Ance, a *Drosophila* angiotensin-converting enzyme homologue, is expressed in imaginal cells during metamorphosis and is regulated by the steroid, 20-hydroxyecdysone

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Ance is a single domain homologue of mammalian angiotensin-converting enzyme (ACE) and is important for normal development and reproduction in *Drosophila melanogaster*. Mammalian ACE is responsible for the synthesis of angiotensin II and the inactivation of bradykinin and *N*-acetyl-Ser-Asp-Lys-Pro, but the absence of similar peptide hormones in insects suggests novel functions for Ance. We now provide evidence in support of a role for Ance during *Drosophila* metamorphosis. The transition of larva to pupa was accompanied by a 3-fold increase in ACE-like activity, which subsequently dropped to larval levels on adult eclosion. This increase was attributed to the induction of *Ance* expression during the wandering phase of the last larval instar in the imaginal cells (imaginal discs, abdominal histoblasts, gut imaginal cells and imaginal salivary gland). *Ance* expression was particularly strong in the presumptive adult midgut formed as a result of massive proliferation of the imaginal midgut cells soon after pupariation. No *Ance* transcripts were detected in the midgut of the fully differentiated adult intestine. Ance protein and mRNA were not detected in imaginal discs from wandering larvae of flies homozygous for the *ecd*^{1} allele, a temperature-sensitive ecdysone-less mutant, suggesting that *Ance* expression is ecdysteroid-dependent. Physiological levels of 20-hydroxyecdysone induced the synthesis of ACE-like activity and Ance protein by a wing disc cell line (Cl.8+), confirming that *Ance* is an ecdysteroid-responsive gene. We propose that the expression of *Ance* in imaginal cells is co-ordinated by exposure to ecdysteroid (moulting hormone) during the last larval instar moult to increase levels of ACE-like activity during metamorphosis. The enzyme activity may be required for the processing of a developmental peptide hormone or may function in concert with other peptidases to provide amino acids for the synthesis of adult proteins.

Key words: imaginal disc, insect development, peptidase.

INTRODUCTION

Ance is a *Drosophila melanogaster* homologue of mammalian angiotensin-converting enzyme (ACE; EC 3.4.15.1), a peptidyl carboxypeptidase originally identified as a key component of the renin-angiotensin system ('RAS'), which regulates blood pressure, fluid and ion balance in mammals [1]. Ance has approx. 40% amino acid sequence identity with each of the two domains of somatic human ACE and displays many of the enzymatic properties described for the human enzyme [2]. For example, *in vitro* studies have shown that Ance is capable of converting angiotensin I into angiotensin II by removing the C-terminal His-Leu, is capable of hydrolysing Phe-Arg from the C-terminus of bradykinin, and is inhibited by human ACE inhibitors [2,3]. However, peptide hormones that are structurally related to mammalian angiotensin and bradykinin are not present in insects, and on this basis it can be concluded that *Drosophila* ACE activity has a novel but, as yet, poorly defined role in insect peptide metabolism. We recently showed that Ance was very efficient at removing pairs of basic residues from the C-terminus of peptide intermediates, which were predicted to be generated by cleavage of proproteins by endoproteases at recognition sites comprising basic residues [4]. Ance may therefore have a general role in the biosynthesis of signalling peptides, since the trimming of C-terminal basic amino acids is an important and common event in the processing of inactive precursors to biologically active peptides [5]. A second *Drosophila* homologue of mammalian ACE, known as Acer, has recently been characterized [6,7]. Acer differs from Ance in that it has very weak activity towards angiotensin I and has a more restricted expression pattern, especially during embryogenesis. At most stages of development, *Acer* appears to be expressed at a much lower level compared with *Ance* [7].

Although only angiotensin I, bradykinin and the haemoregulatory peptide, *N*-acetyl-Ser-Asp-Lys-Pro, have been confirmed as physiological substrates, it is known from *in vitro* studies that mammalian ACE can hydrolyse a wide variety of peptide structures [1]. A broad substrate specificity is also a characteristic of several insect ACEs, which can cleave dipeptides from the C-terminus of many insect and mammalian peptides, in addition to the high-affinity peptides possessing basic C-terminal residues [4,8]. The activity of the insect enzyme, like that of mammalian ACE, even extends to removing C-terminal dipeptideamides from insect peptides that have their C-terminus

Abbreviations used: ACE, angiotensin converting enzyme; Ance, *Drosophila* angiotensin-converting enzyme; LSP, larval serum proteins; PPF, post-puparium formation.

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blocked by an α amide [9,10]. Most insect signalling peptides require an amidated C-terminus for full biological activity and, therefore, Ance may be involved in determining the half-life of peptides, especially those peptides that are released into the haemolymph, where Ance is a major circulating peptidase [11,12].

