# Predicted seminal astacin-like protease is required for processing of reproductive proteins in *Drosophila melanogaster*

**Kristipati Ravi Ram, Laura K. Sirot, and Mariana F. Wolfner***

Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853

Edited by David L. Denlinger, Ohio State University, Columbus, OH, and approved October 12, 2006 (received for review July 21, 2006)

**During mating, males provide females with seminal fluids that include proteins affecting female physiology and, in some cases, reproductive behavior. In several species these male-derived modulators of reproduction are processed upon transfer to the female, suggesting molecular interaction between the sexes. Males could increase their reproductive success by contributing to regulation of this processing; consistent with this hypothesis, seminal fluids are rich in proteolysis regulators. However, whether these molecules carry out processing of male-derived reproductive modulators is unknown. We tested for this role using RNAi to knock down individually 11 *Drosophila* seminal fluid proteases and protease inhibitors. We found that CG11864, a predicted astacin-type metalloprotease in seminal fluid, is necessary to process two other seminal proteins: the ovulation hormone ovulin (Acp26Aa) and the sperm storage protein Acp36DE. This processing occurs only after all three proteins have entered the female. Moreover, CG11864 itself is processed inside males while en route to the female and before its action in processing ovulin and Acp36DE. Thus, processing of seminal proteins is stepwise in *Drosophila*, beginning in the male after the proteins leave their site of synthesis and continuing within another organism, the mated female, and the male-donated protease CG11864 is an agent of this latter processing.**

proteolysis | reproduction | RNAi | seminal proteins | accessory gland proteins

Proteins and other molecules in the seminal fluid can profoundly affect the behavior and/or physiology of the female who receives them, ultimately influencing reproductive success. In addition to these reproductive modulators, the seminal fluid of many organisms contains proteases and protease inhibitors that regulate proteolytic events essential for reproduction: for example, in mammals, a protease inhibitor (protein C inhibitor, PCI) together with a protease (prostate-specific antigen, PSA) coordinates the degradation of semenogelins. This is thought to promote male fertility because it may enhance sperm motility by liquefying the seminal fluid (1–4). Proteolysis regulators are also crucial for protein processing leading to semen coagulation and vaginal plug formation upon copulation (5) and are suggested to function in protecting sperm from premature acrosome reaction or from bacteria (6, 7). Seminal proteolysis regulators could also, in theory, play important roles beyond physical phenomena such as semen coagulation and mating plugs if they acted on proteins critical for physiological changes in the mated female. For example, seminal proteolysis regulators could be critical for keeping reproductive modulators intact and at high levels until they reach the site/target of their action, or to regulate the release of active cleavage products from a precursor at a precise time or place such as the mated female's reproductive tract (8). Here we use *Drosophila* to test the hypothesis that seminal proteases or protease inhibitors regulate the proteolysis of seminal proteins with such functions.

Major components of *Drosophila* seminal fluid are proteins derived from the male's accessory gland. These accessory gland proteins (Acps) are transferred to the female during mating. They enhance the mated female's egg production, increase her rate of ovulation, reduce her sexual receptivity, impact the female's storage of sperm, and also affect her lifespan and eating habits (reviewed in refs. 8–12; also see refs. 13 and 14). Approximately 22% (14) of the 63 Acps annotated (10, 15–18) so far in *Drosophila melanogaster* are predicted proteolytic regulators, including three serine proteases, one cysteine protease, one aminopeptidase, one threonine protease, one metalloprotease, and seven serine protease inhibitors (including a known protease inhibitor, Acp62F) (5, 16, 18, 19). The multiplicity of these enzymes suggests that proteolytic events may be important in *Drosophila* reproduction.

*Drosophila* is a good system to study regulated proteolysis of seminal proteins because cleaved forms of four Acps (thus far) are detected within mated females. Three of these Acps are known to regulate reproductive processes. Ovulin (Acp26Aa) increases the mated female's rate of ovulation (20). Ovulin is cleaved into several peptides (21–23), two of which can independently induce ovulation (24). Acp36DE, a large glycoprotein whose cleaved forms are seen in the mated female, is essential for sperm storage by mated females (25). Sex peptide (Acp70A) increases egg laying and reduces female receptivity to remating for several days after mating (26, 27). Gradual cleavage of sperm-bound sex peptide releases its C-terminal (active) part; this is proposed to underlie the long-term persistence of postmating responses (28). The fourth processed Acp, CG11864, is a predicted astacin-like metalloprotease (5) whose function is not known. In other organisms, the astacin family of metalloproteases is believed to be involved in the processing of biologically active peptides (29) or in several other functions including digestion and breaking down egg envelopes during hatching (30).

Little is known about the regulation and dynamics of the processing of these four Acps, except that none are cleaved in the male's accessory gland and that, in one case (ovulin), both male (i.e., Acp) and female contributions are necessary for cleavage (23). To understand the importance of this male–female interaction at the molecular level, it is essential to identify the molecules involved in the proteolysis of these Acps. Hence, we used RNAi to assess the role(s) of 11 individual Acp proteases/protease inhibitors in postmating processing events of Acps. We tested for the involvement of five predicted Acp proteases [CG6069, CG6168, CG9997, CG11664, and CG11864 (16)] and six predicted Acp protease inhibitors [BG642378, CG6289, CG8137, CG9334, CG10956 (16), and Acp76A (31)] in the

Author contributions: K.R.R., L.K.S., and M.F.W. designed research; K.R.R. and L.K.S. performed research; K.R.R., L.K.S., and M.F.W. analyzed data; and K.R.R., L.K.S., and M.F.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: ASM, after the start of mating; Acp, accessory gland protein.

