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ElaD, a Deubiquitinating Protease Expressed by E. coli

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Background. Ubiquitin and ubiquitin-like proteins (Ubl) are designed to modify polypeptides in eukaryotes. Covalent binding of ubiquitin or Ubls to substrate proteins can be reversed by specific hydrolases. One particular set of cysteine proteases, the CE clan, which targets ubiquitin and Ubls, has homologs in eukaryotes, prokaryotes, and viruses. Findings. We have cloned and analyzed the E. coli protein elaD, which is distantly related to eukaryotic CE clan members of the ULP/SENP protease family that are specific for SUMO and Ned8. Previously misannotated as a putative sulfate/phosphatase, elaD is an efficient and specific deubiquitinating enzyme in vitro. Interestingly, elaD is present in all intestinal pathogenic E. coli strains, but conspicuously absent from extraintestinal pathogenic strains (ExPECs). Further homologs of this protease can be found in Acanthamoeba Polyphaga Mimivirus, and in Alpha-, Beta-and Gammaproteobacteria. Conclusion. The expression of ULP/SENP-related hydrolases therefore extends to plant pathogens and medically relevant strains of Escherichia coli, Legionella pneumophila, Rickettsiae, Chlamydiae, and Salmonella, in which the elaD ortholog sseL has recently been identified as a virulence factor with deubiquitinating activity. As a counterpoint, our phylogenetic and functional examination reveals that ancient eukaryotic ULP/SENP proteases also have the potential of ubiquitin-specific hydrolysis, suggesting an early common origin of this peptidase clan.

INTRODUCTION

Ubiquitin, as well as Ubls such as Ned8 and SUMO, are proteins (almost) exclusively expressed by eukaryotes [1]. Ubiquitination controls many cellular processes, including degradation of proteins by the proteasome and intracellular trafficking. Ned8 is a ubiquitin-like modifier that regulates the rate or extent of ubiquitination, whereas SUMO1 is involved mostly in regulation of transcription factors and in nuclear import. The attachment of ubiquitin or ubiquitin-like modifiers to substrate proteins is covalent, yet reversible [2]. A large family of eukaryotic cysteine proteases is involved not only in generation of ubiquitin-(like) proteins from their precursors, but also in their removal from modified substrates [3,4]. Pathogens can tamper with the ubiquitin-proteasome system to cripple the cell’s defenses [5]. For instance, ubiquitination and proteasomal degradation of p53, initiated by a Human Papillomavirus protein [6], or stabilization of Ikb-2 by Yersinia deubiquitinases [7] have been described. The continuing discovery of new deubiquitinating proteases in viruses broadly hints at how important it is for these pathogens to seize control of posttranslational modifications in host cells [8–10]. Here, we focus on cysteine proteases of the CE clan [11], as defined by the MEROPS database [12]. CE proteases are expressed by viruses, bacteria and eukaryotes. In eukaryotes, this clan represents the family of Ubl-specific proteases (ULP/SENP), which remove SUMO or Ned8 from substrate proteins [13,14]. Viral homologs of ULP/SENP s can act as deubiquitinases, but they also cleave unrelated proteins, as long as a glycine motif is present at the C-terminus of the substrate [15,16]. Examples of bacterial deubiquitinases include YopJ and CslDUBs. First, YopJ is a protease that is secreted into host cells by Y. pestis [17], and homologs to this peptidase can be found in other bacteria, too [18,19]. Injection of YopJ eventually suppresses the inflammatory response in affected cells. The precise molecular mechanism of YopJ family peptidases has not yet been solved, as they lack a hallmark tryptophan following the active-site histidine [20]. Yet, in vitro and in vivo data strongly suggest that these proteases are indeed proteases with specificity for ubiquitin or SUMO [7,18,19], although their effectiveness as virulence factors might depend on additional functions such as acetylation [21,22]. A second example of bacterial CE peptidases can be found in pathogenic Chlamydiae. We have shown that pathogenic Chlamydiae, but not a non-pathogenic environmental strain, express proteases that specifically recognize ubiquitin and Ned8. They do so presumably to remove both modifiers from target proteins of the host cell, as Chlamydiae–like most other bacteria–possess neither a ubiquitin nor a Ned8 homolog [23].

Intrigued by the finding of CE peptidases in bacteria and considering their functional similarity to eukaryotic ULP/SENP proteases, we explored whether additional bacterial homologs with deubiquitinating activity might exist.

