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Neisseria gonorrhoeae Porin Modulates Phagosome Maturation*

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The porin (PorB) of *Neisseria gonorrhoeae* has been implicated in the pathogenesis of this species. Porin is believed to translocate from the bacterial outer membrane into target cell membranes affecting various cell functions. Here we investigated the effect of porin on phagosome maturation. Phagocytosis of latex beads by human macrophages was allowed in the presence or absence of purified porin. Isolation of latex bead-containing phagosomes and subsequent two-dimensional gel electrophoresis revealed substantial differences in the phagosomal protein composition. Immunoblotting detected higher amounts of annexin II and the early endocytic markers Rab5 and transferrin receptor and decreased levels of the late endocytic markers Rab7 and cathepsin D in phagosomes obtained in the presence of porin compared with those obtained in its absence. Furthermore, association of Rab4 with the latex bead-containing phagosomes was revealed by flow cytometry. The amount of this small GTPase was markedly higher in the phagosomes isolated in the presence of porin. The data thus indicate that neisserial porin is itself able to arrest phagosome maturation within macrophages.

Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhea, colonizes the mucosae of the urethra, endocervix, conjunctiva, fallopian tube, rectum, and pharynx. Occasionally, however, gonococci disseminate to cause severe diseases including bacteremia, which may lead to clinical complications such as purulent arthritis, pelvic inflammatory disease, endocarditis, and meningitis (1). Phagocytic cells, such as monocytes, may play a role in the dissemination of gonococci. Gonococci may encounter and survive the intracellular processing of phagocytic cells (2, 3). An important determinant thought to contribute to survival of these gonococci in host cells is porin (PorB). PorB, the most abundant outer membrane protein in *N. gonorrhoeae* (4), functions as a classical porin, serving as an ion and nutrient transport channel in the outer membrane (5). Furthermore, the porin of pathogenic *Neisseria* reportedly translocates from the outer membrane of the gonococci into artificial membranes (6) as well as into that of the host cell (7–9). The notion that PorB plays an important role in virulence comes from epidemiological observations indicating a strong association of certain variant forms of PorB, *i.e.* $PorB_{IA}$,[1] with disseminated gonococcal infections, whereas $PorB_{IB}$-expressing strains have mostly been isolated from patients with local infections (10). This linkage has been supported by *in vitro* observations showing that meningococcal porin transfers into the lipid bilayer at a higher rate (1500 pores/h) compared with the $PorB_{IA}$ (100 pores/h) and $PorB_{IB}$ (5–10 pores/h) porins of *N. gonorrhoeae*. The nonpathogenic *Neisseria sicca* did not transfer porin function to the bilayer (6). These observations suggest that the pore-forming activity may reflect the pathogenesis of a neisserial strain.

Translocation of purified PorB into the host cell membrane affects the antibactericidal activity of the activated polymorphonuclear cells and initiates a series of events that include a transient change in the membrane potential (11), inhibition of host cell granule exocytosis without affecting the NADPH oxidase activity (11–13), and inhibition of actin polymerization and subsequent phagocytosis of meningococci (14). These observations have led to the speculation that PorB may play a role in the modification of the maturation of the phagocytic vacuole, thus facilitating bacterial intracellular survival. Recently, we described an intriguing similarity of PorB to eukaryotic or mitochondrial porins with regard to both structural and physicochemical features and their binding of nucleotide triphosphates (15). Interestingly, purified PorB induces a calcium influx, and its channel activity in the eukaryotic membrane appears to be regulated by ATP/GTP.[2]

Phagosome maturation is a complex process and involves a dynamic exchange of phagosomal components with several intracellular compartments (17). Soon after ingestion of a particle or a microorganism, a series of sequential membrane fusion and budding takes place, which leads to maturation of the phagosome, and delivery of lysosomal markers and enzymes causes destruction of the ingested material. Recent reports have suggested that, during maturation, phagosomes fuse with early (18–20) and late (21) endosomes. Examples of alteration in the membrane composition include the recycling of a range of plasma membrane receptors (19, 20, 22) and the acquisition of endosomal markers such as the mannose 6-phosphate receptor and the lysosome-associated membrane proteins (22–24). The level of soluble contents of maturating phagosomes, including the lysosomal hydrolases cathepsin D and β-glucuronidase, increases markedly over a period of time after internalization (22). Maturating phagosomes were shown to acquire the late endosomal small GTP-binding protein marker Rab7 and to lose

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[1] The abbreviations used are: $PorB_{IA}$ and $PorB_{IB}$, proteins IA and IB (structurally related forms of gonococcal porins, *i.e.* P.IA and P.IB), respectively; LP, latex bead phagosome; LP^+, latex bead phagosome(s) prepared in the presence of PorB; LP^-, latex bead phagosome(s) prepared in the absence of PorB; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TfR, transferrin receptor; HLA-DR, human leukocyte antigen DR-α.

[2] A. Müller, D. Günther, F. Düx, M. Naumann, T. F. Meyer, and T. Rudel, submitted for publication.

