The *Drosophila melanogaster* Seminal Fluid Protein Acp62F Is a Protease Inhibitor That Is Toxic Upon Ectopic Expression

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ABSTRACT

Drosophila melanogaster seminal fluid proteins stimulate sperm storage and egg laying in the mated female but also cause a reduction in her life span. We report here that of eight Drosophila seminal fluid proteins (Acps) and one non-Acp tested, only Acp62F is toxic when ectopically expressed. Toxicity to preadult male or female Drosophila occurs upon one exposure, whereas multiple exposures are needed for toxicity to adult female flies. Of the Acp62F received by females during mating, ~10% enters the circulatory system while ~90% remains in the reproductive tract. We show that in the reproductive tract, Acp62F localizes to the lumen of the uterus and the female's sperm storage organs. Analysis of Acp62F's sequence, and biochemical assays, reveals that it encodes a trypsin inhibitor with sequence and structural similarities to extracellular serine protease inhibitors from the nematode Ascaris. In light of previous results demonstrating entry of Acp62F into the mated female's hemolymph, we propose that Acp62F is a candidate for a molecule to contribute to the Acp-dependent decrease in female life span. We propose that Acp62F's protease inhibitor activity exerts positive protective functions in the mated female's reproductive tract but that entry of a small amount of this protein into the female's hemolymph could contribute to the cost of mating.

IN *Drosophila melanogaster*, secretions from the male accessory glands, the ejaculatory duct, and the ejaculatory bulb comprise the bulk of seminal fluid (Bairati 1968). *Ac*cessory gland *p*roteins (Acps) have been shown to be critical to the male's reproductive success. Acps have diverse effects on the mated female: stimulating a rapid increase in ovulation and egg-laying rate (Chen *et al.* 1988; Aigaki *et al.* 1991; Kalb *et al.* 1993; Herndon and Wolfner 1995; Heifetz *et al.* 2000, 2001; Xue and Noll 2000; Chapman *et al.* 2001), inducing a decrease in receptivity to further mating (Chen *et al.* 1988; Aigaki *et al.* 1991; Xue and Noll 2000), mediating efficient sperm storage (Kalb *et al.* 1993; Neubaum and Wolfner 1999; Tram and Wolfner 1999) and sperm competition (Harshman and Prout 1994; Clark *et al.* 1995; Chapman *et al.* 2000), and causing a dose-dependent decrease in the female's life span (Chapman *et al.* 1995). Since it seems unlikely that evolution would select for male components that function only to decrease female life span, the negative impact of Acps on female life span has been suggested to be a side effect of positive functions of Acps (Chapman *et al.* 1995).

There are ~83 Acps, most of whose functions are presently unknown (Schäfer 1986; DiBenedetto *et al.* 1987; Chen *et al.* 1988; Monsma and Wolfner 1988; Simmerl *et al.* 1995; Wolfner *et al.* 1997; Swanson *et al.* 2001). Mutational analysis demonstrated that Acp26Aa ("ovulin") acts to stimulate egg laying in mated female flies (Herndon and Wolfner 1995; Heifetz *et al.* 2000; Chapman *et al.* 2001) and that Acp36DE is required for efficient sperm storage by females (Neubaum and Wolfner 1999; Chapman *et al.* 2000). While mutants in other Acp genes have not yet been reported, ectopic expression or injection of purified Acp70A ("sex peptide") into unmated females caused progression of oogenesis and decreased sexual receptivity (Chen *et al.* 1988; Aigaki *et al.* 1991; Nakayama *et al.* 1997; Soller *et al.* 1997, 1999). To begin to identify Acps that may contribute to the Acp-dependent decrease in female life span, we ectopically expressed secreted forms of eight different Acps individually during development and assayed for their toxicity to *D. melanogaster*. We report here that ectopic expression of only Acp62F induced increased mortality. Furthermore, when Acp62F was ectopically expressed in adult flies, it led to early mortality.

Acp62F is transferred to females during mating and ~10% of the transferred Acp62F enters the female's hemolymph, thus giving it access to organs outside the reproductive tract (Lung and Wolfner 1999). Here we show that the Acp62F that remains in the female's

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reproductive tract enters the sperm storage organs, placing it in a position to affect sperm.

To ascertain what functions Acp62F might play and why it might be toxic to Drosophila, we used sequence analysis and homology modeling to predict Acp62F's biochemical function. We report here that Acp62F has sequence and structural similarity to a novel class of small extracellular serine protease inhibitors from Ascaris, parasitic roundworms that infect the intestinal tract of mammals (Babin *et al.* 1984). These nematodes secrete protease inhibitors with specificities toward different proteases and these inhibitors may protect the worm from its host's digestive enzymes (Goodman and Peanasky 1982). We demonstrate that recombinant Acp62F indeed has trypsin inhibitory activity and weak chymotrypsin inhibitory activity *in vitro* and that mutating its predicted active site results in predicted changes in its protease inhibitory activity.

Protease inhibitors are present in the seminal fluid of many mammalian species [see, for example, human (Schiessler *et al.* 1976; Fink *et al.* 1990), bull (Cechova and Fritz 1976; Lessley and Brown 1978), horse (von Fellenberg *et al.* 1985), and boar (Veselsky *et al.* 1985)] and they have been shown to play an important role in male fertility. For example, mutant mice lacking the serine protease inhibitor protease nexin-1 (PN-1) in their seminal fluid have impaired fertility (Murer *et al.* 2001), and infertility in some human males is correlated with lack of functional protein C inhibitor (He *et al.* 1999) or with abnormal PN-1 levels in their seminal fluid (Murer *et al.* 2001). Protease inhibitors in seminal fluid have been proposed (1) to protect the reproductive tract and/or sperm from proteolysis by damaged or prematurely acrosome-reacted sperm or (2) to regulate the processing or degradation of seminal fluid proteins and/or coagulation of semen. Protein C inhibitor, for example, is a serpin that forms a complex with prostate-specific antigen (PSA, a serine protease; Watt *et al.* 1986). This complex is thought to prevent degradation of semenogelins and thus liquefaction of the semen coagulum and consequent outflow of spermatozoa from the female's reproductive tract (Kise *et al.* 1996 and reviewed by Robert and Gagnon 1999).

In light of Acp62F's presence in the reproductive tract and its protease inhibitory activity, we propose that Acp62F plays a beneficial role in regulating proteolysis in the genital tract. Given its entry into the female hemolymph (Lung and Wolfner 1999) and toxicity upon ectopic presence in the hemolymph, we propose that Acp62F is a candidate for a molecule that could contribute to the Acp-dependent decrease in female life span (Chapman *et al.* 1995) by inhibiting essential proteolytic events in the hemolymph.

MATERIALS AND METHODS

Fly stocks: All flies were maintained on a 12 hr light/dark cycle at 23° ± 2°. *UAS-Acp* lines for *Acp29AB*, *Acp32CD*, *Acp33A*, *Acp53Ea*, *Acp62F*, *Acp63F/64A*, and *Acp95EF* were generated as described below. *UAS-Acp26Aa* was described in Park and Wolfner (1995). *Hsp70-GAL4/CyO* (Brand and Perrimon 1993) was used as the GAL4 driver. *UAS-GFP* (Yeh *et al.* 1995) was a kind gift from G. Boulianne. Where available, at least two independent lines of each *UAS-Acp* were tested; consistency of results between lines was used to distinguish Acp-specific from line-related effects. All flies for analysis were collected on ice within 4 hr of eclosion and aged for 3–5 days in groups of three to four individuals per vial.

For germline transformation, full-length cDNAs including the predicted signal sequences for the Acps to be tested (Monsma and Wolfner 1988; DiBenedetto *et al.* 1990; Wolfner *et al.* 1997) were cloned into the pUAST transformation vector via *Eco*RI sites (Brand and Perrimon 1993; Kalb *et al.* 1993; Park and Wolfner 1995; this study). Restriction enzyme digestion and sequencing confirmed the cDNAs inserted with their 5′ ends at the predicted position near the 3′ end of the UAS elements. Transgenic flies were generated as in Park and Lim (1995). All lines used in this study carried independent single inserts of *UAS-Acp* as determined by genomic Southern blot analysis (data not shown). All transgenes include sequences that encode the predicted signal sequence to allow secretion of the Acp. Expression of the transgenes was checked by Northern blotting in all cases except for Acp29AB and Acp32CD and confirmed by Western blotting for those proteins for which antibodies were available: Acp26Aa (Monsma and Wolfner 1988), Acp62F (Lung and Wolfner 1999), and green fluorescent protein (GFP; a kind gift from Tom Fox).

