A cysteine protease–like domain enhances the cytotoxic effects of the Photorhabdus asymbiotica toxin PaTox

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The nematode mutualistic bacterium Photorhabdus asymbiotica produces a large virulence-associated multifunctional protein toxin named PaTox. A glycosyltransferase domain and a deamidase domain of this large toxin function as effectors that specifically target host Rho GTPases and heterotrimeric G proteins, respectively. Modification of these intracellular regulators results in toxicity toward insects and mammalian cells. In this study, we identified a cysteine protease–like domain spanning PaTox residues 1844–2114 (PaToxP), upstream of these two effector domains and characterized by three conserved amino acid residues (Cys-1865, His-1955, and Asp-1975). We determined the crystal structure of the PaToxP C1865A variant by native single-wavelength anomalous diffraction of sulfur atoms (sulfur-SAD). At 2.0 Å resolution, this structure revealed a catalytic site typical for papain-like cysteine proteases, comprising a catalytic triad, oxyanion hole, and typical secondary structural elements. The PaToxP structure had highest similarity to that of the AvrPphB protease from Pseudomonas syringae classified as a C58-protease. Furthermore, we observed that PaToxP shares structural homology also with non–C58-cysteine proteases, deubiquitinases, and deamidases. Upon delivery into insect larvae, PaToxP alone without full-length PaTox had no toxic effects. Yet, PaToxP expression in mammalian cells was toxic and enhanced the apoptotic phenotype induced by PaTox in HeLa cells. We propose that PaToxP is a C58-like cysteine protease module that is essential for full PaTox activity.

Photorhabdus asymbiotica is an entomopathogenic bacterium living in mutual relationship with nematodes of the Heterorhabditis species. Upon invasion of the nematodes in insect larvae, Photorhabdus produces several highly active protein toxins, which prevent phagocytosis and autophagy. The toxins destroy the actin cytoskeleton, induce apoptosis, and finally kill the animal (1, 2). The cadaver serves as source of nutrition for both bacteria and nematodes (2–5). Nematodes harboring Photorhabdus species are used as insecticidal agents (6) and toxins produced by the bacteria are of increasing agricultural potential as insecticides (2). Among Photorhabdus species, P. asymbiotica was recognized as an emerging human pathogen and several clinical cases of soft tissue infection and necrotic ulcers were reported predominantly in the United States and Australia (7). One protein toxin that is restricted to P. asymbiotica and not present in other Photorhabdus species is PaTox, which comprises 2957 amino acid residues. Two enzymatic effector domains were identified in the C-terminal part of PaTox substantiating that the toxin has a multidomain structure common for many protein bacterial toxins (8, 9). Most of these large protein toxins have a receptor-binding domain, providing tropism to specific cell types, a translocation domain, an autocatalytic peptidase domain for toxin processing, and one or several effector domains. PaTox harbors a glycosyltransferase domain (PaToxC58), which is guided to the negatively charged inner leaflet of the host cells’ cytoplasmic membrane. There it mono-O-glycosylates a tyrosine in the switch I region of Rho GTPases using UDP-GlcNAc (10). This modification impairs Rho signaling and results in actin cytoskeleton disintegration, blockade of phagocytosis, and death of insect larvae. The second effector domain is a deamidase (PaToxD) that resembles the type III secreted effector SseI from Salmonella typhimurium (11). It deamidates a catalytic glutamine residue of heterotrimeric G proteins, resulting in the permanent activation of the GTPase signaling molecule (10).

In this study, we set out to decipher the role of the region of PaTox upstream of the two effector domains. The function and impact of this region during infection is unknown. Sequence analysis suggested a cysteine protease–like domain, which...
we named PaToxP. It includes a typical cysteine–histidine–aspartate triad. The crystal structure of PaToxP was determined and demonstrated high similarity to the C58-protease AvrPphB (12). Members of the C58-protease family are present in many bacterial toxins and effectors, including Vibrio vulnificus MARTX toxin, Photorhabdus luminescens Mcf2 toxin, and the Histophilus somni effector IbpA (12). We show that an intact catalytic triad of PaToxP is crucial for cell rounding and cell blebbing effects, which resemble apoptotic phenotypes in mammalian cells. Cell intoxication experiments revealed that the protease domain acts in concert with the glycosyltransferase and the deamidase domain.

