A newly identified bacterial cell-penetrating peptide that reduces the transcription of pro-inflammatory cytokines

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Summary

Cell-permeable proteins, also called cell-penetrating peptides (CPPs), have the ability to cross cellular membranes, either alone or in association with bioactive cargo. We identified the Yersinia protein YopM as a novel bacterial cell-permeable protein. Here, we describe the ability of isolated recombinant YopM to enter host cells without a requirement for additional factors. This autonomous translocation of YopM was confirmed in several cell types, indicating that it is an intrinsic property of YopM. Using truncated versions of YopM, we show that either of the two N-terminal α-helices of YopM mediates translocation into the cells. Furthermore, the two α-helices are also able to deliver heterologous cargo, such as GFP or YopE. In addition, we found that, after entering the cells, YopM is functional and efficiently downregulates the transcription of pro-inflammatory cytokines (such as tumor necrosis factor-α and interleukins 12, 15 and 18). This finding suggests the potential use of YopM as a tool for protein delivery. Furthermore, it can lead to important advances in understanding and evaluating the intracellular and molecular function of YopM without the need for infection with Yersinia.

Key words: Cell-penetrating peptide, Pro-inflammatory cytokine, Immunotherapeutic molecule, Effector protein, YopM

Introduction

Cell-penetrating peptides (CPPs), such as the transactivator of transcription (Tat) protein encoded by HIV-1, are relatively short proteins or peptides (5-40 amino acids) with the ability to enter cells by different mechanisms (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Although the biological relevance of such protein transduction needs to be determined, it has attracted considerable interest, because CPPs are capable of intracellular delivery of (non-)covalently conjugated bioactive molecules (Langel, 2005). Nearly 30 CPPs from different species have been described, forming a very heterogeneous group of proteins (Fischer et al., 2005). The uptake mechanism of most CPPs is poorly understood and their mode of action is still mysterious. Thus far, various uptake mechanisms of CPPs have been discussed in the literature, suggesting that different mechanisms might be operative. Internalization mechanisms appear to vary depending on the targeted cell type and the cargo that is conjugated to the CPP (Fischer et al., 2005). Although endocytic uptake mechanisms are clearly favored (Richard et al., 2003), some reports also consider energy-independent internalization as an alternative mechanism for some arginine-rich peptides (Mitchell et al., 2000; Thoren et al., 2003). Recent studies, however, suggest that most CPPs enter host cells by an endocytic mechanism (Richard et al., 2003; Drin et al., 2003; Fotin-Mleczek et al., 2005a). Prior to endocytosis, CPPs interact electrostatically with charged components of the cell surface. Subsequent endocytosis might occur through one of several pathways, depending on the internalized CPP (Langel, 2005). Although most studies focused on clathrin-dependent pathways (Schmid, 1997; Vendeville et al., 2004), clathrin-independent pathways, such as endocytosis via lipid rafts, also seem to be operative for some CPPs (Foerg et al., 2005). Interestingly, endocytosed CPPs are able to escape from endosomes and enter the cell cytosol (Magzoub et al., 2005). Currently, the nature of this endosomal escape mechanism remains elusive, but translocation across the endosomal lipid bilayer appears to be driven by endosome acidification (Potocky et al., 2003; Koch et al., 2003; Fischer et al., 2005).

According to the current model of *Yersinia enterocolitica* infection, YopM is translocated, like other Yersinia outer proteins (Yops), through a type III secretion system (T3SS) into the host cell cytoplasm (for a review, see Cornelis, 2002). YopM of *Yersinia pestis* induces a global depletion of natural killer (NK) cells (Kerschen et al., 2004). In association with this NK-cell depletion, spleen and liver tissues of infected mice and the macrophages and NK cells isolated from these tissues showed decreased expression of several pro-inflammatory cytokines (Kerschen et al., 2004). In contrast to other Yops, YopM is the only effector of Yersinia that does not have a known enzymatic activity and its mode of molecular action is still unknown. The protein consists of two N-terminal helices followed by variable numbers of an approximately 20 amino acid leucine-rich repeat (LRR) motif (12 to 21 LRRs among different Yersinia strains), forming a horseshoe-shaped protein (Evdokimov et al., 2000). After translocation into the host cytosol by T3SS, it has been shown that YopM traffics to the nucleus via a vesicle-associated pathway. Two putative nuclear localization signals (NLSs) have been identified within the *Y. enterocolitica* YopM sequence, LRRs 1-3 and the 32 C-terminal amino acid residues, which do not resemble known NLSs (Benabdillah et al.,

Journal of Cell Science

2004). The mechanism of YopM translocation into the nucleus is not exactly known, although carrier-mediated transport has been suggested (Skrzypek et al., 1998). However, until now, it remained elusive how nuclear localization is related to the function of YopM.

Surprisingly, in this study, we found that *Y. enterocolitica* YopM is able to autonomously translocate into host cells; this is reminiscent of the abilities of some CPPs. Thus far, to our knowledge, this is the first bacterial effector protein demonstrated to act as a CPP.

Results

YopM enters eukaryotic cells autonomously and accumulates intracellularly

Although translocation by T3SS implies an intracellular role for YopM, other studies suggest a putative extracellular role for YopM, such as binding to α-thrombin and to the acute-phase protein α_1-antitrypsin (Hines et al., 2001; Heusipp et al., 2006). To further investigate the putative extracellular role of YopM, we analyzed the effect of isolated and purified recombinant YopM on eukaryotic cells. To this end, HeLa cells were incubated with recombinant YopM and prepared for immunofluorescence microscopy. Surprisingly, when examining the localization of YopM by confocal laser scanning immunofluorescence microscopy, we found YopM to be intracellularly distributed throughout the cytoplasm and apparently also inside the nucleus of the host cell (Fig. 1A). In addition, accumulation of YopM in the perinuclear region was clearly visible.

To confirm cellular uptake, cells were incubated with recombinant YopM and subsequently separated into cytosolic (CF) and membrane protein (MF) fractions. Analysis by western blotting with YopM-specific antiserum revealed that, after 30 minutes, YopM could be detected in the CF but not in the MF of HeLa cells (Fig. 1B). Employing human T84 (epithelial) and HL60 (promyelocytic) cells as well as murine XS52 (epidermal dendritic) cells, we confirmed autonomous translocation of recombinant YopM into the cytosol with comparable efficiency in all cell types investigated, indicating that this is an intrinsic ability of YopM that is not restricted to certain eukaryotic cell types or species (Fig. 1B).

