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The Proteasome Pathway Destabilizes Yersinia Outer Protein E and Represses Its Antihost Cell Activities

Klaus Ruckdeschel, Gudrun Pfaffinger,* Konrad Trülsch,* Gerhardt Zenner,† Kathleen Richter,* Jürgen Heesemann,* and Martin Aepfelbacher†

Pathogenic Yersinia spp. neutralize host defense mechanisms by engaging a type III protein secretion system that translocates several Yersinia outer proteins (Yops) into the host cell. Although the modulation of the cellular responses by individual Yops has been intensively studied, little is known about the fate of the translocated Yops inside the cell. In this study, we investigated involvement of the proteasome, the major nonlysosomal proteolytic system in eukaryotic cells, in Yop destabilization and repression. Our data show that inactivation of the proteasome in Yersinia enterocolitica-infected cells selectively stabilized the level of YopE, but not of YopH or YopP. In addition, YopE was found to be modified by ubiquitination. This suggests that the cytotoxin YopE is physiologically subjected to degradation via the ubiquitin-proteasome pathway inside the host cell. Importantly, the increased levels of YopE upon proteasome inhibition were associated with decreased activity of its cellular target Rac. Thus, the GTPase-down-regulating function of YopE is enhanced when the proteasome is inhibited. The stabilization of YopE by proteasome inhibitor treatment furthermore led to aggregation of the cytotoxic YopE effects on the actin cytoskeleton and on host cell morphology. Together, these data show that the host cell proteasome functions to destabilize and inactivate the Yersinia effector protein YopE. This implies the proteasome as integral part of the cellular host immune response against the immunomodulatory activities of a translocated bacterial virulence protein. The Journal of Immunology, 2006, 176: 6093–6102.

pathogenic Yersinia spp. can cause a broad panel of diseases in humans and rodents. Yersinia pestis is the causative agent of bubonic plague, whereas the enteropathogenic species Yersinia enterocolitica and Yersinia pseudotuberculosis provoke gastrointestinal syndromes, lymphadenitis, and septicemia. Although the Yersinia spp. follow different routes of infection, they share the common ability to counteract host defense mechanisms (1). This enables extracellular survival and multiplication of the bacteria in the host lymphoid tissue. For this purpose, yersiniae engage a common, plasmid-encoded type III protein secretion system (TTSS)† as core virulence determinant (1). The Yersinia TTSS is activated upon host cell contact and specifically mediates the polarized translocation of Yersinia effector proteins (Yersinia outer proteins (Yops)) inside eukaryotic cells. At least six effector Yops are known to be injected (YopE, YopH, YopM, YopT, YopO/YopK, YopP/YopJ). In the host cell, the Yops act on several cellular levels to neutralize a sequence of programmed immune effector functions (1). By interference with the actin cytoskeleton dynamics, yersiniae block their phagocytosis and prevent their killing by phagocytic cells. These effects are largely mediated by YopE, YopH, YopO/YopK, and YopT (2). Interestingly, three of these Yops act on members of the Rho-GTPase family, which critically control rearrangements of the actin cytoskeleton (3, 4). YopE is a GTPase-activating protein (GAP), which inactivates Rho-GTPases by increasing their intrinsic GTPase activity (5, 6). This switches the GTPTases from an active, GDP-bound state to the inactive, GDP-bound state. The preferred target of the YopE GAP activity in cells appears to be the Rac GTPase, although in vitro YopE can operate on Rho and Cdc42 as well (5–7). YopT acts as a cysteine protease that inactivates Rho-GTPase members by cleaving their C-terminal geranylgeranyl isoprenoid moieties (8, 9). The serine/threonine kinase YopO/YopK binds to actin, which promotes autophosphorylation (10). YopO/YopK furthermore interacts with RhoA and Rac, which may lead to inactivation of these molecules (11, 12). YopH is a tyrosine phosphatase that exerts its effects by dephosphorylating host cell proteins, such as p130Cas and the focal adhesion kinase (13, 14). This activity dismantles peripheral focal adhesion complexes of the host cell. Besides these immediate effects on the actin cytoskeleton, Yersinia blocks proinflammatory responses of the infected cell. YopP of Y. enterocolitica and its homologue YopJ of Y. pseudotuberculosis and Y. pestis disrupt the MAPK and NF-κB signaling pathways by targeting members of the MAPK kinase superfamily and the NF-κB-activating IκB kinase-β (reviewed in Ref. 15). Inactivation of these signaling pathways prevents the production of proinflammatory cytokines, such as TNF-α and IL-8. In addition, YopP/YopJ triggers apoptosis in macrophages, a process that involves inhibition of the antiapoptotic NF-κB pathway. Lastly, YopM forms a complex with the kinases protein kinase C-like 2 and ribosomal S6 protein kinase 1 (16). YopM is able to activate these molecules, but the precise function of YopM in the host cell is still unknown. Although a broad panel of modular functions on host cell defense mechanisms has been revealed for the individual Yops, little is known about the fate of the translocated Yops inside the cell.
CONTROL OF YopE ACTION BY HOST CELL PROTEASOME