Preadult lethality assay: To determine the preadult toxicity of each Acp, we induced its ectopic expression during larval and pupal development. All Acps and GFP were tested in parallel. Nine replicates were set for each line. For each replicate, four virgin *UAS-Acp* or *UAS-GFP* females were allowed to mate with four virgin *hsp70-GAL4/CyO* males overnight. The following day, the parents were removed and the number of eggs the females had laid were counted. Each of the nine replicates then followed a different heat-shock regimen. To control for effects resulting from low basal expression of the *hsp70* promoter during development (Mason *et al.* 1984; Glaser *et al.* 1986), one replicate was not heat-shocked. Each of the remaining eight replicates was heat-shocked once on a specified day of development. The animals were heat-shocked by immersing their vial in a 37° water bath for 1 hr at approximately the same time of day each day. The flies were then allowed to develop to adulthood at 23° ± 2°. The number of emerging adults was scored, and the number of dead pupae (which had turned black) was also recorded. Only *hsp70-GAL4; UAS-Acp* animals (non-Curly) are induced to express their Acps. Their *Cy; UAS-Acp* siblings, which do not express the Acps, served as an internal control for the effects of the heat-shock treatments on development and viability. By scoring the proportion of emerging adults that are Curly *vs.* non-Curly, it could be deduced if and when Acp expression was lethal. Chi-square analysis was used to statistically compare the proportion of survivors of each genotype.

Adult lethality assay: To test for effects of Acp62F on adult viability, sibling Curly and non-Curly virgin female progeny from crosses of *UAS-Acp62F* virgin females (lines 2 and 4) to *hsp70-GAL4/CyO* males were subjected to heat shock (as above) on days 2, 4, 6, 8, 10, and 12 posteclosion. The number of surviving Curly and non-Curly flies was counted daily. The statistical significance of the survival distribution was assessed by a log-rank test.

Immunohistochemistry and confocal microscopy: Reproductive tracts were dissected from unmated Canton-S (wild-type) females and from wild-type females 50 min after the start of mating to wild-type males and processed for immunohistochemistry as in Lung and Wolfner (1999).

Recombinant protein production using baculovirus: The entire Acp62F coding region including that of the signal sequence was amplified by PCR using the primers P62F5′Bam (CCGGGGATCCCTTCTATTACTTTTC) and P62F3′H6 (GCT AGCGGCCGCTTAGTGATGGTGATGGTGATGTGAACAGT TGTA). The 5′ primer generated a *Bam*HI site upstream of the start codon and the 3′ primer generated a 6-His tag following the last codon. The PCR product was cloned into the pBacPac8 transfer vector (CLONTECH, Palo Alto, CA) to generate p62FH6BP. Site-directed mutagenesis was performed on p62FH6BP using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) to generate two single mutations (C59A and K61A) and a double mutation (C59A, C63A) with alanine replacements in the Acp62F coding region. The primers used for mutagenesis, with the changes underlined, are the following: C59A (GGCAATGGACCCGCCGTCAAGATGTG); K61A (GACCCTGCGTCGCGATGTGCGGAGC); and C59,63A (GGC AATGGACCCGCCGTCAAGATGGCCGGATCTCCTTG). The entire PCR-derived region and flanking sequences in each construct were sequenced to ensure that no nucleotide substitutions had occurred during PCR.

Recombinant baculovirus generation and protein expression were done as in Hefferon *et al.* (1999) with minor modifications. Recombinant viruses were screened by PCR for the presence of Acp62F coding sequences and by Western blotting for secretion of Acp62F-H6 protein into the culture medium. Acp62F's endogenous signal sequence was sufficient to drive protein secretion in cell lines derived from *Trichoplusia ni* (BTI-5B1-4) and *Spodoptera frugiperda* (Sf9 and Sf21). The protein produced by these cells was secreted and of the same size as observed in *D. melanogaster* males, taking into account the size of the 6-His tag (data not shown). Acp62F-H6 protein was purified from the culture medium of BTI-5B1-4 cells, 4 days after virus infection, with Ni^{+}-agarose resin under nondenaturing conditions as suggested by the manufacturer (QIAGEN, Chatsworth, CA). Eluted samples were run on SDS-PAGE and Acp62F-H6-containing fractions were identified by silver staining, pooled, concentrated, and buffer exchanged to 100 mm NaCl/20 mm Tris, pH 7.5, for protease inhibition assays (see below). Purified Acp62F-H6 protein was either stored in small aliquots at −70° or used immediately.

Sequence comparison and structure prediction: All database searching was performed through the World Wide Web, using the predicted sequence of secreted Acp62F (amino acids 15–115; Wolfner *et al.* 1997) as the query. BLAST searching was performed using the BLASTP v2.0.9 (May 7, 1999) algorithm (Altschul *et al.* 1997) and the nonredundant database set covering GenBank, coding sequence translations, Protein DataBank (PDB), SwissProt, Swiss-Prot updates, and Protein Information Resource. FASTA searching was performed using version 3.2t05 (May 12, 1999) and the nonredundant Swall database set (Pearson and Lipman 1988). FASTA searching was also performed with the SwissProt data set alone. The sequence was also submitted to the PredictProtein server (http: // www.embl-heidelberg.de / predictprotein / predictprotein.html) for secondary structure prediction using the PHD algorithm (Rost and Sander 1993, 1994; Rost *et al.* 1994). Homolog detection using hidden Markov models (Karplus *et al.* 1998) was also performed through the SAM server at the University of California at Santa Cruz (http://www.cse.ucsc. edu/research/compbio/HMM-apps/).

Structural homologs were identified by submitting the Acp62F sequence to the META PredictProtein server (http://www.embl-heidelberg.de / predictprotein / submit_meta.html; Rost 1996) and the UCLA-DOE Structure Prediction Server (http://www.doe-mbi.ucla.edu/people/frsvr/frsvr.html; Fischer and Eisenberg 1996). Threading was performed using the program 123D (http://www-lmmb.ncifcrf.gov/~nicka/123D.html; Alexandrov *et al.* 1996). After potential structural homologs (PDB: 1ata and 1atb) were identified, the Acp62F sequence and coordinate files for the homologs were submitted to SWISS-MODEL (http://www.expasy.ch/swissmod/SWISS-MODEL.html). The homology model returned from SWISS-MODEL was manually adjusted to align the conserved Cys residues, and the resulting model was visualized using the Swiss-PdbViewer software for the Macintosh (Guex and Peitsch 1997). Persistence of vision (POV) files were generated with the Swiss-PdbViewer, and the ray-traced images were rendered using the POV-Ray version 3.1g for the Macintosh, available through the World Wide Web at http://www.povray.org/.

Protease inhibition assay: To test whether Acp62F-H6 could inhibit protease activity, protease inhibition assays were performed at least three times according to Coffman and Goetz (1998), with minor modifications. Bovine pancreatic trypsin (EC3.4.21.4, T-8642), bovine pancreatic chymotrypsin (EC3.4.21.1, C-3142), bovine plasma thrombin (EC3.4.21.5, T-7513), and porcine pancreatic elastase (EC3.4.21.36, E-0258) were assayed with the substrates Na-benzoyl-L-arg *p*-nitroanilide (NA), *N*-succinyl-gly-gly-phe *p*-NA, *N*-succinyl-ala-ala-pro-leu *p*-NA, *N-p*-tosyl-gly-pro-arg *p*-NA, respectively. Briefly, 1 μg of protease (except where specified otherwise) was preincubated with varying concentrations of Acp62F-H6 or control proteins (see below) for 30 min at 22° ± 1° [room temperature (RT)] in 0.95 ml of reaction buffer (0.1 M Tris-HCl, pH 7.5) before addition of 0.5 mm chromogenic protease substrate to a final volume of 1 ml. After addition of substrate, absorbance at 405 nm was measured immediately ($t = 0$) and once every minute for 30 min ($t = 30$) at RT using a Beckman DV40 spectrophotometer. Aprotinin was used as a positive control and lysozyme or bovine serum albumin (BSA) as a negative control for trypsin inhibition assays. All proteases, assay substrates, and control proteins were purchased from Sigma Chemical (St. Louis). Residual protease activity was derived by the following calculation: ($_{\Delta 30,0}$A405 of protein preincubated protease/ $_{\Delta 30,0}$A405 of buffer preincubated protease) × 100%. For assessment of the effects of disulfide bridges in Acp62F-H6, 5 μg of Acp62F-H6 (or buffer as control) was treated with 50 mm dithiothreitol (DTT) for 10 min at RT, prior to preincubation with trypsin.