Evidence for a developmental role for Ance has been obtained from studies of *Ance* mutants and knowledge of the *Ance* expression pattern during *Drosophila* development. Ance expression begins at approx. 4 h of embryogenesis in cells of the amnioserosa, reaching a peak of expresssion at mid- and late-stages of embryogenesis, when expression is seen primarily in the midgut and in the cephalic skeleton ([13], A. D. Shirras and R. E. Isaac, unpublished work). *Ance* has been identified as the target gene of *decapentaplegic* (*dpp*) during embryogensis, and the homoeobox gene *zerknüllt* (*zen*) appears to be one of several transcription factors downstream of *dpp* that directly controls *Ance* expression in the amnioserosa and midgut of embryos [14,15]. Ance protein is secreted from the *Ance*-expressing cells, resulting in a broad extracellular distribution of the enzyme, and the analysis of *Ance* null mutants has shown that the enzyme is required for normal embryonic development (A. D. Shirras and R. E. Isaac, unpublished work). There is evidence that Ance also has important physiological roles in post-embryonic development. *Ance* is expressed in adult testes and appears to be important for spermatogenesis, since trans-heterozygotes of *Ance* mutants, derived by chemical (ethyl methanesulphonate) mutagenesis, are infertile due to the failure of the spermatids to develop beyond the individualization stage [13,16]. ACE activity found in *Drosophila* pupae suggests a functional role for Ance during metamorphosis, a period of development that is defined by large scale destruction of larval tissues and the synthesis of new pupal and adult structures from imaginal cells that are set aside during early embryogenesis [7]. The dramatic changes in morphology, physiology and behaviour occurring during metamorphosis require precise temporal control of gene expression [17]. The major developmental hormones in insects are the ecdysteroid family of hydroxylated steroids, which in *Drosophila* are synthesized and released into the haemolymph from the ring gland. High ecdysteroid titres precede each larval moult and several pulses of the hormone are required for pupal and adult *Drosophila* development [18]. A recent *in vitro* study showed that the ACE-like gene of the silkworm, *Bombyx mori*, is transcribed in wing discs under the direct control of ecdysteroid hormones [19].

We now report that *Ance* expression is induced in most imaginal cells of *Drosophila* during the transition from larva to pupa and we provide evidence that this is a response to ecdysteroids during the last larval instar.

MATERIALS AND METHODS

Insect culture

Drosophila (OregonR and *ecd*^{1}) were maintained in population cages at 25 °C and a 12 h light/12 h dark daily cycle using standard culture conditions [20]. Eggs laid within a 2 h period were used to collect first (L1), second (L2) and third (L3) instar larvae at 30 h, 55 h and 96 h post-egg laying respectively, and pupae were collected at three time-points during pupal/adult development [1 h, 50 h and 96 h, post-puparium formation (PPF)]. Male and female adults were collected within 2 h of eclosion. Feeding third instar larvae were collected using dye coloured food as described by Andres and Thummel [21]. The *ecd*^{1} mutants were kept at the permissive temperature (20 °C). In order to investigate the effect of decreased ecdysteroid titre upon Ance expression, *ecd*^{1} larvae were shifted to the restrictive temperature (29 °C) soon after the second larval moult.

Measurement of the specific activity of ACE during development

Embryos, larvae, pupae and adult OregonR were homogenized in 50 mM Tris/HCl, 1 % (w/v) Triton X-100, pH 7.4. The resulting homogenate was cleared by centrifugation at 13000 ***g*** for 20 min and aliquots were tested for ACE-like activity, using HPLC to measure the release of hippuric acid from the synthetic substrate Hip-His-Leu (5 mM) in 100 mM HEPES, pH 8.5, containing 300 mM NaCl and 10 μM $ZnSO_4$ [22]. Protein was determined by the Bicinchoninic acid method, using BSA as the protein standard in a kit provided by Sigma/Aldrich (Poole, Dorset, U.K.).

In situ hybridization

pBluescript containing Ance cDNA was linearized with *Eco*RI (for the antisense probe) or *Not*I (for the sense probe), and digoxygenin-labelled sense and anti-sense riboprobes were synthesized using T7 and T3 RNA polymerases (Promega) respectively, with digoxygenin-labelled dNTPs (Roche, Lewes, East Sussex, U.K.). Tissues from wandering third instar larvae were dissected and stored in saline [0.7 % (w/v) NaCl; 0.03 % (w/v) Triton X-100] for up to 30 min and *in situ* hybridization was performed as described previously [23].

Immunocytochemistry

Antibodies were raised against recombinant Ance expressed in *Pichia pastoris* [7]. Imaginal discs were dissected in PBS and treated with 2 % (v/v) hydrogen peroxide in methanol for 5 min, then washed 3 times in PBS prior to fixing for 20 min in 4 % (w/v) paraformaldehyde in PBS. Tissues were blocked in 1 % (w/v) BSA, 10 % (v/v) normal goat serum, 0.3 % (w/v) Triton X-100 in PBS for 1 h at 25 °C before addition of primary antibody at 1:2000 dilution. After overnight incubation at 4 °C, discs were washed three times in PBS containing 0.3 % (w/v) Triton X-100, prior to detection with Vectastain ABC kit (Vector Laboratories, Peterborough, U.K.) according to the manufacturer's instructions.

