Mammals possess membrane-associated and cytosolic forms of the puromycin-sensitive aminopeptidase (PSA; EC 3.4.11.14). Increasing evidence suggests the membrane PSA is involved in neuromodulation within the central nervous system and in reproductive biology. The functional roles of the cytosolic PSA are less clear. The genome of the nematode Caenorhabditis elegans encodes an aminopeptidase, F49E8.3 (PAM-1), that is orthologous to PSA, and sequence analysis predicts it to be cytosolic. We have determined the spatio/temporal gene expression pattern of pam-1 by using the promoter region of F49E8.3 to control expression in the nematode of a second exon translational fusion of the aminopeptidase to green fluorescent protein. Cytosolic fluorescence was observed throughout development in the intestine and nerve cells of the head. Neuronal expression was also observed in the tail of adult males. Recombinant PAM-1, expressed and purified from Escherichia coli, hydrolyzed the N-terminal amino acid from peptide substrates. Preferred substrates had positively charged or small neutral amino acids in the N-terminal position. Peptide hydrolysis was inhibited by the metal-chelating agent 1,10-phenanthroline and by the aminopeptidase inhibitors actinomycin, amastatin, and leuvinestin. However, the enzyme was ~100-fold less sensitive toward puromycin (IC50 135 μM) than other PSA homologues. Following inactivation of the enzyme, aminopeptidase activity was recovered with Zn2+, Co2+, and Ni2+. Silencing expression of pam-1 by RNA interference resulted in 30% embryonic lethality. Surviving adult hermaphrodites deposited large numbers of oocytes throughout the self-fertile period. The overall brood size was, however, unaffected. We conclude that pam-1 encodes an aminopeptidase that clusters phylogenetically with the PSAs, despite attenuated sensitivity toward puromycin, and that it functions in embryo development and reproduction of the nematode.

Metalloaminopeptidases catalyze the removal of amino acids from the N terminus of oligopeptides. Aminopeptidases are found in a wide variety of cells; they can be membrane-associated or cytosolic, and they have been implicated in a wide variety of physiological processes, including protein processing, regulation of peptide hormone action, viral infection, and cancer cell proliferation (1). The puromycin-sensitive aminopeptidase (PSA1; EC 3.4.11.14) is a member of the M1 family of metallopeptidases, and, as such, it possesses the archetypal HEXXH(X)18E metal ion coordination site and the GAMEN motif characteristic of this family (2). A membrane-associated PSA is found in rat brain tissue (3), and studies using mouse mutants have shown that this isoform is involved in growth and behaviors associated with anxiety and pain (4). Further characterization has shown that this membrane PSA is essential for fertility in male and female mice (5, 6). The cytosolic isoform of rat PSA is also predominantly found in the brain, although there is also distribution among other organs (3). Neuropeptides, such as enkephalins, have been proposed as substrates for both isoforms of PSA, although the intracellular localization of the cytosolic PSA makes such activity questionable for this isoform. Interestingly, the cytosolic rat brain PSA has been shown to be developmentally regulated, leading to the proposal that the intracellular enzyme could be required for phasic processes related to the development of the central nervous system (7). The recent isolation of a Drosophila melanogaster cytosolic PSA mutant exhibiting defects in spermatogenesis indicates likely structure-function conservation of both isoforms of this aminopeptidase across evolutionary well-diverged species (8).

An aminopeptidase with increased sensitivity toward puromycin, relative to PSA, and with biochemical properties distinct from the ubiquitous PSA, has been shown to localize exclusively to neurons of the central nervous system of rats (9). The predominant intracellular localization of this enzyme and its developmental expression profile suggest a role in neuronal growth and differentiation (10). The in vivo substrates and precise physiological roles of these different aminopeptidases and their isoforms in the central nervous system remain to be elucidated.

Recently, human cytosolic PSA has been shown to hydrolyze cytotoxic T cell epitope precursors and hence contribute to the processing of major histocompatibility complex class I peptides (11, 12). The cytosolic isoform has also been implicated in cell cycle regulation, because puromycin causes cell cycle arrest of murine COS cells (13). Addition of a non-competitive inhibitor of PSA to a human acute lymphoblastic leukemia cell line also

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* This work was supported by a Biotechnology and Biological Sciences Research Council project grant (24/S12813) and forms part of the Medical Research Council Co-operative on Zinc Metallopeptidases in Health and Disease at the University of Leeds. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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caused reduced tumor cell invasion (14, 15). The identification within murine PSA of two motifs, conserved among 26 S pro- tease subunits and microtubule-binding sites, coupled with the subcellular localization of PSA to mitotic spindles in mam- malian cell lines, may offer some insight into the mode(s) of action of the enzyme (13, 16).