Control proteins (BSA and lysozyme at similar concentrations as Acp62F-H6) did not inhibit trypsin activity at all, but instead caused a greater increase in A405 relative to buffer-pretreated trypsin, presumably due to reduced trypsin autolysis (Maroux and Desnuelle 1969). This protection against autolysis was also seen with versions of Acp62F-H6 that do not possess inhibitory activity against the proteases tested (see results).

[^{3}H]Diisopropyl fluorophosphate (DFP) labeling: A total of 10 μl (0.5 μg/μl) of wild-type Acp62F-H6 or K61A mutant protein (or buffer as control) was incubated with 5 μl of trypsin or elastase (0.1 μg/μl) for 30 min at RT. Then 2 μl [^{3}H]DFP (NEN Life Sciences) was added and incubated for another 10 min. The resulting mixture was then subjected to SDS-PAGE analysis on a 12% polyacrylamide gel and to autoradiography, as in Hörler and Briegel (1997).

RESULTS

Acp62F, but not other Acps tested, is toxic to Drosophila: To identify Acps that may cause the reduction in the life span of mated females, we generated transgenic flies that ectopically express individual Acps using the GAL4/UAS system (Brand and Perrimon 1993) and measured their survival. Homozygous *UAS-Acp* females were mated to *hsp70-GAL4/CyO* males (Brand and Perrimon 1993) and their progeny were heat-shocked

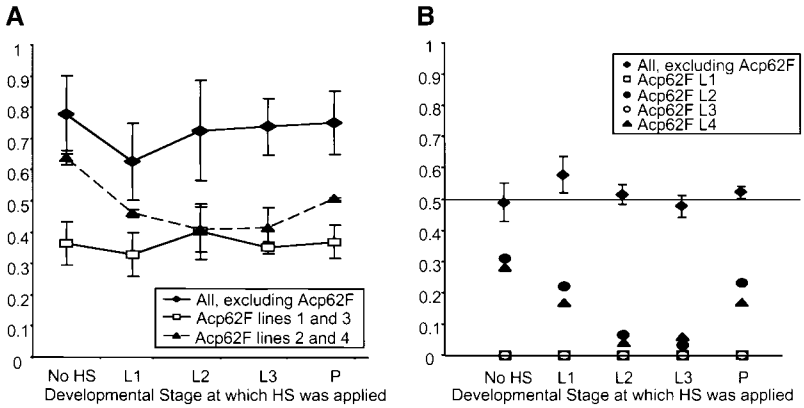

FIGURE 1.—Ectopic expression of only Acp62F during development results in reduced viability. (A) The percentage of all adults that emerged from eggs laid was calculated and plotted to determine whether heat-shock treatment and induction of ectopic expression of Acps in preadults influenced viability. Heat-shock-induced expression of Acp26Aa, Acp29AB, Acp32CD, Acp33A, Acp53Ea, Acp63F/64A, or Acp95EF (solid diamond) or GFP (not shown) during any stage of preadult development (indicated on *x*-axis) did not affect cross-viability, as indicated by the high percentage of eggs that developed to adulthood. In contrast, the percentage of eggs that developed to adulthood in vials that were heat-shocked to induce expression of Acp62F in lines 2 and 4 was reduced relative to control vials that were not heat-shocked (solid triangle). For Acp62F, lines 1 and 3, the percentage of eggs that developed to adulthood, even in vials that were not heat-shocked, was lower than in vials that were induced to express other Acps, indicating that basal expression of Acp62F from the *hsp70* promoter was negatively affecting viability (open squares). Heat shocks were done on days 1, 2, 3, 4, 5, 6, 7, or 9 post-egg laying. Animals that were heat-shocked on days 1–4 were larvae and are grouped as primarily first instar (L1, day 1), second instar (L2, day 2), or third instar (L3, days 3 and 4). Animals heat-shocked on days 5–9 were primarily pupae (P). (B) Decreased viability observed in crosses involving Acp62F is due to the decrease or lack of non-Curly animals, which are induced to express Acp62F. Ectopic expression of Acps other than Acp62F (solid diamond) resulted in 50% Curly and 50% non-Curly progeny under both non-heat-shock and heat-shock conditions, indicating that their expression does not affect viability. In Acp62F, lines 2 (solid circle) and 4 (solid triangle), the percentage of non-Curly was significantly ($P < 0.05$) below 50%, even under non-heat-shock conditions. When heat-shocked during L1, L2, L3, or P, the percentage of non-Curly progeny was further decreased, indicating that expression of Acp62F during development is toxic. In Acp62F, lines 1 (open square) and 3 (open circle), non-Curly progeny, even under non-heat-shock conditions, were absent, further indicating that Acp62F is toxic during development. Error bars present standard errors of the mean.

once on a specified day during postembryonic development to induce expression of Acps. Acp expression was induced only in non-Curly (*hsp70-GAL4; UAS-Acp*) animals. Their Curly (*CyO; UAS-Acp*) sibs did not express Acps and served as controls for the heat-shock treatment (see MATERIALS AND METHODS). Acp toxicity is detected by monitoring the proportion of Curly to non-Curly flies that emerge.

For all non-Acp62F lines, both Curly and non-Curly adult flies emerged under non-heat-shock conditions. Approximately 78% of all eggs laid developed to adulthood (Figure 1A). The proportion of progeny emerging approximated the expected 50% Curly flies and 50% non-Curly flies in each case (Figure 1B and Table 1). In each category, males and females were approximately equally represented. This demonstrated that under non-heat-shock conditions, basal level expression of Acps or GFP, driven by the *hsp70* promoter (GLASER *et al.* 1986) during development, did not affect viability.

Increased mortality was not observed when larvae or pupae were induced to express secretable Acp26Aa, Acp29AB, Acp32CD, Acp33A, Acp53Ea, Acp63F/64A, Acp95EF (Figure 1A), or GFP (data not shown). As under non-heat-shock conditions, the proportion of Curly to non-Curly flies approximated 50:50 in most of these lines when heat shock was applied during L1 (day 1), L2 (day 2), L3 (day 3 or 4), and P (day 5, 6, 7, or 9) of development (Figure 1B). While significant differences from the expected 50:50 ratio of Curly to non-Curly were occasionally observed, they occurred in only one of the two lines tested for each of these Acps (Table 1), suggesting that the effects are line specific rather than Acp specific. Thus, heat shock and expression of any of these seven Acp genes or GFP on any given day of development did not decrease survival to adulthood.

In contrast to the results for non-Acp62F genes, expression of Acp62F produced significantly fewer non-Curly adults or no non-Curly adults (Figure 1B and Table 1), even under non-heat-shock conditions (see below).

UAS-Acp62F lines 2 and 4: In contrast to the results for the other seven Acp genes and GFP, expression of Acp62F during preadult development significantly decreased viability. Under non-heat-shock conditions, the percentage of eggs that developed to adulthood was slightly lower for these lines (61 and 66%, respectively; Figure 1A) than for the other UAS-Acp constructs tested. Very few of the emerging adults were non-Curly (Figure 1B and Table 1), suggesting that low levels of Acp62F expression due to basal activity of the *hsp70* promoter slightly depressed the viability of preadults.