Results

_P. asymbiotica_ toxin PaTox harbors a C58-protease–like domain

Two enzymatically active domains of PaTox, which act in the cytoplasm of the host cell were previously identified by protein sequence similarity analysis (10). Those are the tyrosine-glycosyltransferase PaToxG, which modifies Rho GTPases and influences the regulation of the actin cytoskeleton and the deamidase PaToxD, which modifies heterotrimeric G/H251/H252 proteins. Bioinformatic analyses of full-length PaTox using GlobPlot 2 and DisEMBL 1.5 (13) gave hints for a distinct third globular domain upstream of the glycosyltransferase domain (Fig. 1A). Sequence alignment of this region (amino acids 1844–2114), which was named PaToxP, revealed similarity with peptidases of the C58 YopT peptidase family (Fig. S1). Moreover, the alignment suggested that the catalytic center of PaToxP is formed by the 3 amino acid residues Cys-1865, His-1955, and Asp-1975, building a catalytic triad typical for cysteine proteases. We cloned extended fragments of this region comprising amino acids 1701–2114 (called henceforth WT protein PaToxP) and a variant where the nucleophilic cysteine residue from the catalytic triad was replaced by alanine (C1865A, called PaToxPCA). We produced and purified PaToxPCA (Fig. S2) and performed crystallization experiments.

Crystal structure of PaToxP

We obtained initial crystals of the PaToxPCA variant under several conditions and of varying quality. Crystallization conditions were optimized and phases were obtained by native single-wavelength anomalous diffraction of sulfur atoms (native-SAD) (Table 1). The structure was refined at 2.0 Å resolution using a high-resolution native data set (Table 1). PaToxPCA crystallized in space group P212121 with one molecule in the asymmetric unit. Refinement converged with an R_factor of 0.175 and R_free of 0.214. The main chain dihedral angles of all residues are in the allowed regions of the Ramachandran plot, with 96.6% of residues in preferred regions, 1.4% in allowed regions, and no outliers. Statistics of diffraction data and refinement are summarized in Table 1.

The structure of PaToxP comprises two domains (Fig. 1B). Amino acid residues 1844–2032 form a globular domain with an α–β–α structural fold (green). The N-terminal domain comprises amino acids 1730–1843 (gray). Amino acid residues 1921–1926 and 1964–1967 were poorly resolved and thus omitted from the structure. In addition, 184 water and few ethylene glycol molecules were included. The N-terminal domain belongs to a yet unidentified
part of the toxin and comprises five α-helices and a small two-stranded β-sheet. The structure of the globular protease domain consists of a central 6-stranded antiparallel β-sheet that divides 8 helices in two parts, with α-helices α1–α5 and α8 on one side and α-helices α6–α7 on the other side of the β-sheet (Figs. 1B and 2A). The order of the β-strands is β1–β5–β4–β3–β2–β6, which is a conserved central feature in most papain-like proteases. The protease domain of PaToxP\(^{2\*}\)CA shows high similarity to structure and active site architecture of the AvrPphB protease (Table 2, Fig. 2, A and B), which is the only structurally characterized representative of the C58-protease family of YopT peptidases (14). The alanine substitution depicted in Fig. 3B shows an elongated crevice to both sides of the catalytic nucleophile position, which might form the binding surface for regulatory or substrate proteins (Fig. 3). The electrostatic potential presentation of the surface (Fig. 3A) shows that this groove is mostly electroneutral.

The electrostatic potential surface of entire PaTox\(^{P}\) is depicted in Fig. 3B. Noteworthy is the pronounced basic surface that is mainly contributed by the elongated α-helix and the β-sheet of the N-terminal domain (Fig. 3B). This strong electropositive surface is the result of the presence of 18 lysine and arginine residues forming a large cluster of basic residues.

**Table 1**

| Data collection | S-AD | Native |
|-----------------|------|--------|
| **PaTox\(^{P}\)** |      |        |
| Space group     | P 2\(_1\)2\(_1\)2\(_1\) | P 2\(_1\)2\(_1\)2\(_1\) |
| Cell dimensions |       |        |
| a, b, c (Å)     | 63.50 75.96 76.33 | 63.41 76.06 76.33 |
| α, β, γ (°)     | 90.00 90.00 90.00 | 90.00 90.00 90.00 |
| Number of crystals | 2 | 2 |
| Wavelength      | 2.066 | 1.2037 |
| Resolution (Å)  | 48.85–2.40 (2.49–2.40) | 53.86–2.0 (2.05–2.0) |
| Number of unique reflections | 14,941 (1,594) | 25,589 (1,856) |
| R\(_{merge}\) | 0.247 (3.733) | 0.261 (2.298) |
| I/σ\(_{I}\) | 27.1 (2.9) | 14.7 (2.5) |
| CC1/2 | 0.999 (0.923) | 0.997 (0.772) |
| Completeness (%) | 99.4 (97.9) | 100.0 (99.9) |
| Multiplicity | 110.2 (78.9) | 95.5 (62.0) |

\( ^{a} \) Values in parentheses are for highest-resolution shell.