The sequencing of the entire genome of the nematode Caenorhabditis elegans (17), and the powerful molecular and ge- netic techniques applicable to this organism, make it an excel- lent model system for the study of biological processes. The C. elegans genome potentially encodes ~320 peptidases, which have been classified into five families on the basis of their catalytic mechanisms (18). The metallopeptidase family con- tains most members, because all are brain DEF genes, which are defined by classic genetic approaches to reveal diverse roles in develop- ment, growth, and behavior (19–23).

Materials and Methods

Nematode Culture and Transformation—The C. elegans Bristol strain (N2) was grown at 20 °C on NGM agar (1.7% (w/v) agar, 25 mM potassium phosphate, pH 6.0, 50 mM NaCl, 2.5 μg ml−1 peptone, 5 μg ml−1 chitin, 1 mM MgCl2, 1 mM CaCl2) supplemented with Esche- richia coli OP50. Young adult hermaphrodites were transformed by microinjection of the syncytial gonad with plasmid pLS47 (see below) and pRF4, which encodes the dominant selectable marker rol-6 in C. elegans. The recombinant enzyme was eluted from the soluble fraction by Ni-NTA affinity chromatography as described by the manufacturer. Subsequent DNA sequence analysis confirmed the integrity of the mutated plasmid, designated pLS35.

Expression and Purification of Recombinant PAM-1—E. coli TOP10 harboring pLS35 was grown at 37 °C to mid-log phase, and expression of PAM-1 was induced by addition of 0.2% (w/v) arabinose. Four hours post-induction, cells were harvested and recombinant PAM-1 was pu- rified from the soluble fraction by Ni-NTA affinity chromatography as described by the manufacturer (Qiagen, Crawley, West Sussex, UK). The recombinant enzyme was eluted from the Ni-NTA column with 50 mM imidazole, and 4 × 1-m1 fractions were collected and analyzed by SDS-PAGE. The eluate containing purified PAM-1 was passed through a 30 μm disposable concentrator (Flowgen, Lichfield, Staffordshire, UK) to allow buffer exchange with 100 mM Tris-HCl, pH 7.6. The recombinant enzyme was stored at −20 °C in 50% (v/v) glycerol. Protein quantification was carried out using the bicinchoninic acid assay (Pierce, Rock- ford, IL) and bovine serum albumin as the standard.

Immunoelectrophoretic Blot Analysis—Samples were electroblotted onto a polyvinylidene difluoride membrane and blocked using TBS/0.1% (v/v) Tween 20/5% (v/v) milk powder. Detection utilized goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:10,000 in TBS/0.1% (v/v) Tween 20) (Sigma-Aldrich), followed by enhanced chemiluminescence (ECL Plus) as described by the manufac- turer (Amersham Biosciences, Uppsala, Sweden).

Biochemical Assays—Amino acid methyl coumarin (AMC) substrates were purchased from Sigma-Aldrich and Bachem (Bubendorf, Switzer- land). Assays were performed at ambient temperature in a Wallac Victor2 (PerkinElmer Life Sciences, Beaconsfield, Buckinghamshire, UK) microplate reader adapted with emission and excitation filters of 380 and 460 nm, respectively. Each assay was performed in 50 mM Tris-HCl, pH 7.6, and comprised 10 ng of recombinant PAM-1 and a range of substrate concentrations between 1 μM and 0.3 μM in the presence of 1 mg ml−1 bovine serum albumin. Inhibitors were purchased from Sigma-Aldrich and preincubated for 15 min at varying concentrations with enzyme in the assay buffer. Inhibitor assays were initiated by addition of 10 μM Arg-AMC. Enzyme kinetics was determined using WinPlot (PerkinElmer Life Sciences) and EnzPack (Biosoft) software. The IC50 values were calculated from semi-log plots with a minimum of nine different concentrations of inhibitor.

