Synergistic Pore Formation by Type III Toxin Translocators of *Pseudomonas aeruginosa*†

Eric Faudry,‡ Grégory Vernier,§ Emmanuelle Neumann,|| Vincent Forge,§ and Ina Attree*,‡

Laboratoire de Biochimie et Biophysique des Systèmes Intégrés (UMR 5092 CNRS/CEA/UJF), 17 rue des Martyrs, 38054 Grenoble Cedex 09, France, Laboratoire de Biophysique Moléculaire et Cellulaire (UMR 5090 CNRS/CEA/UJF), 17 rue des Martyrs, 38054 Grenoble Cedex 09, France, and Institut de Biologie Structurale Jean-Pierre Ebel (UMR 5075 CNRS/CEA/UJF), Grenoble, France

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ABSTRACT: Type III secretion/translocation systems are essential actors in the pathogenicity of Gram-negative bacteria. The injection of bacterial toxins across the host cell plasma membranes is presumably accomplished by a proteinaceous structure, the translocon. In vitro, *Pseudomonas aeruginosa* translocators PopB and PopD form ringlike structures observed by electron microscopy. We demonstrate here that PopB and PopD are functionally active and sufficient to form pores in lipid vesicles. Furthermore, the two translocators act in synergy to promote membrane permeabilization. The size-based selectivity observed for the passage of solutes indicates that the membrane permeabilization is due to the formation of size-defined pores. Our results provide also new insights into the mechanism of translocon pore formation that may occur during the passage of toxins from the bacterium into the cell. While proteins bind to lipid vesicles equally at any pH, the kinetics of membrane permeabilization accelerate progressively with decreasing pH values. Electrostatic interactions and the presence of anionic lipids were found to be crucial for pore formation whereas cholesterol did not appear to play a significant role in functional translocon formation.

Most of the Gram-negative pathogenic bacteria (*Salmonella* spp, *Shigella* spp, *Yersinia* spp, *Pseudomonas aeruginosa*, ...) rely on a type III secretion system to cause life-threatening diseases. These "molecular syringes" inject toxic products (also called effectors) into the host cell cytoplasm, in order to facilitate host−microbe interactions (*1, 2*). Effectors are secreted through a needlelike hollow conduit, and the crossing of the eukaryotic plasma membrane is facilitated by a proteinaceous structure believed to form a pore (*3*). This structure, called a translocon, is made up of two bacterial proteins inserted into the host membrane during the infection and possessing α-helical transmembrane domains (*4*−*7*). In ex vivo infection models, type III translocators of *Yersinia* (YopB and YopD) and *P. aeruginosa* (PopB and PopD) provoke cellular membrane damage and channels whose diameter was estimated to be 1.2−3.5 nm by osmoprotection and dye-exclusion experiments (*8*−*10*). Furthermore, the damage caused in the cell membrane by membrane-inserted translocators could also participate in the bacterial virulence by modifying cell signaling, as recently proposed for *Yersinia pseudotuberculosis* (*11, 12*).

The homologous proteins of *Pseudomonas* translocators in *Shigella* (IpaB and IpaC), *Salmonella* (SipB and SipC), and *Yersinia* (YopB and YopD) were shown to interact with artificial membranes (*13*−*18*). In particular, IpaB has been shown to be able to induce either membrane leakage or membrane fusions (*13, 16*). Interactions between the translocators have been reported (*6, 14, 19*−*22*). However, the respective roles of the translocators, the steps that require their interactions and the molecular mechanism of toxin translocation across the host plasma membrane remain unknown.

Recent studies focused on the importance of host cell cholesterol and lipid rafts in the context of the type III secretion-dependent cell intoxication. The lipid rafts, lipid-ordered structures enriched in cholesterol, are able to induce the type III secretion in *Shigella* (*23*). Moreover, the translocators IpaB from *Shigella* and SipB from *Salmonella* were shown to directly bind cholesterol, the cholesterol being essential for virulence effector delivery into host cell (*24*). However, the relevance of cholesterol in each successive step necessary to the passage of the toxins through the host membrane, and particularly the pore formation, is still speculative (*25*).

