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Functional domains of the human epididymal protease inhibitor, eppin

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Eppin has two potential protease inhibitory domains: a whey acid protein or four disulfide core domain and a Kunitz domain. The protein is also reported to have antibacterial activity against Gram-negative bacteria. Eppin and its whey acid protein and Kunitz domains were expressed in *Escherichia coli* and their ability to inhibit proteases and kill bacteria compared. The Kunitz domain inhibits elastase (EC 3.4.21.37) to a similar extent as intact eppin, whereas the whey acid protein domain has no such activity. None of these fragments inhibits trypsin (EC 3.4.21.4) or chymotrypsin (EC 3.4.21.1) at the concentrations tested. In a colony forming unit assay, both domains have some antibacterial activity against *E. coli*, but this was not to the same degree as intact eppin or the two domains together. When bacterial respiratory electron transport was measured using a 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay, eppin and its domains caused an increase in the rate of respiration. This suggests that the mechanism of cell killing may be partly through the permeablization of the bacterial inner membrane, resulting in uncoupling of respiratory electron transport and consequent collapse of the proton motive force. Thus, we conclude that although both of eppin's domains are involved in the protein's antibacterial activity, only the Kunitz domain is required for selective protease inhibition.

Proteases are important in the regulation and modulation of a variety of biological processes. These include protein turnover, apoptosis, blood coagulation and the inflammatory response. Clearly, these processes must be carefully regulated to ensure that they are not inappropriately activated. One level of regulation is through the action of small proteins which function as protease inhibitors [1–3]. These molecules are often co-expressed with the molecules that they regulate and they have attracted interest as potential antiviral [4], antibacterial [5], antiparasitic [6], anticancer [7,8] and anti-inflammatory agents [4,9,10].

One recently discovered protease inhibitor is epididymal protease inhibitor (eppin). This protein is expressed in mammalian epididymal tissue [11,12] and also in the trachea [13]. The epididymis is a tubular structure in the male reproductive tract in which sperm mature and are stored. Three mRNAs encoding eppin (eppin-1, eppin-2 and eppin-3) are transcribed from a single gene. Eppin-1 and eppin-3 are translated to give

Abbreviations
CFU, colony forming units; elafin, elastase specific inhibitor; pNA, *p*-nitroanilide; SLPI, secretary leukocyte protease inhibitor; WAP, whey acidic protein; XTT, 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

identical protein sequences, whereas eppin-2 gives rise to a protein with a 22 amino acid sequence at the N-terminus, which is believed to act as a secretory signal sequence. Eppin-1 is expressed in the testis and epididymis, eppin-2 is expressed in the epididymis only and eppin-3 is expressed in the testis only [11]. The isoform of eppin that is expressed in the trachea remains to be determined. Functional studies carried out on eppin have focused on the protein lacking the signal sequence [14,15]. Two putative protease inhibitor domains can be identified in the protein by sequence analysis: an N-terminal whey acidic protein (WAP; also known as four disulfide core) domain and a C-terminal Kunitz domain [11]. In addition to these putative protease inhibitory motifs, eppin has been shown to have antimicrobial activity against Gram-negative bacteria [15]. Interestingly, the human eppin gene is located on chromosome 20 in a cluster of 13 other WAP domain containing gene sequences [13,16]. Some of the proteins expressed from these genes also have antibacterial activity. Both secretary leukocyte protease inhibitor (SLPI) and elastase (EC 3.4.21.37) specific inhibitor (elafin) have been found to kill Gram-positive and Gram-negative bacteria, suggesting that these proteins may play a role in the innate immune response [17,18]. These proteins have a dual role and also act as protease inhibitors [19,20]. The relationship between these activities is not well understood. Of the eleven remaining WAP domain proteins encoded on chromosome 20, nine have not yet been characterized [16]. *In vivo*, eppin is associated with the surface of ejaculated spermatozoa through a protein complex consisting of semenogelin 1, lactotransferrin and clusterin [14,21]. It has been speculated that this may enable eppin to provide protection for the spermatozoa against both bacteria and proteases [14,15].

Eppin has been suggested as a target for novel male contraceptive methods [22–26]. Immunization of *Macaca radiata* monkeys against eppin resulted in temporary infertility in seven out of nine animals tested; the infertility was reversible in five out of the seven cases [27]. The mechanism of this infertility is not known, but it suggests that the presence of functional eppin is required for successful fertilization.