RESULTS

To search the sequenced genomes of bacteria for new members of the CE protease clan, we employed PHI-BLAST [24] to find proteins with the typical catalytic triad of histidine, aspartate (or glutamate or asparagine), and the active-site cysteine [25]. Candidate proteins were subjected to a second round of analysis, in which we excluded proteins without the hallmark oxyanion-stabilizing group, consisting of at least one glutamine (or glutamate) following the active-site cysteine.

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asparagine) close to the active-site cysteine. Lastly, the predicted secondary structure of candidate proteases was compared to the solved structure of eukaryotic CE clan homologs [26,27]. Apart from YopJ and the ULP/SENP homologs in *Chlamydiae*, we found potential CE peptidases in Alpha-, Beta-, and Gammaproteobacteria (Figure 1), but not in bacteria from other branches. We also found a peptidase homolog in the giant Acanthamoeba Polyphaga Mimivirus [28] and in African Swine Fever Virus, the first virus in which a ubiquitin conjugating enzyme was discovered and for which deubiquitinating activity has been suggested [29–32]. The bacteria we identified share a close (symbiotic or pathogenic) relationship with eukaryotes, and all viruses with CE proteases possess a dsDNA genome. The sequence variations among these peptidases are too extensive to allow for significant bootstrap values and it is therefore not possible to faithfully infer phylogeny from this dataset. Yet, this is also true for the eukaryotic ULP/SEPNPs, which fall into three functional classes, specific for either SUMO (ULP1 and ULP2 group) or Nedd8 (SENP8 group). While best reciprocal BLAST hits and consensus phylogram trees tend to correctly predict to which functional class a particular ULP/SENP homolog belongs, bootstrap support is generally not significant [33].

We were especially interested in the protein elaD (belonging to “group II”, see Figure 1), expressed by *E. coli* and with orthologs in *Legionella pneumophila* and in all currently sequenced strains of *Salmonella* (Figure 2). *E. coli* is an abundant commensal in the human gut and also a relevant pathogen. Nonetheless, little is known about genes that define pathogenicity of various *E. coli* strains, apart from those that encode obvious toxins [34–36]. We furthermore chose elaD, because it represents one of the more distantly related hits in our bioinformatics screen and we aimed to test the robustness of our prediction by examining this protein’s function.

Figure 1. Phylogram representation of CE clan proteases in viruses, bacteria, and eukaryotes. Eukaryotic peptidases (in blue) belong to the C48 subfamily and can be separated into three groups: ULP1 (including the mammalian proteases SENP1, 2, 3, and 5), ULP2 (including SENP6 and 7), and the SENP8 group with proposed specificity for SUMO (ULP1 and ULP2 group) and Nedd8 (SENP8 group), respectively. Bacterial proteins are indicated with a preceding “B”, viral proteases with a “V”. We have further divided microbial protease homologs by color: green for biochemically tested proteases, red indicating the absence of published data on the function of these putative proteases, and yellow for the group representing elaD and its orthologs. The C5 family contains Adenovirus proteases with deubiquitinating activity, C55 comprises the bacterial YopJ homologs, and C57 the Vaccinia virus I7 peptidases. Based on sequence similarity, two bacterial C48 family groups can be distinguished: a group of Proteobacteria (located at one o’clock) which appear to be closely related to fungal SENP8 homologs (common node indicated with a circle, bootstrap support~60%), and *Chlamydiae*, for which we had previously shown the presence of deubiquitinating and deneddylating activity. Three additional groups have not yet been assigned to specific CE clan subfamilies in the MEROPS database, including Mimivirus (“group I”), Gammaproteobacteria (“group II”), and *Rickettsiae* (“group III”). The African Swine Fever Virus protease and the I7 Vaccinia virus protease have not been tested for deubiquitinating or Ubl-specific activity, but they both require a glycine-based motif at the C-terminus of the substrate, as found in ubiquitin or Ubls. The unrelated CD clan peptidase Clostripain is used as outgroup in this phylogram. For clarity, this tree does not contain all orthologs and paralogs of the different groups or families. Sequence information is provided in Table 2.

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The goal of our first experiment was to confirm protease activity and to determine the substrate specificity of elaD. Because of its relationship to ULP/SENP8, we hypothesized that elaD might recognize UbIs or ubiquitin. We expressed elaD by in vitro transcription/translation in rabbit reticulocyte lysate and incubated the metabolically labeled polypeptide with electrophilic probes, in which a Michael acceptor was added to the C-terminus of ubiquitin or the UbIs SUMO1, Nedd8, and ISG15 [14]. As shown in Figure 3, elaD readily forms a covalent adduct with the ubiquitin probe and to a much lesser extent also with the Nedd8 probe, but not detectably with SUMO1 or ISG15. Moreover, when mutating the putative active-site cysteine at position 313 to serine, covalent binding to the electrophile is abolished. This indicates that the cysteine residue in elaD is essential for catalytic activity, as has been observed for YopJ [7], for the Chlamydia protease CT868 [23] and for the eukaryotic ULP/SENP8s [2]. To date, specific labeling of putative proteases with activity-based probes has shown excellent correlation with enzymatic activity [14,37,38].

