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From the inside out – processing of the Chlamydial autotransporter PmpD and its role in bacterial adhesion and activation of human host cells

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Summary

Polymorphic membrane protein (Pmp)21 otherwise known as PmpD is the longest of 21 Pmps expressed by *Chlamydophila pneumoniae*. Recent bioinformatical analyses annotated PmpD as belonging to a family of exported Gram-negative bacterial proteins designated autotransporters. This prediction, however, was never experimentally supported, nor was the function of PmpD known. Here, using 1D and 2D PAGE we demonstrate that PmpD is processed into two parts, N-terminal (N-pmpD), middle (M-pmpD) and presumably third, C-terminal part (C-pmpD). Based on localization of the external part on the outer membrane as shown by immunofluorescence, immuno-electron microscopy and immunoblotting combined with trypsinization, we demonstrate that N-pmpD translocates to the surface of bacteria where it noncovalently binds other components of the outer membrane. We propose that N-pmpD functions as an adhesin, as antibodies raised against N-pmpD blocked chlamydial infectivity in the epithelial cells. In addition, recombinant N-pmpD activated human monocytes *in vitro* by upregulating their metabolic activity and by stimulating IL-8 release in a dose-dependent manner. These results demonstrate that N-PmpD is an autotransporter component of chlamydial outer membrane, important for bacterial invasion and host inflammation.

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Introduction

The family *Chlamydiaceae* comprise a diversified group of obligate intracellular Gram-negative bacteria infecting a wide range of different cell types in their eukaryotic hosts, causing a variety of acute and chronic diseases (Blanchard and Mabey, 1994; Kuo *et al*., 1995; Hahn and McDonald, 1998; Kalayoglu *et al*., 2002). They share a characteristic, biphasic cycle of development with infectious, spore-like elementary bodies (EB) and intracellular dividing, metabolically active reticulate bodies (RB) that inhabit a non-fusogenic inclusion (Moulder, 1991).

For a productive chlamydial infection, adhesion of EB leading to invasion must take place. Adhesion is often mediated *via* receptor–ligand interactions, where a receptor on a host cell surface is bound by a ligand on the bacterial surface. Presence or absence of receptors and ligands facilitates bacterial entry into specific tissues, being responsible for tissue tropism (Meyer, 1999). In chlamydial infections, host cell membrane components like mannose-receptor or the oestrogen receptor complex were shown to support the infection (Su *et al*., 1996; Mamelak *et al*., 2001; Taraktchoglou *et al*., 2001; Davis *et al*., 2002). In addition, inhibition of infection after proteolytic treatment further pointed at protein components to function as cellular receptor(s) (Byrne and Moulder, 1978; Vretou *et al*., 1989). Differences in pathogenicity between chlamydial species and biovars are linked to a diverse tissue tropism. This can partly be explained by different abilities to cope with the challenges in the respective microenvironment but also with the prerequisite to attach to and invade susceptible cell types (Rasmussen-Lathrop *et al*., 2000; Belland *et al*., 2001; Fehlner-Gardiner *et al*., 2002; Kuo *et al*., 2002).

Evaluation of the chlamydial attachment mechanism(s) revealed a stepped effect by treating EB with mild heat and heparin or heparan sulphate or by preincubating host cells with polycationic chemicals (Kuo and Grayston, 1976; Zhang and Stephens, 1992; Chen and Stephens, 1997; Wuppermann *et al*., 2001). No single adhesin could be identified on EB, but a variety of molecules like MOMP, Hsp70 or OmcB have been suggested to be involved in adhesion (Su *et al*., 1990; Raulston, 1995; Kuo *et al*., 1996; Stephens *et al*., 2001; Raulston *et al*., 2002).

In this context, the polymorphic membrane proteins (Pmps) form an interesting group of chlamydial proteins defined by the presence of highly repetitive motifs of four amino acids (GGAI and FxxN) (Everett and Hatch, 1995). The importance of the Pmp superfamily is underscored by the fact that intracellular bacteria collapse the size of their genome in a process called 'evolution by reduction'. Nevertheless, more than five per cent of the total coding capacity in *C. pneumoniae* and almost 22% absent in *C. trachomatis* consist of *pmp*-genes with *C. pneumoniae* having 21 members compared to only nine in *C. trachomatis* (*pmpA-I*). Functionally, Pmps were suggested to take part in binding or docking to eukaryotic cells, a task attributed to proteins containing more than one of GGAI and FxxN motifs [i.e. bacterial rOmpA (*Rickettsia* spp.) or eukaryotic zonadhesin (*Mus musculus*)] (Grimwood and Stephens, 1999; Kalman *et al*., 1999; Read *et al*., 2000; Dobrindt and Hacker, 2001).

Bioinformatically, all members of the heterogeneous Pmp superfamily were classified as autotransporters (Grimwood and Stephens, 1999; Henderson and Lam, 2001). Autotransporters use a secretion mechanism, in which they pass the outer membrane (OM) of Gram-negative bacteria and reach the surface without an assistance of other proteins. In detail, after Sec-dependent delivery into the periplasm, conserved C-terminal transporter domain spontaneously forms a pore-like beta-barrel in the OM through witch the N-terminal passenger domain leaves the bacterium (Pohlner *et al*., 1987; Henderson, *et al*., 1998). In case of *C. trachomatis* and *C. psittaci*, several Pmps [also called (P)Omps] were shown to be expressed late during the infection cycle and were present in OM-complexes or on the bacterial surface, eliciting strong immune response in the course of natural infection (Knudsen *et al*., 1999; Tanzer *et al*., 2001; Tanzer and Hatch, 2001; Vretou *et al*., 2003).

In *C. pneumoniae*, all 21 Pmps were shown to be transcribed (Grimwood *et al*., 2001). Strain and even single clone diversity caused by a different number of tandem repeats in *pmp6* and a potential frame shift provided by a slip(ped) strand mechanism with a poly(G) stretch in *pmp10*, respectively, was considered to provide a high level of functional and antigenic diversity on the surface of *C. pneumoniae* (Christiansen *et al*., 1999; Shirai *et al*., 2000; Grimwood *et al*., 2001; Pedersen *et al*., 2001; Rocha *et al*., 2002).

Pmp21 (PmpD) was shown to be post-translationally cleaved/processed resulting in N-terminal surface exposure, which is in agreement with the proposed autotransporter-export mechanism (Vandahl *et al*., 2002). Recently, NF-κB-mediated induction of the inflammatory mediators IL-6, IL-8 and MCP-1 was observed in human endothelial cells incubated with recombinant Pmp20 or Pmp21 (Niessner *et al*., 2003).

Despite existing *in silico* predictions, experimentally verified function of PmpD remained to date unclear. Here, we wanted to determine the detailed processing and subcellular localization of PmpD and the functional properties of this protein. To address this, we raised a polyclonal serum against PmpD and performed series of experiments to determine its temporal expression pattern during infection, mechanism of transport and the processing and localization along with its function as a chlamydial adhesin.

Results

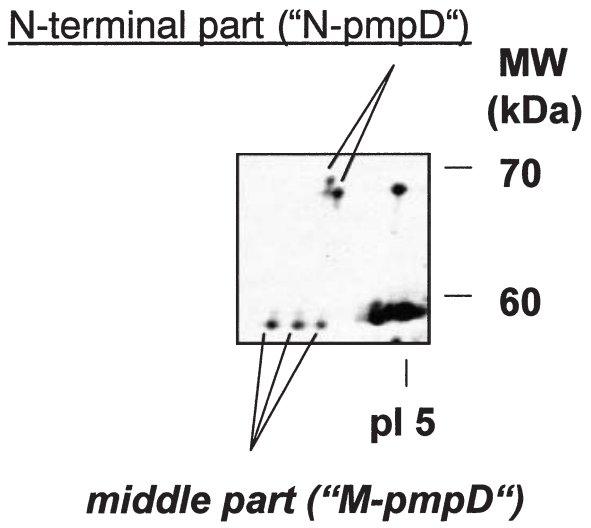
Post-translational processing of C. pneumoniae *PmpD*

First, we wanted to correlate a pattern of protein species with a certain stage in the developmental cycle of *C. pneumoniae.* To achieve this we performed bacterial proteome analyses at different times during chlamydial infection in HEp-2 cells. Total lysates of urografin-purified EB and RB were separated using 2D-gel technology. Next, large 2D-gels were stained with Coomassie G-250, individual spots were excised and proteins were identified by MALDI-Peptide Mass Fingerprinting (PMF).

Two prominent features present in the lysate of RB at four days post infection (p.i.) in the molecular weight range of <70 kDa contained peptides from the N-terminal part of PmpD (Pmp21), N-pmpD, and peptides from a series of 2×3 features in the size of about 55 kDa matched to theoretical trypsin cleavage-products from the middle part of PmpD, M-pmpD, with peptides from amino acid 122–655 and 670–1114 respectively (Fig. 1A and B). The predicted pI for the N-terminal fragment is 4.6–4.7 and for the middle part 4.4. The occurrence of 2 and 2×3 spots indicates post-translational modification of the two fragments (Fig. 1A). Schematic representation of the possible cleavage sites and deduced molecular weight for PmpD protein fragments is shown in Fig. 1C.