A reduced percentage of Acp62F-expressing progeny survived to adulthood when the crosses were heat-shocked to induce Acp62F expression during L1 (day 1), L2 (day 2), L3 (day 3 or 4), and P (day 5, 6, 7, or 9). Compared to a 61–66% survival rate under non-heat-shock conditions, the survival rate for heat-shocked animals was 41 to 51% (Figure 1A). Moreover, the majority (69–97%) of adults emerging in these vials were Curly (rather than the 50% expected; Figure 1B and Table 1), suggesting that the reduction in survival was due to non-Curly ani-

TABLE 1

Summary of results from hs-inducedAcp expression study in preadults

	Eggs	Adults	Cy (%)	Non-Cy (%)		Eggs	Adults	Cy (%)	Non-Cy (%)
	Acp26Aa (103.1)					Acp53Ea (N1)			
No HS	38	26	60.0	40.0	No HS	41	43	60.4	39.6*
L1 (Day 1)	75	40	42.3	57.7	L1 (Day 1)	69	47	32.8	67.2*
L2 (Day 2)	54	42	49.3	50.7	L2 (Day 2)	68	62	47.9	52.1
L3 (Day 3, 4)	97	77	55.5	44.5	L3 (Day 3, 4)	151	119	55.4	44.6
P (Days 5, 6, 7, 9)	220	172	50.3	49.7	P (Days 5, 6, 7, 9)	197	176	43.3	56.7*
	Acp26Aa (107.1)					Acp63F/64A (L2)			
No HS	46	37	61.7	38.3*	No HS	74	68	50.6	49.4
L1 (Day 1)	40	25	35.5	64.5*	L1 (Day 1)	72	60	28.4	71.6*
L2 (Day 2)	69	53	46.6	53.4	L2 (Day 2)	88	78	49.4	50.6
L3 (Day 3, 4)	126	85	47.9	52.1	L3 (Day 3, 4)	167	121	61.0	39.0*
P (Days 5, 6, 7, 9)	264	205	46.7	53.3	P (Days 5, 6, 7, 9)	261	236	49.0	51.0
	Acp29AB (29.3)					Acp95EF (J1)			
No HS	75	56	47.4	52.6	No HS	129	83	59.0	41.0
L1 (Day 1)	63	40	53.2	46.8	L1 (Day 1)	202	108	38.9	61.1*
L2 (Day 2)	77	53	56.3	43.8	L2 (Day 2)	125	61	49.2	50.8
L3 (Day 3, 4)	109	89	51.2	48.8	L3 (Day 3, 4)	253	178	49.4	50.6
P (Days 5, 6, 7, 9)	250	168	51.1	48.9	P (days 5, 6, 7, 9)	459	238	49.2	50.8
	Acp29AB (36.4)					Acp95EF (J5)			
No HS	56	45	48.8	51.2	No HS	55	22	40.9	59.1
L1 (Day 1)	75	46	42.1	57.9	L1 (Day 1)	101	39	41.0	59.0
L2 (Day 2)	63	48	37.2	62.8*	L2 (Day 2)	126	32	46.9	53.1
L3 (Day 3, 4)	112	94	47.9	52.1	L3 (Day 3, 4)	190	75	56.0	44.0
P (Days 5, 6, 7, 9)	263	196	46.5	53.5	P (Days 5, 6, 7, 9)	482	230	46.9	53.1
	Acp32CD (3.4)					Acp62F (LUL1)			
No HS	79	51	42.9	57.1	No HS	60	26	100.0	0.0*
L1 (Day 1)	69	53	42.7	57.3	L1 (Day 1)	65	26	100.0	0.0*
L2 (Day 2)	60	46	51.2	48.8	L2 (Day 2)	59	29	100.0	0.0*
L3 (Day 3, 4)	96	68	46.3	53.7	L3 (Day 3, 4)	159	53	100.0	0.0*
P (Days 5, 6, 7, 9)	204	152	48.4	51.6	P (Days 5, 6, 7, 9)	257	109	100.0	0.0*
	Acp32CD (4.4)					Acp62F (LUL2)			
No HS	46	41	51.7	48.3	No HS	65	43	69.0	31.0*
L1 (Day 1)	54	18	48.1	51.9	L1 (Day 1)	80	38	78.0	22.0*
L2 (Day 2)	39	20	45.7	54.3	L2 (Day 2)	65	22	93.5	6.5*
L3 (Day 3, 4)	125	85	50.5	49.5	L3 (Day 3, 4)	133	47	96.8	3.2*
P (Days 5, 6, 7, 9)	271	206	47.6	52.4	P (Days 5, 6, 7, 9)	268	135	76.9	23.1*
	Acp33A (T4A)					Acp62F (LUL3)			
No HS	63	54	44.2	55.8	No HS	68	20	100.0	0.0*
L1 (Day 1)	40	29	52.7	47.3	L1 (Day 1)	65	17	100.0	0.0*
L2 (Day 2)	54	53	48.8	51.2	L2 (Day 2)	73	23	100.0	0.0*
L3 (Day 3, 4)	153	131	54.1	45.9	L3 (Day 3, 4)	65	24	100.0	0.0*
P (Days 5, 6, 7, 9)	255	207	44.2	55.8*	P (Days 5, 6, 7, 9)	208	66	100.0	0.0*
	Acp53Ea (N2)					Acp62F (LUL4)			
No HS	54	47	45.8	54.2	No HS	70	43	71.6	28.4*
L1 (Day 1)	73	62	49.7	50.3	L1 (Day 1)	78	35	82.9	17.1*
L2 (Day 2)	39	36	53.8	46.2	L2 (Day 2)	54	26	95.7	4.3*
L3 (Day 3, 4)	134	119	52.7	47.3	L3 (Day 3, 4)	131	63	94.0	6.0*
P (Days 5, 6, 7, 9)	222	208	50.6	49.4	P (Days 5, 6, 7, 9)	250	128	82.7	17.3*

Ectopic expression of Acp62F is lethal to preadult Drosophila. Progeny from UAS-Acp females and hsp70-Gal4/CyO males were heat-shocked once for 1 hr on a specified day during development and allowed to develop to adulthood. These data have been grouped into developmental stages during which the animals were heat-shocked, as indicated in the table. Animals that were heat-shocked on day 1 were classified as L1, on day 2 as L2, on days 3 or 4 as L3, and on days 5, 6, 7, or 9 as P. The proportion of emerging adults that were Curly versus non-Curly was scored to determine if expression of the Acp during development is lethal. Deviations from the expected 50/50 ratio of Curly to non-Curly was tested by chi-square analysis and significant differences ($P < 0.05$) are indicated by *. Only those animals expressing Acp62F showed enhanced lethality during all stages of development and in all lines.

mals being killed by expression of Acp62F prior to adulthood. When heat shock was applied during L3 (day 4) and P (day 5, 6, or 7), dead pupae were evident, a situation not observed in other *hsp70-GAL4/CyO* × *UAS-Acp* crosses. This indicated that expression of Acp62F on days 4 through 7 caused pupal lethality while expression earlier may have prevented the formation of pupae. More than 90% of the progeny were Curly when heat shock was applied during L2 (day 2), L3 (day 3 and 4) for line 2 and during L2 (day 2), L3 (day 3 and 4), and P (day 5, 6, 7, or 9) for line 4 (Table 1). Thus, overexpression of Acp62F on any day (1 through 7) of preadult development is toxic to *D. melanogaster*.

UAS-Acp62F lines 1 and 3: Surprisingly, progeny from *hsp70-GAL4/CyO* × *UAS-Acp62F* lines 1 or 3, even under non-heat-shock conditions, displayed much lower survival rates (43 and 29%, respectively) than other *hsp70-GAL4/CyO* × *UAS-Acp* crosses (Figure 1A). With *UAS-Acp62F* lines 1 and 3, only Curly adults emerged and dead pupae were not observed (Figure 1B and Table 1), indicating that lethality occurred prior to the pupal stage. The absence of non-Curly flies under non-heat-shock conditions suggested that basal expression from the *hsp70* promoter might have killed these animals. However, since non-Curly *hsp70-Gal4; UAS-62F* lines 2 and 4 flies were obtained under non-heat-shock conditions, expression of Acp62F driven by the basal activity from the *hsp70* promoter could not have been solely responsible for the death of *hsp70-GAL4; UAS-62F* animals from lines 1 or 3. Since the *UAS-Acp62F* lines 1 and 3 stocks themselves are viable, it is unlikely that the *UAS-Acp62F* construct inserted into the genome near an enhancer or promoter that drove its expression independently of GAL4 in these lines. It is more likely that the transgene inserted near a regulatory element that enhanced expression from the UAS element when it was activated at a low level, *e.g.*, by low basal amounts of *hsp-70*-driven GAL4. In summary, ectopically expressed Acp62F is toxic to preadult *D. melanogaster*, and it is the only one of eight Acps tested (and GFP) to show this activity.

