otherwise indicated, standard overnight cultures of *L. monocytogenes* and Enteropathogenic *E. coli* were grown in BHI (Brain Heart Infusion) or Luria-Bertani (LB) as described previously.[21,29] Typically, overnight cultures of bacteria were grown and subcultured to mid log phase growth. One ml of bacteria was centrifuged and suspended in 1.0 ml cell culture media, and 5 μl of the bacteria culture used to infect 35mm plates of cultured cells. Cultured cells (PtK2, Caco-2, and HeLa) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum (GIBCO BRL, Grand Island, NY). Prior to apoptosis assay experiments, cells were trypsinized and seeded at 5×10^4 cells/ml onto either 35-mm glass bottom tissue culture dishes, or 12-mm diameter, 0.13–0.17-mm-thick glass cover slips.

Staurosporine, (Molecular Probes, Inc., Eugene, OR) was used as a positive control for induction of apoptosis. A final concentration of 1.0 μM was added to the cell media and the cells incubated between 20 minutes and 4 hours. Rhodamine labeled phalloidin was purchased from Molecular Probes Inc. (Eugene, OR). Anti-Tir I was prepared as described by Freeman *et al*., 2000. Anti-GST antibody was a gift from Dr. Margaret Chou (University of Pennsylvania School of Medicine). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Quantitation of DNA fragmentation

Strains of bacteria (EPEC E2348/69, *L. monocytogenes*) were allowed to infect dishes of cultured cells for various incubation times. Growth medium was aspirated, the cells washed with fresh medium and the cultured cells fixed using standard TUNEL cell fixation procedures for the APO-BRDU™ (Pharmingen, San Diego, CA) or APO-Direct kit™ (Molecular Probes, Inc., Eugene, OR), using manufacturer's recommendations. Apoptosis induces the fragmentation of DNA into pieces as short as 200 base pairs.[30] Many 3′-hydroxyl ends are present in the DNA as a result of the large number of DNA fragments that occur in apoptotic nuclei. Bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) adds on to the free 3′-hydroxyl ends.[31] Apoptotic nuclei can then be identified by staining the DNA with anti-BrdU monoclonal antibody that is covently conjugated to fluorescein. Cultures infected with *L. monocytogenes* were treated 1.5 hrs post infection times with gentamycin (10 μg/ml, GIBCO BRL, Grand Island, NY) to kill residual organisms, washed, cultured for 4 hrs, and fixed as described above. Minor modifications to the protocol were made for use with adherent cultured cells as opposed to cell suspensions, and the use of propidium iodide/RNase was omitted. All infection incubations were done at 37°C, with one dish of cells left uninfected as a negative control, and one dish exposed to staurosporine as a positive control. Multiple trials were performed and numbers of apoptotic cells quantified using Microsoft Excel.

For assessment of induced apoptosis by secreted proteins, EPEC (E2348/69) were grown to an optical density

at 600 nm of ~0.8 in protein-free DMEM. The bacterial growth medium was centrifuged twice, and the supernatant processed through a second 0.22 micron filter (Nalgene™, Rochester, NY), balanced to pH of 7.4, and processed again through a 0.22 micron filter (Nalgene™, Rochester, NY). Cultured cells were incubated with the filtered, and bacteria-cleared medium for various periods of time. Multiple trials were performed with the filtered protein-free medium and each cultured cell line. Prior to treatment, cultured cells were rinsed 5× with protein free DMEM to remove residual protein from the cell media. Similar controls as described above were performed with each assay, and treated cultured cells were fixed and assayed for apoptotic nuclei as described above.

Fractionation and concentration of EPEC secreted proteins in protein free medium

For protein analysis of EPEC secreted proteins, 600 ml of EPEC (E2348/69) growth medium was processed. Freshly inoculated protein free DMEM media was incubated with wild type EPEC at 37°C with agitation for ~20 hours. The media was centrifuged at 1500 × g, and the supernatant filtered using a 500 ml Stericup 0.22 micron filter (Millipore Corp., Bedford, MA). Aliquots of 200 ml of the filtered media were dialyzed twice overnight against 2 liter volumes of stabilizing buffer (30 mM sodium phosphate monobasic, pH 7.5, 1% sodium azide/1% Pen-Strep antibiotic). Fractions intended for apoptosis assays on living cultured cells were dialyzed against 500 ml of phosphate buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl, adjusted to pH 7.4). For concentration of the EPEC secreted proteins, the dialysis bag was immersed in Aquacide (Calbiochem Inc., San Diego, CA). Trichloroacetic acid (TCA) was added to a sample of the protein fraction and the pellet suspended in SDS sample buffer BioRad, LaJolla CA). Proteins were separated on 12.5% SDS/PAGE and either stained with Coomassie Blue, for visual viewing of the proteins, or electrophoretically transferred to nitrocellulose for western blot analysis. Protein aliquots were assayed for the ability to induce apoptotic activity at concentrations of total protein 0.03 mg, 0.06 mg, 0.12 mg, or 0.24 mg. Protein samples retaining apoptotic activity were further fractionated to eliminate low molecular weight metabolites and other molecules by centrifugation in a 10 K Centricon® centrifugal filters (Millipore, Danvers, MA). Cultured cells were incubated with the protein fractions at 37°C for 24 hours, and then fixed and assayed for apoptosis as described above. Concentrated proteins were separated by 12.5% SDS/PAGE and electrophoretically transferred to nitrocellulose for western blot analysis.