In the present study, we express and characterize eppin lacking the N-terminal signal sequence, WAP and Kunitz domains from eppin in order to assign functions to them. We demonstrate that the Kunitz domain is solely responsible for elastase inhibitory activity of the molecule. In contrast, although both domains exhibit some antibacterial activity against Gram-negative bacteria, it appears that both are required for full activity.

Results

Expression and purification of eppin and its domains

Eppin, the WAP domain and the Kunitz domain could all be expressed in *Escherichia coli* (Fig. 1A–C). The WAP domain proved to be soluble and could be purified under native conditions. Typical yields were 1–2 $mg \cdot L^{-1}$ of original culture. Both eppin and the Kunitz domain were insoluble following expression and had to be extracted under denaturing conditions (6 M guanidine hydrochloride). The proteins were refolded by dialysis into $NaCl/P_i$. Final yields of soluble protein were approximately 0.2 $mg \cdot L^{-1}$ of bacterial culture. The structural integrity of the proteins was assessed using CD spectroscopy (Fig. 1D–F, Table 1). In all cases, spectral features were observed that were consistent nonrandom coil structures, suggesting that the proteins had been successfully refolded. Addition of the WAP and Kunitz domain spectra results in a spectrum similar to that obtained with eppin (Fig. 1G). An alternative test for folding and disulfide bond formation in proteins is to compare their mobilites on SDS/PAGE under reducing and nonreducing conditions [28,29]. The expressed proteins have different mobilities on tris-tricine gels depending upon whether they are pre-incubated in dithithreitol, or not (Fig. 1H).

Protease inhibition activity of eppin and its domains

Eppin and the two domains were compared for their ability to inhibit the proteases elastase, chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4). Both eppin and the Kunitz domain were able to inhibit elastase to a similar extent ($IC_{50} = 2.9 \pm 0.4$ μM and 3.5 ± 0.6 μM, respectively; Fig. 2). The limited solubility of these proteins meant that concentrations greater than approximately 12 μM were not possible, which accounts for some of the uncertainty in these values. No inhibition was observed with the WAP domain up to the highest possible concentration of this domain (50 μM). No inhibition of trypsin or chymotrypsin activity was observed with eppin or either of the domains (data not shown).

Antibacterial activity of eppin and its domains

The survival of *E. coli* XL-Blue cells exposed to eppin and its domains was assessed by a colony forming unit (CFU) assay. As previously reported

