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The proprotein convertase *amontillado* (*amon*) is required during *Drosophila* pupal development

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ABSTRACT

Peptide hormones governing many developmental processes are generated *via* endoproteolysis of inactive precursor molecules by a family of subtilisin-like proprotein convertases (SPCs). We previously identified mutations in the *Drosophila amontillado* (*amon*) gene, a homolog of the vertebrate neuroendocrine-specific Prohormone Convertase 2 (PC2) gene, and showed that *amon* is required during embryogenesis, early larval development, and larval molting. Here, we define *amon* requirements during later developmental stages using a conditional rescue system and find that *amon* is required during pupal development for head eversion, leg and wing disc extension, and abdominal differentiation. Immuno-localization experiments show that *amon* protein is expressed in a subset of central nervous system cells but does not co-localize with peptide hormones known to elicit molting behavior, suggesting the involvement of novel regulatory peptides in this process. The *amon* protein is expressed in neuronal cells that innervate the corpus allatum and corpora cardiaca of the ring gland, an endocrine organ which is the release site for many key hormonal signals. Expression of *amon* in a subset of these cell types using the GAL4/UAS system in an *amon* mutant background partially rescues larval molting and growth. Our results show that *amon* is required for pupal development and identify a subset of neuronal cell types in which *amon* function is sufficient to rescue developmental progression and growth defects shown by *amon* mutants. The results are consistent with a model that the *amon* protein acts to proteolytically process a diverse suite of peptide hormones that coordinate larval and pupal growth and development.

Introduction

Many peptide hormones and neuropeptides are produced by limited proteolysis of inactive precursor peptides. The release of an active peptide hormone from its precursor molecule typically occurs by proteolytic cleavage after paired basic amino acids and is mediated by a family of proteases known as the subtilisin-like proprotein convertases (SPCs) (Bergeron et al., 2000). Following convertase processing, most peptides undergo removal of the C-terminal basic residues *via* a carboxypeptidase (Fricker and Leiter, 1999) and subsequent α-amidation of the C-terminal glycine residue (Kulathila et al., 1999). Processing of precursor proteins may liberate a single or multiple bioactive products (Sossin et al., 1989; Zhou et al., 1999), and a given precursor may be differentially processed in a cell-specific fashion depending on the SPC processing enzymes expressed (Furuta et al., 1997; Rouille et al., 1995). Thus processing of peptide hormone precursors may serve as an important regulatory step to modulate peptide and neuropeptide signaling.

Prohormone Convertase 2 (PC2) is one of seven vertebrate SPCs identified to date and displays a neuroendocrine expression pattern that implicates the enzyme in the activation of peptide hormones and neuropeptides (Muller and Lindberg, 2000). The *Drosophila* PC2 homolog, *amontillado* (*amon*), was identified based on sequence similarity to conserved regions between yeast Kex2, human furin, and human PC2 (Siekhaus and Fuller, 1999). *amon* is expressed throughout the life cycle of the fly, and *amon* transcripts localize to the larval central nervous system and regions of the gut (Siekhaus, 1997; Siekhaus and Fuller, 1999), suggesting that *amon* acts in neuroendocrine tissues. The *amon* protein has been shown to be an active protease on a KR containing synthetic peptide when expressed in *Drosophila* S2 cells with the *Drosophila* 7B2 protein, a homolog of the 7B2 protein that functions in maturation of vertebrate PC2 (Hwang et al., 2000). Isolation and analysis of a series of EMS induced *amon* mutants showed that *amon* is required during embryogenesis and early larval development and suggests that the *amon* protein may act to process peptide hormones that control hatching, larval growth, and larval molting (Rayburn et al., 2003).