*To whom correspondence should be addressed. E-mail: mfw5@cornell.edu.





www.pnas.org/cgi/doi/10.1073/pnas.0606228103

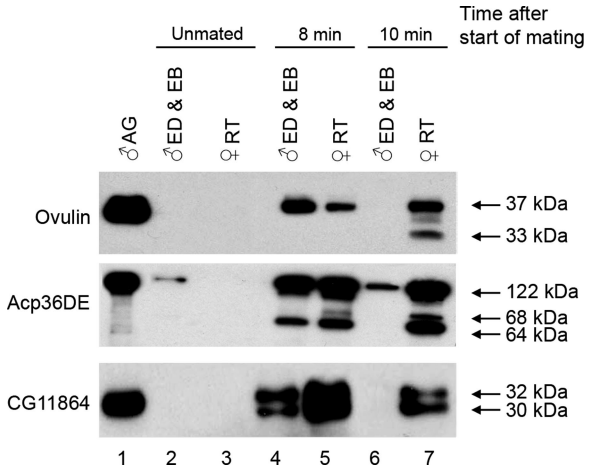


**Fig. 1.** Processing of ovulin, Acp36DE, and CG11864 in the male ejaculatory duct and bulb (lane ED & EB) or in the female reproductive tract (lane ♀-RT). At 8 min ASM only full-length ovulin is seen. Acp36DE shows the full-length 122-kDa band and a cross-reactive band at 64 kDa (36) (data not shown) at 8 min ASM in the ED & EB samples, and the 122- and 64-kDa bands and a 68-kDa processed product in the mated female. Previously, the cross-reactive 64-kDa band was suggested to be derived from the female (36), but our result clearly shows that it is a male-derived non-Acp (thus, from either sperm or other seminal proteins cross-reacting with Acp36DE antibody), because it is seen in ejaculatory duct/bulb samples. For CG11864 both the full-length protein (32 kDa) and the processed 30-kDa band (35) are detected in male and female (ED & EB and ♀-RT) by 8 min ASM. Protein equivalent to eight male ejaculatory ducts/bulbs or four mated female reproductive tracts from 8 min ASM or 10 min ASM were loaded in each lane.

processing of three Acps (CG11864, ovulin, and Acp36DE) in the mated female reproductive tract. We observed that the cleavage of seminal fluid proteins includes events that occur in the male and subsequent events that occur only in the female. Furthermore, we identified one seminal protease of that stepwise process, CG11864, which is necessary for normal processing of Acp26A and Acp36DE. This study also provides evidence for the role of seminal proteolysis regulators in the processing of reproductive molecules.

## Results and Discussion

**One Acp, CG11864, Is Processed Before Transfer to Females During Mating.** In mammals, passage through the male reproductive tract results in modifications to proteins on the sperm surface (32, 33). To determine whether *Drosophila* seminal proteins are processed during passage through the male reproductive tract, we interrupted matings at 8 and 10 min after the start of mating (ASM) [mating between wild type (Canton-S) flies typically lasts for 20 min] and analyzed proteins from the seminal fluid mixture in the mating male's ejaculatory duct/bulb. Although none of the three Acps we were able to examine (see *Materials and Methods*) is cleaved while residing in the male's accessory gland before mating, CG11864 is processed within the male after Acps have mixed with sperm and/or other proteins in the male ejaculatory duct/bulb (Fig. 1). In contrast, only full-length ovulin and Acp36DE are detected in the accessory gland and the seminal fluid mixture from the ejaculatory duct/bulb samples taken during mating (Fig. 1). Processed forms of ovulin and Acp36DE were detected only after transfer to the female (Fig. 1), consistent with earlier reports of these forms in the mated female [ovulin (21, 23) and Acp36DE (25)]. Therefore, our results demonstrate that CG11864 is modified in the ejaculatory duct/bulb during its passage through the male reproductive tract.

CG11864 is a predicted astacin-like metalloprotease. Comparative structural modeling showed perfect threading of CG11864 to astacin, and the active site motif (HEXXH) is also intact (5). Most astacins are secreted proteases and require proteolytic removal of a signal peptide and a propeptide for optimal activity (reviewed in ref. 34). To determine whether CG11864 could contain a propeptide, we aligned the N-terminal amino acid sequence of CG11864 and other proteases in the astacin family around the experimentally proven propeptide cleavage site of astacin (Fig. 2). This alignment revealed that CG11864 is enriched in basic residues like arginine around its sequence that aligns with astacin's propeptide cleavage site; such motifs act as potential recognition/binding sites for processing enzymes like trypsin. The alignment suggests that this region is a putative propeptide cleavage site in CG11864. Furthermore, the difference in gel mobility of CG11864 from the accessory gland and the ejaculatory duct/bulb is on the order of that expected from release of CG11864's predicted propeptide (3