To determine the kinetics of YopM internalization, uptake and accumulation of YopM-Cy3 (10 μg/ml) were followed by fluorescence-activated cell sorting (FACS) analysis for 24 hours. The mean cellular fluorescence (MCF) values indicate a high internalization rate of YopM-Cy3 during the initial 5 hours of incubation. After 20 hours, the internalization rate reaches a plateau that persists for at least another 4 hours (Fig. 1C). In addition to the kinetics of YopM internalization, Fig. 1C shows a dose-dependence curve (3 hours incubation time) of YopM-Cy3 uptake. Treatment of HeLa cells with YopM at low concentration (1 μg/ml) leads to a significant increase in MCF. Incubation with higher concentrations of Cy3-labeled protein results in an increase in MCF in a concentration-dependent manner (Fig. 1C). For some CPPs, such as the HIV Tat protein, altered uptake characteristics have been reported following conjugation to a chromophore (Maiolo et al., 2005). Therefore, we followed cellular uptake of YopM-Cy3 by immunofluorescence microscopy. HeLa cells incubated with YopM-Cy3 show a similar cellular distribution to unlabeled YopM when analyzed by immunofluorescence microscopy employing a YopM-specific antiserum (Fig. 1C). From this, we conclude that conjugation to Cy3 does not significantly influence autonomous YopM delivery.

To investigate a putative temperature or energy dependence of YopM-Cy3 translocation into HeLa cells, internalization and accumulation of YopM-Cy3 (10 μg/ml) were followed by FACS analysis for 1 hour at 4°C and 12°C. The data indicate that YopM-Cy3 can enter HeLa cells at low temperatures (4°C or 12°C; Fig. 1D), but with less efficiency than at 37°C (Fig. 1D).

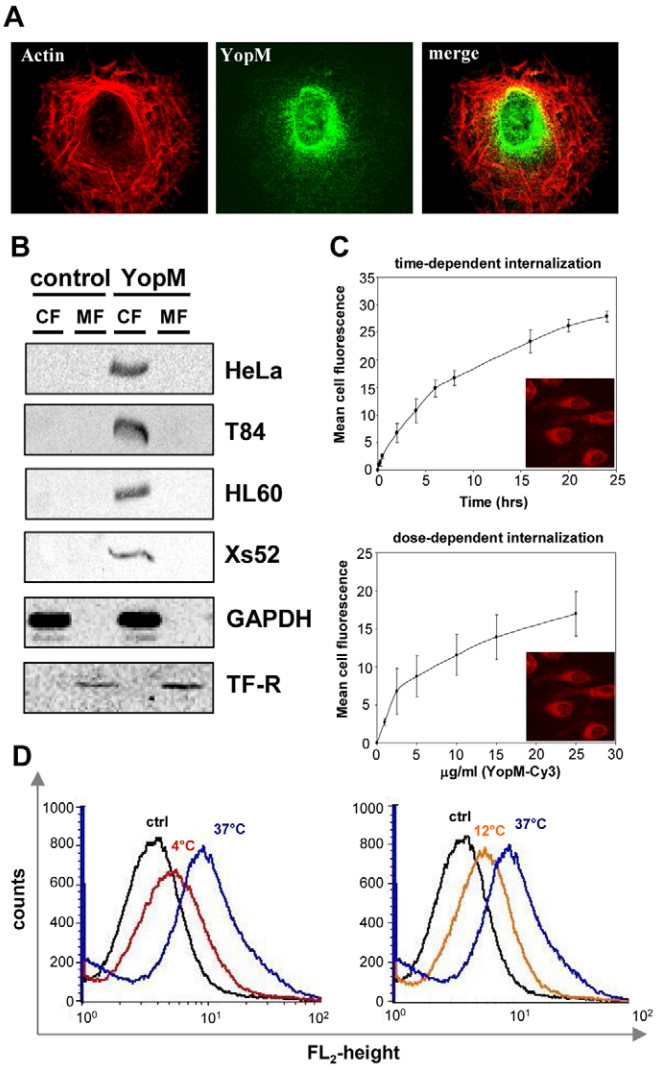

Fig. 1. Recombinant YopM enters host cells independent of T3SS. (**A**) Confocal immunofluorescence microscopy reveals cytoplasmic localization of YopM (25 μg/ml) (green) in HeLa cells after 3 hours incubation with recombinant protein. Actin was stained with phalloidin-Texas Red (red). Overlay of both channels (merge). Pictures consisted of one optical section of a z-series with a pinhole of 1 airy unit (AU). Magnification 100×. (**B**) Immunoblot of cell fractionations of different eukaryotic cells after incubation with YopM (25 μg/ml) using YopM-specific antiserum. (**C**) HeLa cells incubated with Cy3-labeled YopM for different times were analyzed by flow cytometry. The image in the inset shows cells incubated for 1 hour with YopM-Cy3 (10 μg/ml). In addition, HeLa cells were incubated with different amounts of Cy3-labeled YopM (1-25 μg/ml) for 3 hours and analyzed by flow cytometry. (**D**) FACS analysis of HeLa cells (10^5) after incubation with YopM-Cy3 (10 μg/ml) at different temperatures (4°C, 12°C and 37°C) for 1 hour. FL_2 height corresponds to YopM-Cy3 fluorescence.

The N-terminal α-helices of YopM mediate cellular uptake and are able to deliver GFP as cargo

YopM is a horseshoe-shaped protein consisting of two N-terminal α-helices followed by a variable number of LRRs (12 to 21 LRRs