Tir eukaryotic expression constructs

Full length Tir was cloned into *Hind*III/*Bam*HI (New England Biolabs, Inc., Beverly, MA) digested pEYFP N-1 (Clonetech/BD Biosciences, Palo Alto, CA) using primer sets (restriction sites underlined); 5′: AAGCTTATGCCTATTGGTAA and 3′: GGATCCCGAACGAAACGTACTGG for polymerase chain reaction (PCR) amplification of full length Tir (1658 base pair) using Vent polymerase (New England Biolabs, Inc., Beverly, MA) and EPEC genomic DNA.[23] Tir, expressing the second and third domain (pGST-eTir2-3) was amplified using primer sets 5′: AAGCTTATGCAGGCGTTGGCTTTGACA and 3′: GGATCCCGAACGAAACGTACTGG, and subcloned into the eukaryotic expression vector pEBG.[32] Both constructs (pTirFL-YFP and pGST-eTir2-3) were transformed into DH5alpha, and the plasmids purified and sequenced (Napcore, The Children's Hospital of Philadelphia, Philadelphia, PA). Host cells were transfected with the plasmids using Lipofectamine 2000 (Invitrogen/Life Technologies, Carlsbad, CA), and insoluble and soluble protein cell fractions analyzed by 12% SDS/PAGE, transferred to nitrocellulose and analyzed by Western blot for an expressed fusion protein of predicted size using an anti-Tir1 antibody,[23] and a biotin-labeled anti-rabbit secondary antibody (Molecular Probes, Beverly, MA) and visualized with chemiluminescence (Amersham, Piscataway, NJ). Bacterially expressed and purified GST-Tir1 and GST-Tir3 were prepared as described by Freeman *et al*.[23]

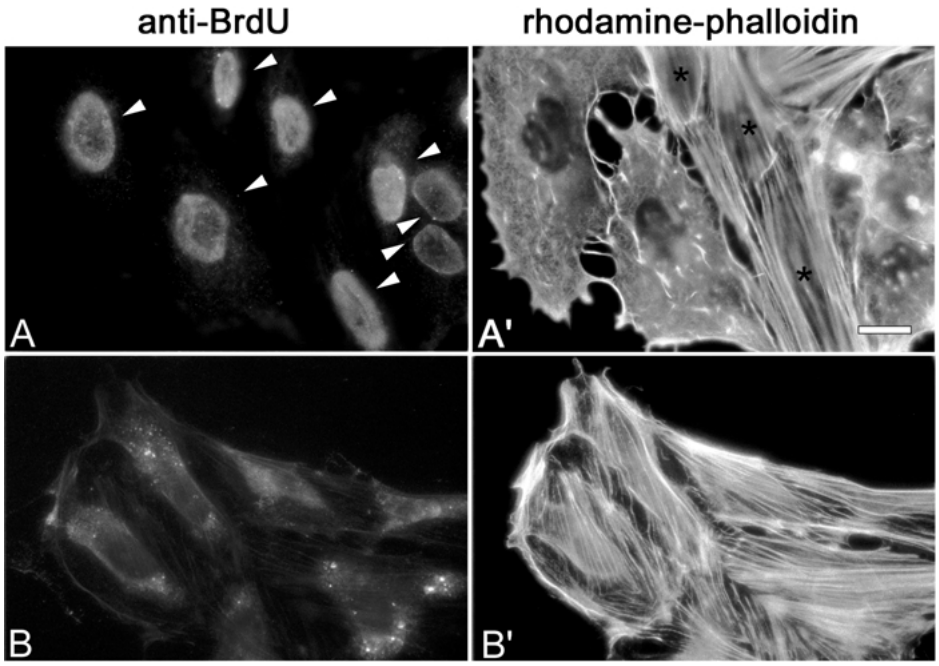
Assessment of mitochondrial localization

Transfected TirFL-YFP was visualized in live and fixed cells for localization. Co-localization to the mitochondria was visualized with MitoTracker® (Molecular Probes, Inc., Eugene, OR) using manufacturers recommendations. Briefly, TirFL-YFP transfected cultures were incubated with 1 μM (final concentration) MitoTracker® and visualized live after a 20 minute incubation. Transfected cells were assessed for apoptotic effects as described above using APO-Direct tunnel kits (Molecular Probes, Inc., Eugene, OR). Fluorescent images of live cells were obtained using a Nikon Diaphot 200 inverted fluorescence microscope.

Immunfluorescence, labeling of actin cytoskeleton, and microscopy

Images of fixed cells were obtained as previously described.[21] Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.01% IGEPAL (Sigma, St. Louis, MO) and incubated with phalloidin-tetramethyl rhodamine B isothiocynate conjugate (Fluka Biochemicals, Ronkokoma, N.Y.) and mounted in Mowiol.

Figure 1. (**A, B**) Apoptotic and control cells. Detection of apoptotic DNA fragmentation following ultraviolet radiation (UV) exposure. Cultured PtK2 cells were exposed to UV (at wavelength 254 nm) radiation using the Stratalinker UV crosslinker. Apoptotic nuclei were detected using a standard TUNEL kit which includes bromolated deoxyuridine triphosphate nucleotide (Br-dUTP) and a fluorescently labeled antibody that recognizes Br-dU incorporated into DNA fragments generated during apoptosis (**A**; arrows). Co-staining with rhodamine phalloidin revealed disruption to the actin cytoskeleton in the majority of cells (**A′**). Three cells with BrdU-positive nuclei still have a normal disposition of actin fibers (black astrisk). Untreated PtK2 control cells have no fluorescent nuclei with non-specific cytoplasmic fluorescence (**B**) and a normal, intact actin cytoskeleton as seen by co-staining with rhodamine phalloidin (**B′**). Scale bar = 20 μm.