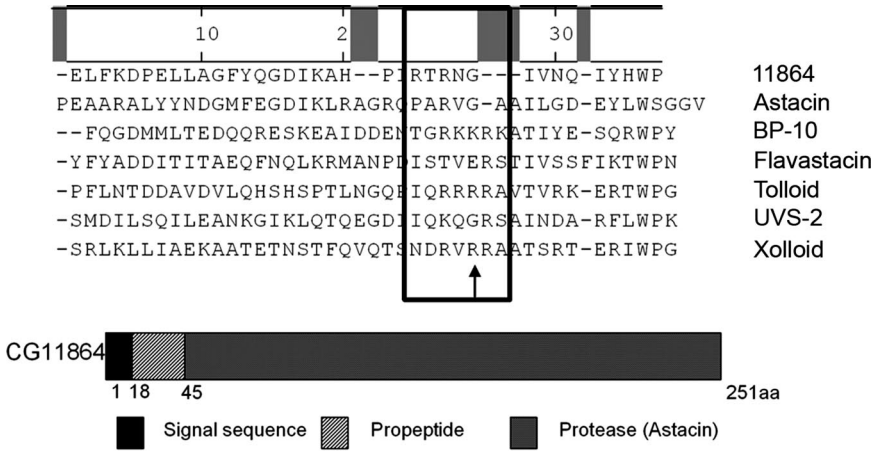


**Fig. 2.** Alignment of the N-terminal sequence predicted for the secreted form of CG11864 with the propeptide cleavage region of astacin and other astacin-like metalloproteases. Astacin family members are cleaved at ≈30 aa from N terminus (42) of their secreted form at a basic region (boxed). Based on the sequence alignment and its basic residue cluster, CG11864's propeptide cleavage site is predicted to be after amino acid 45 in the CG11864 sequence [amino acid 27 in the figure; because CG11864's signal peptide is predicted to be amino acids 1–18 (as per by SignalP 3.0), amino acid 19 is indicated as ''1'' in this figure]. This size is consistent with the 3-kDa difference seen between full-length and processed CG11864 (35). Alignments were done by using the Clustal W algorithm of the Megalign program (Lasergene; DNASTAR) and used the sequences of astacin-family members from the GenBank database (accession nos. CAA64981, CAA46637, 2112204A, AAC46482, P42664, and CAA70854).

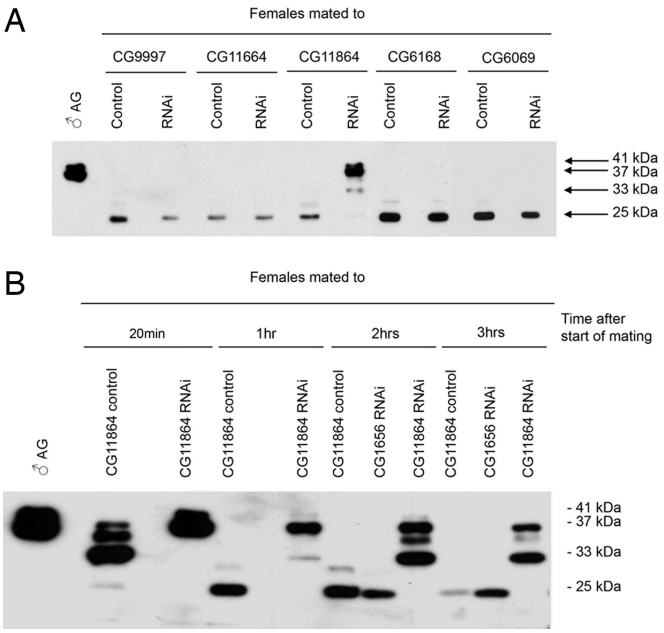


**Fig. 3.** Processing of ovulin in mates of RNAi and control males. (*A*) Processing of ovulin at 45 min ASM in females mated to knockdown males (lane RNAi) of different Acp protease lines in comparison with their controls (lane Control). Female reproductive tracts were dissected at 45 min ASM, and protein equivalents of one pair of accessory glands (lane ♂ in this and subsequent figures) or two female reproductive tracts were loaded in each lane and processed for Western blotting with anti-ovulin. (*B*) Analysis of female reproductive tract samples from later time points after mating confirm that ovulin (and similarly Acp36DE; data not shown here) processing is blocked in females mated to CG11864 knockdown males. Mated female reproductive tracts were dissected at the indicated times ASM from mates of knockdown males (lane CG11864 RNAi) or control males (lane CG11864 control) and probed with anti-ovulin. Samples from females mated to males knocked down for a different Acp that is not a proteolysis regulator (CG1656) were run as a control to rule out the effects of the *tubulin-GAL4* background.

kDa) (35). Therefore, our observation that only full-length CG11864 is detected in the accessory glands, with a shorter processed product in the seminal fluid mixture, is consistent with the hypothesis that CG11864 is produced as an inactive form in the accessory gland and is activated en route to the female reproductive tract.

**Of Five Proteases Tested, CG11864 Is Necessary for the Cleavage of Other Acps.** Processed forms of ovulin and Acp36DE are detected within the mated female [ovulin (21, 23) and Acp36DE (25)] but not within males (Fig. 1). A prior study indicated that Acps play a role in processing ovulin within the mated female (23). To test whether knockdown of any Acp protease affected the processing of other Acps within the mated female, we generated males with $<2.5\%$ of five predicted Acp proteases (individually; see *Materials and Methods*). Proteins from the reproductive tracts of females mated to individual-protease-knockdown males were probed with antibodies against ovulin, Acp36DE, and CG11864. Processing of both ovulin and Acp36DE was affected only in mates of CG11864 knockdown males (Fig. 3*A*). Protein extracts from mates of CG11864 control males and all other knockdown males had the expected final processing product of 25 kDa for ovulin (23) at 45 min ASM (Fig. 3*A*). However, at this time mates of CG11864 knockdown males showed only full-length 41-kDa and 37-kDa, and an intermediate 33-kDa processing product and lacked the 25-kDa product (Fig. 3*A*, lane RNAi for CG11864). Processing of Acp36DE was similarly affected when females were mated to CG11864 knockdown males: mates of CG11864 control (and all other control and RNAi) males displayed the full-length 122-kDa and the expected 68-kDa processing product (25, 36) whereas mates of CG11864 knockdown males had full-length 122-kDa protein along with only a very faint 68-kDa band (data not shown). These results suggested that the processing of ovulin and Acp36DE was prevented or delayed in mates of males deficient in CG11864.