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the early endosomal marker Rab5 (24).

Members of the Rab subfamily are believed to govern vesicular transport (vesicle docking/fusion) in the exocytic and endocytic vesicles in mammalian cells (25–27) by regulating interaction between targeting molecules on the transport vesicles and the acceptor membrane (27). Several studies have suggested that fusion and budding in the phagocytic pathway are regulated by the Rab subfamily since proteins such as Rab5 and Rab7 and other small GTPases were found to be localized on phagosomes isolated at different time points during maturation of phagosomes to phagolysosomes (24, 28).

To determine whether PorB modifies intracellular processing of the phagosome, we reconstituted the process of gonococcal phagocytosis with latex beads after treatment of primary human macrophages with purified PorB. Latex bead phagosomes (LPs) were recovered from cells at various time points, and their protein composition and the distribution of cellular markers were analyzed. These were compared with phagosomes formed in the absence of PorB. Significant differences in the phagosomal protein composition were found. Furthermore, we detected higher amounts of annexin II and early endocytic markers and decreased levels of late endocytic markers in phagosomes obtained in the presence of porin compared with those obtained in its absence. These data show that association of PorB with the plasma membrane and LPs modifies the phagosomal processing.

EXPERIMENTAL PROCEDURES

Preparation and Cultivation of Human Macrophages—Monocytes were isolated from "buffy coat" blood of healthy human blood donors by density gradient centrifugation over Ficoll-Isopaque (Biochrom KG Seromed, Berlin, Germany). Buffy coat blood bags (8–10 bags/experiment, 50–120 ml/bag; obtained from the Blood Transfusion Section of the University Clinic in Tübingen, Germany) were diluted in PBS without Ca^{2+} and Mg^{2+} (PBS^-), layered over Ficoll, and centrifuged ($277 \times g$, 25 °C, 30 min). The mononuclear cells at the interfaces between the plasma and Ficoll were collected and washed twice with PBS^-. The pellets of different buffy coat blood fractions were resuspended together in 40 ml of McCoy's medium supplemented with 20% human AB serum (29), and monocytes were allowed to adhere to tissue culture Petri dishes for 2–3 h. Non-adherent cells (lymphocytes) were washed with PBS containing Ca^{2+} and Mg^{2+} (PBS^+), and the remaining adherent cells (predominantly monocytes) were supplemented with new fresh medium containing 20% human AB serum. During the next 3–5 days, the medium was changed daily. Macrophages started to arise on the third or fourth day of cultivation. Macrophage maturation was judged morphologically using phase-contrast microscopy according to criteria adopted by Hanifin and Cline (29).

Neisserial Porin—Porin was purified from the disseminating *N. gonorrhoeae* isolate VP1, producing a $PorB_{IA}$ type of porin, and reconstituted in detergent as described previously (15). Purity of purified porin was checked by SDS-PAGE.

Formation and Isolation of $PorB^+$ and $PorB^-$ LPs—To isolate $PorB^+$ and $PorB^-$ LPs (referred to here as LP^+ and LP^-), one batch of macrophages was prepared as described above. One-half of the recovered cells was treated with 6 μg/ml PorB for 5 min, whereas the other half was treated with the same buffer excluding porin. Latex beads (0.8-μm diameter, 10% suspension, blue-dyed; Sigma) were then added to the cells at a dilution of 1:100 in McCoy's medium supplemented with 20% human AB serum. After a 55-min pulse (internalization), the cells were washed and homogenized (0 chase) or washed, warmed to 37 °C, and incubated (chased) for 1.5 and 7 h before homogenization. LPs were then isolated by centrifugation on a discontinuous sucrose gradient as described by Desjardins *et al.* (24).

Transmission Electron Microscopy (TEM)—Human macrophages and isolated LPs were fixed in 2.5% cacodylate-buffered glutaraldehyde (pH 7.2) containing 5 mM $CaCl_2$ for 1 h, washed with cacodylate buffer, embedded in 2% agar, and post-fixed in the same fixative. After washing, agar blocks containing the pellets of cells or phagosomes were immersed in 1% OsO_4, dehydrated stepwise in ethanol (50–100%), and embedded in Durcupan (Fluka). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM109 electron microscope.

Association of Porin with the Plasma Membrane of Human Macrophages and LPs—Macrophages were incubated for 5 min with purified PorB at a concentration of 6 μg/ml in McCoy's medium plus 20% human AB serum. The cells were then washed three times with PBS^+, scraped with a rubber policeman, and washed three times by centrifugation ($277 \times g$) with 3% bovine serum albumin-containing PBS^- to dissociate any nonspecific interaction between PorB and the plasma membrane. Total integral proteins from the plasma membrane were first extracted by Triton X-114 (30) and precipitated by methanol/chloroform (31). LPs were isolated in the presence and absence of porin as described above. All samples were separated by SDS-PAGE on 12% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and analyzed by Western blotting using the $PorB_{IA}$-specific monoclonal antibody (9).

