YopM of *Yersinia enterocolitica* specifically interacts with α 1-antitrypsin without affecting the anti-protease activity

Gerhard Heusipp,[†] Katrin Spekker,[†] Sabine Brast, Stefan Falker and M. Alexander Schmidt

Correspondence

Gerhard Heusipp
heusipp@uni-muenster.de

Institut für Infektiologie, Zentrum für Molekularbiologie der Entzündung, Universitätsklinikum Münster, von-Esmarch-Str. 56, 48149 Münster, Germany

It was previously shown that α 1-antitrypsin (AAT) interacts with the type III secreted (T3S) EspB and EspD proteins of enteropathogenic *Escherichia coli* (EPEC), resulting in reduced functionality of the proteins. To determine if AAT is also able to interact with T3S proteins of other pathogens, the binding of AAT to Yop proteins of *Yersinia enterocolitica* was analysed. AAT did not interact with YopB or YopD, which have functions in type III translocation similar to EspB and EspD in EPEC, but specifically interacts with YopM, a member of the leucine-rich repeat (LRR) family of proteins, in overlay and pull-down assays. To determine regions of YopM involved in AAT binding, various N- and C-terminally truncated versions of YopM were recombinantly expressed, and their ability to interact with AAT analysed. All versions tested were able to bind AAT, indicating that at least eight LRR of YopM are sufficient for AAT interaction. The main physiological role of AAT is to inhibit neutrophil elastase; however, elastase was efficiently inhibited by AAT in the presence and absence of YopM, indicating that YopM does not interfere with the anti-protease inhibition activity of AAT, and that the domain of AAT interacting with YopM is not identical to AAT's protease interaction domain. Furthermore, it was shown that elastase efficiently degrades YopM and other Yop proteins. The data suggest that AAT has additional functions in the host response against bacterial infections that are not related to its anti-protease activity.

Received 18 November 2005

Revised 24 January 2006

Accepted 6 February 2006

INTRODUCTION

The genus *Yersinia* consists of three species pathogenic to humans, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*. Although they differ in their route of infection, all pathogenic *Yersinia* species show a tropism for lymphoid tissues. While *Y. pestis* is transmitted to the bloodstream of the host via a fleabite, *Y. pseudotuberculosis* and *Y. enterocolitica* are food-borne pathogens, and cross the intestinal epithelium at Peyer's patches. All *Yersinia* species are able to survive and replicate extracellularly in lymphoid tissues. This is made possible by a virulence plasmid-encoded type III secretion system (T3SS) that translocates effector proteins through the bacterial and host membranes directly into the host cell cytosol, where the effectors have anti-phagocytic and anti-inflammatory effects (Cornelis, 2002). T3SSs have been described in various Gram-negative human, animal and plant-pathogenic bacteria, and are

devoted to establishing and maintaining a successful infection (Hueck, 1998). The plasmid-encoded *Yersinia* Ysc T3SS has been exceptionally well characterized. The secreted proteins include the two translocators, YopB and YopD, that insert into the host membrane, and six effector Yop proteins (YopE, YopT, YopO/YpkA, YopM, YopP/YopJ, YopH) that interfere with host signalling cascades resulting in the inhibition of phagocytosis, down-regulation of the immune response, and induction of apoptosis (Cornelis, 2002). A biochemical function for each of the effector Yop proteins has been established, although thus far the role in pathogenesis has not been elucidated sufficiently for all of them (reviewed by Viboud & Bliska, 2005). The effectors YopE, YopT and YopO interact with Rho GTPases and thereby disrupt the actin cytoskeleton to prevent phagocytosis. YopH has a similar function, but acts by targeting signalling pathways activated by phagocytic mechanisms. The role of YopP during infection is to suppress cytokine production and induce apoptosis. YopP is a protease that inhibits MAPK and NF- κ B signalling by interfering with ubiquitination.

Although YopM has an important role in pathogenesis, its function has until now not been determined satisfactorily (Leung *et al.*, 1990; Mulder *et al.*, 1989). YopM belongs to a

[†]These authors contributed equally to this work.

Abbreviations: AAT, α 1-antitrypsin; EPEC, enteropathogenic *Escherichia coli*; LRR, leucine-rich repeat; Ni-NTA, nickel-nitrilotriacetate; SAAAPNA, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide; T3S, type III secreted; T3SS, type III secretion system.

family of proteins containing leucine-rich repeat (LRR) motifs that have been described as participating in protein–protein interactions (Kobe & Kajava, 2001). Determination of the crystal structure revealed that YopM is a horseshoe-shaped protein and that four YopM monomers interact to form a hollow cylinder (Evdokimov *et al.*, 2001). In earlier experiments it was shown that YopM of *Y. pestis* interacts with α -thrombin and inhibits platelet aggregation, but these properties are dispensable for virulence (Hines *et al.*, 2001; Leung *et al.*, 1990; Nemeth & Straley, 1997; Reisner & Straley, 1992). As for the other effector proteins, YopM is translocated into the cytoplasm of the host cell, but interestingly can also be found in the nucleus of infected cells (Skrzypek *et al.*, 1998). More recently, transcriptional analysis of mouse macrophages after infection with *Y. enterocolitica* indicated a putative role for YopM in the control of cell cycle and cell growth (Sauvonnet *et al.*, 2002), while in another study no effect of YopM on host cell transcription could be detected (Hoffmann *et al.*, 2004). Using a different approach, McDonald *et al.* (2003) identified the kinases PRK2 and RSK1 as intracellular targets of YopM, but again the biological significance of this interaction remains to be determined. The most recent data indicated that YopM targets the innate immune system, as it was required for depletion of NK cells after intravenous infection of mice with *Y. pestis* (Kerschen *et al.*, 2004). Interestingly, in contrast to all other Yop proteins, an enzymic activity could not be identified for YopM (Viboud & Bliska, 2005).