In order to exclude artificial cleavages by the density-step ultracentrifugation procedure or sample preparation for 2D-analysis, we determined the molecular size of PmpD in total lysates of infected HEp-2 cells. Samples were placed directly into Laemmli SDS lysis-buffer and processed by immunoblotting with the rabbit polyclonal antiserum raised against the denatured, purified $(His)_6$-tagged recombinant N-terminal part of PmpD (without the signal sequence, amino acid 17–670). In the infected HEp-2 cells ('+') collected sequentially over 4 days of infection, three protein bands reacted with the antiserum (Fig. 2A): a band of 170 kDa corresponding to the size of full-length PmpD, one of about 120–130 kDa correlating in size with the N-terminal plus the middle part connected together and cleaved from the C-terminal part and a band of 70 kDa in the size of the N-terminus only. Figure 2B

A



B

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   1 mvakktvrsy rssfshsviv ailsagiafe AHSLHSSELD LGVFNKQFEE
  51 HSAHVEEAQT SVLKGSDPVN PSQKESEKVL YTQVPLTQGS SGESLDLADA
 101 NFLEHFQHLF EETTVFGIDQ KLVWSDLDTR NFSQPTQEPD TSNAVSEKIS
 151 SDTKENRKDL ETEDPSKKSG LKEVSSDLPK SPETAVAAIS EDLEISENIS
 201 ARDPLQGLAF FYKNTSSQSI SEKDSSFQGI IFSGSGANSG LGFENLKAPK
 251 SGAAVYSDRD IVFENLVKGL SFISCESLED GSAAGVNIVV THCGDVTLTD
 301 CATGLDLEAL RLVKDFSRGG AVFTARNHEV QNNLAGGILS VVGNKGAIVV
 351 EKNSAEKSNG GAFACGSFVY SNNENTALWK ENQALSGGAI SSASDIDIQG
 401 NCSAIEFSGN QSLIALGEHI GLTDFVGGGA LAAQGTLTLR NNAVVQCVKN
 451 TSKTHGGAIL AGTVDLNETI SEVAFKQNTA ALTGGALSAN DKVIIANNFG
 501 EILFEQNEVR NHGGAIYCGC RSNPKLEQKD SGENINIIGN SGAITFLKNK
 551 ASVLEVMTQA EDYAGGGALW GHNVLLDSNS GNIQFIGNIG GSTFWIGEYV
 601 GGGAILSTDR VTISNNSGDV VFKGNKGQCL AQKYVAPQET APVESDASST
 651 NKDEKSLNAC SHGDHYPPKT VEEEVPPSLL EEHPVVSSTD IRGGGAILAQ
 701 HIFITDNTGN LRFSGNLGGG EESSTVGDLA IVGGGALLST NEVNVCSNQN
 751 VVFSDNVTSN GCDSGGAILA KKVDISANHS VEFVSNGSGK FGGAVCALNE
 801 SVNITDNGSA VSFSKNRTRL GGAGVAAPQG SVTICGNQGN IAFKENFVFG
 851 SENQRSGGGA IIANSSVNIQ DNAGDILFVS NSTGSYGGAI FVGSLVASEG
 901 SNPRTLTITG NSGDILFAKN STQTAASLSE KDSFGGGAIY TQNLKIVKNA
 951 GNVSFYGNRA PSGAGVQIAD GGTVCLEAPG GDILFEGNIN FDGSFNAIHL
1001 CGNDSKIVEL SAVQDKNIIF QDAITYEENT IRGLPDKDVS PLSAPSLIFN
1051 SKPQDDSAQH HEGTIRFSRG VSKIPQIAAI QEGTLALSQN AELWLAGLKQ
1101 ETGSSIVLSA GSILRIFDSQ VDSSAPLPTE NKEETLVSAG VQINMSSPTP
1151 NKDKAVDTPV LADIISITVD LSSFVPEQDG TLPLPPEIII PKGTKLHSNA
1201 IDLKIIDPTN VGYENHALLS SHKDIPLISL KTAEGMTGTP TADASLSNIK
1251 IDVSLPSITP ATYGHTGVWS ESKMEDGRLV VGWQPTGYKL NPEKQGALVL
1301 NNLWSHYTDL RALKQEIFAH HTIAQRMELD FSTNVWGSGL GVVEDCQNIG
1351 EFDGFKHHLT GYALGLDTQL VEDFLIGGCF SQFFGKTESQ SYKAKNDVKS
1401 YMGAAYAGIL AGPWLIKGAF VYGNINNDLT TDYGTLGIST GSWIGKGFIA
1451 GTSIDYRYIV NPRRFISAIV STVVPFVEAE YVRIDLPEIS BQGKEVRTFQ
1501 KTRFENVAIP FGFALEHAYS RGSRAEVNSV QLAYVFDVYR KGPVSLITLK
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1601 FNGGIRIIF
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C

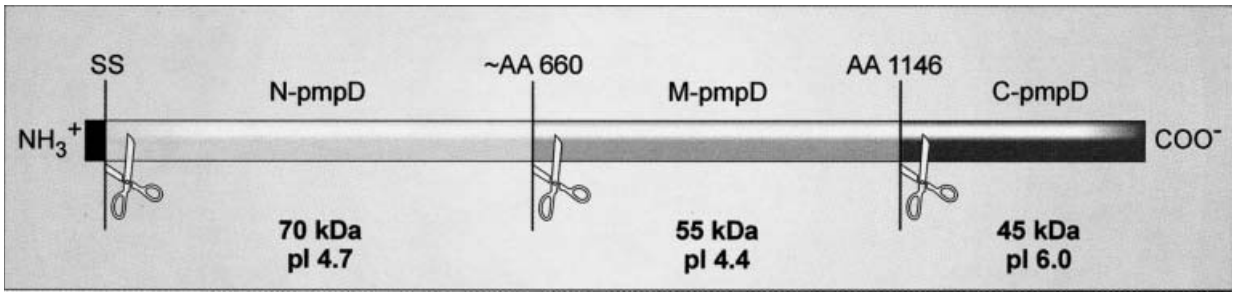

Fig. 1. PmpD is post-translationally modified – analysis using 2D-gels. *Chlamydophila pneumoniae* RB were harvested at 2 and 4 days p.i., purified over an Urografin density-step gradient and separated using two-dimensional gel electrophoresis. Positions of the spots identified as N-terminal (amino acid 122–655) and middle (amino acid 670–1114) parts of PmpD by MALDI-PMF are indicated in a small part of the acidic region of a silver-stained gel (A).Identified peptides matching the N-terminus and the middle part are shown underlined and in bold/italic, respectively. Positions of the signal sequence (small caps) and the C-terminal part (grey background) as shown earlier (Vandahl *et al*., 2002) are highlighted (B). C. Illustrates schematic representation of the whole PmpD molecule with its possible cleavage sites and respective molecular weights.

shows the same blot stripped and incubated with a mouse antiserum against *C. pneumoniae* Hsp70 as a loading control. Lower amount of Hsp70 on day 1 p.i. could be explained by less bacteria present at this time of infection. Interestingly, regardless of the time p.i., the strongest band was that of 70 kDa (Fig. 2A). The 130 kDa band was most abundant at 2 days p.i., as compared to the later phases of the infection cycle. Characteristic for the majority of polyclonal antibodies, non-specific bands suggestive of cross-reaction with proteins in uninfected HEp-2 cells ('–') could be observed, but clearly distinguished from the pattern in infected cells. This was also true for the region of 60–70 kDa where a cross-reactive protein runs beneath the 70 kDa fragment of PmpD. Isolation of total IgG-fraction from the rabbit serum or affinity purification of antibodies with the native recombinant N-terminal part of PmpD coupled to CNBr-activated sepharose 4B had not improved the reaction pattern (data not shown).

In addition, we determined the expression of PmpD during persistent infection induced by the iron chelator DAM (50 μg ml^{-1}). However, there were no quantitative differences between acute and persistent infection at 3 days p.i. after normalization with Hsp70 as an internal control (data not shown).

Localization and surface accessibility of N-pmpD

Based on structural composition and homology, two groups predicted PmpD to be a member of the autotransporter system (also called type V secretion system) using *in silico* analysis (Henderson *et al*., 1998; Yen *et al*., 2002). We therefore tested whether N-pmpD is indeed

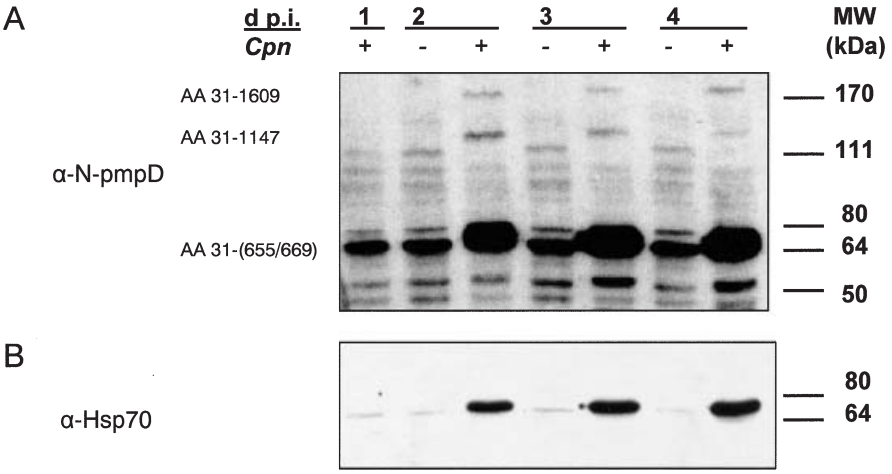

Fig. 2. PmpD is processed in the course of an *in vitro* infection. HEp-2 cells were infected with *C. pneumoniae* (MOI = 1) and incubated for 1, 2, 3 and 4 days under standard conditions. Total cell lysates were separated on a 6% SDS-gel and immunoblotted. Using the rabbit polyclonal serum against N-pmpD, three bands of about 170, 130 and 70 kDa were detected in the infected cells (*Cpn* +) (A). All bands were present as of 2 days p.i. with the 70 kDa isoform being the predominant one during whole infection and 120 kDa giving the most intense staining on day 2 p.i. The blot was stripped and re-probed with a mouse antiserum against chlamydial Hsp70 for normalization (B).