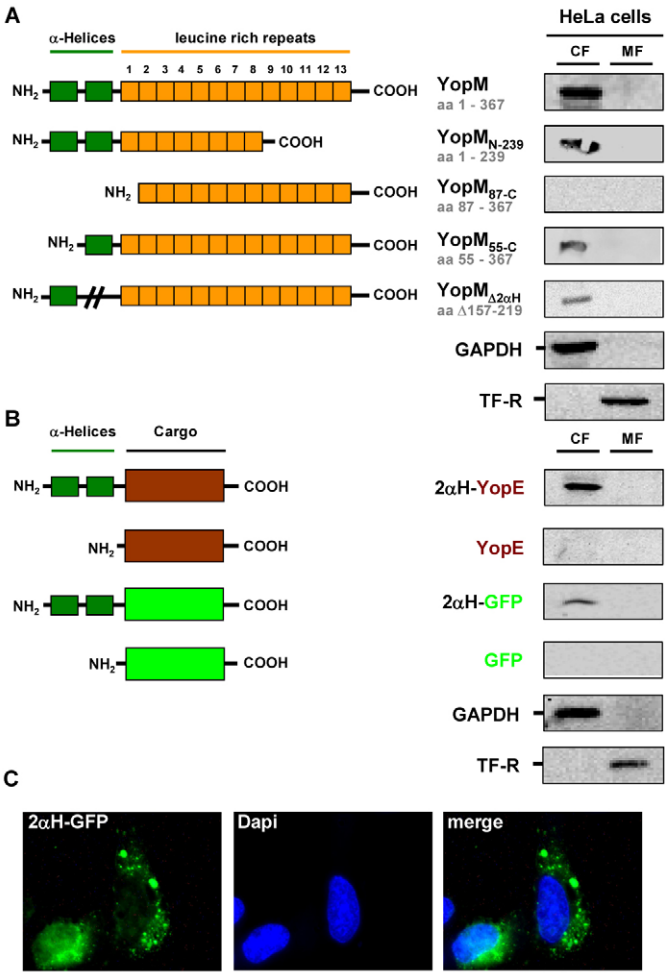

Fig. 2. The N-terminal α-helices of YopM mediate cellular uptake and are able to deliver proteins as cargo. (**A**) Schematic overview of different truncated YopM versions and detection of these proteins by cellular fractionation of HeLa cells after 30 minutes incubation with the respective recombinant protein (25 μg/ml). (**B**) Schematic overview of YopE and GFP fused to the N-terminal domain of YopM. The ability of 2αH-YopE and 2αH-GFP to enter host-cell membranes was analyzed by immunoblot analysis of fractionated HeLa cells after 30 minutes incubation with the fusion proteins (25 μg/ml) and control proteins (YopE and GFP; 25 μg/ml). (**C**) Fluorescence microscopy of HeLa cells after 1 hour incubation with recombinant 2αH-GFP (green) (15 μg/ml). DNA was stained with DAPI (blue). Overlay of both channels (merge).

among different Yersinia strains) that are generally implicated in protein-protein interactions (Evdokimov et al., 2000; Kobe and Kajava, 2001).

To define the YopM domains that mediate cellular delivery, we constructed C- and N-terminally truncated versions of YopM and analyzed their translocation into HeLa cells by cellular fractionation. As shown in Fig. 2A, the truncated recombinant $YopM_{N\text{-}239}$ protein, which lacks 128 amino acids at the C terminus, can be found in the CF following incubation of HeLa cells for 30 minutes. By contrast, incubation of HeLa cells with $YopM_{87\text{-}C}$, which lacks the N-terminal 86 amino acids (including the two α-helices), results in the loss of cellular translocation (Fig. 2A). Interestingly, YopM variants with deletion of either one of the two α-helices (Fig. 2A; $YopM_{55\text{-}C}$ and $YopM_{\Delta 2\alpha H}$) are still able to enter cells; both $YopM_{55\text{-}C}$ and $YopM_{\Delta 2\alpha H}$ are detected in the cytosolic fraction (Fig. 2A). This indicates that both helices can function independently of each other in enabling YopM to enter target cells. These results indicate that the N-terminal α-helical domain is necessary and sufficient to mediate the uptake of YopM. Therefore, we were interested to investigate whether this domain might also be able to act as a 'cargo transporter' in mediating the cellular delivery of unrelated proteins. To this end, the Yersinia effector protein YopE and GFP were used as model cargo proteins, as they are not able to enter host cells on their own. We fused the N-terminal α-helical domain of YopM (2αH) to the N termini of YopE and GFP, and incubated HeLa cells with the resulting fusion proteins (2αH-YopE or 2αH-GFP) for 30 minutes. Cellular uptake was analyzed by western blotting (Fig. 2B). In contrast to the unconjugated YopE and GFP proteins, the 2αH-YopE and 2αH-GFP fusion proteins were detected in CFs of incubated HeLa cells by immunoblot analysis (Fig. 2B). Furthermore, fluorescence microscopy revealed that the 2αH-GFP fusion protein is distributed all over the cytoplasm of the cells, but apparently does not appear inside the nucleus, even after incubation for up to 3 hours (Fig. 2C).

In summary, these results clearly demonstrate that the N-terminal α-helical domain of YopM is necessary and sufficient for uptake of the effector protein into the host-cell cytosol. In addition, the N-terminal α-helical domain of YopM is able to deliver cargo proteins into eukaryotic cells, but most probably not into the nucleus. This is most probably because of the lack of the two NLSs (comprising LRRs 1-3 and the 32 C-terminal amino acid residues of YopM).

Cellular uptake of YopM is lipid-raft dependent

The internalization of a wide variety of extracellular proteins and other factors into eukaryotic cells occurs through distinct mechanisms of endocytosis (Conner and Schmid, 2003). This also holds true for most CPPs, which initially traverse the cytoplasmic membrane by common endocytic mechanisms (Richard et al., 2003; Lundberg et al., 2003). To elucidate whether the cellular uptake of YopM follows a specific endocytic pathway, we employed different inhibitors of different cellular uptake mechanisms (Fig. 3A). Pretreatment of HeLa cells with cytochalasin D (5 μM, 1 hour), an inhibitor of F-actin elongation, results in 32.5% inhibition of YopM uptake, whereas uptake is not significantly altered by dynasore (80 μM, 1 hour), a specific inhibitor of dynamin and clathrin-mediated endocytosis (Macia et al., 2006). The role of microtubules in cellular uptake of YopM was assessed with the inhibitors colchicine (10 μM, 1 hour) and nocodazole (20 μM, 1 hour). Whereas pretreatment of HeLa cells with colchicine does not lead to significant inhibition of YopM uptake, nocodazole inhibits YopM uptake by 25%. Interestingly, cytochalasin D and nocodazole show a strong synergistic inhibitory effect on YopM internalization; this is perhaps not too surprising, as microtubule and actin dynamics are essential for vesicle formation and transport in eukaryotic cells. These results indicate that invagination processes at the cytoplasmic membrane and vesicular transport mediated by microtubules seem to contribute to YopM uptake into HeLa cells, whereas clathrin-dependent endocytosis only plays a minor role. Therefore, different inhibitors of lipid-raft-associated endocytosis were employed, as lipid rafts are involved in specific endocytic pathways, including receptor-independent macropinocytosis and caveolae-mediated endocytosis (Hanzal-Bayer and Hancock, 2007; Anderson, 1998). Whereas treatment of HeLa cells with amiloride (10 mM, 1 hour), an inhibitor of