Western Blot Analysis of Cellular Markers in LPs—Proteins from the same number of LP^+ and LP^- (100×10^6, estimated using a counting chamber with a 10× objective lens and correction of the phase-contrast disc that illuminates the blue-dyed beads on a dark background) isolated at two time points (55-min pulse + 0-min chase and 55-min pulse + 7-h chase) were separated by SDS-PAGE on 12% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and checked for association of cellular markers with the appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) or goat anti-mouse IgG (1:5000). Blots were developed by ECL (Amersham Pharmacia Biotech). Different cellular markers were analyzed using affinity-purified anti-Rab4, anti-Rab5, and anti-Rab7 antibodies prepared as described by Zerial *et al.* (32); a rabbit polyclonal anti-cathepsin D antibody (a kind gift of Dr. K. Figura, Institut für Biochemie II, Göttingen, Germany); a monoclonal antibody against the C-terminal end of the HLA-DR-α chain (a generous gift of Dr. J. Trowsdale, Imperial Cancer Research Fund, London, United Kingdom); and monoclonal antibodies against the transferrin receptor (TfR) and annexin II purchased from Calbiochem and Transduction Laboratories, respectively.

Flow Cytometry—Flow cytometry was employed as an alternative method to check for association of Rab4 with LP^+ and LP^-. Analysis of single organelles after disruption of cells using flow cytometry is now known as single organelle fluorescence analysis (for review, see Ref. 33). Single organelle fluorescence analysis was performed as follows. LP fractions (2×10^6 phagosomes/fraction) were incubated for 30 min on ice with the above-described rabbit polyclonal anti-Rab4 antibody at a final concentration of 7 μg/ml. Rabbit IgG (7 μg/ml; Sigma) was used as a negative control. The LPs were then incubated for 30 min on ice with goat anti-rabbit Ig conjugated with phycoerythrin (Dianova) as a second antibody at a dilution of 1:100. LP^+ and LP^- were separated from the rest of the solution by floating on a sucrose step gradient (62, 40, 35, 25, and 10%). The gradients were centrifuged using a Beckman TL-100 centrifuge at 4 °C in a Beckman TLS-55 swinging bucket rotor for 10 min at 35,000 rpm. The LP bands were collected from the interface between the 10 and 25% sucrose concentrations and analyzed using a FACS Vantage (Becton Dickinson) equipped with a Coherent Enterprise laser turned to 120 milliwatts of output at 488 nm. Fluorescence was measured using a 575 ± 26-nm filter. Phagosomes (2×10^4) of each sample were analyzed.

Two-dimensional PAGE—The same numbers of LP^+ and LP^- isolated at 0, 1.5, and 7 h after phagocytosis were solubilized in lysis buffer, and the proteins were separated on the basis of their molecular properties in each dimension as described by Bjellqvist *et al.* (34). In the first dimension, nonlinear immobilized pH gradient strips ranging from pH 4 to 9 were used, and the running conditions were as described previously (35). In the second dimension, SDS-PAGE was performed with 1.5-mm thick gradient gels (9–16%). Two-dimensional polyacrylamide gels were stained with ammoniacal silver (36) and scanned using a laser densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) connected to a workshop station power point (Macintosh). The amount of a polypeptide spot was estimated by the relative volume and the relative density in the two-dimensional gel images using Melanie II analysis software (Bio-Rad). The two parameters reflect the amount of each spot in LP^+ and LP^- polypeptide patterns computed after correction of the differences in gel staining.

RESULTS

Preparation of LP^+ and LP^- from Human Macrophages—Maturation of macrophages from primary human monocytes isolated from buffy coat blood required only the addition of serum factors provided by the human AB serum. No antibiotics were needed to obtain contamination-free cultures. In each experiment, cells from 8–10 bags of buffy coat blood were

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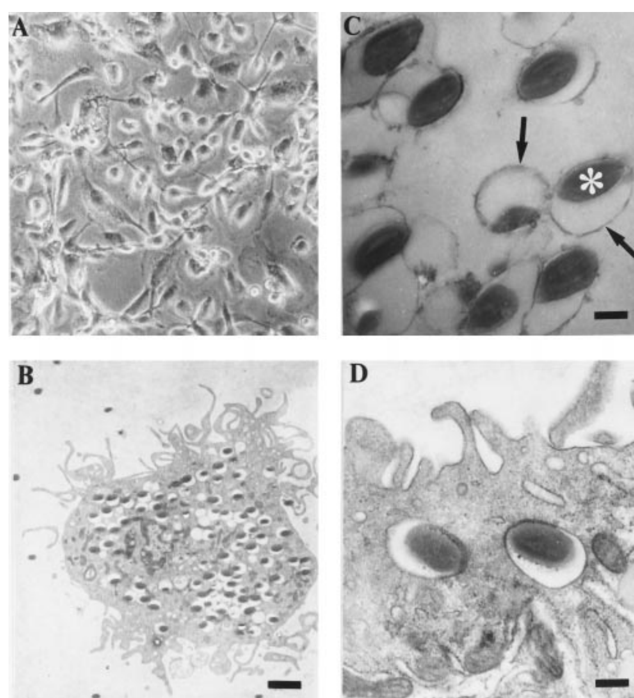