located to the outside of bacteria, suggestive of transport across both bacterial membranes. *Chlamydia pneumoniae* was grown for 2 days and infected HEp-2 cells were fixed and incubated with glass beads (diameter 425–600 μm) in order to permeabilize eukaryotic membranes while leaving the bacterial OM intact. PmpD was accessible to the antibodies without permeabilization using Triton X-100 (in red, Fig. 3A), proving that it must be exposed to the surface of bacteria. As a control, we used a polyclonal serum raised against the intracellular Fur transcription factor (ferric uptake regulator) from *E. coli* that also recognizes an intracellular factor in *C. pneumoniae* (our unpublished data) (kindly provided by Michael Vasil, University of Colorado Health Sciences Center, Denver, Colorado). Unlike serum reacting with PmpD, anti-Fur serum gave strong positive staining only after permeabilization with Triton X-100, confirming the reliability of our experimental system thus unequivocal localization of PmpD to the external surface of chlamydial cells. (Fig. 3B and D).

To verify the immunofluorescence data, we performed limited trypsin digestion. In this experiment, purified EB were incubated with increasing concentrations of trypsin and next washed and analysed by immunoblotting. We observed degradation of N-pmpD starting at 10 μg ml^{-1} of trypsin, indicative of its external localization on EB (Fig. 4A). At the same time, levels of Hsp70 remained intact (Fig. 4B). Additionally, a gradual degradation of N-pmpD observed using proteinase K confirmed the results of limited trypsin digestion (data not shown).

Despite cleavage from the full-length molecule, the N-terminal part of PmpD remained attached to the chlamydial outer membrane. The topology predictions have not allocated N-pmpD as an integral part of the membrane, thus we hypothesized that it must interact with other OM structures. For Antigen49 from *E. coli*, a non-covalent binding with its previously connected C-terminal β-barrel was described. This binding could be broken by mild heat treatment (incubation at 60°C for 10 min) (Caffrey and Owen, 1989). In our experiments, N-pmpD could not be released from the bacterial surface by heat treatment, neither by incubation in phosphate buffer with high ionic strength (3 M NaCl) or using the reducing medium alone (10 mM DTT and β-mercaptoethanol, data not shown). Figure 5 shows the amount of N-pmpD attached to EB (Fig. 5A) or membrane pellets (Fig. 5B) after incubation with different conditions. The interaction with EB seemed not to be mediated by divalent cations (no effect with 60 mM EDTA) and could not be disturbed by a pH shift to pH 3 (incubation in 100 mM glycine) or pH 9.5 (200 mM KH_2CO_3) but only at a pH of more than 12 (in 100 mM NaOH) (Fig. 5A).

To examine whether the binding partner is an integral outer membrane protein, we isolated the OM-fraction (insoluble in 2% Sarkosyl) as well as the Chlamydial outer membrane complexes (COMC) (insoluble in 2% SDS). Without reducing agents, a small portion of N-pmpD was found in the pellet (<5%) in both conditions, but adding β-mercaptoethanol and DTT resolved N-pmpD completely, suggestive of association of N-pmpD with OM and COMC (Fig. 5B).

Many autotransporters are cleaved off and released from their C-terminal beta-barrel in the OM, even only under certain growth conditions or during an infection *in vivo* (Goldberg *et al.*, 1993). We asked whether N-pmpD could be released and secreted to the inclusion lumen or to the cytoplasm of the host cell. Confocal analysis after

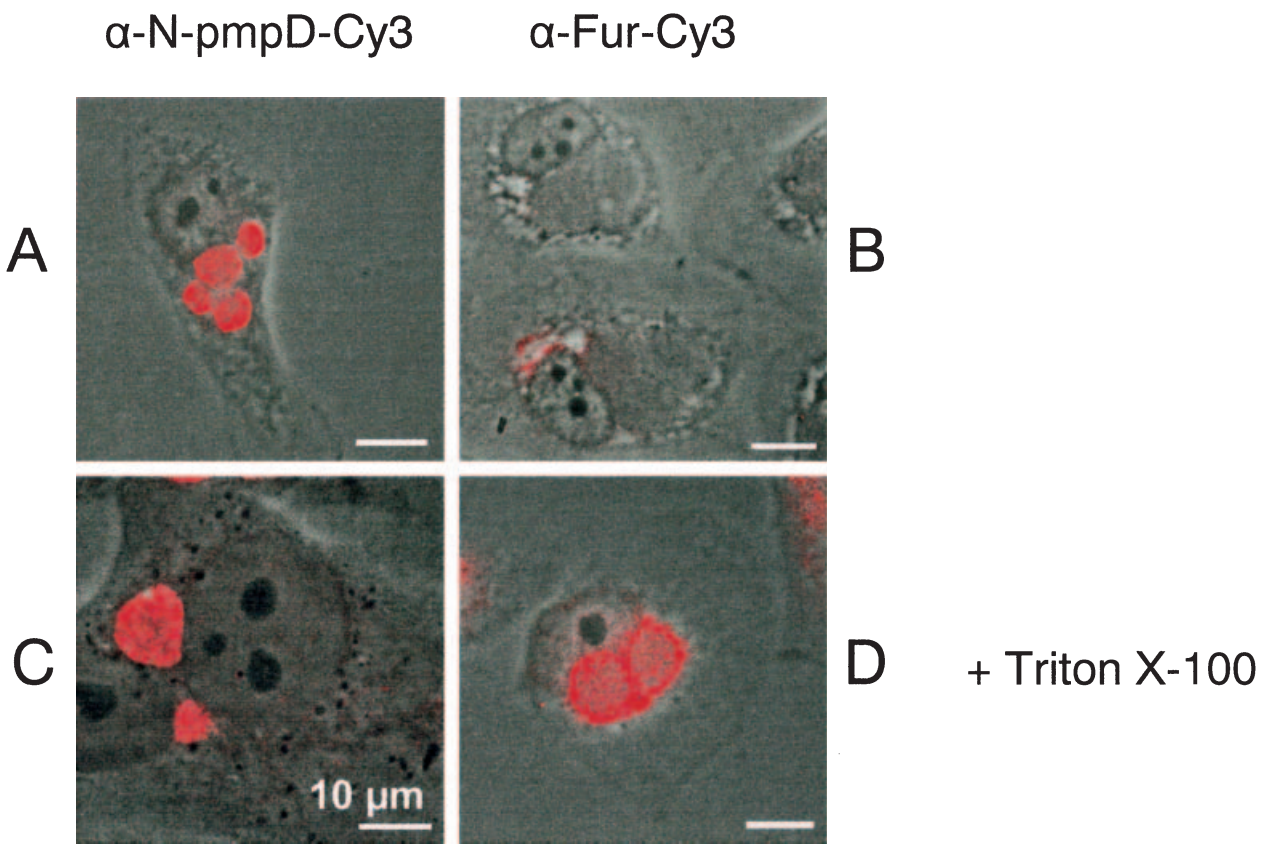

Fig. 3. PmpD is surface exposed – staining with and without Triton X-100 permeabilization. After 2 days of infection, *C. pneumoniae*-infected HEp-2 cells were either fixed using STF and permeabilized with glass beads (425–600 µm) leaving the bacteria intact (A and B) or fixed with 2% PFA and incubated in 0.5% Triton X-100 for total permeabilization (C and D). The samples were then blocked in 0.2% BSA and stained with the anti-N-pmpD serum (red, A and C) or an anti-*E. coli* Fur protein serum, which recognizes an intracellular factor also in *C. pneumoniae* (red, B and D). Only N-pmpD could be detected without Triton X-100 permeabilization (A) suggestive of its surface localization. After permeabilization, both proteins could be detected (C and D).

immunofluorescence staining at 1, 2 and 3 days p.i. indicated distribution of PmpD strictly inside the inclusions of *C. pneumoniae* (Fig. 6A, C and E). Interestingly, the honeycomb-like pattern was different than the staining pattern of Hsp60 (large bodies) and PmpD seemed to form a cluster surrounding the bacteria (Fig. 6B, D and F).