ACE-like activity and Ance expression in discs from *ecd*^{1} third instar larvae

Discs from five 5-day-old larvae (*ecd*^{1}) maintained at the permissive temperature, or five wandering third instar insects (*ecd*^{1}) maintained at the restrictive temperature, were dissected into 10 μl of *Drosophila* Ringer's Solution [182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris/HCl (pH 7.2)] containing 1 % (w/v) Triton X-100, and incubated at 4 °C for 16 h. The volume was made up to 200 μl with 10 mM Tris/HCl (pH 7.4) containing 1 % (w/v) Triton X-100, and the samples were sonicated (5 × 20 s) in an iced water bath and then centrifuged at 10000 rev./min in a bench-top centrifuge for 10 min. The supernatant was retained for measuring ACE activity [22] and for immunoelectrophoresis.

Immunoelectrophoresis

Proteins from either a single wing disc or a pool of detergent-solubilized discs (wing, haltere, leg and eye-antennal discs from five larvae) were separated by SDS/PAGE (10 % gel) according

to standard procedures [24], transferred to a PVDF membrane, and then incubated with the anti-Ance antibody diluted at 1:5000 in PBS containing 0.05 % (v/v) Tween 20 and 5 % (w/v) non-fat dried milk powder [7]. Bound anti-Ance antibody was detected using a horseradish peroxidase-conjugated sheep anti-(rabbit Fc) antibody and the ECL® detection kit (Amersham International, Little Chalfont, Bucks., U.K.), as described in the manufacturer's instructions.

Cl.8+ cell culture

The cell line Cl.8+ was derived from the wing imaginal disc cell line CME W1 [25]. The cells used in the following experiments were passage 53 (23 passages after cloning). Cells were cultured in 5 cm culture dishes in modified Shield's and Sang's medium, containing 2 % (v/v) heat-inactivated foetal calf serum and 2.5 % (v/v) heat-inactivated fly extract, prepared as described previously [26], and were incubated at 25 °C in a 5 % (v/v) CO_2 atmosphere. For studying the effects of 20-hydroxyecdysone, cells were seeded at 3×10^6 cells per dish in 5 ml of medium supplemented with 0, 5×10^{-9}, 10^{-8} or 5×10^{-8} M 20-hydroxyecdysone (Sigma–Aldrich). The cells were incubated for 12, 24 or 48 h, at which point the medium was removed and stored at −20 °C until needed for measuring ACE activity. For each culture dish, four individual cell counts were carried out using a haemocytometer, and the mean counts recorded. Three separate dishes were employed for each treatment.

RESULTS

ACE activity during *Drosophila* development

The ACE specific activity of cleared homogenates of whole insects at various developmental stages was measured using the synthetic substrate Hip-His-Leu (Figure 1). ACE specific activity increased 10-fold between early embryogenesis and the first larval instar, followed by a 2.5-fold increase between the first and second instars. The specific activity in third instar larvae did not differ greatly from the level found in second instars, but increased after pupariation reaching a peak at 50 h PPF, which is a 3-fold increase compared with the specific activity of second and third instar larvae. Thereafter, the ACE specific activity declined during the later stages of metamorphosis, with newly eclosed adults possessing around 60 % less activity relative to the mid-pupal peak. There was no apparent difference in ACE specific activity between homogenates of male and female adults. Over 90 % of the ACE activity at the various *Drosophila* stages was inhibited by the ACE inhibitor, trandolaprilat (10 μM), confirming the specificity of the ACE assay.

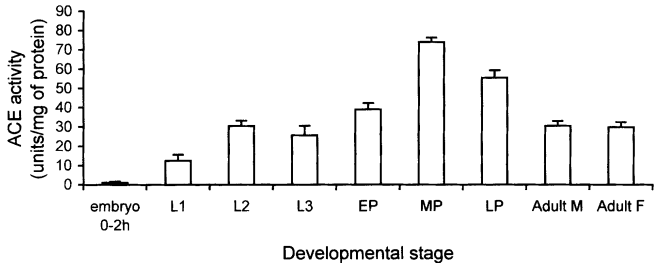

Figure 1 Changes in ACE specific activity during *Drosophila* development

Enzyme activity was measured in homogenates of various life stages using the substrate Hip-His-Leu as described in the Materials and methods section. The data are presented as the mean total ACE activity (nmol of Hip-His-Leu hydrolysed/min) ± S.E.M. L1, first larval instar; L2, second larval instar; L3, third larval instar; EP, early pupal stage (1 h PPF); MP, mid-pupal stage (50 h PPF); LP, late pupal stage (96 h PPF); M, male; F, female.