Yersinia strains, cell lines, and infection conditions

The Y. enterocolitica strains used in this study were the serotype O8 wild-type strain WA-314 and its virulence plasmid-cured derivative WA-C (30). Furthermore, we used two Yersinia strains that produced only a restricted pattern of effector Yops. Our data show that the proteasome activity destabilizes YopE, but does not apparently affect the cellular levels of YopH and YopP. These results give rise to the assumption that translocated YopE is targeted to proteasomal degradation in the host cell. The destabilization of YopE aids to terminate the YopE GAP activity, which concomitantly counteracts YopE-mediated cytoskeletal alterations. Together, these results indicate that the ubiquitin-proteasome pathway can play a direct role in nullifying the activities of a bacterial effector protein. This may finally help the organism to endure immunomodulation by Yersinia.

Materials and Methods

Western blot (WB), immunoprecipitation (IP), and cell transfection

For assessment of the cellular Yop levels, infected cells were solubilized at the time points indicated with a buffer containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, and phosphatase and protease inhibitors (Roche). The lysates were cleared by centrifugation, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with polyclonal Abs directed against YopE, YopH, or YopP (19, 33). Immunoreactive bands were visualized using appropriate secondary Abs and ECL detection reagents (Amersham Biosciences). To determine the cellular Yop levels in time course studies, the infected cells were treated with 1% digitonin (Sigma-Aldrich), a nonionic detergent that preferentially disrupts cholesterol-containing membranes. This ensures preferable solubilization of the eukaryotic plasma membrane, but not of bacteria (34), which helps to limit Yop release from cell-associated yersiniae. To overexpress ubiquitin for the YopE and ubiquitin IP experiments, HEK293 cells were seeded in six-well cell plate and transfected with a human cDNA construct encoding an octameric tandem fusion of hemagglutinin-ubiquitin (35) or empty control vector. Transfections were conducted by the calcium-phosphate transfection method, as described (36). The ubiquitin expression vector was provided by M. Treier (European Molecular Biology Laboratory, Heidelberg, Germany). Eighteen hours after transfection, the cells were infected with Yersinia strains and processed for WB. Accordingly, the cells were lysed 90 min after onset of infection with a lysis buffer containing 50 mM Tris (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 μM PMSF, 10 μM MG-132, and protease inhibitors (29). Eventually, the deubiquitinating inhibitor N-ethylmaleimide (10 μM) was additionally included (29, 37). The lysates were preabsorbed to protein A/G-agarose (Santa Cruz Biotechnology) for 1 h at 4°C and then incubated with rabbit polyclonal Abs directed against YopE or YopP for 16 h at 4°C to precipitate the respective Yop from the infected cells. The immune complexes were collected and washed with lysis buffer containing 50 mM Tris (pH 6.7), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 μM MG-132, 2% SDS for 30 min at 50°C, and reused for a second immunoblotting procedure. This stripping method was also applied to remove the membranes for successive labeling with anti-Yop Abs and for controlling equal protein loading of the gels by detecting β-tubulin with mouse mAb (D-10; Santa Cruz Biotechnology) in the cellular lysates. The shown data are from one experiment representative for at least three performed.

Immunofluorescent labeling of actin filaments

F-actin was stained with Alexa 568-labeled phallolidin (Molecular Probes) in accordance with previous publications (7). Briefly, cells were fixed in PBS with formaldehyde 10 min after onset of infection and then lysed with ice-cold acetone for 5 min, washed, and stained with phallolidin for 20 min. The preparations were analyzed by fluorescence and phase-contrast microscopy. Assays were repeated at least three times.