The electrostatic potential surface of entire PaTox\(^{P}\) is depicted in Fig. 3B. Noteworthy is the pronounced basic surface that is mainly contributed by the elongated α-helix and the β-sheet of the N-terminal domain (Fig. 3B). This strong electropositive surface is the result of the presence of 18 lysine and arginine residues forming a large cluster of basic residues.

**Structural similarity of PaTox\(^{P}\) to proteases, deubiquitinases, and deamidases**

Similar structures in the Protein Data Bank were searched with the DALI protein structure comparison server (15) using the protease domain only as query structure. Similar structures belong to members of the CA clan of the cysteine protease family (EC 3.4.22). This group of enzymes is present in prokaryotes, eukaryotes, and viruses and contains a great diversity of enzymatic functions. The type III-secreted effector AvrPphB
Protease-like domain of PaTox

Figure 2. Catalytic domain of PaTox\textsuperscript{P}. A, the protease domain without N-terminal extension is shown in cartoon representation with a 6-stranded \(\beta\)-sheet separating the catalytic domain in two parts. The catalytic center is located in the middle of the sheet. The inset of the active site, catalytic triad C1865A, His-1955, and Asp-1975 and Gln-1854 from the supposed oxyanion hole are shown as green sticks with their \(2\sigma\)–\(F\) electron density map contoured at 1.5\(\sigma\). B, comparison of structurally aligned PaTox\textsuperscript{P} (green) with AvrPphB (yellow) (PDB code 1UKF), staphopain (cyan) (PDB code 1CV8), and papain (light brown) (PDB code 1PPN). The catalytic triads and the conserved amino acid residues stabilizing the negatively charged transition state are shown as sticks. Corresponding amino acid numbering of PaTox\textsuperscript{P} is indicated. The position of the oxyanion hole location is marked with red dot.

Table 2
DALI search showing unique proteins with a \(Z\)-score of 5 or higher for the catalytic protease domain (amino acid residues 1843–2114)

| PDB entry | Z-score* | RMSD  | lali | nres | \%id | Protein | Organism            |
|-----------|----------|-------|------|------|------|---------|---------------------|
| 1 1ukf    | 11.5     | 2.9   | 141  | 188  | 13   | Avirulence protein AvrPphB | Pseudomonas         |
| 2 27q   | 10.0     | 3.2   | 142  | 232  | 8    | Herpesvirus tegument protein M48 protease (htpM48P) | Human herpesvirus 1 |
| 3 4ip3    | 6.4     | 3.8   | 125  | 192  | 12   | Uncharacterized protein ORF169B | Shigella flexneri    |
| 4 5xe9    | 5.8     | 3.2   | 101  | 136  | 16   | Peptidase domain of ABC-transporter ComA | Streptococcus       |
| 5 4ry2    | 5.4     | 3.5   | 105  | 699  | 9    | Peptidase domain of ABC-transporter PCAT1 | Clostridium         |
| 6 2ffg    | 5.2     | 2.3   | 69   | 80   | 9    | Uncharacterized protein YkU | Bacillus subtilis    |
| 7 1yh4    | 5.1     | 3.4   | 102  | 173  | 10   | Staphopain B | Staphylococcus aureus |
| 8 5kdg    | 5.0     | 3.4   | 103  | 186  | 8    | Gifsy-2 prophage protein GtgE | S. typhimurium      |
| 9 4d8i    | 5.0     | 4.3   | 122  | 254  | 7    | Cysteine protease SpeB | Streptococcus pyogenes |
| 10 3bba   | 5.0     | 4.6   | 128  | 246  | 9    | Cysteine protease Interpain A | Prevotella intermedia |

* Z-score, the statistical significance of the similarity between catalytical domain of PaTox\textsuperscript{P} and related proteins; RMSD, root mean square deviation of \(C\) atom; lali, number of structurally equivalent residues; nres, total number of amino acid residues in the hit protein; \%id, percentage of identical over structurally equivalent amino acid residues.

from Pseudomonas syringae shows the highest structural similarity to the PaTox\textsuperscript{P} domain with a \(Z\)-score of 11.5. AvrPphB belongs to the Yop1-like cysteine protease family (Table 2) and shows dual proteolytic activity: autolysosomal cleavage and proteolysis of an intracellular substrate, the serine/threonine kinase PBS1 in the eukaryotic host cell (16). Other structures with high similarity include the herpesviruses tegument protein M48\textsuperscript{ISP} that has deubiquitinating activity (\(Z\)-score 10.0) (17) and Ospl/ORF169B, a type III secretion effector from Shigella flexneri that deamidates the ubiquitin ligase Ubc13 (\(Z\)-score 6.4) (18). In all structures identified, the 6-stranded \(\beta\)-sheet, the conserved N-terminal helix harboring the catalytic cysteine, and the arrangement of the histidine and aspartic acid (in some cases glutamic acid) of the catalytic triad are structurally well aligned (Fig. S4). The surrounding helices diverge and result in the low overall sequence similarity among these enzymes.

