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Meningococcal Porin PorB Prevents Cellular Apoptosis in a Toll-Like Receptor 2- and NF-κB-Independent Manner▽

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Meningococcal porin PorB is an inhibitor of apoptosis induced via the intrinsic pathway in various cell types. This effect is attributed to prevention of mitochondrial depolarization and of subsequent release of proapoptotic mitochondrial factors. To determine whether apoptosis is globally inhibited by PorB, we compared the intrinsic and extrinsic pathways in HeLa cells. Interestingly, PorB does not prevent extrinsic apoptosis induced by tumor necrosis factor alpha plus cycloheximide, suggesting a unique mitochondrial pathway specificity. Several intracellular factors regulated by NF-κB, including members of the Bcl-2 family and of the inhibitor of apoptosis (IAP) family, play major roles in controlling apoptosis, and some of them are thought to contribute to the antiapoptotic effect of the gonococcal porin, PIB. However, most of the members of the Bcl-2 family and the IAP family are not induced by meningococcal PorB in HeLa cells, with the exception of Bfl-1/A1. Interestingly, PorB does not induce NF-κB activation in HeLa cells, likely due to a lack of Toll-like receptor 2 (TLR2) expression in these cells. Bfl-1/A1 expression is also regulated by CBF1, a nuclear component of the Notch signaling pathway, independent of NF-κB activation. Since HeLa cells are protected by PorB from intrinsic apoptosis events, regardless of TLR2 and NF-κB expression, the possibility of a contribution of alternative signaling pathways to this effect cannot be excluded. In this paper, we describe an initial dissection of the cascade of cellular events involved in the antiapoptotic effect of PorB in the absence of TLR2.

Apoptosis, or programmed cell death, is characterized by morphological events, including membrane blebbing and nuclear and chromatin condensation, and by intracellular events, such as activation of cytosolic proteins and DNA degradation (26). A variety of different intracellular stress signals can trigger apoptosis, including bacterial infections, excessive calcium, chemical substances, DNA-damaging agents (intrinsic or mitochondrial pathway), and cell surface death receptor activation (extrinsic pathway). Both pathways are divided into three basic phases: (i) initiation, (ii) commitment, and (iii) execution, ending with cell death (71).

Several intracellular protein families, such as the Bcl-2 family (40), caspases (8), and the inhibitors of apoptosis (IAPs) (16), play important roles in controlling apoptosis. Bcl-2 proteins have a dual role; they trigger apoptosis (Bax, Bak, and Bid [1]) or block it (Bcl-2, Bcl-xL, Bfl-1, and Mcl-1 [39]). Proapoptotic Bcl-2 proteins can induce release of mitochondrial factors, including cytochrome *c* (36), apoptosis-inducing factor (AIF) (70), and Smac/DIABLO (18), in both a mitochondrial membrane potential-dependent manner and a mitochondrial membrane potential-independent manner (2, 25, 74). These events lead to activation of caspase 9 and 6 (intrinsic pathway) and subsequent DNA degradation. Alternatively, proapoptotic Bcl-2 proteins can also directly activate caspase 8 (extrinsic pathway) (1, 68), but the two pathways converge at a downstream event, caspase 3 activation (24). Antiapoptotic Bcl-2 proteins act mostly by modulating mitochondrial functions directly by interacting with mitochondrial components of the permeability transition pore or indirectly by neutralizing proapoptotic Bcl-2 proteins (7, 69, 72). IAPs are a family of proteins that directly inhibit caspase activation (16, 32) and, similar to Bcl-2 proteins, are also regulated by NF-κB (9).

Modulation of apoptosis by several intracellular and extracellular bacteria, mostly to avoid normal host defense responses, has been described previously. Many bacteria induce and/or prevent apoptosis, depending on the host cell type, growth conditions, or bacterial life cycle. Some examples of bacteria that inhibit apoptosis are *Chlamydia* (20, 22, 79), *Shigella flexneri* (11), *Brucella* (28), *Porphyromonas gingivalis* (56, 58), *Neisseria meningitidis*, and *Neisseria gonorrhoeae* (4, 23, 31, 42, 50, 55, 62, 65, 73). Our group and other workers have reported that live *N. meningitidis* and purified meningococcal porin inhibit apoptosis (15, 49, 50, 62, 75), potentially via multiple mechanisms. While meningococcal infection induces NF-κB-mediated upregulation of antiapoptotic genes, purified PorB and PorB from live bacteria directly interact with mitochondria and modulate their membrane potential, preventing release of cytochrome *c*. *N. gonorrhoeae* and purified gonococcal porin PIB induce NF-κB-mediated upregulation of antiapoptotic genes (4, 5, 23, 33, 55, 65), which could also contribute to prevention of apoptosis.

A correlation between the antiapoptotic effect of PorB and activation of NF-κB has not been shown so far, although our group has demonstrated that PorB activates NF-κB in a Toll-like receptor 2 (TLR2)-dependent manner (43, 46, 48, 52). Interestingly, various human and murine cell types are protected from apoptosis by PorB (23. 49, 50, 51) regardless of TLR2 expression. To clarify the role of this receptor in the

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antiapoptotic effect of PorB, this work focused in particular on naturally TLR2-deficient HeLa cells (78) and aimed at dissecting the potential cascade of cellular events elicited by PorB leading to protection from apoptosis.

MATERIALS AND METHODS

Cell cultures and reagents. HeLa cells were cultured in Dulbecco modified Eagle medium (Cellgro, Mediatech) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Meningococcal PorB was purified by column chromatography as previously described (51) and, for some experiments, was labeled with Alexa Fluor 594 (Invitrogen) as previously described (52). Staurosporine (STS), recombinant human tumor necrosis factor alpha (TNF-α), cycloheximide (chx), propidium iodide (PI) (Sigma), rhodamine 123 (rh123) (Invitrogen), and parthenolide (PA) (EMD Biosciences) were used as described below.

Induction of apoptosis and flow cytometry. Apoptosis was induced by incubating HeLa cells (10^5 cells/ml) with 1 μM staurosporine or with 20 ng/ml TNF-α plus 10 μg/ml of cycloheximide for 24 h. The mitochondrial membrane potential was measured by flow cytometry using cells stained with 1 μM rhodamine 123, as previously described (49). DNA degradation was determined by propidium iodide staining of permeabilized cells, also as previously described (49). Cells were analyzed by flow cytometry with a FACScan flow cytometer using CellQuest acquisition and analysis software (Becton Dickinson, Mountain View, CA). Gating was used to exclude cellular debris. Individual experiments were repeated at least five times. Anti-TLR2 fluorescein isothiocyanate (FITC)-labeled antibody (eBioscience) was used for flow cytometry detection of surface expression of TLR2 on HeLa cells.