To determine whether processing was delayed or fully blocked in these females, we examined the processing of ovulin and Acp36DE in mates of CG11864 knockdown males at later time points. In protein samples taken at 1, 2, and 3 h ASM we detected only full-length and an intermediate processing product but not the final 25-kDa processed product of ovulin (Fig. 3*B*). Similarly, only very low levels of the Acp36DE processed product of 68 kDa were detected in these extracts even at 3 h ASM (data not shown). The partial processing of ovulin and Acp36DE in mates of CG11864 knockdown males could be due to the 1–2% of CG11864 protein remaining (seen when the Western blots are exposed for longer durations; data not shown). Another possible explanation for the partial processing may be the involvement of other proteases in processing of these Acps. Nonetheless, our results clearly demonstrate that CG11864 is required for the normal processing of ovulin and Acp36DE in mated females.

Members of the astacin family have been suggested to serve a variety of physiological functions, including digestion in crayfish (37), egg hatching in *Xenopus* (30), morphogenesis, pattern formation in mammals (38) and *Drosophila* (39), and peptide processing in the small intestine and kidney tubules of mammals (29, 40). We report here the involvement of an astacin-like protease in regulating the processing of male-derived molecules necessary for critical female reproductive behaviors in *Drosophila* (i.e., ovulation and sperm storage).

Because ovulin and Acp36DE are not cleaved until they enter the mated female, females must provide a molecule(s) or conditions for this proteolytic processing. Previously, the extent of ovulin processing seen in mated females was shown to depend on the amount of Acps that a female received from her mate (23). Our results indicate that CG11864 is the Acp (or at least one of the Acps) responsible for this.

Interestingly, CG11864's role in processing ovulin and Acp36DE is supported by these proteins' tissue targeting patterns. Within the mated female, CG11864 remains confined to the reproductive tract (35). Acp36DE, which also remains within the reproductive tract (25), is cleaved there. This is also true for a subset of ovulin molecules that remain in the reproductive tract after mating. However, those ovulin molecules that leave the mated female's reproductive system and enter her circulatory system (which lacks CG11864) are not processed.

Mates of males knocked down for any other Acp predicted protease we tested (CG6069, CG6168, CG9997, and CG11664) showed no abnormalities or delays in processing of ovulin, Acp36DE, or CG11864 (ovulin is shown as the representative example in Fig. 3*A*).

**Mates of CG11864 Knockdown Males Lay Eggs, Ovulate, Store Sperm, and Remate at Levels Similar to Controls.** Cleavage of Acps by a mechanism involving CG11864 might be activational [as for prohormones like ELH (41) and astacin-like metalloproteases (42)]. Alternatively, such cleavages might be degradational, perhaps to limit the time in which a female is exposed to Acps. Ovulin enhances ovulation (20), and Acp36DE is essential for efficient sperm storage (36). If the cleavage of these Acps is activational, we would expect mates of CG11864 knockdown males to have lower levels of these measures than mates of controls. To determine whether incomplete processing of ovulin and/or Acp36DE affects these processes, we tested the levels of ovulation, egg laying, fertility, hatchability, sperm storage, and remating of mates of CG11864 knockdown or control males.

**Table 1. Reproductive phenotypes of mates of CG11864 knockdown or CG11864 control males**

| Assay | Females mated to control males (*n*) | Females mated to knockdown males (*n*) |
|---|---|---|
| Egg laying* | 30.0 ± 1.74 (18) | 31.8 ± 1.63 (17) |
| Ovulation† | 0.5 ± 0.1 (49) | 0.7 ± 0.1 (68) |
| Hatchability‡ | 93.1 ± 1.08 (28) | 93.8 ± 1.2 (27) |
| Fertility§ | 27.1 ± 1.59 (18) | 30.6 ± 1.6 (17) |
| Receptivity¶ | 3 of 15 | 3 of 15 |
| Sperm storage‖ | | |
| SR 2 h ASM | 288.3 ± 13.5 (25) | 285.6 ± 14.5 (25) |
| SP 2 h ASM | 201.9 ± 11.9 (25) | 207.6 ± 17.8 (21) |
| SR 4 days ASM | 226.7 ± 20.2 (25) | 217.3 ± 20.7 (25) |
| SP 4 days ASM | 41.2 ± 20.5 (5) | 38.0 ± 23.8 (5) |

Values given are mean ± SE with sample sizes in parentheses.
*Number of eggs laid per day by a mated female for 10 days ASM ($P = 0.31$).
†Number of oocytes released by the ovary of the mated female 3 h ASM ($P = 0.27$).
‡Percentage of laid eggs reaching adult stage ($P = 0.66$).
§Number of progeny per day produced by a singly mated female for 10 days ASM ($P = 0.23$).
¶Ratio of females that remated relative to total number of females tested ($P = 0.67$).
‖Number of sperm stored in the single seminal receptacle (SR) or two spermathecae (SP) of the mated female at 2 h ASM ($P = 0.90$ and 0.79 for SR and SP, respectively) and 4 days ASM ($P = 0.75$ and 0.56 for SR and SP, respectively).