Next, we assessed enzyme kinetics by measuring hydrolysis of fluorogenic substrates derived from ubiquitin, SUMO1 and Nedd8. For these experiments, we expressed elaD in E. coli. The growth rate of the bacteria was unaffected when overexpressing elaD, but we only recovered about 50% of the wildtype protein when compared to the amounts of C313S mutant (Figure 4A). As demonstrated by release of the fluorophore 7-amino-4-methylcoumarin (AMC), elaD cleaves ubiquitin-AMC, but not SUMO1-AMC or Nedd8-AMC, and the C313S mutant of elaD fails to cleave either substrate (Figure 4B and data not shown). The initial rate of hydrolysis with 100 nM ubiquitin-AMC and 50 nM elaD is in the order of 0.3–0.6 per minute, defining elaD as a moderately active deubiquitinase, compared to the rapid Isopeptidase T with a rate of ca. 8 per minute [39] or to the much slower ubiquitin-protease USP14 with a rate of ca. 0.01 per minute (data not shown). It should be noted that we could not assess V_max because the enzymatic rate increased linearly with substrate concentration (we tested up to 20 μM ubiquitin-AMC; 50 nM elaD then hydrolyzed ubiquitin-AMC at an initial rate of 5 per minute). Similar observations have been made with the SARS virus deubiquitinase [9]. Overall, our functional analyses confirm the prediction of our bioinformatic screening and define elaD as a deubiquitinating protease.

Why do eukaryotic CE peptidases show specificity distinct from their prokaryotic counterparts? Could this indicate a profound discrepancy, hinting towards a separate origin of these two protease groups? We set out to challenge the presently held notion that ULP/SENP proteases do not exhibit ubiquitin-specific activity [13], while most tested bacterial homologs apparently do. To this end, we chose to biochemically define CE clan members of a more deeply branched class of eukaryotes. Pezizomycotina, a subgroup of fungi that includes A. fumigatus, A. nidulans, M. grisea, N. crassa, and G. zeae encode putative SENP8 proteases that are related in amino acid sequence to a group of yet uncharacterized bacterial C48 homologs (Figure 1). In our phylogram, the common node between the respective prokaryote C48 group and SENP8 homologs of Pezizomycotina replicates with a bootstrap support of >60%. We cloned, expressed and tested the putative SENP8 protease of G. zeae, as a representative of Pezizomycotina. Unlike mammalian SENP8, the G. zeae ortholog displays dual activity towards Nedd8 and ubiquitin, similar to the previously defined CE clan protease CT868.
Figure 4. A. Expression of recombinant elaD and elaD<sub>C313S</sub> in bacteria. Coomassie staining of a 10% SDS-PAGE, showing the relatively higher expression levels of elaD<sub>C313S</sub>, compared to wildtype elaD. The purity of elaD was increased to 30–40% after a second round of size exclusion chromatography (not shown). Similar to <i>in vitro</i> translated elaD, the bacterially expressed protein also reacts with ubiquitin-VME. Molecular mass indicated at the left (in kDa). B. Biochemical assay for hydrolytic activity of elaD. His-tagged elaD was expressed in and purified from <i>E. coli</i> and incubated with fluorogenic substrates based on ubiquitin, SUMO1 and Nedd8. No hydrolytic activity of elaD was observed on SUMO1-AMC and Nedd8-AMC (data not shown). In contrast, the C-terminal peptide bond of ubiquitin-AMC is efficiently cleaved by elaD, leading to release and dequenching of the fluorophore AMC. Depicted is a representative experiment in which variable concentrations of purified elaD were incubated with 500 nM ubiquitin-AMC for 40 min. The C313S mutant of elaD has no proteolytic activity and wildtype elaD can be inhibited by alkylation of its active-site cysteine with 5 mM N-ethylmaleimide (data not shown). The y-axis shows relative fluorescence units, the x-axis represents the time scale with measurements every 2 minutes.
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of the prokaryote *C. trachomatis* (Figure 5) [23]. This result indicates that ubiquitin-specificity is not restricted to bacterial or viral CE peptidases, but also exists in some ancient eukaryotic members of this protease clan.