In a recent study we identified the acute-phase protein α 1-antitrypsin (AAT) as a binding partner for the type III secreted (T3S) EspB and EspD proteins of enteropathogenic *Escherichia coli* (EPEC). This interaction resulted in reduced virulence of EPEC as analysed by EspB/EspD-dependent haemolysis of red blood cells and pedestal formation on infected HeLa cells (Knappstein *et al.*, 2004). AAT is the most abundant circulating serine protease inhibitor in human serum. Its main role is to protect the upper respiratory tract from destruction by neutrophil elastase. It is synthesized primarily in the liver, but also is expressed in, and secreted by, extra-hepatic tissues, intestinal enterocytes, macrophages and monocytes (Molmenti *et al.*, 1993; Perlmutter *et al.*, 1985). To analyse if AAT is able to interact with T3S effector proteins of other human pathogens besides EPEC, we analysed the interaction of AAT with Yop proteins of *Y. enterocolitica*. We show that AAT binds specifically to YopM in overlay and pull-down assays, and that the AAT–YopM interaction does not interfere with AAT's activity to inhibit elastase. It can be inferred from our data that there is a novel role for AAT in host defence that is not related to its anti-protease activity.

METHODS

Bacterial strains, tissue culture cell lines and culture conditions. We used the *Y. enterocolitica* O8 strain JB580v as the wild-type strain (Kinder *et al.*, 1993). Unless otherwise indicated, all strains were grown in Luria–Bertani (LB) broth or on agar plates, at 26 °C for *Y. enterocolitica* or 37 °C for *E. coli*. The following antibiotics

were used at the final concentrations indicated: for *Y. enterocolitica*, nalidixic acid (20 $\mu\text{g ml}^{-1}$), chloramphenicol (12.5 $\mu\text{g ml}^{-1}$); for *E. coli*, kanamycin (50 $\mu\text{g ml}^{-1}$), chloramphenicol (25 $\mu\text{g ml}^{-1}$). For the induction of type III secretion, an overnight culture of *Y. enterocolitica* grown in brain–heart infusion (BHI) medium was diluted to an OD₆₀₀ 0.1 in fresh BHI medium supplemented with 20 mM MgCl₂ and 20 mM sodium oxalate (BHI_{MOX}) to deplete Ca²⁺ from the medium, grown at 26 °C for 2 h and then shifted to 37 °C for an additional 4 h. Yop proteins were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 10% (v/v) to bacteria-free supernatants, washed with acetone and analysed by SDS-PAGE.

HeLa cells (ATCC CCL 2) were routinely grown at 37 °C in a 10% CO₂ atmosphere in DMEM supplemented with 10% (v/v) fetal calf serum, 1 mM glutamine, penicillin (100 U ml⁻¹) and streptomycin (100 $\mu\text{g ml}^{-1}$). J774.A1 cells (ATCC TIB-67) were routinely grown at 37 °C in a 10% CO₂ atmosphere in RPMI medium supplemented with 10% (v/v) fetal calf serum, 1 mM glutamine, penicillin (100 U ml⁻¹) and streptomycin (100 $\mu\text{g ml}^{-1}$).

Construction of plasmids and protein purification. The primers used for the amplification of *yopM* fragments by PCR are listed in Table 1. For the recombinant expression of YopM with a C-terminal 6 × His-tag, the *yopM* coding sequence was amplified by PCR using the primer pair KS-yopM1/KS-yopM2 and *Pfu* polymerase, and ligated into *NheI/XhoI*-digested pET24b(+) (Novagen) resulting in plasmid pET-yopM. N- or C-terminally truncated versions of YopM were similarly constructed using primer pairs KS-yopM5/KS-yopM2 (pET-yopM_{87-C}), KS-yopM7/KS-yopM2 (pET-yopM_{172-C}), KS-yopM1/KS-yopM6 (pET-yopM_{N-239}) and KS-yopM1/KS-yopM8 (pET-yopM_{N-286}) (Fig. 3). The plasmids were transferred to *E. coli* BL21(DE3) (Novagen) by electroporation to allow the induction of expression of the recombinant proteins. *E. coli* BL21(DE3) cells carrying the respective expression plasmids were grown to OD₆₀₀ 0.6. After addition of 1 mM IPTG to induce YopM expression, the cultures were incubated for an additional 3 h at 37 °C. Subsequently, bacteria were harvested by centrifugation. His-tagged proteins were purified by Ni-nitrilotriacetate (Ni-NTA)-agarose affinity purification under non-denaturing conditions as recommended by the supplier (Qiagen). Proteins were eluted in buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, 250 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100 and analysed by SDS-PAGE. Protein concentrations were assessed by the Bradford assay (Bradford, 1976).