None of these postmating responses differed in magnitude between mates of CG11864 knockdown males and mates of control males (Table 1). Although these results superficially fit the prediction for a nonactivational role of the cleavage, we do not believe that they can distinguish conclusively between activational and nonactivational roles. First, processing of both Acps is not completely inhibited in mates of CG11864 knockdown males; we see partial processing of ovulin and a small amount of the 68-kDa cleavage product of Acp36DE. This partial processing could be due to the residual amounts of CG11864 present in the knockdown males or to the presence of redundant proteolytic activities. Second, if the cleavages are activational but the phenotypic effects of activation are very small, our study may not have had the power to detect these effects. For example, experiments with ovulin have shown that the full-length form has ovulation-stimulatory activity although one of ovulin's cleavage products shows slightly higher activity (24) (this has not been fully tested for Acp36DE). Thus, further studies are required to determine the precise role of processing in the postmating functions of ovulin and Acp36DE.

**None of the Acp Predicted Protease Inhibitors Tested Affects the Processing of Ovulin, Acp36DE, or CG11864.** Protease inhibitors made in the accessory gland could potentially protect Acps from premature or inappropriate proteolysis in the accessory gland before mating (8). If so, loss of that protease inhibitor could result in premature cleavage of Acps in the unmated male. Protease inhibitors from male accessory glands may also regulate Acp proteolysis in mated females. In this case, knockdown of such a protease inhibitor could either speed up processing or cause abnormal Acp processing in mated females. To test whether absence of male-derived protease inhibitors affects Acp proteolysis, protein samples from the accessory glands of protease inhibitor knockdown males or control males and reproductive tracts of their mates were separately probed with antibodies against ovulin, Acp36DE, or CG11864. We did not detect any processing differences from control in the accessory glands of males or the reproductive tracts of their mates upon knockdown of CG6289, CG8137, CG9334, CG10956, Acp76A, and BG642378 (Fig. 4*B*). We did not detect any enhancement or acceleration of Acp processing in females mated to any protease inhibitor knockdown males relative to controls (Fig. 4*A*).

There are several explanations for the lack of an effect of knocking down male-derived protease inhibitors on proteolysis of Acps. First, these protease inhibitors may not be involved in Acp proteolysis. Second, it is possible that these protease inhibitors might be redundant in function. Some redundancy is also noticed in their targeting within the mated female (35). Third, because these males are knockdowns only, presence of residual levels of targeted protease inhibitors may still be sufficient to inhibit proteases involved in Acp cleavage. It is important to note that all of the predicted Acp protease inhibitors that we tested here are predicted serine protease inhibitors (serpins) whereas CG11864 is a predicted zinc metalloprotease (5), and we know of no reports implicating serpins in zinc metalloprotease inhibition. Further studies will be required to see whether molecules like phosphinic peptides, potent inhibitors of zinc metalloproteases (43), are present in the accessory gland.

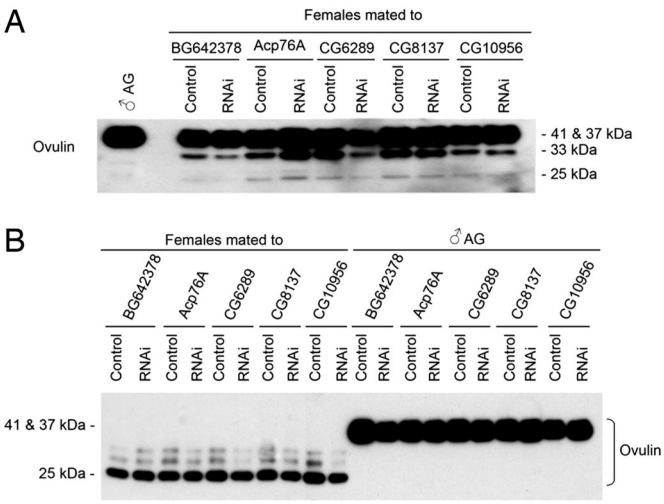


**Fig. 4.** Processing of ovulin in females mated to knockdown males (lane RNAi) of different Acp protease inhibitor lines in comparison with their controls (lane Control) at 15 min ASM (*A*) and 45 min ASM (*B*) (left side). To test whether knockdown of any of the protease inhibitors results in the premature processing of Acps in males, protein samples from male accessory glands (AG) were probed from knockdown males (RNAi) and control males. Analogous to the results shown here, the processing of Acp36DE and CG11864 was also not affected in any of the protease inhibitor knockdown males and their mates (data not shown). Protein equivalents of one pair of male accessory glands or two female reproductive tracts were loaded in each lane.

## Conclusion

We have identified the molecular nature of a male contribution to the processing of Acps that occurs within the mated females. This provides a molecular handle for (at least part of) the male "side" of the male–female cooperation that was proposed for the processing of Acps (23). In addition, identification of this role for CG11864 provides demonstration of an important function for seminal proteases: regulated proteolysis of a prohormone in the seminal fluid. We found that processing of seminal proteins occurs at different times during the mating process, and in different organisms (i.e., male and female of the mating pair). None of the Acps we examined are processed at their site of synthesis, the male accessory gland. CG11864 is processed in the male as the protein transits through the ejaculatory duct on its way to the female. This leads to an intriguing hypothesis, given CG11864's membership in the astacin family of proteases. The prototype of the astacin family, astacin, is usually synthesized as an inactive pro-molecule; cleavage of its propeptide activates the protease. CG11864's sequence is consistent with the presence of a propeptide of the typical size, and its size change as it passes through the male reproductive tract is consistent with removal of the propeptide. If CG11864 is activated by removal of its propeptide during transit through the male, this modification alone is still insufficient for CG11864-mediated processing of ovulin or Acp36DE, because neither target is processed until entry into the female. This suggests that processing of these Acps requires molecule(s) and/or physiological or temporal conditions available in the female that assist CG11864 or remove barriers to the processing of its two direct/indirect targets. Such stepwise proteolysis, beginning in one organism and continuing in another, provides the opportunity for two individuals to regulate the proteolysis of the same molecule and presents an interesting system for investigating the molecular and evolutionary dynamics of proteolytic cascades.