The regulation of molting and metamorphosis in insects has its roots in an endocrine axis and relies on a delicate interplay between steroid and peptide hormones (Ewer, 2005; Mesce and Fahrbach, 2002; Nijhout, 1994; Riddiford, 1993; Truman, 1992; Zitnan et al., 2007). This axis includes the brain, the corpus allatum (CA), and the prothoracic gland which act as sources of peptide and steroid

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hormones, the effectors of molting and metamorphosis. Pulses of the steroid hormone 20-hydroxyecdysone, hereafter referred to as ecdysone, act to initiate larval molting (Riddiford, 1993). One manner in which ecdysone may exert its regulatory effect on molting and metamorphosis is by affecting the expression of genes whose products are part of the ecdysis cascade. For example, in both *Manduca* and *Drosophila* the peptide hormone gene *ecdysis triggering hormone* (*eth*) contains a putative ecdysone response element upstream of the transcription start site (Park et al., 1999; Zitnan et al., 1999). Studies in *Manduca* show that rising ecdysteroid levels prior to ecdysis induce *eth* gene expression in the Inka cells and that a drop in the ecdysteroid titer is required for ETH release (Zitnan et al., 1999).

Two additional peptide hormones, eclosion hormone (EH) and crustacean cardioactive peptide (CCAP) are known to interact with ETH in an ecdysis cascade to elicit the behavioral outputs that characterize molting and metamorphosis in insects. Low levels of circulating ETH trigger the release of EH from the brain. This initial EH release induces a subsequent and exhaustive release of ETH from the Inka cells which in turn cues exhaustive release of EH (McNabb et al., 1997). In *Manduca*, EH acts through a second messenger system to cause elevated levels of cGMP in cells that express CCAP (Ewer et al., 1997). CCAP release elicits ecdysis motor burst while suppressing the pre-ecdysis behaviors initiated by EH (Gammie and Truman, 1997). Notably, the ETH and CCAP pro-peptides of *Drosophila* contain putative dibasic processing sites (Park et al., 1999, 2003) suggesting they require endoproteolytic activation. The *Drosophila* EH pro-peptide also contains a possible dibasic processing site (Horodyski et al., 1993), although processing at this site may not be necessary for production of the bioactive EH peptide.

Here we have used a conditional rescue system to ask whether *amon* is required during postembryonic developmental transitions in *Drosophila*. *amon* mutants rescued past earlier embryonic and larval requirements by heat-shock induced expression of an *amon* cDNA and then removed from the rescue regime at the late third instar stage display defects in head eversion, leg and wing extension, and abdominal differentiation, indicating that *amon* activity is required for these aspects of metamorphosis. Although *amon* mutants show similar phenotypes to *eth* mutants and to pupae resulting from CCAP-cell ablation, the *amon* protein does not co-localize with ETH, CCAP, or EH, suggesting the existence of novel peptide hormones that regulate molting and metamorphosis. Interestingly, the *amon* protein localizes to cells that innervate the CA and corpora cardiaca (CC) sections of the ring gland, suggesting that *amon* protein may regulate the endocrine activity of this gland. Finally, expression of *amon* in a subset of neuronal cell types in an *amon* mutant background is sufficient to partially rescue developmental progression and growth.

Materials and methods

Conditional rescue of amon mutants

One hundred fifty *yw; hs-amon/hs-amon; Df(3R)Tl-X e/TM3 Sb Ser* y^+ *e* virgin females were crossed to 150 *yw;* $amon^{C241Y}$ or $amon^{Q178st}$ *e/TM3 Sb Ser* y^+ *e* males in an egg collection chamber and allowed to lay eggs on 100 mm grape juice agar plates spotted with yeast paste for 3 days at 25 °C. On the third day, a 4 or 8 h egg collection was taken and *yellow* larvae (*yw; hs-amon/+;* $amon^{C241Y}$ or $amon^{Q178st}$ *e/Df(3R)Tl-X e*) were collected at 36 h after egg laying (ael) and placed on a fresh plate. Animals were heat shocked for 30 min at 37 °C every 24 h beginning at 36 h ael and subsequently scored for survival until all the animals on the positive control plate eclosed. Experimental animals were heat shocked as above until 108 h ael. After each scoring, larvae were transferred to a fresh plate unless the animal had pupariated. Dead larvae were mounted in polyvinyl lactophenol for examination. Pupae from the experimental plate were removed from the pupal case in water under a Leitz dissecting scope once a majority of the control animals had successfully eclosed. Once out of the pupal case, animals were photographed using a digital camera (Hamamatsu 3CCD) mounted to a Leitz dissecting scope. Control animals failing to eclose were also removed from the pupal case and photographed at later time points. Percent pupariated and eclosed values were calculated by dividing the number of animals that had pupariated or eclosed by the number of animals collected at 36 h ael.