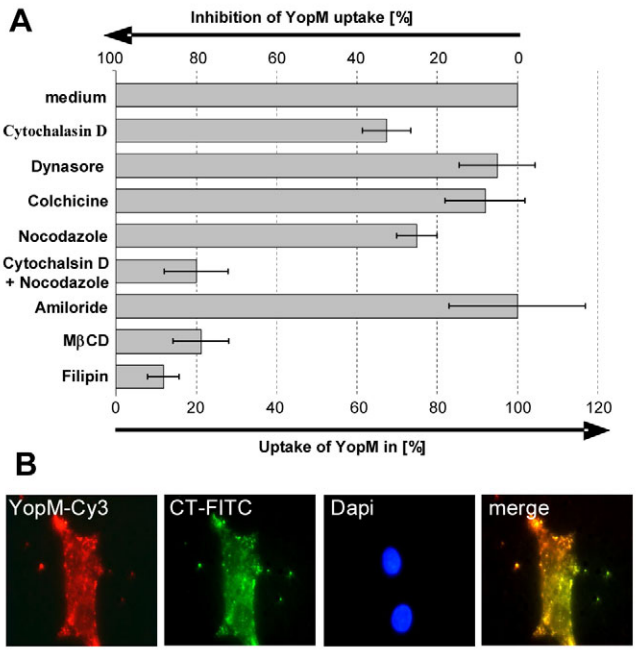

Fig. 3. The cellular uptake of YopM is lipid-raft dependent. (**A**) The influence of inhibitory drugs on YopM uptake was determined by pre-incubation of HeLa cells with the inhibitors for 1 hour before the addition of recombinant YopM (25 µg/ml, see text for details). Data were generated by quantifying signal intensities in immunoblots of fractionated HeLa cells incubated with YopM and the respective inhibitor, and developed with YopM-specific antiserum using Lumi-Imager T1 and Lumi Analyst software. Data are expressed as means ± s.d. and represent at least three different experiments. (**B**) Colocalization experiments of YopM-Cy3 and FITC-labeled CT-B (CT-FITC) by fluorescence microscopy. HeLa cells were incubated with YopM-Cy3 (red) and CT-FITC (green) (10 µg/ml, each) for 30 minutes and prepared for fluorescence microscopy. DNA was stained with DAPI (blue). Overlay of both channels (merge). Magnification 100×.

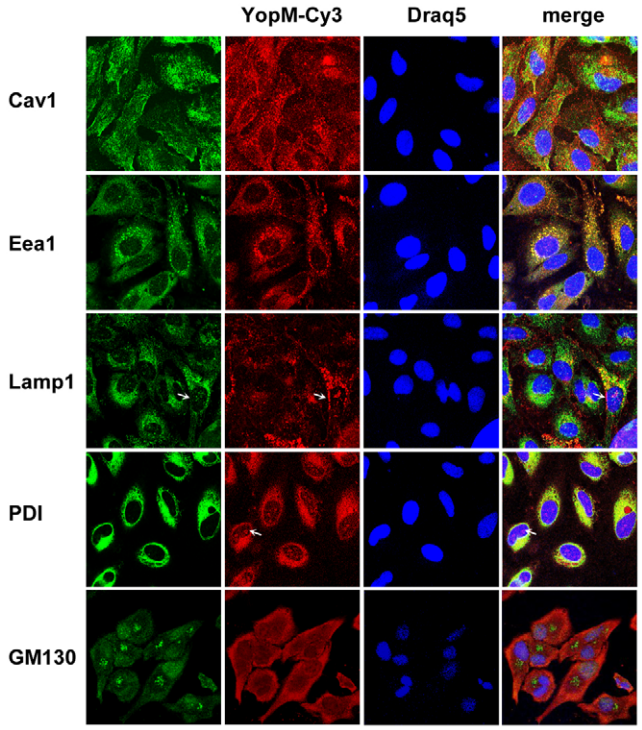

Fig. 4. Intracellular YopM is transported towards the ER. HeLa cells incubated with YopM-Cy3 (10 µg/ml) were processed for immunofluorescence microscopy after different times (5 minutes to 3 hours). The cellular distribution of YopM-Cy3 (red) was compared to signals obtained for marker proteins of different vesicular and cellular compartments (caveolae: Cav1, 5 minutes; early endosomes: Eea1, 15 minutes; late endosomes: Lamp1, 30 minutes; Golgi: GM130, 1 hour; ER: PDI, 3 hours). Marker proteins were detected using Cy2-labeled secondary antibodies (green). DNA was stained with DRAQ5 (blue). Overlay of both channels (merge). The white arrows indicate a YopM signal distinct from Lamp1-positive vesicles and inside the cell nucleus (PDI). Pictures consisted of one optical section of a z-series with a pinhole of 1 airy unit (AU). Magnification 100×.

macropinocytosis, does not significantly affect YopM uptake into HeLa cells, the disruption of lipid-raft structures by methyl-β-cyclodextrin (MβCD, 5 mM, 1 hour) and filipin (10 mM, 1 hour) (Shogomori and Futerman, 2001; Bergy and Eble, 1968) reduced YopM uptake to 10% compared with untreated cells (Fig. 3). To exclude that the inhibitory effects of MβCD or filipin result from direct interactions with YopM, causing its precipitation, YopM was incubated with the inhibitors (1 hour, in equimolar amounts) prior to adding the protein to cells. FACS analysis revealed that no significant effect of preincubation was detectable, underlining the specific effect of the lipid-raft-disrupting pharmacological inhibitors on the internalization of YopM (supplementary material Fig. S1). From these data, we conclude that lipid rafts play a major role in T3SS-independent translocation of YopM and suggest caveolae-dependent uptake of YopM into HeLa cells. To confirm uptake via caveolae, we used fluorescein isothiocyanate (FITC)-labeled cholera toxin B-subunit (CT-B FITC) as a tracer of caveolae-dependent and lipid-raft-associated endocytosis (Fotin-Mleczek et al., 2005a). Fluorescence microscopy of HeLa cells after incubation with CT-B FITC and YopM-Cy3 for 30 minutes shows colocalization of the fluorophore-labeled proteins (Fig. 3B), supporting our finding that YopM is internalized into HeLa cells by a caveolae-dependent mechanism.

Intracellular YopM is transported towards the ER

CT and other bacterial protein toxins are routed to the endoplasmic reticulum (ER) by retrograde transport before release into the cytosol (el Bâya et al., 1997; Sandvig and van Deurs, 2005; Lencer and Tsai, 2003). Colocalization of YopM with CT-B and the observed lipid-raft-dependent uptake of YopM suggested similar trafficking of YopM towards the ER. To evaluate this possibility, colocalization analysis of YopM with cell-compartment-specific markers in HeLa cells was performed in a time-course experiment (Fig. 4). Cells incubated with YopM for 5-15 minutes show partial colocalization of YopM with caveolin 1 (Cav1) close to the cytoplasmic membrane (Fig. 4; Cav1). The merged image shows the presence of YopM in caveolae and caveosomes, supporting the results of our experiments using the inhibitors MβCD and filipin (Fig. 3). Whereas Cav1 is transported back through recycling endosomes to the plasma membrane (supplementary material Fig. S2), YopM colocalizes with early endosomal antigen 1 (Eea1) (Fig. 4; Eea1) after 15-30 minutes incubation. This shows that YopM reaches the early endosome (EE) after internalization by means of caveolae and then further follows the endocytic pathway to late endosomes (LEs), as indicated by the colocalization of YopM with lysosome-associated membrane protein 1 (Lamp1)-