Assessment of apoptosis by fluorescence microscopy

Apoptotic cells were detected and quantified by an assay based on the detection of phosphatidylserine exposed on the outer leaflet of apoptotic cells (18). Unfixed cells were stained with fluorescein-conjugated annexin...
V. which has high affinity to membrane-exposed phosphatidylserine, according to the manufacturer’s instructions (Annexin-V-Fluos; Roche). The simultaneous application of the DNA stain propidium iodide (Sigma-Aldrich) allowed the discrimination of apoptotic from necrotic cells, because the nuclei of necrotic cells retain their shapes, but the nuclei of apoptotic cells appear strongly condensed (18). The percentages of apoptotic cells were determined by analyzing a minimum of 200 cells/sample by fluorescence microscopy. Experiments were repeated three times.

Precipitation of active Rac

Pull-down assays for precipitation of active Rac were performed with the Cdc42/Rac1 interactive binding (CIB) motif of p21-activated kinase (PAK) fused to GST, as described in detail elsewhere (38). The expression vector for GST-PAK-CIB was kindly provided by J. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Briefly, HEK293 cells were lysed 3.5 h after onset of infection for 5 min in a buffer containing 10% glycerol, 50 mM Tris (pH 7.4), 100 mM NaCl, 1% Nonidet P-40, and 2 mM MgCl2. rGST-PAK-CIB was freshly prepared from Escherichia coli, coupled to glutathione-Sepharose beads, and added to the cellular lysates for 30 min for Rac precipitation. The precipitated complexes were washed three times in lysis buffer and subjected to WB using mAb against Rac1 (BD Transduction Laboratories). The assay was repeated three times.

Results

The proteasome differentially controls the levels of translocated Yop effectors

The proteasome is an essential nonlysosomal proteolytic system in eukaryotes that critically controls the turnover of intracellular proteins. To ascertain whether the proteasome pathway also contributes to regulate the stability of Yop effectors, we examined the protein levels of a number of translocated Yops in HEK293 cells that were infected with Yersinia in the absence or presence of the proteasome inhibitory peptide MG-132. Cellular lysates were prepared 1 h after a 90-min infection with the Y. enterocolitica serogroup O8 wild-type strain WA-314. The lysates were processed for immunoblotting with Abs directed against YopE, YopH, or YopP. Fig. 1A shows that addition of the proteasome inhibitor markedly enhanced the protein level of YopE, whereas the levels of YopH and YopP were not affected by MG-132 and remained constant. This suggests that the stability of YopE, but not of YopH or YopP, is governed by a proteasome-dependent pathway. Addition of the proteasome inhibitor did not increase the levels of YopE in bacterial control samples that were processed for immunoblotting in parallel to the samples of Yersinia-infected cells (Fig. 1B). Furthermore, the addition of chloramphenicol to inhibit bacterial protein synthesis did not prevent the accumulation of YopE in Yersinia-infected cells (Fig. 1C). Chloramphenicol on the contrary efficiently suppressed growth of the bacteria at the used concentration of 20 μg/ml (data not shown), which reflects its inhibitory activity on the bacterial protein metabolism. These data indicate that the accumulation of YopE in proteasome inhibitor-treated cells is not the result of increased bacterial protein synthesis, but indeed depends on host cellular processes. MG-132 furthermore stabilized YopE in a dose-dependent manner (Fig. 1D, left panel). The replacement of MG-132 by the proteasome inhibitor epoxomicin, which acts highly selective and does not inhibit nonproteasomal proteases (39), mediated a comparable effect (Fig. 1D, right panel). Interestingly, the increase in the amounts of YopE remained constant in proteasome inhibitor-treated cells for at least 6 h, whereas the YopE levels decreased at that time point in the absence of MG-132 (Fig. 1E). This indicates that treatment of the cells with the proteasome inhibitor counteracts destabilization of YopE by the host cell proteasome upon prolonged incubation. We were unable to verify complete disappearance of YopE from the cells in the absence of the proteasome inhibitor, because minor, but incremental amounts of YopE were released from cell-associated, gentamicin-killed bacteria at the later time points (Fig. 1E and data not shown). However, the selective accumulation of YopE in the proteasome inhibitor-treated cells indicates that the proteasome machinery critically determines the levels of YopE in the host cell. This confirms the concept that the destabilization of YopE mediated by the host cell at the later time points (Fig. 1E) results from YopE degradation through the proteasome pathway. In this regard, the proteasome appears to directly counteract intoxication of the host cell with this bacterial virulence protein.