Results

Sequence Analysis of the C. elegans Gene Encoding PAM-1—A full-length expressed sequence tag (yk348g1) derived from a him-8 (high incidence of males) mixed-stage cDNA library, was completely sequenced by primer walking, confirming the 884-amino acid PAM-1 sequence prediction (CE10790) in the C. elegans database (www.wormbase.org). Examination using SignalP (version 1.1, www.cbs.dtu.dk/services/SignalP)
of the genomic sequence upstream of the designated start methionine codon did not reveal the presence of a potential signal peptide sequence, and hence PAM-1 is predicted to be an intracellular aminopeptidase. The amino acid sequence contained the active site residues HEXXH[X]18E and GAMEN, as expected for a member of the M1 family of aminopeptidases (Fig. 1A). Residues 227–251 of PAM-1 show 46% identity, 71% similarity, to the putative \( \alpha \)-type proteasome subunit motif identified in the murine PSA (13). The microtubule-binding site motif identified in mouse PSA (13) is poorly conserved in PAM-1, and an additional 0.5 kb of genomic sequence upstream of the designated start met-exon 1 and 2 of the \( \text{pam-1} \) gene. The F1 larvae appeared to develop normally to adulthood. During the initial 24-h time period of self-fertility, a small number of oocytes were laid by RNAi-treated nematodes, whereas oocytes were never observed on control plates during this time period (data not shown).

**Expression Pattern Analysis of PAM-1**—The spatial/temporal expression pattern of PAM-1 was studied by generation of transgenic \( \text{C. elegans} \) expressing extrachromosomal arrays of the \( \text{pPD95.69} \) plasmid engineered to comprise 4.6 kb of sequence immediately 5' of \( \text{pam-1} \) and an additional 0.5 kb of \( \text{pam-1} \) sequence, generating a second exon translational fusion to GFP (pLS47). Independent lines of transgenic \( \text{C. elegans} \) expressing the reporter transgene showed fluorescence in embryo, larval, and adult stages (Fig. 2). Embryonic reporter gene activity was observed around the time of gastrulation and continued throughout development (Fig. 2, A–D). Strong fluorescence was observed in the intestinal cells of larvae and adults, and this was particularly enhanced in the cells of the posterior gut (Fig. 2E). Larval and adult fluorescence was also observed in neuronal cell bodies around the nerve ring and in processes extending toward the tip of the nose, indicative of them being the amphid sensory neurons (Fig. 2F). The expression pattern in males was determined by mating males (N2, Bristol strain) with hermaphrodites expressing the \( \text{pam-1} \)-\( \text{gfp} \) transgene. In addition to the components described above, adult males showed transgene neuronal expression in the developing and mature male tail (Fig. 2, G and H).

**\( \text{pam-1} \) Has Roles in Embryogenesis and Reproduction**—RNA interference was carried out by allowing the \( \text{RNAi-sensitive mutant} \) \( \text{rrf-3} \) to feed on bacteria expressing dsRNA targeting exons 1 and 2 of the \( \text{pam-1} \) gene. The F1 larvae appeared to develop normally to adulthood. During the initial 24-h time period of self-fertility, a small number of oocytes were laid by \( \text{RNAi-treated nematodes} \), whereas oocytes were never observed on control plates during this time period (data not shown).

During the second and third 24-h time periods of self-fertility, the frequency of oocytes laid by \( \text{RNAi-treated animals} \) was significantly greater (Student’s \( t \) test, \( p < 0.0001 \)) than by \( \text{RNAi-free nematodes} \) not expressing dsRNA (Fig. 3A). The penetrance of the phenotype was \( -60\% \), and overall, \( -10 \)-fold more oocytes were laid by animals subjected to dsRNA (243 \( \pm \) 7, \( n = 20 \)) than controls (24 \( \pm \) 3, \( n = 7 \)). The brood size for the
The C. elegans Puromycin-sensitive Aminopeptidase

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Effect of Inhibitors—The inhibitor profile of PAM-1 was determined by measuring the rate of hydrolysis of Arg-AMC in the presence of different inhibitors (Table II). The recombinant PAM-1 was insensitive to inhibition by the serine protease inhibitor, phenylmethylsulfonyl fluoride, and the cysteine protease inhibitor, E-64. In contrast, the enzyme was sensitive to the chelating agent, 1,10-phenanthroline, as would be expected for a metallopeptidase. The aminopeptidase inhibitors actinonin, amastatin, and leuhalten were the most potent inhibitors of PAM-1 (IC50 values 0.1–0.6 μM). Puromycin inhibited the enzyme with an IC50 value of 135 μM (Fig. 5).

Metal Ion Reactivation of PAM-1—Following complete inactivation of PAM-1 in 4 mM 1,10-phenanthroline, enzyme activity was recovered by addition of Zn2+, Ni2+, and Co2+ (Fig. 6). Zn2+ was the most effective ion, restoring the greatest enzyme activity at all concentrations between 1 and 180 μM. No restoration of enzyme activity was obtained using Cu2+, Mn2+, Mg2+, Fe2+, and Hg2+ at concentrations between 0.001 and 2 mM.