PaTox\textsuperscript{P} delivered into insect larvae is not sufficient for their intoxication

Injection experiments revealed that PaTox is toxic for Galleria mellonella larvae (Fig. 4) (10). After injection of full-length PaTox, the majority of larvae died within 8 days. Larvae injected with a C-terminal–truncated version of PaTox lacking the deamidase domain (PaTox\textsubscript{4,D}) still died, yet the death of larvae was significantly delayed. Further deletion of the glycosyltransferase domain (PaTox\textsubscript{4,GD}) or mutation of the glycosyltransferase DXD motif, a di-aspartate motif essential for glycosyltransferase activity and necessary for binding of the donor substrate UDP-GlcNAc, showed no significant toxic activity and larvae behaved as injection control animals. The specific deamidase and glycosyltransferase activities of the truncated constructs were confirmed in vitro assays shown in Fig. S5. Because the construct PaTox\textsubscript{4,GD} still harbors the protease (P) domain PaTox\textsuperscript{P}, we anticipate that PaTox\textsuperscript{P} alone is not able to kill insects. In larval experiments, the Rho-inactivating glycosylsulfatase domain seems to play the most important role in toxicity. Whether the PaTox\textsuperscript{P} domain is important for the translocation into insect cells, remains unclear and needs to be clarified in future experiments.

Expression of PaTox\textsuperscript{P} is toxic for mammalian cells

To assess the activity of the PaTox\textsuperscript{P} domain in mammalian cells, we expressed PaTox\textsuperscript{P} as an EGFP fusion protein in HeLa cells. After 48 h, PaTox\textsuperscript{P} expressing cells showed a significantly increased cell rounding (Fig. 5, A and B). When the catalytic cysteine residue (Cys-1865) was mutated to alanine (PaTox\textsuperscript{CA}) cell rounding strongly decreased. This indicated that an intact protease domain is crucial for the intracellular effects of PaTox in HeLa cells. Furthermore, staining of the actin cytoskeleton by TRIC-phalloidin revealed a strong cell membrane blebbing phenotype with WT PaTox\textsuperscript{P} (Fig. 5C). In most cases the inner membrane of these bleb vesicles were decorated with cortical actin filaments indicating early events of programmed cell death (Fig. 5C, inset). In contrast to this, the active site
cysteine mutant PaToxPCA exhibited strongly reduced cell membrane bleb formation and behaved as the EGFP control. Taken together, ectopic expression of PaToxP seems to be toxic for mammalian cells and results in cell rounding and the induction of membrane bleb formation. Cell morphological effects are strongly supportive for early steps of programmed cell death and seem to be dependent on a catalytically intact protease domain. The catalytic cysteine mutant showed no toxic effects. Interestingly, expression of PaToxP in Saccharomyces cerevisiae under an inducible galactose promoter influences neither yeast growth nor does it lead to a toxic phenotype. This was also observed when PaToxP-expressing yeast were cultivated under growth stress conditions as osmotic stress (1 M sorbitol), ER stress (20 mM DTT), salt stress (1 M NaCl, 1 M KCl), signaling stress (0.15% caffeine), and temperature stress (5 °C, 37 °C) conditions (data not shown). Unlike metazoans, induction of apoptosis in unicellular organisms like S. cerevisiae is differently regulated (19). Lack of growth phenotypes in PaToxP-expressing yeast might argue toward an apoptosis induction by PaToxP in mammalian cells, which would corroborate our overexpression experiments in HeLa cells. It is worth mentioning that the related C58-domain of MARTX is also not toxic in yeast but in mammalian cells (12). In the case of MARTX, a missing toxin processing has been suggested.