We conducted additional experiments with a Yersinia strain that produces a YopE-YopP hybrid protein in which the N-terminal 138 aa of YopE were fused to the N terminus of YopP (strain WA-ΔyopP/YopE138-YopP). Fig. 1F demonstrates that both YopE and the YopE-YopP constructs were stabilized in a MG-132-dependent manner, as opposed to original YopP (Fig. 1A). Neither Yop was detected in control cells infected with the virulence plasmid-cured strain WA-C. This strain is unable to set up the Yersinia TTSS and to produce any effector Yop. These results indicate that the targeting of YopE for destruction by the proteasome depends on the first 138 aa of YopE. Because the YopE N terminus harbors the protein secretion and translocation domains (40, 41), this suggests that the intrinsic Yop secretion and translocation properties can also determine the t1/2 of the respective Yops inside the host cell by regulating their susceptibility to proteasomal degradation.

Translocated YopE is subjected to ubiquitination inside the host cell

The conjugation of cellular proteins with ubiquitin moieties is a well-characterized posttranslational modification event that targets proteins to the proteasome for degradation (20–22). This mechanism could similarly be involved in the destabilization of YopE by the host cell. We thus explored the possibility that translocated YopE is modified inside the host cell by ubiquitination. The detection of protein ubiquitination requires a careful methodological approach because ubiquitination is a dynamic process that involves both ubiquitinating and deubiquitinating enzymes. Consequently, only a small fraction of the cellular pool of a protein that is destined for proteasomal degradation is modified by ubiquitin residues at a given time (42–45). To increase the likelihood of detection of ubiquitin-modified YopE species, we transiently transfected HEK293 cells with a eukaryotic plasmid that expresses high levels of ubiquitin (35). The transfected cells were infected 18 h later with a Yersinia strain that overexpresses YopE as single effector Yop. This strain harbors two plasmids, one encoding the structural and regulatory components of the Yersinia type III secretion system, the other encoding the effector protein YopE (strain WA-TTSS/YopE). This strain produces higher amounts of YopE compared with the wild-type strain WA-314 (Fig. 2A). The YopE product of this strain was stabilized by proteasome inhibitor treatment similar to YopE of the wild-type strain WA-314 (Fig. 2A). The cellular effects of this strain were compared with those conferred by the virulence plasmid-cured strain WA-C. A YopE-negative mutant or a Yersinia strain that expresses only the secretion-translocation module without YopE is inappropriate to serve as adequate control, because these strains cause necrotic cell death (46) (data not shown). These nonspecific effects result from pore formation in the host cell membrane by the Yersinia type III protein secretion apparatus in the absence of YopE. YopE inhibits this process by preventing actin cytoskeleton rearrangements that otherwise cause damage to the host cell membrane (46). The cells

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were additionally treated with the proteasome inhibitor MG-132 before infection, a procedure that mediates the accumulation of ubiquitinated proteins that are destined for proteasomal degradation. The YopE proteins were immunoprecipitated from the lysates of the infected cells with anti-YopE Abs and immunoblotted with anti-ubiquitin to detect ubiquitin-modified YopE species. Fig. 2B, left panel, shows that a set of anti-ubiquitin Ab immunoreactive bands specifically precipitated with the YopE Ab. These bands were found in neither precipitates prepared from WA-C-infected cells nor in control samples using an anti-YopP Ab instead of anti-YopE. The ubiquitin-modified protein species migrated with slower electrophoretic mobilities than original YopE (23 kDa). They were separated from original YopE by increasing distances, starting from ~8 kDa. This could be consistent with the modification of YopE by mono- as well as polyubiquitination. In fact, the majority of the ubiquitinated proteins was detected also by the anti-YopE Ab (Fig. 2B, right panel), which gives evidence that the ubiquitin-modified proteins represent ubiquitinated YopE species. In line with the stabilization of YopE by proteasome inhibitor treatment accumulated the YopE-ubiquitin conjugates in precipitates from MG-132-treated cells (Fig. 2B). The concomitant increase of the levels of unmodified YopE (Fig. 2B) presumably results from the action of deubiquitinating enzymes. These proteases mediate processing of ubiquitin conjugates when proteasomal degradation of the respective proteins is blocked, which leads to accumulation of the unmodified protein species (47). Our data furthermore showed that the detection of polyubiquitinated YopE was prominent for the YopE-overexpressing strain WA-TTSS/YopE, but less obvious for the wild-type strain WA-314 (data not shown). This could be consistent with the requirement of increased...
YopE levels for the detection of YopE polyubiquitination. In fact, only a small fraction of YopE was polyubiquitinated in comparison with unmodified YopE (Fig. 2B). The polyubiquitinated YopE protein species were furthermore sensitive to both deubiquitinating enzymes and proteasomal degradation because addition of the protease inhibitors N-ethylmaleimide and MG-132 to the lysis buffer increased the appearance of polyubiquitinated YopE (Fig. 2C). The higher protein levels produced by strain WA-TTSS/YopE probably outbalance YopE deubiquitination and degradation, which then facilitates the detection of YopE polyubiquitination. Together, these results show that translocated YopE is subjected to ubiquitination inside the Yersinia-infected host cell. In agreement with these observations, we detected polyubiquitinated YopE in cellular lysates of cells infected with the YopE-overproducing strain WA-TTSS/YopE (Fig. 2B).