## Materials and Methods

**Fly Stocks.** We used 3- to 5-day-old virgin females from Canton-S strain of *D. melanogaster* for all matings. Knockdowns were generated by crossing appropriate transgenic flies (see below) to *tubulin-GAL4/TM3*, *Sb* (stock no. BL5138; Bloomington Stock Center, Indiana University). All flies were maintained on yeast–glucose medium at room temperature (22 ± 1°C) and 12:12 light/dark cycle.

**Generation of Transgenic Strains to Knock Down 11 Protease or Protease Inhibitor Acps Through RNAi.** We tested for the involvement of five predicted Acp proteases [CG6069, CG6168, CG9997, CG11664, and CG11864 (16)] and six predicted Acp protease inhibitors [BG642378, CG6289, CG8137, CG9334, CG10956 (16), and Acp76A (31)] in the processing of Acps in the mated female reproductive tract. The sympUAST-*w* P-element vector [UAS-*w*-UAS (44)] was modified by insertion of "gateway cassette reading frame A" (Invitrogen, Carlsbad, CA) at its EcoRI site in place of the *white* gene (*w*) in the original vector through standard cloning methods; the sequence of the resulting sympUAST-GW vector (UAS-GW-UAS) was confirmed (Bio Resource Center, Cornell University). Full-length cDNAs for the above-mentioned protease and protease inhibitor genes (16) were cloned into this vector to generate sympUAST-Acp clones (UAS-Acp-UAS). Transgenic lines were generated upon injecting DNA of each of these sympUAST-Acp (UAS-Acp-UAS) clones into embryos of the $w^{1118}$ strain of *D. melanogaster* following standard procedures (45).

**Confirmation of Acp Knockdowns.** Experimental (knockdown) males (*tubulin-GAL4*; UAS-Acp-UAS) and control males (*TM3*, *Sb*; UAS-Acp-UAS) were obtained by crossing *tubulin-GAL4/TM3*, *Sb* females to UAS-Acp-UAS males. Experimental and control males were separated from females within 3–4 h of eclosion and aged separately for 3–5 days in vials containing fresh yeast–glucose medium.

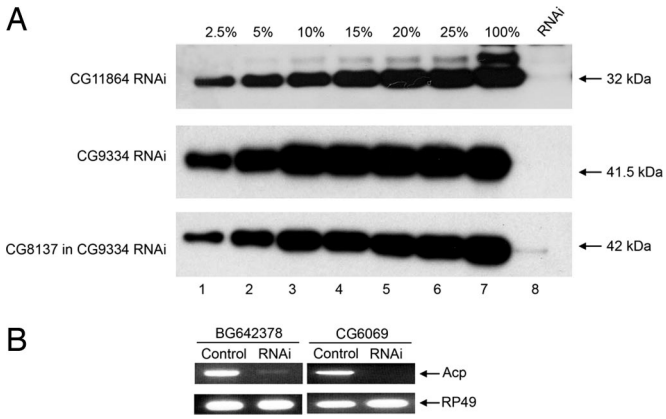


**Fig. 5.** Example Western blots (*A*) and RT-PCR (*B*) showing the levels of Acp knockdown in experimental males (RNAi) compared with control males. (*A*) In Western blots, the levels were quantified by running serial dilutions to the level of 2.5% (lane 1), 5% (lane 2), 10% (lane 3), 15% (lane 4), 20% (lane 5), and 25% (lane 6) of Acps from control male (100%; lane 7) in parallel with Acps equivalent to one experimental male (RNAi). In all but one case, knockdowns were specific to the targeted Acp. The one exception was for the sequence related protease inhibitors (CG8137 and CG9334). Knockdown of CG8137 knocks down CG9334 and vice versa; an example is shown (CG8137 in CG9334 RNAi). (*B*) For PCR amplification, cDNA prepared from RNA extracts of 20 control males or experimental males was used. RP49 primers (48) were used as positive control for the quality/quantity of cDNA. The figure shows exemplar of data for one protease and one protease inhibitor. We observed similar results for all other proteases and protease inhibitors tested.

Levels of Acp knockdown were determined by Western blotting (see *Analyzing the Processing of Ovulin, Acp36DE, Sex Peptide, and CG11864* for brief methodology) for the four Acps (CG6289, CG8137, CG9334, and CG11864) for which sufficiently specific, affinity-purified antibodies are available (35). Relative to the amounts in control males, each of these Acps was knocked down to <2.5% of normal levels (see Fig. 5*A* for examples). The remaining seven Acp lines (CG6069, CG9997, CG11664, CG6168, Acp76A, CG10956, and BG642378) were assessed for the knockdown of specific Acps' transcript levels by using RT-PCR. Total RNA was isolated from ≈20 3- to 5-day-old experimental and control males by TRIzol extraction according to the manufacturer's instructions (GIBCO, Bethesda, MD). cDNA was synthesized by using the SuperScript II first-strand synthesis system for RT-PCR (Invitrogen). Transcript levels were measured semiquantitatively through PCR amplification by using gene-specific primers (16) with subsequent analysis using 1% agarose gel electrophoresis by comparing to PCR products obtained from control males' extracts. We observed no or little amplification of mRNAs from the RNAi-targeted genes in knockdowns compared with control males (see Fig. 5*B* for examples).