Although the translocation channel has not yet been visualized in vivo, oligomeric ringlike structures are formed in vitro when the *P. aeruginosa* translocators PopB or PopD are incubated with lipid vesicles (*26*). Here, we present evidence that these oligomeric structures are able to provoke membrane permeabilization. Moreover, PopB and PopD act synergistically to form pores of a defined size. While the binding of the translocators to lipid vesicles takes place at neutral pH, the membrane permeabilization is much more

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* Corresponding author. Tel: 33 4 38 78 34 83. Fax: 33 4 38 78 44 99. E-mail: iattreedelic@cea.fr.

‡ Laboratoire de Biochimie et Biophysique des Systèmes Intégrés.

§ Laboratoire de Biophysique Moléculaire et Cellulaire.

|| Institut de Biologie Structurale Jean-Pierre Ebel.

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effective at mild acidic pH and requires the presence of anionic phospholipids.

EXPERIMENTAL PROCEDURES

Materials. L-α-Phosphatidylcholine and L-α-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3-diazole) were from Avanti Polar. L-α-Phosphatidylserine, cholesterol, sphingomyelin, cerebrosides, and the FITC-labeled dextrans were from Sigma. Sulforhodamine B was from Molecular Probes. Lipids and liposomes were stored under argon and liposomes used within 5 days after preparation.

Protein Expression and Purification. Soluble PopB and PopD, both released from their chaperon PcrH, and PcrV were prepared essentially as described previously (*6*, *26*) with minor modifications: after Ni^{2+}-affinity purification, PopB/PcrH and PopD/PcrH complexes were buffer exchanged on PD10 columns (Amersham) instead of 16/60 Superdex 200 to obtain pure PopB and PopD. The proteins freed from their chaperon were kept in 25 mM sodium acetate pH 5.0 and added extemporaneously to the liposome suspension buffered at the desired pH.

Liposome Leakage Assay. LUVs[1] composed of either PC and PS (8/2, mol/mol); PC, PS, and cholesterol (11/4/5, mol/mol); or PC, cholesterol, sphingomyelin, and cerebrosides (2/2/1/1, mol/mol) (mimicking lipid-rafts (*23*)) were prepared with 25 mM Tris-HCl pH 7.2 containing 50 mM sulforhodamine B by standard reverse phase evaporation. Briefly, lipids were dried in a rotative evaporator and resuspended by sonication in ether−buffer [1/1, vol/vol], and ether was slowly removed by increasing the vacuum prior to extrusion through 0.4 μm (five times) and 0.2 μm (10 times) filters. Unincorporated dye was removed by size-exclusion chromatography on a PD10 column (Amersham) equilibrated with 25 mM Tris, 50 mM NaCl, pH 7.2. Dye efflux was monitored by the increase in fluorescence on a Jasco FP-6500 after the addition of 10 nM protein to a 2 mL suspension of 10 μM LUV in Tris or acetate buffer at different pHs (excitation at 565 nm, emission at 586 nm with slits of 3 and 5 nm, respectively). SRB was selected as fluorescent probe because of its high quantum yield independently of the pH. Fluorescence was normalized with the following equation:

$$F(t)_{\text{norm}} = (F(t) - F_0)/(F_{\text{max}} - F_0)$$

where F_0 is the fluorescence level before protein addition and F_{max} the level after addition of 0.4% Triton X100 at the end of each assay. The initial rate (V_0) was deduced from a linear regression over the first 5 s of dye efflux. The exact pH of the solution within the cuvette was measured after each assay.

A similar procedure was used for detecting the release of FITC-dextrans with mean molecular masses of 4.4, 19.4, and 77 kDa except that the dye concentrations were 15, 4, and 0.5 mM, respectively, and excitation was at 480 nm and emission at 520 nm with slits of 3 and 5 nm, respectively.