positive vesicles after 30-60 minutes incubation (Fig. 4; Lamp1). Additionally, YopM is located in perinuclear structures that are distinct from LEs (Fig. 4; Lamp1). After incubation for 1 hour, YopM colocalizes with protein disulfide isomerase (PDI), an ER-resident enzyme (data not shown). The amount of YopM associated with the ER seems to increase during prolonged incubation for up to 3 hours and small amounts of YopM seem to enter the nucleus (Fig. 4; PDI). How YopM reaches the ER and the nucleus remains elusive. Interestingly, during the whole time course (up to 3 hours), YopM was never identified in the Golgi apparatus (GA) (Fig. 4; GM130). In comparison, toxins using classical retrograde transport pathways, such as CT or Shiga toxin, travel by a Rab9-dependent pathway from LEs to the GA and finally by the *trans*-Golgi network to the ER (Sandvig and van Deurs, 2005; Lencer and Tsai, 2003). This indicates that YopM does not follow the classical retrograde pathway from LEs via the GA to the ER.

Autonomously translocated YopM is functional and downregulates transcription of TNFα

At present, the molecular function of YopM is not known. However, in a murine infection model, it has been shown that a *Y. pestis yopM*-deficient mutant is no longer able to reduce the expression of certain pro-inflammatory cytokines in NK cells, macrophages, and in spleen and liver tissues (Kerschen et al., 2004). Therefore, we wondered whether autonomously translocated YopM is able to interfere with the expression of pro-inflammatory cytokines. To this end, we analyzed the transcription of tumor necrosis factor-α (TNFα) after treatment of HL60-derived macrophages with recombinant YopM (25 μg/ml) using quantitative real-time PCR (qRT-PCR) (Fig. 5A). We found the transcription of TNFα to be strongly reduced (to 40% that of control cells) after 6 hours incubation with YopM. Similar results could also be obtained with other cell lines, including HeLa and T84 cells (data not shown). The downregulation of TNFα transcription specifically depends on the cellular uptake of YopM, as incubation with the translocation-inactive derivative $YopM_{87\text{-}C}$ or with YopE did not reduce TNFα transcription. By contrast, incubation with control proteins results in increased levels of mRNA encoding TNFα (up to 50% compared with untreated cells; Fig. 5A). This increase in TNFα mRNA is attributed to residual lipopolysaccharides (LPS) in the purified protein preparation. This conclusion is supported by the effect of mock treatment of HL60 cells with fractions of lysates of *Escherichia coli* carrying the empty expression vector that had been eluted from a Ni-NTA affinity column. This treatment also increased the amount of TNFα mRNA. Quantification of the possible endotoxin content by the LAL assay revealed that the YopM preparation contained approximately 3.65 EU/mg LPS. CPPs have been reported to interfere with signaling cascades by inducing receptor internalization during uptake (Fotin-Mleczek et al., 2005b). Therefore, to address this possibility, we investigated YopM-dependent receptor internalization as a possible source of TNFα downregulation using the 2αH-GFP fusion protein as a translocated test protein. If cell penetration of YopM via receptor internalization mediated by the N-terminal α-helical domain is the cause of TNFα downregulation, the protein transduction domain (PTD) of YopM, which is included in the 2αH-GFP fusion protein, should also reduce transcription of the cytokine. Although able to enter cells, the 2αH-GFP fusion protein and GFP alone did not reduce TNFα transcription (Fig. 5A). This clearly excludes receptor internalization as the cause of reduced TNFα transcription and, furthermore, shows that downregulation of TNFα transcription is an intrinsic property of YopM that is probably associated with its LRR domain.

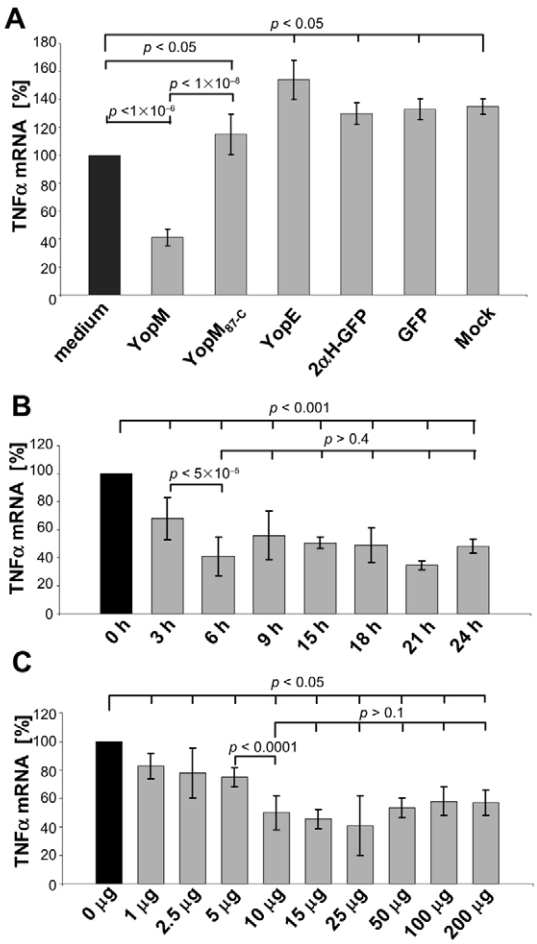

Fig. 5. YopM downregulates TNFα transcription in HL60-derived macrophages. (**A**) HL60 cells were differentiated into macrophages and incubated with recombinant YopM, $YopM_{87\text{-}C}$, YopE, 2αH-GFP, GFP or 'mock' (25 μg/ml, each) for 6 hours. (**B**) Time-course analysis of TNFα mRNA after incubation of HL60 macrophages with recombinant YopM (25 μg/ml) for 0-24 hours. (**C**) Concentration-dependent effects on TNFα transcription after incubation of HL60 cells with 0-50 μg/ml recombinant YopM for 6 hours. After RNA isolation and cDNA construction, all qRT-PCR data were obtained using SYBR green and TNFα-specific primers. The mRNA level of medium-treated cells was set as 100%. Data represent means and s.d. of at least three independent experiments, each performed in duplicate. The *P* values are given to indicate statistical significance of differences ($P<0.05$).