FIG. 1. **Phase-contrast microscopy of human macrophages and electron microscopy of intracellular and isolated latex beads.** *A*, phase-contrast micrograph of a monolayer of 3-day-old human macrophages (with a 20× objective lens) derived from peripheral blood monocytes. *B*, TEM of a macrophage after internalization of latex beads for 90 min. *C*, TEM of an isolated latex bead phagosome fraction devoid of contamination by other cellular organelles. Note the intact membrane around the beads similar to intracellular morphology shown in *D*. *Bars* represent 2.5 μm in *B* and 0.4 μm in *C* and *D*.

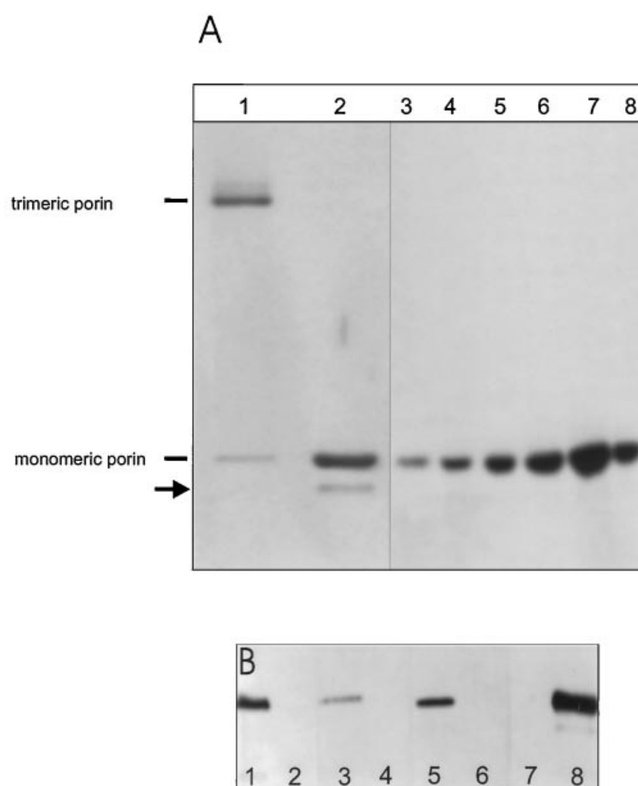

FIG. 2. **Purity of PorB fractions and association of PorB with human macrophage plasma membrane and LPs.** *A*, SDS-PAGE analysis showing a PorB preparation containing trimeric (native) and monomeric porins (*lane 1*). *Lane 2*, same sample as in *lane 1*, but boiled (100 °C) prior to electrophoresis, showing the existence of traces of P.III (*arrow*) in this preparation; *lanes 3–8*, boiled PorB samples of a panel of fractions from pure preparations pooled and used in this study. Boiling converts the native trimeric form of porin to the monomeric form and dissociates existing traces of P.III from this putative complex. *B*, immunoblot analysis of PorB incubated with human macrophages. *Lane 1*, cells treated with PorB; *lane 2*, cells treated with buffer; *lane 3*, integral membrane protein fraction isolated after treatment of macrophages with PorB; *lane 4*, integral membrane protein fraction isolated from cells treated with buffer; *lane 5*, LP fraction (150 × 10^6 phagosomes) isolated from cells treated with PorB; *lane 6*, LP fraction (150 × 10^6 phagosomes) isolated from cells treated with buffer; *lane 7*, last wash of *lane 1* (last wash of lanes *2–6* showed a similar result); *lane 8*, purified PorB as a positive control.

necessary to prepare several hundreds of millions of LP^+ and LP^- at a certain time point. Three to five days after monocyte adhesion, monolayers of macrophages were obtained (Fig. 1*A*). Macrophages obtained by this method showed a high potential in internalizing latex beads, as determined by TEM (Fig. 1*B*). LPs isolated as described above were surrounded by a continuous and distinct membrane (Fig. 1*C*) similar to that seen in an intact cell (Fig. 1*D*). Morphological investigation by TEM of different LP^+ and LP^- fractions showed that these fractions were devoid of contamination by other organelles (Fig. 1*C*).

Since morphological investigation may not reveal contamination with fragments of broken organelles, we used high resolution two-dimensional PAGE to detect protein markers of unrelated organelles in our fractions. The presence or absence of these protein markers was revealed by matching with the image of the two-dimensional gel of human liver available in the SWISS-2DPAGE Program data base.[3] The following markers were investigated: cytochrome *c* oxidase for mitochondria; vesicular integral membrane protein (VP36) for the Golgi complex; and protein-disulfide isomerase, calreticulin, and immunoglobulin heavy chain-binding protein (BiP) for the endoplasmic reticulum. This approach revealed the presence of traces of the tested mitochondrial and endoplasmic reticular markers and the absence of the Golgi marker (data not shown), consistent with the results of Desjardins *et al.* (23), who characterized LPs isolated from human myeloid leukemia U937 cells. In contrast to phagosomal proteins, these unrelated markers were not enriched in our LP fractions and were equally distributed in LP^+ compared with LP^- (data not shown).