To determine the subcellular localization of N-pmpD we also performed immunogold EM studies. The samples were stained on the third day p.i. The rabbit antiserum reacted with RB, EB and intermediate forms. Interestingly, N-pmpD was localized preferentially on the surface of bacteria and on vesicular structures in the inclusion lumen

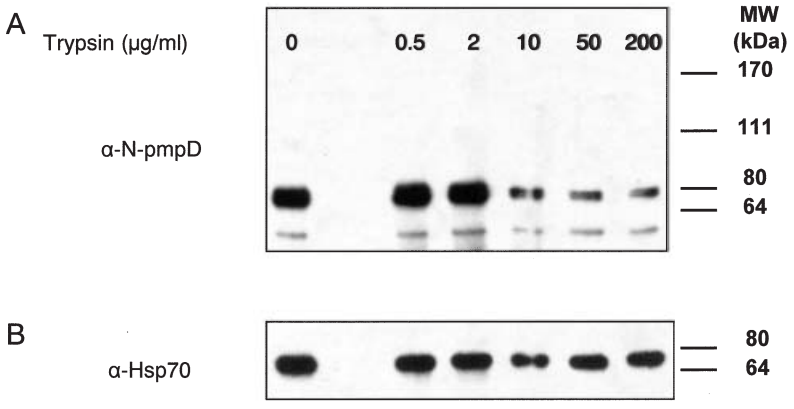

Fig. 4. PmpD is surface exposed – limited trypsin digestion of EB. Twenty µl of highly concentrated EB (1×10^7 IFU) from *C. pneumoniae* harvested 4 days p. i. were incubated with the indicated concentrations of trypsin in PBS at 37°C for 30 min. EB were collected by centrifugation, washed and resuspended in Laemmli SDS-sample buffer. After separation, the samples were immunoblotted with rabbit serum against N-pmpD (A) and next re-probed with mouse serum against chlamydial Hsp70 (B). Starting at 10 µg ml^{-1}, trypsin removed N-pmpD but not Hsp70 from the surface of EB confirming its surface localization.

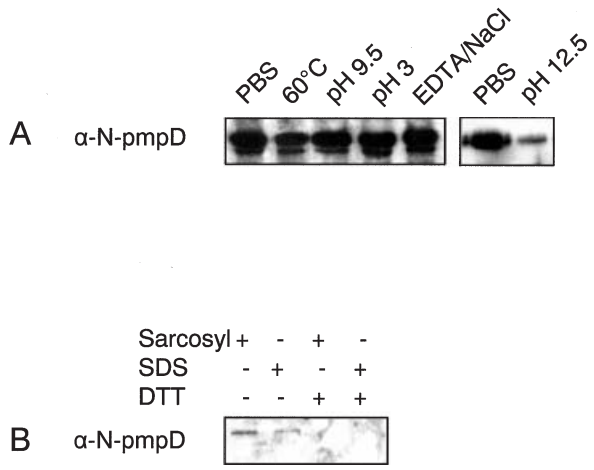

Fig. 5. N-pmpD interacts with the components of OM on EB. Twenty µl EB stock (1×10^7 IFU) from *C. pneumoniae* harvested 4 days p. i. were incubated in a volume of 1 ml at 37°C for 60min (or at 60°C in PBS for 20min as indicated) in different buffers [200 mM KH_2CO_3 (pH 9.5), 100 mM glycine (pH 3.0), 60 mM EDTA + 3 M NaCl, PBS and 100 mM NaOH (pH 12.5)], centrifuged and processed by immunoblotting with anti-N-PmpD (A). Only basic pH disrupted the connection of N-pmpD with the surface of bacteria. Chlamydial membrane fractions insoluble in 2% Sarkosyl or in 2% SDS were incubated in presence or absence of the reducing agents (10 mM DTT + 10% 2-ME). The remaining insoluble fractions were then processed by immunoblotting with anti-N-PmpD (B). Addition of reducing agents disrupted the association of N-pmpD with Sarkosyl and SDS-insoluble membrane fractions.

that seemed to be pinched off from chlamydial cells or membranes (Fig. 7). There was no staining in the cytoplasm of infected cells. Thus, corroborating the confocal analyses, PmpD was not found to be secreted into the host cell or inserted in the inclusion membrane but remained associated with bacteria and their membranes.

Bioactivity of N-pmpD – infection and activation of human cells

In addition to homology with known autotransporters and despite the long phylogenetic distance between *Chlamydiaceae* and the proteobacteria, PmpD is homologous to a variety of bacterial adhesins. Members of the unique polymorphic family of Pmps have in common characteristic repeats of four amino acids (GGAI/L/V, 2–12 times and FxxN, 4–23 times). To date, all proteins containing more than one GGAI/L/V repeat were shown to be involved in adhesion [i.e. binding to eukaryotic cell surfaces either as bacterial adhesins (*rickettsiae* rOmpA) or as eukaryotic docking- and recognition partners (*mouse* zonadhesin) (Grimwood and Stephens, 1999)]. We therefore assumed that if the physiological function of N-pmpD would be to mediate binding to and/or entry into eukaryotic host cells, antibodies against N-pmpD should neutralize chlamydial infectivity. To investigate this, affinity purified serum or IgG fraction from the polyclonal rabbit serum against N-pmpD were incubated with EB at 37°C 1 h before infection of HEp-2 cells. After centrifugation for 1 h and incubation for 2 days, the inclusions were visualized by immunostaining. Infectivity was calculated as the mean value of inclusion numbers in three different dilutions for each condition. Already 0.5 mg ml^{-1} (10% v/v) α-N-pmpD serum had a decreasing effect on chlamydial infectivity, 2.5 mg ml^{-1} _-N-pmpD had a dramatic effect lowering infectivity down to about 40% whereas 4.5 mg ml^{-1} α-N-pmpD abolished the infection almost completely (Fig. 8). No difference was seen in the blocking activity between the two types of antibodies used (affinity purified or IgG fraction). Control antibodies against MOMP or LPS had no decreasing effect on chlamydial infectivity.

To determine if the attachment of PmpD could activate immune host cells we determined the metabolic activity of human monocytes (cell line THP-1) in response to the recombinant protein. The THP-1 cells were arrested in growth by starvation for 40 h in 0.2% FBS and next incubated with the recombinant N-pmpD. Twenty-four hours later metabolic activity was measured. Recombinant N-pmpD had a strong metabolism-enhancing effect in a concentration-dependent manner with values of almost twice of the background activity exceeding the effect of THP-1 incubation with PMA as a positive control for differentiation and hence stimulation of monocytic cells (Fig. 9A). In addition, the supernatants from THP-1 incubated with N-pmpD contained IL-8 as measured by ELISA. The concentration of secreted IL-8 increased in a dose-dependent manner in response to the recombinant N-pmpD (Fig. 9B). The response to rN-pmpD was not abolished by the addition of polymyxin B (100 µg ml^{-1}, incubated for 30 min at 37°C), therefore excluding the possibility of artificial activation by contaminating LPS from *E. coli*.

Discussion

The main finding of the presented study is that PmpD from *C. pneumoniae* is a cleaved, surface exposed protein mediating the early interaction of EB with the host cell and inducing activation and cytokine release from monocytes. Our data are consistent with observations and predictions of previous investigators and significantly extend our understanding of the processing and the function of this chlamydial protein.

Our observations suggest extensive processing of PmpD (Fig. 1C). In the first step, the full length PmpD is exported to a periplasmic space possibly by the Sec-machinery. Next, the signal sequence is most likely cleaved off in agreement with a recent finding by Vandahl *et al.* (2002) who identified the N-terminal amino acid as ^{30}Ala from their 66 kDa fragment corresponding to our fragment in the size of <70 kDa. Then, a β-barrel may be