Construction of a *Y. enterocolitica yopM* mutant strain. For the construction of the *Y. enterocolitica yopM* mutant strain GHY189 (*yopM::pEP-yopM*), an internal fragment of the *yopM* gene was amplified by PCR using primers KS-yopM3 and KS-yopM4 (Table 1), digested with *Clal/XbaI*, and ligated into the *Clal/XbaI*-digested suicide plasmid pEP185.2 (Kinder *et al.*, 1993) resulting in plasmid pEP-yopM. This plasmid was transferred to *Y. enterocolitica* JB580v by conjugation using *E. coli* S17-1 λ pir (Miller & Mekalanos, 1988) as donor. The resulting *Y. enterocolitica* strains resistant to nalidixic acid and chloramphenicol were analysed for proper insertion of the suicide plasmid by Southern blotting, and for the loss of YopM in supernatants after growth under conditions inducing type III secretion of Yop proteins.

Overlay assay, pull-down assay and preparation of cell lysates. To investigate the interaction of Yop proteins with AAT, supernatants of *Y. enterocolitica* grown at 37 °C in BHI_{MOX} were TCA precipitated, separated by SDS-PAGE and transferred to nitrocellulose membrane by Western blotting. The membrane was incubated with AAT and subsequently probed with AAT-specific antiserum as previously described (Knappstein *et al.*, 2004). Pull-down assays were performed as previously described (Knappstein *et al.*, 2004) with the following exception. Recombinant YopM proteins were

Table 1. Primers

Primer	Sequence (5'→3') (restriction site is underlined)	Recognition site
KS-yopM1	CTAGCTAGCTTTATAACTCCAAGAAATGTATCT	<i>NheI</i>
KS-yopM2	CCGCTCGAGCTCAAATACATCATCTTCAAGTTT	<i>XhoI</i>
KS-yopM3	CCATCGATCGACAAGCCCATGAGCTAGAA	<i>Clal</i>
KS-yopM4	GCTCTAGATCTTATTCCATTGCTGGATGC	<i>XbaI</i>
KS-yopM5	CTAGCTAGCCCGGAATTACCTCCGCATTTAGAG	<i>NheI</i>
KS-yopM6	CCGCTCGAGGAAGGTTAACTCTGCGGTAATTC	<i>XhoI</i>
KS-yopM7	CTAGCTAGCCCTTCACTGGAATTTCTTGCTGCT	<i>NheI</i>
KS-yopM8	CCGCTCGAGCTGATTATCTCTGACATCAAGTTC	<i>XhoI</i>

eluted from the Ni-NTA agarose column to determine the amount of protein. After dialysis against Tris-buffered saline, approximately 3 µg recombinant YopM was incubated with Ni-NTA agarose and subsequently incubated with purified AAT (Sigma) or cell lysates as previously described (Knappstein *et al.*, 2004). Eluted proteins were analysed by SDS-PAGE and immunoblotting using AAT- and YopM-specific antibodies.

Elastase activity assay. The activity of elastase (from human leukocytes; Sigma) was analysed with the chromogenic substrate *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (SAAAPNA; Sigma). Briefly, 1 mM SAAAPNA was incubated with 0.008 U elastase in buffer containing 50 mM Tris pH 8.0, 100 mM NaCl, 0.5% (v/v) Triton X-100, for various time points at 37 °C in a microtitre plate. Substrate conversion was measured in a microplate reader as the increase in absorbance at a wavelength of 405 nm. Inhibition of elastase was achieved by the addition of 10 µM AAT (final concentration). To analyse the effect of YopM on AAT's ability to inhibit elastase, the elastase activity assay was performed in the presence or absence of various concentrations of recombinant YopM. To analyse the effect of elastase on YopM, recombinant YopM or the supernatant of *Y. enterocolitica* grown at 37 °C in BHI_{MOX} was incubated with elastase (0.008 U) in the presence or absence of inhibitory concentrations of AAT (10 µM) at 37 °C for 2 h. Samples were mixed with SDS-PAGE sample buffer, and analysed by Western blotting and YopM-specific antibodies, or Coomassie staining of SDS-polyacrylamide gels.

RESULTS

AAT interacts with YopM in an overlay assay

In a previous study we identified AAT as a binding partner for the T3S translocator proteins EspB and EspD of EPEC. As the translocators have a central conserved role in T3SSs of forming a pore in the host cell membrane through which effector proteins are translocated into the host cytosol, we hypothesized that AAT might also be able to interact with translocator proteins from other pathogens employing T3SSs. Therefore, we analysed supernatants of *Y. enterocolitica* grown under conditions inducing the secretion of Yop proteins (37 °C, BHI_{MOX}) in an overlay assay with AAT. As shown in Fig. 1, a specific interaction occurred with a protein with an approximately 50 kDa molecular mass. After comparison with the molecular masses of all T3S proteins of *Y. enterocolitica* we assumed that the protein interacting with AAT might be YopM. This was confirmed by using Yop supernatant from a *yopM* mutant strain (GHY189) and recombinant YopM with a C-terminal

His-tag in an overlay assay. These experiments clearly showed an interaction of AAT with recombinant YopM and YopM from supernatants of wild-type *Y. enterocolitica*, while no interaction of AAT with proteins from the *yopM* mutant strain could be detected (Fig. 1). These data show that AAT specifically interacts with YopM of *Y. enterocolitica*, while it does not interact with the translocation pore-forming proteins YopB and YopD as might be expected from data obtained with EPEC.