Cell activation assays. HeLa cells were treated with purified PorB (10 μg/ml or 50 μg/ml) or with TNF-α (20 ng/ml) for various periods of time, as described below. NF-κB inhibition studies were performed by adding parthenolide (10 μM) (79) at 2 h prior to stimulation. The inhibitor concentration was determined experimentally as the concentration at which NF-κB was inhibited without induction of cell toxicity (using phase-contrast microscopy to examine cell morphology). In some assays, cells were also treated with Pam3CSK4 (100 ng/ml) or heat-killed *N. meningitidis* at a multiplicity of infection (MOI) of 50.

Subcellular fractionation. Mitochondrial fractions were prepared as previously described (49). For Golgi membrane fractions, the cells were lysed in 200 mM sucrose, 10 mM Tris (pH 7.2), homogenized, mixed with 62% sucrose (final sucrose concentration, 37.5%), and overlaid with 2 ml of 35% sucrose and 1 ml of 29% sucrose in an SW55 centrifuge tube. After 90 min of centrifugation at 50,000 rpm, Golgi membranes were harvested at the interface between 29% sucrose and 35% sucrose (41). Late and early endosome fractions were obtained using a sucrose step gradient as described previously (54). For nuclear and cytosolic fractionation, cells were resuspended in lysis buffer B (10 mM HEPES [pH 7.5], 10 mM KCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, protease inhibitor cocktail) for 30 min and then centrifuged at 800 × *g* for 5 min at 4°C. The supernatants were designated cytosolic fraction and stored. The nuclear pellets were washed once with lysis buffer C (lysis buffer B containing 0.1% Nonidet P-40), designated the nuclear fraction, and stored. The total protein concentration of the cell fractions was determined by using the Bio-Rad protein concentration assay (Bio-Rad, Hercules, CA). Whole-cell lysates were prepared by resuspending cells in lysis buffer A (10 mM Tris [pH 8.0], 10 mM EDTA, 0.5% Tween 20).

Western blotting. For evaluation of intracellular proteins, equal amounts of proteins were separated by SDS-PAGE, which was followed by Western blotting. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T) and incubated in 5% bovine serum albumin in TBS-T overnight at 4°C with the following antibodies: anti-PorB rabbit serum (49), anti-cytochrome *c* (clone 7H8.2C12; Pharmingen), anti-Golgin, anti-Rab4 (early endosome marker), anti-KDEL (late endosomes marker), anti-pro-caspase 3, anti-pro-caspase 7, anti-Bcl-xL, anti-Bcl-2, anti-Bax, anti-AIF, and anti-p65/RelA (Stressgen); anti-Bfl-1 (Abcam); anti-Bid (Biosource); and anti-cIAP-2, anti-HSP70, anti-c-Rel, anti-Rel-B, anti-p100/p52, anti-p105/p50, anti-IκBα, and anti-H3 (Cell Signaling). Horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were used, and the immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

Fluorescence microscopy. HeLa cells (10^4 cells/ml) were plated in complete medium on glass coverslips placed into six-well plates, allowed to adhere, and incubated at 37°C for 1 h, 4 h, and 24 h with 10 μg/ml of PorB labeled with Alexa Fluor 594. The cells were then washed five times, fixed with 3.7% paraformaldehyde in warm medium for 15 min at 37°C, washed again, and permeabilized with 100% ice-cold acetone for 5 min. After further washing, the cells were incubated with anti-cytochrome monoclonal antibody (1:1,000) for 30 min at room temperature and then with anti-mouse FITC-labeled secondary antibody (1:5,000; Sigma). Following a final wash, the coverslips were mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and stored in the dark at 4°C. The slides were examined by fluorescence microscopy using an Olympus BX41 fluorescent microscope attached to computerized imaging equipment, and the images were processed with Olympus DP Controller software.

NF-κB luciferase reporter assay. HeLa cells (10^5 cells/ml) were plated in 24-well plates and allowed to adhere overnight. The next day, the cells were transfected with an NF-κB luciferase reporter vector as previously described (10). Briefly, 450 μl of fresh medium containing 50 μl of GeneJuice (Novagen) plus 2 μl of the reporter plasmid preparation was incubated for 15 min at 25°C prior to addition to the cells for overnight transfection. The following day, PorB (10 μg/ml), Pam3CSK4 (100 ng/ml), TNF-α (20 ng/ml), or heat-killed *N. meningitidis* at an MOI of 50 in fresh medium was added and incubated for 18 h, and this was followed by measurement of luciferase activity using commercial reagents (Promega) according to the manufacturer's protocol. The luminescence in triplicate wells was assessed using a Wallac Victor2 luminometer.

IL-8 ELISA. Supernatants from HeLa cells or BEAS-2B cells incubated as described above were collected, and interleukin-8 (IL-8) production was measured by an enzyme-linked immunosorbent assay (ELISA) using a BD OptEIA h-IL-8 ELISA set (Pharmingen) according to the manufacturer's protocol.

RESULTS

Mitochondrial localization of PorB is a time-sensitive, TLR2-independent event. Our group has previously shown that mitochondrial colocalization of PorB occurs in HeLa cells after 24 h of incubation and has demonstrated that there is a direct association between PorB and the mitochondrial protein VDAC (49, 50). We have also shown that TLR2 is a surface receptor for PorB (52), which might enhance intracellular delivery of PorB in cells that express this receptor. However, HeLa cells lack TLR2 expression (78), and thus internalization of PorB in these cells is likely to be TLR2 independent. To examine the kinetics of mitochondrial localization of PorB independent of a (known) potential receptor-mediated interaction, HeLa cells were incubated with 10 μg/ml of PorB at 37°C for 30 min, 1 h, 4 h, and 24 h, and mitochondrial and cytosolic fractions were separated. Aliquots of equivalent protein concentration were examined by Western blotting with an anti-PorB antibody, which demonstrated that there was a time-dependent association of PorB with mitochondria as soon as after 30 min of incubation, which increased over time (Fig. 1A). The PorB accumulation in the cell cytosol at the same time points was negligible (Fig. 1A). Cytochrome *c* was used as a mitochondrial marker (Fig. 1A) and was not detected in the cytosolic fractions (not shown). Anti-HSP70 antibody was used as a loading control (Fig. 1A). As a control for PorB internalization, whole-cell lysates were examined by Western blotting with anti-PorB antibody (Fig. 1B). The potential interaction of PorB with other intracellular compartments, such as the Golgi membranes or early or late endosomes, was also examined by Western blotting of isolated organelles. PorB did not colocalize with the Golgi membranes after 24 h of incubation, while it was widely associated with the fraction containing the remaining cellular membranes, including mitochondria (Fig. 1C). Golgi membranes were identified with an anti-Golgin antibody (Fig. 1C). Similar results were obtained when both early- and late-endosome fractions were examined (not shown). The colocalization of PorB with mitochondria after 24 h of incubation has been established previously by Western blotting and by