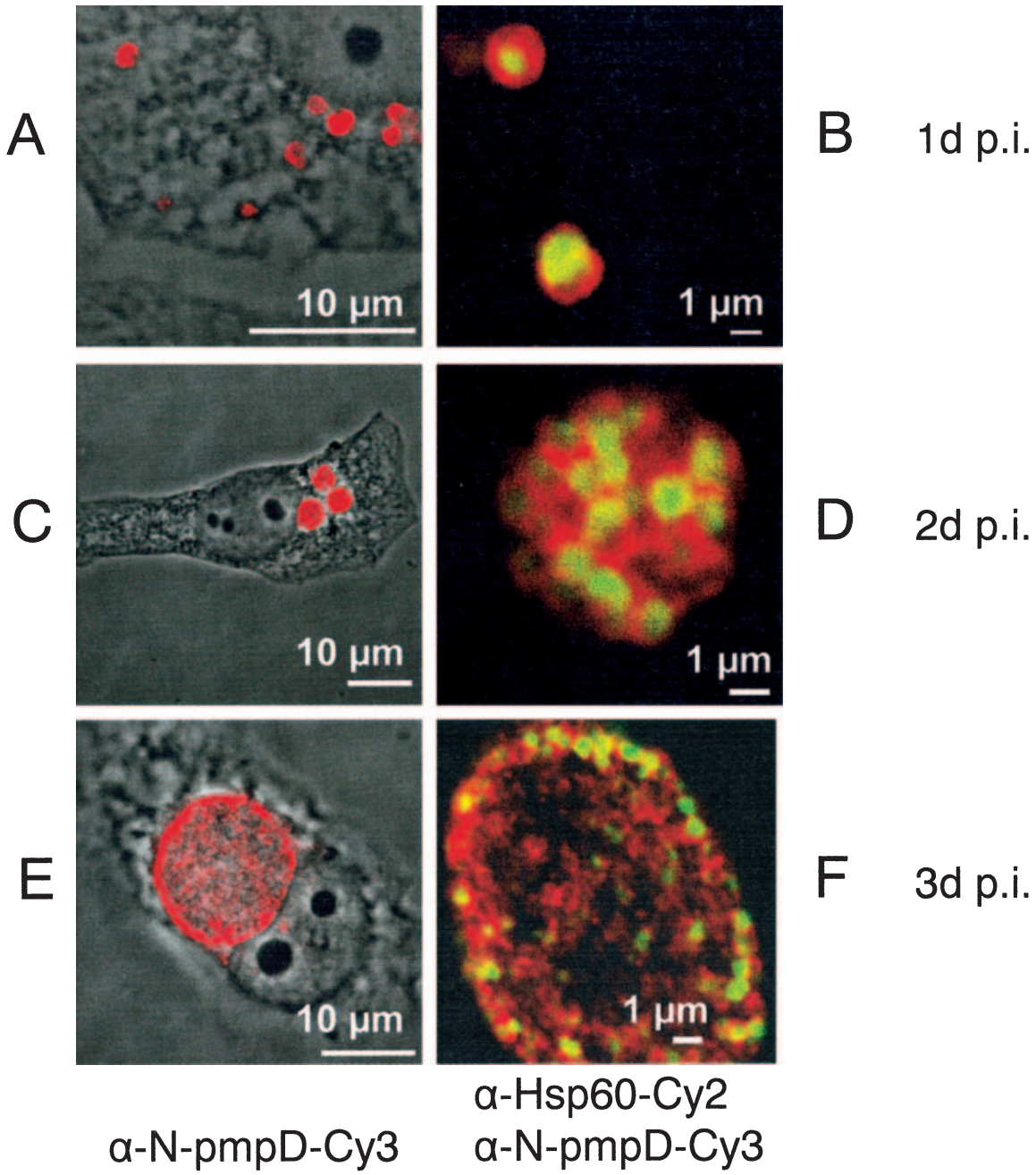

Fig. 6. PmpD associates with bacteria – confocal microscopy study. HEp-2 cells were infected with *C. pneumoniae* and incubated for 1, 2 and 3 days (panel A/B, C/D and E/F, respectively). Next, the cells were washed, fixed with STF, permeabilized with 0.5% Triton X-100 and stained using the polyclonal rabbit serum raised against N-pmpD (red) and the monoclonal mouse antibody against Hsp60 (green). N-pmpD could not be found in cellular compartments outside the chlamydial inclusion (fluorescence + phase-contrast in A, C, D). Staining pattern indicates colocalization of N-pmpD with individual bacteria in a clustered structure surrounding larger forms stained by Hsp60 and reminiscent of an honeycomb (B, D, F).

formed by the C-terminal part and the N-terminal passenger domain is most likely exported outside and cleaved from its translocation unit (~45 kDa) referring to the model autotransporter. The last step, in which presence of two fragments of 70 and 55 kDa was observed in our 2D-analysis, would be a cleavage in the N-terminal passenger domain. Immunoblot analysis of the infected HEp-2 cells confirmed these processing events from full length PmpD (170 kDa) *via* the intermediate pro-protein (N-pmpD + M-pmpD, 125 kDa) finally leading to N-pmpD (70 kDa).

Processing of PmpD is supported by immunoblotting of the infected cell lysate with antiserum gained by immuni-

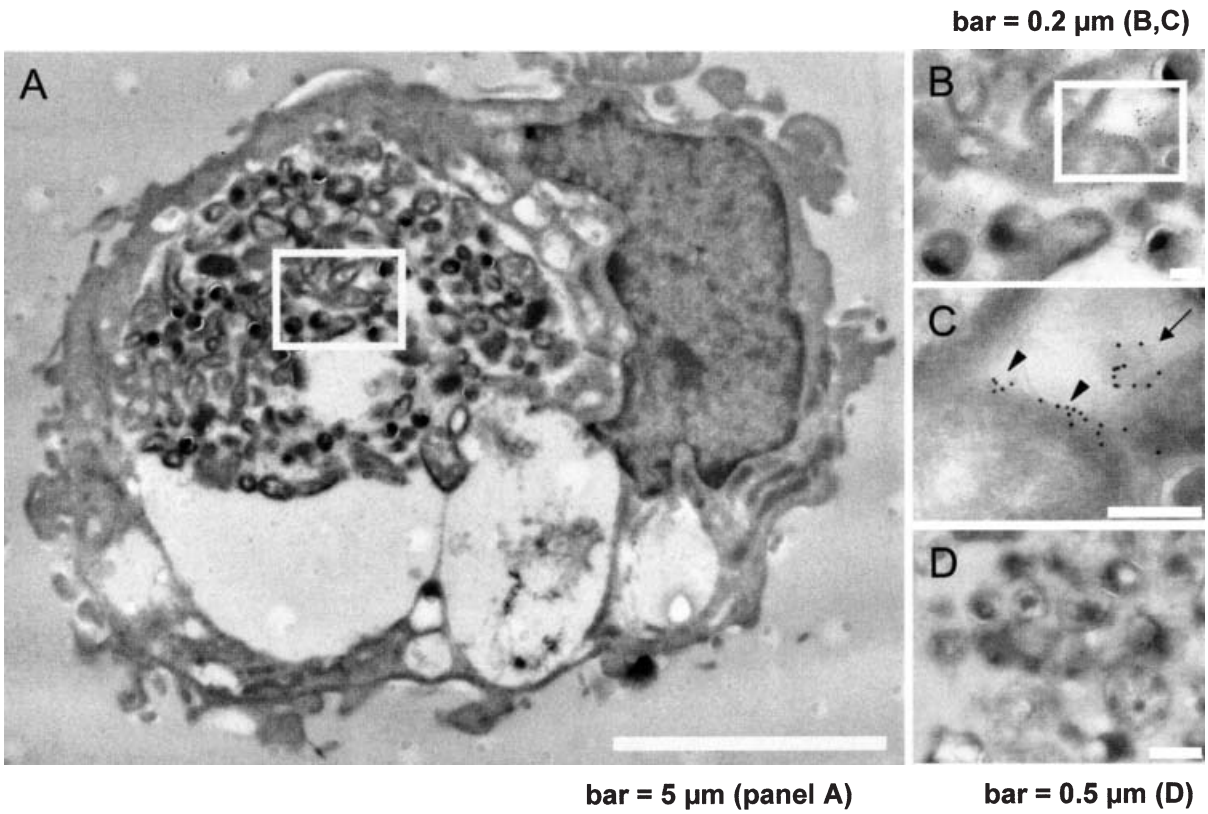

Fig. 7. PmpD localizes to the surface of bacteria – EM immunogold labelling. HEp-2 cells were infected with *C. pneumoniae* and incubated for 3 days.Specimens were fixed (4% paraformaldehyd/0.1% glutaraldehyde), infiltrated with 1.6 M saccharose/25% polyvinylpyrollidone, mounted on aluminum stubs and frozen. Ultrathin cryosections were incubated with the rabbit antiserum against *C. pneumoniae* N-pmpD followed by a goat anti-rabbit antibody coupled to 12 nm gold colloids. Gold particles indicating presence of N-pmpD localized to RB, EB and intermediate forms. A shows the whole infected cell, B is a magnification of an area inside the inclusion and C is a further magnification of B (positions indicated by white boxes). D shows part of an inclusion stained with preimmunization serum as a negative control. The predominant staining was on the surface of bacteria (arrowheads) or associated with vesicle-like structures of unknown nature in the inclusion lumen (arrow) (C).

zation with synthetic PmpD peptide, which revealed two weakly reacting bands (40 and 60 kDa) that could correspond to the C-terminal and middle part (Grimwood *et al.,* 2001). Another PmpD fragment of 66 kDa was identified with peptides matching the N-terminal part. In addition, the first amino acid of the C-terminal part was determined by N-terminal sequencing (^{1146}Ser, Fig. 1B) (Vandahl *et al*., 2002). The authors suggested a further cleavage consistent with the idea of PmpD to be a member of the autotransporter family but in contrast to the serum we used, their polyclonal antiserum raised against a larger portion of PmpD (recombinant protein with AA 52–1129) could neither detect the middle part or full length PmpD. The reason might be a different epitope recognition by our antiserum that was raised specifically against the recombinant N-pmpD (AA 16–670). However, both antibodies reacted with the surface of *C. pneumoniae*.

In our 2D-analysis, we could not identify a spot with a PMF corresponding to C-pmpD in the range of 45 kDa. On the other hand, Vandahl *et al*. (2001) identified only the C-terminal fragment of PmpD (47 kDa, pI 5.5) in the proteome of *C. pneumoniae* EB. This could be explained by using different procedures in purification, sample preparation and staining of 2D-gels (silver and Coomassie versus radioactive labeling by ^{35}S-Met/Cys incorporation). In addition, we used purified RB while the previous proteome map (Vandahl *et al*., 2001) was obtained by using purified EB. C-pmpD being an integral part of the OM could have been better preserved than the non-covalently bound fragments. Thus, our data are complementary rather than contradictory to previous results and the presence of cleavage products and unprocessed PmpD observed in total cell lysates during the course of infection argues against artificial cleavage.