Antibody development

A peptide corresponding to the final 27 amino acids of the *amon* protein with three additional amino terminal residues (CKC) was synthesized by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia. The peptide was coupled *via* M-Maleimidobenzoyl N-Hydroxysuccinimide Ester (MBS, Pierce) to bovine serum albumin as described below and then injected into five mice at the Monoclonal Antibody Facility at the University of Georgia. Following an initial subcutaneous injection with Freund's complete adjuvant (Sigma Chemical), mice were boosted intraperitoneally using Freund's incomplete adjuvant every 21 days and bleeds were taken approximately 7 days after each boost. Beginning with the third bleed, serum was tested *via* Western analysis for its ability to recognize the *amon* protein. Following the sixth boost one mouse produced serum specific for *amon* protein and was subsequently boosted a total of 15 times over the course of about 1 year. Bleeds taken after the seventh boost were used in the immunocytochemistry and Western analyses as described below.

Antigen generation

Five milligrams of carrier protein (Bovine Serum Albumin, Sigma) was dissolved in 1 mL of phosphate buffered saline (PBS). 50 μL of MBS in N, N dimethylformamide (DMF) was added drop by drop to 500 μL of the carrier protein solution and then stirred at room temperature for 30 min. Excess MBS was removed using Micro Bio-Spin P-30 Tris Chromatography Columns (Bio-Rad) equilibrated with PBS. A 20-fold excess of synthetic peptide was added to the carrier protein–MBS solution and stirred at room temperature for 3 h. To make sure that peptide was coupled to the carrier protein, the solution was run on a 10% polyacrylamide gel next to a control solution (carrier protein–MBS solution alone) and stained with Coomassie.

Immunocytochemistry

Wandering third instar larvae (*Canton S*) were dissected, fixed (2 h in PBS and 175 mM NaCl, pH 7.4, with 4% paraformaldehyde), dehydrated, and rehydrated according to the protocol of Cao and Brown (2001). Tissues were washed in TBS (25 mM Tris, 137 mM NaCl, 0.27 mM KCl) containing 0.5% Triton X-100 (TBST) two times for 5 min each and then blocked in TBST+ 5% goat serum overnight at 4 °C. All subsequent steps were performed at 4 °C. Primary antiserum was added in TBST+ 2% BSA at a 1/200 dilution and left overnight. Following a wash of at least 1 h in TBST+ 2% BSA, the secondary antibody (anti-mouse IgG conjugated to Alexa Fluor 488, 568 or 594, Molecular Probes, Inc.) was added at a dilution of 1:500–1:2000 in TBST+ 2% BSA and tissues were incubated overnight in the dark. Tissues underwent a final wash in TBST and were then mounted on slides in a 1:1 TBST/glycerol solution. Images were captured on an Olympus BX60 microscope using a JVC digital camera (model KYF70BM) and the AutoMontage (Syncroscopy) software. To obtain *amon* mutant third instar CNS tissues for use as a control, *amon* mutant larvae (*yw; hs-amon/+;* $amon^{Q178st}$ *e /Df(3R)Tl-X e*) were rescued past early developmental requirements for *amon* function by periodic expression of a *hs-amon* transgene for 45 min at 37 °C every 24 h, beginning at 36 h AEL until 84 h AEL. The CNS was dissected at