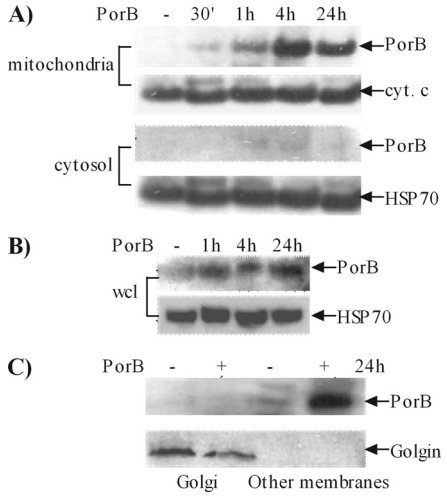

FIG. 1. Time-dependent intracellular localization of PorB. HeLa cells were incubated with PorB (10 μg/ml) for different lengths of time, and intracellular PorB localization was examined by Western blotting with an anti-PorB polyclonal antibody. (A) Mitochondrial or cytosolic fractions; B) whole-cell lysates (wcl); (C) Golgi membranes. Anti-cytochrome *c*, anti-Golgin, and anti-HSP70 antibodies were used to identify organelle fractions and as loading controls.

fluorescence microscopy (50). Here, the time-dependent association of PorB with HeLa cells was examined by fluorescence microscopy of cells incubated with 10 μg/ml of PorB labeled with the red fluorochrome Alexa Fluor 594 (52) for 1 h, 4 h, and 24 h; the cells were counterstained with an anti-cytochrome *c* monoclonal antibody, followed by a FITC-labeled secondary antibody, and were examined by fluorescence microscopy (magnification, ×60). Figure 2 shows the time-dependent increase in the association of red fluorescent PorB with HeLa cells after 1 h, 4 h, and 24 h of incubation (Fig. 2B, 2E, and 2H, respectively). FITC-stained cells are shown in Fig. 2A, 2D, and 2G, and merged fluorescence images are shown in Fig. 2C, 2F, and 2I.

PorB prevents mitochondrial depolarization in a time-dependent manner. To examine whether protection from apoptosis correlates with time-dependent PorB colocalization with mitochondria, HeLa cells were incubated simultaneously with 1 μM staurosporine (STS) and PorB (10 μg/ml) for 24 h or were pretreated with PorB for 1 h, 4 h, and 24 h prior to induction of apoptosis. The mitochondrial membrane potential was measured by flow cytometry as function of uptake of rho-

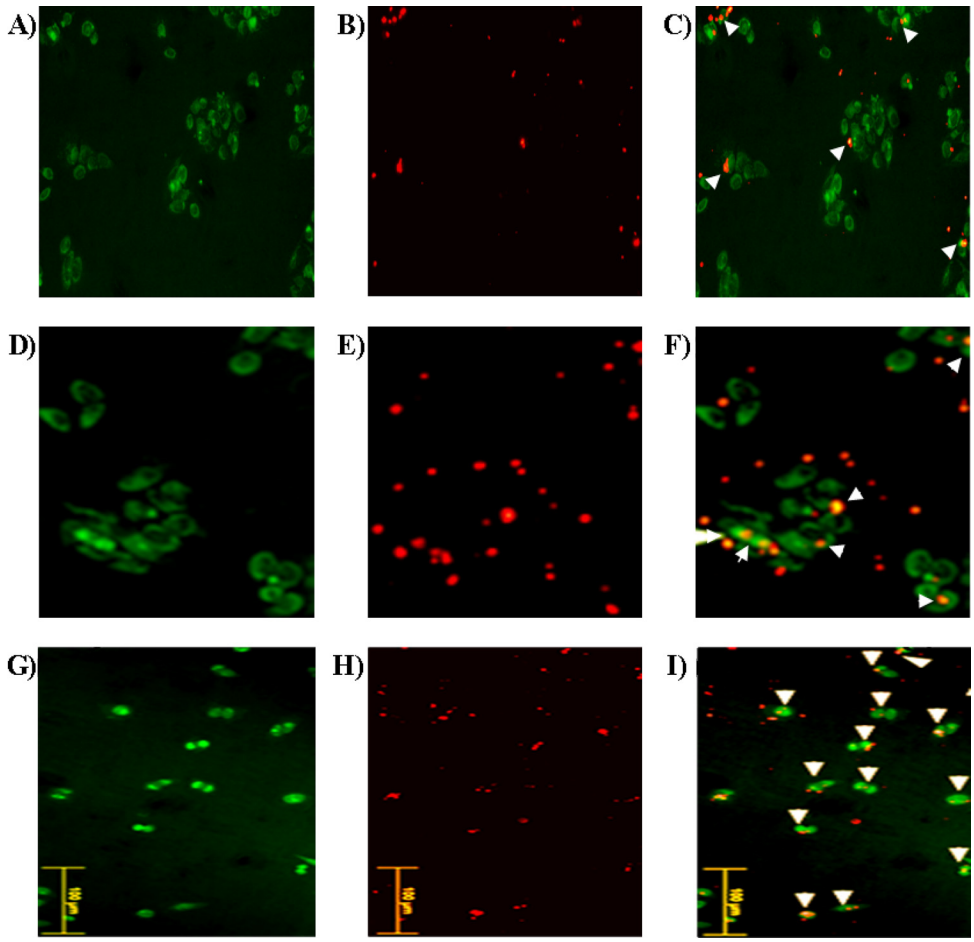

FIG. 2. Fluorescence microscopy of time-dependent PorB association with HeLa cells. Cells were plated on glass coverslips placed in six-well plates and incubated with PorB (10 μg/ml) labeled with the red fluorochrome Alexa-Fluor 594. The cells were permeabilized, fixed, and counterstained with an anti-cytochrome *c* monoclonal antibody followed by a FITC-labeled anti-mouse secondary antibody. The coverslips were mounted on glass slides and examined by fluorescence microscopy. (A, D, and G) FITC- and cytochrome *c*-stained cells; (B, E, and H) cells incubated with red fluorescent PorB for 1 h, 4 h, and 24 h, respectively; (C, F, and I) merged images. Magnification, ×60.

