Targeting of the pro-apoptotic VDAC-like porin (PorB) of *Neisseria gonorrhoeae* to mitochondria of infected cells

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Infection of cell cultures with *Neisseria gonorrhoeae* results in apoptosis that is mediated by the PorB porin. During the infection process porin translocates from the outer bacterial membrane into host cell membranes where its channel activity is regulated by nucleotide binding and voltage-dependent gating, features that are shared by the mitochondrial voltage-dependent anion channel (VDAC). Here we show that porin is selectively and efficiently transported to mitochondria of infected cells. Prevention of porin translocation also blocked the induction of apoptosis. Mitochondria of cells treated with porin both *in vitro* and *in vivo* were depleted of cytochrome *c* and underwent permeability transition. Overexpression of Bcl-2 blocked porin-induced apoptosis. The release of cytochrome *c* occurred independently of active caspases but was completely prevented by Bcl-2. Our data suggest that the *Neisseria* porin can, like its eukaryotic homologue, function at the mitochondrial checkpoint to mediate apoptosis.
Keywords: apoptosis/mitochondria/*Neisseria*/permeability transition/voltage-dependent anion channel (VDAC)-like porin

Introduction

Apoptosis is a special form of programmed cell death that plays a pivotal role during developmental morphogenesis and cell homeostasis. The basic mechanism of apoptosis regulation is evolutionarily conserved from nematodes to man; in fact, some major lessons were learned by studies of apoptosis in the nematode *Caenorhabditis elegans* (Metzstein *et al*., 1998). The major executioners of apoptosis are the caspases (cysteinyl aspartate-directed proteases), which comprise a family of 15 members exerting different functions in inflammation and apoptosis (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997).

The pathways by which caspases are activated have been pinned down to two major branches: one is initiated by cell surface receptors, the other by mitochondria. Ligation of receptors of the tumour necrosis factor (TNF) receptor family (Wallach *et al*., 1997)

results in the recruitment and activation of initiator caspases, e.g. caspase-8 (Boldin *et al*., 1996; Muzio *et al*., 1996). Active caspase-8 is able to process and activate other caspases, which initiate apoptosis by cleavage of cellular substrates (Salvesen and Dixit, 1997). Substrates include the DNA-repair enzyme poly(ADP-ribose) polymerase (PARP) (Tewari *et al*., 1995) and the cytoskeleton-associated protein fodrin (Cryns *et al*., 1996; Janicke *et al*., 1998), both of which are cleaved by caspase-3.

For many of the pathways induced by diverse stimuli such as cytotoxic or environmental stress or toxic cell metabolites, the mitochondria appear to be the main integrators of apoptotic signalling. They respond to these stimuli with the release of caspases-2 and -9 (Susin *et al*., 1999a) or caspase-activating proteins like cytochrome *c* (Liu *et al*., 1996) and apoptosis initiator factor (AIF) (Susin *et al*., 1999b). Cytochrome *c* is required as cofactor in a complex with the adapter molecule Apaf-1 and dATP for caspase-9 activation (Zou *et al*., 1997). Like caspase-8, active caspase-9 cleaves and activates effector caspases that coordinate the execution phase of the death programme (Slee *et al*., 1999). Thus, although the initial phase of receptor-mediated and mitochondria-mediated apoptotic pathways appears to be rather different, a similar set of caspases is eventually activated and executes the death programme.

Although the major molecular effects of apoptotic activation of mitochondria have been elucidated, the trigger initiating the release of cytochrome *c* and other factors is still not well understood. Many investigators find a collapse of the mitochondrial inner membrane potential ($\Delta\psi m$) during apoptosis, indicating the opening of a large conductance channel generally known as the permeability transition (PT) pore complex (Zoratti and Szabo, 1995; Green and Reed, 1998). The complex involved in PT regulation consists of several factors including the mitochondrial porin, also known as voltage-dependent anion channel (VDAC) (Zoratti and Szabo, 1995; Beutner *et al*., 1998), the adenine nucleotide translocator (ANT) (Brustovetsky and Klingenberg, 1996), the peripheral benzodiazepine receptor (Pastorino *et al*., 1994) and probably kinases like hexokinase and/or creatine kinase (Beutner *et al*., 1998). Interestingly, PT pore opening is induced by several pro-apoptotic second messengers like Ca^{2+}, pro-oxidants, nitric oxide, ceramide and caspases (Zamzami *et al*., 1995; Bernardi and Petronilli, 1996; Marzo *et al*., 1998a), suggesting a direct involvement of PT pore opening in apoptosis induction. Moreover, it is regulated by the anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-X_L, which stabilize mitochondrial membranes (Decaudin *et al*., 1997; Kroemer, 1997a) and by the pro-apoptotic member, Bax, which induces PT (Xiang *et al*., 1996).

Recently, we demonstrated the induction of apoptosis during the infection of epithelial cells and phagocytes by *Neisseria gonorrhoeae* (*Ngo*), a human-specific bacterial pathogen (Müller *et al.*, 1999). Gonococci attach to their target cells via pili, hair-like protein appendages (Swanson *et al.*, 1987) or by Opa proteins, which in addition to adhesion induce the receptor-mediated uptake by the host cell (Makino *et al.*, 1991; Gray-Owen *et al.*, 1997; Hauck *et al.*, 1998). Although adhesion or invasion is a prerequisite, neither pili nor Opa proteins, but the PorB porin, induces apoptosis of target cells, when added in its purified form (Müller *et al.*, 1999). Porins form integral diffusion channels in the outer membrane of Gram-negative bacteria. Some porins contain binding sites for certain substrates found in the environment of these bacteria (Benz, 1995). The binding of nucleotides by porin is unusual in this respect (Rudel *et al.*, 1996) since nucleotides are not a natural substrate for *Neisseria*. However, during an infection porin translocates into the membrane of target cells (Weel and van Putten, 1991), where its channel activity is tightly regulated by cytosolic nucleotides that increase the voltage-dependent gating of the porin channel (Rudel *et al.*, 1996). The properties of PorB such as voltage dependence and nucleotide binding closely resemble mitochondrial VDAC functions (Benz, 1994) and are not found in other porins of Gram-negative bacteria.

Since PorB exhibits similar properties to mitochondrial VDACs and since both are involved in apoptosis induction, we investigated the influence of PorB on mitochondrial function during apoptosis induction. Here we show that infection or PorB treatment causes loss of membrane potential and the release of cytochrome *c* from mitochondria of intact cells. Surprisingly, PorB also induces the release of cytochrome *c* from purified mitochondria. The release of cytochrome *c* as well as PT and the induction of apoptosis is blocked by Bcl-2 in intact cells. Furthermore, in porin-treated cells as well as in infected cells PorB is specifically and efficiently targeted to the mitochondria.