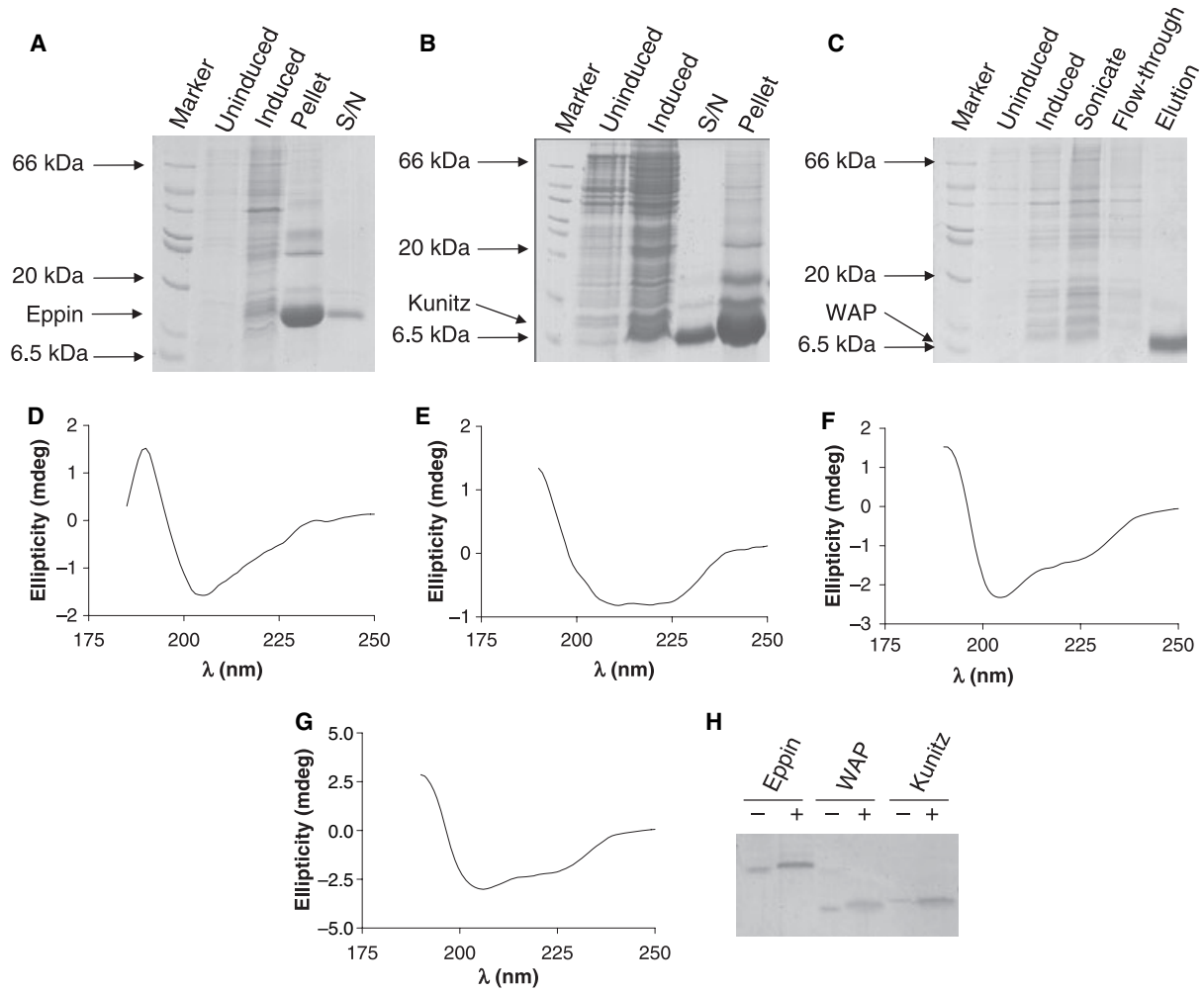

Fig. 1. Expression and purification of recombinant (A) eppin, (B) Kunitz domain and (C) WAP domain. Eppin and the Kunitz domain were purified under denaturing conditions followed by dialysis to remove the denaturing agent. The WAP domain was purified under native conditions. Uninduced and induced refer to cell extracts from a 1 mL sample of cells taken immediately before addition of isopropyl thio-β-D-galactoside and before harvesting. S/N refers to the supernatant following dialysis to remove guanidine hydrochloride and was the soluble sample used in further experiments with eppin and the Kunitz domain. The insoluble material following dialysis is referred to as the pellet. The sonicate is the material present after sonication and the flow through is the material that passed through the column. The elution is the soluble material present on elution of the WAP domain by 250 mM imidazole and was, following dialysis, used in further experiments with this fragment. CD spectra were obtained for: (D) eppin (10 μM), (E) the Kunitz domain (10 μM) and (F) the WAP domain (90 μM). Addition of the spectra (G) obtained for the WAP and Kunitz domains (dotted line) gives a similar spectrum to that obtained for eppin (solid line). To permit comparison, the spectra were normalized such that the highest positive ellipticity was set to equal 1.0. The three proteins have different mobilities on 15% tris-tricine SDS/PAGE (H) depending on the presence (+) or absence (–) of 130 mM dithiothreitol.

[15], eppin kills bacterial cells at a concentration of 3.5 μM after exposure for 180 min (Fig. 3). Equimolar amounts of either the WAP or Kunitz domain also killed the cells, but not to as great an extent as eppin. When both the WAP and Kunitz domains were incubated with the bacteria, the level of killing observed with eppin was restored. Longer exposure to the proteins (360 min) resulted in less apparent killing in all cases, but the overall trend was preserved (Fig. 3). This is probably due to some of the cells in the control samples dying, thus partly masking the effects of the proteins. Interestingly, these killing effects could only be observed in 10 mM sodium phosphate buffer; exposure of the cells to eppin (or its domains) in LB media resulted in no detectable reduction in cell viability (data not shown). This may be because actively growing cells in the presence of nutrients are more able to repair the damage caused by eppin (and its domains) than those maintained in phosphate buffer.