To determine the amount of YopM and the incubation time necessary for induction of reduced TNFα transcription in HeLa cells, we quantified TNFα mRNA after different time intervals of YopM incubation. After 3 hours incubation with 25 μg/ml YopM, the transcription of TNFα was slightly reduced and reached a minimum after 6 hours (60% reduction). Prolonged incubation times did not enhance the effect on TNFα mRNA levels (Fig. 5B). Furthermore, a concentration of 1-5 μg/ml YopM significantly reduces TNFα mRNA levels after 6 hours incubation (Fig. 5C). Using increasing amounts of YopM (up to 10 μg/ml) resulted in even less TNFα transcription, but a further increase to 10-50 μg/ml YopM did not additionally reduce TNFα mRNA levels (Fig. 5C).

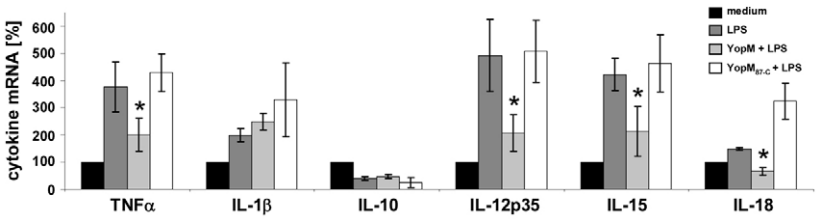

Fig. 6. Effect of YopM on the transcription of pro-inflammatory cytokines in HL60-derived macrophages after stimulation with LPS. HL60 cells differentiated into macrophages were pre-incubated with recombinant YopM and $YopM_{87\text{-}C}$ (25 μg/ml, each) for 3 hours before stimulation with 1 μg/ml LPS for 16 hours. After RNA isolation and cDNA construction, the mRNA levels of TNFα, IL-1β, IL-10, IL-12p35, IL-15 and IL-18 were assessed by qRT-PCR. All data were obtained using SYBR green and cytokine-specific primers. The mRNA level of medium-treated cells was set as 100%. Data represent means and s.d. of at least three independent experiments, each performed in duplicate. The asterisk (*) indicates significant differences (P<0.05) in the YopM-dependent reduction of LPS stimulation of pro-inflammatory cytokines.

Effect of YopM on the transcription of pro-inflammatory cytokines

The reduction in TNFα transcription by autonomously translocated YopM prompted us to investigate whether YopM might also influence the expression of additional pro-inflammatory cytokines. Infection of mice with *Y. pestis* results in YopM-dependent downregulation of TNFα, interleukin (IL)-1β, IL-12, IL-15 and IL-18 messages, whereas transcription of the anti-inflammatory cytokines IL-4 and IL-10 is not affected (Kerschen et al., 2004). Therefore, we analyzed transcription of these cytokines after pre-incubation of HL60-derived macrophages for 3 hours with either recombinant YopM or the truncated derivative $YopM_{87\text{-}C}$, followed by 16 hours stimulation with LPS (1 μg/ml). Recombinant YopM did significantly reduce mRNA levels for TNFα, IL-12p35, IL-15 and IL-18 and, therefore, inhibited LPS-induced transcription (Fig. 6). These findings indicate that autonomously translocated YopM is functional and has an impact on the transcription of pro-inflammatory cytokines other than TNFα. By contrast, pre-incubation of HL60-derived macrophages with the truncated protein $YopM_{87\text{-}C}$, which was used as control prior to LPS stimulation, did not result in a significant decrease in the transcription of the cytokines analyzed, indicating that cellular uptake of YopM, mediated by the N-terminal α-helical domain, is necessary to counteract the pro-inflammatory response induced by LPS. After incubation with YopM or $YopM_{87\text{-}C}$ and subsequent stimulation with LPS, transcription of IL-1β was not significantly changed in HL60-derived macrophages (Fig. 6). Interestingly, the transcription levels of the anti-inflammatory IL-10 after pre-incubation with YopM or $YopM_{87\text{-}C}$ are not significantly changed compared with LPS-stimulated cells. In all experiments, IL-10 transcription did not increase, indicating that YopM-mediated downregulation of pro-inflammatory cytokines is not caused by the induction of IL-10. The anti-inflammatory cytokine IL-4 is not expressed in HL60 cells. Taken together, these results suggest that autonomously translocated YopM specifically reduces the expression of several pro-inflammatory cytokines.

Discussion

We identified the *Y. enterocolitica* T3SS-secreted effector protein YopM as a novel CPP and demonstrated the ability of YopM to autonomously enter different eukaryotic cell types, including immune and epithelial cells, by caveolin-dependent endocytosis. Furthermore, treatment of cells with recombinant YopM leads to the downregulation of pro-inflammatory cytokines such as TNFα. The mechanism of receptor-independent uptake and vesicular transport shows characteristics of the mechanism postulated for some CPPs (Fotin-Mleczek et al., 2005a). These proteins harbor a PTD that mediates the traversal of biological membranes in a receptor-independent way (Richard et al., 2003). Furthermore, the PTD of CPPs possesses the ability to transport (non-)covalently linked cargo into the target cell (Dietz and Bähr, 2004; Langel, 2005; Richard et al., 2003). Our studies revealed that the N-terminal α-helical domain of YopM (amino acids 1-86) is necessary and sufficient for cellular uptake. Although each α-helix seems to be sufficient to mediate cellular uptake alone, the α-helices most probably work synergistically to promote YopM uptake. In the crystal structure of YopM, residues 1-33 could not be defined; therefore, the most N-terminal part of this YopM domain presumably remains in a flexible conformation (Evdokimov et al., 2000). The following residues 34-86 constitute two α-helices. However, except for the presence of several arginine and lysine residues, there is no obvious similarity to known PTDs of CPPs, indicating that YopM might have specific features enabling cellular uptake. The N-terminal α-helical domain of YopM could also be used to deliver suitable cargo proteins (YopE and GFP) into the cytoplasm of target cells. Moreover, the fact that GFP and especially YopE, as yet another effector protein of pathogenic Yersinia, were not able to enter HeLa cells by themselves demonstrates that autonomous translocation is apparently not a common feature of T3SS effectors, but rather an intrinsic property of YopM. In addition, the N-terminal α-helical domain of YopM represents a PTD that is useful for the delivery of cargo into eukaryotic cells, but most probably not into the nucleus. This is most probably because the NLSs, comprising LRRs 1-3 and the 32 C-terminal amino acid residues of YopM, are missing (Skrzypek et al., 1998; Skrzypek et al., 2003; Benabdillah et al., 2004). Thus far, it could not be resolved whether YopM and its putative cargo are able to escape from the endocytic vesicles to the cytosol, because YopM might be liberated from vesicular structures into the CF during cell fractionation. To date, it is also not known whether or not and by which mechanism CPPs and CPP-conjugated cargo can escape from vesicles following endocytosis. Most CPPs initially traverse the cytoplasmic membrane by common endocytic mechanisms (Johannes and Goud, 2000; Richard et al., 2003). Potocky et al. reported the localization of Tat conjugates in the cytoplasm and in the nucleus, which suggests that the internalized cargo could, at least to some extent, escape from endocytic vesicles (Potocky et al., 2003).