Detection of Protein−Liposome Interaction by FRET. Experiments were performed as previously described (*27*) with some adaptations. Briefly, PC−PS LUVs were prepared by reverse phase evaporation (see above) with the addition of NBD-phosphatidylethanolamine (NBD-PE) to the lipid mixture at 1% final concentration and no entrapped dye. Interaction was monitored as an increase of NBD fluorescence on a PTI QM-4 (excitation at 280 nm, emission at 532 nm with both slits of 5 nm) due to energy transfer from the tryptophan residues to the labeled headgroups of the lipids. Measurements were performed at room temperature with 10 μM lipids and 10 nM proteins. Experimental curves were fitted with single-exponential equations using SigmaPlot software.

Binding to Lipids. Overlay assays were adapted from Dowler et al. (*28*). Briefly, 25 μg of lipids in chloroform (PC, PS, cholesterol, and sphingomyelin) were spotted on nitrocellulose membranes (Hybond C, Amersham). The membranes were soaked in a solution of 2% dry skimmed milk dissolved in PBS containing 0.05% Tween 20 (PBS-T) for 1 h. Membranes were then incubated with PopB and PopD (0.1 μM and 0.3 μM, respectively) in PBS-T for 1 h at room temperature. After washing with PBS-T, the presence of the proteins was detected by standard immunoblot techniques using primary antibodies directed against PopB or PopD (*6*).

Transmission Electron Microscopy. Samples prepared as for monitoring membrane permeabilization were incubated for 2 h at room temperature and applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 2% uranyl acetate. A grid was placed on top of the carbon film, which was subsequently air-dried. Micrographs were taken under low-dose conditions with a Philips CM12 microscope operating at 120 kV and a nominal magnification of 13000.

RESULTS

PopB and PopD Interact with Lipid Vesicles Independently of pH. Purified PopB and PopD proteins, detached from their common chaperon PcrH, were obtained by a two-step purification procedure (see Experimental Procedures). The binding of PopB and PopD to lipid vesicles was monitored by the method of fluorescence resonance energy transfer (FRET). When protein tryptophan residues, the donors, approach the lipid headgroups labeled with the fluorochrome *N*-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD), the acceptors, FRET is detected as an increase of the acceptor fluorescence. Figure 1A shows representative records of the FRET time course. When large unilamellar vesicles (LUVs) made up of phosphatidylcholine (PC) and phosphatidylserine (PS) were used, the FRET took place within 80 s, and similar kinetics were observed with PopB, PopD, and an equimolar mixture of both proteins. When LUVs containing only PC were used, no FRET was detected in any condition and whatever the proteins present (Figure 1A). As a control, the experiment was performed with PcrV (Figure 1A), a third partner of Pop proteins in vivo involved in translocation but not expected to interact with membranes (*6*). In that case no FRET was detected. PopB, PopD, and PcrV contain 1, 2, and 3 tryptophan residues, respectively.

[1] Abbreviations: LUVs, large unilamellar vesicles; SRB, sulforhodamine B; NBD, *N*-(4-nitrobenzo-2-oxa-1,3-diazole); FRET, fluorescence resonance energy transfer; PC, phosphatidylcholine; PS, phosphatidylserine; Cer, cerebrosides; PE, phosphatidylethanolamine.