The protease domain augments the apoptotic phenotype of PaTox in HeLa cells

To analyze the function of the protease domain of PaTox in context of cell intoxication, we cloned and expressed PaToxP separately or in combination with the other C-terminal enzymatic domains as His-tagged proteins (Fig. 6A) and performed cell intoxication experiments (Fig. 6B). As the protein delivery system into mammalian cells, we used the protective antigen (PA), the binding and translocation domain of anthrax toxin (20). Microscopic analyses of HeLa cells after incubation with the toxin fragments for 18 h showed cellular retraction and collapse of the cell body only with constructs containing the Rho-inactivating tyrosine glycosyltransferase (G domain) (Fig. 6B). The deamidase domain (D) or the protease domain (P) alone showed no significant alterations under these conditions. Notably, a combination of all three domains (PaToxPGD) showed in addition to rounding a strong blebbing phenotype compared with the tandem fusion construct, consisting of the glycosyltransferase and the deamidase domain (PaToxGD) or the glycosyltransferase (PaToxG) alone. When we used the active site cysteine mutant of the protease domain (PaToxPGDC) membrane blebbing strongly decreased. This indicates that the cysteine protease domain PaToxP is involved in the formation of blebs in mammalian cells and might enhance apoptotic effects.
Protease-like domain of PaTox

Figure 5. PaToxP expression is cytotoxic to HeLa cells. A, EGFP fusion protein of WT PaToxP and its mutant C1865A (EGFP-PaToxPCA) were expressed in HeLa cells. Cell rounding was analyzed by fluorescence microscopy after 2 days. pEGFP-C1 vector served as control. B, quantification of rounded HeLa cells after ectopic expression of EGFP-PaToxP or the active site mutant EGFP-PaToxPCA for 2 days. The percentage of rounded cells to total transfected cells was calculated. Data represent the average of three independent experiments. For each experiment >50 cells were counted. Error bars, S.D. Asterisk represents significant p values, ***, <0.001, Student’s t test. C, fluorescent micrographs of HeLa cells expressing the protease domain of PaTox (EGFP-PaToxP) or the active site cysteine mutant (PaToxPCA) (green) for 2 days. Bottom row shows TRITC-phalloidin staining (red) of the actin cytoskeleton. Inset shows a magnification of the plasma membrane blebbing phenotype of EGFP-PaToxP expressing cells. Scale bars, 10 μm.

Discussion

In this study, we identified a new domain in the bacterial protein toxin PaTox from the entomopathogen and emerging human pathogen *P. asymbiotica*. The crystal structure of the PaTox region covering amino acids 1729–2114 was determined at 2.0 Å resolution. It revealed a protease domain with highest structural similarity to C58-proteases of the clan CA (nomenclature of proteases according to the MEROPS peptidase database (14)) as well as an adjacent N-terminal domain. Enzymes of the family of C58-proteases comprise bacterial toxins including MARTX (multi-functional autoprocessing repeats in toxins), Mcf2 from *P. luminosum*, IbpA from *H. somni*, the type III-secreted effectors YopT from *Yersinia* species, and AvrPphB protease from *P. syringae* (Fig. 7 and Fig. S1). From these proteins the protease activity was confirmed with MARTX, AvrPphB, and YopT. The additional domain comprises the 112-amino acid residues N-terminal to the protease and has a mainly α-helical structure. This region may be part of the protease or may belong to a yet uncharacterized domain of the multiprotein toxin PaTox. For this N-terminal domain, BLAST analysis and search for structural homologies using the DALI server revealed sequence similarity to hypothetical proteins and poor structural homology with a Z-score below 4 (data not shown). The presence of an enzyme-specific N-terminally linked propeptide like in AvrPphB protease (16) is not likely, as no autocatalytic activity could be detected (data not shown).

The surface of the PaToxP structure displays a substantial positively charged surface area contributed by the N-terminal domain (Fig. 3). This might indicate a binding surface for regulatory proteins or substrates or an interaction site with a membrane surface. The possibility, that this domain is not related to protease activity of PaToxP but rather involved in other, yet unknown functions of the protein toxin like membrane permeation and effector protein translocation, should also be considered.

*G. mellonella* injection studies revealed that the PaToxP-domain alone is not toxic for insects. An intact glycosyltransferase domain (G) of PaTox seems to be the crucial toxic part of the toxin. Mutation of the essential DxD motif results in abrogation of toxic phenotypes (Fig. 4). Nevertheless, overexpression of the PaToxP-domain as EGFP fusion protein in HeLa cells results in cell rounding and extensive bleb formation resembling early events of apoptosis (Fig. 5). Cell intoxication experiments using anthrax PA and His-tagged PaTox fragments comprising different active domains showed that all three domains (glycosyltransferase, deamidase, and protease domain) are important to induce strong cell rounding and membrane blebbing effects in HeLa cells (Fig. 6). Although cell rounding was observed in all cases when the Rho-inactivating glycosyltransferase was present, the blebbing phenotype was augmented by the presence of the protease domain. This effect was not observed when the catalytic cysteine was mutated to alanine. These results corroborate the findings with ectopic expression of EGFP-PaToxP and give a hint for the induction of apoptosis. Unfortunately, we were not able to detect PARP-1 cleavage or caspase 3/7 processing, classical indicators for apoptosis (data not shown).