AAT interacts with YopM in a pull-down assay

To analyse if AAT is also able to bind YopM in solution, we performed pull-down assays with recombinant YopM and purified AAT. His-tagged YopM was bound to Ni-NTA agarose matrix, incubated with AAT, washed, eluted from the matrix and analysed by SDS-PAGE and immunoblotting using AAT-specific antibodies. Fig. 2 shows that YopM is able to bind to AAT in solution; binding of AAT to the matrix in the absence of YopM was not detected. To further show that the interaction of YopM with AAT is specific, we performed pull-down assays with recombinant YopM and cell lysates from HeLa cells and J774.A1 macrophage-like

Fig. 1. AAT specifically interacts with YopM in an overlay assay. Supernatants of *Y. enterocolitica* JB580v (lane 1) or of *yopM* mutant strain GHY189 (lane 2) grown at 37 °C in BHI_{MOX} to induce Yop protein secretion were separated by SDS-PAGE, transferred to nitrocellulose and incubated with AAT. As a control, recombinant His-tagged YopM was included (lane 3). Binding of AAT to YopM was detected with anti-AAT antiserum. Molecular masses of standard proteins are indicated in kDa (lane M).

Fig. 2. AAT specifically interacts with YopM in a pull-down assay. Purified recombinant YopM bound to Ni-NTA agarose via a C-terminal His-tag was incubated with purified AAT (a, lane 1), lysate of HeLa (b, lane 1) or J774.A1 cells (b, lane 2). Binding of AAT to the Ni-NTA matrix was not detected in the absence of YopM (a, lane 2). After elution from the matrix, proteins were separated by SDS-PAGE and transferred to nitrocellulose. Binding of AAT to YopM was detected using AAT-specific antiserum. Molecular masses of standard proteins are indicated in kDa (lane M).

cells. *Y. enterocolitica* interacts with both cell types during an infection, and Yop proteins specifically target macrophages in the host. As shown in Fig. 2, AAT can be detected after a

pull-down assay with YopM and cellular lysates, indicating that YopM interacts not only with purified AAT, but also with AAT from HeLa and J774.A1 cell lysates in a pull-down assay.

Analysis of AAT interaction with N- and C-terminally truncated versions of YopM

The main function of AAT is to interact with serine proteases and to inhibit their activity. However, in this and a previous study (Knappstein *et al.*, 2004) we could show that AAT also interacts with non-proteolytic bacterial T3S proteins. We were, therefore, interested in determining if a specific binding domain exists in YopM that mediates interaction with AAT. The YopM protein is heterogeneous between different *Yersinia* strains, and variants differing in the amount of LRR have been reported (Boland *et al.*, 1998). The YopM protein of *Y. enterocolitica* O8 strain JB580v used in our study contains 13 LRR. An additional characteristic structural feature of YopM is its N-terminal tandem pair of α -helices (Evdokimov *et al.*, 2001). Different recombinant YopM versions were constructed, which were truncated at the N- or the C-terminus and expressed in *E. coli* with a C-terminal His-tag. After purification of the proteins by affinity chromatography, we analysed their interaction with AAT in overlay and pull-down experiments (Figs 3 and 4). The recombinant YopM_{87-C} protein is missing the N-terminal α -helices and the first half of LRR1. As this protein is still able to bind AAT, we conclude that the α -helices are not involved in AAT interaction. Further N-terminal deletion up to LRR6 (YopM_{172-C}) did not abolish AAT binding, although nearly half of the protein is deleted,

Fig. 3. Schematic overview of the different N- and C-terminally truncated YopM protein versions. The locations of the primers used to construct the corresponding expression plasmids are indicated. Grey rectangles represent LRRs. The amino acids represented in the truncated YopM versions are indicated corresponding to full-length YopM (367 aa). As a result of the repeat structure of *yopM*, primer KS-yopM7 is able to anneal to two regions in *yopM* during PCR, but only the PCR product resulting from binding in the region encoding LRR5 was cloned and expressed.

Fig. 4. AAT binds to N- and C-terminally truncated versions of YopM in overlay and pull-down assays. The different N- and C-terminally truncated YopM versions were affinity purified, separated by SDS-PAGE, and either Coomassie stained to document the amount of each protein (a) or transferred to nitrocellulose and incubated with AAT (b). Asterisks indicate the location of YopM_{87-C} and YopM_{172-C}. Binding of AAT to the YopM versions was detected with anti-AAT antiserum (b). For the pull-down assay, full-length YopM and truncated versions were bound to Ni-NTA agarose and incubated with AAT. After elution from the affinity matrix, proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Ni-NTA agarose without bound YopM was used as a control for unspecific binding of AAT to the matrix (no protein). AAT was detected using AAT-specific antibody (c). Molecular masses of standard proteins are indicated in kDa (lane M).

indicating that the N-terminus of YopM is not necessary for AAT binding. Similar results were obtained with the C-terminal deletions YopM_{N-239} and YopM_{N-286}, showing that, in addition, no specific C-terminal motif necessary for

AAT interaction exists. All YopM variants tested were able to bind AAT in overlay as well as in pull-down assays (Fig. 4b, c). The weak signals from YopM_{87-C} and YopM_{172-C} in the overlay assay (Fig. 4a, b) are not due to inefficient AAT binding, but to the instability of these variants, resulting in only small amounts of protein in the assay. Attempts to further concentrate both proteins were not successful. However, YopM_{87-C} as well as YopM_{172-C} bound AAT with efficiencies comparable to the other YopM versions in pull-down experiments (Fig. 4c). The shortest YopM variants used in our study still contained eight LRRs (YopM_{N-239} and YopM_{172-C}), indicating that the LRRs are involved in AAT–YopM interaction and that eight LRRs from the N- or the C-terminus of YopM are sufficient for interaction with AAT. Our results might indicate either that AAT is able to interact with any LRR irrespective of its location in YopM, or that LRRs 6–8, which are included in all the truncated versions, constitute a binding motif.