Assuming autotransporter-like translocation and processing for other members of the Pmp superfamily, earlier results of others appear in new light: for PmpG8, -G11 and -G8 in *C. pneumoniae,* smaller polypeptides were observed (Grimwood *et al*., 2001). For other Pmps, positive reacting bands at different sizes could be observed depending on the probe being denatured by boiling or not, which is in agreement with the known temperature-

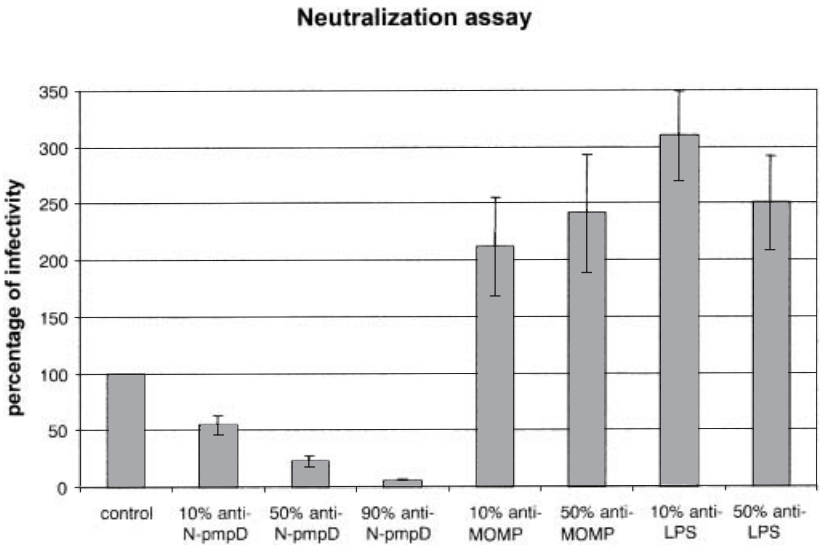

Fig. 8. Neutralization assay with antibodies against N-pmpD. Different volumes (as indicated by percentage; V/V) of antigen-purified rabbit-antibodies or total rabbit IgG fraction (5 mg ml^{-1} each) were incubated with 5×10^5 IFU for 1 h at 37°C in PBS without addition of complement. Aliquots were taken to infect HEp-2 cells seeded on coverslips in 24-well plates and inclusions stained and counted at 2 days p.i. Mouse monoclonal α-MOMP or genus-specific α-LPS antibodies were used as controls and the results normalized for infection with EB incubated with PBS only (= 100% infectivity). Incubation of EB with 0.5 mg ml^{-1} of the α-N-pmpD antiserum ('10%' V/V) reduced the infectivity by about 50%, whereas incubation of EB with 2.5 mg ml^{-1} ('50%') or 4.5 mg ml^{-1} ('90%') of the anti-N-pmpD antiserum reduced the infectivity by about 80% and 95%, respectively. Incubation of EB with the respective amounts of control antibodies has not decreased infectivity but induced greater uptake and development of bacteria, instead.

dependent running behaviour of proteins containing pore-like beta-barrel structures (McCafferty *et al*., 1995; Christiansen *et al*., 1999; Knudsen *et al*., 1999). In *C. psittaci,* only the N-terminus of Pmps was found to be surface exposed forming a beta helix immunogenic in ovine infection, consistent with the model of a C-terminal translocator unit incorporated in the OM (Everett and Hatch, 1995; Longbottom *et al*., 1998; Vretou *et al*., 2003).

Our EM and immunofluorescence experiments complemented by limited proteolysis show unequivocally that the N-terminal part of PmpD is present on the surface of chlamydial cells. The ring-like pattern in Fig. 6 sometimes appearing in cubical or spherical configuration indicates presence of N-pmpD on RB. Surface localization is in agreement with the differential fixation method used for Pmp6, −20 and −21 (Vandahl *et al*., 2002). However, unlike the above authors, we focused majority of our work on the N-terminal, passenger portion of PmpD.

In the limited trypsin digestion, we used Hsp70 as a negative control, though it has been shown by other groups to be surface-exposed in *C. trachomatis*. However, accessibility of the substrate-binding domain occurred only after incubation with reducing agents disturbing disulphide bridges in the membrane (Raulston *et al*., 2002). Similarly, it might be protected or less accessible than N-pmpD at least at the trypsin concentrations used. In addition, there may be structural differences between *C. trachomatis* and *C. pneumoniae*.

Binding of N-pmpD to the membrane was strong and could not be disrupted by heat treatment, high ionic strength, chelation of divalent cations or moderate pH-shifts (Fig. 5A). In isolated OM-fractions (insoluble in 2% Sarkosyl) as well as in Chlamydial outer membrane complexes (COMC) (insoluble in 2% SDS) a proportion of N-pmpD could be recovered in the absence of reducing agents (Fig. 5B). The COMCs are a cluster of cysteine-rich proteins highly cross-linked by disulphide bridges forming a stabilizing lattice below the OM. Partial release of N-pmpD from the OM (solubilized by 2% Sarkosyl) and from COMC (solubilized by 2% SDS) under non-reducing conditions indicates an interaction with one or more proteins involved in the COMC-cluster only for the small percentage of PmpD molecules (N-terminus) that co-isolate with OM and COMCs, whereas the majority has other interaction partners. Alternatively, the non-covalent binding to the OM is weakened by detergent treatment in general and sensitive only to Sarkosyl and SDS to a similar extend. However, incubation of EB with several classes of detergents had different effects on recovery of N-pmpD in the insoluble fraction with 2% zwittergent or NP-40, bringing more N-pmpD into solution than Triton X-100, Tween, CHAPS or the surfactant saponin, independent from reducing or non-reducing conditions (data not shown).

Although the passenger domain of autotransporters are often secreted, the mature N-pmpD could not be observed on the outside of the chlamydial inclusion membrane nor in the cytoplasm. Therefore, we assume that N-pmpD must act at the surface of EB where it stays strongly attached to the components of the OM. The staining pattern visualized by electron microscopy showed additionally a strong association of N-pmpD with vesicle-like structures (Fig. 7) that could possibly be derived by fission or simply by shedding from the chlamydial OM. This type of chlamydial vesicles was observed before but so far no physiological role was ascribed (Heuer *et al*., 2003).

To date, no function was assigned to PmpD or to other members of Pmp family. PmpD shares homology with known adhesins [OmpA (*Rickettsia* spp.), TibA (*E. coli* ETEC), FN0291 (*F. nucleatum*), HMWA (*Y. pestis*)]. Pres-

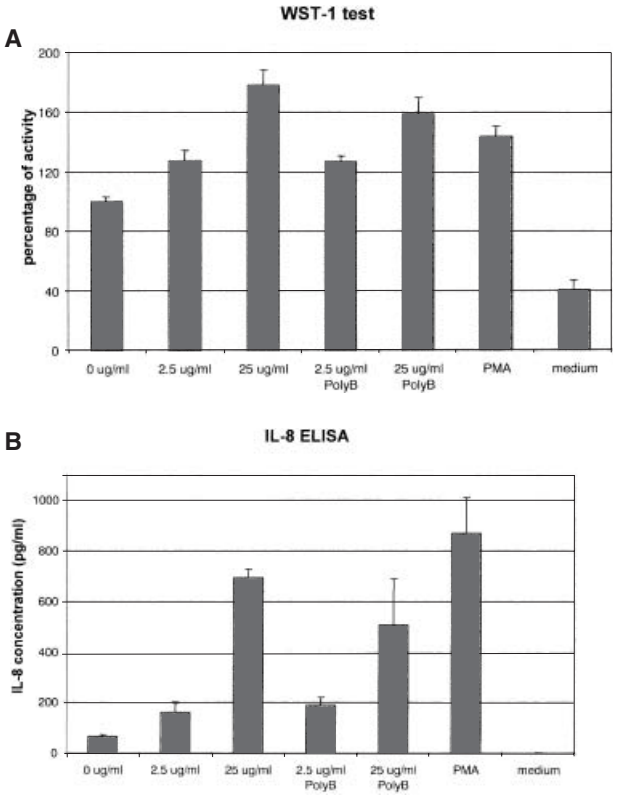

Fig. 9. N-pmpD activates human monocytes as indicated by upregulated mitochondrial activity and IL-8 release assays. THP-1 cells (4×10^4) were synchronized for 40 h in medium containing 0.2% FBS, resuspended in 10% FBS and incubated in 200 µl with: medium only (0 µg), medium that contained 2.5 and 25 µg $ml^{-1}$of the recombinant N-pmpD, 2.5 and 25 µg ml^{-1} of the recombinant N-pmpD pretreated with 100 µg ml^{-1} polymyxin B for 30 min at 37°C and 100 nM PMA. Cells were centrifuged at 920 *g* for 1 h and incubated for 24 h at 35°C. Metabolic activity was determined using the colorimetric assay with WST-1 (A_{450} after 120 min incubation at 37°C) (A). Supernatants from the cell culture were subjected to IL-8 sandwich ELISA (B). We observed increased metabolic activity in THP-1 cells incubated with 0.5 µg and 5 µg of the recombinant N-pmpD which had a more pronounced effect than PMA as an activating agent (positive control). The N-pmpD-induced activity correlated with the IL-8 release in a dose-dependent manner.

ence of the highly repetitive tetra-amino acid motifs in Pmps suggests their possible role in adhesion to membranes of different host cell types (Everett and Hatch, 1995). Our experiments demonstrated reduction in the chlamydial infectivity by anti-N-pmpD antibodies for up to 95% in a concentration-dependent manner. In contrast, monoclonal antibodies against *C. pneumoniae* MOMP and genus-specific antibodies against chlamydial LPS showed no reduction in infectivity but increased uptake of EB instead, possibly because of a Fc-receptor mediated uptake of opsonized chlamydial EB (Su *et al*., 1991; Scidmore *et al*., 1996). This makes a simply sterical effect of the antibodies blocking the interaction of a second chlamydial adhesin with the receptor of the host cell unlikely, but it cannot be fully excluded. Neutralizing capacity of antibodies and antiserum have been demonstrated for other OM-components of *Chlamydiaceae* with indications for a role in adherence as for PorB (*C. trachomatis*), Hsp70 (*C. trachomatis*), cell wall-associated glycosaminoglycan (*C. trachomatis and C. pneumoniae*), and MOMP (*C. trachomatis*) (Caldwell and Perry, 1982; Rasmussen-Lathrop *et al*., 2000; Mamelak *et al*., 2001; Kawa and Stephens, 2002). However, in contrast to *C. trachomatis*, MOMP of *C. pneumoniae* is not immunodominant and neutralizing antibodies were only reported against a conformation-dependent epitope. Antiserum against peptides representing variable domains of MOMP failed to neutralize infection and a monoclonal antibody against *C. pneumoniae* LPS was effective only for the strain it was raised against (Peterson *et al*., 1996; 1998; Wolf *et al*., 2001). Furthermore, immunoreactive surface exposed structures like a glycolipid exoantigen (GLXA, *C. psittaci* and *C. trachomatis*), a 76 kDa and a 54 kDa protein (*C. pneumoniae*) as well as two unknown antigens (*C. pneumoniae*) served as targets for protective antibodies (Girjes *et al*., 1993; Gran *et al*., 1993; Perez *et al*., 1994; Puolakkainen *et al*., 1995; An *et al*., 1997; Wiedmann-Al-Ahmad *et al*., 1997). However, we could not differentiate between an inhibition of binding and uptake/invasion and a negative effect of bound antibodies on the course of the chlamydial development after entry into the eukaryotic cell.