So far, our results support a model in which extracellularly added recombinant YopM undergoes caveolae-dependent endocytosis into early or sorting endosomes, which then sort to late endosomes. At later time points, YopM is enriched in the

perinuclear region in particular, colocalizes with the ER and can also be found – at least to some extent – inside the nucleus. As YopM cannot be detected in the GA, transport of YopM from the cytoplasmic membrane to the ER is distinct from the classical retrograde transport that has been described for bacterial protein toxins such as CT, pertussis toxin and Shiga toxin (el Bâya et al., 1997; Sandvig and van Deurs, 2005). At present, however, it still remains elusive how YopM might be associated with different vesicles during intracellular trafficking and whether association with the outside of vesicles might be feasible as well. After translocation of the effectors into the host-cell cytosol by the T3SS during infection with Yersinia, it has been shown that YopM traffics to the nucleus by means of a vesicle-associated pathway (Skrzypek et al., 1998). Whether this intracellular pathway is also followed by autonomously translocated YopM needs to be addressed in further investigations. Intracellular transport of YopM by association with the outside of vesicles could explain why YopM is only present in cytosolic and not in membrane fractions of cells and why transport of YopM from the cytoplasmic membrane to the ER is distinct from the classical retrograde pathway.

Obviously, to reach the nucleoplasm, YopM either has to directly cross the plasma membrane followed by association with the outside of endosomes for vesicle-associated trafficking towards the nucleus or has to escape from endosomes after vesicular transport towards the nucleus. This process might involve induced opening of transient pores, back fusion of intralumenal vesicles from multivesicular bodies or endosomal escape (Medina-Kauwe, 2007). However, intracellular transport processes seemed to be important for intracellular accumulation of YopM. Uptake of the protein is less efficient at lower temperatures than internalization at 37°C, which indicates the involvement of an energy-dependent intracellular trafficking process for the cytosolic accumulation of YopM. Furthermore, the strong synergistic inhibitory effect of cytochalasin D and nocodazole on the internalization of YopM underlines this assumption. Polymerization of actin is involved in vesicle formation and transport, whereas microtubuli serve as 'rails' for vesicle trafficking. Inhibition of both cytoskeletal facilitators of intracellular trafficking processes most probably leads to retention of YopM at the plasma membrane and disrupted transport towards the cytosol of the cell.

Our results clearly demonstrate that autonomously translocated YopM is functional in being able to downregulate the expression of various pro-inflammatory cytokines, including TNFα. This is reminiscent of a study by Kerschen et al. in which the observed downregulation of pro-inflammatory cytokines during *Y. pestis* infection of mice had been linked to YopM (Kerschen et al., 2004). Further analyses of TNFα transcription after treatment of cells with proteins $YopM_{87\text{-}C}$ and YopE showed that these two proteins were not able to reduce transcription of TNFα and that downregulation of TNFα transcription is an intrinsic property of YopM. Because $YopM_{87\text{-}C}$ (which is not able to enter cells) also did not reduce transcription of any other cytokine, the immunomodulating property is apparently directly linked to cellular uptake of YopM mediated by the N-terminal α-helical domain. Given that 2αH-GFP enters cells but does not have immunomodulatory activities, the N-terminal α-helical domain is clearly not involved in modulating cytokine transcription. Instead, the activity of YopM seems to depend on its LRR domain. Similar to YopM, the effector proteins SspH1 of *Salmonella enterica* and IpaH9.8 of *Shigella flexneri* are T3SS-translocated LRR-containing effector proteins that localize to the host-cell nucleus (Haraga and Miller, 2003; Rohde et al., 2007). SspH1 and IpaH9.8 are members of a ubiquitin ligase family of bacterial effectors that supposedly use the LRR domain as a scaffold (Rohde et al., 2007; Zhu et al., 2008). SspH1 binds with its LRR to PKN1 kinase, which also serves as a substrate for SspH1-mediated ubiquitylation (Haraga and Miller, 2003; Rohde et al., 2007). Interestingly, YopM also interacts with two cellular kinases, RSK1 and PRK2 (McDonald et al., 2003), but lacks a ubiquitin ligase domain. Therefore, despite these similarities, the mechanism YopM uses to downregulate transcription of pro-inflammatory cytokines is different from that of SspH1 and IpaH proteins, and might instead rely on protein scaffolding by its LRR. In this context, our findings open the possibility to study interactions of YopM with host cells functionally without interference from additional bacterial components. However, whether our findings might be directly relevant to Yersinia pathogenesis cannot be judged by the presently available in vitro data.

Beyond the newly identified function and effect of YopM on host cells, our findings suggest a potential new tool for protein delivery, open the possibility to study molecular interactions of YopM without interference from additional bacterial components and, moreover, immediately suggest a potential therapeutic application. YopM can be considered a novel locally applicable, self-delivering immunotherapeutic molecule, especially for the treatment of autoimmune diseases characterized by elevated levels of pro-inflammatory cytokines such as TNFα. Acute and chronic inflammatory diseases are associated with the production of inflammatory cytokines, such as TNFα, which is a potent mediator of septic shock and a therapeutic target in chronic inflammatory disorders, including, for example, rheumatoid arthritis and Crohn's disease (Weckmann and Alcocer-Varela, 1996; Le Buanec et al., 2006). So far, there have been several reports of CPP conjugates that can attenuate inflammatory-disease-associated responses, particularly in models of rheumatoid arthritis. One example of this is an apoptosis-inducing fusion protein that reduces synovial inflammation when administered to rabbit joints (Mi et al., 2003). Another example is a fusion protein derived from the NEMO-binding domain (NBD) and the PTD of Tat, which was used to block nuclear factor-κB activation, with consequent reduced cytokine production and inhibition of joint inflammation (May et al., 2000; Dai et al., 2004; Jimi et al., 2004). Although these are very promising approaches using engineered peptides, YopM, by contrast, has been optimized during pathogen-host co-evolution to reduce several pro-inflammatory cytokines simultaneously. These simultaneous effects on the transcription of certain pro-inflammatory cytokines could be quite beneficial, especially for the treatment of autoimmune diseases that are caused by the deregulation of inflammatory mediators. This makes YopM attractive as a potential novel self-delivering immunotherapeutic molecule. We are following up on these potential applications of YopM, so far with encouraging results.