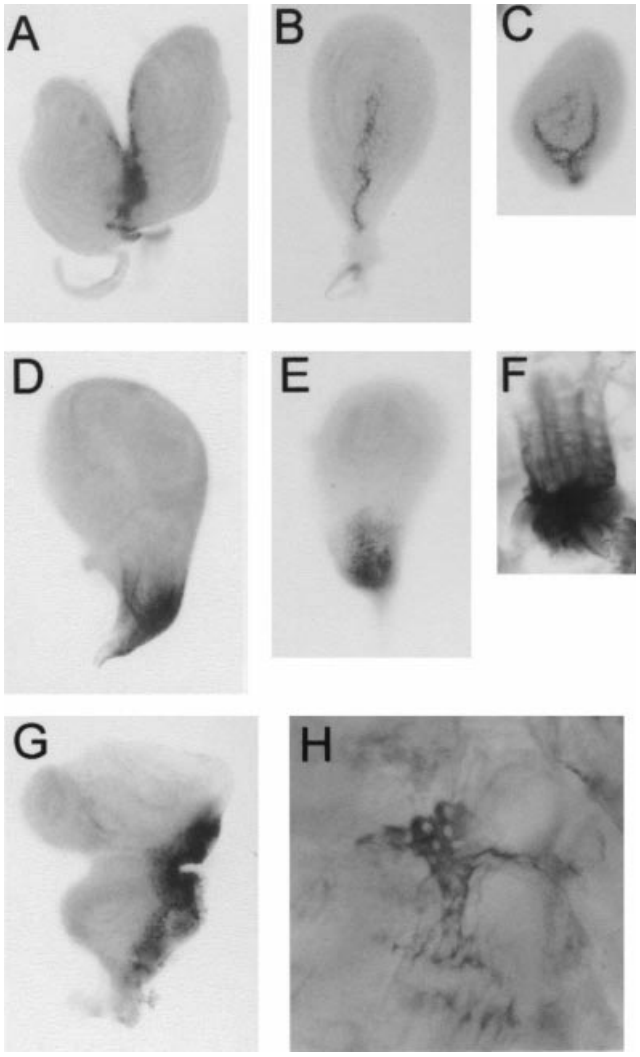

Figure 2 Ance expression in the imaginal cells of wandering third instar larvae of *Drosophila*

Ance mRNA was detected by whole mount *in situ* hybridization using a digoxigenin-labelled *Ance* riboprobe. (**A**), (**B**) and (**C**) First, second and third leg imaginal discs respectively. *Ance* expression is restricted to a subset of peripodial membrane cells. (**D**) and (**E**) Wing and haltere discs respectively. *Ance* transcripts are localized in the presumptive thoracic regions of the discs. (**F**) Dorsal prothoracic disc. (**G**) Eye-antennal disc. Strong expression was localized in eye and antennal regions in a zone of cells adjacent to where the two eye-antennal discs abut. (**H**) Ventral abdominal histoblast nest of small diploid cells. *Ance* expression is evident in the histoblast cells, but absent from the surrounding larval epidermal cells.

Imaginal cells of third instar larvae begin to express *Ance* at the wandering stage

The expression of *Ance* was examined by *in situ* hybridization in tissues dissected from wandering larvae. Strong *Ance* expression was detected in imaginal cells, but not in the differentiated larval cells (Figure 2). Within each imaginal disc, *Ance* transcripts were detected in subsets of cells. In each case, there was a distinctive and reproducible pattern; however, the identity of the cells was not clear. In the case of the second and third leg disc, the *Ance*-

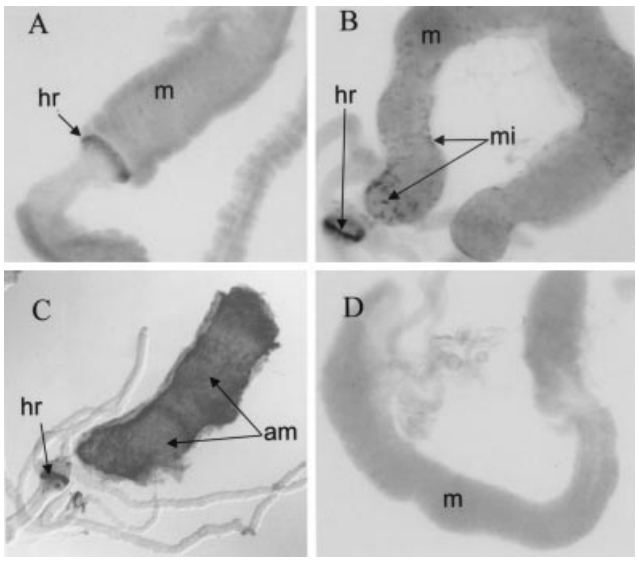

Figure 3 Ance expression in hindgut imaginal ring cells and midgut (m) imaginal islands from early (feeding) third larval instar to adulthood