Images of double-labeled cells were obtained as previously described.[33] Fluorescent images were obtained using a Nikon Diaphot 200 inverted fluorescence microscope. Photographic images were assembled using Metamorph (Universal Imaging Corp., West Chester, PA) and Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

Results

EPEC induced DNA fragmentation and cytoskeletal effects

The cleavage of genomic DNA into numerous, random sized fragments is characteristic of apoptotic cell death.[34–37] This event is measurable by treating cells with a BrdU-nucleotide and detecting its incorporation into DNA fragments with a fluorescently labeled anti-BrdU antibody. To standardize detection of apoptotic cells, we exposed cells to ultraviolet radiation (Figure 1) and staurosporine treatment, both well-established to induce programmed cell death. Changes in the actin cytoskeleton in apoptotic cells can be seen when fluorescent phalloidin is used together with antibody detection of BrdU incorporation (Figure 1; A and A′). Control cells did not display apoptotic nuclei or cytoskeletal alteration (Figure 1; B and B′). Cultures of Caco-2, the human colon carcinoma line, or the renal epithelial PtK2 cells from rat kangaroo showed similar apoptotic effects when infected with EPEC. Nuclei showed BrdU incorporation, and the actin cytoskeleton was disrupted (Figure 2; A; a, a′). Infection of

Figure 2. PtK2 cells infected with (**A; a** and **a′**) EPEC (E2348/69) and (**A; b, b′**) *L. monocytogenes*. PtK2 cells infected with EPEC (E2348/69) and assayed for apoptotic nuclei. An 8 hour infection of PtK2 cells with EPEC showed apoptotic nuclei in the host cells (**A; a**), The actin network of same cells, fixed and stained with rhodamine phalloidin showed staining of the polymerized actin in EPEC pedestals (white arrows) with complete disruption of actin network (**A; a′**). No apoptotic nuclei were observed in PtK2 cells incubated with *L. monocytogenes* for six hours. The bacteria, however, stain positively for BrdU incorporation. In trials for 4, 6, 8, 10, 12, and 24 hours apoptotic nuclei were not seen in PtK2 nuclei (**A; b**). Cells co-labeled with rhodamine phalloidin display the typical actin comet tails associated with *L. monoctyogenes* (white arrows), as well as an actin cytoskeleton that becomes increasingly disrupted with time of infection (**A; b′**). Scale bar = 20 μm. (**B**) Summation of extent of apoptosis at selected time points each representing four trials with PtK2 cells and EPEC. After 10 hours of infection many cells detached from the substrate and were lost. The remaining cells were enriched for non-apoptotic cells. Data point symbols for each trial are indicated in the enclosed box.

host cells with *L. monocytogenes* also induced rearrangement of the actin cytoskeleton in the host cell and association of actin filaments with the bacteria. There was no indication from BrdU incorporation, however, that *L. monocytogenes* induced apoptotic effects (Figure 2; A; b, b′). As infection times increased and more bacteria attached, most host cell actin filament arrays disassembled and actin was concentrated beneath the attached EPEC. These apoptotic effects were completely abolished when EPEC was chemically inactivated using 3.8% paraformaldehyde.

Despite the variability in the time when apoptotic nuclei were detected, all trials resulted in apoptosis