Table 1. Deconvolution of CD spectra of the three fragments. Spectra were collected as described in the Experimental procedures and deconvolved using the CDDSTR [50] as modified by Sreerama and Woody [51] within the CDPRO suite of software. Each protein was deconvolved with reference to the SMP50 basis set, which contains 43 soluble proteins and 13 membrane proteins. The estimated percentages of each secondary structure type are shown.

Protein	α-helix	β-sheet	Turn	Random
Eppin	46.1	25.6	10.6	17.3
Kunitz domain	21.1	22.7	17.3	33.9
WAP domain	68.0	11.2	7.2	13.0

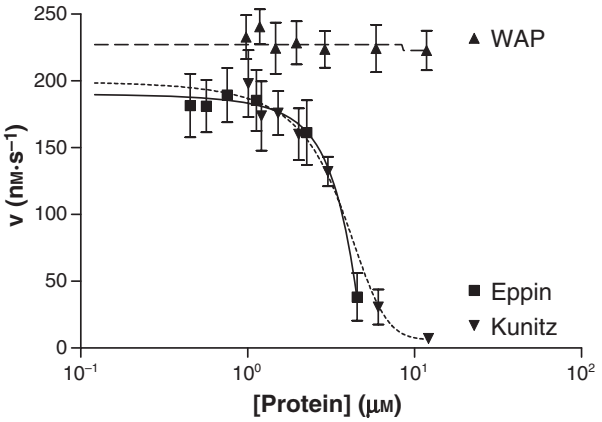

Fig. 2. Elastase (concentration, 50 nM) inhibition activity of eppin and its domains. Rates of Suc-Ala-Ala-Pro-Val-pNA cleavage were measured spectrophotometrically at various concentrations of eppin (■, solid line), WAP domain (▲, dashed line) and Kunitz domain (▼, dotted line). Lines were fitted by nonlinear curve fitting.

Effects of eppin and its domains on respiratory electron transport

It has been previously reported that exposure of *E. coli* cells to eppin results in permeablization of the bacterial cell membrane, which can be observed by electron microscopy [15]. Such permeablization is likely to lead to a disruption of the proton electrochemical gradient across this membrane and possible uncoupling of respiratory electron transport from proton translocation and, ultimately, ATP synthesis. This uncoupling may be a contributory factor in cell death. Therefore, the activity of respiratory electron transport was assessed by measuring 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction by live cells (Fig. 4). At all concentrations tested (0.7–3.5 μM), eppin and the Kunitz domain caused a substantial increase in the rate of XTT reduction. The lowest concentration of the WAP domain had a small, but detectable, effect. This effect increased in a concentration

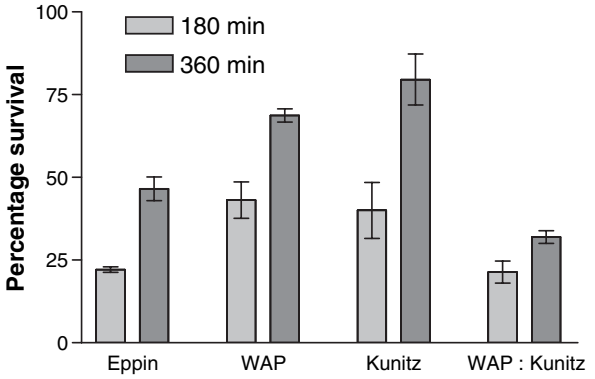

Fig. 3. Antibacterial activity of eppin and its domains. The survival of *E. coli* XL1-Blue cells exposed to 3.5 μM protein for 180 and 360 min was assessed using a CFU assay as described in the Experimental procedures. The bars represent the mean percentage survival of two samples of bacteria and the error bars the SDs of these means. Percentage survival was calculated from the fraction of treated cells surviving compared to a control sample (i.e. no added proteins) carried out in parallel. The SDs of the colony counts for the control samples were no greater than 16% of their means.

dependent manner. Following 24 h treatment of the bacteria with the proteins, no reduction of the XTT was observed (data not shown). This suggests that the bacteria were now dead and also that the reduction of XTT was due to enzymes of the bacterial respiratory electron transport chain and was not spontaneous or due to contaminants.