AAT interaction with YopM does not interfere with protease inhibition

The main physiological role of AAT is to inhibit elastase in the lung of humans (Crystal, 1990). However, as AAT is expressed in various human tissues, and cells like enterocytes, monocytes and macrophages, its protease inhibition function is not restricted to the lung epithelium (Perlmutter *et al.*, 1985, 1989). As *Y. enterocolitica* survives and replicates in lymphatic tissues after infection, it interacts with macrophages and neutrophils that express various proteases. It has been shown that neutrophil elastase specifically cleaves virulence factors of *Shigella*, *Salmonella* and *Yersinia* (Weinrauch *et al.*, 2002). The specific inhibition of proteolytic enzymes by anti-proteases such as AAT is an important mechanism for regulating proteolysis. However, studies suggest that protease inhibitors can be inactivated by different mechanisms involving cellular as well as bacterial factors, leading to microenvironments in which proteolysis is facilitated even in the presence of inhibitors (Owen & Campbell, 1999). Therefore, we hypothesized a role for a YopM–AAT interaction during infection might be the obstruction of AAT's inhibition activity against proteases like elastase, resulting in, for example, tissue destruction or interference with host signalling. We developed an assay employing human leukocyte elastase and the synthetic substrate SAAAPNA to characterize the effect of YopM on AAT's ability to inhibit elastase. The results are shown in Fig. 5. Conversion of the substrate by elastase was detected as an increase in absorbance at a wavelength of 405 nm. AAT (10 μ M final concentration) efficiently inhibited elastase activity in this assay. When different concentrations of YopM were added to the assay, this did not interfere with elastase inhibition by AAT. However, addition of YopM alone resulted in a reduced conversion of the SAAAPNA substrate. This could be the result of elastase inhibition by YopM; alternatively, YopM could be a competitor for the substrate. From these data we conclude that YopM does not interfere with AAT's ability to inhibit elastase.

Fig. 5. YopM does not interfere with AAT's ability to inhibit elastase. Elastase was incubated with the chromogenic substrate SAAAPNA in the presence (+) or absence (-) of 10 μ M AAT, and substrate conversion was detected as increase in absorbance at 405 nm. In addition, various amounts of YopM (black bars, no YopM; white bars, 28 μ M YopM; grey bars, 14 μ M; hatched bars, 7 μ M YopM) were added to analyse the effect on substrate conversion. AAT efficiently inhibits elastase activity irrespective of the presence or absence of YopM in the assay. Addition of YopM in the absence of AAT also resulted in a decreased substrate conversion. Means and SDs of a representative experiment performed in duplicate are shown.

Elastase efficiently degrades YopM and other Yop proteins

As it has been shown that neutrophil elastase is able to degrade T3S virulence factors of *Shigella*, *Salmonella* and *Yersinia* (Weinrauch *et al.*, 2002), and as we observed a decreased conversion of a synthetic substrate by elastase in the presence of YopM, we investigated whether YopM itself

might be degraded by elastase. When recombinant YopM or T3S supernatant of *Y. enterocolitica* was incubated with elastase, YopM was efficiently degraded. This degradation was inhibited by the addition of 10 μ M AAT (Fig. 6). However, in the presence of other Yop proteins, YopM is degraded less efficiently than recombinant YopM alone. Residual amounts of YopM can still be detected after 2 h incubation with elastase, while a higher amount of recombinant YopM is completely degraded in the same assay. Furthermore, not only YopM, but also other Yop proteins from supernatants were degraded, implicating substrate competition as a possible explanation for the less efficient degradation of YopM from supernatants. The results are in accordance with other observations indicating a role for neutrophil elastase in the specific degradation of bacterial virulence factors (Weinrauch *et al.*, 2002).

Fig. 6. Elastase efficiently degrades Yop proteins. After incubation of recombinant YopM or Yop proteins from supernatants with or without 0.008 U elastase for 2 h at 37 °C, proteins were separated by SDS-PAGE and transferred to nitrocellulose. YopM was detected using specific antibodies (a). Alternatively, proteins were Coomassie stained after SDS-PAGE (b). Recombinant His-tagged YopM (lanes 1–3) as well as YopM from bacterial supernatants (lanes 4–6) is efficiently degraded by elastase in the absence, but not in the presence, of AAT. Lanes 1 and 4, YopM and Yop protein supernatant, respectively, without elastase; lanes 2 and 5, YopM and Yop protein supernatant, respectively, incubated with elastase; lanes 3 and 6, YopM or Yop protein supernatant, respectively, incubated with elastase and 10 μ M AAT. Molecular masses of standard proteins are indicated in kDa (lane M).