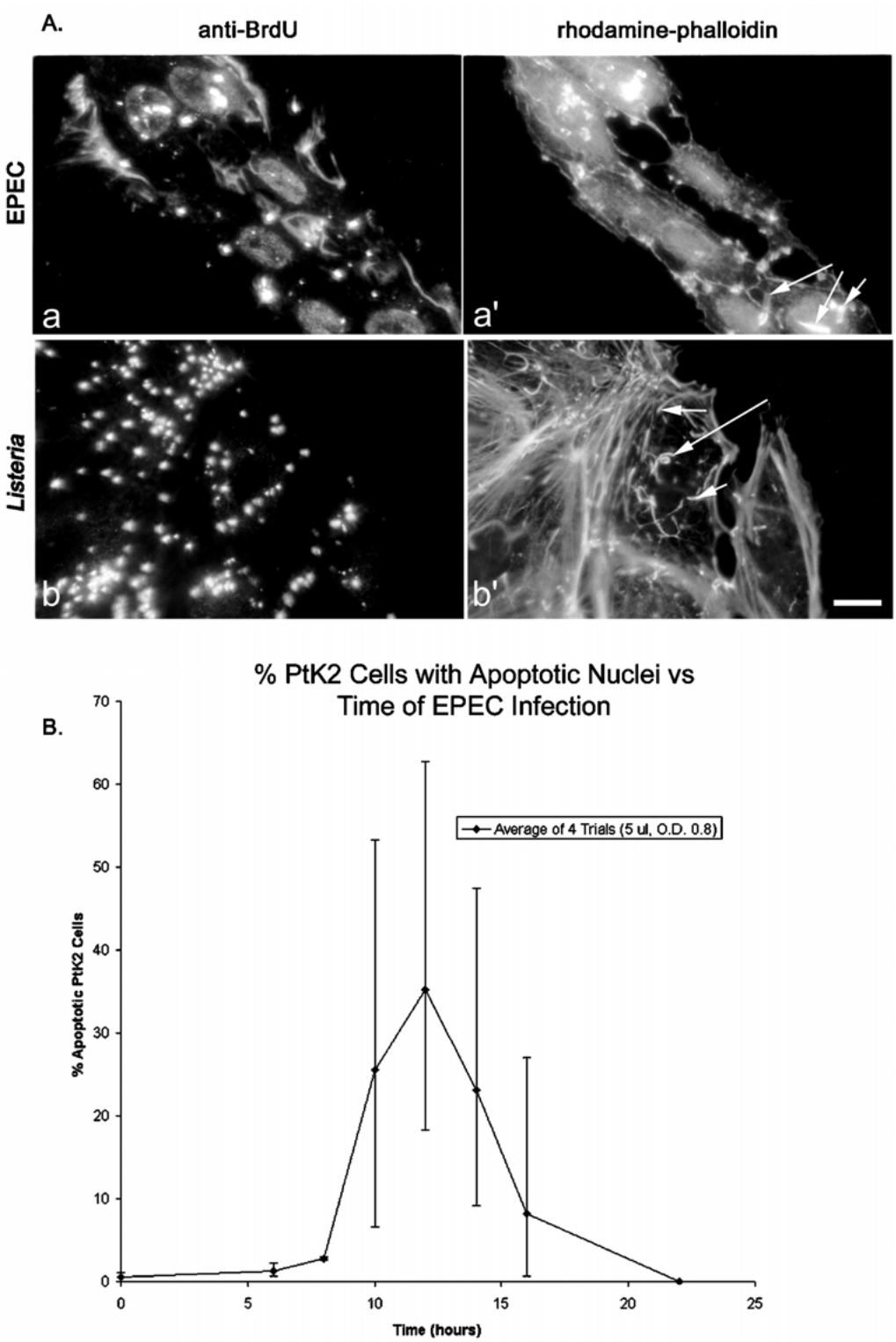
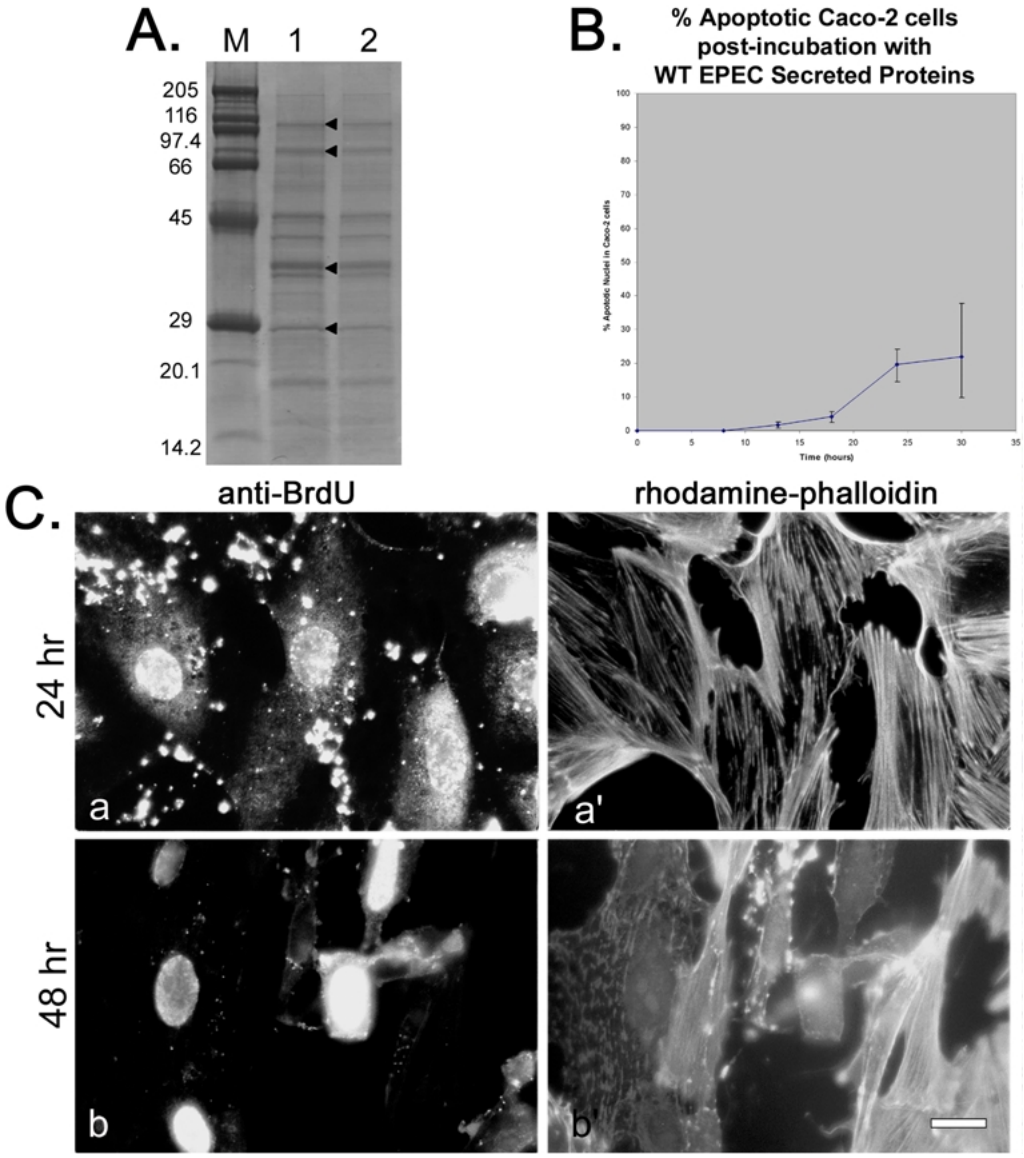


Figure 3. Bacterial adherence is not required for EPEC-induced apoptosis. Media in which EPEC were cultured and then cleared of organisms induced apoptotic effects. (**A**) Proteins from the conditioned medium were concentrated and analyzed by 12.5% SDS/PAGE and Coomassie Blue. Lanes 1 and 2 indicate protein profiles from ~45 μg and ~30 μg, respectively, of concentrated bacterial proteins. The profile is similar to previously published EPEC secreted protein profiles. Arrows indicate bands corresponding to the mobility's of previously reported proteins: EspC (110 kDa); Tir (56.8 kDa; gel mobility ~78 kDa); a triplet containing EspB, EspD, and a homolog of *E. coli*'s GAPDH (37–40 kDa); EspA (25 kDa).[60] Lane M indicates protein markers. (**B**) Percentage of apoptotic nuclei in Caco-2 cells treated with EPEC conditioned medium for the indicated time periods. Graph represents a standard sampling of four experiments, using Caco-2. (**C**) Apoptosis in PtK2 cells incubated for 24 hours with EPEC-conditioned medium. Equal volumes of medium containing either 0.12 mg total protein (**C; a, a′**) or 0.24 mg total EPEC-secreted protein (**C; b, b′**). Cultured cells incubated with medium unexposed to EPEC proteins did not show apoptotic nuclei (data not shown). Scale bar = 20 μm.