Ance mRNA was detected by whole mount *in situ* hybridization. (**A**) Feeding third instar larva; weak staining is seen in the hindgut imaginal ring cells (hr). (**B**) 0–2 h PPF showing *Ance* staining in the hindgut imaginal ring cells (hr) and the midgut imaginal islands (mi). (**C**) 4–6 h PPF; staining can be seen in the differentiating cells of the adult midgut (am) which surround the old larval midgut and in the hindgut imaginal ring cells (hr). (**D**) Midgut (m) from a mature adult; no *Ance* expression could be detected.

expressing cells clearly lie in the peripodial membrane, rather than the columnar epithelium. In addition to the imaginal discs, *Ance* expression was also observed in abdominal histoblasts (Figure 2H) and the salivary gland imaginal ring cells (results not shown).

Ance transcripts were detected in the hindgut imaginal ring cells and in midgut imaginal islands of wandering third instar larvae (Figure 3). No Ance expression was observed in the imaginal island cells of the midgut of insects during the feeding stage of the third instar, and only weak expression could be found in the hindgut imaginal ring cells of these animals (Figure 3A). *Ance* transcripts appear in the groups of four midgut island cells during the wandering and gut clearing stages of the third instar, which develops into a strong expression pattern by 2 h PPF as these cells undergo proliferation (Figure 3B). At 4–6 h PPF, *Ance* expression can be seen in the layer of adult midgut cells surrounding the larval midgut (Figure 3C). In contrast, fully differentiated adult midgut cells do not show any signs of *Ance* expression (Figure 3D).

Localization of Ance protein in imaginal discs

Imaginal discs from wandering last instar larvae were subjected to immunoelectrophoresis and immunocytochemistry, in order to determine the localization of Ance protein. The 72 kDa Ance was readily detected by immunoelectrophoretic analysis of single wing discs using the Ance-specific antibody (Figure 4F). Immunocytochemical staining was apparent in all imaginal discs examined (Figure 4). In all cases, Ance appeared to be present on the surface of the discs with an essentially uniform distribution, although some concentration of staining was observed in the morphogenetic furrow of the eye disc (Figure 4D).

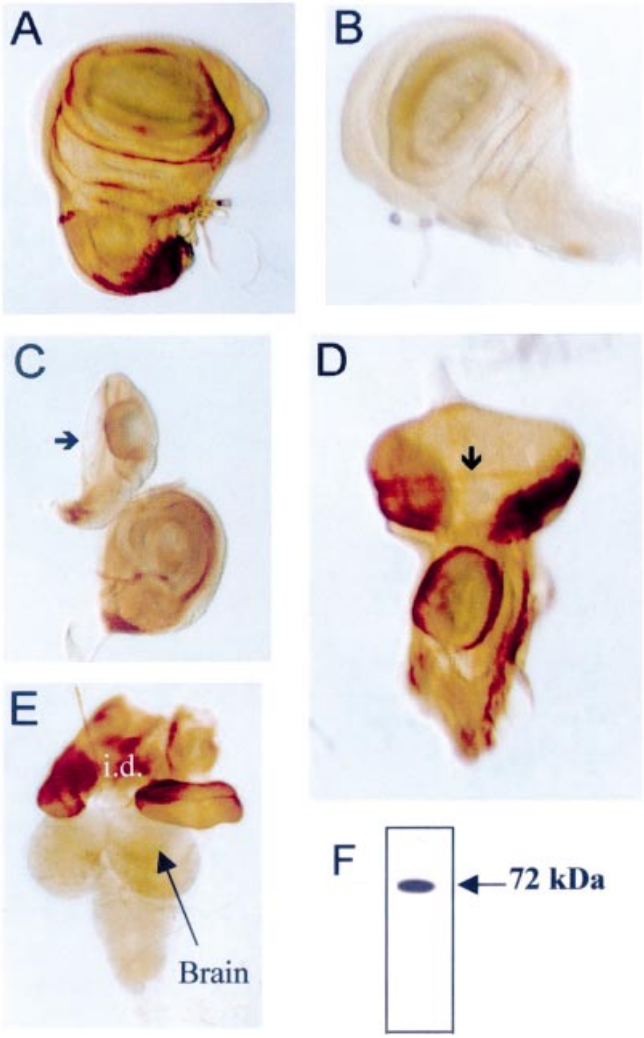

Figure 4 Immunocytochemical localization of Ance in imaginal discs

Ance protein was detected using an anti-Ance antibody and visualized using a peroxidase-conjugated secondary antibody and Vectastain ABC kit. Ance is present on the surface of all discs examined. (**A**) Wing disc. (**B**) Wing disc, control serum. (**C**) Upper, haltere. Lower, third leg. There is an absence of staining in the peripodial membrane of the haltere disc (arrow). (**D**) Eye-antennal disc. The morphogenetic furrow is visible (arrow). (**E**) Brain–imaginal disc complex; i.d., imaginal discs. There is no detectable staining in the brain. (**F**) Immunoelectrophoretic detection of Ance in a single wing disc using an Ance-specific antibody.