DISCUSSION

The acute phase protein AAT is a well-characterized anti-protease that inhibits the proteolytic activity of serine proteases. Its main target in humans is neutrophil elastase in the lung, and AAT deficiency may result in emphysema due to tissue destruction by elastase (Crystal, 1990). In a recent study we could show that AAT has an additional function presumably not related to its anti-protease activity: binding to the EPEC proteins EspB and EspD, which reduced the virulence of the bacteria by interfering with type III secretion (Knappstein *et al.*, 2004). As T3SSs are common in bacterial pathogens, we analysed if AAT might be able to interact with T3S proteins of other pathogens. We were able to identify an interaction between YopM of *Y. enterocolitica* and AAT in overlay assays, as well as in pull-down assays. This interaction is probably mediated by the LRR structure of YopM, but does not interfere with AAT's role as an anti-protease, implying that different domains of AAT are involved in interacting with proteases and with YopM.

When we first analysed the interaction of AAT with Yop proteins in overlay assays, we were surprised that we did not detect binding to YopB or YopD. As for EspB and EspD in EPEC, these Yop proteins are involved in the formation of a pore in the eukaryotic host membrane, through which T3S effector proteins are translocated into the host cytosol (Ide *et al.*, 2001; Neyt & Cornelis, 1999). In contrast to our previous results showing interaction of AAT with the EspB and EspD translocator proteins, AAT interacted with the effector protein YopM, indicating that AAT does not recognize a general motif that might be present on translocator proteins of T3SSs. The specificity of the binding of AAT to YopM was confirmed by various complementary approaches. First, AAT did not interact with proteins from supernatants of a *yopM* mutant strain; second, AAT bound to recombinantly expressed and affinity-purified YopM; and third, YopM bound to purified AAT as well as AAT from HeLa and J774.A1 cell lysates in pull-down assays. It was especially important to show binding of YopM to AAT from cellular lysates, as this experiment showed the specificity of the interaction most convincingly. McDonald *et al.* (2003) used a similar approach to identify host proteins binding to YopM. The two protein kinases PRK2 and RSK1 co-immunoprecipitated with, and were activated by, YopM (McDonald *et al.*, 2003). However, the biological function of the interaction remains unknown. In different studies it was shown that YopM of *Y. pestis*, which contains two more LRR compared to YopM of *Y. enterocolitica* O8 used in our study, is able to bind the protease α -thrombin (Leung *et al.*, 1990; Reisner & Straley, 1992). This suggests that YopM is a versatile protein that is able to interact with various host proteins, presumably via its LRR. Depending on the bound substrate, YopM monomers might interact with each other to assemble distinct protein regions into an overall structure capable of binding a specific ligand. This is supported by the crystal structure of the *Y. pestis* YopM protein, where four YopM monomers interact to form a hollow cylinder (Evdokimov *et al.*, 2001). Furthermore, the LRR structure

allows the presentation of variable loop regions and might thereby facilitate various protein–protein interactions.

To analyse which regions of YopM were important for AAT binding, we constructed various N- and C-terminally truncated versions of YopM. Our analysis revealed that neither the N- nor the C-terminus is necessary for AAT binding. Even when approximately half of the protein from the N- or the C-terminus is deleted, YopM is still able to bind AAT. This indicates that either the LRR itself, or a motif presented by LRR6–8, is recognized by AAT. Future analysis will be aimed at defining the binding region of YopM, but also of EspB and EspD from EPEC, more closely, to shed more light on the interaction of AAT with virulence proteins. These investigations should, of course, also include analysis of the mechanism by which AAT is able to interact with non-protease proteins, as this interesting new aspect of AAT interaction with pathogen proteins has not been studied until now.

The main questions that remain to be answered are, first, where does the YopM–AAT interaction take place (intracellularly or extracellularly), and second, what is the biological significance of the YopM–AAT interaction in the context of a natural infection? AAT is mainly a secreted protein that is very abundant in human serum, but it is also secreted from macrophages, monocytes and enterocytes (Molmenti *et al.*, 1993; Perlmutter *et al.*, 1985), cells with which *Y. enterocolitica* interacts during infection. YopM is translocated by *Y. enterocolitica* directly into the host cytosol. This might indicate that both proteins interact either intracellularly, before AAT is secreted, or extracellularly, assuming some YopM protein might be secreted. It has been discussed that YopM of *Y. pestis* might have a role in pathogenesis after secretion in addition to a role after translocation, as it interacts with α -thrombin, but this hypothesis could not be proven experimentally (Hines *et al.*, 2001). In addition, there is experimental evidence suggesting that YopM and possibly other Yop effectors might not only be translocated, but also be secreted during an infection. For example, Cheng & Schneewind (2000) quantified the amount of apolarly secreted YopM during an infection of HeLa cells to be approximately 7 %. A different study shows that there is a strong humoral immune response of mice infected with *Y. pestis* to YopM, but not to, for example, YopE, implying that YopM might have an extracellular function *in vivo* (Benner *et al.*, 1999). It is, therefore, quite possible that YopM has intracellular and extracellular functions during an infection, and that the extracellular function is related to YopM's interaction with AAT. While we favour a model where YopM interacts with AAT extracellularly, YopM might also interact with intracellular AAT to prevent or at least reduce its secretion. In response to inflammatory mediators like interleukin 6 or endotoxin, the amount of secreted AAT is increased (Kalsheker *et al.*, 2002), and YopM might interfere with this process. However, although YopM interacts with AAT from cellular lysates, we were not able to detect intracellular interaction by co-immunofluorescence analysis, but this might also be due to the relatively low amounts of both

proteins in the cell after infection, and to the relatively low specificity of the antisera used in the experiment. The interaction of YopM with PRK2 and RSK1 was, for example, identified after transfecting YopM expression constructs into eukaryotic cells, thereby experimentally increasing the amount of intracellular YopM (McDonald *et al.*, 2003).