(Figure 2; B). The BrdU assays indicated that EPEC induced apoptosis in both PtK2 and Caco-2 cells.

EPEC-secreted proteins can induce apoptosis

EPEC, cultured in BHI medium, are known to secrete a number of proteins, several of which have been identified and characterized (Figure 3; A).[19] To address whether these proteins were capable of inducing apoptotic effects in the absence of bacteria, cultured cells were incubated with growth medium after EPEC had been removed. Following incubation with the EPEC-conditioned medium for 24 hours, 33% of PtK2 cells had detectable apoptotic nuclei. This effect was proportional to the protein concentration of the conditioned medium applied, suggesting

that a higher concentration of EPEC secreted proteins was responsible for the apoptotic effects. The majority of the apoptotic cells also displayed actin cytoskeletal disassembly. Similar results were obtained with Caco-2 cells. The average percentage of apoptotic Caco-2 nuclei detected from 4 trials at similar time points are shown in Figure 3; B. The error bars indicate the highest and lowest percentages of apoptotic nuclei detected in multiple trials of separate cultures at the stated time point within the same trial. By 30 hours, significant numbers of cells had been lost. Treatment with conditioned medium also induced actin disassembly, though this occurred at a much slower rate than with live bacterial infection. Treated PtK2 cells (Figure 3; C) clearly showed apoptotic nuclei (Figure 3; C; a and b) and actin cytoskeletal disorganization that increased with increasing amounts of conditioned medium (Figure 3; C; a′ and b′). Cultured cells treated with growth medium, unexposed to EPEC, or exposed to chemically inactivated bacteria, failed to display apoptotic nuclei (data not shown). Taken together, these results suggest live, adherent EPEC are not required for induced apoptosis.

Tir is present in apoptotic inducing protein fractions

To identify component(s) capable of triggering apoptotic events in the absence of bacterial adherence, we concentrated and partially purified the EPEC-secreted proteins further. Small molecules less than 10 kDa, were eliminated by size exclusion filtration. Western blot analysis of the filtered sample using an anti-Tir1 antibody,[23] identified an immunoreactive band at the predicted molecular weight (Figure 4; A, lane 5-arrow). Exposure of cultured Caco-2 cells to EPEC secreted protein sample induced similar apoptotic effects (Figure 4; B; b and b′), although increased protein concentrations and longer sample incubation periods were needed to observe similar numbers of apoptotic nuclei and alteration of the actin cytoskeleton. Caco-2 cells incubated with DMEM (Figure 4; A; lane 6) showed no apoptotic nuclei and no aberrant organization to the actin cytoskeleton (Figure 4; B; a and a′).

Mitochondrial localization of Tir

To assess the functional relationship between Tir and programmed cell death events specifically, we expressed Tir