**DISCUSSION**

We have found previously undescribed CE peptidase homologs in several bacterial and in one viral species (Figure 1). We have furthermore proven that one of the more distantly related homologs – the protein elaD in *E. coli* – can act as a deubiquitinating enzyme *in vitro*. Earlier, we had shown that related *Chlamydia* proteases also have deubiquitinating activity and similar observations had been made regarding the YopJ protease family. This suggests that the yet undefined bacterial CE peptidases could also display ubiquitin-or Ubl-specificity, especially considering their even closer sequence relationship to eukaryotic ULP/SENP proteases. These findings raise two important questions. First, are these proteases really specific for ubiquitin *in vivo*, or are we simply measuring an off-target artifact, with the true substrates merely resembling ubiquitin? Second, why are these proteases—which are present in every eukaryote—so widely distributed in bacteria and viruses as well, and what can we learn about their genetic origin?

One might argue that the bacterial CE clan peptidases have distinct specificity for bacterial substrates. For instance, it has been
is indeed eukaryotic ubiquitin. Moreover, the ortholog of elaD in a homolog of ubiquitin, making it likely that the substrate of elaD and to display deubiquitinating activity exception of ubiquitin by elaD reflects a highly specific interaction. With the structure among these Ubls, we conclude that hydrolysis of clearly distinguish elaD from the more promiscuous viral CE bond in ubiquitin-AMC, but not in Nedd8-AMC. These features vinylsulfone was significantly weaker than to the ubiquitin probe.

critical C-terminal region. We observed no reactivity between sequence similarity to ubiquitin, and ISG15 is even identical at the present in ubiquitin, but also in certain viral substrates [15,16].

CE protease has relaxed specificity for a consensus site that is the true substrate is of bacterial origin. Similarly, the Adenovirus strain 53638 also encodes elaD as a pseudogene, while careful inspection suggests that this is just the result of sequencing slippage in an adenosine-rich region of the elaD gene. APEC, NMEC, and UPEC belong to the group of extraintestinal pathogenic strains (ExPEC).

The gene encoding elaD can be found in all intestinal pathogenic E. coli strains, and in the commensal strain K12. The gene is absent in APEC, NMEC, and two uropathogenic strains, and encoded as a pseudogene in three other strains. Interestingly, all pseudogenes in E. coli and Shigella begin with the same single-nucleotide deletion, resulting in a frame shift and downstream sequence deterioration. Formally, the strain 53638 also encodes elaD as a pseudogene (indicated with an asterisk), but careful inspection suggests that this is just the result of sequencing slippage in an adenosine-rich region of the elaD gene. APEC, NMEC, and UPEC belong to the group of extraintestinal pathogenic

Table 1. Expression profile of elaD in different E. coli strains.

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<th>Group</th>
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<td>EPEC</td>
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<tr>
<td></td>
<td>E22</td>
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<td>commensal</td>
<td>K12/W3110</td>
<td>Yes</td>
</tr>
<tr>
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<td>APEC O1</td>
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</tr>
<tr>
<td>NMEC</td>
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The gene encoding elaD can be found in all intestinal pathogenic E. coli strains, and in the commensal strain K12. The gene is absent in APEC, NMEC, and two uropathogenic strains, and encoded as a pseudogene in three other strains. Interestingly, all pseudogenes in E. coli and Shigella begin with the same single-nucleotide deletion, resulting in a frame shift and downstream sequence deterioration. Formally, the strain 53638 also encodes elaD as a pseudogene (indicated with an asterisk), but careful inspection suggests that this is just the result of sequencing slippage in an adenosine-rich region of the elaD gene. APEC, NMEC, and UPEC belong to the group of extraintestinal pathogenic strains (ExPEC).

The notion of a dynamic horizontal gene transfer involving deubiquitinases is further underscored by the distribution of ubiquitin-specific proteases in Chlamydiae, all pathogenic strains express CE peptides, except for C. pneumoniae, which instead encodes a homolog of the eukaryotic otubain-type deubiquitinating enzymes [47]. Also, one group of bacterial C48 peptidases in particular clusters close to the SENP8 homologs of Pezizomycotina (Figure 1). The sequence relationship and the shared habitat of these organisms-plant symbionts and phytopathogens vs. environmental fungi-raises the possibility of gene transfer between them [48]. From a functional perspective, we could show that a homolog of SENP8 in Pezizomycotina does exert ubiquitin-specific hydrolase activity, like previously characterized bacterial CE proteases (Figure 5). The dual specificity of SENP8 from the fungus G. zea towards ubiquitin and Nedd8 is not trivial. Although Nedd8 is arguably the closest relative to ubiquitin, there are sequence differences that require distinct conjugating machineries and deconjugating proteases [27,49]. In particular position 72-an arginine in ubiquitin, and an alanine in Nedd8-can act as a compatibility switch and a single replacement at this side chain can cause ubiquitin to mimic Nedd8 and vice versa [50,51]. In this respect, the recognition of both ubiquitin and Nedd8 by the SENP8 homolog of G. zea is in contrast to what has been observed in mammalian SENP8 [27].