Discussion

The activities of the two putative protease inhibitory domains of human eppin have been assessed. Elastase inhibitory activity resides in the Kunitz domain with the WAP domain having no inhibitory activity against the proteases tested. This observation correlates with the finding that the amino-terminal WAP domain of SLPI similarly has no antiprotease activity [30]. As judged by the SIM alignment tool [31], the WAP domain of eppin has 42% sequence similarity with the N-terminal WAP domain of SLPI and only 36% sequence similarity with the C-terminal antiprotease WAP domain of SLPI [30]. This observation, combined with the theory that the WAP domain of eppin may be more defensin-like in function than other WAP domains [24], suggests that the lack of antiprotease activity by eppin's WAP domain is not surprising. This defensin-like function is also supported by our CD data, which suggest a higher percentage of α-helix than would be expected of a typical WAP domain. Although WAP domains are typically

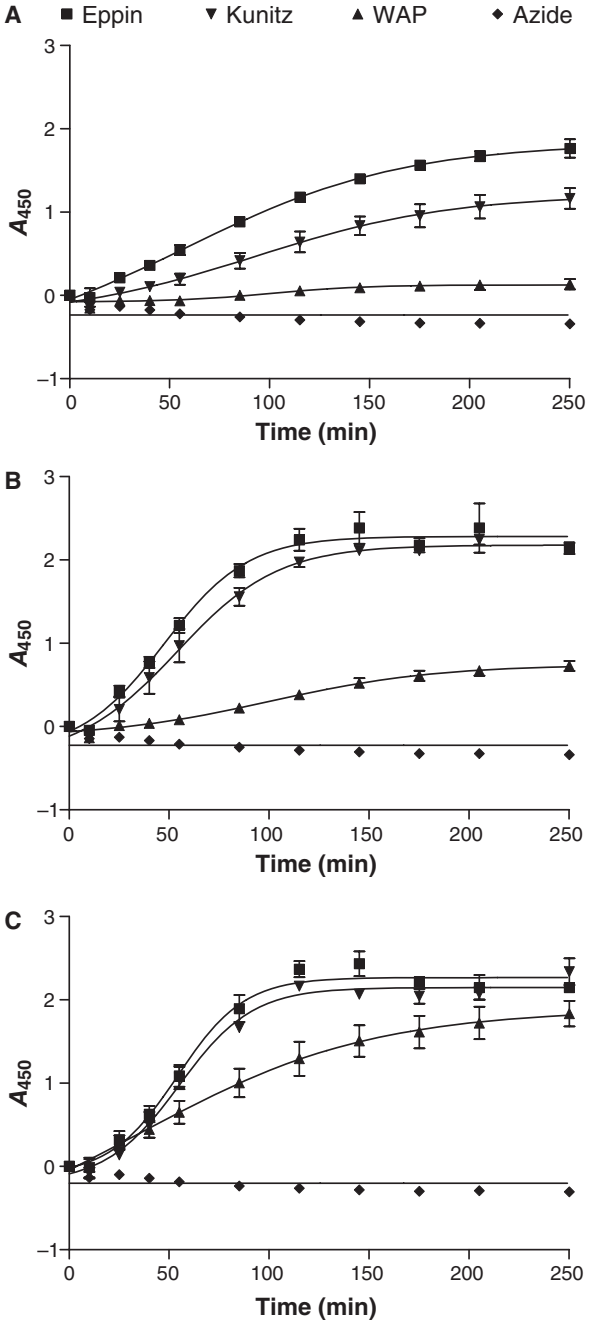

Fig. 4. Effects of eppin and its domains on respiratory electron transport. Electron transport was measured by monitoring the reduction of XTT as described in the Experimental procedures. The points represent the mean of four independent measurements and the error bars calculated as one SD of these means. The experiment was carried out at three different concentrations of protein or sodium azide (an inhibitor of electron transport): (A) 0.7 μM, (B) 1.7 μM and (C) 3.5 μM.

composed of β-sheet and random coil, some defensins have α-helical content [32–34]. WAP domains in other proteins, however, have been shown to have protease inhibitory activity. For example, the C-terminal domain of avian WAP domain and Kunitz domain-containing protein exhibits protease inhibitory activity, although this activity is restricted to the microbial proteases subtilisin and proteinase K [35,36].