To determine whether Acp62F is toxic to adult female flies, we heat-shocked *hsp70-GAL4; UAS-Acp62F* (lines 2 and 4) adult virgin females on days 2, 4, 6, 8, 10, and 12 posteclosion. We observed no difference in mortality between Curly and non-Curly female adults when heat shock was applied only once. However, upon repeated heat shocks every other day, a significant decrease in viability is seen in the non-Cy flies ($P < 0.0003$, line 2; $P < 0.0001$, line 4; Figure 2). Results of lines 2 and 4 were equivalent in all respects. We cannot test lines 1 and 3 because non-Curly adults were not generated, even under non-heat-shock conditions. Preliminary results indicate that Acp62F is similarly toxic to males, indicating that males are not immune to Acp62F; normally this protein does not gain access to their hemolymph. Ectopic expression of Acp26Aa (Park and Wolfner 1995), Acp32CD (Y. Heifetz and M. F. Wolfner, unpublished results), and GFP after either single or multiple heat shocks (Yeh *et al.* 1995; Edwards *et al.* 1997) in adults is not toxic, suggesting that Acp62F's toxicity to adults is specific. Thus, lethality to adults requires repeated exposure to Acp62F, even though a single exposure is sufficient to kill preadults.

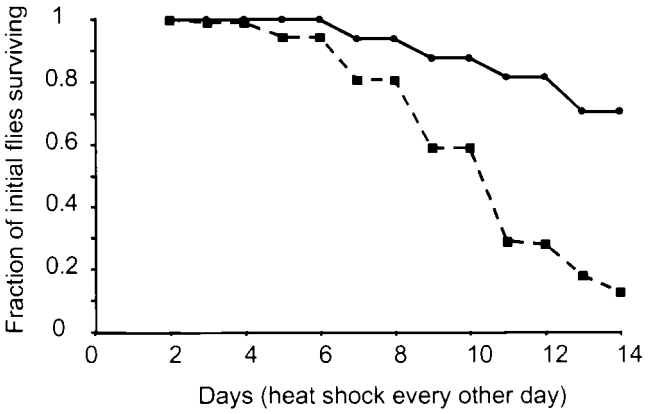

Figure 2.—Repeated expression of Acp62F decreases the viability of adult female *D. melanogaster*. Two-day-old virgin female progeny from the cross of *hsp70-GAL4/CyO* × *UAS-Acp62F*, line 4, were heat-shocked on days 2, 4, 6, 8, 10, and 12 posteclosion, as described in materials and methods. The number of surviving flies was counted daily and results are presented as percentage of initial flies. Solid squares, non-Curly flies; *n*, 110. These *hsp70-GAL4; UAS-Acp62F* flies express Acp62F upon heat shock (data not shown). Solid circles, Curly flies; *n*, 65. These *CyO; UAS-Acp62F* flies do not express Acp62F upon heat shock (data not shown). The curves are significantly different ($P < 0.0001$) based on a log-rank test.

Acp62F localization in the female reproductive tract: The localization of a protein can provide insight into its function. We previously reported that ~90% of the Acp62F transferred during mating remains within the female reproductive tract, while ~10% enters the female's hemolymph from a specialized region of the reproductive tract (Lung and Wolfner 1999). By 7 min after the start of mating (the last time point examined in that study), Acp62F in the female reproductive tract had localized to the posterior vagina and the lumen of the uterus (Lung and Wolfner 1999). Immunostaining of the genital tracts of mated females at 50 min after the start of mating shows Acp62F in the lumen of the major female sperm storage organ (the seminal receptacle, Figure 3C), in addition to the lumen of the uterus (Lung and Wolfner 1999). Western blotting with anti-Acp62F antibody shows that Acp62F is also present in the other sperm storage organs, the spermathecae (M. Bloch Qazi, S. Cleland and M. F. Wolfner, unpublished results). Acp62F's localization in the lumen of the uterus and in the sperm storage organs suggests that it plays a role in protecting sperm during deposition and storage (Gilbert 1981).

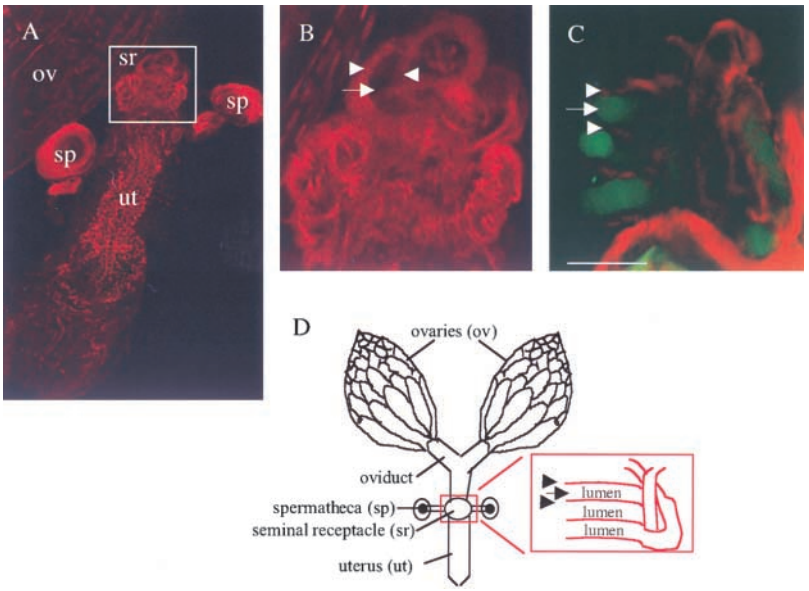

FIGURE 3.—Acp62F localizes to the seminal receptacle of the mated female at 50 min after the start of mating. Confocal micrograph of whole-mount reproductive tract from unmated female (A and B) and mated female (C) double stained with anti-Acp62F antibody (green) and with propidium iodide (red) to stain nuclei. (A) Entire reproductive tract of unmated female (minus most of the ovaries) to show the relative organization of the various reproductive organs. (B) Larger view of the seminal receptacle of the unmated female (sr, boxed region in A) and (C) optical longitudinal section across the seminal receptacle from a mated female. Note that Acp62F (in green) is clearly in the lumen (arrow) of the coiled spaghetti-like seminal receptacle of the mated female, but not those of the unmated female. The seminal receptacle wall is demarcated by cellular staining in red (arrowhead). Bar, ~100 μm. (D) Schematic diagram of female reproductive tract showing the exposed seminal receptacle lumen lined by the seminal receptacle cellular layer.

Sequence similarity, secondary structure, and threading reveal Acp62F's high similarity to Ascaris extracellular protease inhibitors: To address which functions Acp62F might play in the female reproductive tract and why its ectopic expression might be toxic, we examined the Acp62F sequence for clues to its biochemical function (results summarized in Table 2). BLAST searching of the protein sequence databases using the sequence of secreted Acp62F (amino acids 15–115) returned hits for proteins of dissimilar size, function, and/or distribution. It is apparent that the sequence similarity shared between Acp62F and the high-scoring hits, zonadhesin, Von Willebrand factor, and metallothioneins, was a result of the high Cys content (12%) in Acp62F. Because these proteins are either much larger than Acp62F or possess a different intracellular *vs.* extracellular location, we surmised that they do not represent true homologs.

In contrast, FASTA searching returned serine protease inhibitors from the nematode Ascaris as high-scoring hits. Four additional sequence comparison methods (see MATERIALS AND METHODS) also returned the Ascaris trypsin inhibitor as the top-scoring hit (Table 2), with scores for each method lending confidence to the significance of the match. The Acp62F sequence was also searched against known protein structures using the threading algorithm 123D (ALEXANDROV *et al.* 1996). When the threading comparison was performed with sequence and secondary structure evenly weighted and contact capacity potential (roughly equivalent to hydrophobicity) weighted at zero, the Ascaris trypsin inhibitor structure 1ata appeared as the top-scoring hit (Table 2, 123 Da). This approach was used because hydrophobicity is not expected to play a significant role in the folding of the Ascaris trypsin inhibitors, because they lack a hydrophobic core (HUANG *et al.* 1994) and because Acp62F may also lack a hydrophobic core (see below). When sequence similarity, secondary structure prediction, and contact capacity potentials were equally weighted for this algorithm, the Ascaris trypsin inhibitor received the second highest score. The fact that six methodologies, involving sequence comparison, secondary structure comparison, or threading, each returned one of the Ascaris isoinhibitors as the top-scoring hit strongly suggests that these proteins were structural counterparts of Acp62F.