With the data currently at hand, whether the AAT–YopM interaction is beneficial for the host or for the pathogen cannot be determined. In one scenario, the interaction might provide an advantage for the pathogen. This is not linked to AAT's anti-protease activity, as elastase was still efficiently inhibited by AAT in the presence of YopM. Furthermore, binding to AAT did not protect YopM against proteolytic degradation. This could imply that YopM might target a different function of AAT, as it is becoming evident that AAT has a much broader activity than previously anticipated that is not related to its antiprotease activity. This includes a role of AAT as a signalling molecule for the expression of pro-inflammatory cytokines (Aldonite *et al.*, 2004; Brantly, 2002; Hiemstra, 2002). Furthermore, various studies indicate that YopM targets the innate immune response of the host (Kerschen *et al.*, 2004; Marketon *et al.*, 2005). As AAT is an acute-phase protein, interaction of YopM with AAT is well in agreement with the idea that this interaction might interfere with the host's unspecific immune response. The best-characterized effect of YopM is the depletion of NK cells after systemic infection with *Y. pestis*, which is not detectable after infection with a *yopM* mutant strain (Kerschen *et al.*, 2004). How YopM acts at the molecular level in this process remains elusive. Interestingly, AAT is able to inhibit NK cell activity (Laine *et al.*, 1990; Okumura *et al.*, 1985), implying a putative link between YopM–AAT interaction and NK cell depletion.

In an alternative scenario, the AAT–YopM interaction is beneficial for the host, comparable to the effect of AAT on EPEC virulence (Knappstein *et al.*, 2004). However, as it is currently not known how YopM contributes to virulence at the molecular level, it cannot be assessed how AAT could interfere with YopM's function in virulence. Although our data provide new insights into AAT's and YopM's function, the role of YopM during infection remains enigmatic, and future experiments have to be aimed at determining the biological role of the molecular interaction between AAT and YopM during an infection.

ACKNOWLEDGEMENTS

We thank Erwin Bohn for the gift of YopM antiserum and Silke Michgehl for help with experiments. This work was supported by Innovative Medical Research grant (IMF: HE110401) of the Medical School of the University of Münster and in part by grants of the Deutsche Forschungsgemeinschaft (DFG SFB293/B5, SCHM770/10).

REFERENCES

- Aldonite, R., Jansson, L. & Janciauskiene, S. (2004). Concentration-dependent effects of native and polymerised α 1-antitrypsin on primary human monocytes, in vitro. *BMC Cell Biol* 5, 11.
- Benner, G. E., Andrews, G. P., Byrne, W. R., Strachan, S. D., Sample, A. K., Heath, D. G. & Friedlander, A. M. (1999). Immune response to *Yersinia* outer proteins and other *Yersinia pestis* antigens after experimental plague infection in mice. *Infect Immun* 67, 1922–1928.
- Boland, A., Havaux, S. & Cornelis, G. R. (1998). Heterogeneity of the *Yersinia* YopM protein. *Microb Pathog* 25, 343–348.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.
- Brantly, M. (2002). α 1-Antitrypsin: not just an antiprotease. Extending the half-life of a natural anti-inflammatory molecule by conjugation with polyethylene glycol. *Am J Respir Cell Mol Biol* 27, 652–654.
- Cheng, L. W. & Schneewind, O. (2000). *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J Bacteriol* 182, 3183–3190.
- Cornelis, G. R. (2002). The *Yersinia* Ysc-Yop 'type III' weaponry. *Nat Rev Mol Cell Biol* 3, 742–752.
- Crystal, R. G. (1990). α 1-Antitrypsin deficiency, emphysema, and liver disease. Genetic basis and strategies for therapy. *J Clin Invest* 85, 1343–1352.
- Evdokimov, A. G., Anderson, D. E., Routzahn, K. M. & Waugh, D. S. (2001). Unusual molecular architecture of the *Yersinia pestis* cytotoxin YopM: a leucine-rich repeat protein with the shortest repeating unit. *J Mol Biol* 312, 807–821.
- Hiemstra, P. S. (2002). Novel roles of protease inhibitors in infection and inflammation. *Biochem Soc Trans* 30, 116–120.
- Hines, J., Skrzypek, E., Kajava, A. V. & Straley, S. C. (2001). Structure-function analysis of *Yersinia pestis* YopM's interaction with alpha-thrombin to rule on its significance in systemic plague and to model YopM's mechanism of binding host proteins. *Microb Pathog* 30, 193–209.
- Hoffmann, R., van Erp, K., Trulzsch, K. & Heesemann, J. (2004). Transcriptional responses of murine macrophages to infection with *Yersinia enterocolitica*. *Cell Microbiol* 6, 377–390.
- Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62, 379–433.
- Ide, T., Laarmann, S., Greune, L., Schillers, H., Oberleithner, H. & Schmidt, M. A. (2001). Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. *Cell Microbiol* 3, 669–679.
- Kalsheker, N., Morley, S. & Morgan, K. (2002). Gene regulation of the serine proteinase inhibitors α 1-antitrypsin and α 1-antichymotrypsin. *Biochem Soc Trans* 30, 93–98.
- Kerschen, E. J., Cohen, D. A., Kaplan, A. M. & Straley, S. C. (2004). The plague virulence protein YopM targets the innate immune response by causing a global depletion of NK cells. *Infect Immun* 72, 4589–4602.
- Kinder, S. A., Badger, J. L., Bryant, G. O., Pepe, J. C. & Miller, V. L. (1993). Cloning of the *YenI* restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O:8 and construction of a transformable R^-M^+ mutant. *Gene* 136, 271–275.
- Knappstein, S., Ide, T., Schmidt, M. A. & Heusipp, G. (2004). α 1-Antitrypsin binds to and interferes with functionality of EspB from atypical and typical enteropathogenic *Escherichia coli* strains. *Infect Immun* 72, 4344–4350.
- Kobe, B. & Kajava, A. V. (2001). The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol* 11, 725–732.
- Laine, A., Leroy, A., Hachulla, E., Davril, M. & Dessaint, J. P. (1990). Comparison of the effects of purified human α 1-antichymotrypsin