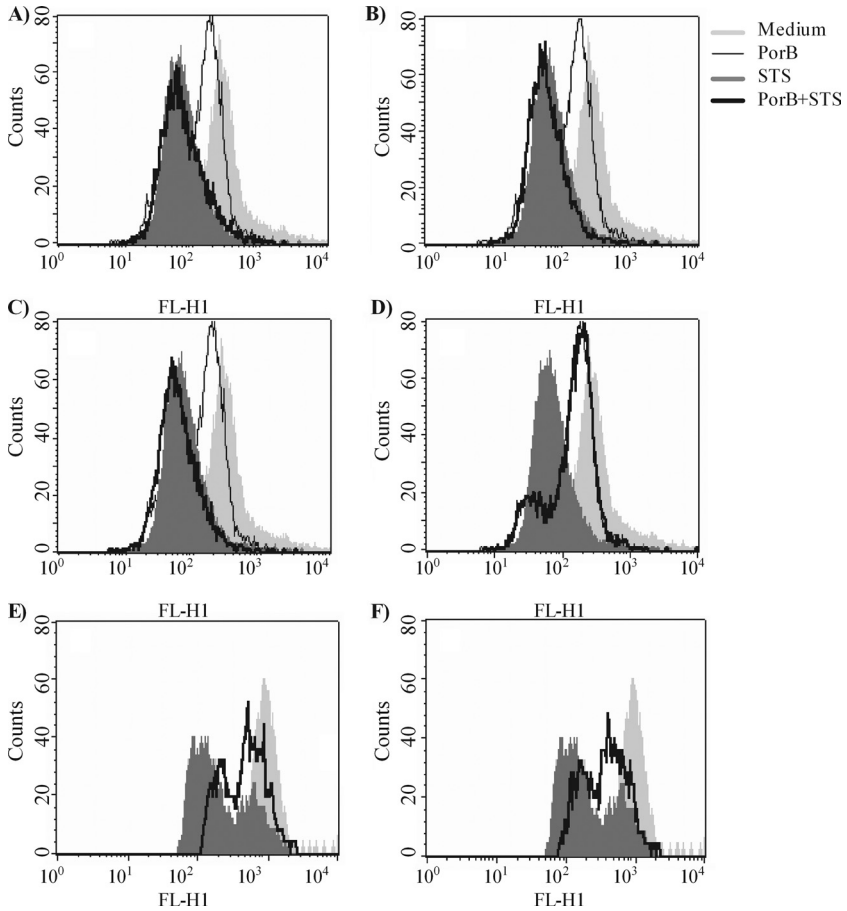

FIG. 3. Time-dependent prevention of mitochondrial depolarization by PorB. Mitochondrial depolarization was examined by flow cytometry of HeLa cells stained with rhodamine 123, a mitochondrial potential-dependent fluorescent dye. Cells were (A) incubated with PorB (10 μg/ml) and 1 μM STS simultaneously, (B to D) preincubated with 10 μg/ml of PorB for (B) 1 h, (C) 4 h, or (D) 24 h, or (E and F) preincubated with 50 μg/ml of PorB for (E) 4 h and (F) 24 h prior to addition of STS. In all the histograms, the fluorescence of cells treated with medium alone is indicated by the light gray area, the fluorescence of cells treated with PorB alone is indicated by the thin line, the fluorescence of cells treated with STS is indicated by the dark gray area, and the fluorescence of cells treated with PorB and STS is indicated by the thick line. Gating was used to exclude cellular debris. The results are representative of the results of at least four independent experiments.

damine 123 (rh123), a mitochondrial potential-dependent fluorescent dye (49). As shown in all of the histograms in Fig. 3, HeLa cells incubated with medium alone displayed high levels of rh123 fluorescence, indicating that there was an intact mitochondrial membrane potential (Fig. 3, light gray area), similar to that of cells incubated with PorB alone (Fig. 3, thin line). Incubation with STS for 24 h induced mitochondrial depolarization, as shown by a low level of rh123 fluorescence, due to the inability of the cells to retain the dye (Fig. 3, dark gray area). Simultaneous addition of STS and PorB (Fig. 3A, thick line) or incubation of PorB for 1 h and 4 h prior to the addition of STS (Fig. 3B and 3C, respectively, thick line) did not prevent mitochondrial depolarization, while this effect was reversed by 24 h of preincubation with PorB (Fig. 3D, thick line). Mitochondrial depolarization was also prevented by PorB in human airway epithelial cell line BEAS-2B, a naturally TLR2-expressing human cell line (not shown) relevant for airborne organisms, such as *N. meningitidis*. Since PorB appears to colocalize with mitochondria after 30 min of incubation (Fig. 1A), the delay in protection could suggest that a critical concentration of mitochondrial PorB may be necessary. To address this possibility, HeLa cells were incubated with a 5-fold-higher concentration of PorB (50 μg/ml) for 4 h or 24 h prior to induction of apoptosis. When 50 μg/ml of PorB was used, protection from mitochondrial depolarization was observed after 4 h of incubation with PorB (Fig. 3E, thick line), as well as after 24 h (Fig. 3F, thick line).

Selectivity of PorB for the intrinsic apoptosis pathway. Cellular apoptosis can be induced by a variety of different stimuli, which can activate the intrinsic or extrinsic pathway. Staurosporine is an intrinsic inducer of apoptosis, and our previous studies have shown that, due to protection from mitochondrial depolarization, PorB prevents or reduces apoptotic events downstream of mitochondria, such as caspase cleavage and DNA degradation (49, 50). In agreement with our previous results, PorB prevented cleavage of pro-caspase 7 induced by STS in HeLa cells, as shown by Western blotting of whole-cell lysates with an anti-caspase 7 antibody (Fig. 4A). However, when apoptosis was induced via the extrinsic apoptosis pathway with TNF-α (20 ng/ml) plus cycloheximide (chx) (10 μg/ml) for 24 h, PorB did not prevent cleavage of pro-caspase 3 and only minimally reduced the cleavage of pro-caspase 7 (Fig.