Association of Purified PorB with the Macrophage Plasma Membrane and LPs—The isolated porin used in this study was highly pure. As previously shown (15), an additional band became visible in some preparations of native trimeric porin after boiling (Fig. 2*A*, *lane 2*). This additional band represents the outer membrane protein P.III, which appears to be complexed with PorB. In our experiments, we used porin preparations devoid of this additional P.III and other proteins and with a low content of lipopolysaccharide (*e.g.* Fig. 2*A*, *lanes 3–8*).

Association of purified PorB with the plasma membrane of human macrophages of the culture system and with LPs isolated from these cells was examined by Western blotting using a $PorB_{IA}$ type-specific monoclonal antibody. Integral proteins isolated from membranes of cells treated with PorB contained a protein that reacted with the antibody (Fig. 2*B*, *lane 3*), whereas the integral proteins from cells treated with the buffer did not (*lane 4*). Similarly, LPs isolated from cells treated with PorB (LP^+) contained a reacting protein (Fig. 2*B*, *lane 5*), whereas LP^- did not (*lane 6*). Whole macrophages incubated with the buffer as well as the last wash collected after incubation of the cells with PorB contained no protein detectable with the antibody (Fig. 2*B*, *lanes 2* and *7*, respectively), whereas cells incubated with PorB and collected in the last pellet con-

[3] http://expasy.hcuge.ch/ch2d/ch2d-top.html.

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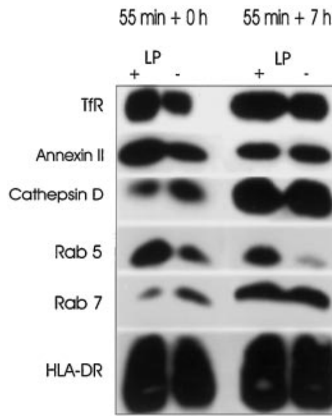

FIG. 3. **Western blot analysis of cellular markers in LP$^+$ and LP$^-$ at 0 and 7 h of chase.** The blot was probed with a monoclonal antibody to TfR, a monoclonal antibody to annexin II, a polyclonal anti-cathepsin D antibody, an affinity-purified polyclonal anti-Rab5 antibody, an affinity-purified polyclonal anti-Rab7 antibody, and a monoclonal antibody against the C-terminal chain of HLA-DR-α. Component proteins from the same number of LP$^+$ and LP$^-$ at the two time points were separated on a 12% SDS-polyacrylamide gel.

tained a protein that reacted with the antibody (*lane 1*).

Association of Cellular Markers with LP$^+$ and LP$^-$—To address the question of whether PorB affects the maturation process of LPs, the association of cellular markers that have been localized to endosomes and phagosomes, such as Rab4, Rab5, Rab7, annexin II, cathepsin D, TfR, and HLA-DR, was analyzed. The association of these markers was followed up in LP$^+$ and LP $^-$ isolated at two time points (55-min pulse + 0-min chase and 55-min pulse + 7-h chase). At each time point, an equal number of phagosomes from LP$^+$ and LP$^-$ were resolved by one-dimensional gel electrophoresis and analyzed by Western blotting using specific antibodies. All markers, except Rab4, were detected on both LP$^+$ and LP$^-$ using this technique; however, significant differences in the association pattern of some markers were revealed (Fig. 3). At 0 chase, the amount of annexin II and TfR present in LP$^+$ clearly exceeded that of LP$^-$, in contrast to cathepsin D, which was reduced in LP$^+$ (Fig. 3). After 7 h, the difference in the amount of these proteins in LP$^+$ and LP$^-$ decreased to a minimum (Fig. 3). HLA-DR associated equally with LP$^+$ and LP$^-$ at the two time points tested (Fig. 3). Marked differences in the amounts of Rab5 and Rab7 associated with LP$^+$ and LP$^-$ were revealed. The amount of Rab5 was significantly higher in LP$^+$ compared with LP$^-$ at 0 chase and decreased at a higher rate in LP$^-$ compared with LP$^+$ after 7 h (Fig. 3). Rab7 showed a pattern opposite to that of Rab5 at 0 chase (Fig. 3). After 7 h, Rab7 was equally enriched in LP$^+$ and LP$^-$ (Fig. 3).