**FIGURE 2.** YopE is subjected to ubiquitination. A, Increased YopE quantities produced by *Yersinia* strain WA-TTSS/YopE. HEK293 cells were infected with wild-type *yersiniae* (WA-314) or with strain WA-TTSS/YopE, which produces YopE as sole effector Yop, in the absence or presence of MG-132. Three hours after onset of infection, cellular lysates were prepared and subjected to immunoblotting using anti-YopE Abs. Equal loading of the gel with cellular lysates was controlled by successive immunoblotting against β-tubulin. B, Precipitation of ubiquitinated YopE protein species. HEK293 cells were transfected with a ubiquitin expression plasmid and infected 20 h later with virulence plasmid-cured *yersiniae* (WA-C) or the *Yersinia* strain that overproduces YopE as sole effector Yop (WA-TTSS/YopE), in the presence or absence of MG-132. Cellular extracts were prepared 90 min after onset of infection and immunoprecipitated using anti-YopE or anti-YopP Abs, as indicated (IP). The precipitates were immunoblotted with the anti-ubiquitin Ab P4D1 for the detection of ubiquitin-modified proteins (WB ubiquitin; left panel). The overall content of ubiquitinated proteins in the cellular lysates before precipitation was estimated by anti-ubiquitin immunoblotting using ∼10% of the initial lysates (cell lysate, WB ubiquitin; left panel, bottom). To control successful and specific precipitation of YopE, the membrane with the separated immunoprecipitates was stripped and reprobed with anti-YopE Abs (WB YopE; right panel). The production and translocation of YopE under the different infection conditions were assessed in cellular lysates by immunoblotting with anti-YopE Abs (cell lysate, WB YopE; right panel, bottom). C, Augmented detection of YopE polyubiquitination. HEK293 cells were transfected with the ubiquitin expression plasmid and left uninfected or infected 20 h later with strain WA-TTSS/YopE in the presence of MG-132. Cellular extracts were prepared 60 min after onset of infection with a lysis buffer containing MG-132 and the deubiquitinase inhibitor N-ethylmaleimide (10 μM). Immunoprecipitates were performed using anti-YopE or anti-YopP Abs, as indicated (IP). The precipitates were first immunoblotted with the anti-ubiquitin Ab FK2 for the detection of ubiquitin-modified proteins (WB ubiquitin; left panel), and then stripped and reprobed with anti-YopE Abs for the detection of YopE protein species (WB YopE; middle panel). The production and translocation of YopE by strain WA-TTSS/YopE under the different infection conditions were assessed in cellular lysates by immunoblotting with anti-YopE Abs (cell lysate, WB YopE; right panel). The apparent ubiquitin-modified forms of YopE are denoted in B and C (YopE-Ub, polyubiquitinated Ub; YopE-ub mono, monoubiquitinated Ub mono). The asterisks in B and C denote the position of the H chain of the precipitating Ab. The molecular mass of standard marker proteins are indicated in kDa. The negative control (ø) shows the result of uninfected cells.
with the role of polyubiquitin moieties in targeting a selected protein for proteasomal destruction (20, 22, 48, 49), the polyubiquitination of YopE probably contributes to regulate the degradation of YopE through the proteasome pathway. The monoubiquitination of YopE could play an additional proteasome-independent role, for instance determining intracellular YopE localization (50).

