vations on the absence of metabolism of these phosphinic peptides in vivo can be generalized, this will considerably extend the utility of these compounds to the probing of many aspects of the function of this family of proteases in vivo.

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Conserved roles for peptidases in the processing of invertebrate neuropeptides
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Abstract
Invertebrates use a wide range of peptides as transmitters and hormones to regulate complex behaviour, physiology and development. These animals, especially those that are amenable to genetic study and are the subject of genome-sequencing projects, provide powerful model systems for understanding the functions of peptidases in controlling the bioactivity of peptides. Neprilysin, a zinc metallopeptidase and a key enzyme in the metabolism of mammalian peptides, is also implicated in the inactivation of peptides at synapses and of circulating peptide hormones in insects and nematodes. A family of neprilysin-like genes are present in the genomes of both Drosophila melanogaster and Caenorhabditis elegans; in C. elegans it seems that individual family members have evolved to take on different physiological functions, because they are expressed in a tissue-specific manner. Angiotensin I-converting enzymes (peptidyl dipeptidase A, angiotensin-converting enzyme) are another group of zinc metallopeptidases found in some invertebrates that lack angiotensin peptides. In D. melanogaster there are two functional angiotensin-
converting enzymes that are essential for normal development. One of these (Acer) is expressed in the embryonic heart, whereas the second enzyme (Ance) is expressed in several tissues at different stages of the life cycle. The accumulation of Ance within secretory vesicles of some peptide-synthesizing cells suggests a role for the enzyme in the intracellular processing of insect peptides. Ance is very efficient at cleaving pairs of basic residues from the C-terminus of partly processed peptides, suggesting a novel role for the enzyme in prohormone processing. Invertebrates will continue to provide insights into the evolutionarily conserved functions of known peptidases and of those additional family members that are expected to be identified in the future from genome-sequencing projects.

Introduction

Regulatory peptides are the largest and structurally the most diverse class of signalling molecules in invertebrates. They serve as transmitters and modulators in the nervous system and as hormones controlling key physiological processes, complex behaviours and development. In some invertebrates the expression of peptide genes is so common in the central nervous system that it is sometimes difficult to find a non-peptidergic neuron (e.g. in nematodes at least 70% of the total number of approx. 300 neurons are believed to be peptidergic). It is likely that the mechanisms central to the functioning of peptides as signalling molecules have been conserved during the course of metazoan evolution; it is therefore widely accepted that invertebrates with relatively simple nervous and endocrine systems can be exploited to increase our understanding of how peptides can regulate behaviour and development. This is especially true for invertebrates that are amenable to genetic studies and are the subject of genome-sequencing projects (i.e. Caenorhabditis elegans and Drosophila melanogaster). Such a comparative approach might prove useful in identifying conserved fundamental mechanisms of biosynthesis, inactivation and receptor-mediated signalling of regulatory peptides.

Peptidases have long been recognized as key components of the peptidergic system, being involved in both the biosynthesis of active peptide from the prohormone polypeptide and in peptide inactivation after release from the nerve terminal or endocrine cell. We have been studying the biochemistry and molecular biology of peptidases, especially zinc metallopeptidases, responsible for the processing and inactivation of regulatory peptides in insects and nematodes. Neprilysin (NEP; neutral endopeptidase 24.11, enkephalinase, EC 3.4.24.11) and angiotensin-converting enzyme (ACE) are two prototypical mammalian zinc metallopeptidases involved in the metabolism of neuropeptides in the brain and peptide hormones in the circulation [1,2]. The sizes of these families are increasing, particularly as a result of the genome-sequencing projects, and it is clear that the next major challenge will be to assign physiological functions to the more recently discovered zinc metallopeptidases. Here we review what is known about the NEP and ACE families in insects and nematodes and speculate about their function.

Regulatory peptides in insects and nematodes

More than 200 bioactive peptides have been identified from numerous insect species; most can be grouped into classes of closely related structures that have been conserved in a variety of insect species (reviewed in [3]). Many of these peptides are small (5–15 residues) and have an amidated C-terminus that is normally vital for full biological activity and provides protection from degradation by carboxypeptidases present in the extracellular environment. Some peptides are also protected at the N-terminus from general aminopeptidases by the presence of pyroglutamate (e.g. the adipokinetic and hypertrehalosaemic peptides) or a proline residue in position 2 (e.g. many of the insect tachykinins) [3]. Larger peptides (20–40 residues) are also well represented and include peptides showing structural similarity to mammalian insulin and corticotropin-releasing factor (CRF)-like peptides [3,4].