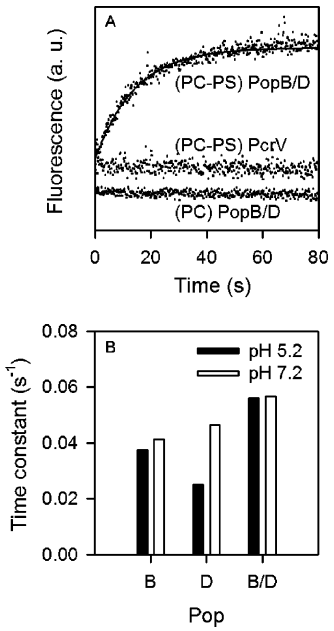

FIGURE 1: Interaction of Pop proteins with liposomes detected by FRET. Liposomes (PC−PS 8/2, mol/mol, or PC alone) labeled with 1% L-α-phosphatidylethanolamine-NBD were incubated at t_0 with Pop proteins or the control protein PcrV, at a lipid/protein molar ratio of 1000, and the fluorescence resulting from energy transfer from tryptophan to labeled headgroup was recorded. (A) Representative time course data set for the PopB−PopD equimolar mixture with PC−PS and PC vesicles and PcrV with PC−PS vesicles as a negative control. The signal recorded in the case of PC alone was downshifted for sake of clarity. The continuous line shows the fit curve described below. (B) The fluorescence increase corresponding to FRET was recorded and the data fitted with a single-exponential equation. Time constants are presented for PopB, PopD, and the PopB−PopD mixture, at pH 5.2 (black columns) and 7.2 (white columns).

Fluorescence time courses were fitted with single-exponential equations in order to estimate their time constants (Figure 1A, continuous line). The values obtained for the various combinations of Pop proteins at two pHs are reported in Figure 1B. The time constants range from 0.025 s^{-1} to 0.057 s^{-1}. The rate of binding of PopB is similar for both pHs investigated, while the binding of PopD is slower at acidic pH. The binding detected upon the addition of an equimolar mixture of PopB and Pop D is slightly faster at both pHs. Finally, the binding rate of the PopB−PopD mixture is not sensitive to the pH

PopB and PopD Synergistically Permeabilize Vesicles. We then examined membrane permeabilization by measuring the release of a fluorescent dye, sulforhodamine B (SRB), entrapped at self-quenching concentration within the PC−PS LUVs. When the bilayer integrity is disrupted, the encapsulated dye leaks out and is immediately diluted in the external medium, leading to an increase in fluorescence proportional to the amount of released fluorochrome (*29*).

The release of the dye from the PC−PS vesicles induced by PopB and PopD was very slow at neutral pH (data not shown). In contrast, the SRB release is readily observed at pH 5.2 (Figure 2A). At this pH, we compared membrane permeabilization caused by PopB, PopD, and a mixture of both proteins. SRB efflux was markedly slower with PopD than with PopB, although all kinetics tended toward the same plateau. Of interest, the SRB efflux in the presence of an equimolar mixture of PopB and PopD was significantly faster

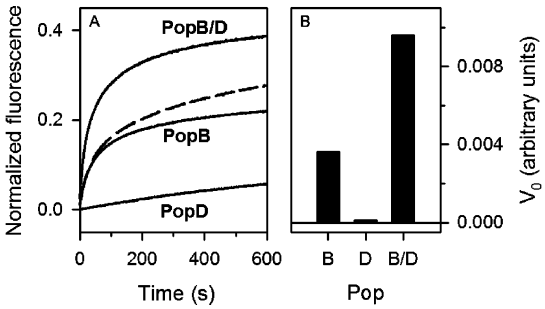

FIGURE 2: Membrane permeabilization by Pop proteins. Liposomes (PC−PS) were incubated with Pop proteins, and the increase in fluorescence reflecting the release of sulforhodamine B (SRB) loaded in liposome at self-quenching concentration was recorded. (A) Comparative kinetics of liposome permeabilization by Pop proteins. Proteins were added to the liposome suspension at pH 5.2, and fluorescence was monitored. The dashed line represents the sum of the PopB plus PopD kinetics. (B) Comparison of the initial rates of SRB release obtained upon incubation with PopB, PopD, and the equimolar mixture of PopB and PopD.