Stabilization of YopE by the proteasome inhibitor aggravates the effects of YopE on the host cell actin cytoskeleton

It is well established that delivery of YopE inside the host cell mediates disruption of the actin microfilament structure. This results from the blockade of Rho-GTPase activities (5, 6). This effect of YopE, together with the action of YopH, YopT, and YopO/ YpkA, provides *Yersinia* with the ability to resist phagocytosis. However, it has been shown that the host cell neutralizes the antiphagocytic Yop effects when constant Yop supply by adhering bacteria is impaired by antibiotic treatment (17–19). The degradation of YopE by the proteasome could be one of the mechanisms that help to shut down Yop action. From this it could be concluded that stabilization of YopE by inhibition of the proteasome would enhance the YopE activity. So, we wondered whether addition of MG-132 could intensify the cytotoxic impact of YopE on host cell morphology. To ensure preferable analysis of the function of YopE, we infected HEK293 cells with *Yersinia* strains that expressed only a restricted Yop pattern (31, 32). These were strain WA-TTSS/YopE, which produces only YopE, and strain WA-TTSS/YopE + YopH, which additionally synthesizes YopH. The YopE proteins of both strains were stabilized by proteasome inhibitor treatment similar to YopE of the wild-type strain WA-314 (Fig. 2A and data not shown). The cellular effects of these strains were compared with those conferred by the virulence plasmid-cured strain WA-C after 4 h of infection.

Fig. 3 shows that both YopE-producing strains WA-TTSS/YopE + YopH and WA-TTSS/YopE induced a characteristic rounded cell morphology that is associated with cytoskeletal disruption by YopE in MG-132-treated cells. The altered cell morphology was considerably less pronounced in the absence of MG-132. A similar effect was observed on cells infected with the wild-type strain WA-314 or by usage of epoxomicin instead of MG-132 (data not shown). Addition of the proteasome inhibitor did not alter the morphology of WA-C-infected cells. This suggests that the increased stability of YopE in proteasome-defective cells enables enhancement of the YopE-induced cytoskeletal alterations. To rule out that the observed cellular effects may result from the induction of apoptosis, which could also be mediated by proteasome inhibitor treatment, we determined the extent of apoptosis by fluorescence microscopy 7 h after onset of infection. Accordingly, the cells were labeled with fluorescein-conjugated annexin V, an apoptosis marker that functions to detect phosphatidylserine exposure in dying cells as early stage apoptotic event. The simultaneous application of the red DNA stain propidium iodide allowed discrimination of apoptotic from necrotic cells (18). More than 95% of the MG-132-treated cells were viable in these conditions independently by which *Yersinia* strain the cells were infected. The viability of the cells exceeded 98% in the absence of the proteasome inhibitor. This shows that only a small portion of the cells has lost its viability due to MG-132 treatment. Staurosporine (5 μM) served as positive control in these experiments mediating 67 ± 12% cell death. These data indicate that intensification of the YopE-dependent cell alterations in presence of MG-132 does not result from onset of apoptosis, but may be a YopE-specific effect on the host cell.

To directly link the activity of YopE to its effects on the actin microfilament structure, we conducted additional experiments with HBMECs. The flat morphology of endothelial cells allows a detailed illustration of actin microfilaments by fluorescent staining (7). It is shown in Fig. 4 that, similar as in HEK293 cells, stabilization of YopE by the proteasome inhibitor was associated with increased morphological cell alterations. Furthermore, the stabilization of YopE enforced the disruption of actin microfilaments. This indicates that the proteasome activity has direct influence on the capacity of YopE to exert its antihost functions. The aggravation of the YopE effects in presence of the proteasome inhibitor suggests that the proteasome serves to down-regulate the activity of YopE in *Yersinia*-infected cells under physiological conditions.

The proteasome indirectly controls the activity of YopE in the host cell

YopE is thought to mediate its cellular effects by acting as GAP for Rho-GTPases, which confers Rho-GTPase inactivation, in particular that of Rac (3, 7). YopE increases the intrinsic GTPase activity of Rac, which converts Rac from the active GTP- to the inactive GDP-bound state. Consequently, one should be able to measure less GTP-bound, activated Rac in the proteasome inhibitor-treated cells. We conducted a Rac activation assay that takes advantage of the fact that only the GTP-bound, but not the GDP-bound form of Rac specifically interacts with the CRIB domain of PAK (51). The rGST-PAK-CRIB protein can therefore specifically precipitate the GTP-bound form of Rac from cellular lysates (38). Fig. 5 shows that a considerable amount of GTP-loaded Rac was detected in uninfected cells, indicating basal Rac activity in HEK293 cells. This activation status was not reduced by addition of the proteasome inhibitor alone. As expected, when the cells were infected with the YopE-producing *Yersinia* strain WA-TTSS/YopE, the amount of active Rac was diminished. Furthermore, presence of the proteasome inhibitor additionally suppressed the appearance of activated Rac in WA-TTSS/YopE-infected cells. This demonstrates that blockade of the proteasome enforces the
inhibitory effect of YopE on Rac activation. Thus, by governing YopE stability, the proteasome controls YopE activity. This conversely suggests that the host cell counteracts YopE activities by mediating its degradation through the proteasome pathway.