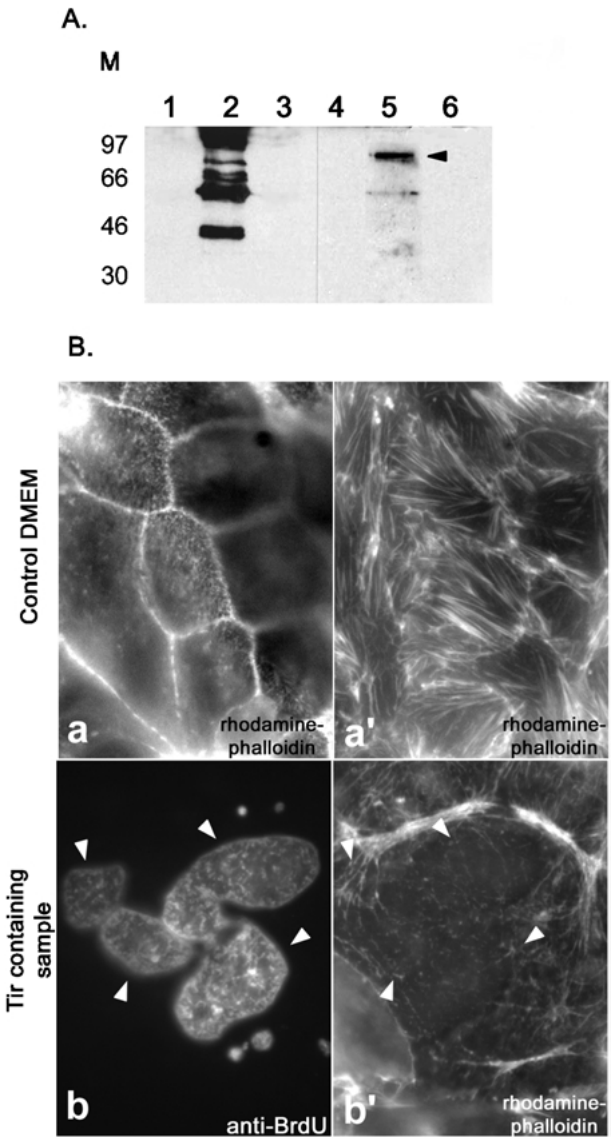


Figure 4. (**A**) Western blot of Tir in EPEC-conditioned medium. Medium was filtered using Centricon® 10 Kd filter units to remove molecules smaller than 10 Kd. The medium was then concentrated and the proteins were separated by SDS/PAGE, electrophoretically transferred to nitrocellulose and incubated with an anti-Tir1 polyclonal antibody. Lane 1. Bacterially expressed and purified GST (∼.5 μg total protein); Lane 2. Bacterially expressed and purified GST-Tir 1 (∼.5 μg total protein); Lane 3. Bacterially expressed and purified GST-Tir3 (∼.8 μg total protein). Multiple bands visualized in Lane 2 are a result of degradation of the fusion protein. Lane 4. Chicken gizzard extract (∼15 μg total protein); Lane 5. Concentrated EPEC (E2348/69) secreted protein (∼.15 μg total protein); Lane 6. Concentrated DMEM (∼10 mg/ml). M indicates protein marker sizes. Arrow indicates an immunoreactive band corresponding to Tir. (**B**) Caco-2 cells exposed to aliquots of the concentrated, partially purified protein sample, containing Tir, for 24 hours. Control Caco-2 cells were incubated with protein-free DMEM growth medium fractionated and concentrated in parallel with the EPEC conditioned-medium. Control cells showed no apoptotic nuclei, and two different focal planes identified normal microvilli and stress fibers (**B; a** and **a**′). In contrast, Caco-2 cells incubated with the concentrated EPEC conditioned-medium show apoptotic nuclei (**B; b**, arrows) and a disrupted actin network in the corresponding cells (**B; b**′, arrows). Scale bar = 20 μm.

ectopically from two different eukaryotic expression vectors (Figure 5; A): one a full length Tir fusion protein tagged with a fluorescent YFP (TirFL-YFP) for live cell visualization, and another truncated Tir fragment missing the Tir1 domain fused to a GST tag (GST-eTir2-3). The YFP construct expressed a fusion protein in transfected cells of predicted mobility (Figure 5; B; lane 6, 7) from both soluble and insoluble protein fractions. Both TirFL-YFP (Figure 5; C; TirFL-YFP and merge panels) and GST-eTir2-3 (data not shown) localized to the mitochondria, as co-localized with MitoTracker® (Figure 5; C; merge panels). In cells transfected with the YFP vector plasmid alone, the expressed YFP was diffuse and did not localize to mitochondria (Figure 5; D; YFP panel) as detected by MitoTracker (Figure 5; D; MitoTracker panel).

DNA fragmentation in cells with mitochondrially localized Tir

In order to examine if apoptotic activity was associated with the ectopic expression of Tir, we analyzed PtK2, Caco-2, and HeLa cells expressing the truncated Tir, GST-eTir2-3, which also localizes to mitochondria (not shown). The cells transfected with GST-eTir2-3 were fixed and stained with anti-GST and anti-BrdU antibodies. In Figure 6, three merged panels show Tir-transfected cells with apoptotic nuclei (arrows). About 30% of transfected cells had apoptotic nuclei as compared with $<\sim 2\%$ in vector alone transfections. Not all transfected cells displayed apoptotic nuclei, but apoptotic nuclei were found only in transfected cells. Other transfected cells had normal morphology and mitochondrial localization (Figure 6; arrows with asterisk). These data suggest apoptotic effects result from intracellularly expressed Tir, and pose a Tir dependent mediating role in EPEC induced apoptosis.

Discussion

Programmed cell death is emerging as a trademark effect of bacterial pathogenesis with evidence also accumulating that some bacteria can prevent apoptotic mechanisms.[14,38,39] Three reports have demonstrated that EPEC induce pro-apoptotic events in host cells, and we present evidence consistent with those observations.[26–28] Two reports demonstrated EPEC-induced apoptosis, but did not find that the apoptotic events were reproduced when uninfected cells were incubated in medium in which EPEC were cultured and subsequently removed.[26,28] In one of these studies HeLa and a human colon carcinoma cell line, T84,[28] were exposed to the EPEC-conditioned filtrate for 6 hours. Our experiments demonstrated filtrate exposure was needed for more than 6 hours (Figure 3B) before apoptosis could be detected. In the other study[26] using HeP2, HeLa, and Caco-2 cells, it is unclear how the filtrate was prepared or the length of incubation periods with uninfected cells. Thus, while both of these reports demonstrated EPEC-induced apoptotic effects, they did not find any specific EPEC-secreted proteins to be putative inducers of apoptosis. The suggestion in this study of a potential role for Tir in apoptosis is based on its presence in EPEC-conditioned filtrates that induce apoptosis, its localization to mitochondria when ectopically expressed in host cells, and the fact that cells transfected with mitochondrial localized Tir exhibit apoptosis.

Tir is a bacterial protein secreted via a Type III secretion apparatus.[40,41] It is one among several encoded proteins including, intimin, CesD, EspB/EspD and CesT (Tir specific chaperone molecule) of the LEE (locus for Enterocyte Effacement) pathogenicity island.[42] Upstream from chromosomal Tir is an open reading frame encoding, Map (mitochondrial associated protein, formerly ORF19), an effector molecule that localizes to the host cell mitochondria. There is no evidence of a direct binding of Tir and Map, but cellular fractionation studies of mitochondrial fractions that contain Map, also contain Tir.[43] This would suggest host cell compartmentalization of Tir to regions other than the plasma membrane. Although the predicted Map sequence contains features suggestive of mitochondrial import proteins, the Tir amino acid sequence does not. This is consistent with the lack of predicted membrane signal in several pathogen-encoded, mitochondrially localized proteins.[43,44] At present, we have not detected Tir localization in mitochondria cells infected with EPEC with immunocytochemistry with Tir1 antibody. Relatively low levels of Tir are expressed in comparison to the other Lee effectors, and specific mechanisms of Tir insertion into host plasma membrane are not clear.[19,45–47] In view of the induction of apoptosis in cells where ectopically expressed Tir localized to mitochondria, it may be possible that during EPEC infection a subpopulation of Tir becomes compartmentalized to these organelles. Our data indicate that this association does not require the Tir 1 domain. Localization after EPEC infection may also be difficult to detect given the probable low amount of mitochondrial association of Tir and the morphological changes the cell undergoes during apoptotic events.