One possible explanation for these observations is that CE proteases originally derived from a deubiquitinase, a protease specific for this most conserved eukaryotic protein. As CE peptides in eukaryotes structurally diversified to accommodate the evolving family of Ubls, protease counterparts in bacteria, viruses, and some deeply branched eukaryotes retained their specificity for the “ur-substrate” ubiquitin.

Together with the published literature, our data supports the notion that the clan of CE proteases was acquired by bacteria and viruses via horizontal gene transfer from eukaryotes. Why this family of enzymes forms such a particularly attractive substrate for genetic exchange is an intriguing question. The distribution of CE proteases in symbiotic and pathogenic prokaryotes and viruses is suggestive of a general role in host-microbe interactions, as exemplified by the Salmonella protease sseL [42].

MATERIALS AND METHODS

Phylogenetic Analysis

Protein and DNA sequence data were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), the Institute for Genomic Research (www.tigr.org), and the University of Wisconsin E. coli Genome Project (www.genome.wisc.edu). Protein sequence identifiers are listed in Table 2. Sequences containing and surrounding the catalytic core of the proteases were aligned with the ClustalX algorithm (default parameters) (bips.u-strasbg.fr/fr/Documentation/ClustalX) [52], manually edited with GeneDoc (www.psc.

The evolving family of Ubls, protease counterparts in bacteria, viruses, and some deeply branched eukaryotes retained their specificity for the “ur-substrate” ubiquitin.
Cloning, expression, and biochemical analysis of elaD and G. zeae SENP8

The full-length elaD gene (NCBI protein sequence identifier GI: 16130204) was amplified by PCR from the K12 strain BL21 (Novagen) and cloned into pET28a (Novagen). PCR was performed with the Platinum Supermix (Invitrogen), following the manufacturer’s instructions (Ta 55°C, 32 cycles) and with these primers for NdeI/XhoI insertion into pET28a: Forward: 5’ GCGGATCCGAATTCCAGTGAATGTTGCTGGATGACAGCTGACAGTTTGGATCACCTTG 3’; Reverse: 5’ GCTGCGAGGCGGTTTGGATCACCTTG 3’. The final cloning products were fully sequence confirmed with primers for the T7 promoter and terminator site of pET28a (Novagen primers 69348-3 and 69337-3). All three proteins were expressed in E. coli BL21(DE3) (Novagen) after induction with 1 mM IPTG for 3–4 hours at 37°C, and isolated with nickel NTA-Agarose under native conditions (see “The QIAexpressionist” handbook; http://www.qiagen.com/HP/QIAexpressionist). The initial purity was 5–10% (elaD and elaD313S) and 70% (G. zeae SENP8), respectively and improved after size exclusion chromatography (Superdex75-prep, Amersham) to a final purity of 30–40% (elaD and elaD313S) and >90% (G. zeae SENP8), as assessed by Coomassie staining. Importantly, the quality of the final preparations was identical for elaD and elaD313S. For the specificity screen, the proteases were expressed by in vitro transcription/translation in reticulocyte lysate (TNT T7 Quick coupled IVT kit, Promega) in the presence of 35S-methionine (PerkinElmer NEG709A), and then diluted 3–4× with a buffer containing Tris (50 mM, pH 7.5), NaCl (150 mM) and DTT (2 mM or 5 mM N-ethylmaleimide for the negative control), before 0.1 μg of the electrophilic probes were added for 20–40 min at room temperature. The AMC hydrolysis screen was conducted in the same dilution buffer as described above, with an additional 1 mg/ml bovine serum albumin, 100 nM to 20 μM of ubiquitin-AMC, SUMO1-AMC, and Nedd8-AMC (Boston Biochem) were incubated with various concentrations of bacterially expressed elaD or elaD313S at 27°C for the indicated time points. The G. zeae SENP8 experiments were conducted as endpoint measurements (100 nM AMC substrates, 60 nM protease, and 16 hours reaction time). Fluorescence was analyzed with a Spectramax M2 multi-detection microplate reader (Molecular Devices).

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Author Contributions

Conceived and designed the experiments: SM AC. Performed the experiments: SM GK AC. Analyzed the data: SM AC. Contributed reagents/materials/analysis tools: SM GK AC. Wrote the paper: HP SM GK AC. Other: Provided financial support and mentored the project: HP.

REFERENCES