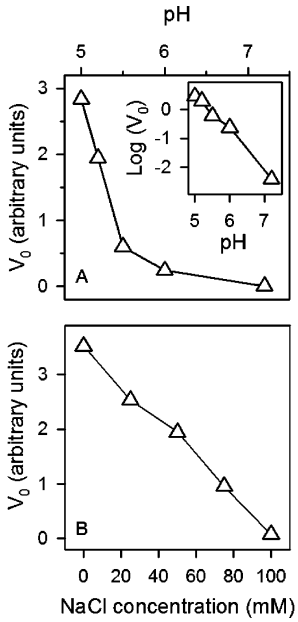

FIGURE 3: pH and NaCl dependence of membrane premeabilization. (A) pH dependence of permeabilization by the equimolar mixture of PopB and PopD. The initial rate of fluorescence increase (V_0) was plotted against the pH values. Inset shows the same graph with logarithmic *y* scale. (B) Effect of NaCl concentration on permeabilization by the equimolar mixture of PopB and PopD. The initial rate of fluorescence increase (V_0) was plotted against the NaCl concentration.

than those induced by individual proteins and even faster than the sum of the individual effects of PopB and PopD (Figure 2A; dashed line), uncovering a synergistic effect. Preliminary experiments using different molar ratios of PopB and PopD indicated that the more marked synergy effect was observed with an equimolar mixture (data not shown). Since the SRB efflux kinetics did not obey a simple equation such as a single or double exponential, the initial speed (V_0) was used to compare the release kinetics under different conditions (Figures 2B and 3). At pH 5.2, the value of V_0 obtained for the PopB−PopD mixture is about 2.6 times higher than that for PopB alone and than the sum of the values observed for PopD and PopB individually (Figure 2B), demonstrating the synergy between the two translocators in membrane permeabilization. In a similar way as for the liposome

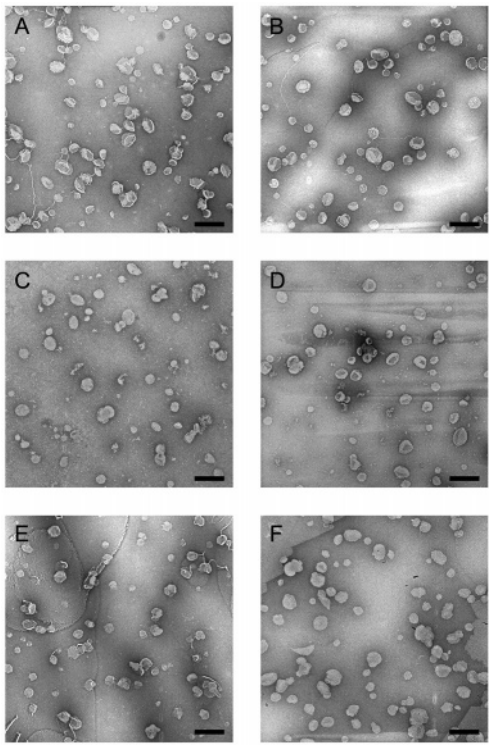

FIGURE 4: Pore formation does not induce vesicle collapse. Liposomes made of PC−PS (A, C, E) or PC−PS−Chol (B, D, F) were observed by negative-stain electron microscopy. Prior to stain with 2% uranyl acetate, liposomes were left untreated (A, B) or incubated with a PopB−PopD equimolar mixture at pH 7.2 (C, D) or at pH 5.2 (E, F). Scale bars correspond to 500 nm.

binding experiments, PcrV was used in control experiments. In the case of PcrV, no permeabilization could be detected at any pH (data not shown).

The pH dependence of V_0 is shown for the PopB−PopD mixture only (Figure 3A). In contrast to the Pop−lipid interaction monitored by FRET (Figure 1B), dye efflux was dependent upon pH (Figure 3A). Notably, lowering the pH from 7.2 to 5.2 resulted in a 500 times increase in the initial rate of dye release (Figure 3A; inset), whereas Pop−LUV interaction was similar at both pH's (Figure 1B), showing that membrane association can take place without the formation of a functional pore.

According to the FRET experiments, used to monitor the binding of the Pop proteins to LUVs, the presence of anionic phospholipids (PS) is determinant for protein−lipid binding, suggesting that electrostatic interactions between the proteins and the membrane interface are necessary. The importance of these interactions in membrane permeabilization was further examined by monitoring the dye efflux from PC−PS vesicles in the presence of increasing salt concentrations. The initial rates of dye release decreased as the salt concentration increased (Figure 3B), showing that electrostatic interactions are indeed involved in the pore formation process.