Using Western blotting, we failed to detect Rab4 in LPs (consistent with the results of Desjardins *et al.* (24) and Via *et al.* (37), who used the same technique); therefore, we further investigated its association with LP$^+$ and LP$^-$ by flow cytometry (single organelle fluorescence analysis). Using this technique, we were able to detect a differential association of this small GTPase with LP$^+$ and LP$^-$ early after internalization (Fig. 4). The amount of protein associated with LP$^+$ isolated at 0 chase was markedly higher in comparison with that associated with LP$^-$, and 7 h after incubation, Rab4 was no longer detectable in both phagosome fractions (LP$^+$ and LP$^-$) (Fig. 4).

Kinetics of the Protein Composition of LP$^+$ and LP$^-$—Next we asked whether the differences in the rate of acquisition and loss of the regulatory small GTPases in LP$^+$ and LP$^-$ were accompanied by differences in the protein composition of phagosomes. The same numbers of LP$^+$ and LP$^-$ isolated at 0, 1.5, and 7 h after phagocytosis were separated by two-dimensional PAGE. The gels of a set of LP$^+$ and LP$^-$ isolated at the same time point showed distinct patterns, and these patterns changed over time. Although the intensities of a large number of protein spots remained constant, the amounts of several other proteins either decreased or increased with time (data not shown). Comparison between the images of two counterpart silver-stained gels of LP$^+$ and LP$^-$ isolated at a given time point revealed significant differences in the association of many proteins with LP$^+$ compared with LP$^-$. The counterpart spots that differed by at least 4-fold in their amount, as estimated by densitometry (see "Experimental Procedures"), are presented in Fig. 5 (*A* and *B*).

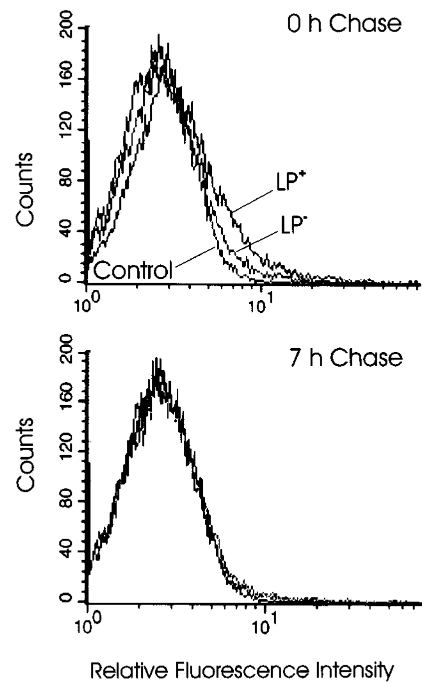

FIG. 4. **Flow cytometry analysis of Rab4 association with LP$^+$ and LP$^-$.** These histograms show the effect of PorB on the association of the small GTP-binding protein Rab4 to LPs isolated from human macrophages in the presence and absence of PorB at 0 and 7 h after a 55-min internalization. Phagosomes (2 × 10^4) of LP$^+$ and LP$^-$ were analyzed. The control is a sample in which LPs were treated with unspecific rabbit IgG as a first antibody.

In an attempt to identify the proteins that were present in different amounts in LP$^+$ compared with LP$^-$, images of two-dimensional gels loaded with LP$^+$ and LP$^-$ isolated at 0, 1.5, and 7 h after internalization were compared with images of master two-dimensional gels of human macrophage-like U937 cells, human lymphoma cells, and human liver. These master gels are available in the SWISS-2DPAGE Program data base.[3] By matching with the SWISS-2DPAGE data base, spots 1 and 2 (Fig. 5*B*) were identified as the lysosomal enzyme cathepsin D heavy chain. Using a polyclonal anti-cathepsin D antibody, spot 2 was confirmed by immunoblotting after separation of the proteins of the entire human macrophage by two-dimensional PAGE (data not shown). The antibody did not react with spot 1. The other unknown spots are candidates for protein identification, and mass spectrometry is a feasible approach. At later time points (1.5 and 7 h), the amount of cathepsin D in LP$^+$ and LP$^-$ was found to be equal (data not shown), similar to our finding in Western blotting at 7 h after internalization.

DISCUSSION

Previous reports demonstrated that insertion of neisserial porins into eukaryotic cells selectively affects several cellular