YopE is ubiquitinated at Lys\(^75\)

To specify the impact of ubiquitination on the stability of YopE, we intended to identify the lysine residues involved in YopE polyubiquitination. The result shown in Fig. 1 indicated that the targeting of YopE for proteasomal destruction depends on the first 138 YopE aa. We therefore created a number of YopE point mutants in which single lysine residues of this region have been replaced by arginine. The YopE mutant proteins were then used to replace wild-type YopE in strain WA-TTSS/YopE. The cellular effects of the different strains were compared. These experiments revealed that the polyubiquitination of YopE was significantly diminished when lysine at position 75 was mutagenized (Fig. 6A; strain WA-TTSS/YopE(K75R)). In contrast, the ubiquitination patterns were not altered for a Lys62 YopE mutant (WA-TTSS/YopE(K62R)). Thus, although ubiquitination of a protein is often not restricted to a specific lysine residue, Lys75 appears to be an important YopE polyubiquitin acceptor site. Interestingly, the cellular protein levels of YopE(K75R) were increased in comparison with wild-type YopE and did not significantly change upon MG-132 pretreatment (Fig. 6B). This indicates that YopE(K75R), unlike wild-type YopE, appears protected against proteasomal destabilization. The K75R mutation therefore appears to impair both the polyubiquitination of YopE and its degradation by the host cell proteasome. Microscopic evaluations moreover revealed that the cells infected with YopE(K75R)-producing Yersinia displayed a pronounced rounded phenotype in both the presence and absence of MG-132 (Fig. 6C). Thus, the morphological cell alterations induced by YopE(K75R) became independent of MG-132 pretreatment in correlation to the protein levels. These data reconfirm our hypothesis that YopE polyubiquitination and its targeting to the proteasome pathway determine the stability and activity of YopE.

**Discussion**

In this study, we investigated whether the ubiquitin-proteasome pathway could play a role in controlling infectious diseases by regulating the \(\mu_1/2\) of bacterial virulence factors that are delivered inside the host cell. The presented data show that the proteasome critically determines the stability and activity of YopE in \(Y.\) *enterocolitica*-infected cells. Treatment of the cells with a proteasome inhibitor substantially increased the quantity of YopE in the
cells, which was accompanied by enhanced GAP activity toward its cellular target Rac. This intensified the YopE-mediated disruption of the host cell actin cytoskeleton. Together with the observation that YopE succumbs to polyubiquitination, these findings strongly suggest that YopE is degraded in the host cell via the ubiquitin-proteasome pathway. This could finally help the cells to regain control over actin cytoskeleton rearrangements and to counteract the immunomodulatory activity of \textit{Yersinia} (17–19).