One of the closest sequence homologs of the PaToxP-domain with 22% sequence identity is the C58-protease like Mcf (makes caterpillars floppy) domain of the MARTX toxin from *V. vulnificus* (Fig. 7 and Fig. S1) (12). This domain is present in all clinically important *V. vulnificus* biotype 1 clade C strains (21).
Figure 6. PaTox toxicity is enhanced by the P domain. A, schematic representation of the His-tagged PaTox truncation constructs (PaToxPGD, PaToxGD, PaToxP, PaToxG, and PaToxD) used for cell intoxication experiments. B, Normarski microscopy of HeLa cells incubated without (control) or with the His-tagged constructs PaToxP, PaToxG, PaToxGD, PaToxPGD, and PaToxPGDCA each 20 nM in combination with PA (20 nM) as a delivery system. Pictures were taken 18 h after treatment. Representative images of at least three independent experiments are shown. Scale bar, 10 μm.

Figure 7. C58-protease domain is conserved in several families of bacterial toxins and effectors. A, schematic representation of toxins and effectors containing a similar C58-protease domain (P): P. asymbiotica PaTox (accession number C7BK9P9), V. vulnificus MARTX (P = Mcf2-domain) (accession number NP_762440), P. luminescens Mcf2 (accession number AAR21118), H. somni IbpA (accession number Q06277), Yersinia pestis YopT (accession number NP_395155), and P. syringae avirulence protein AvrPphB (accession number AAA25727). Known autocleavage sites are marked by a scissor; α/β, hydrolase; RID, Rho-inactivation domain; RRSP, Ras/Rap1-specific endopeptidase; PMT, Pasteurella multocida toxin C1/C2 domain; CPD, cysteine protease domain; HRMA, type III effector-like avirulence protein; FHA-like, filamentous hemagglutinin-like domain; Fic, filamentation induced by cAMP domain. B, partial sequence alignment harboring the residues of the catalytic triade. C, phylogenetic tree of the indicated C58-protease domains.
Protease-like domain of PaTox

The Mcf$_{\text{v}}$ domain was shown to induce apoptosis in a cysteine protease-dependent manner and seems to lead to a loss of mitochondrial membrane potential, release of cytochrome c, activation of Bax and Bak, and the processing of PARP-γ (22). Notably, the intracellular Mcf$_{\text{v}}$-substrate inducing apoptosis is still not known. Agarwal et al. (12) assumed that the Mcf$_{\text{v}}$ domain does not have a CHD catalytic triad and proposed instead a RCD tripeptide motif essential for catalytic activity. The crystal structure of PaTox$^p$ from the present study clearly defines a catalytic CHD motif consisting of Cys-1865, His-1955, and Asp-1975. A RCD motif similar to Mcf is not present in our PaTox$^p$ structure. Interestingly, Mcf$_{\text{v}}$ is autocatalytically processed and processing is induced by a yet unknown heat-stable host cell protein (12). Also other toxin cysteine proteases from the C80-protease family are dependent on host cell factors. For example, the autoprocessing of TcdB from Clostridium difficile and other clostridial glycosylating toxins is induced by inositol hexakisphosphate (InsP$_6$) (23). Whether PaTox$^p$ activity is also dependent on a host cell factor needs to be determined. Autocatalytic assays in the presence of cell lysate and various host cell factors including InsP$_6$ did not indicate autoprocessing activity of PaTox (data not shown).

DALI searches for structural homologs of PaTox$^p$ identified the avirulence protein AvrPphB from P. syringae, the smallest homolog of the C58 family of proteases (Fig. 7). AvrPphB is a plant pathogen type III effector, which cleaves itself autocatalytically and additionally cleaves the intracellular substrate PBS1, a serine/threonine kinase. In plant cells, cleavage fragments of PBS1 are recognized by the pattern recognition receptor RPP55, which results in the hypersensitive response, a kind of localized cell death response to defend infections of plant tissue (16). Mutation of the catalytic cysteine blocks autoprocessing and also the induction of hypersensitive response in Arabidopsis thaliana (24). In contrast to Mcf$_{\text{v}}$, AvrPphB does not need a host cell factor for the autocatalytic cleavage. Cleavage occurs under in vitro conditions with recombinant proteins (24).