Size-Defined Pores Are Responsible for Vesicle Permeabilization. Membrane permeabilization and the subsequent dye efflux could result from the formation of a pore as well as from other membrane disruption mechanisms leading to liposome lysis, such as phopsholipase activity or membrane micellization. In a previous study, we found that cholesterol-containing vesicles lysed upon incubation with

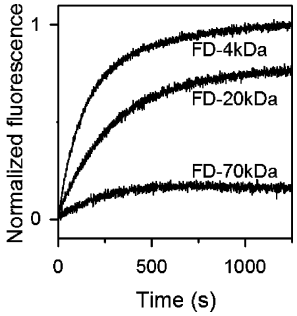

FIGURE 5: Pop proteins form size-selective pores. Release of FITC-dextrans (FD) of 4, 20, and 70 kDa by an equimolar mixture of PopB and PopD. Liposomes (PC−PS) were incubated with the Pop protein mixture at pH 5.2. The increase in fluorescence reflects the release of FITC-dextrans loaded in liposome at self-quenching concentration.

PopB and/or PopD using proteins prepared in acidic conditions (*26*). To examine the Pop/liposome mixtures used in the present work, we performed observations by negative-stain electron microscopy (EM) after 2 h of incubation (Figure 4). As already suggested by dye-release kinetics from liposome of various lipid compositions and light scattering experiments (not shown), no vesicles collapse or fusion could be observed upon incubation with Pop proteins. Similar populations of vesicles were observed by EM independently of the pH, the presence of proteins, and the addition of cholesterol in the lipid composition.

To further test the hypothesis of the formation of a pore and to estimate its diameter, leakage assays were performed with entrapped labeled molecules of increasing sizes (*30*, *31*). Fluorescein-labeled dextrans of various sizes were entrapped in vesicles at self-quenching concentrations, and membrane permeabilization was monitored as described for the SRB release experiments. Dextran molecules of 4 and 20 kDa (hydrodynamic radius, R_H, of 2.3 and 3.4 nm, respectively) were readily released from lipid vesicles in the presence of the Pop mixture, the 20 kDa dextran release being somehow slower (Figure 5). In contrast, the 70 kDa dextran (R_H of 6.1 nm) was retained (Figure 5), strongly supporting that PopB and PopD formed functional size-defined channels of a diameter between 3.4 and 6.1 nm within the membrane vesicles. The same size selectivity was observed for PopB and PopD individually. The limited increase in fluorescence observed with the 70 kDa dextran in the first 500 s is markedly different from those observed with the smaller labeled dextrans and may be a consequence of the lipid vesicles swelling due to the formation of a pore allowing the entry of water but not the release of the 70 kDa dye. As expected, no release of the 70 kDa dextran was observed with vesicles containing cholesterol (data not shown).

Cholesterol Is Not Essential for Pore Formation. In a recent study, IpaB and SipB (the *Shigella* and *Salmonella* counterparts of PopB) were shown to bind cholesterol (*24*). The experiments presented above were performed with vesicles devoid of cholesterol, indicating that binding and pore formation do not require cholesterol in the *P. aeruginosa* type III secretion/translocation model, at least in vitro. Thus, we tested whether Pop proteins could directly interact with cholesterol and/or other phospholipids.

In protein−lipid overlay assay, the presence of the proteins bound to the lipids was revealed by specific antibodies

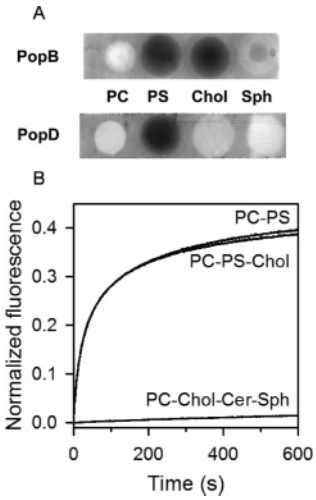

FIGURE 6: Importance of lipid composition for pore formation. (A) Phosphatidylcholine (PC), phosphatidylserine (PS), cholesterol (Chol), and sphingomyelin (Sph) were spotted on nitrocellulose membranes, which were subsequently incubated with PopB or PopD. Binding of Pop proteins to the lipids was detected using PopB and PopD specific antibodies. (B) Liposomes with different lipid compositions were incubated with a PopB−PopD equimolar mixture at pH 5.2. The increase in fluorescence reflects the release of sulforhodamine B loaded in liposome at self-quenching concentration. Abbreviation: cerebrosides (Cer).