Results

Porin induces structural and biochemical changes of mitochondria that are typical of apoptotic cells

When epithelial or immune cells are treated with porin purified from *N.gonorrhoeae* they undergo apoptosis (Müller *et al.*, 1999). The underlying mechanism involves release of Ca^{2+} into the cytosol and the activation of proteases of the caspase and calpain families. Since mitochondria play a central integrative role in the regulation of apoptosis (reviewed by Kroemer *et al.*, 1998) we investigated the effects that porin treatment has on the structural and biochemical integrity of mitochondria (Figure 1). Jurkat cells were treated with 7 µg/ml porin for 15 h, the mitochondria were isolated and the cytochrome *c* content was determined by western blot analysis. Mitochondria of untreated and buffer-treated cells (Figure 1A) contained the same amount of cytochrome *c* while mitochondria of porin-treated cells had lost all their cytochrome *c* (Figure 1A). To ensure equal loading of proteins the western blots were also
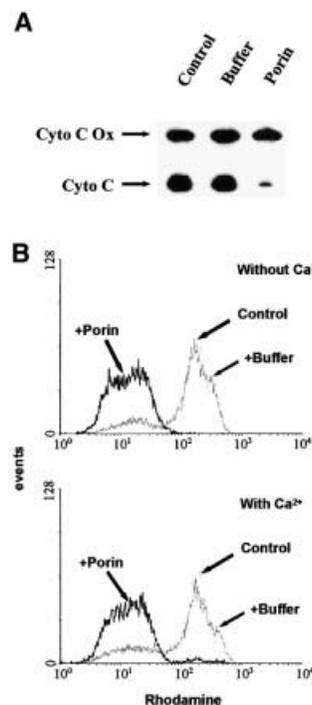

Fig. 1. Effects of porin on the release of cytochrome *c* and PT of mitochondria *in vivo*. (**A**) Mitochondria were isolated from Jurkat T cells treated with either 7 µg/ml purified porin or an equal volume of porin purification buffer for 15 h. Mitochondrial lysates were subjected to SDS–PAGE followed by western blotting using monoclonal antibodies against human cytochrome *c* oxidase subunit II (Cyto C Ox) and denatured human cytochrome *c* (Cyto C), respectively. (**B**) Jurkat T cells were treated with porin or an equal volume of porin purification buffer for 15 h in the presence and absence of 1 mM Ca^{2+} as indicated, stained with rhodamine 123 for 30 min at 37°C and analysed by flow cytometry. The histogram shows the analysis of 10 000 cells per sample.

developed with an antibody directed against cytochrome *c* oxidase. The same result was obtained with mitochondria isolated from porin-treated monocytic and epithelial cell lines (not shown). Redistribution of cytochrome *c* was also analysed microscopically by double staining Jurkat cells with an antibody directed against native cytochrome *c* and MitoTracker, a potential-sensitive dye specific for mitochondria (Figure 2). In control or buffer-treated cells both dyes colocalized completely, indicating an intact mitochondrial membrane potential and a cytochrome *c* distribution typical of healthy cells (Figure 2, vector control and vector + buffer). In contrast, upon porin treatment a large population of cells (80–90%) no longer stained with MitoTracker and in addition had lost the granular staining of cytochrome *c* (Figure 2, vector + porin). These cells also showed clear signs of apoptosis, i.e. apoptotic body formation, condensation of the cytoplasm and cell shrinkage. Similarly, infection of HeLa cells with *N.gonorrhoeae* strain N242 also resulted in PT, the release of cytochrome *c* from mitochondria and the typical morphological alterations of apoptotic cells (Figure 3). This occurred in ~40–50% of the population.

We confirmed the loss of the mitochondrial membrane potential after porin treatment by staining live cells with the potential-sensitive dye rhodamine 123 for flow-cytometric analysis (Figure 1B). This dye is reportedly
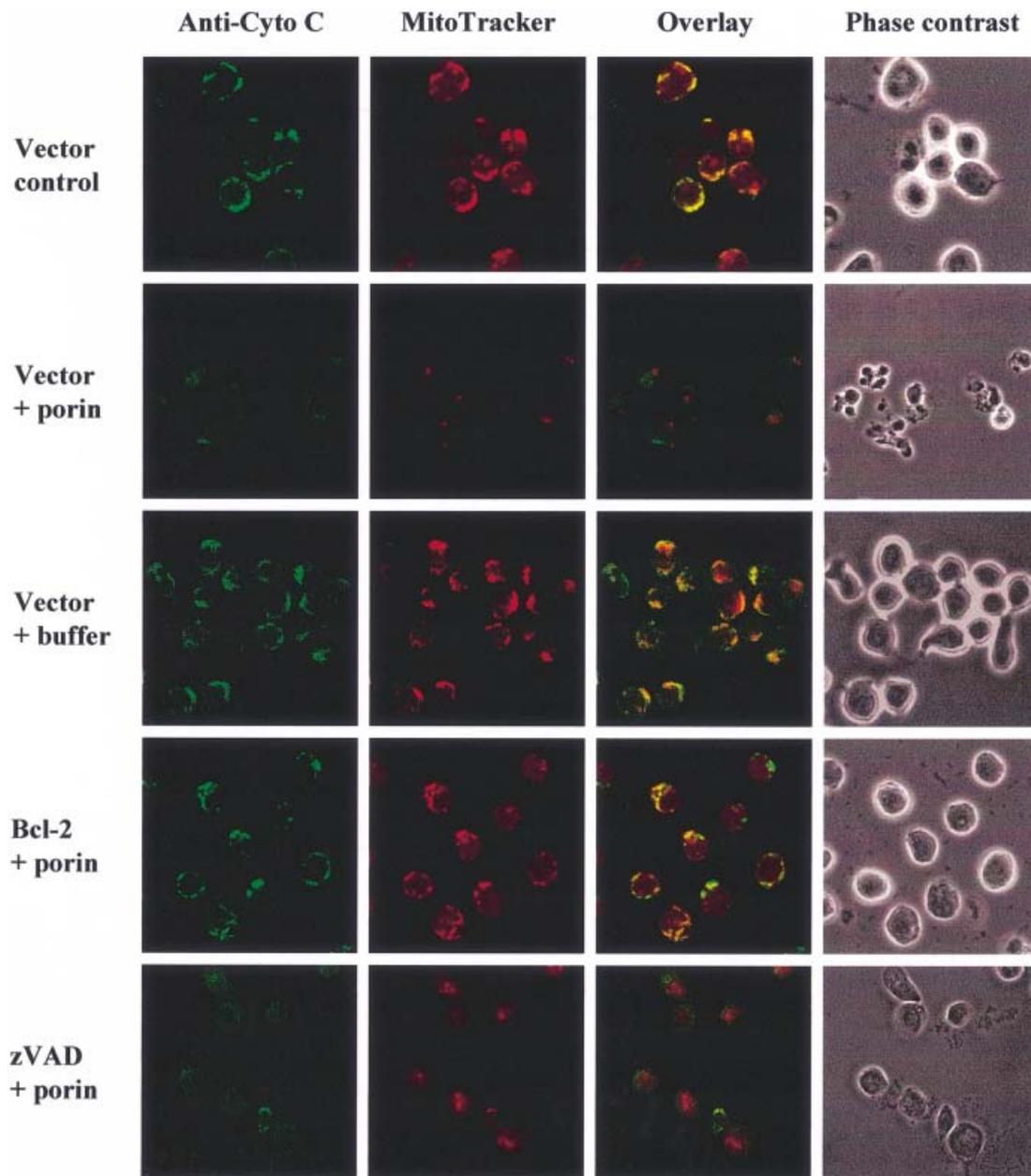
Fig. 2. Bcl-2 overexpression and caspase inhibition block porin-induced apoptosis by different mechanisms. Jurkat T cells either expressing Bcl-2 or carrying an empty vector were treated with 7 µg/ml porin or purification buffer for 15 h or left untreated. They were then subjected to immunocytochemistry using an anti-cytochrome *c*-specific antibody and an Alexa-488-coupled secondary antibody. Cells were stained with MitoTracker before fixation. A phase contrast image, single colours and overlays are shown for every section. Cells in the lowest panel were treated with 50 µM zVAD-fmk (Bachem) for 1 h before addition of porin.

only incorporated into mitochondria with intact membrane potential (Van der Heiden *et al.*, 1997). Cells that were treated with porin for 15 h showed a shift towards lower intensity (Figure 1B), the cells have therefore undergone PT. Since porin provokes a rapid influx of extracellular Ca^{2+} in treated cells (Müller *et al.*, 1999) and Ca^{2+} alone is sufficient to induce PT in other systems *in vitro* (Marzo *et al.*, 1998a), we tested whether Ca^{2+} is required for PT induced by porin. PT occurred with the same kinetics in cells treated with porin in Ca^{2+}-free medium compared with the control with Ca^{2+} (Figure 1B), thus excluding a direct effect of Ca^{2+} on porin-induced PT.