Rounding of HeLa cells induced by overexpression of EGFP-PaTox$^p$ might indicate that Rho GTPases might be affected by PaTox$^p$. The fact, that toxin delivery by anthrax PA was insufficient to visualize cell rounding might be attributed to the low amount of proteins transported into the cell compared with intracellular expression of EGFP-PaTox$^p$. It was shown for another C58-peptidase, YopT from Yersinia species (Fig. 7) that it cleaves the C-terminal isoprenyl moiety from Rho GTPases, which results in the displacement of Rho from the plasma membrane. Consequently, Rho GTPase signaling is blocked and actin filaments are depolymerized (16). To study a similar activity of PaTox$^p$, we analyzed the processing and modification of the Rho GTPases RhoA, Rac1, and Cdc42 in cell lysates and recombinant proteins, but we were not able to see proteolytic, deamidation, or transglutaminylation activity of PaTox$^p$. Additionally, we did not observe the cleavage of the EGFP products in Western blotting experiments after ectopic expression of various N-terminal EGFP constructs in HeLa cells (data not shown). Furthermore, unspecific cleavage reactions using artificial substrates as fluorescent-quenched casein, or a fluorescent-labeled peptide mixture, or deamidation and transglycinylation of the peptide Z-Gln-Gly in the presence of hydroxylamine with the detection of ammonia release did not reveal any catalytic activity of PaTox$^p$. It seems that PaTox$^p$ has a strict substrate specificity, which complicates the substrate identification.

We assume that PaTox$^p$ displays a highly stringent substrate specificity, which seems to be common for most of the C58-protease domains in bacterial toxins and effectors. Ongoing studies will decipher the identity and function of the substrates of PaTox$^p$, which will extend the spectrum of the few known C58-family substrates.

Experimental procedures

Strains, vectors, and materials

Escherichia coli DH10B, TG1 were used for cloning and E. coli BL21 (DE3) for recombinant protein production (Invitrogen). Haploid S. cerevisiae MH272–3α (ura3, leu2, his3, trp1, ade2) or diploid MH272–3α/a (ura3/ura3, leu2/leu2, his3/his3, trp1/trp1, ade2/ade2) were used for yeast genetic studies (25). The following commercial E. coli and S. cerevisiae/ E. coli shuttle vectors were used: pET28a, pET11 (Novagen, Madison, WI), and pRS313[His3] (26). For yeast transformation, standard genetic techniques were applied (27). All sequences of the corresponding plasmids and site-directed mutations were confirmed by sequencing (GATC Inc., Konstanz, Germany). DNA-modifying enzymes, Pfu DNA polymerase, 5-fluoroorotic acid, kits for plasmid DNA and PCR products isolation were purchased from Fermentas (St. Leon-Rot, Germany). Components of liquid media for S. cerevisiae and E. coli were from Difco (Becton Dickinson GmbH, Heidelberg, Germany) and Roth (Carl Roth GmbH, Karlsruhe, Germany) correspondingly. Yeast S. cerevisiae were cultivated in YPD (1% yeast extract, 2% peptone, and 2% glucose) or minimal (0.67% yeast nitrogen base without amino acids and with ammonium sulfate, 2% glucose, or 2% galactose and the corresponding supplement) media at 30 °C. Isopropyl β-D-thiogalactopyranoside (IPTG) was from Roth. All other reagents were of analytical grade and purchased from commercial sources.

Antibodies

Anti-GST (catalog number 27-4577-01, dilution 1:2,000) was from GE Healthcare and horseradish peroxidase linked antimouse antibody from Rockland Immunochemicals.

Cloning of PaTox genes and site-directed mutagenesis

The cloning, recombinant expression of PaTox and PaTox$^p$ fragments from genomic DNA of P. asymbiotica (PAU_02230) was performed from a pET28a or pGEX4T vector as described previously (10) or ligated into the vector pEGFP C1 for mammalian expression. QuikChange Kit™ (Stratagene La Jolla, CA) in combination with Pfu Ultra HF DNA polymerase was used for the replacement of one to three nucleotides. All sequences of corresponding plasmids were confirmed by sequencing (GATC Inc., Konstanz, Germany).

Recombinant protein expression

E. coli BL21* CodonPlus cells (Stratagene) transformed with the desired plasmid were grown in LB broth supplemented with
the corresponding antibiotics on a shaker at 37 °C until $A_{600} = 0.8$. Protein expression from the pET28-based plasmids was induced by 1 mM IPTG (Roth) for 4 h (PaTox$^\beta$) or overnight (PaTox full-length, PaTox$^\Delta$D, and PaTox$^\Delta$GD) at 22 °C, and pGEX-based expression was induced with 0.2 mM IPTG for 6 h at 37 °C. Bacterial cells were harvested by centrifugation at 6000 $\times$ g for 15 min, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM imidazole, 30 $\mu$g/ml DNase I, 1 mM $\beta$-mercaptoethanol, and protease inhibitor mixture (Roche Applied Science)) and lysed by French press or ultrasonic treatment. The cleared lysate was subjected to chromatography on a GSH-Sepharose or nickel-equilibrated HisTrap column connected to an AKTA Purifier (GE Healthcare, Freiburg, Germany). Bound proteins were eluted with 10 mM reduced GSH or 0.5 M imidazole depending on the construct used. Further purification and removal of protein impurities or small molecular weight components as reduced GSH, or EDTA was achieved by size-exclusion chromatography using a Superdex 75 10/300 column.