ACE activity is reduced in *ecd*^{1} homozygous third instar larvae

Flies homozygous for the *ecd*^{1} allele demonstrate a temperature-sensitive recessive lethal phenotype. At the permissive temperature of 20 °C, the flies develop normally and normal peaks of ecdysteroid occur during development. However, if third instar larvae are shifted to the non-permissive temperature of 29 °C, the normal ecdysteroid peaks do not occur and the ecdysteroid titre remains low [27]. We therefore used flies homozygous for the *ecd*^{1} allele to investigate the role of ecdysteroids upon *Ance* expression during the wandering larval phase. ACE specific activity of whole *ecd*^{1} third instar kept at the permissive temperature (31 ± 3 nmoles of Hip-His-Leu hydrolysed/min/larva; rate of hydrolysis $\pm$ S.E.M., $n = 7$) was 150 % higher than the activity of larvae shifted to the restrictive

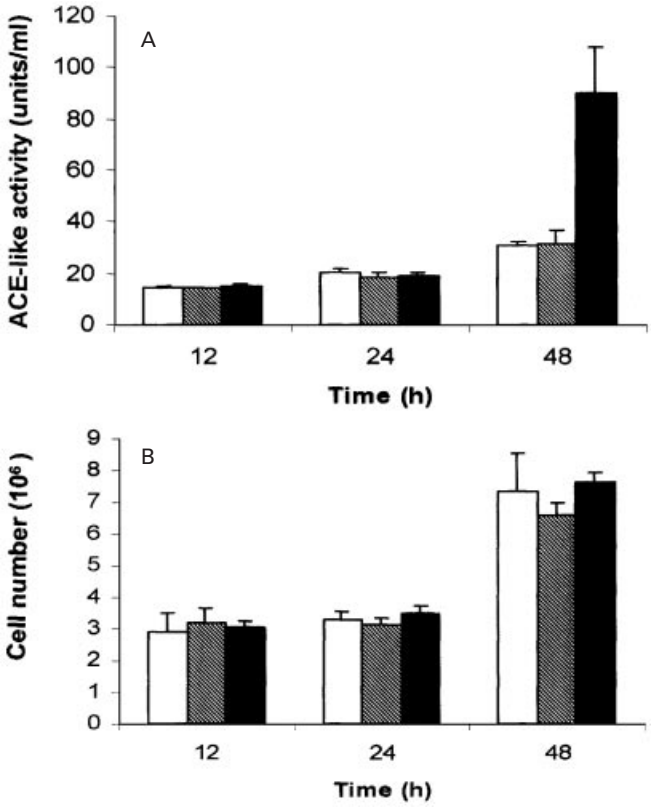

Figure 5 The effect of 20-hydroxyecdsyone on the secretion of Ance by cultured Cl.8+ cells

Cells were cultured in absence of 20-hydroxyecdsyone (white bars), or in presence of 5 nM (hatched bars) or 10 nM 20-hydroxyecdysone (black bars), for 12 h, 24 h and 48 h. (**A**) Mean ACE activity (nmol of Hip-His-Leu hydrolysed/min; ±S.E.M., $n = 3$) secreted into the medium by the Cl.8+ cells at each time point (see Materials and methods section). (**B**) Mean cell numbers (±S.E.M., $n = 3$) for each experiment.

temperature (21±1 nmoles of Hip-His-Leu hydrolysed/min/larva; rate of hydrolysis±S.E.M., $n = 7$) at the second/third larval instar transition. When ACE activity levels in imaginal discs were determined, the results were far more striking. Strong ACE activity (113 nmol of Hip-His-Leu hydrolysed/min) was detected in imaginal discs from *ecd*^{1} third instar larvae maintained at the permissive temperature, which contrasted greatly with the very low level of ACE activity (< 1 nmol of Hip-His-Leu hydrolysed/min) in discs obtained from *ecd*^{1} third instar insects, which had been subjected to a shift to the restrictive temperature. This marked difference in the expression of *Ance* was confirmed by the failure to detect by immunoelectrophoresis Ance protein in *ecd*^{1} discs kept at 29 °C, using a protocol that gave a strong 72 kDa immunoreactive band from discs of *ecd*^{1} larvae kept at 20 °C. We also examined *Ance* expression in *ecd*^{1} larval tissues by *in situ* hybridization. The distribution of *Ance* mRNA in wandering larvae of the *ecd*^{1} mutants maintained at the permissive temperature, was indistinguishable from the expression pattern observed with the OregonR strain (Figures 2 and 3). In contrast, we were unable to detect *Ance* transcripts in imaginal discs, abdominal histoblasts, the salivary gland imaginal ring cells, the hindgut imaginal ring cells nor in midgut imaginal islands of wandering *ecd*^{1} larvae which had been shifted to the non-permissive temperature (images not shown).