To check the specificity of targeting, gene sequences used to generate dsRNA were subjected to the dsCheck program (http://dscheck.rnai.jp), and we confirmed that there are no potential off-targets except for CG8137 and CG9334 (see below). To further confirm that the knockdowns were specific to the targeted Acp, we examined levels of other Acps (e.g., CG14560, ovulin, Acp36DE, and Acp29AB) in knockdown males and their mates relative to control males. With the exception of CG8137 and CG9334, we observed that the knockdown males produced, and transferred to females, normal amounts of all untargeted Acps tested (data not shown). The exceptions were instructive: CG8137 and CG9334 are gene duplicates [≈85% identical at DNA level (5)]. In *tubulin-*

*GAL4*; UAS-8137-UAS males, not only were CG8137 levels knocked down (to <2.5%), but levels of CG9334 were also knocked down to 15–25% of the control males. In *tubulin-GAL4*; UAS-9334-UAS males, both CG9334 and CG8137 levels were knocked down to <2.5% (Fig. 4*A*, see CG8137 and CG9334, lane RNAi). In both cases, no other Acp tested was affected.

**Analyzing the Processing of Ovulin, Acp36DE, Sex Peptide, and CG11864.** To examine protein processing during mating, 3- to 5-day-old Canton-S virgin females and unmated males were mated but then interrupted at 8 min and 10 min ASM. Protein samples from ejaculatory ducts and bulbs of males, or from the reproductive tracts of their mates, were extracted as in Ravi Ram *et al.* (35). To analyze the effect of protease/protease inhibitor knockdown on Acp processing in mated females, 3- to 5-day-old unmated knockdown and control males were mated to 3- to 5-day-old Canton-S virgin females. Female reproductive tracts were dissected at the time indicated in the text (usually 15 min or 45 min ASM). Proteins were extracted for sample preparation as in Ravi Ram *et al.* (35) and subjected to Western blotting as described below. If abnormalities in Acp processing were detected (resulting in the appearance of anomalous bands or the persistence of unprocessed proteins), additional samples from 1, 2, and 3 h ASM were analyzed. Protein equivalents of one pair of accessory glands or eight ejaculatory ducts/bulbs or two female reproductive tracts were loaded in each lane. Samples were separated on 15% SDS/polyacrylamide gels, and Western blotting was performed as described by Ravi Ram *et al.* (35) with a blocking solution containing primary and secondary antibodies diluted 1:1,000 and 1:2,000, respectively. Western blots were probed with antibodies against candidate Acps that are proteolytically processed/cleaved in the mated female reproductive tract: ovulin (21), Acp36DE (25), sex peptide (28), and CG11864 (35). A minimum of two independent lines per construct and four independent sets of samples were probed for each result. We were unable to detect any mobility shift in sex peptide on Western blots under conditions in which it was expected to be processed (ref. 28 and data not shown), and therefore we did not include this Acp in further study.

For the knockdown lines for which we detected abnormality in Acp processing, we carried out egg-laying, hatchability, and fertility assays (for 10 days ASM), receptivity assays (1 day ASM) (following refs. 46 and 47), and ovulation assays (at 3-h ASM following ref. 20) and estimated levels of sperm storage in the female sperm storage organs, namely seminal receptacle and spermathecae (following ref. 36). We used a *t* test to compare the number of sperm in storage between females mated to knockdown and control males, because these data were normally distributed. We used a Mann–Whitney *U* test to analyze egg-laying, ovulation, hatchability, and fertility because the data for these traits were not normally distributed and could not be transformed to normality. We used Fisher's exact test to analyze receptivity to mating (i.e., whether a female was willing to remate).

We thank our past and present laboratory members for their helpful comments on the manuscript and also for their help during the course of this work, especially N. A. Buehner for assistance in making transgenic flies. We give special thanks to Dr. E. Giordano (University of Naples, Naples, Italy) for kindly providing the symPUAST vector. This work was supported by National Institutes of Health Grant HD38921 (to M.F.W.) and National Research Service Award Fellowship 1F32GM074361 (to L.K.S.).