There are several examples of bacterially encoded effector proteins targeting the mitochondria. *Listeria* ActA and *Neisseria* PorB have both been reported to be mitochondria-associated.[14,44,48–50] *Neisseria*, depending on the species can either induce or prevent programmed cell death, but *Listeria* induction of apoptosis appears to be cell type specific.[14,51] *Helicobacter pylori*, a pathogen of gastroduodenal diseases, has also been shown to induce apoptosis.[13] *H. pylori* releases a vacuolating cytotoxin Vac A, which gets cleaved into an N-terminal (34 kDa) and

Figure 5. Mitochondrial localization of EPEC Tir. (**A**) Illustration of two Tir constructs: full length Tir cloned into the pEYFP eukaryotic expression vector (**A**, top half of figure) and a truncated Tir encoding the 2nd and 3rd domain of Tir in a eukaryotic GST expression vector (**A**, bottom half of figure). (**B**) Western blot of protein lysates. Cells (PtK2, Caco-2 and HeLa) were transfected and protein lysates assayed with either a polyclonal anti-Tir 1 antibody (shown) or anti-GST polyclonal antibody. The TirFL-YFP construct (shown) expressed a fusion protein of predicted size as well as the GST-eTir2-3 (not shown). Lane 1. Bacterially expressed and purified GST-Tir 1; Lane 2. Bacterially expressed and purified GST; Lane 3. YFP vector alone transfected cell lysate; Lane 4. TirFL-YFP transfected cell soluble lysate; Lane 5. TirFL-YFP transfected cell insoluble lysate. Bacterially expressed and purified GST-Tir1 has an aberrant mobility due to degradation of the purified protein (Lane 1). Both cytosol and membrane fractions were assayed to ensure detection of the fusion protein (Lanes 4 and 5). (**C**) Intracellular localization of full length Tir (**C**; TirFL-YFP panel, arrows). Transfected cells are distinguished by comparing TirFL-YFP panels and MitoTracker® panels (**C**; MitoTracker® panels). Merged panels indicate co-localization of Tir and host mitochondria displaying yellowish-green cells (**C**; merged panel). (**D**) YFP expression alone gives a diffuse pattern (**D**; YFP panel, arrows) and merged panels do not indicate mitochondrial co-localization (**D**; merged panel). Scale bar = 20 μm.

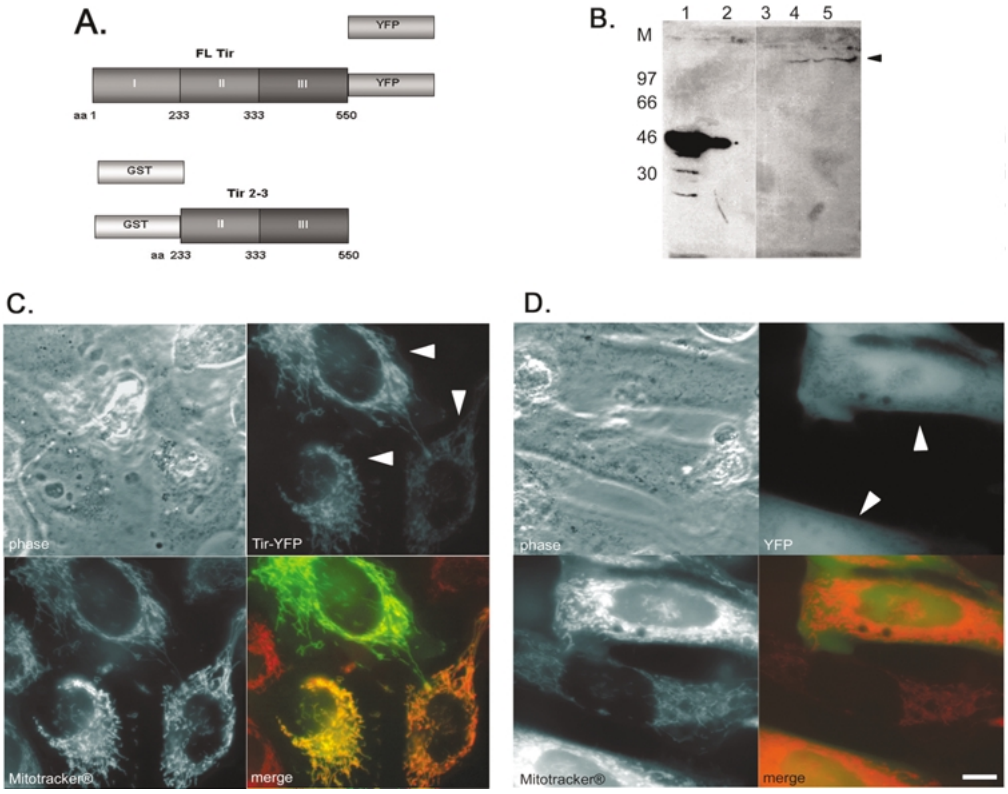


Figure 6. Ectopically expressed Tir induces apoptotic effects. Three separate experiments illustrating PtK2 cells transfected with GST-eTir2-3, fixed and assayed for apoptotic effect, are shown. BrdU is detected with a fluorescein conjugate (green color) and Tir2-3 is detected using the polyclonal GST antibody and a secondary Texas Red conjugate (red color). The three panels show merged images of apoptotic nuclei (arrows) in transfected cells. Transfected cells showing no apoptotic nuclei (arrows containing "*") also retain a more normal morphology. Scale bar = 20 μm.