Materials and Methods

Plasmid construction and protein purification

The construction of $YopM_{N\text{-}367}$, $YopM_{N\text{-}239}$, and $Yop_{M87\text{-}C}$ has been described (Heusipp et al., 2006). $YopM_{55\text{-}C}$ was constructed similarly using primer pairs F-$yopM_{55\text{-}C}$ and R-$yopM_{55\text{-}C}$ (supplementary material Table S1) to delete the coding region for amino acid residues 1 to 55. Deletion of the second α-helix of YopM was achieved by inverse PCR with primer pairs F-$yopM_{\Delta\alpha2H}$ and R-$yopM_{\Delta\alpha2H}$ (supplementary material Table S1), and pET-yopM as template. The 2αH-GFP fusion protein with a C-terminal 6×His tag was constructed by first deleting the DNA fragment encoding the LRR of YopM by inverse PCR of plasmid pET-yopM using primers F-2αH and R-2αH (supplementary material Table S1) and subsequently ligating the GFP-coding

region amplified by PCR with primers F-GFP and R-GFP (supplementary material Table S1) from GFP mutant 2 as template in frame 3′ of the yopM sequence coding for the N-terminal α-helical domain. For expression and affinity purification of GFP fused with a C-terminal 6×His tag, the same GFP PCR fragment was ligated into expression plasmid pET24b(+). The 2αH-YopE fusion protein was constructed in the same manner as described for the fusion protein 2αH-GFP, using primer pairs F-YopE(2αH) and R-YopE (supplementary material Table S1) for the amplification of yopE from genomic DNA of *Y. enterocolitica* O8 strain JB580v. For the expression and affinity purification of YopE fused with a C-terminal 6×His tag, yopE was amplified by PCR using primer pair F-YopE and R-YopE (supplementary material Table S1) and genomic DNA of *Y. enterocolitica* O8 strain JB580v as a template, and ligated into the expression plasmid pET24b(+).

Expression and purification of recombinant proteins was performed as described (Heusipp et al., 2006). Affinity-purified proteins were dialyzed against PBS and concentrated using Centricon centrifugal filters (Millipore). The LPS content of the protein preparations was analyzed by Profos AG (Regensburg, Germany) with the Limulus Amoebocyte Lysate (LAL) assay using a representative sample of 2.5 mg/ml YopM protein in PBS, pH 7.0.

FACS analysis and microscopy

FACS and fluorescence microscopy were employed to investigate autonomous translocation by YopM. For this purpose, HeLa cells were seeded in 6-well plates or on glass coverslips in 24-well plates, and grown to about 70% confluency. The cell-culture medium was exchanged before incubation with YopM-Cy3 [10 μg/ml for 1 hour, if not indicated otherwise; Cy3 labeled using the Cy3 AB labeling kit (GE Healthcare)]. Cells were then washed three times with PBS, trypsinized to remove surface-bound YopM and washed again with PBS. An acid-buffer wash (0.2 M glycin, pH 2) was used to remove any residual surface-bound proteins (Langel, 2005). Subsequently, cells were fixed in 4% PFA (w/v) and washed with PBS twice. Finally, the cells were analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences). Fluorescence was assessed from cells judged to be intact by their forward and side scatter. For fluorescence microscopy, HeLa cells grown on glass coverslips were incubated with recombinant YopM (10 μg/ml) and FITC-conjugated CT (10 μg/ml) in fresh media for the indicated times. After incubation, cells were washed as depicted above and prepared for immunofluorescence microscopy as described (Michgehl et al., 2006). YopM was visualized either by Cy3 labeling or by immunofluorescence using a YopM-specific mouse polyclonal antiserum and a Cy2-labeled goat anti-mouse secondary antibody (Dianova). Cellular structures were visualized by primary antibodies against Cav-1 (caveolae), Eea1 (early endosomes), LAMP-1 (late endosomes), GM130 (*cis*-Golgi matrix protein) and PDI (ER) according to the manufacturer's instructions (Cell Signaling Technology), and a Cy2-labeled goat anti-rabbit secondary antibody (Dianova). Filamentous actin was stained with Texas-Red-conjugated phalloidin (Sigma). DNA was stained with 300 nM DAPI (Sigma) or with DRAQ5 (Biostatus) for confocal laser scanning microscopy.

Fractionation of eukaryotic cells

For cell fractionation, HL60, HeLa, T84 and Xs52 cells were seeded in 10 cm cell-culture dishes. Prior to cell fractionation, HL60 cells were differentiated into macrophages by incubation with TPA (Sigma). Prior to incubation with recombinant proteins (25 μg/ml) for 30 minutes, cells were washed with PBS and fresh media added. After incubation, the cells were washed twice with PBS, followed by an acid-buffer wash and a PBS wash. Finally, cells were prepared for fractionation and analyzed as described (Kenny and Finlay, 1997; Michgehl et al., 2006). As internal controls, both fractions were analyzed for the presence of soluble cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the membrane transferrin receptor (TF-R), with antibodies raised against the marker proteins (GAPDH, Santa Cruz; TFR, Abcam).

The influence of inhibitory drugs on YopM uptake was determined by pre-incubation of HeLa cells with 5 μM cytochalasin D, 80 μM dynasore, 10 μM colchicine, 20 μM nocodazole, 10 mM amiloride, 5 mM MβCD or 10 mM filipin for 1 hour before the addition of recombinant YopM (25 μg/ml). Uptake of YopM in the presence of inhibitors was assessed by immunoblotting and by measuring the band intensity in comparison to YopM uptake in control cells, with normalization to β-actin as a loading control, using Lumi-Imager T1 and Lumi Analyst software (Roche). Experiments were repeated at least three times. Data are expressed as means ± s.d.

Cytokine expression analysis by qRT-PCR

For qRT-PCR, HL60 cells were grown and subsequently differentiated into macrophages as described above. After differentiation, cells were incubated with the different recombinant proteins (25 μg/ml, if not indicated otherwise) for the indicated times. For the induction of pro-inflammatory cytokines, HL60 cells were stimulated with 1 μg/ml LPS from *E. coli* O111:B4 (Sigma) for 16 hours after pre-incubation with recombinant YopM (25 μg/ml) for 3 hours. After incubation, cells were washed with PBS, and total RNA was extracted using the Roche RNA extraction kit (Roche Molecular Biochemicals) and transcribed into cDNA. Changes in cytokine expression were measured by qRT-PCR employing the LightCycler amplification and detection system (Roche Molecular Biochemicals). Specific primer pairs for human cytokines were derived from the Universal ProbeLibrary (UPL, Roche Applied Science). The *HPRT1* gene (hypoxanthine phosphoribosyltransferase 1), a low-abundance housekeeping gene, was used as reference. Data were analyzed by application of the Roche quantification program software (version 3.5, Roche Molecular Biochemicals) and expressed as means ± s.d. Significance analysis was done by a two-sample unequal variance *t*-test with two-tailed distribution (post test). Significance was set as $P<0.05$.

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