Porin induces cytochrome c release and PT in purified mitochondria

Isolated mitochondria have been used previously to demonstrate apoptogenic effects of purified proteins such as Bax on cytochrome *c* content and membrane potential (Jürgensmeier *et al.*, 1998). In the case of porin-induced apoptosis it is especially relevant to investigate the effects of the porin on isolated mitochondria because of its similarities to mitochondrial VDAC, which is known to participate in PT and cytochrome *c* release (Beutner *et al.*, 1998; Shimizu *et al.*, 1999). Purified mitochondria from 1×10^7 Jurkat T cells were treated with 2 µg of porin and
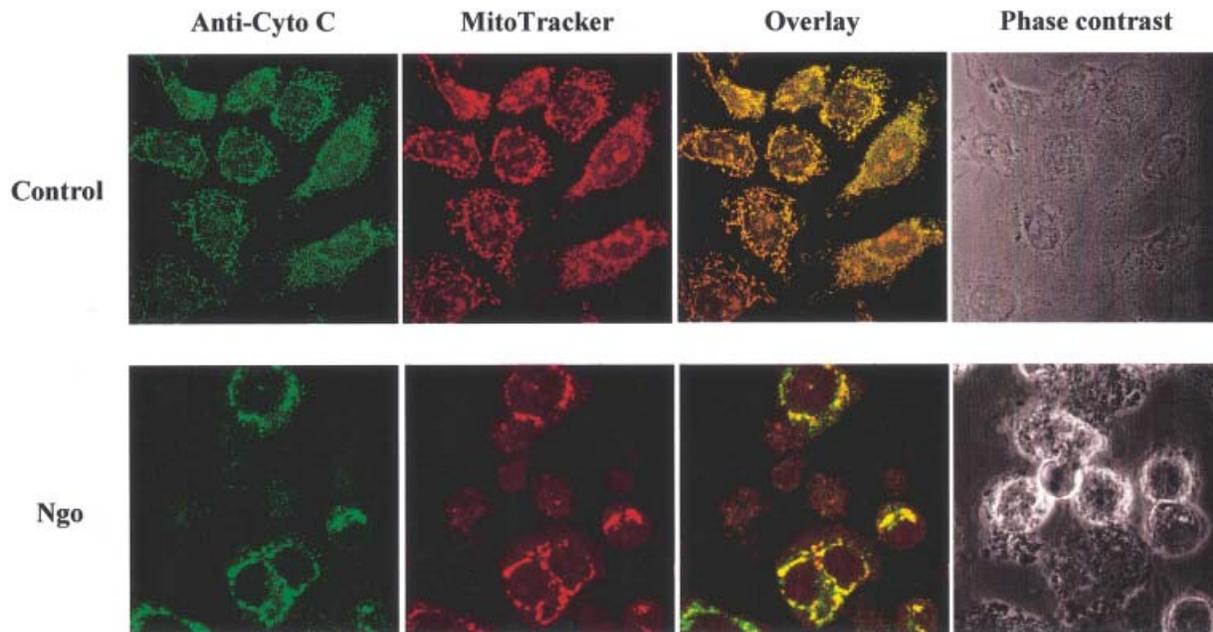
Fig. 3. Cytochrome *c* release and PT also occurs in HeLa cells infected with *N.gonorrhoeae*. HeLa cells were infected with gonococcal strain N242 for 15 h at an m.o.i. of 1. Cells were then subjected to immunocytochemistry using an anti-cytochrome *c*-specific antibody and an Alexa-488-coupled secondary antibody. MitoTracker staining was performed before fixation. A phase contrast image, single colours and overlays are shown for infected cells and uninfected control cells. Note the swollen mitochondria in infected, non-apoptotic cells. Loss of or diffuse cytochrome *c* staining in apoptotic cells is indicative of cytochrome *c* release.

cytochrome *c* release was monitored by western blotting (Figure 4A). Mitochondria treated with porin were completely depleted of cytochrome *c*, whereas addition of porin purification buffer alone did not trigger this release. Interestingly, amounts of porin (0.5 µg) that were insufficient to induce a cytochrome *c* release reproducibly had an opposite effect: the mitochondria were protected from the spontaneous loss of cytochrome *c* that is otherwise observed after extended incubation at 37°C (not shown). We also looked for PT as a putative response of isolated mitochondria to porin treatment. For this purpose, the mitochondria were stained with rhodamine 123 and fluorescence intensity was monitored by flow cytometry (Figure 4B). Indeed, a clear shift to lower intensity is observed in mitochondria treated with porin as compared with the control, which is indicative of loss of membrane potential. Also, purified mitochondria treated with porin increased in volume compared with an untreated control, which was monitored in a swelling assay over time (Figure 4C). These data therefore provide clear evidence that neisserial porin alone is sufficient for eliciting an *in vitro* mitochondrial response similar to that typically observed in the course of apoptosis *in vivo*.

Overexpression of Bcl-2 or Bcl-X$_L$ blocks porin-induced apoptosis

Bcl-2 and *Bcl-X*$_L$ are anti-apoptotic members of the Bcl family. They are localized to organelle membranes as a result of their C-terminal membrane anchor (Yang and Korsmeyer, 1996) and Bcl-X$_L$ in addition is able to form ion channels in synthetic lipid membranes (Antonsson *et al.*, 1997; Minn *et al.*, 1997). Overexpression of these proteins has repeatedly been shown to affect all the apoptotic phenotypes seen at the mitochondrial level, yet it

remains unclear if this is due to a direct function or if it is a consequence of inhibition of an earlier step in the death pathway (Zamzami *et al.*, 1996; Kluck *et al.*, 1997). Jurkat T cells stably expressing Bcl-2 and the control cell line were treated with porin for 15 h. Control transfected Jurkat cells showed clear morphological signs of apoptosis including cell shrinkage and extensive apoptotic body formation (Figure 2, phase contrast). These features were completely abolished by overexpression of Bcl-2. In order to investigate whether the observed protection from apoptosis induction was due to an inhibition of cytochrome *c* release by Bcl-2, the cells were subjected to immunocytochemistry using an anti-cytochrome *c* antibody and additional staining of the mitochondria by MitoTracker (Figure 2). While the control cell line completely released cytochrome *c* upon porin treatment and had undergone PT, Jurkat–Bcl cells retained cytochrome *c* and membrane potential just like the untreated control (Figure 2). These results were confirmed with two additional cell lines, CEM-Bcl-X$_L$ and SKW6-Bcl-2, which both stably expressed anti-apoptotic Bcl proteins (data not shown).