The protease inhibitors exhibiting similarity to Acp62F belong to a small family of structurally unique proteins with five disulfide bridges (GRASBERGER *et al.* 1994; HUANG *et al.* 1994). The prototypical members of this inhibitor family are proteins secreted by *Ascaris lumbricoides* that range from 62 to 65 residues (BABIN *et al.* 1984), 35 to 38 residues shorter than predicted for secreted Acp62F. Alignment of the sequences of several members of this family with Acp62F (Figure 4) shows that the region of similarity lies roughly in the middle of Acp62F and spans the entire length of the Ascaris proteins. Acp62F shares 31% identity in its overlap with the Ascaris trypsin inhibitor (ITR1_ASCU) and 35% identity in its overlap with the Ascaris chymotrypsin/elastase inhibitor (ICE1_ASCU). The conserved residues include all 10 Cys residues involved in forming the five disulfide bridges characteristic of this inhibitor family (see Figure 4). Relative to the Ascaris chymotrypsin/elastase inhibitor, secreted Acp62F has a 14-residue N-terminal and 23-residue C-terminal extension (including 2 additional Cys residues near its C terminus) that are not present in other known members of the Ascaris protease inhibitor family.

The structures of Ascaris chymotrypsin/elastase in-

TABLE 2

Summary of results from sequence comparison

Method of comparison[a]	Database	First hit[b] and % identity/aa overlap	Description	Score	Confidence level
Sequence					
BLASTP	Nonredundant[c]	ZAN_PIG 38%/67 residues	Zonadhesin precursor, a 2476-residue transmembrane sperm protein from pig	E-value: 2×10^{-5}	10^{-10}–10^{-5}
FASTA	SWALL[d]	SWALL Q18157 38%/58 residues	2.2 Mb of contiguous nucleotide sequence from chromosome III of *Caenorhabditis elegans*	E-value: 0.0052	NA
FASTA	SWISSPROT	ICE1_ASCU 35%/59 residues	Ascaris chymotrypsin/elastase inhibitor, a 63-residue secreted protein	E-value: 0.0073	NA
HMM search	HMM library of sequences in PDB	1ata 34%/58 residues	Ascaris trypsin inhibitor, a 62-residue secreted protein	−47.17	NA
GONNET	PDB	1ata 34%/58 residues	Ascaris trypsin inhibitor, a 62-residue secreted protein	*Z*-score: 4.26	4.8 ± 1.0
Sequence + secondary structure					
GONNET + PREDSS	PDB	1ata 34%/58 residues	Ascaris trypsin inhibitor, a 62-residue secreted protein	*Z*-score: 4.53	4.8 ± 1.0
GONNET + PREDSS + MULT	PDB	1ata 34%/58 residues	Ascaris trypsin inhibitor, a 62-residue secreted protein	*Z*-score: 4.66	5.0 ± 1.0
GONNET + PREDSS + LOCAL	PDB	1ata 34%/58 residues	Ascaris trypsin inhibitor, a 62-residue secreted protein	*Z*-score: 5.29	6.0 ± 1.0
Threading					
123D a	Subset of PDB	1ata 37%/58 residues	Ascaris trypsin inhibitor, a 62-residue secreted protein	*Z*-score: 1.70	NA
123D b	Subset of PDB	4mt2 31%/61 residues	Rat metallothionein-2, a 61-protein intracellular protein used to sequester toxic metal ions	*Z*-score: 3.21	NA

NA, not applicable.

[a] BLASTP: version 2.0.9 (May 07, 1999), FASTA: version 3.2t05 (May 12, 1999), GONNET (Dec. 17, 1998), GONNET + PREDSS (Dec. 17, 1998), MULT + GONNET + PREDSS (Dec. 17, 1998), and GONNET + PREDSS + LOCAL (Dec. 17, 1998).

[b] Highest-scoring non-Acp62F sequence.

[c] Nonredundant search of GenBank CDS translations + PDB + SwissProt + SPupdate + PIR.

[d] SWALL: Non-Redundant Protein sequence database, Swissprot + Trembl + TremblNew.

FIGURE 4.—Multiple sequence alignment of mature Acp62F, Ascaris trypsin inhibitor (ITR1), Ascaris chymotrypsin/elastase inhibitor (ICE1), Anisakis inhibitor (SP1-3; LU *et al.* 1998), Bombina trypsin inhibitor (BSTI; MIGNOGNA *et al.* 1996), and *Apis mellifera* cathepsin G/chymotrypsin inhibitor (AMTR1; BANIA *et al.* 1999). The P1 residue of each inhibitor's active site is indicated in boldface. A shorter region of Acp62F also has similarity (51.7% sequence identity in boxes) to a 28-amino-acid region of the 48-amino-acid insecticidal spider venom neurotoxin PhTx2-6 (WOLFNER *et al.* 1997). However, out of the 10 Cys residues conserved in all members of the PhTx2 neurotoxin family (CORDEIRO *et al.* 1995) only 7 Cys can be aligned with those in Acp62F (see WOLFNER *et al.* 1997; Figure 4), suggesting that Acp62F may have a different structure due to different disulfide topography and thus a different function. Consistent with this, we were not able to detect any neuromodulatory activity of Acp62F in several independent electrophysiological and behavioral assays (O. LUNG, unpublished results).

hibitor (ICE1_ASCU; HUANG *et al.* 1994) and trypsin inhibitor (ITR1_ASCU; GRASBERGER *et al.* 1994) have been solved by X-ray crystallography and NMR, respectively. To evaluate the structural similarity of Acp62F to the Ascaris protease inhibitors, we constructed an Acp62F homology model (Figure 5A). Like the Ascaris inhibitors, the Acp62F model has a flattened structure, with an overall similarity closer to that of the trypsin inhibitor than that of the chymotrypsin/elastase inhibitor, and lacks a hydrophobic core. Acp62F residues 29–89 and the Ascaris trypsin inhibitor both have a hydrophobic residue content of 21%, and the structures of both appear to have a small area of buried hydrophobic residues in the β-sheet formed by the last two β-strands in each structure. In our model, all the Cys residues that Acp62F shares with the nematode inhibitors participate in disulfide bonds. This arrangement of disulfide bridges is consistent with the prediction of HUANG *et al.* (1994): lacking a true hydrophobic core, the Ascaris protease inhibitors may depend on their disulfide bridges to maintain their folding; note that the model in Figure 5A includes five disulfide bridges. In addition, the reactive site loop of the Acp62F model shows good structural similarity to the reactive sites of the Ascaris protease inhibitors (Figure 5B). The fact that the Ascaris trypsin inhibitor reactive site changes conformation in response to pH, with the P1 residue assuming active and inactive conformations at pH 2.4 and 4.75, respectively (GRASBERGER *et al.* 1994), suggests that Acp62F's activity *in vivo* may also be regulated by changes in pH.

The multiple sequence alignment allowed identification of Acp62F's P1 reactive site residue, the primary determinant of protease inhibitor specificity (SCHECHTER and BERGER 1967). Acp62F shares with the Ascaris trypsin inhibitor a positively charged residue at the P1 position (Figure 4). The P1 lysine residue of Acp62F (K61) would be predicted to interact favorably with the negatively charged Asp found at the bottom of trypsin's specificity pocket (BRANDEN and TOOZE 1991). Chymotrypsin and elastase, in contrast, have nonpolar residues in their specificity pockets and, accordingly, the Ascaris inhibitor for these enzymes has an uncharged Leu at the P1 position (PEANASKY *et al.* 1984).