FIGURE 6. Lys$^{75}$ controls YopE polyubiquitination, stability, and activity. A, Repression of YopE polyubiquitination by mutation of Lys$^{75}$. HEK293 cells were transfected with the ubiquitin expression plasmid and left uninfected or infected 20 h later in the presence of MG-132. The investigated \textit{Yersinia} strains produced either wild-type YopE (WA-TTSS/YopE) or YopE with mutations in Lys$^{62}$ (WA-TTSS/YopE(K62R)) or Lys$^{75}$ (WA-TTSS/YopE(K75R)). Cellular extracts were prepared 60 min after onset of infection with a lysis buffer containing MG-132 and N-ethylmaleimide. Immunoprecipitates were performed using anti-YopE or anti-YopP Abs, as indicated (IP). The precipitates were first immunoblotted with the anti-ubiquitin Ab FK2 to detect ubiquitin-modified proteins (WB ubiquitin; left panel), then stripped and reprobed with anti-YopE Abs to detect YopE protein species (WB YopE; right panel). The apparent ubiquitin-modified forms of YopE are denoted (YopE-Ub, polyubiquitinatated Ub$_n$, monoubiquitinated Ub$_{mono}$). The asterisk marks the position of the H chain of the precipitating Ab. The molecular mass of standard marker proteins are indicated in kDa. A relatively strong background probably resulting from Ab degradation impedes the detection of YopE-ubiquitin conjugates with the anti-YopE Ab in the molecular mass ranges below 50 kDa (right panel). B, Proteasome inhibitor-independent stability of YopE(K75R). HEK293 cells were left uninfected or infected with yersiniae producing either wild-type YopE (WA-TTSS/YopE) or the YopE Lys$^{75}$ mutant (WA-TTSS/YopE(K75R)), in the absence or presence of MG-132. Two hours after onset of infection, cellular lysates were prepared and subjected to immunoblotting using anti-YopE Ab. Equal loading of the gel with cellular lysates was controlled by successive immunoblotting against β-tubulin. The negative control (φ) shows the result of uninfected cells. C, Proteasome inhibitor-independent reinforcement of the activity of YopE on the host cell by the K75R mutation. HEK293 cells were infected with virulence plasmid-cured yersiniae (WA-C) or \textit{Yersinia} strain producing wild-type (WA-TTSS/YopE) or Lys$^{75}$-mutagenized YopE (WA-TTSS/YopE(K75R)), in the absence or presence of MG-132. Four hours after onset of infection, the cells were fixed and the cellular morphologies were microscopically evaluated.
of YopE by the proteasome. Processed YopE then could itself serve as target for T cell recognition and mediate protective priming of CD8 T cells. This could be one of the mechanisms that provide T cells with the ability to confer host resistance to Yersinia (53).

Thrillingly, proteosomal degradation of bacterial virulence factors may not always be advantageous to the host. It has been shown that rapid destabilization of Salmonella SopE through the proteasome pathway helps salmonellae to avoid excessive induction of an inflammatory immune response (29). Translocated SopE initially activates Rac and Cdc42, which mediate cytoskeletal rearrangements that trigger cellular entry of salmonellae and escape of the bacteria from the host immune system. However, prolonged action of SopE activates proinflammatory signals that may counteract infection. Consequently, the deactivation of SopE by the proteasome after initial induction aids to suppress the production of inflammatory response mediators that finally supports bacterial survival. From these observations, it could be speculated that the inactivation of YopE by the proteasome may be beneficial also for Yersinia. Thus, it could be possible that the termination of the YopE effects regulates the accessibility of YopT or YopO/YpkA to the Rho-GTPases. In fact, it has been shown that YopE and YopT can compete for the same reservoir of Rac1 and generate two spatially distinct, differentially activated pools of Rac (54). This effect of sequential Yop action may be important for the infection process. In addition, because YopE also possesses a regulatory role in Yop translocation by stabilizing the translocation channel (46), an exceeding activity of YopE may be counterproductive for efficient Yop translocation. From these findings, it appears possible that the regulation of YopE stability by the proteasome may help Yersinia to accommodate Yop delivery inside the host cell or to fine-tune the Yop effects on their cellular targets. This could finally contribute to determine the progression of Yersinia infection in the compromised host. It has been revealed that YopE is required for prolonged colonization of Y. enterocolitica-infected mice (55). This suggests that the cellular effects conferred by YopE primarily play a role in maintaining persistent Yersinia infection, rather than in triggering acute overwhelming disease. The restriction of YopE activity by the proteasome may thus aid to dampen overshooting Yersinia virulence that could otherwise cause premature consumption of the infected host organism and thereby limit host-to-host dissemination of the bacteria. The balancing of the YopE levels may instead support the establishment of persistent, productive Yersinia infection. This suggests that the inactivation of YopE by the proteasome, which appears to be an integral part of the host defense against the immunomodulatory activities of Yersinia, could concomitantly influence the course and outcome of Yersinia infection.

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Disclosures

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References

12. Barz, C., T. N. Abahi, K. Trülzsch, and J. Heesemann. 2000. The Yersinia SerT phosphorylation activity by the proteasome may thus aid to dampen overshooting Yersinia virulence that could otherwise cause premature consumption of the infected host organism and thereby limit host-to-host dissemination of the bacteria. The balancing of the YopE levels may instead support the establishment of persistent, productive Yersinia infection. This suggests that the inactivation of YopE by the proteasome, which appears to be an integral part of the host defense against the immunomodulatory activities of Yersinia, could concomitantly influence the course and outcome of Yersinia infection.