Although immunocytochemical studies indicate the presence of a variety of neuropeptides in nematodes, only the FaRPs (FMRFamide-related peptides) have been studied in any detail. Many members of this peptide family have been extracted from both parasitic and free-living nematodes and a search of the C. elegans genome sequence has led to the identification of 20 genes (fpr) coding for possibly more than 50 distinct FaRPs [5]. Some of these peptides have a role in locomotion, feeding behaviour, touch sensitivity and osmotic avoidance [6]. The RFamide C-terminus is essential for potent activity on the nematode somatic musculature. In addition to the
Neuropeptide-degrading NEPs

NEP is the prototypical member of the M13 family of zinc metallopeptidases, which also includes endothelin-converting enzymes (ECE-1 and ECE-2), XCE (a peptidase of unknown function), Kell (a blood-group antigen), PEX (involved in the regulation of bone metabolism) and SEP (a soluble secreted endopeptidase) [7,8]. NEP is responsible for the metabolic inactivation of peptides (e.g. substance P, enkephalin peptides and natriuretic peptides) in the brain and circulation of mammals [1]. Apart from SEP, they are type II integral membrane glycoproteins of around 700 amino acid residues that are anchored to cellular membranes by an uncleaved signal peptide [7]. The active site of NEP is positioned extracellularly and is well positioned to hydrolyse peptide substrates at the cell surface. A characteristic common to all NEP/ECE family members is inhibition by phosphoramidon, a property that has proved to be a useful tool in the characterization of NEP-like activities from invertebrate species. NEPs from both vertebrate and invertebrate tissues have very similar substrate specificities, cleaving peptide bonds that comprise the amino group of a hydrophobic residue [9–13].

A membrane associated NEP-like activity has been found in tissues from the housefly (Musca domestica), D. melanogaster, the gypsy moth (Lymantria dispar), the locust (Schistocerca gregaria) [13–16] and the cockroach (Leucophaea maderae) (D. R. Nassel and R. E. Isaac, unpublished work). The enrichment of this activity in synaptic membranes from S. gregaria is consistent with a possible role for the neural enzyme in the inactivation of neuropeptide transmitters [15]. Histochemical staining for NEP in the brain of the locust and cockroach has established that the enzyme is localized to neuropile regions containing terminals of interneurons. These interneurons contain tachykinin-related peptides, suggesting that insect NEP is involved in the metabolism of these peptides at synapses. As in mammals, insect NEP has a broad tissue distribution and it is therefore not surprising to find that NEP, associated with peripheral tissues, has been implicated in the metabolism of circulating locust adipokinetic hormone, a metabolic hormone responsible for mobilizing triacylglycerols during flight [17].

The metabolism of peptides has been studied in the parasitic nematode Ascaris suum [12] and the free-living C. elegans. In both species a NEP-like activity has been identified that, like NEP, recognizes peptide bonds incorporating the amino group of hydrophobic amino acids and is inhibited by phosphoramidon. The C. elegans enzyme cleaved the nematode neuropeptide PF1 (Ser-Asp-Pro-Asn-Phe-Leu-Arg-Phe-NH₂) at multiple cleavage sites, each comprising a hydrophobic residue (Asn⁴-Phe⁴, Phe⁵-Leu⁶ and Arg⁷-Phe⁸). Although associated with a membrane fraction, the C. elegans NEP-like activity did not partition into Triton X-114, suggesting that it is not an integral membrane protein (R. E. Isaac, unpublished work).

The sequencing of the entire genome of the fly D. melanogaster and the nematode C. elegans has provided a complete inventory of the M13 class of NEP/ECE-like genes in these two animals and an insight into the structural diversity of this family of proteins. BLAST searches for NEP/ECE-like proteins identified 24 and 16 genes with high TBLASTN scores in the fly and worm genomes respectively. Many, but not all, of the predicted proteins encoded by these genes contain the highly conserved active-site residues and motifs of the prototypic mammalian NEP. It is not clear at present whether the members of this relatively large family of NEP-like peptidases are all functional but 15 and 10 of the family members in D. melanogaster and C. elegans respectively are known to be expressed and are therefore presumed to have a physiological function. The expression patterns of some of the C. elegans NEP genes have been studied by using promoter-reporter fusion genes revealing tissue-specific expression, which probably reflects differences in their physiological roles (P. Stancombe, unpublished work).