20-Hydroxyecdysone induces secretion of ACE activity in the imaginal disc cell line Cl.8+

As an alternative approach to investigate the role of ecdysone in *Ance* expression, we made use of the ecdysone-responsive wing disc cell line Cl.8+. Cells were cultured for up to 48 h, either in the absence or presence of 20-hydroxyecdysone. At each time point the medium was assayed for ACE activity and the cells were counted (Figure 5). In the presence of 10 nM 20-hydroxyecdysone, a three-fold increase in ACE activity occurred by 48 h of culture, compared with cells grown in the absence of ecdysteroid or in the presence of 5 nM 20-hydroxyecdysone (Figure 5A). Cell numbers were not affected by these ecdysteroid concentrations (Figure 5B), although typical morphological changes associated with ecdysteroid response were seen: after 48 h, cells treated with 10 nM 20-hydroxyecdysone showed the formation of smaller cell aggregates than controls, with thinner cell cover between these and cuticle-secreting cells within the aggregates. Immunoelectrophoresis of the culture medium from cells grown for 48 h in the presence of 10 nM 20-hydroxyecdsyone, using antisera raised to Ance and Acer respectively, showed that Ance, but not Acer, had been secreted (results not shown).

DISCUSSION

A previous study showed that *Drosophila* pupae contained ACE-like activity that fluctuated during pupal development and that a peak of activity, attributed to Ance, occurred at around stage P6 (25–46 h, PPF) [7]. We have now extended that study to include an early embryo stage, all three larval stages, and adult insects. The very low levels of enzyme activity in 2-h embryos is less than 10% of the activity found 28 h later in first instar larvae, and is consistent with the start of *Ance* expression in the embryo at 4–8 h post-egg-laying [13]. After the second instar, there appears to be little change in ACE specific activity until pupariation has taken place. During pupal development, ACE specific activity peaks at a mid-pupal stage before declining in adults to around 40% of the peak activity. There was no difference between the level of enzyme activity found in newly ecdysed adult male and female *Drosophila*.

The high ACE activity occurring during metamorphosis in *Drosophila* suggests that the dipeptidyl carboxypeptidase activity has a stage-specific role in the metabolism of peptides during this period of re-modelling of not only the external cuticular structures, but also of most internal organs of the insect, and which involves diverse cellular processes such as cell proliferation, programmed cell death and extensive autophagy [28]. Preparation for the transition from larva to pupa begins with a change in the behaviour of the last larval instar. In the second half of the third instar, *Drosophila* larvae enter a wandering phase, cease to feed and clear food from the gut in preparation for pupariation. There are several ecdysteroid peaks around the time of pupariation: in the last larval instar there is a small peak followed by a larger ecdysteroid peak, required for the larval–pupal moult, which occurs approx. 4–8 h before puparium formation, at around the time that the gut contents are cleared [29,30]. The expression of Ance in imaginal cells from wandering third instar larvae in the process of clearing food from the intestine, but not from feeding larvae of the same instar, coincides with the pulses of larval ecdysteroid occurring during the latter half of the third instar. The induction appears to be temporally co-ordinated in imaginal cells of the discs, salivary glands, mid- and hind gut and abdominal histoblasts. Expression in the imaginal ring cells of the foregut was difficult to determine, since background colour

development in the region of the proventriculus, where these cells are located, interfered with our observations.

Induction of *Ance* expression in the midgut imaginal cells occurs prior to the massive cell proliferation that occurs 0–4 h PPF. In the wandering last larval stage, these imaginal cells are distributed throughout the midgut and occur as clusters of four cells that have arisen from embryonic progenitor cells by two rounds of cell division during larval development [31]. Soon after pupariation, the quartet of midgut imaginal cells begin to proliferate to form the adult midgut, which is constructed around the larval gut tissue now undergoing histolysis and contraction [32]. Although the imaginal hindgut ring strongly expresses Ance, these cells do not differentiate to form the adult hindgut until much later in metamorphosis (> 25 h) [31]. All the progenitor cells of the adult midgut appear to be expressing *Ance* during the pre-pupal stage (0–12 h PPF) of metamorphosis and, considering the cell numbers involved, it is safe to conclude that the growing adult midgut is a major site of synthesis of Ance in *Drosophila* pupae. This co-ordinated expression of *Ance* in most, if not all, imaginal cell types is evidence for a stage-specific role for the peptidase during metamorphosis.