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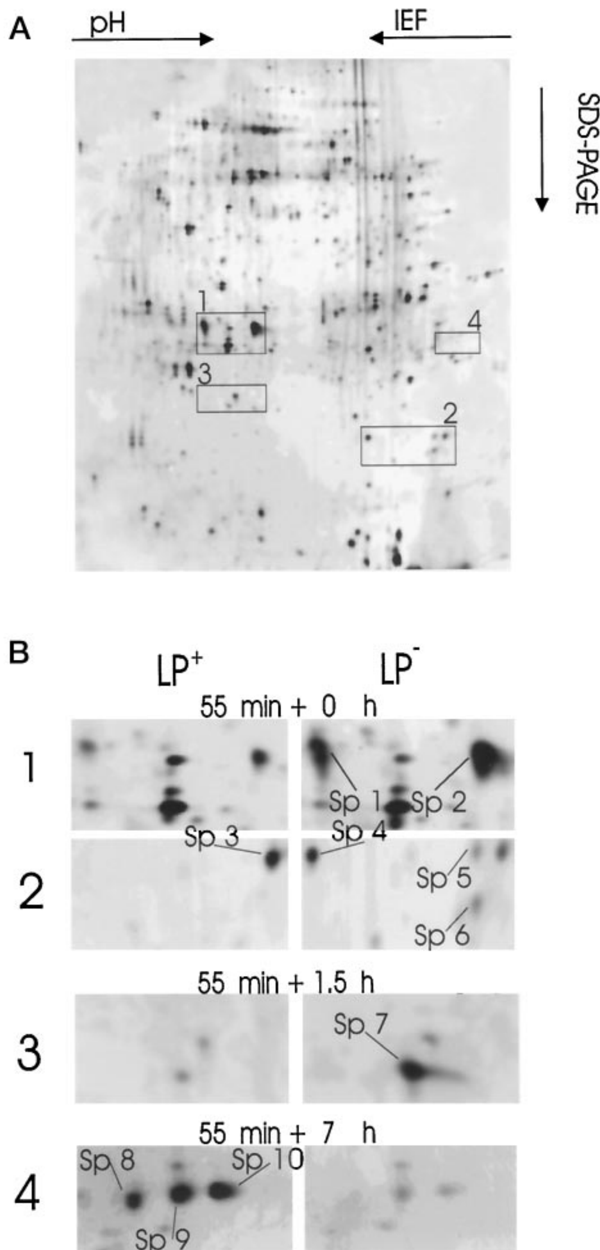

FIG. 5. **Two-dimensional gel electrophoresis analysis of LP$^+$ and LP$^-$.** *A*, silver-stained two-dimensional gel of LPs isolated directly after a 55-min pulse (0-h chase). *Boxes* indicate the location of spots that differed by at least 4-fold in their intensity between LP$^+$ and LP$^-$ at 0, 1.5, and 7 h of chase. *Boxes 3* and *4* indicate the areas in which spots 7–10 depicted in *B* will appear at 1.5 or 7 h of chase. *B*, magnification of the areas in *boxes 1–4* showing the difference in intensities of LP$^+$ and LP$^-$ counterpart spots (*Sp*). In each case, the same number of LPs (1×10^8) were loaded on 9–16% gradient polyacrylamide gels. *IEF*, isoelectric focusing.

functions *in vitro* (11, 13, 14). These studies provided evidence for a possible role of PorB in the intracellular survival of *N. gonorrhoeae*. Inhibition of degranulation (11, 13, 14) and changes in membrane potential of activated neutrophils (11) indicate that PorB may interfere with the vesicle transport pathway and signal transduction. In this study, we have examined the effect of PorB on intracellular processing of phagosomes. Using latex beads, we reconstituted the process of gonococcal phagocytosis after treatment of primary human macrophages with purified PorB. The developed human macrophage culture system proved an efficient source of LPs. Using a $PorB_{IA}$ type-specific monoclonal antibody, we demonstrated the association of purified PorB with the nascent phagosomes and the plasma membrane of the human macrophage. Purified porin has been shown to translocate into the plasma membrane of red blood cells (7), neutrophils (11), and an epithelial cell line (38). These observations are supported by patch clamp studies (15).

Since newly formed phagosomes are modified during the maturation process, which involves the sequential appearance and disappearance of plasma membrane and phagosome markers (for review, see Ref. 39), one objective of our study was to investigate the effect of PorB on the association of cellular markers (including the regulatory small GTPases Rab4, Rab5, and Rab7; annexin II; the lysosomal enzyme cathepsin D; TfR; and HLA-DR) with LP$^+$ and LP$^-$ at different time points after internalization. Members of the Rab protein family, including Rab5 and Rab7, are known to sequentially exchange onto the phagosomal membrane during phagosome maturation (24). Here we show that association of purified PorB with the plasma membrane and the subsequent association with isolated LPs caused marked differences in the amounts of Rab proteins associated with LP$^+$ and LP$^-$. The small GTPase Rab5, which regulates endocytosis and delivery to endosomes (40) and homotypic fusion in the early endocytic compartment (41), was significantly enriched in LP$^+$ compared with LP$^-$ and was hardly detectable in LP$^-$ at 7 h of chase compared with LP$^+$, which retained a considerable amount of the protein at this time point. This protein is suggested to play a similar regulatory role in the phagocytic pathway (24), and recently, using an *in vitro* assay, Alvarez-Dominguez *et al.* (42) demonstrated that Rab5 regulates the fusion between *Listeria* phagosomes and endosomes.