Caspases are known inducers of cytochrome *c* release and PT (Marzo *et al.*, 1998b) and are known to be activated during porin-induced apoptosis (Müller *et al.*, 1999). We therefore assessed their role in porin-mediated mitochondrial damage. Interestingly, the inhibition of caspases by the broad range caspase inhibitor zVAD-fmk blocked all described morphological alterations but could not prevent the release of cytochrome *c* and PT in treated cells (Figure 2, zVAD + porin).

In order to quantify the anti-apoptotic effect of the Bcl proteins (Figure 5A, left panels) we measured phosphatidylserine (PS) exposure. The majority (>90%) of
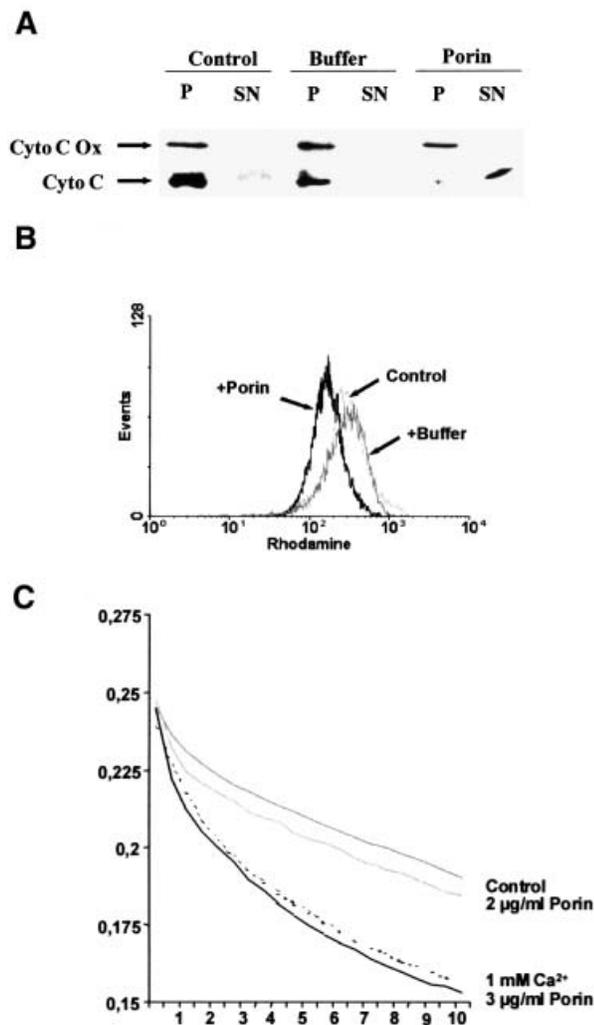
A

B

C

Fig. 4. Effects of porin on the release of cytochrome *c*, PT and swelling of mitochondria *in vitro*. (**A**) Mitochondria were isolated from 5×10^7 Jurkat cells. Porin (2 µg) or an equal volume of porin purification buffer was applied for 30 min at 37°C *in vitro*. The pellet (P) and the supernatant (SN) of every sample were then analysed by western blotting using monoclonal antibodies against human cytochrome *c* oxidase subunit II (Cyto C Ox) and denatured human cytochrome *c* (Cyto C), respectively. (**B**) Mitochondria obtained as described in (A) were stained with rhodamine 123 for 30 min at 37°C and analysed by flow cytometry. (**C**) Mitochondria were purified from mouse liver and subjected to a swelling assay as described in Materials and methods. The increase in mitochondrial volume was monitored by determining the optical density at 600 nm. The increase in volume after addition of the stimulus is visible as a decline in the OD_{600} over 10 min. Note that the untreated mitochondria swell spontaneously, a process that is not increased by low amounts of porin. However, higher concentrations of porin lead to an increase in the volume of mitochondria similar to treatment of the mitochondria with Ca^{2+}, a well known inducer of mitochondrial swelling.

vector-transfected Jurkat cells treated with porin for 15 h bound annexin V, which is indicative of PS exposure on their surface (upper left panel), whereas no increase in the annexin V-positive population was observed with Jurkat–Bcl (lower left panel). These results were confirmed by quantifying DNA degradation via propidium iodide incorporation (not shown).

When the same cells were monitored for mitochondrial membrane potential by staining with rhodamine 123, no porin-induced shift towards lower fluorescence intensity was observed in cells expressing Bcl-2, indicating that PT did not occur in these cells (Figure 5A, right panels) and thereby confirming the conclusions drawn from microscopic analysis. Downstream caspases were not active in Jurkat–Bcl-2, as judged by the absence of substrate cleavage of PARP (data not shown) and fodrin (Figure 5B). Interestingly, whereas the generation of the 120 kDa caspase signature fragment of fodrin (Nath *et al.*, 1996) was completely blocked, the 150 kDa calpain fragment did appear, indicating that calpains are active in the presence of overexpressed Bcl-2, but their activity is not sufficient to drive the cells into apoptosis.