Unlike in specimens analysed 2 days p.i., we were unable to detect full length PmpD in purified EB (Fig. 4A). This suggested that the 170 kDa protein serves as a precursor and the N-terminal passenger part localized on the surface of EB is in fact the functional domain of PmpD. Cleavage after passing the outer membrane could be a trigger to fold into the right conformation. However, a possible contribution of M-pmpD that also contains multiple tetra-amino acid-motifs and other components of the EB OM mediating adhesion cannot be excluded. Interestingly, recombinant N-pmpD stimulated metabolic activity and IL-8 secretion in THP-1 cells in a concentration dependent manner (Fig. 9). Induction of a proinflammatory cytokine response (IL-1β, TNF-α, IL-6) after infection with *C. pneumoniae in vitro* has been demonstrated for both, the monocytic cell line Mono Mac 6 and freshly isolated human peripheral blood mononuclear cells (PBMC) and IL-8 production was induced in alveolar macrophages (Heinemann *et al*., 1996; Kaukoranta-Tolvanen *et al*., 1996; Redecke *et al*., 1998). Cytokine release including IL-8 from human PBMC and mouse macrophages could be attributed to acellular components without the need of viable *Chlamydiae* and Hsp60 respectively (Netea *et al*., 2000; Bulut *et al*., 2002). At present, it is unclear whether the observed stimulating property of recombinant N-pmpD could be modulated by additional surface components of EB or by factors secreted into the target host cell during

early infection. NF-κB-dependent induction of the inflammatory mediators IL-6, IL-8 and MCP-1 in human endothelial cells with recombinant Pmp20 or Pmp21 was recently demonstrated (Niessner *et al*., 2003).

Given that PmpD interacts directly with the host cells and mediates early immunostimulatory events during an ongoing infection and inflammation, passive administration of antibodies or stimulation of the humoral response against PmpD could possibly be used as therapeutic strategies. Furthermore, the demonstrated neutralizing effect of antibodies against N-pmpD supports a protective role of the humoral immune system during the early infection and makes PmpD an important target for anti-chlamydial vaccination.

Experimental procedures

Media

Growth medium for eukaryotic cells was RPMI 1640 (Gibco BRL) supplemented with 300 mg ml^{-1} L-glutamine, 10% FBS (heat-inactivated, Biochrome, Berlin, Germany), 25 mM HEPES, and 10 μg ml^{-1} gentamicin.

Unless otherwise specified, during the infection FBS concentration was reduced to 5% and 1 μg ml^{-1} cycloheximide was added.

Cell line, bacterial strains and propagation

The human epithelioid cell line HEp-2 (ATCC-CCL23) derived from a larynx carcinoma was used as host cells. *Chlamydophila pneumoniae* strain CWL029 (ATCC strain VR1013, a kind gift from Dr Gunna Christiansen) was propagated in HEp-2 cell line by centrifugation at 920 *g* for 1 h at 35°C (Al Younes *et al*., 1999). Presence of *Mycoplasma* spp. was excluded using PCR, mycoplasma detection system (Roche Diagnostics GmbH, Mannheim, Germany) and DAPI staining. The infection was done using one infectious EB/host cell (MOI = 1).

Purification of C. pneumoniae

EB and RB were purified from HEp-2 cells grown in six-well plates. The infected HEp-2 cells were harvested at 2 or 4 days post infection (p.i.) and disrupted using glass beads (3 mm, Roth, Germany). HEp-2 cell debris was removed by centrifugation (10 min at 500 *g*). The supernatant was then centrifuged at 4°C for 40 min at 48 000 *g* and the pellet was resuspended in 5 ml PBS-buffer containing 0.25 M sucrose (SPG).

Elementary bodies and RB were separated and purified from host organelles by ultracentrifugation at 50 000 *g* for 1 h through a discontinuous gradient consisting of 30, 35, 40 and 45% Urografin (Schering, Germany). Upon centrifugation, the two layers (an EB layer at the 40–45% and an RB layer at the 35–40% interface) were transferred to separate vials, washed in SPG and pelleted.

Purity of the preparations was confirmed by electron microscopy (data not shown).

Two-dimensional gel electrophoresis

Elementary body- or RB-pellets were solubilized in five volumes of a buffer containing 9 M urea, 25 mM Tris/HCl, pH 7.1, 50 mM KCl, 3 mM EDTA, 70 mM DTT, 100 nM pepstatin, 1 mM PMSF, 2% CHAPS and 2% carrier ampholytes (Servalyte pH 2–4; Serva, Heidelberg, Germany). After 30–60 min of stirring and vortexing at room temperature (RT), the samples were ultracentrifuged at 100 000 *g* (Optima TLX; Beckman, Palo Alto, CA, USA) for 30 min at RT. The clear supernatant was frozen at −70°C. The proteins were separated by a large gel 2-D technique (gel size 30 cm × 23 cm) (Klose and Kobalz, 1995). The IEF rod gels (diameter of 1.5 or 2.5 mm for preparative gels, 0.9 mm for analytical gels) contained 3.5% acrylamide, 0.3% piperazine diacrylamide (Bio-Rad, Richmond, CA, USA) and a mixture of 4% w/v carrier ampholytes (Klose and Kobalz, 1995). For preparative gels and analytical gels 250 μg, and 60 μg of protein, respectively, were applied to the anodic side of the gel and focused at 8870 Vh. After focusing, the gels were equilibrated for 10 min in a buffer containing 125 mM Tris/phosphate, pH 6.8, 40% glycerol, 70 mM DTT, and 3% SDS. The equilibrated gels were frozen at −70°C (or directly run in the second dimension). After thawing, the IEF gels were immediately applied to SDS-PAGE gels, which contained 15% w/v acrylamide and 0.2% bisacrylamide. The SDS-PAGE system of Laemmli was used, replacing the stacking gel by the equilibrated IEF gel. Electrophoresis was performed using a two-step increase of current, starting with 15min at 120 mA and 65 mA for preparative gels and analytical gels, respectively, followed by a run of about 6 h at 150 mA and 85 mA for preparative gels and analytical gels, respectively, until the front reached the end of the gel.

Analytical gels were stained with silver nitrate and dried for 2 h at 75°C between cellophane membranes using a gel dryer (Model 585; Bio-Rad) (Jungblut and Seifert, 1990). Preparative gels were stained with Coomassie brilliant blue G-250 (Serva), equilibrated in water and stored sealed in plastic bags (Doherty *et al*., 1998).

Tryptic digestion

The in-gel direct measurement procedure was used (Lamer and Jungblut, 2001). The Coomassie brilliant blue G-250 stained single gel spots were excised with a scalpel and equilibrated by addition of 75 μl 200 mM ammonium bicarbonate (pH 7.8) for 30 min at 30°C while shaking. By addition of 105 μl acetonitrile, gel pieces were shrunk for another 30 min. Subsequently the solution was exchanged with 75 μl 50 mM ammonium bicarbonate (pH 7.8), for re-swelling of the gel piece for 30 min at 30°C while shaking. After shrinking, equilibrating and removing the buffer the gel spots were dried in a SpeedVac Concentrator (Eppendorf, Hamburg, Germany). Trypsin (0.1 μg; Promega, Madison, WI, USA) dissolved in 1 μl 50 mM acetic acid and 19 μl 50 mM ammonium bicarbonate (pH 7.8) was added and incubated at 37°C overnight. The supernatant was collected and the gel pieces were washed with 25 μl 0.25% aqueous TFA/acetonitrile (mixed 2:1, respectively) and again the supernatant was collected. The combined supernatants were evaporated in the SpeedVac Concentrator and dissolved in 2 μl 0.5% aqueous TFA/

acetonitrile (mixed 2:1, respectively) for mass spectrometric analysis.

Peptide mass fingerprinting by MALDI-MS and identification of the proteins

The mass spectra were recorded by using a time-of-flight MALDI mass spectrometer (Voyager-Elite; PerSeptive Biosystems, Framingham, MA, USA). The samples were mixed in an Eppendorf tube with equal volume (0.4 μl each) of the matrix solution: 20 mg ml^{-1} of α-cyano-4-hydroxycinnamic acid (CHCA) in 0.3% aqueous TFA/acetonitrile (mixed 1:1, respectively) or 50 mg ml^{-1} of 2,5-dihydroxybenzoic acid (DHB) in 0.3% aqueous TFA/acetonitrile (mixed 2:1, respectively) were used as matrices. The mixtures were applied to a gold-plated sample holder and introduced into the mass spectrometer after drying. The spectra were obtained in the reflectron mode by summing 100–200 laser shots with an acceleration voltage of 20 kV, 70% grid voltage, 0.05 guide wire voltage, 100 ns delay, and low mass gate at 500 m/z.