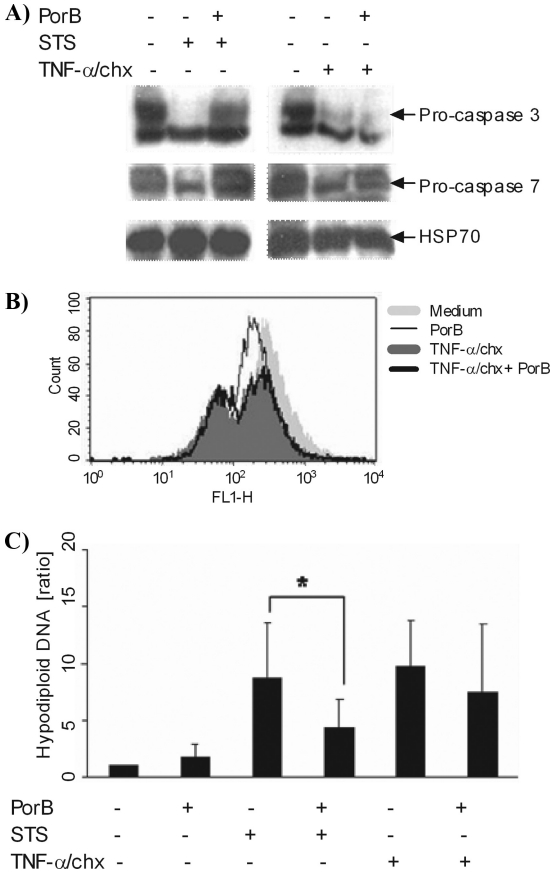

FIG. 4. PorB does not prevent extrinsic apoptosis. HeLa cells were incubated with PorB (10 μg/ml) for 24 h prior to induction of apoptosis with 1 μM STS or with 20 ng/ml of TNF-α plus 10 μg/ml chx. (A) Cleavage of caspase 3 and 7 induced by STS is prevented by PorB. Caspase 3 cleavage induced by TNF-α and chx is not inhibited by PorB, and caspase 7 cleavage appears to be slightly reduced, as determined by Western blotting of whole-cell lysates with anti-caspase 3 and anti-caspase 7 antibodies. Anti-HSP70 antibody was used as a loading control. (B) PorB does not protect HeLa cells from mitochondrial depolarization induced by TNF-α and chx, as measured by rh123 fluorescence and FACS analysis as previously described. Light gray area, medium alone; thin line, treatment with PorB alone; dark gray area, treatment with TNF-α and chx; thick line, treatment with PorB, TNF-α, and chx. The results are representative of the results of at least four independent experiments. (C) DNA degradation induced by STS or by TNF-α and chx as determined by propidium iodide (PI) staining of cells incubated as described above, followed by FACS analysis. The results are expressed as percentages of the hypodiploid DNA in the gated cell population normalized to the value for the medium control cells, and the data are the averages and standard deviations of multiple experiments. *, $P < 0.05$ (Student's t test).

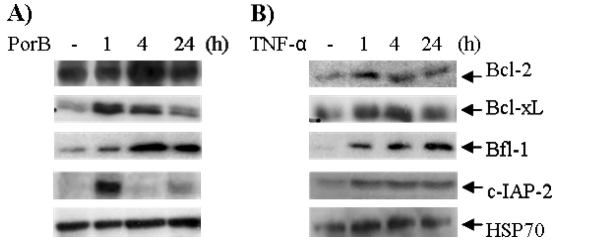

FIG. 5. Effect of PorB on expression of Bcl-2 and IAP family members. HeLa cells were incubated with (A) PorB (10 μg/ml) and (B) TNF-α (20 ng/ml) for 1 h, 4 h, and 24 h, and expression of Bcl-2, Bcl-xL, Bfl-1, and c-IAP2 was examined by Western blot analysis of cell lysates. Bcl-2, Bcl-xL, and c-IAP-2 were transiently expressed in response to PorB after 1 h or 4 h of incubation, and expression decreased after 24 h of incubation, while expression of Bfl-1 increased in a time-dependent manner. TNF-α was used as a positive control for induction of protein expression, and HSP70 was used as a loading control.

4A). An anti-HSP70 antibody was used to demonstrate equal loading of the samples.

To determine whether PorB can prevent TNF-α- and chx-dependent mitochondrial depolarization, rh123 staining was examined using fluorescence-activated cell sorting (FACS) as described above. Mitochondrial depolarization induced by TNF-α and chx (Fig. 4B, dark area) was not prevented by incubation with PorB for 24 h (Fig. 4B, thick line). As expected, mitochondrial depolarization was not detected in cells incubated with medium alone or with PorB alone (Fig. 4B, light gray area and thin line, respectively). Since PorB can rescue HeLa cells from mitochondrial depolarization induced by STS, a specific effect on the intrinsic apoptosis pathway is hypothesized. Apoptotic DNA degradation was also examined by using incorporation of propidium iodide (PI), followed by quantification of cells with hypodiploid DNA by FACS analysis. PorB significantly prevented STS-induced DNA degradation (approximately 50%) (Fig. 4C), while it did not reduce DNA degradation induced by TNF-α and chx. Figure 4C shows the results of multiple experiments ($n = 5$), expressed as ratios of DNA degradation compared to the data for medium control cells.

Effect of PorB on cellular proteins involved in apoptosis. The phenomena that describe prevention of intrinsic apoptosis by PorB might also suggest a potential contribution of the cellular apoptotic machinery, which is heavily regulated by both proapoptotic and antiapoptotic members of the Bcl-2 family. To address whether PorB has an inhibitory effect on proapoptotic Bcl-2 proteins, the time-dependent expression and intracellular localization of Bak, Bax, and Bid were examined by Western blotting of lysates of whole cells incubated with PorB (10 μg/ml) for 1 h, 4 h, and 24 h. Incubation with PorB for up to 24 h did not affect the expression of the mitochondrial proapoptotic protein Bak, did not induce mitochondrial accumulation of Bax, and did not induce cleavage of the cytosolic, full-length, inactive form of Bid (not shown). In addition, the lack of mitochondrial cytochrome *c* and the lack of apoptosis-inducing factor (AIF) released in the cell cytosol were further indications of mitochondrial membrane integrity (not shown).

Next, the effect of PorB on antiapoptotic Bcl-2 proteins was examined. A modest increase in expression of Bcl-2 was detected after 4 h of incubation with PorB, but the level returned to the baseline level after 24 h (Fig. 5A), consistent with our previous results (50). Similar transient expression of Bcl-xL was also detected (Fig. 5A). Only expression of Bfl-1 increased in a time-dependent manner (Fig. 5A), in agreement with what was previously shown for gonococcal porin PIB in male urethral cells (5). As both live gonococci and purified PIB induce early upregulation of c-IAP-2 gene expression (5, 23), the c-IAP-2 protein levels in response to PorB were examined.