Porin is targeted to the mitochondria

Due to the similarities between eukaryotic VDAC and neisserial porin it seemed a promising hypothesis that porin is targeted to the mitochondria where it then exerts its cytotoxic effects directly by inducing PT and cytochrome *c* release. When isolated mitochondria were treated with porin *in vitro*, the vast majority of the porin amount applied was detected in the mitochondrial pellet, whereas hardly any porin was found in the supernatant (not shown). Thus, porin associated with mitochondria *in vitro*. We then addressed the question of whether porin is localized to mitochondria of porin-treated cells. Cells were either treated with porin purification buffer or with porin for 15 h and washed extensively before preparation of whole cell lysates and mitochondria. We also treated cells for the same period of time with purified *Staphylococcus aureus* α-toxin which, like porin, consists of amphipathic β-barrels and induces apoptosis by forming pores in the cytoplasmic membrane of the cells (Jonas *et al.*, 1994; Song *et al.*, 1996). Purified gonococcal PilC adhesin, which binds to several human cell lines, served as additional control. Porin, as well as PilC and α-toxin, were present in whole cell lysates, indicating their insertion into or tight attachment to membranes (Figure 6A and B). However, large amounts of porin purified with the mitochondrial fraction whereas PilC and α-toxin were always absent from this compartment. Pro-cathepsin D (Figure 6A) and transferrin receptor (not shown) did not copurify with the mitochondrial compartment, suggesting the absence of endosomal and cytoplasmic membranes. To test whether porin is also transported to mitochondria during infection with *N.gonorrhoeae*, HeLa cells were infected with either the adherent and invasive Opa-expressing strain N242 (Figure 6C) or the adherent, piliated strain N138 (Figure 6D), both of which induce apoptosis in HeLa cells (Müller *et al.*, 1999). Highly purified mitochondria from these cells contain large amounts of porin protein. Indeed, porin was the only neisserial antigen detectable in mitochondria from infected cells. Neither Opa outer membrane proteins (Figure 6C) nor PilC (Figure 6D) were found in the same mitochondrial preparations, suggesting a selective transport of porin to the mitochondria of infected cells. Porin was not present in mitochondria of HeLa cells infected with non-adherent gonococcal strains, e.g. N898 (Figure 6D) and commensal *Neisseria* spp. (not shown), which are unable to induce apoptosis. Confocal immunomicroscopy of porin-treated or infected cells using purified antiserum against porin revealed the presence of porin in mitochondria that
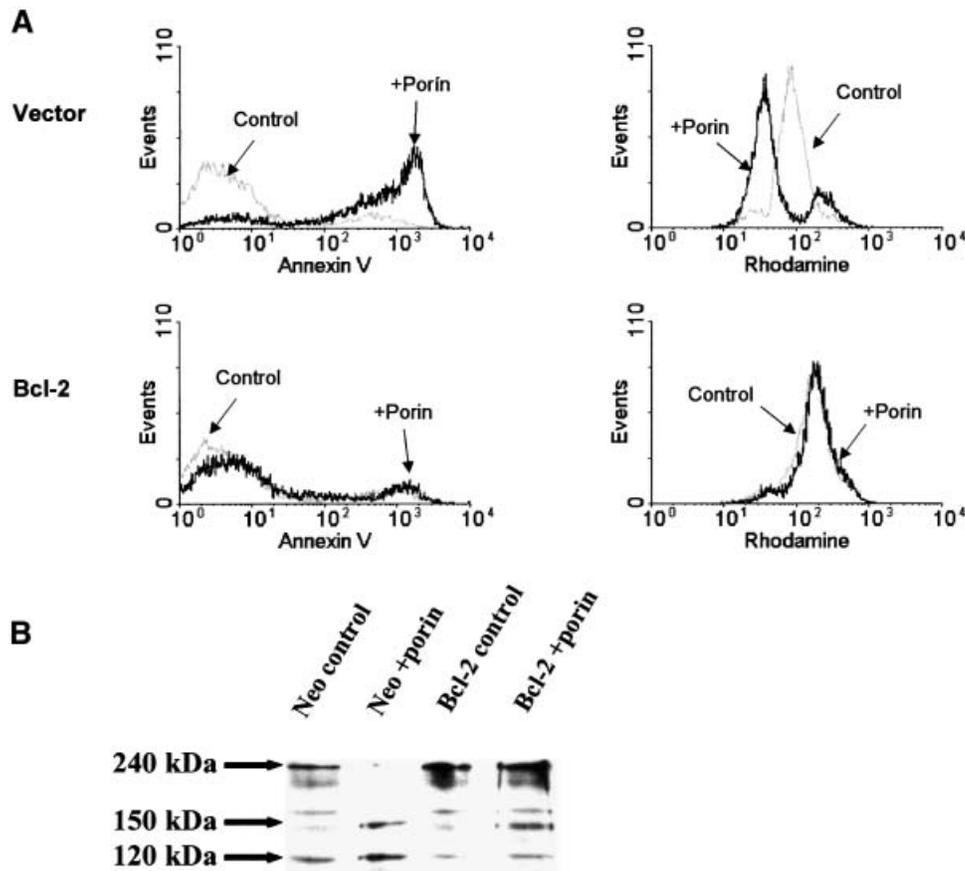
Fig. 5. Bcl-2 overexpression inhibits porin-induced PT, caspase activation and apoptosis. (**A**) Jurkat cells either expressing Bcl-2 or carrying an empty vector were treated with 7 µg/ml porin (as indicated in the histograms) for 15 h and split: one half was subjected to an annexin V binding protocol in order to determine PS exposure, the other was stained with rhodamine 123 to assess mitochondrial membrane potential. Both samples were analysed by flow cytometry. (**B**) Aliquots of the samples in (A) were lysed and subjected to western blotting for assessment of caspase activation. Cleavage of full-length fodrin (240 kDa) into signature fragments of 120 and 150 kDa was monitored by using a monoclonal antibody against fodrin that recognizes all three forms.

appeared extremely enlarged (Figure 7). In summary, porin is efficiently and selectively transported to the mitochondria of porin-treated or infected cells undergoing apoptosis.

Interference with porin translocation prevents apoptosis

We noticed that induction of apoptosis by porin is highly sensitive to serum. In the presence of 10% heat-inactivated fetal calf serum (FCS) the strong pro-apoptotic effect of porin was completely blocked (Figure 8A). The inhibitory effect of serum was not due to degradation since the overall amount of porin was similar during incubation in the absence or presence of serum (data not shown). However, in the presence of serum, porin was no longer present in the cell lysate and therefore was also absent from mitochondria prepared from treated cells (Figure 8B). Thus, translocation of porin is an absolute prerequisite for its pro-apoptotic effect.

Discussion

It has become increasingly apparent that mitochondria participate in the regulation of those forms of apoptotic cell death that do not involve activation of upstream caspases via signalling cascades triggered by death receptor ligation (Scaffidi *et al.*, 1998; Yoshida *et al.*, 1998). However, the significance of single events observed at the mitochondrial level for the overall regulatory process and especially the exact sequence of events is still controversially debated (for a review see Green and Reed, 1998). Here we describe a new mechanism of mitochondria-dependent apoptosis induction occurring naturally during infection of human cells by *N.gonorrhoeae*.