and chemiluminescence. PopB was able to bind cholesterol immobilized on nitrocellulose (Figure 6A). In addition, both PopB and PopD bound to PS but not to PC nor to sphingomyelin (Figure 6A). No binding was observed with the control protein PcrV, neither to phospholipids nor to cholesterol (data not shown). To further examine the importance of cholesterol in the pore formation, membrane leakage was monitored from vesicles with various lipid compositions. The kinetics of dye release from LUVs made of PC−PS−cholesterol were not markedly different from those observed with PC−PS vesicles (Figure 6B), exhibiting similar pH and charge dependences (data not shown). Substituting PS by other negatively charged phospholipids, i.e., phopshatidylglycerol or phosphatidic acid, did not modify the pore-formation kinetics (data not shown). In contrast, LUVs with various lipid compositions but lacking anionic phospholipids were not prone to pore formation. As an example, fluorescence measurement obtained with raftlike vesicles made of PC−cholesterol−sphingomyelin−cerebrosides (*23*) is presented in Figure 6B. Moreover, no Pop binding was detected to LUVs made up of the same lipids, as assessed by FRET experiments (data not shown). Therefore, negatively charged phospholipids are absolutely required for binding to and perforation of vesicles by Pop proteins.

DISCUSSION

The molecular mechanisms underlying the passage of type III secreted effectors across the host−cell plasma membrane are still unknown. The subclass of type III secreted proteins, named translocators, possessing α-helical transmembrane domains and being associated with eukaryotic cells during infection has been identified. However, their respective functions in translocation remain speculative. *P. aeruginosa* translocators PopB and PopD are able to form ringlike structures with inner and outer diameters of 4 and 8 nm, respectively, strongly suggesting that these structures may correspond to translocation pores inserted within host−cell membranes (*26*). We set up experiments with the purpose of functionally characterizing PopB and PopD, and we show that they form size-defined pores within liposome membranes.

The membrane leakage observed upon incubation with an equimolar mixture of PopB and PopD was faster than the mathematical sum of PopB- and PopD-induced dye releases (Figure 2), disclosing that the two translocators operate in synergy. Models of infection with mutant bacteria showed that both proteins are necessary for the translocation process (*4*, *6*, *8*, *32*, *33*), and the translocators were shown to interact with each other in liposome extracts as well as in solution (*6*, *14*, *19*−*22*). These elements led to the hypothesis that the translocation is performed by the two proteins working in concert. The synergy documented here in liposome perforation assay is direct functional evidence that validates this hypothesis. From our experiments it cannot be inferred that PopB and PopD form equimolar complexes within the membrane, because an unknown amount of self-associated species may also interact with the vesicle membranes, making the interpretation of the synergy effect in terms of stoichiometry difficult. Further experiments are underway to unravel the exact pore composition.

Using FITC-labeled dextrans, we showed that the membrane perturbations caused by PopB and PopD in vesicle membranes allow the release of molecules with an average R_H of 3.4 nm, while those with a R_H of 6.1 nm are retained (Figure 5). Therefore, type III translocators form genuine size-selective pores. This size selectivity is consistent with the 4 nm internal diameter of the described ringlike structures formed by PopB and PopD in liposomes (*26*) and the size of the translocon estimated by osmoprotection experiments in vivo: between 2.8 and 3.5 nm for *P. aeruginosa* (*9*) and between 1.2 and 3.5 nm concerning other bacteria species (*3*). The apparent larger size observed in our experiment in comparison to the in vivo findings could be related to the differences in the techniques employed: release of dextran versus protection against lysis by external osmoprotectants. In addition, these dimensions are consistent with the sizes of the pores formed by other toxins (usually 0.5 to 3 nm), with the exception of the cholesterol-dependent cytolysins that induce pores larger than 15 nm (*29*, *34*).