By contrast, the C-terminal Kunitz domain of eppin is responsible for inhibition of the protease activity of elastase, as exhibited by the similar IC_{50} values of 2.9 μM and 3.5 μM recorded for eppin and the Kunitz domain, respectively. Isolated Kunitz domains from other proteins have also been found to exert antiprotease activity [37]. Therefore, the data presented here suggests that eppin, through the action of its Kunitz domain, may act as an antiprotease *in vivo*. The relatively weak inhibition towards elastase and the lack of detectable inhibition of trypsin and chymotrypsin suggests that the physiological target of eppin's antiprotease activity has yet to be discovered. By contrast, two well characterized elastase inhibitors, eglin C and ecotin, display nM and pM inhibition constants respectively [38,39]. Furthermore, the lack of observed protease inhibition by the WAP domain does not rule out the possibility that this domain may have activity against other proteases.

The location of eppin's antibacterial activity is less clear-cut. Both domains appear to retain some activity, but not at the same level as the intact protein. Furthermore, both domains appear to contribute to the up-regulation of respiratory electron transport, albeit with the Kunitz domain having a higher activity at lower concentrations compared to the WAP domain. The CFU assays, carried out over 3 and 6 h periods, suggest that intact eppin, rather than one distinct domain, is essential for the full antibacterial potential of the protein to be exerted. Bacterial survival dropped to approximately 20% following exposure of the bacteria to eppin at 3 h, whereas with exposure to either WAP or Kunitz domain, survival dropped to only 45% and 40% respectively. Interestingly, when bacteria were exposed to the two domains of eppin in solution together, survival again dropped to approximately 20%. This suggests that the individual domains of eppin, when exposed to each other in solution, may either be capable of reassociation to form an intact complex, or may act additively.

The up-regulation of respiratory electron transport, observed by the XTT assays, is consistent with a mechanism that involves uncoupling of proton translocation from electron transport. Similar results are observed with well characterized uncoupling agents such as 2,4-dinitrophenol [40]. We speculate that the permeablization of the bacterial cell membrane observed in other studies in relation to eppin and other WAP domain proteins [15,41] will permit the bidirectional diffusion

of protons and thus prevent the build up of a proton electrochemical gradient. The initial response of the cells (as observed in the present study) is to increase the rate of electron transport in an attempt to pump more protons across the membrane to compensate for the collapse in the proton electrochemical gradient. Eventually, the electron transport chain is unable to provide enough energy to maintain the proton electrochemical gradient, ATP production falls and, ultimately, the cells die, as demonstrated by 24 h incubation of the bacteria with eppin and its domains. Similar mechanisms have been proposed for other antibacterial proteins, such as magainin [42]. The mechanism by which eppin causes permeablization of the cell membrane remains to be discovered. The results obtained in the present study are interesting because they indicate that both domains are capable of causing respiratory uncoupling.

These data suggest that eppin acts as an antibacterial agent capable of killing Gram-negative bacteria through cell membrane permeabilization mechanisms. The WAP and Kunitz domains of eppin, although both capable of carrying out this function, cannot do so to the same extent as the intact protein. Conversely, eppin and the Kunitz domain can inhibit leukocyte elastase activity but the WAP domain does not share this function. This evidence suggests that eppin shares characteristics with SLPI and elafin, two other dual role WAP domain proteins. Eppin may have a role in innate male (and possibly female) immunity. Clarification of this role will be required before the molecule can be targeted by novel male contraceptives because it may not be desirable to reduce the potency of a component of innate immune system.

Experimental procedures

Expression and purification of eppin and its domains

An IMAGE clone [43] encoding the full length eppin gene (IMAGE clone ID 5165509) was used as a PCR template for the amplification of the three regions: the region encoding the WAP motif (residues 29–73); a second region encoding the Kunitz domain (residues 77–127) and the region incorporating both these domains and the spacer sequence between them (residues 22–133), thus encoding the intact eppin molecule, excluding the signal sequence (Fig. 5). The primers for these amplifications were designed such as to incorporate *Nco*I and *Xho*I restriction enzyme sites at the 5′ and 3′ ends of the amplification products respectively. The forward primers also incorporated codons encoding six histidine residues to facilitate subsequent purification of the