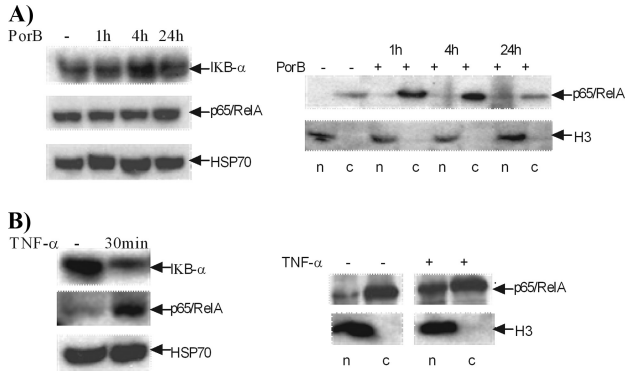

FIG. 6. Lack of NF-κB expression and activation in HeLa cells by PorB. HeLa cells were incubated with PorB (10 μg/ml) for 1 h, 4 h, and 24 h or with TNF-α (20 ng/ml) for 30 min, and NF-κB activation was measured by Western blotting. (A) Whole-cell lysates (left panel) and nuclear (n) or cytosolic (c) fractions (right panel) of PorB-treated cells examined with anti-IκBα and anti-p65/RelA antibodies. (B) Whole-cell lysates (left panel) and nuclear or cytosolic fractions (right panel) of cells incubated with TNF-α. Anti-HSP70 antibody was used as a loading control, and anti-histone 3 was used to determine the purity of the nuclear fraction.

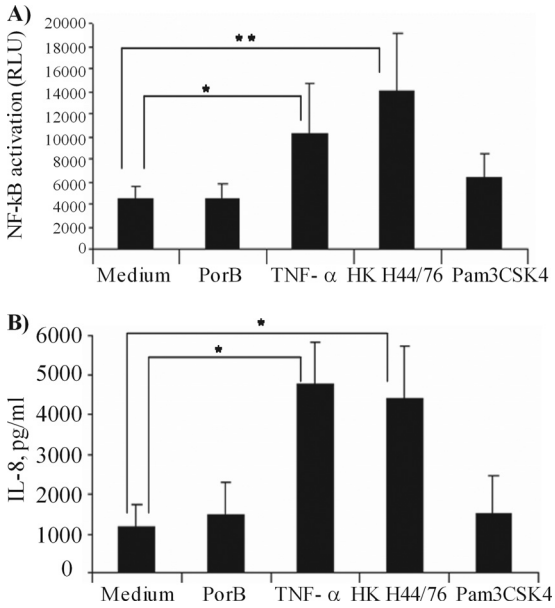

FIG. 7. Lack of NF-κB activity in HeLa cells in response to PorB. (A) HeLa cells transfected with a luciferase vector under control of NF-κB were incubated with PorB (10 μg/ml), Pam3CSK4 (100 ng/ml), TNF-α (20 ng/ml), and heat-killed *N. meningitidis* H44/76 (MOI, 50) for 24 h. NF-κB activation was measured by the luciferase assay. The results are the means and standard deviations for duplicate wells in quadruplicate experiments. *, $P = 0.02$ (Mann-Whitney test); **, $P = 0.05$ (Mann-Whitney test). (B) HeLa cells were incubated as described above, and the IL-8 concentrations in the cell supernatants were measured by an ELISA. The results are the means and standard deviations of multiple experiments. *, $P = 0.0005$ (Mann-Whitney test).

Figure 5A shows that transient expression of c-IAP-2 was detected, similar to expression of Bcl-2 and Bcl-xL.

As a positive control for expression of these proteins upon cell activation, HeLa cells were incubated with TNF-α alone (20 ng/ml), which did not affect Bax, t-Bid, cytochrome *c*, and AIF (not shown), since TNF-α does not induce apoptosis in the absence of chx. In agreement with previous findings, treatment with TNF-α alone induced increased expression of Bcl-2, Bcl-xL, Bfl-1, and c-IAP2 in a time-dependent manner in HeLa cells (Fig. 5B). Loading of equal amounts of proteins was determined with an anti-HSP70 antibody.

Lack of NF-κB activation by PorB in the absence of TLR2. Most intracellular antiapoptotic factors are under transcriptional control of NF-κB, which therefore plays a major role in prevention of cell death (30). NF-κB activation is induced by whole neisseriae and neisserial porins in various cell systems (5, 13, 21, 23, 27, 35, 59, 77); in particular, PorB induces NF-κB activation in a manner dependent on TLR2 (46, 48, 52), a receptor that is not present in HeLa cells (78). To determine whether PorB could induce TLR2-independent NF-κB activation in these cells, multiple approaches were used. First, cell fractionation and Western blotting were employed to examine NF-κB protein levels in response to PorB. HeLa cells were incubated with 10 μg/ml of PorB for 1 h, 4 h, and 24 h or with TNF-α (20 ng/ml) for 30 min as a TLR-independent positive control. PorB did not induce expression and degradation of IκBα (essential for release of active NF-κB) or expression of total intracellular NF-κB (p65/RelA) in whole-cell lysates in the absence of TLR2 (Fig. 6A, left panel). As expected, IκBα degradation and p65/RelA expression were detected in response to TNF-α (Fig. 6B, left panel). An HSP70 antibody was used as a loading control.

To verify our findings, nuclear translocation of p65/RelA was also examined by Western blotting of nuclear and cytosolic fractions. PorB did not induce p65/RelA nuclear translocation at early time points and appeared to slightly induce nuclear translocation at 24 h (Fig. 6A, right panel). Incubation with TNF-α alone for 30 min was sufficient to promote p65/RelA nuclear translocation (Fig. 6B, right panel). An antihistone antibody was used to determine the purity of the nuclear fractions. NF-κB subunits other than p65/RelA were also examined by Western blotting. A small, time-dependent increase in total p100 and p105 expression was detected in response to PorB after 24 h of incubation, but there was no significant nuclear translocation of the active NF-κB subunits (not shown). Collectively, these data suggest that PorB does not induce significant NF-κB activation in HeLa cells, although intracellular accumulation of p105 and p100 precursors may occur.

To correlate the biochemical evidence for a lack of TLR2-dependent NF-κB activation by PorB with functional activation, HeLa cells were transfected with a luciferase reporter vector under control of the NF-κB promoter (10), and luciferase production was examined. This approach has been used previously to characterize TLR-dependent NF-κB activation by PorB in other cell systems (48, 52). Transfected cells were incubated for 18 h with PorB (10 μg/ml), TNF-α (20 ng/ml) as a TLR-independent positive control, Pam3CSK4 (100 ng/ml) as a TLR2-dependent control, and heat-killed *N. meningitidis* (MOI, 50) as an additional control. PorB and Pam3CSK4 did not induce NF-κB-mediated luciferase production (Fig. 7A), while TNF-α and heat-killed *N. meningitidis* induced robust luciferase production (Fig. 7A). The results were expressed in

relative luciferase units compared to duplicate wells in triplicate experiments.