Cholesterol and lipid rafts seem to be involved in several steps of type III mediated cell intoxication, but their roles in pore formation per se remain to be elucidated (*25*). Unlike PopD, PopB specifically binds cholesterol immobilized on a nitrocellulose membrane and their *Salmonella* and *Shigella* counterparts exhibit similar features (*24*). However, in present experiments PopB and PopD were able to interact with and perforate cholesterol-free vesicles and inclusion of cholesterol did not substantially modify the pore-formation kinetics, indicating that cholesterol is not central for in vitro pore formation. Although PopB and its counterparts bind cholesterol adsorbed to a solid phase or in complex with cyclodextrin (this work and ref *24*), the orientation and accessibility of cholesterol inserted within lipid bilayers do not allow proper protein binding (FRET and dye-release experiments). In our previous work we found that cholesterol-containing

vesicles lysed upon incubation with Pop proteins. This was not the case in the present work, as observed by EM (Figure 4). Since we used the same proteins, it appears that differences in vesicle preparation technique (reverse evaporation vs detergent dialysis) could be responsible for this difference. Indeed, one could not exclude the presence of residual detergent in the membrane of vesicles prepared by detergent dialysis (*35*), which would lead to a lower stability of cholesterol-containing vesicles.

Other studies already reported interactions of the Pop homologues with model membranes devoid of cholesterol (*13*, *15*−*17*, *20*, *36*, *37*). It is conceivable that cholesterol plays an indirect role in the translocation process, for example via the lipid rafts and the receptors they harbor. Alternatively, lipid vesicles may be too distant models to decipher the role of cholesterol, because they lack the membrane potential and the asymmetric lipid distribution found in cellular membranes.

Charged phospholipids exhibited a crucial role regarding the binding of proteins to lipid vesicles and formation of functional pores. Both proteins, PopB and PopD, were able to interact directly with PS in an overlay assay. In functional assays, neither significant dye release nor interaction could be detected with liposomes devoid of anionic lipids (this work and ref *26*). Consistently, IpaB- and IpaC-induced liposome permeabilization is dependent on charged phospholipids (*15*, *16*, *37*). Like the α-pore forming toxins exotoxin A, colicin, and the translocation domain of the diphtheria toxin (*34*, *38*), the Ipa and Pop proteins induce faster pore formation at mild acidic conditions (Figure 3A and refs *15* and *16*). Several reports showed that the preincubation of the translocators with urea increases their ability to permeabilize vesicle membranes (*15*, *16*). Indeed, we could observe significant membrane leakage even at neutral pH when PopB and PopD are incubated with chaotropic salts prior to contact with liposomes (data not shown). Thus, it appears that mild denaturing conditions (acidic pH or chaotropic salts) improve pore formation, presumably by exposing hydrophobic stretches and lowering the energy required for the conformational changes.

We show here that interaction of Pop translocon with the membrane can occur independently of functional pore assembly (Figures 1B and 3A), as pore formation, but not membrane association, is pH-sensitive. In vivo, the signal for this switch from prepore to pore can be either a cellular receptor or a bacterial protein passing through the needle. Alternatively, the interaction with a "helper protein" such as PcrV may facilitate proper PopB/D translocon assembly in host membranes. Indeed, PcrV is required for translocon assembly in vivo (*7*) and is presumably localized at the tip of the needle (*39*). Otherwise, local physicochemical conditions, such as the pH, as suggested by our experiments, may be influencing translocon formation.

The identification of distinct stages in pore formation is a valuable tool for structural studies aimed at uncovering the pore formation mechanisms (*40*, *41*). The structural modifications taking place during the translocation pore opening are currently being investigated by biochemical and biophysical approaches.

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