As in most other complex apoptosis scenarios, cells either infected with *N.gonorrhoeae* or treated with neisserial porin display a release of cytochrome *c* into the cytosol and a complete loss of the mitochondrial membrane potential (PT). We did not investigate the sequence of these events in detail and thus do not know whether PT and cytochrome *c* release are functionally connected. In such diverse systems as CD95(Fas/Apo-1)-induced apoptosis in Jurkat T cells (Van der Heiden *et al.*, 1997) and UVB irradiation or staurosporine treatment of HeLa cells (Bossy-Wetzel *et al.*, 1998), cytochrome *c* release was observed before membrane depolarization. Under the experimental conditions cited, PT does not take place until several hours after cytochrome *c* release, thereby ruling out the possibility that loss of cytochrome *c* results immediately in disconnection of the respiratory
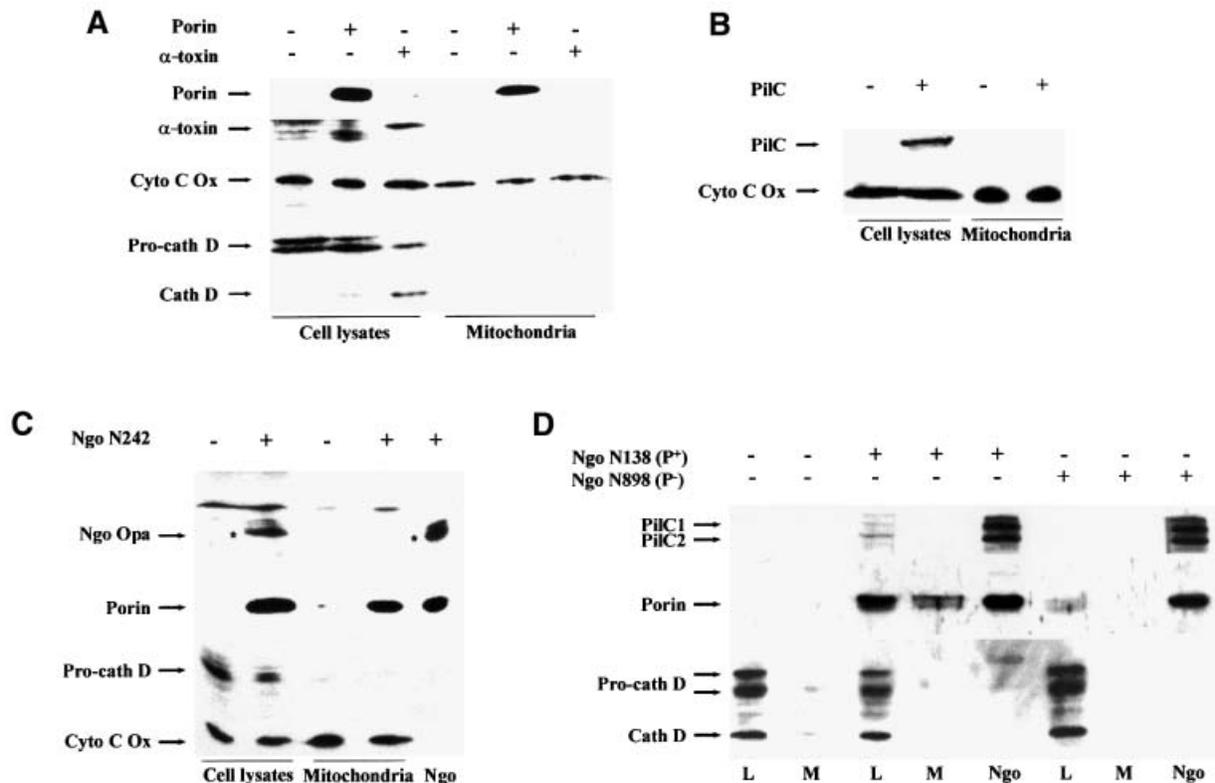
Fig. 6. Porin is targeted to the mitochondria of porin-treated and infected cells. (**A**) HeLa cells were treated with 7 μg/ml porin or respective amounts of staphylococcal α-toxin for 15 h. Lysates were prepared from an aliquot of every sample. In parallel, mitochondria were prepared and both cell lysates and mitochondrial preparations were subjected to western blotting using specific antibodies against porin, α-toxin, cathepsin D and cytochrome *c* oxidase. Detection was performed using the ECL system (Amersham) according to the manufacturer's instructions. (**B**) HeLa cells were treated with 5 μg/ml purified PilC for 15 h. Cell lysates and mitochondria were prepared from treated and control cells and were subjected to western blotting using specific antibodies against PilC and cytochrome *c* oxidase. Detection was performed as described above. (**C**) HeLa cells were infected with gonococcal strain N242 for 15 h at an m.o.i. of 1. Lysates and mitochondria were prepared from infected and non-infected control cells and subjected to western blotting using specific antibodies against Opa proteins, porin, cathepsin D and cytochrome *c* oxidase. Detection was performed as described above. (**D**) HeLa cells were infected with piliated (N138) and non-piliated (N898), isogenic derivatives of gonococcal strain MS11 for 15 h at an m.o.i. of 1. Lysates and mitochondria were prepared from infected and non-infected control cells and subjected to western blotting using specific antibodies against PilC, porin and cathepsin D. Detection was performed as described above. L, lysate; M, mitochondria.

chain and membrane depolarization. PT here occurs rather as a result of caspase activation by the 'apoptosome', a cytosolic complex consisting of dATP, cytochrome *c*, Apaf-1 and caspase-9. This is in contrast to our observation of PT in porin-treated cells in the absence of caspase activity (Figure 2). It is currently not known whether porin-induced PT is a prerequisite for cytochrome *c* release. A remarkable example of PT playing a role in downstream caspase activation is given by the HIV-1 viral protein R (Vpr), which targets mitochondria if added as purified protein or after overexpression in transfected cells (Jacotot *et al.*, 2000). Inhibition of Vpr-induced PT, which is accomplished via interaction with the ANT, also prevented caspase activation and apoptosis.

Overexpression of anti-apoptotic Bcl family members such as Bcl-2 or Bcl-X$_L$ completely inhibited porin-induced apoptosis as determined by morphological criteria and PS exposure. Active caspases were not detected and PT as well as the release of cytochrome *c* was completely blocked in cell lines stably expressing Bcl-2 or Bcl-X$_L$ upon porin treatment. These observations are in accordance with the results obtained in other systems, which suggest that anti-apoptotic Bcl family members act in mitochondria at the level of cytochrome *c* release (Kluck

et al., 1997; Van der Heiden *et al.*, 1997; Yang *et al.*, 1997; Shimizu *et al.*, 1999).

Several models attempting to explain the mechanism of the release of cytochrome *c* and other caspase-activating proteins are currently discussed. One hypothesis suggests that hyperpolarization-induced mitochondrial swelling results in outer membrane rupture followed by release of mitochondrial components from the intermembrane space into the cytosol (Van der Heiden *et al.*, 1997). Another possibility is the formation of large pores by Bcl family proteins such as Bax (Antonsson *et al.*, 2000) and still another model favours rapid loss of inner membrane potential followed by PT (Kroemer *et al.*, 1997). All models are capable of explaining the massive leakage of not only cytochrome *c* and AIF, but also an increasing number of other constituents of the mitochondrial intermembrane space into the cytosol. Among these are active caspases-2 and -9 (Susin *et al.*, 1999a), caspase-3 (Mancini *et al.*, 1998; Samali *et al.*, 1998), AIF (Susin *et al.*, 1999b) and the mitochondrial chaperones HSP60 and HSP10 (Samali *et al.*, 1999; Xanthoudakis *et al.*, 1999). However, none of the models fully explains the phenotype we obtained. The model proposing mitochondrial swelling and outer membrane rupture is in agreement with the
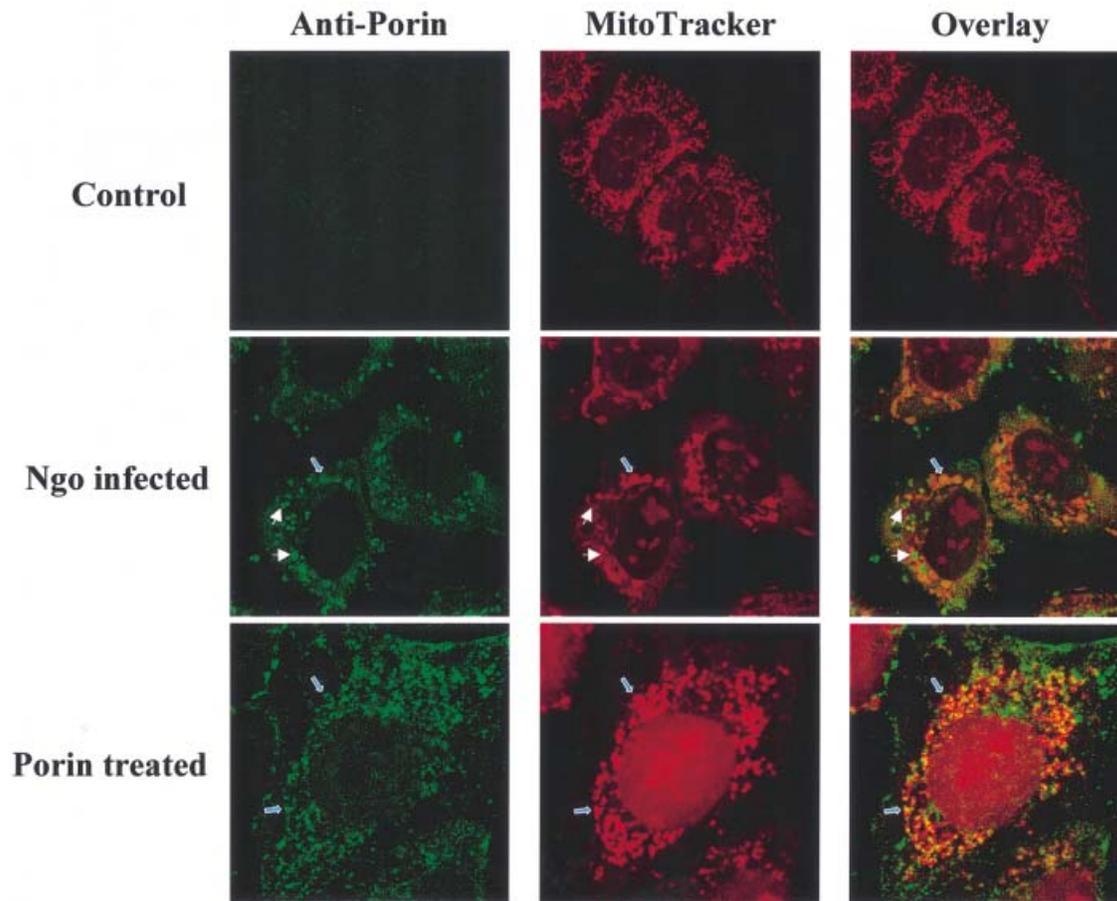
Fig. 7. Porin is targeted to the mitochondria of porin-treated and infected cells. HeLa cells were infected with the non-piliated, Opa-positive gonococcal strain VP1 (N242) for 3 h at an m.o.i. of 1 or treated with 7 µg/ml porin for the same time. Infected, treated and control cells were double stained with MitoTracker and a polyclonal, porin-specific antiserum followed by an Alexa-488-labelled secondary antibody. Single fluorescence pictures and overlays are shown. White arrows point to bacteria, blue arrows to mitochondria.