Lastly, IL-8 secretion was measured by an ELISA using supernatants of HeLa cells incubated as described above. PorB and Pam3CSK4 did not induce IL-8 secretion (Fig. 7B) due to the lack of TLR2 expression in these cells. TLR2-independent IL-8 production was induced by TNF-α and heat-killed *N. meningitidis* (Fig. 7B), in agreement with previous results. Induction of IL-8 by PorB was also measured using BEAS-2B cells, which respond to TLR2 ligands, including PorB (not shown).

Control of mitochondrial potential by PorB is not dependent on NF-κB transcriptional activity. Although it appears that NF-κB is not required for the antiapoptotic effect of PorB in HeLa cells, to determine whether inhibition of NF-κB activation could interfere with this property, the sesquiterpene lactone parthenolide (PA) was used in the apoptosis assay. Various concentrations of PA were tested for cell toxicity, and 10 μM was chosen as a nontoxic concentration with significant NF-κB inhibition (not shown). As a control for the ability of PA to inhibit NF-κB activation, HeLa cells were preincubated with 10 μM PA for 2 h prior to incubation with 20 ng/ml of TNF-α for 30 min, and whole-cell lysates were examined by Western blotting. IκBα degradation induced by TNF-α was inhibited by PA (Fig. 8A), similar to p65/RelA nuclear translocation (not shown). Anti-HSP70 antibody was used as a loading control.

The effect of PA on the mitochondrial potential was examined by using rh123 fluorescence and FACS. The mitochondrial potential was not affected by treatment with PA (10 μM) alone for 24 h (Fig. 8B, gray dotted line) or by treatment with PA for 2 h followed by treatment with 10 μg/ml of PorB (Fig. 8B, black dotted line), similar to the results for PorB alone (Fig. 8B, black line) and for medium alone (Fig. 8B, light gray area). Induction of mitochondrial depolarization by STS (Fig. 8C, dark gray area) was prevented by treatment with PorB alone (Fig. 8C, thin line) and by treatment with PA for 2 h followed by treatment with PorB for an additional 24 h (Fig. 8C, thick line), demonstrating that inhibition of NF-κB does not affect the ability of PorB to prevent mitochondrial depolarization.

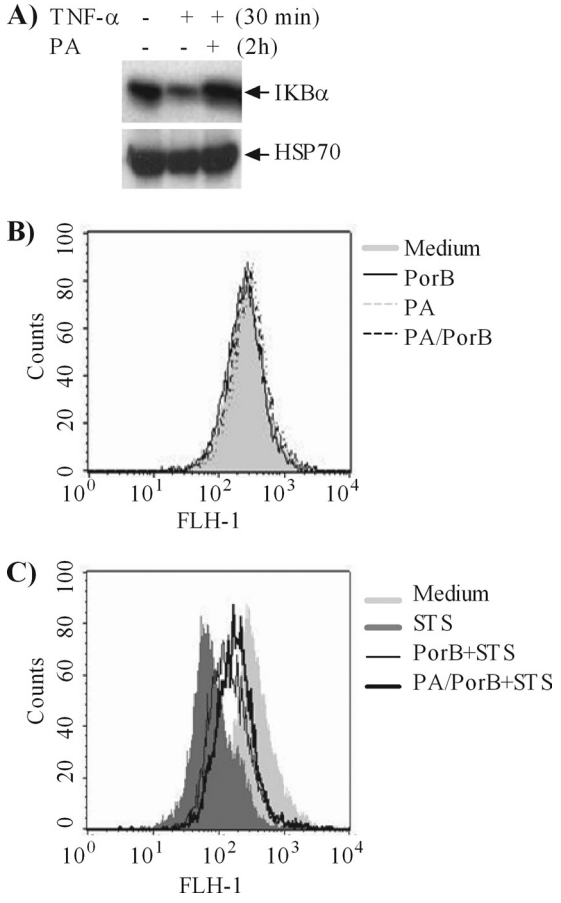

FIG. 8. Inhibition of NF-κB does not affect prevention of mitochondrial depolarization by PorB. (A) HeLa cells were incubated with the NF-κB inhibitor parthenolide (PA) (10 μg/ml) for 2 h and then with TNF-α (20 ng/ml) for 30 min, and inhibition of IκBα degradation was examined by Western blotting of whole-cell lysates with an anti-IκBα antibody. Anti-HSP70 antibody was used as a loading control. (B) Analysis of the mitochondrial potential of HeLa cells incubated with PA as described above and then with PorB (10 μg/ml) for 24 h. The mitochondrial potential was measured by rh123 fluorescence and FACS analysis as previously described. Gray area, medium control cells; black line, incubation with PorB alone for 24 h; dotted gray line, incubation with PA alone for 24 h; dotted black line, incubation with PA for 2 h and with PorB for 24 h. (C) HeLa cells were incubated with PA and PorB as described above in the presence or absence of STS (1 μM) for 24 h, and the mitochondrial membrane potential was measured by rh123 staining. Light gray area, medium control cells; dark gray area, incubation with STS; black line, incubation with PorB and STS; thick line, incubation with PA, PorB, and STS.

DISCUSSION

Previously, our group correlated the antiapoptotic effect of PorB with its direct interaction with the mitochondrial protein VDAC and subsequent modulation of mitochondrial membrane potential (49, 50), an upstream event in the intrinsic apoptotic pathway. Since these initial observations, further evidence that purified neisserial porins and live *Neisseria* infection inhibit apoptosis in various types of cells has been reported (4, 5, 23, 50, 55, 62, 65, 75). While the mechanism of protection from apoptosis by the gonococcal porin PIB appears to involve activation of NF-κB and expression of antiapoptotic genes (5), it is not clear whether similar cellular and molecular mechanisms also regulate the antiapoptotic effect of PorB. For example, PorB elicits NF-κB activation in a TLR2- and TLR1-dependent manner (48, 52) but prevents apoptosis in various cell types, regardless of the TLR2 expression level or tissue origin. Our group and others have reported PorB-mediated prevention of apoptosis in TLR2-competent cells (murine primary B cells, B cell lines, and human female cervical epithelial cells), in TLR2-negative cells (HeLa cells), and in cells expressing low physiological levels of TLR2 (human airway epithelial BEAS-2B cells) (53; P. Massari, unpublished observations). The latter, a relevant cell type for infection with airborne pathogens, such as *N. meningitidis*, are susceptible to activation by PorB (P. Massari, unpublished observations). Collectively, our observations so far suggest that PorB can prevent mitochondrial depolarization regardless of TLR2 expression and signaling, likely via multiple mechanisms of cell activation. These mechanisms include the known direct effect

of PorB on mitochondria and potential activation of cellular antiapoptotic responses. To specifically exclude the contribution of TLR2-dependent activation pathways to the antiapoptotic effect of PorB, here we examined the potential contribution of the host cell machinery to this phenomenon using HeLa cells, which lack TLR2 expression (78).