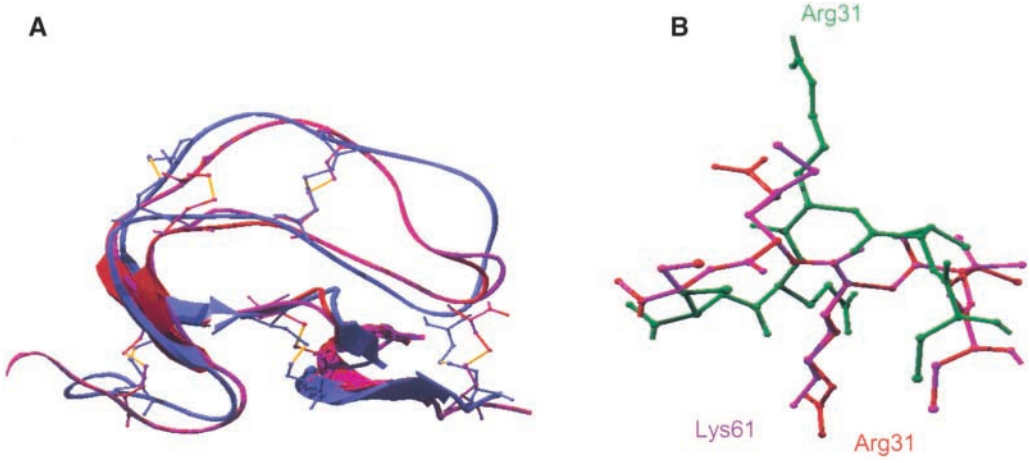

FIGURE 5.—(A) Frontal view of ribbon diagrams showing the predicted structure of Acp62F superimposed over the Ascaris inhibitors. Acp62F (purple), Ascaris trypsin inhibitor at pH 4.75 (1ata, red), and Ascaris chymotrypsin/elastase inhibitor (aceC, blue). The conserved cystines participating in intramolecular disulfide bridges (yellow) are depicted in the model. The reactive site loop is located between the two disulfide bridges seen at the top of each structure. (B) Frontal view of predicted structure of Acp62F (purple) overlaid on the active sites of 1ata (inactive form at pH 4.75, red) and 1atb (active form at pH 2.4, green). The active P1 residue for each inhibitor is indicated. Note that the P1 residue of the Ascaris trypsin inhibitor flips up and away from the molecule's core at the pH corresponding to inhibition activity.

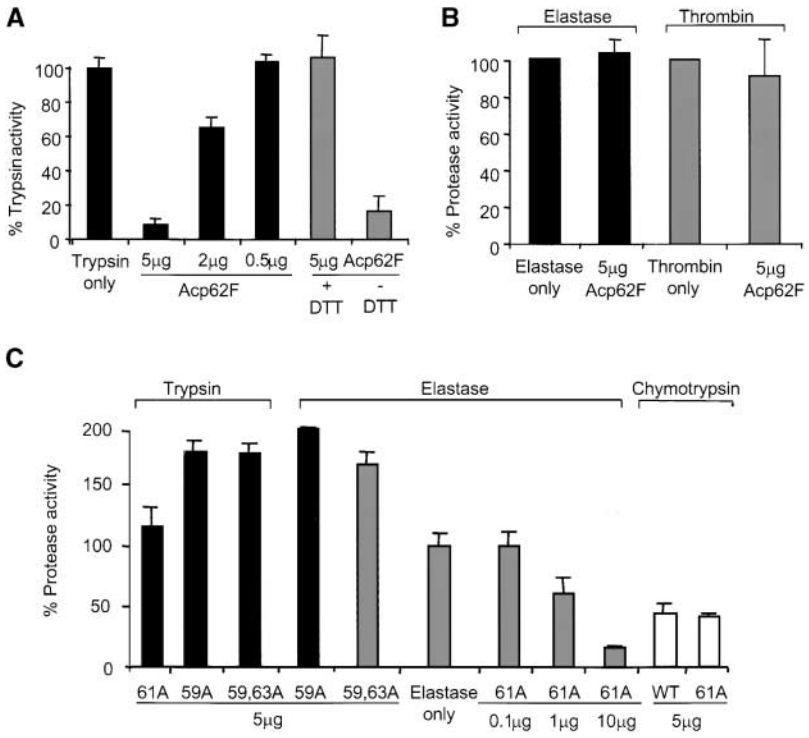

FIGURE 6.—(A) Acp62F-H6 is a trypsin inhibitor whose activity is dependent on its disulfide bridges. Trypsin activity decreases after preincubation of 1μg trypsin with increasing amounts of Acp62F-H6 protein (solid bars). Acp62F's trypsin inhibitory activity is abolished if Acp62F is first treated with DTT (+DTT), but not with buffer alone (−DTT), before preincubation with trypsin (shaded bar; see MATERIALS AND METHODS). (B) Acp62F-H6 does not inhibit 0.5 μg elastase (solid bar) or 1 μg thrombin (shaded bar). (C) Acp62F-H6 C59A and C59,63A mutants, in which one or both cysteines flanking the reactive site were mutated to alanine, no longer inhibit trypsin activity (solid bar). Similar mutagenesis of the predicted P1 lysine residue to alanine (K61A) abolished Acp62F-H6's trypsin inhibitor activity (solid bar), but generated a dose-dependent elastase inhibition activity (shaded bar; compare with elastase only) not present in the wild-type protein (B) or the C59A (solid bar) and C59,63A mutant proteins (shaded bar). Both wild-type Acp62F-H6 and the K61A mutant protein had low levels of chymotrypsin inhibitor activity (open bar). The fact that the K61A mutant maintained chymotrypsin inhibitor activity suggests that its failure to inhibit trypsin is a direct consequence of the site-specific mutation and not due to gross protein misfolding. Plots are averages of three or four experiments with error bar of one standard deviation.

Acp62F inhibits trypsin *in vitro*: To determine whether the sequence analysis results reflected biological activity, we tested whether Acp62F-H6 inhibits proteases *in vitro*. Our model above predicts that Acp62F would inhibit trypsin-like proteases particularly well and other proteases less well or not at all.

Preincubating trypsin with increasing concentrations of Acp62F-H6 prior to addition of substrate results in a dose-dependent inhibition of trypsin activity (Figure 6A). This inhibition is not seen with BSA and lysozyme controls (data not shown), indicating that trypsin inhibition is not due to substrate competition and that Acp62F-H6 is a trypsin inhibitor. However, in contrast to aprotinin, which can completely inhibit trypsin at a 1:1 molar ratio (data not shown), a higher 10:1 molar ratio of Acp62F to trypsin is needed for 95% inhibition (see Figure 6A). In similar assays using a 10:1 molar ratio of Acp62F to the protease, Acp62F-H6 showed a lower level of chymotrypsin inhibition (~50% inhibition, Figure 6C), and it did not inhibit elastase or thrombin (Figure 6B).

The lack of a hydrophobic core in the Ascaris protease inhibitors and the involvement of all 10 conserved Cys residues in disulfide bond formation suggest these disulfide bridges are structurally and functionally important. Consistent with this, reducing Acp62F-H6's disulfide bonds with DTT abolished its trypsin inhibitory activity (Figure 6A). To further confirm our prediction that Acp62F has a structure similar to that of Ascaris protease inhibitors, we generated site-directed mutations in Acp62F-H6 at the predicted P1 residue (K61A) or at one (C59A) or both of the cysteines flanking the predicted reactive site loop (C59,63A). Both the C59A and C59,63A mutations abolished Acp62F-H6's trypsin inhibitory activity *in vitro* (Figure 6C), indicating the importance of the conserved cysteine residues in Acp62F's protease inhibition activity. On the basis of the structure of the active site pockets of trypsin, chymotrypsin, and elastase, we predicted that substitution of Acp62F's P1 lysine with alanine would result in abolition of inhibition activity against trypsin and an increase in inhibition activity against elastase. The P1 position is occupied by an alanine in the *Anasakis simplex* elastase inhibitor AsPI-1 (LU *et al.* 1998) and by a leucine in the *A. lumbricoides* chymotrypsin/elastase inhibitors (PEANASKY *et al.* 1984). As predicted, the Acp62F-H6 K61A mutant lost its trypsin inhibition activity, retained its chymotrypsin activity, and gained elastase inhibitory activity (Figure 6C) not possessed by wild-type Acp62F-H6 protein or the C59A and C59,63A mutant proteins (Figure 6, B and C). These observations strongly suggest that K61 is the P1 residue for Acp62F.