The proteins were identified by using the peptide mass fingerprinting analysis software MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm), ProFound (http://129.85.19.192/prowl-cgi/ProFound.exe?FORM=1) or Mascot (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF). The NCBInr database was used for the searches, considering maximum one missed cleavage site, pyro-glutamate formation at N-terminal residues, oxidation of methionine, acetylation of N-terminus, and modification of cysteines by acrylamide.

Chemicals, fluorescent probes and antibodies

Genus-specific rabbit polyclonal antibodies were from Milan Analytica AG (La Roche, Switzerland) and used in a dilution of 1:60. Anti-*C. pneumoniae* Hsp60-antibodies from Alexis Biochemicals (San Diego, USA) were diluted 1:5000 for immunoblot analysis and 1:500 for immunofluorescence experiments respectively.

The polyclonal rabbit antiserum against N-pmpD (amino acid 16–670) was produced by BioGenes (Berlin, Germany) and diluted 1:300 for immunoblot analysis and 1:100 for confocal microscopy. Antisera were raised against the recombinant, denatured protein (amino acid 16–670 overexpressed in *E. coli* BL21) purified from preparative SDS-PAGE gels; antibodies for neutralization experiments were either affinity-purified in a batch procedure using the recombinant native N-terminal part of PmpD coupled to CNBr-activated sepharose 4B (Sigma, Germany) or total IgG was isolated by protein A-affinity chromatography following the manufacture's instructions.

Hsp70 in immunoblot analysis was detected with a polyclonal mouse serum raised against recombinant $(His)_6$-tagged protein (BioGenes) and diluted 1:5000. Secondary antibodies coupled with fluorochromes were from Molecular Probes (Leiden, the Netherlands) and diluted 1:100–1:200. The secondary antibody used in Western blots in the peroxidase linked species-specific whole antibodies from Amersham Biosciences (UK) (dilutions of 1:4000 was used).

Cloning and purification of C. pneumoniae *N-pmpD*

The N-terminal part of PmpD without the signal sequence (amino acid 16–670) was cloned in the IPTG-inducible vector pET43a using the primers 5′-TTG ATG CAT TCC GTA ATA GTA GCA ATA TTG TCA G (5′ end) and 5′-CTG CTA AGT TTT AGG AGG ATA ATG ATC TCC ATG (3′end) using standard procedures.

The $(His)_6$-tagged native N-terminus was overexpressed in *E. coli* BL21 and purified using Ni-NTA Agarose (Qiagen, Germany) following the manufacture's instructions.

Cell staining and immunoblot analyses

For confocal microscopy, cells were seeded and infected on glass coverslips in 24-well plates (Al Younes *et al*., 1999). At indicated days p.i., the cells were washed with PBS, fixed for 30 min with 4% paraformaldehyde (Sigma-Aldrich, Munich, Germany), permeabilized if indicated using 0.5% Triton X-100 (Merck) for 5 min and blocked for 20 min with 2% BSA in PBS. For differential staining of surface-localized N-pmpD infected cells at 2 days p.i. were fixed with STF (Streck Laboratories, Omaha, USA) for 20 min and permeabilized with glass beads (425–600 μm, Sigma, Germany).

The respective antibodies were diluted in 2% BSA, incubated for 1 h at RT and washed 3 × with PBS. Incubation with the secondary antibodies diluted in PBS was also for 1 h at RT. After washing, the glass slides were dried and mounted in Mowiol mounting media on glass microscopic slides. The labelled preparations were analysed using a Leica TCS NT laser scanning confocal microscope equipped with krypton-argon mixed gas laser. Images were obtained and processed using Adobe Photoshop 6.0.

Immunoblotting was done according to standard procedures with ECL detection system (Amersham and Perkin Elmer Life Sciences).

EM and immunogold labelling

Infected HEp-2 cells (*C. pneumoniae* or *C. trachomatis* as indicated) were fixed with 4% paraformaldehyde/0.1% glutaraledhyde, infiltrated with 1.6 M saccharose/25% polyvinylpyrollidone, mounted on aluminum stubs and frozen. Ultrathin cryosections were incubated with the rabbit antiserum against *C. pneumoniae* N-pmpD followed by a goat anti-rabbit antibodies coupled to 12 nm gold colloids (Jackson). For the evaluation of staining, a Leo 906 transmission electron microscope was used.

Preparation of detergent-insoluble complexes and outer membrane

Chlamydial outer membrane complexes (COMC) and OM fractions were isolated based on their insolubility in 2% SDS under non-reducing conditions and 2% Sarkosyl respectively (Caldwell *et al*., 1981). Elemenary bodies from *C. pneumoniae* strain VR1310 (1×10^7 IFU) harvested 4 days post infection were incubated in 1 ml of either 2% Sarkosyl (Sigma, Germany), 2% SDS, 2% Sarkosyl + 10 mM DTT + 10% β-

mercaptoethanol (2-ME) and 2% SDS + 10 mM DTT + 10% 2-ME. OM and COMC fractions were pelleted at 250 000 *g* for 30 min and resuspended in Laemmli sample buffer for SDS-PAGE.

Interaction studies of PmpD with the EB-OM

Binding of the N-pmpD to EB was evaluated by incubating EB (1×10^7 IFU) at different conditions; either for 20 min at 60°C in PBS or for 60 min at 37°C in PBS, 200 mM KH_2CO_3 (pH 9.5), 100 mM glycine (pH 3.0), 60 mM EDTA + 2 M NaCl, 2% Sarkosyl (Sigma), 2% Zwittergent (Sigma), 2% SDS (Biomol), Tween 20 and 80 (Merck), 2% Triton X-100 (Calbiochem) and 2% saponin (Sigma) in PBS. Intact EB and insoluble complexes were collected by centrifugation at 20 000 or 250 000 *g*, respectively, and resuspended in Laemmli sample buffer for SDS-PAGE and immunoblotting.

Limited trypsin and proteinase K digestion

To determine the surface exposure of N-pmpD EB (1×10^7 IFU) were incubated with 0, 0.5, 2, 10, 50 and 200 μg ml^{-1} trypsin (Difco Laboratories, USA) at 37°C for 30 min. Intact EB were collected by centrifugation, washed and resuspended in Laemmli sample buffer for SDS-PAGE.

EB were also incubated with proteinase K (Merck, Germany) in the concentration range of 0.01–1 μg ml^{-1} at RT for 10 min and processed the same way for immunoblotting.

Neutralization studies

Different volumes (as indicated by percentage; V/V) of antigen-purified rabbit antibodies or total rabbit IgG fraction (5 mg ml^{-1} each) were incubated with EB (5×10^5 IFU) in a volume of 50 or 100 μl for 1 h at 37°C in PBS without addition of complement. Aliquots were taken to infect HEp-2 cells seeded on coverslips in 24-well plates overnight. HEp-2 cells were centrifuged (920 *g* for 1 h at 35°C) and incubated for 2 days in infection medium containing 1 μg ml^{-1} cycloheximide. Inclusions were visualized and counted at 2 days p.i. Mouse monoclonal α-MOMP (Dako Ltd, Ely, UK) or genus-specific α-LPS antibodies (Progen GmbH, Heidelberg, Germany) were used as controls and the results normalized for infection with EB incubated with PBS only (= 100% infectivity). Three different dilutions were used for each condition giving approximately 100, 50 and 25 inclusions per microscopic field (magnification: × 40). Ten or more fields were counted.

WST-1 activity assay

The monocytic cell line THP-1 was placed in RPMI medium containing 0.2% FBS for 40 h before the experiment. For the proliferation assay the cells were washed and resuspended in medium containing 10% FBS. In 96-well plates, 200 μl containing 4×10^4 cells each were with incubated with different amounts of the recombinant N-pmpD (untreated or incubated with 100 μg ml^{-1} polymyxin B (Sigma-Aldrich Chemie, Germany) for 30 min at 37°C) or 100 nM PMA as indicated, centrifuged at 920 *g* for 1 h and incubated for further 24 h at 35°C. Metabolic activity was determined using a colorimetric assay with the cell proliferation reagent WST-1 (Roche, Germany) following the manufacture's instructions. Shortly, after aspirating the supernatant for IL-8 measurement 10 μl of the reagent and 40 μl medium was added to the remaining THP-1 cells (in 40 μl) in each well, incubated for 120 min and the absorbance at 450 nm determined with an ELISA-reader (Molecular Devices, CA, USA).

Probability levels of $P < 0.05$ calculated with the Student's *t*-test were considered to be significant.

IL-8 ELISA

Interleukin-8 (IL-8) was measured in the supernatants after 24 h with a sandwich enzyme-linked immunosorbent assay (ELISA, Biosource) according to the manufacture's instructions. In this assay a monoclonal mouse goat anti-IL-8 antibody was bound to the wells of 96-well microtitre plates (Nunc, Maxisorp) at 4°C for 20 h. Non-specific binding sites were blocked with 0.5% BSA. Supernatants and recombinant human IL-8 standard (BioSource) were added and incubated for 2 h, followed by incubation for 90 min with the biotinylated monoclonal mouse anti-IL-8 detection antibody. After incubation with peroxidase-conjugated streptavidin for 30 min, plates were developed using freshly prepared tetramethyl benzidine for 20–40 min and read at 450 nm. Results were obtained by interpolation from the standard curve.

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