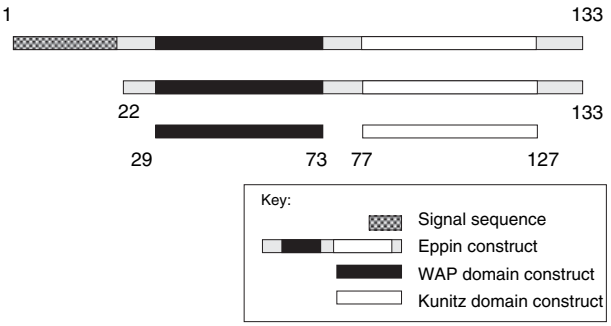

Fig. 5. Domain structure of eppin and constructs used in the present study. All the constructs were produced with N-terminal hexahistidine fusion tags to facilitate purification.

expressed proteins by Ni^{2+}-affinity chromatography. Following purification, the PCR products were cloned into the corresponding sites in pET21d (Novagen, Nottingham, UK). The DNA sequence of all constructs was verified (MWG Biotech, Ebersberg, Germany).

For expression, recombinant plasmids were transformed into *E. coli* BL21(DE3)[pLysS] cells. Overnight cultures of these cells (5 mL) were grown in LB (Miller) medium supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin and 34 $\mu g \cdot mL^{-1}$ chloramphenicol at 37 °C with shaking. These cultures were added to 1 L of fresh LB (plus 100 $\mu g \cdot mL^{-1}$ ampicillin and 34 $\mu g \cdot mL^{-1}$ chloramphenicol) and grown, shaking at 37 °C until A_{600} was in the range 0.6–1.0 (typically 3–5 h). The cultures were then induced with isopropyl thio-β-D-galactoside (final concentration 1 mM) and allowed to grow for a further 2–3 h. Cells were harvested by centrifugation (4200 ***g*** for 15 min) and resuspended in a buffer containing 50 mM Hepes-OH, pH 7.8; 150 mM NaCl; 10% (v/v) glycerol. Cell resuspensions were stored, frozen at −80 °C until required.

Recombinant proteins were purified as follows. Frozen cell suspensions were thawed and then sonicated on ice (three pulses of 30 s at 100 W with 15 s intervals between pulses for cooling). The sonicate was centrifuged at 27 000 ***g*** for 15 min and the resulting supernatant applied to a 1 mL nickel affinity column (His-Select; Sigma, Poole, UK) that had been previously equilibrated in wash buffer [50 mM Hepes-OH, pH 7.8; 500 mM NaCl; 10% (v/v) glycerol]. The column was then washed with 20 mL of wash buffer and the protein eluted in three 2 mL aliquots of wash buffer supplemented with 250 mM imidazole. Fractions containing protein (as judged by 15% SDS/PAGE) were dialysed overnight at 4 °C into NaCl/P_i (10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4). Purified proteins were stored in aliquots at −80 °C. Where purification under denaturing conditions was required, the pellet isolated following sonication was resuspended in buffer [50 mM Hepes-OH, pH 7.8; 150 mM NaCl; 10% (v/v) glycerol; 6 M guanidine hydrochloride] and the same procedure was followed as

above, except that all the subsequent buffers contained 6 M guanidine hydrochloride. In all cases, eluted fractions containing protein were dialysed overnight against NaCl/P_i.