1. He S, Lin YL, Liu YX (1999) *Mol Hum Reprod* 5:513–519.
2. Malm J, Hellman J, Hogg P, Lilja H (2000) *Prostate* 45:132–139.
3. O'Rand MG, Widgren EE, Sivashanmugam P, Richardson RT, Hall SH, French FS, VandeVoort CA, Ramachandra SG, Ramesh V, Jagannadha Rao A (2004) *Science* 306:1189–1190.
4. O'Rand MG, Widgren EE, Wang Z, Richardson RT (2006) *Mol Cell Endocrinol* 250:157–162.
5. Mueller JL, Ripoll DR, Aquadro CF, Wolfner MF (2004) *Proc Natl Acad Sci USA* 101:13542–13547.
6. Veselsky L, Cechova D (1980) *Hoppe-Seyler's Z Physiol Chem* 361:715–722.
7. Wojtczak M, Dietrich GJ, Ciereszko A (2005) *Fish Shellfish Immunol* 19:387–391.
8. Wolfner MF (2002) *Heredity* 88:85–93.
9. Chapman T, Davies SJ (2004) *Peptides* 25:1477–1490.
10. Gillott C (2003) *Annu Rev Entomol* 48:163–184.
11. Ravi Ram K, Ramesh SR (2003) *Indian J Exp Biol* 41:1372–1383.
12. Wolfner MF, Applebaum S, Heifetz Y (2004) *Comprehensive Insect Physiology, Biochemistry, Pharmacology, and Molecular Biology* (Elsevier, Amsterdam).
13. Carvalho GB, Kapahi P, Anderson DJ, Benzer S (2006) *Curr Biol* 16:692–696.
14. Wigby S, Chapman T (2005) *Curr Biol* 15:316–321.
15. Holloway AK, Begun DJ (2004) *Mol Biol Evol* 21:1625–1628.
16. Mueller JL, Ravi Ram K, McGraw LA, Bloch Qazi MC, Siggia ED, Clark AG, Aquadro CF, Wolfner MF (2005) *Genetics* 171:131–143.
17. Swanson WJ, Clark AG, Waldrip-Dail HM, Wolfner MF, Aquadro CF (2001) *Proc Natl Acad Sci USA* 98:7375–7379.
18. Walker MJ, Rylett CM, Keen JN, Audsley N, Sajid M, Shirras AD, Isaac RE (2006) *Proteome Sci* 4:9.
19. Lung O, Tram U, Finnerty CM, Eipper-Mains MA, Kalb JM, Wolfner MF (2002) *Genetics* 160:211–224.
20. Heifetz Y, Lung O, Frongillo EA, Jr, Wolfner MF (2000) *Curr Biol* 10:99–102.
21. Monsma SA, Harada HA, Wolfner MF (1990) *Dev Biol* 142:465–475.
22. Monsma SA, Wolfner MF (1988) *Genes Dev* 2:1063–1073.
23. Park M, Wolfner MF (1995) *Dev Biol* 171:694–702.
24. Heifetz Y, Vandenberg LN, Cohn HI, Wolfner MF (2005) *Proc Natl Acad Sci USA* 102:743–748.
25. Bertram MJ, Neubaum DM, Wolfner MF (1996) *Insect Biochem Mol Biol* 26:971–980.
26. Chapman T, Bangham J, Vinti G, Seifried B, Lung O, Wolfner MF, Smith HK, Partridge L (2003) *Proc Natl Acad Sci USA* 100:9923–9928.
27. Liu H, Kubli E (2003) *Proc Natl Acad Sci USA* 100:9929–9933.
28. Peng J, Chen S, Busser S, Liu H, Honegger T, Kubli E (2005) *Curr Biol* 15:207–213.
29. Dumermuth E, Eldering JA, Grunberg J, Jiang W, Sterchi EE (1993) *FEBS Lett* 335:367–375.
30. Katagiri C, Maeda R, Yamashika C, Mita K, Sargent TD, Yasumasu S (1997) *Int J Dev Biol* 41:19–25.
31. Coleman S, Drahn B, Petersen G, Stolorov J, Kraus K (1995) *Insect Biochem Mol Biol* 25:203–207.
32. Anakwe OO, Sharma S, Hoff HB, Hardy DM, Gerton GL (1991) *Mol Reprod Dev* 29:294–301.
33. Eddy EM, Vernon RB, Muller CH, Hahnel AC, Fenderson BA (1985) *Am J Anat* 174:225–237.
34. Zwilling R, Stöcker W (1997) *The Astacins: Structure and Function of a New Protein Family* (Kovac, Hamburg).
35. Ravi Ram K, Ji S, Wolfner MF (2005) *Insect Biochem Mol Biol* 35:1059–1071.
36. Neubaum DM, Wolfner MF (1999) *Genetics* 153:845–857.
37. Stocker W, Gomis-Ruth FX, Bode W, Zwilling R (1993) *Eur J Biochem* 214:215–231.
38. Fukagawa M, Suzuki N, Hogan BL, Jones CM (1994) *Dev Biol* 163:175–183.
39. Ferguson EL, Anderson KV (1992) *Development (Cambridge, UK)* 114:583–597.
40. Gorbea CM, Marchand P, Jiang W, Copeland NG, Gilbert DJ, Jenkins NA, Bond JS (1993) *J Biol Chem* 268:21035–21043.
41. Newcomb R, Fisher JM, Scheller RH (1988) *J Biol Chem* 263:12514–12521.
42. Mohrlen F, Baus S, Gruber A, Rackwitz HR, Schnolzer M, Vogt G, Zwilling R (2001) *Eur J Biochem* 268:2540–2546.
43. Dive V, Georgiadis D, Matziari M, Makaritis A, Beau F, Cuniasse P, Yiotakis A (2004) *Cell Mol Life Sci* 61:2010–2019.
44. Giordano E, Rendina R, Peluso I, Furia M (2002) *Genetics* 160:637–648.
45. Rubin GM, Spradling AC (1982) *Science* 218:348–353.
46. Herndon LA, Wolfner MF (1995) *Proc Natl Acad Sci USA* 92:10114–10118.
47. Kalb JM, DiBenedetto AJ, Wolfner MF (1993) *Proc Natl Acad Sci USA* 90:8093–8097.
48. Fiumera AC, Dumont BL, Clark AG (2005) *Genetics* 169:243–257.