DISCUSSION

The present study describes the role of the puromycin-sensitive aminopeptidase, PAM-1, in the biology of the free-living nematode C. elegans. The expression pattern analysis shows that PAM-1 is expressed throughout development, and the neuronal component is consistent with PSA localization reported for other organisms (4, 31). This may reflect an evolutionary conserved role for PSA in the processing of neuropeptides. The precise nature of such a role remains controversial, although studies with mammalian brain thimet oligopeptidase, and neurolysin, demonstrate that cytosolic metallopeptidases are able to function in neuropeptide metabolism (32). One possibility is that PAM-1, which appears to lack a signal sequence, may enter the secretory pathway by a non-classic mechanism, as described for thimet oligopeptidase (33) and neurolysin (34). The alternative possibility is that neuropeptides are internalized into neuronal cells by surface receptors, as demonstrated for neurotensin (35) and opioids (36), and following release, they are processed by cytosolic peptidases. The strong intestinal reporter gene expression indicates that PAM-1 may also function in the processing of peptides, absorbed by the intestinal cells following breakdown of proteins in the gut lumen. Such a function has been proposed for the C. elegans homologues of aminopeptidase P (37) and leucine aminopeptidase (23). Indeed, it is not surprising that a plethora of proteases, including cathepsin B-like (38, 39) and cathepsin L-like (40) cysteine proteases and aspartic proteases (41), is expressed in the intestine, where they are able to function in digestion.

The family of Phe-Met-Arg-Phe amide-related neuropeptides (FaRPs) are extensively expressed in neurons and gland cells of C. elegans (42, 43), and members are therefore potential PAM-1 substrates. Disruption of one FaRP encoding gene, frp-1, results in multiple behavioral defects, including nose-touch insensitivity (44, 45). Recent work using a combination of fluorescence-activated cell sorting and microarray analyses
indicates that PAM-1 might be present within *C. elegans* touch receptors (46), which is consistent with the *pam-1* expression pattern reported herein, and hence the possibility exists that it may metabolize FaRP-1. However, examination of the *C. elegans* RNAi-sensitive mutant *rrf-3* (28) following *pam-1 (RNAi)* did not reveal an observable phenotype related to touch receptor deficiency. Nevertheless, the refractory nature of neurally expressed genes to RNAi is well documented, and it remains a formal possibility that PAM-1 may be involved in sensory perception. RNAi did, however, result in two observ-

\[\text{TABLE I}\]

| Kinetic constants for the hydrolysis of amino acid AMC substrates by PAM-1 |
|-----------------|------|----------|-----------------|
| AMC substrate  | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
| Arg             | 34   | 0.2      | 6.7             |
| Lys             | 78   | 0.5      | 6.2             |
| Leu             | 63   | 0.4      | 6.1             |
| Ala             | 97   | 0.4      | 4.2             |
| Met             | 59   | 0.3      | 3.1             |
| Tyr             | 73   | 0.1      | 1.8             |
| Phe             | 160  | 0.2      | 1.1             |
| Gly             | 390  | 0.4      | 0.9             |
| Ser             | 840  | 0.3      | 0.3             |

\[\text{TABLE II}\]

| Effect of inhibitors on the activity of PAM-1 toward Arg-AMC |
|-----------------|------|
| Inhibitor       | IC$_{50}$ |
| Actinonin       | 0.1 |
| Amastatin       | 0.2 |
| Leuhistin       | 0.6 |
| Apstatin        | 22  |
| Bestatin        | 25  |
| Puromycin       | 135 |
| Arphamenine     | 131 |
| Phenanthroline  | 155 |

\[\text{Fig. 3.} \text{ Silencing pam-1 by RNAi shows that it has roles in reproduction and embryogenesis. Numbers of oocytes, embryos, and progeny were recorded throughout the adult hermaphrodite self-fertile period, showing that the RNAi-treated animals laid a high proportion of oocytes (A), whereas brood sizes were not significantly different (B). RNAI-treated, } n = 20; \text{ control, } n = 7. 
\text{ The experiment was repeated in triplicate. C, brightfield images of a typical embryo following arrest during the early stages of gastrulation due to pam-1 (RNAi) (i) and a wild-type embryo undergoing gastrulation (ii).}\]

\[\text{Fig. 4.} \text{ Expression and purification of the 10× His tag/Xpress leader epitope/PAM-1 fusion protein in E. coli. A, SDS-PAGE analysis of insoluble (1) and soluble (2) lysates prepared 4 h post-induction with 0.2% (w/v) arabinose. PAM-1 is highlighted with the arrow. B, post-purification analysis of the soluble lysate by Coomassie Brilliant Blue staining of an SDS-polyacrylamide gel; C, Western blot of the purified extract using the anti-6× His tag antibody.}\]

\[\text{D. R. Brooks, unpublished data.}\]
Inositol triphosphate signaling pathway results in ovulation of oocytes available for fertilization. Disruption of the activation pattern confirms that pam-1::gfp treated and control animals. The reporter gene expression in germ line cells was not unexpected given that C. elegans reporter genes rarely express in these cells (52). Microarray analyses of C. elegans mutants defective in development of either the male or female germ line indeed suggest pam-1 as a candidate for expression in both germ lines (53).