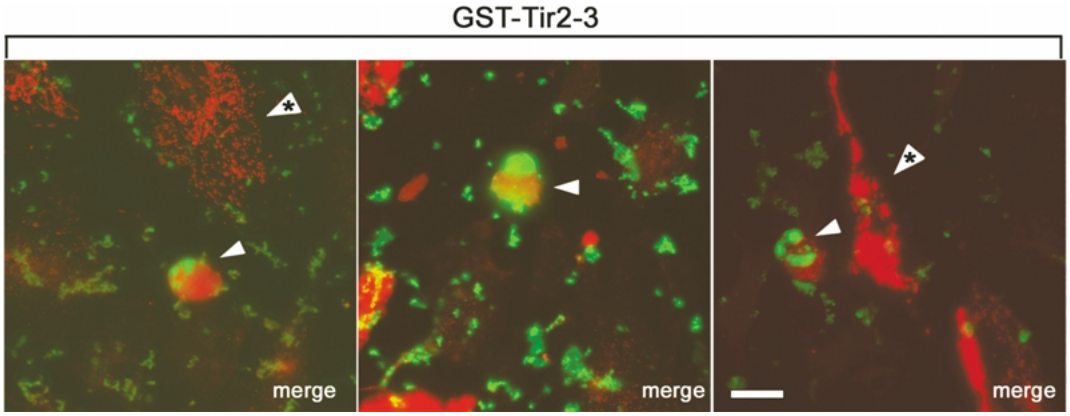
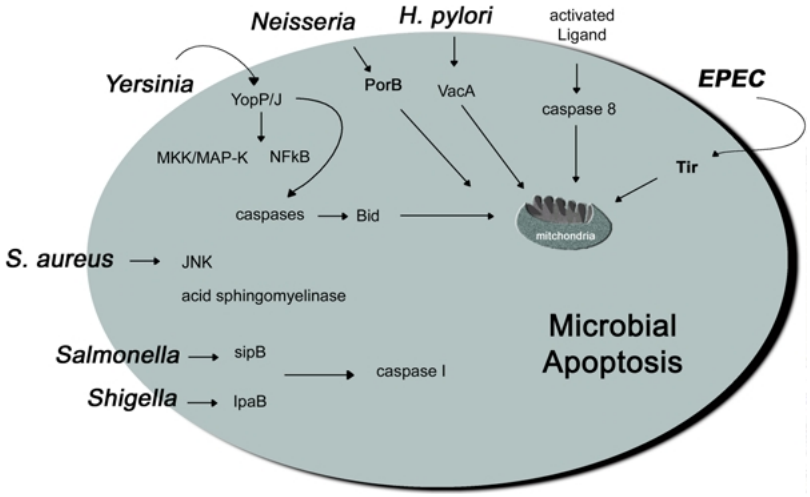


Figure 7. Microbial apoptosis. Illustration of several organisms and potential pathways associated with programmed cell death activities.



C-terminal (58 kDa) subunit. This N-terminal subunit gets targeted to the mitochondria and induces characteristic apoptotic events.[52,53] Overall, these data suggest the ability of bacterial pathogenesis to manipulate the centralized role of mitochondria in programmed cell death mechanisms (Figure 7).[38]

Bacterial infection, in general, may be simply a trigger that increases processes normally underway in the host. It is known that the lining of the small intestine is renewed in animals over several days. This turnover of cells is thought to be a tissue response to the harsh environment of digestive enzymes and the resident bacteria in the gut. The cells are lost near the tips of the villi by a combination of shedding and apoptosis.[54,55] The rate of renewal of the intestinal lining can be detected by labeling the stem cells at the base of the villi with tritiated thymidine. Autoradiographs of these cells at different time points show that progeny of the mitotic stem cells move along the villi to exit at the tips of the villi.[55] The time for movement by the differentiating cells to the villus tip is faster when bacteria are present. In germ-free mice, the transit time for the renewal of the intestinal lining is about four days, and in mice with a normal microflora, the transit time is about two days.[56,57] In rats infected with *S. typhimurium* and in pigs infected with a gastrointestinal virus, the transit times were faster than in uninfected animals.[58] These data are consistent with a bacterially mediated stimulation of normal host apoptosis that facilitates the elimination of invading microbes. Hall *et al*. (1994) have advanced the hypothesis that apoptosis is the major process that is responsible for the renewal of the intestine and that the extent of it is underreported because the macrophages digest the cells before apoptotic nuclei can be detected using histological staining assays.[59] The attachment of EPEC to an apoptotic cell destined to be shed would tie the bacteria to the cell's fate of phagocytosis by macrophages or flushing from the intestine. The ability of EPEC to translocate on the surfaces of infected host cells might enable the pathogen to move from an apoptotic cell to an adjacent uninfected cell, however cell to cell transfer has not been observed.[21]

In contrast to this report and several previous reports on EPEC-induced apoptosis in tissue culture cells,[26–28] there is a single report that apoptosis was not promoted in rabbits infected with rabbit EPEC (REPEC).[8] Heczko *et al*. (2001) suggested their results may indicate an actual reduction in the rate of apoptosis in the REPEC infected ileum and ileal Peyer's Patches. It is possible that in the complex *in vivo* system multiple intersecting cellular responses result in an outcome very different from that in a homogeneous population of cultured cells. An alternative interpretation could also be taken when comparing the images of the REPEC-infected ileum with the uninfected ileum arguing the reduced height of the villi seen in the infected ileum is due to loss of apoptotic cells that, *in vivo,* in the animal, are cleared by immune cells or lost in a general flushing of the intestine (diarrhea). Hall *et al*. (1994) have reported the clearing of cells undergoing apoptosis takes place in about one to two hours. It may be that the REPEC infected animals lost approximately two-thirds of their villous lining, prior to the histochemical apoptotic assays being performed. This interpretation would explain the differences the four reports (including this report) using a tissue culture model where apoptotic measurements were taken every few hours versus a collection of samples over days and weeks in the intact animal.[8]

Acknowledgments

The first two authors made equal contributions to this manuscript. This work was supported by a grant from

the United States Department of Agriculture to JMS (Research Institute Competitive Grants Program of the USDA, agreement 99-35204-7864).

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