ACE

ACEs are primarily dipeptidyl carboxypeptidases, removing dipeptides from the C-terminus of oligopeptides. From the comparative biochemical standpoint it is helpful to define the properties of an ACE, on the basis of the known properties of the mammalian enzyme [18]. In addition to being a dipeptidyl carboxypeptidase, our definition of an ACE includes the ability to convert angiotensin I into angiotensin II as well as the hydrolysis of bradykinin and the inhibition of peptidase activity by low concentrations (less than 1 µM) of mammalian ACE inhibitors (e.g. captopril, lisinopril, trandolapril and fosinoprilat). The housefly (M. domestica) enzyme was the first insect ACE to be studied and it was clear that the enzyme could be
bradykinin-like peptides in insects. Moreover, a classified as an ACE on the basis of the definition set out above [19,20]. However, the physiological substrates for insect ACE still remain largely unknown. Despite the isolation and characterization of over 200 insect peptides, there is no evidence for the existence of angiotensin I and bradykinin-like peptides in insects. Moreover, a search of the D. melanogaster genome does not reveal any potential homologues of angiotensinogen and kininogen, the precursor proteins of angiotensin I and bradykinin.

There are two D. melanogaster ACEs, known as Ance and Acer. Unlike mammalian somatic ACE, they are single-domain proteins without a membrane anchor [21-23]. The sequencing of the Drosophila genome identified a further four D. melanogaster ACE-like genes on the second chromosome. Of these six genes, only two (Ance and Acer) are known to code for active zinc metallopeptidases. Very little is known about the properties of Acer, apart from the following: (1) its expression during embryogenesis is restricted to the developing heart, (2) it is expressed during late pupal development, and (3) it fails to convert angiotensin I into angiotensin II. Thus, unlike Ance, Acer only partly satisfies our definition of ACE. Another difference between Ance and Acer is that Acer is much more efficient at removing C-terminal dipeptideamide from partly processed oligopeptides possessing a C-terminal dipeptideamide. Recently we reported a novel prohormone-processing activity of insect ACE, which might also be relevant to the role of the enzyme in reproduction in insect and mammalian species. Both Drosophila Ance and human germinal ACE efficiently remove basic dipeptides from partly processed oligopeptides possessing a C-terminal GKR and GRR extension. GKR or GRR-extended peptides representing sequences of the prohormones of mammalian gastrin, cholecystokinin, luteinizing-hormone-releasing hormone, enkephalins, thyrotropin-releasing hormone and the locustamyotropin/leucopyrokinin/pheromone-biosynthesis-activating neuropeptide ('PBAN')/diapause hormone family of insect peptides are high-affinity substrates for both insect and mammalian ACE [27-29]. Because the cleavage of pro-proteins at pairs of basic residues is a very common event in both insects and mammals, it is conceivable that ACE is a general processing enzyme responsible for the final stages of the maturation of peptides required for meiosis, sperm maturation, the successful fertilization of ova, the facilitation of egg-laying or the modulation of reproductive behaviour.

A P-element insertion in the Acer gene is embryonic lethal, indicating an important role for the enzyme at this stage of development. Our laboratories, in collaboration with other groups, are currently defining the role of this alternative form of insect ACE with genetic and biochemical methods. However, at present we have few clues as to the identity of the Acer substrates and the processes regulated by the enzyme, because most insect peptides, including the GKR and GRR-extended peptides, are very poor substrates indeed [24].

No ACE-like activity has been found in nematode tissues, although a single copy of an ACE-like gene is present in the C. elegans genome and an EST for the gene has been recorded. However, a closer inspection of the C. elegans genome reveals the substitution of amino acids that are normally crucial for enzymic activity; we therefore predict that the protein encoded by the gene cannot function as a typical ACE. The absence of an enzymically active ACE in C. elegans might reflect a less sophisticated peptidergic signalling system in comparison with that in the insects.

In conclusion, it seems that the protein
structure and substrate specificity of NEP have been conserved throughout the evolution of metazoans and is a fundamental mechanism for terminating the activity of regulatory peptides in the central nervous system and in the circulation. Insect ACE has many of the biochemical properties of the mammalian enzymes and has an important role in embryogenesis and reproduction. However, the peptide signalling pathways in insects that depend on ACE have not yet been defined but might well involve both the processing and inactivating properties of the enzyme.

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Exploring the Caenorhabditis elegans and Drosophila melanogaster genomes to understand neuropeptide and peptidase function

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Abstract
Comparison of peptidase gene families in the newly released Drosophila melanogaster and Caenorhabditis elegans genomes highlights important differences in peptidase distributions with relevance to the evolution of both form and function in these two organisms and can help to identify the most appropriate model when using comparative studies relevant to the human condition.

Introduction
The publication and availability of the annotated genome of Drosophila melanogaster on 27 March 2000 [1] opens up a new era in comparative studies. With two metazoan genomes now available,