swelling we observed when isolated mitochondria were treated with porin *in vitro* or when cells were infected with *N.gonorrhoeae*. The effects of porin on isolated mitochondria argue that cytochrome *c* release occurs as a result of channel formation. The release of cytochrome *c* might involve the opening of a pre-existing channel, e.g. by direct binding to one of its components, as was recently described for an interaction of Bax and ANT (Marzo *et al.*, 1998c) or Bax and VDAC (Shimizu *et al.*, 1999).

Several caspases have been shown to induce the release of cytochrome *c* (Marzo *et al.*, 1998b). However, since mitochondria of porin-treated cells completely lost their cytochrome *c* in the presence of the caspase inhibitor zVAD we can exclude this possibility.

Another completely different explanation for the observed mitochondrial changes involves elevations in cytosolic Ca^{2+}. Porin induces a very rapid transient increase in intracellular Ca^{2+}, thereby activating the Ca^{2+}-dependent protease calpain (Müller *et al.*, 1999). One can speculate that increased Ca^{2+} levels may also be sufficient to induce PT, as was shown previously *in vitro* (Marzo *et al.*, 1998a) and *in vivo* (Bernardi and Petronilli, 1996). However, porin induced PT in the absence of extracellular Ca^{2+}, indicating that the influx of Ca^{2+} is not the primary trigger for PT.
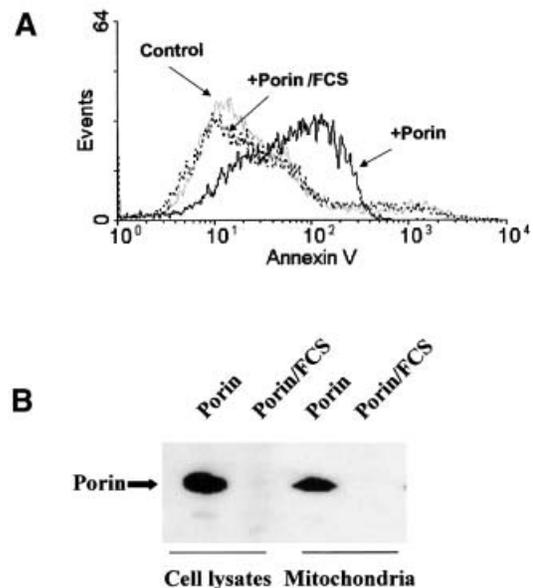

Fig. 8. FCS blocks apoptosis and porin insertion. (**A**) HeLa cells were treated with 7 µg/ml porin for 15 h in the presence or absence of 10% FCS. Cells were harvested and stained with annexin V. (**B**) HeLa cells were treated with porin for 15 h in the presence or absence of 10% FCS. Lysates and mitochondria were prepared and subjected to western blotting using a specific antibody against gonococcal porin variant P.IA.

It is an intriguing feature of the neisserial porin that it translocates from the outer bacterial into artificial membranes and host cell membranes (Lynch *et al.*, 1984; Weel and van Putten, 1991; Rudel *et al.*, 1996). Biochemical analysis of purified mitochondria from porin-treated as well as infected cells clearly reveals that ~50% of all porin found in the cell is localized to these organelles, whereas other neisserial antigens tested were absent. Among these were Opa proteins, very abundant integral proteins in the outer membrane of the gonococcal strains used in this study, which also form several amphipathic β-strands. PilC is found in pili (Rudel *et al.*, 1995a) as well as in the outer membrane (Rudel *et al.*, 1995b; Rahman *et al.*, 1997). Both proteins can thus be considered adequate controls for elucidating artificial fusion of outer membrane vesicles with mitochondria. However, neither Opa nor PilC was found in the mitochondrial preparations of infected cells. To our knowledge, this is the first example ever of a bacterial antigen being transported to host cell mitochondria. Interestingly, the capability of a strain to translocate its porin into host cell membranes is dependent on adherence and correlates with its ability to induce apoptosis. This and the fact that FCS blocks both porin insertion and apoptosis induction argues for a functional connection between these processes.

While the pro-apoptotic effects of porin targeting on the organelles could be demonstrated, the mechanism of the targeting process is completely unknown. One might hypothesize that porin residing in the plasma membrane is somehow internalized by a process similar to recycling of surface receptors and, once inside, couples to the regular intracellular vesicle transport. This would require active participation of the cytoskeleton. However, inhibitors of actin reorganization (cytochalasin D) or microtubules (colchicin) were unable to block targeting of porin to mitochondria (not shown). Furthermore, cross-linking experiments revealed that porin located in mitochondria forms multimers consisting of two and three monomers, i.e. the same complexes that can also be observed in the outer membrane of gonococci. In addition, the process of intracellular trafficking seems to be very fast, since traces of porin were already present in purified mitochondria as early as 20 min post-infection. We therefore assume that the porin moves intracellularly either by means of a soluble intermediate or that it actually has the ability to 'jump' from one membrane to another by an as yet undefined mechanism. Recently, the M11L protein of myxoma virus was shown to target mitochondria via a C-terminal targeting signal sequence that conforms to a newly described consensus sequence present in Bcl family members (Everett *et al.*, 2000). Experiments are currently under way to identify the essential epitopes for mitochondrial targeting of porin. The molecular basis of the insertion of mitochondrial porin into the outer mitochondrial membrane has only recently been elucidated (Schleiff *et al.*, 1999). Integration into the lipid bilayer is accordingly mediated by the mitochondrial receptor Tom20 but bypasses the requirement for the channel-forming complex Tom40. It will be interesting to see if gene deletions in essential components of the mitochondrial import machinery have an effect on neisserial porin insertion similar to that on mitochondrial porin insertion (Sollner *et al.*, 1989).