The RNAi data reported in this study also demonstrate that pam-1 is required for embryogenesis, hence extending the physiological roles attributed to PSA. A role in embryo development is consistent with the embryonic reporter gene expression pattern reported herein, and it may reflect an essential role in the cell cycle for pam-1, despite the lack of a recognizable microtubule-binding site motif (13). It seems reasonable to speculate that pam-1 processes bioactive peptides involved in signaling events within the germ lines and subsequently during embryogenesis. The presence of a potential α-type protease som subunit motif within pam-1 may offer an insight into its function. As previously proposed (13) and recently demonstrated with mammalian cells (11, 12), PSA may function downstream of the proteasome, with the resulting peptides targeted for release at the cell surface. During C. elegans embryogenesis, the yolk proteins, or vitellogenins, are the major sources of nourishment (54), and hence an alternative explanation for the embryonic lethal phenotype is that pam-1 may be required to complete digestion of these vital nutrients.

The expression and purification of recombinant pam-1 allowed analysis of the biochemical properties of the peptidase. With respect to substrate specificity, the enzyme exhibited similarity to cytosolic PSAs of mammals (13, 31, 55, 56), preferring peptides with either a positive charge or small neutral amino acid at the N terminus. The determined Km and catalytic efficiency (kcat/Km) values of pam-1 and human liver PSA (56) show good agreement, whereas pam-1 substrate binding affinity is approximately an order of magnitude less than reported for rat brain PSA (9). This discrepancy in catalytic efficiencies might reflect limitations of the recombinant pam-1 enzyme preparation, such as incomplete folding, relative to the rodent PSA, which was purified to homogeneity from rat brain (9).

The inhibitor profile of pam-1 is very similar to other PSAs (9, 56) with the notable exception of inhibition by puromycin. The Pam-1 IC50 constant for puromycin (135 μM) indicates that the nematode enzyme has significantly reduced sensitivity toward this drug. Indeed, human PSA is completely inhibited by 100 μM puromycin (56), and IC50 values are reported to be of the order of 1 μM (57). In this respect, pam-1 is more like aminopeptidase N (Km ~ 100 μM) (58). However, we cannot exclude the possibility that the N-terminal tag present on the recombinant nematode enzyme is influencing binding of puromycin, even though it appears not to affect interactions with the other inhibitors tested.

Divalent cations are essential for metallopeptidase activity, and they bind to the two histidine residues and the distal glutamate present within the active site motif, HEXXH/XH18E, of the M1 class of metallopeptidases. Different metal ions can be exchanged within the active site of metallopeptidases, resulting in modulation of catalytic efficiency (59). Following inactivation by metal ion chelation, the activity of Pam-1 could be recovered using Zn2+, Ni2+, and Co2+. Both Zn2+ and Co2+ were able to restore activity to metal ion-depleted mammalian PSA (57). Physicochemical analysis would be required to determine which of these metal ions resides within the active site, although the profile of recovery observed with Pam-1 would suggest Zn2+ as the most likely candidate.

In summary, we have demonstrated using RNAi that the
C. elegans PSA orthologue, PAM-1, is involved in reproduction and embryogenesis. The gene expression pattern analysis shows that pam-1 is expressed throughout development, and spatio components indicate that it is also likely to function in the nematode nervous system and in digestion. The biochemical characterization of recombinant PAM-1 shows that many properties are similar to mammalian PSA, with the exception that inhibition of the nematode peptidase by puromycin is attenuated.

Acknowledgments—We thank Alan Coulsen (Sanger Centre, Hinxton, Cambridgeshire, UK) for cosmids F49E5, Yuji Kohara (National Institute of Genetics, Mishima, Japan) for the expressed sequence tag clone yk348g1, Andrew Fire (Carnegie Institute of Washington, Baltimore, MD) for the laboratory vector kits containing plasmids pPD95.69 and pPD129.36, and Theresa Stiernagle (C. elegans Genetics Centre, University of Minnesota, MN) for the RNAi-sensitive mutant rrf-3.

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