Rab7, which has been specifically localized to late endosomes (43), is found in high levels in late latex bead compartments (24), and is thought to regulate endocytic membrane transport from the early to late endosomal compartment (44), was associated in a pattern opposite from that of Rab5 at 0 chase. After 7 h, Rab7 associated equally with LP$^+$ and LP$^-$. In both LP$^+$ and LP$^-$, Rab7 was detected soon after internalization. A similar finding describing the acquisition of Rab7 and other late endosomal markers like cathepsin D by phagosomes of fixed *Staphylococcus aureus* 15 min after internalization has previously been reported (22). Rab4, the protein that was localized to early endosomes (45) and that controls the early sorting events through the recycling compartments (46), was found to be markedly increased in LP$^+$ at 0 chase compared with LP$^-$. The differences in the amount of Rab proteins acquired by LP$^+$ and LP$^-$ show that association of porin with the phagosomal membrane modifies phagosomal processing. These results provide evidence that LP$^+$ acquire and lose these regulatory proteins at a different rate compared with LP$^-$ and that LP$^-$ acquire the characteristics of late endosomes more rapidly.

Changes in the Rab protein composition of phagosomes due to bacterial factors have recently been reported in the literature. For example, purified phagosomes of live non-hemolytic *Listeria monocytogenes* were enriched with Rab5 as compared with those of dead *L. monocytogenes* (42). Furthermore, survival of pathogens residing in phagosomes has been at least partially attributed to the ability of the pathogen to selectively exclude Rab proteins. Via *et al.* (37) reported that *Mycobacterium bovis* phagosomes do not acquire Rab7 even 7 days post-infection, whereas this GTP-binding protein is already detectable a few hours after phagocytosis of latex beads. Proteins regulating membrane trafficking, such as the small GTP-binding proteins, can be influenced by bacterial products as reported for the vacuolating cytotoxin of *Helicobacter pylori*. Cellular vacuoles induced by this pathogen in epithelial cells were

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found to be enriched with Rab7, but excluded Rab5 and other early endocytic markers (47).

Two-dimensional PAGE analysis of LP^+ and LP^- isolated at a given time point revealed that PorB had a profound effect on the protein composition of LP^+ compared with LP^-. These modifications may be for the benefit of the bacterium and may facilitate prolonging intracellular survival. Phagolysosome formation has been shown to be required for killing of gonococci (48, 49). The significantly reduced delivery of the lysosomal enzyme cathepsin D to LPs following interaction with PorB, detected early after internalization, indicates a significant delay in phagosome maturation and impairment of oxygen-independent killing mechanisms. Intracellular survival of certain other pathogens has previously been attributed to their ability to alter their course inside the phagocytic cells, and they are thought to do so by producing molecules that modify their phagosome. Examples of these pathogens include the *Mycobacterium* complex, *Toxoplasma gondii*, and *Salmonella typhimurium* (for review, see Refs. 50–52).

The equal distribution of the late endocytic markers cathepsin D and Rab7 in both LP^+ and LP^- late in the process of phagosome maturation (7-h chase) indicates that LP^+ begin eventually to mature. It is not clear whether LP^+ start to mature early after formation and at a different rate compared with LP^- or whether they mature simultaneously via an alternate pathway. Acquisition of late endocytic markers at a later time point (7-h chase) by LP^+ suggests that PorB acquired by the phagosome during formation may not remain active over a longer period of time, which may then enable the phagosome to undergo maturation. The delay in the maturation of phagosomes initiated by PorB may therefore depend on the amount of active PorB present in the phagosome. If gonococci present within the phagosome are able to continuously synthesize porin, which then translocates into the phagosomal membrane, this observed effect could be maintained over an extended period of time. Although LP^+ isolated at the late time point (7-h chase) display some late endocytic characteristics, they differ from LP^- in that they still contain high levels of Rab5. Hence, the effect of PorB is at least partially maintained at late time points after internalization.

It is currently unknown how PorB modifies phagosomal processing, but the differences in annexin II content in LP^+ and LP^- found in Western blotting invite speculation. Annexin II is a member of a protein family thought to interact with cellular membranes (53). The function of this protein has not yet been defined, but due to its ability to bind phospholipids and cytoskeletal elements in a calcium-dependent manner, a cross-linking function in the cell cortex has been postulated (53). The presence of a higher amount of annexin II in LP^+ compared with LP^- suggests that these phagosomes will bind more strongly to the submembranous cytoskeletal elements around the phagosomes. Such binding may then lead to a decrease of phagosome movement. The presence of higher amounts of TfR, an abundant protein of the plasma membrane, recycling vesicles, and early endosomes (for review, see Refs. 54 and 55), and the early endocytic markers Rab4 and Rab5 in LP^+ isolated directly after a 55-min internalization compared with LP^- support this speculation. Vice versa, fast moving LP^- will interact more extensively with other vesicles and hence will gradually mature to late endosomes. We do not know how PorB causes such an increase in the amount of annexin II bound to the phagosomes. A possible explanation is the transient calcium influx measured directly after treatment of eukaryotic cells with PorB, which could be inhibited by addition of ATP.[2] ATP was shown to cause closing of the pore (15). The increase in intracellular calcium may promote binding of additional amounts of annexin II to the phagosomal membranes. Evans and Nelsestuen (16) have reported that annexins can respond quickly to a calcium signal by rapidly associating with membranes.

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