and α_1 -proteinase inhibitor on NK cytotoxicity: only α_1 -proteinase inhibitor inhibits natural killing. *Clin Chim Acta* **190**, 163–173.

Leung, K. Y., Reisner, B. S. & Straley, S. C. (1990). YopM inhibits platelet aggregation and is necessary for virulence of *Yersinia pestis* in mice. *Infect Immun* **58**, 3262–3271.

Marketon, M. M., DePaolo, R. W., DeBord, K. L., Jabri, B. & Schneewind, O. (2005). Plague bacteria target immune cells during infection. *Science* **309**, 1739–1741.

McDonald, C., Vacratsis, P. O., Bliska, J. B. & Dixon, J. E. (2003). The *Yersinia* virulence factor YopM forms a novel protein complex with two cellular kinases. *J Biol Chem* **278**, 18514–18523.

Miller, V. L. & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**, 2575–2583.

Molmenti, E. P., Perlmutter, D. H. & Rubin, D. C. (1993). Cell-specific expression of α_1 -antitrypsin in human intestinal epithelium. *J Clin Invest* **92**, 2022–2034.

Mulder, B., Michiels, T., Simonet, M., Sory, M.-P. & Cornelis, G. (1989). Identification of additional virulence determinants on the pYV plasmid of *Yersinia enterocolitica* W227. *Infect Immun* **57**, 2534–2541.

Nemeth, J. & Straley, S. C. (1997). Effect of *Yersinia pestis* YopM on experimental plague. *Infect Immun* **65**, 924–930.

Neyt, C. & Cornelis, G. R. (1999). Insertion of a Yop translocation pore into the macrophage plasma membrane by *Yersinia enterocolitica*: requirement for translocators YopB and YopD, but not LcrG. *Mol Microbiol* **33**, 971–981.

Okumura, Y., Kudo, J., Ikuta, T., Kurokawa, S., Ishibashi, H. & Okubo, H. (1985). Influence of acute-phase proteins on the activity of natural killer cells. *Inflammation* **9**, 211–219.

Owen, C. A. & Campbell, E. J. (1999). The cell biology of leukocyte-mediated proteolysis. *J Leukoc Biol* **65**, 137–150.

Perlmutter, D. H., Cole, F. S., Kilbridge, P., Rossing, T. H. & Colten, H. R. (1985). Expression of the α_1 -proteinase inhibitor gene in human monocytes and macrophages. *Proc Natl Acad Sci U S A* **82**, 795–799.

Perlmutter, D. H., Daniels, J. D., Auerbach, H. S., De Schryver-Kecsckemeti, K., Winter, H. S. & Alpers, D. H. (1989). The α_1 -antitrypsin gene is expressed in a human intestinal epithelial cell line. *J Biol Chem* **264**, 9485–9490.

Reisner, B. S. & Straley, S. C. (1992). *Yersinia pestis* YopM: thrombin binding and overexpression. *Infect Immun* **60**, 5242–5252.

Sauvonnet, N., Pradet-Balade, B., Garcia-Sanz, J. A. & Cornelis, G. R. (2002). Regulation of mRNA expression in macrophages after *Yersinia enterocolitica* infection. Role of different Yop effectors. *J Biol Chem* **277**, 25133–25142.

Skrzypek, E., Cowan, C. & Straley, S. C. (1998). Targeting of the *Yersinia pestis* YopM protein into HeLa cells and intracellular trafficking to the nucleus. *Mol Microbiol* **30**, 1051–1065.

Viboud, G. I. & Bliska, J. B. (2005). *Yersinia* outer proteins: role in modulation of host cell signalling responses and pathogenesis. *Annu Rev Microbiol* **59**, 69–89.

Weinrauch, Y., Drujan, D., Shapiro, S. D., Weiss, J. & Zychlinsky, A. (2002). Neutrophil elastase targets virulence factors of enterobacteria. *Nature* **417**, 91–94.