Although the precise composition of the PT complex is not yet defined it is remarkable that nearly all components identified so far have functional and structural homologues in the bacterial kingdom. The adenine-nucleotide translocator and the peripheral benzodiazepine receptor homologue are found in *Rhodobacter capsulatus* (Armstrong *et al.*, 1989; Carmeli and Lifshitz, 1989). Interestingly, cytochrome *c*, one of the major effectors of mitochondria-mediated caspase activation, is generally found localized to the outer side of the inner membrane in aerobic bacteria. Furthermore, members of the Bcl-2 family exhibit structural homology to colicins, pore-forming toxins produced by *Escherichia coli* (Muchmore *et al.*, 1996). Porins of Gram-negative bacteria (Mannella, 1998) exhibit structural and, in the case of the *Neisseria* porin (Rudel *et al.*, 1996), also functional homologies to eukaryotic porins. Recently, the apoptosis-inducing factor was identified and shown to be homologous to bacterial oxidoreductases (Susin *et al.*, 1999b). Thus, on the basis of these findings there is overwhelming support for the recently raised hypothesis that endosymbiosis of bacteria brought not only aerobic life to the early eukaryotes but also the basic machinery for programmed cell death, a critical step from unicellular to multicellular life (Frade and Michaelidis, 1997; Kroemer, 1997b).

The system of apoptosis induction described in this and our previous study (Müller *et al.*, 1999) employs an infection model of human cells cocultured with the bacterial pathogen *N.gonorrhoeae.* The main advantage of this system for investigating basic questions related to mitochondrial function is that all of the observed morphological and biochemical effects linked to apoptosis induced by the pathogen can be achieved by a single bacterial factor, the neisserial porin. This factor is unique in that it acts directly on purified mitochondria, exerting the same effects (i.e. cytochrome *c* release and loss of membrane potential) as *in vivo*. Since the porin can be detected in the mitochondria of infected cells, this *in vitro* system simplifies the situation without rendering it completely artificial. In addition, the similarities between the neisserial porin and the mitochondrial porin, which is reportedly involved in mitochondrial regulation of apoptosis, invite speculations on aspects of the evolution of the apoptotic machinery.

Materials and methods

KH_2PO_4, 0.3 mM mannitol, 0.1% BSA pH 7.2) and loaded on to a sucrose gradient consisting of 3 ml of SA (lower) buffer (1.6 M sucrose, 10 mM KH_2PO_4, 0.1% BSA pH 7.5) and 3 ml of SB (upper) buffer (1.2 M sucrose, 10 mM KH_2PO_4, 0.1% BSA pH 7.5). The gradient was centrifuged at 72 000 g in a swinging bucket rotor for 1 h. The mitochondria that had accumulated in the interphase were harvested, supplemented with 4 vols of MSM buffer to dilute the sucrose and pelleted by centrifugation at 45 000 g for 10 min. The resulting pellet was resuspended either in sample solution (for electrophoresis and immunoblotting) or in MSM buffer without BSA (for *in vitro* experiments).

Electrophoresis and immunoblotting

Proteins were separated under reducing conditions for 1 h at 160 V in 7, 10 or 12% SDS–polyacrylamide gels and then blotted overnight at 100 mA on to a PVDF membrane (Millipore). The membrane was blocked for 1 h in 3% BSA in Tris-buffered saline (TBS)–0.1% Tween, and then incubated with monoclonal antibodies against cytochrome c (clone 7H8.2C12; Pharmingen, San Diego, CA), cytochrome c oxidase subunit II (Molecular Probes, Eugene, OR), fodrin (Chemicon, Temecula, CA), α-toxin (a generous gift from S.Bhakdi, Mainz, Germany), Opa (a generous gift from M.Achtman, Berlin, Germany), gonococcal porin P.IA and polyclonal antisera against cathepsin D (DAKO, Copenhagen, Denmark) and gonococcal PilC. After three washes in TBS–0.1% Tween, the filter was incubated for 1 h with peroxidase-coupled secondary antibody and bound antibody was detected by enhanced chemiluminescence (Amersham).

Assessment of mitochondrial membrane potential

Cells were incubated with 7 µg/ml porin for 15 h and stained with 5 µg/ml rhodamine 123 (Molecular Probes, Eugene, OR), which was added to the culture medium for 30 min at 37°C. The stained cells were harvested, washed once with PBS and analysed on a FACS-Calibur flow cytometer (Becton Dickinson). Rhodamine fluorescence was monitored in channel 2.

Mitochondrial swelling assay

Mitochondrial swelling assays were performed as described (Uyemura *et al.*, 1997). Briefly, mouse liver mitochondria were purified and resuspended in 125 mM sucrose, 65 mM KCl, 5 mM succinate, 5 µM rotenone, 20 mM $CaCl_2$, 10 mM HEPES pH 7.5, at 0.3 mg/ml. Porin or Ca^{2+} was added to the mitochondria suspension and the OD_{600} of the suspension was measured every 30 s for 10 min. The decrease in optical density correlates with the increase in mitochondrial volume. Since the untreated mitochondria swell spontaneously the same volume of low concentrated porin, which had no effect on swelling, was used as a buffer control. The comparison of the untreated or buffer-treated mitochondria and the porin or Ca^{2+}-treated mitochondria indicates the relative potential of these substances to induce swelling.

In vitro assay for mitochondrial cytochrome c release and PT

Mitochondria were isolated from 5×10^7 cells as described and resuspended in MSM buffer without BSA. A stimulus (e.g. porin or buffer) was applied for 30 min at 37°C to an aliquot of the preparation. The mitochondria were then pelleted at 18 000 g for 5 min and resuspended in sample buffer, whereas the proteins contained in the supernatant were precipitated with trichloracetic acid before resuspension in sample buffer. SDS–PAGE was performed as described above. Alternatively, mitochondria were stained with 5 µg/ml rhodamine 123 and analysed by flow cytometry.

Quantification of PS exposure

The flipping of PS from the inner to the outer leaflet of the plasma membrane is assessed by *in vitro* binding of the serum factor annexin V, which is coupled to FITC for detection by flow cytometry. Cells were treated with porin for 15 h, harvested, washed twice in PBS and suspended in 100 µl of binding buffer (10 mM HEPES–NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V–FITC was added and cells were incubated for 15 min in the dark. After one wash, cells were resuspended in 200 µl of binding buffer and counterstained with 1 µg/ml propidium iodide for determination of permeable (necrotic) cells. Ten thousand cells per sample were analysed with a Becton Dickinson FACS-Calibur equipped with a 15 mW, 488 nm air-cooled argon laser using Cell Quest software. Histograms showing the FITC fluorescence intensity in 1024 channels revealed the amount of annexin V–FITC-positive cells that were determined as apoptotic.

Immunofluorescence microscopy

Cells were seeded on coverslips, treated with porin for 15 h or infected with *N.gonorrhoeae*, and fixed in 3% paraformaldehyde. Fixed cells were permeabilized using 0.2% Triton X-100; non-specific binding was blocked by 30 min of incubation in goat serum, and staining was performed using a monoclonal antibody against native cytochrome c (Pharmingen, San Diego, CA) or a purified polyclonal anti-P.IA serum followed by an Alexa-488-coupled secondary antibody. Colocalization experiments required staining of live mitochondria with 150 nM MitoTracker (Molecular Probes, Eugene, OR) for 30 min at 37°C before fixation. In the case of suspension cells, the staining procedure described above was performed before cytospinning of the cells on to coverslips. Samples were analysed on a Leica confocal microscope using TCS software.

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