**Yeast growth assay**

To analyze the toxic effects on yeast growth, *S. cerevisiae* cells expressing PaTox$^\alpha$ and the corresponding mutants were titrated 5-fold from the starting value of $A_{600} = 1.0$. From each dilution, an aliquot of 5 $\mu$l of suspension was dropped onto SD agar, supplemented with the corresponding marker substances. Where indicated, the pH was adjusted correspondingly or additional stress agents were included (20 mM DTT, 1 M sorbitol, 1 M NaCl, 1 M KCl, or 0.15% caffeine). Petri plates were incubated for 3–5 days at 30 °C (or at the temperature mentioned in figure legends) before photography.

**Crystallization**

His-tagged PaTox$^\alpha$ C1865A (amino acids 1701–2114) was crystallized by the sitting-drop vapor-diffusion method at 20 °C. Protein (21 mg/ml in 10 mM Tris-HCl, pH 7.4, and 100 mM NaCl) was mixed 2:1 with reservoir solution (25.7% (w/v) PEG 8000, 150 mM HEPES, pH 7.5, 60 mM potassium sodium tartrate), 20% Ethylene glycol was supplemented for cryo-protection prior to crystal harvesting.

**Data collection and refinement**

Diffraction data were collected at 100 K on beamlines ID23-1 (28) as well as ID30b of the European Synchrotron Radiation Facility (ESRF) and P13 (29) at the European Molecular Biology Laboratory (EMBL). Final data sets used for sulfur SAD phasing were prepared using Phenix AutoSol. A total of 10 sites of the 14 sulfur atoms potentially present in the asymmetric unit (one protomer of the crystallized construct has 14 methionine and no cysteine residues) were found. AutoBuild from Phenix was used to obtain an initial model (33). Several alternated cycles of model building using COOT (34) and phenix.refine (33) were required to obtain the final model. Quality of the structure was checked with MolProbity (35). Figures were prepared using PyMol, the Molecular Graphics System, version 1.5.0.4 Schrödinger, LLC. For data collection and refinement statistics see Table 1.

**Cell culture**

HeLa (ATCC CCL-2) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, 4 mM penicillin, 4 mM streptomycin, and 1% sodium pyruvate (Biochrom, Berlin, Germany). Cells were cultivated in a humidified atmosphere of 5% CO$_2$ at 37 °C. Where necessary, cells were starved overnight in Dulbecco’s modified Eagle’s medium without fetal calf serum. Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) for 2 days according to the manufacturer’s instructions. Cells were analyzed by confocal microscopy. For intoxication of cells, PaTox fragments (20 nm) were applied into the medium or a combination of *Bacillus anthracis* PA (20 nm) and incubated for 18 h at 37 °C if not mentioned elsewhere.

**Microscopy**

Fluorescence images were acquired with an inverted Axiovert 200M microscope (Carl Zeiss, Jena, Germany) and a Cool-snap HQ II (Photometrics) digital camera, driven by Metamorph imaging software (Universal Imaging, Downingtown, PA). The microscope was equipped with planapochromat objectives, a Yokogawa CSU-X1 spinning disk confocal with an emission filter wheel, and 488- and 561-nm laser lines. Image processing was performed using the Metamorph imaging software.

G. mellonella injection

Latest-instar *G. mellonella* larvae were selected by similar size and absence of any melanization. Larvae were randomly chosen, chilled on ice for 5 min, and the site of injection was surface-disinfected with 70% ethanol. Hemocoel injection was performed with 10 $\mu$l of PaTox toxin or PaTox-fragment solutions (50 ng/larva) through the last left pro-leg using a microsyringe with a 26-gauge needle. Control insects were injected with 10 $\mu$l of PBS or left untreated. Larvae from each replicate were placed onto Greiner 6-well plates (one larvae per well) and incubated in the dark at 28 °C for 11 days. Loss of larvae reaction on poking was the indication for death. Differences in survival was analyzed by the Kaplan-Meier method using the software SigmaPlot 10.0 (Systat Software, Inc.).

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Protease-like domain of PaTox

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