Protease purification

The purification of human elastase was based on the method of [44]. Three volumes of sucrose extraction buffer (0.1 M sodium phosphate, 0.2 M sucrose, 1 M NaCl, pH 6.0) were added to 50 mL of human blood cells. The cells were homogenized and sonicated (six pulses of 30 s at 100 W with 30 s intervals between pulses). The lysed cells were kept for 1 h on ice and then centrifuged at 25 000 ***g*** for 40 min. The supernatant was retained and contaminating DNA was removed by the addition of DNase I (EC 3.1.21.1) (Calbiochem, Nottingham, UK) to a final concentration 33 000 unit·mL^{-1} (manufacturer's unit definition) and incubated at room temperature for 2 h. The pH of the mixture was adjusted to 8.0 with 2 M Tris and it was then centrifuged at 4200 ***g*** for 10 min. The supernatant was loaded onto a Sepharose T column (bed volume 7 mL) at 1 mL·min^{-1}. The column was washed with 1 L of washing buffer (0.05 M Tris-HCl, 1.0 M NaCl, pH 8.0) and then 60 mL of buffer D (0.05 M sodium acetate, 0.1 M NaCl, pH 5.0) was used to elute elastase in 3 mL fractions. The protein-containing fractions were pooled and dialysed against buffer E (0.02 M sodium acetate, 0.6 M NaCl, pH 5.5) with three changes at 1 h intervals and then overnight at 4 °C. An SP-sepharose column (bed volume 7 mL) was washed over with 30 mL of buffer E (0.02 M sodium acetate, 0.6 M NaCl, pH 5.5) at 1 mL·min^{-1} and the dialysate was loaded onto the SP column at the same flow rate. The flow-through was collected in 1 mL fractions and protein-containing fractions were pooled. These were then reapplied to the SP column pre-equilibrated in 50 mL of buffer G (0.02 M sodium acetate, 0.35 M NaCl, pH 5.5) and a further 30 mL of buffer G was then washed through the column. The elastase was eluted in 1 mL fractions with a linear NaCl gradient (60 mL) in the range 0.35–0.85 M. Protein-containing fractions were pooled and the purity assessed by SDS/PAGE. Bovine pancreatic trypsin and chymotrypsin were purchased from Sigma.

Analytical methods

Protein concentrations were estimated by the method of Bradford [45] using BSA (New England Biolabs, Hitchin, UK) as a standard.

CD spectroscopy

Measurements of CD spectra were made using a JASCO J810 spectropolarimeter (Jasco, Tokyo, Japan). Each experiment was carried out at 20 °C with the sample held in a demountable quartz cuvette. The pathlengths for each experiment were chosen to maximize the signal to noise for each sample: Eppin, 0.02 cm; WAP domain, 0.05 cm; Kunitz domain, 0.05 cm. All proteins were dissolved in NaCl/P_i.

Protease inhibition assays

The rate of elastase, chymotrypsin or trypsin hydrolysis of peptide bonds was measured using the chromagenic substrates Suc-Ala-Ala-Pro-Val-pNA, Suc-Ala-Ala-Pro-Phe-pNA and Bz-Phe-Val-Arg-pNa (Bachem, Weil am Rhein, Germany), respectively. Cleavage of these compounds results in the release of *p*-nitroanilide (pNA), which was measured spectrophotometrically using a LabSystems 352 platereader (Labsystems, Vienna, VA, USA) with a 405 nm filter. The K_m value for the appropriate substrate was determined for each enzyme under the conditions of the experiment. This was to ensure selection of a substrate concentration that would give a reproducibly measurable rate (i.e. not too low), which is likely to be affected by inhibitors (i.e. not too close to the maximal rate where the effects of competitive inhibitors would be minor). Inhibition assays were carried out in triplicate at 22 °C over a 5 min period using substrate concentrations equal to the experimentally determined K_m and enzyme concentrations of 50 nM in a total reaction volume of 250 μL. Initial rates of hydrolysis were calculated and IC_{50} values estimated using nonlinear curve fitting [46] as implemented in the program GRAPHPAD PRISM 3.0 (Graphpad Software, San Diego, CA, USA). All points were weighted equally.

Antibacterial assays

CFU assays were based on previously described methods [15,47,48]. Briefly, mid-log phase bacteria (*E. coli* XL1-Blue) were washed twice and resuspended in 10 mM sodium phosphate buffer, pH 7.4. The bacterial suspension was diluted in the same buffer to approximately 1×10^6 CFU·mL^{-1}. The resuspended *E. coli* cells were incubated with 0.7 μM, 1.7 μM and 3.5 μM of the proteins, at 37 °C. Aliquots were removed at 180 and 360 min and serially diluted with 10 mM sodium phosphate buffer; 100 μL of the diluted samples were spread on LB plates and incubated at 37 °C overnight. The following day, the resulting colonies were counted. Bacterial survival was calculated as the mean CFU in the presence of the proteins expressed as a percentage of the CFU of control samples (i.e. that had been incubated in buffer alone).

XTT assays

XTT (Sigma) assays were based on the method of McCluskey *et al.* [49] and used to measure the rates of respiratory activity of *E. coli* XL1-Blue. The bacteria were exposed to eppin or its domains at 37 °C and XTT reduction was measured spectrophotometrically using a LabSystems 352

platereader with a 450 nm filter for 250 min in 96-well plates (reaction volume 300 μL).

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