First, we described a rapid, time-dependent interaction of PorB with mitochondria. This interaction is not detected in other intracellular compartments, such as Golgi membranes or the endoplasmic reticulum, in agreement with previous findings (50). Once PorB colocalizes with mitochondria, it prevents apoptotic mitochondrial membrane depolarization (49) by interacting with VDAC, a component of the permeability transition pore (12) and possibly by interfering with the formation of contact sites between the inner and outer mitochondrial membranes (47). Since PorB colocalizes with mitochondria rapidly during incubation, one could imagine that its ability to modulate mitochondrial potential is also forthcoming. However, PorB did not prevent mitochondrial depolarization induced by STS prior to a 24-h preincubation. The delay in protection might be explained by the necessity to accumulate a critical amount of PorB for mitochondrial membrane stabilization, and a 5-fold-higher dose of PorB prevented mitochondrial depolarization at earlier time points.

While mitochondrial depolarization has a primary role in the intrinsic apoptosis pathway, in the extrinsic pathway this process is either bypassed immediately (type I cells) or may amplify the death signal via certain proapoptotic Bcl-2 family members (type II cells) (45, 63). We examined the effect of PorB on extrinsic apoptosis induced in HeLa cells by TNF-α in the presence of the protein synthesis inhibitor cycloheximide. Our results demonstrate that PorB has a nonessential role in prevention of caspase activation upstream and downstream of mitochondria in the extrinsic apoptosis pathway. Importantly, PorB did not rescue cells from secondary mitochondrial membrane depolarization, likely due to previous major apoptotic events. These observations, combined with the evidence that PorB nevertheless protects HeLa cells from STS-induced apoptosis, indicate that there is a specific effect only on the intrinsic apoptosis pathway. Obviously, given that protection from mitochondrial depolarization by PorB appeared to increase over time, the possibility that PorB induces intracellular factors that have not been described so far cannot be excluded, despite the uncharacteristically long time necessary for cellular events possibly regulated by signal transduction pathways. The key cellular players during apoptosis include Bcl-2 and IAP family members, which are activated mostly by cleavage or a change in intracellular localization and regulate release of apoptotic mitochondrial factors in both a mitochondrial potential-dependent manner and a mitochondrial potential-independent manner. Since PorB induces neither expression and translocation of proapoptotic Bax, Bak, and Bid nor release of cytochrome *c* and AIF, it is plausible that its antiapoptotic effect is not due to inhibition of these factors. Nevertheless, antiapoptotic proteins could contribute to the properties of PorB. However, PorB induced early and transient expression of Bcl-2, Bcl-xL, and c-IAP-2 in the first 1 h to 4 h of incubation, in agreement with previous findings for gonococcal PIB in male urethral epithelial cells (5). Whether these proteins are induced only at early time points or are degraded at later time points is not clear. Bcl-2, Bcl-xL, and c-IAP-2 thus do not play a major role in PorB-mediated prevention of apoptosis. Interestingly, PorB induced stable, time-dependent expression of Bfl-1 (17), a suppressor of both intrinsic and extrinsic apoptosis (64, 67, 76, 81) expressed in bone marrow and, at low levels, in other tissues. It has been shown that the gonococcal porin PIB induces early upregulation of *bfl-1* gene expression in a male urethral epithelial cell model (5), similar to the findings for whole *N. gonorrhoeae* incubated with female cervical epithelial cells (23). It is tempting to speculate that neisserial factors, including porins, are important for modulation of apoptosis in tissues that are relevant for infection by these microorganisms, such as the reproductive tract or the airway epithelium. The effects of PorB and of whole *N. meningitidis* cells on cells of the human airway epithelia are currently being investigated. Bfl-1 expression is transcriptionally regulated by an NF-κB-dependent complex containing AP-1 and C/EBPβ and is dependent on a c-Rel/p50 heterodimer (19, 29, 38, 81), and NF-κB also directly regulates a number of other cellular factors involved in apoptosis (3, 9, 57). Although PorB is known to activate NF-κB through TLR2 and TLR1 signaling (48, 52), we found that it did not induce p65/RelA expression or IκBα degradation and induced only minimal p65/RelA nuclear translocation in HeLa cells. In addition, only some cytosolic accumulation of p105 and p100 precursors was detected, but no nuclear translocation of these subunits was detected. The lack of functional NF-κB activation in response to PorB and other TLR2 ligands was confirmed by the absence of NF-κB-dependent luciferase production and IL-8 secretion. The lack of TLR2 expression in HeLa cells may explain the inability of PorB to activate these cells. In addition, PorB also did not activate NOD-transfected HEK cells (66; R. Ingalls and P. Massari, unpublished observations). Collectively, similar to findings for *Chlamydia trachomatis* (20, 22, 79, 80), these observations argue against a major role for NF-κB transcriptional activation in the antiapoptotic effect of PorB in HeLa cells (which does not induce IκBα degradation or p65/RelA nuclear translocation but induces p65 cleavage to interfere with the host inflammatory response) (37). Although the lack of NF-κB activation might explain the lack of Bcl-2, Bcl-xL, and c-IAP-2 expression by PorB, induction of Bfl-1 expression might be induced via NF-κB-independent transactivation of the *bfl-1* gene, similar to what has been described for Burkitt's lymphoma cells with EBV nuclear antigen 2 via CBF1 (or RBP-J kappa), a nuclear component of the Notch signaling pathway (60). Regulation of Bfl-1 in epithelial cells in the absence of NF-κB has not been described so far, and this regulation in response to PorB should be investigated further.

Inhibition of NF-κB with pharmacologic reagents can increase the susceptibility to apoptosis. However, the NF-κB inhibitor parthenolide (6) did not affect the antiapoptotic properties of PorB, suggestiing that NF-κB has a marginal role for PorB in this process. The possibility that other signaling pathways (including the p38 mitogen-activated protein kinase, PI3K/AKT [14], and Notch [34] pathways) contribute cannot be excluded, since regulation of Bcl-2 family members has been hypothesized for these pathways (44, 61). Further analysis of the TLR2-independent signaling induced by PorB might identify a novel contribution of intracellular factors involved in prevention of apoptosis.

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