The final destination of pupal Ance will determine the function of the peptidase during metamorphosis. Ance is a secreted protein, which unlike mammalian ACE, does not possess a C-terminal hydrophobic domain that serves as a membrane anchor [2]. Since the internal tissues of *Drosophila* are bathed in haemolymph, we expect that much of the Ance synthesized by differentiating and proliferating imaginal cells will be destined for the circulation. Indeed, we have shown in another study that Ance is a major peptide-degrading enzyme present in haemolymph of *Drosophila* larvae [12]. Our present immunocytochemical studies show that some of the Ance protein becomes associated with the external surfaces of imaginal discs by an unknown mechanism.

We have provided evidence that *Ance* expression in imaginal cells at this stage of development is under the control of the moulting hormone, 20-hydroxyecdysone. The ecd^1 allele is a temperature-sensitive recessive lethal mutation, which results in the loss of the ability to synthesize ecdysteroids, when insects are shifted to the restrictive temperature of 29 °C during the third instar [33]. The failure of imaginal discs taken from ecd^1 insects shifted to 29 °C to make *Ance* RNA and protein, suggests that *Ance* expression in imaginal discs is absolutely dependent upon prior exposure to 20-hydroxyecdysone. Direct evidence for *Ance* induction by 20-hydroxyecdysone was obtained using the *Drosophila* Cl.8+ cloned cell line derived from wing imaginal discs [25]. This cell line exhibits several responses to exposure to 20-hydroxyecdysone, including changes in cell morphology and increased rate of chitin synthesis, the slowing and cessation of cell division and some cell death. All of these responses are also observed in intact discs which are exposed to 20-hydroxyecdysone and reflect the morphological changes and apoptosis that accompany natural differentiation of the imaginal discs during pupal development. Our studies using Cl.8+ cells indicate that Ance expression is sensitive to physiological concentrations of 20-hydroxyecdysone. The morphological changes associated with 10 nM 20-hydroxyecdysone treatment, notably changes in cell aggregation and cuticle secretion, are clearly visible after 48 h, whereas the beginnings of a reduction in the rate of cell proliferation induced by this hormone level is not seen until 72 h.

The timing of the induction of *Ance* expression in the imaginal cells of third instar larvae suggests that this might be a direct response to a pulse of ecdysteroids occurring during the last larval instar. This suggestion is supported by a recent study of ecdysone-inducible genes expressed in the imaginal wing discs of the silkworm, *B. mori*, which identified *BmAcer*, a homologue of *Drosophila Ance*, as a primary response gene [19]. The fact that ACE gene expression is induced in imaginal cells during the transition of the last larval instar to the pre-pupa of two species from different insect orders, indicates that this stage-specific expression is conserved in holometabolous insects and is likely to be of fundamental importance for development during the early stages of metamorphosis.

Ance is not the only peptidase to be expressed in imaginal discs and up-regulated during metamorphosis. Serine peptidases are expressed in imaginal discs of *Drosophila* and are probably required for proteolysis of extracellular matrix proteins during ecdysone-dependent morphogenesis of bristles, legs and wings [34]. One of these enzymes (Stubble-stubbloid) is a type II membrane peptidase that might also transmit signals from the extracellular surface to the intracellular domain to facilitate changes in cell shape [35]. A gene encoding a prolyl endopeptidase (*DPEP*) is expressed in imaginal discs of *Drosophila* and the fleshfly, *Sarcophaga peregrina*, and has been implicated in the ecdysone-induced differentiation of *S. peregrina* imaginal discs [36–38]. Recently, a second ecdysone-inducible peptidase gene has been shown to be expressed in the wing disc of *B. mori* [39]. The predicted gene product has high sequence homology to mammalian neprilysins, a family of zinc metallopeptidases that hydrolyse regulatory peptides [40]. Other *Drosophila* peptidases (aminopeptidase and dipeptidase) have been characterized biochemically and shown to have high specific activities during metamorphosis and, in the case of the aminopeptidase, the enzyme activity can be induced by 20-hydroxyecdysone [41–43]. Peptidases like Ance, aminopeptidase, prolyl endopeptidase and dipeptidase will preferentially hydrolyse small peptides and may have roles in cellular differentiation by either processing or inactivating regulatory peptides. Alternatively, these enzymes may be involved in the recycling of amino acids from peptides generated by hydrolysis of larval proteins during the destruction of larval tissues and the larval serum proteins (LSP-1 and LSP-2), found sequestered in storage granules of the fatbody towards the end of larval development [44]. The catabolism of LSP-1 and LSP-2 during metamorphosis is believed to be an important source of amino acids for use as energy fuel and for the synthesis of new pupal and adult proteins [45]. The high levels of Ance and dipeptidase activity present during metamorphosis could work in concert to convert larval peptides to free amino acids.

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