Thus our mutagenesis and structural modeling results show that Acp62F most likely has a structure similar to the extracellular Ascaris protease inhibitors and that K61 is its reactive site P1 residue. To further confirm that residue 61 of Acp62F can interact directly with the active site of serine proteases, we examined whether Acp62F-H6 or the K61A mutant protein can inhibit binding of a specific active site inhibitor of serine esterases (diisofluorophosphate, or DFP) to trypsin and elas-

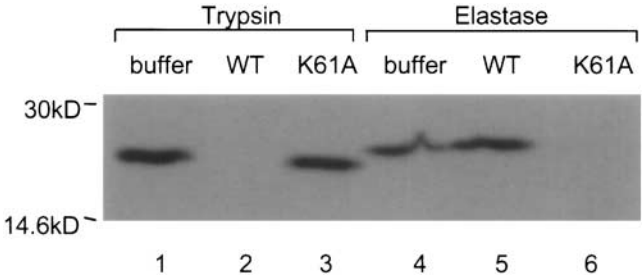

Figure 7.—Acp62F-H6 and the K61A mutant protein inhibit [^{3}H]DFP labeling of trypsin and elastase, respectively. Autoradiography of trypsin (lanes 1–3) or elastase (lanes 4–6) preincubated with buffer (lanes 1 and 4), Acp62F-H6 (WT, lanes 2 and 5), or Acp62FK61A-H6 (K61A, lanes 3 and 6) prior to [^{3}H]DFP labeling. Note that Acp62F-H6 inhibited [^{3}H]DFP labeling of trypsin, but not elastase, while Acp62FK61A-H6 inhibited [^{3}H]DFP labeling of elastase, but not trypsin.

tase, respectively. Consistent with our *in vitro* protease inhibitor assays, wild-type Acp62F-H6, but not the K61A mutant protein, inhibited [^{3}H]DFP binding to trypsin (Figure 7, lanes 2 and 3). The reverse is true for elastase, where the K61A mutant, but not the wild-type protein, inhibited [^{3}H]DFP binding (Figure 7, lanes 5 and 6). These results indicate that residue 61 is the P1 residue that interacts with the active sites of the proteases we tested.

DISCUSSION

Acp62F is the only Acp out of eight tested that is toxic to *D. melanogaster* when ectopically expressed. Ectopically expressed Acp62F is toxic to larvae and to pupae upon single exposure and to adults upon multiple exposure. In attempting to identify a molecular function for Acp62F that could account for this toxicity, we determined that Acp62F has both sequence and predicted structural similarity to a family of extracellular protease inhibitors from Ascaris. We showed *in vitro* that Acp62F has trypsin inhibitory activity and weak chymotrypsin inhibitory activity consistent with the molecular predictions. Given the importance of serine proteases in regulating biological processes, we propose that Acp62F's toxicity in the ectopic expression assay may be due to its protease inhibitor activity.

On the basis of its localization in the female reproductive tract and its predicted molecular function, we propose that Acp62F could perform a positive function in the reproductive tract, such as protecting sperm or other seminal fluid proteins from protease attack. Acp62F's presence in the female sperm storage organs suggests that it may function in protecting sperm. Seminal fluid protease inhibitors have been reported in several vertebrates, and they were proposed to perform regulatory roles. For example, bovine seminal plasma inhibitor BUSI-II localizes to the acrosomal region of epididymal spermatozoa and is thought to prevent premature acrosome reaction (Veselsky and Cechova 1980). Murine seminal fluid proteinase inhibitor SVI binds to the acrosomal cap region of the sperm head at ejaculation but not after *in utero* incubation, suggesting that it may play a role in the fertilization process, perhaps as a decapacitation factor (Irwin *et al.* 1983; Robinson *et al.* 1987). An interesting parallel to this idea of extracellular protease inhibitors exerting a protective function comes from work on the Ascaris protease inhibitors that are similar in sequence and predicted structure to Acp62F. These extracellular protease inhibitors are secreted by, and bind to, the worms and have been suggested to thereby protect these intestinal parasites from digestion by the host's digestive enzymes (Goodman and Peanasky 1982; Peanasky *et al.* 1984; Hawley and Peanasky 1992).

In contrast to its potentially beneficial functions in the female reproductive tract, Acp62F's toxicity upon ectopic expression and its entry into the mated female's hemolymph make it a candidate for contributing to the cost of mating in females. Multiple matings decrease the life span of the mated female (Fowler and Partridge 1989), in part due to multiple exposures to Acps (Chapman *et al.* 1995). We showed here that multiple exposures of adult *D. melanogaster* females to Acp62F decrease their viability. Preliminary results of similar assays show that multiple ectopic expression of Acp62F similarly decreases male viability, suggesting that males are not immune to their own deleterious peptides. However, in males, Acp62F is confined within the male reproductive tract and thus would not normally be in position to negatively affect male viability. We hypothesize that the presence of Acp62F in the hemolymph of mated females might contribute to the decrease in life span by interfering with essential protease-regulated processes that occur extracellularly. There are several such processes. One example among several is the proprotein activation integral to the immune response (reviewed by Kanost 1999), such as the proteolytic cascades that lead to drosomycin expression (Levashina *et al.* 1999) or prophenoloxidase activation (Chosa *et al.* 1997; Jiang *et al.* 1998; Green *et al.* 2000). *D. melanogaster* mutants in the *necrotic* (*nec*) gene, which encodes a serpin, die in late pupal stage or within a few days of eclosion (Green *et al.* 2000). This is thought to be due to activation of proteolytic cascades involved in the immune response (Green *et al.* 2000) and indicates the importance of maintaining the delicate balance between protease and protease inhibitor activity. We hypothesize that the presence of a protease inhibitor where it should not be (*i.e.*, Acp62F in the hemolymph of mated females) might contribute to the Acp-dependent decrease in a mated female's life span by interfering with essential protease-regulated processes. Inhibition of cascades involved in protective responses might make mated females weaker or more vulnerable to infection.

If a cost of mating is associated with protease inhibition, it most likely would involve more than just Acp62F. At least eight potential secreted protease inhibitors are produced by the accessory glands (Wolfner *et al.* 1997; Swanson *et al.* 2001; see also Schmidt *et al.* 1989; Coleman *et al.* 1995) and thus potentially delivered into the female's hemolymph. Their specificities and thus effects could differ. It is not possible to make absolute quantitative inferences from the experiments in this article to the conditions of a normal mating. The ectopic expression system drives >50-fold higher levels of Acp62F than those encountered in the hemolymph of a female after a single mating (Lung and Wolfner 1999; data not shown) and the ectopic expression of Acp62F from an induced mRNA can presumably be produced over a longer period of time than the normal limited delivery time (<20 min) of Acps during a mating. Thus, it will be important to determine if additional protease inhibitors are transferred to females during mating and whether they also incur a cost of mating to females.

The hypothesis that Acp62F, and potentially other protease inhibitors from seminal fluid, are toxic to a Drosophila upon entry into her hemolymph raises the question of why this (these) seminal fluid Acp(s) have not been eliminated during evolution. It is interesting in this light that 20% of predicted Acps are predicted regulators of proteolysis (nine predicted proteases, eight predicted inhibitors) in 83 predicted Acps (Swanson *et al.* 2001). It seems likely that the proteolysis regulators exert essential positive functions, formally analogous to that reported by Murer *et al.* (2001) for the mouse seminal fluid protease inhibitor PN-1, that would make it disadvantageous to eliminate them. It might therefore have been more advantageous to evolve some resistance to the negative effects of "toxic" Acps in the hemolymph, thereby "tuning" the amount or activity of seminal fluid protease inhibitors in the hemolymph (or the female's reaction to them) to a tolerable level. Such a model would be consistent with the predictions of some sexual conflict hypotheses (*e.g.*, Rice 1996).

One method to tune the amount or activity of seminal fluid protease inhibitors in the hemolymph would be to regulate Acp entry into the hemolymph. Preventing all Acp entry into hemolymph, however, would likely be disadvantageous, since at least some Acps appear to act through the hemolymph (Chen *et al.* 1988; Aigaki *et al.* 1991). It is not clear though whether Acp entry into the hemolymph is regulated and if so how it is regulated. Comparison of the amino acid sequences of Acps that enter the hemolymph did not reveal any obvious "hemolymph entry signal" sequence (Lung and Wolfner 1999). The variety of sequences of Acps that enter hemolymph (Monsma *et al.* 1990; Lung and Wolfner 1999) and rapid sequence variation among Acps (Aguade *et al.* 1992; Cirera and Aguade 1997; Tsaur and Wu 1997; Aguade 1998, 1999; Tsaur *et al.* 1998, 2001; Begun *et al.* 2000; Swanson *et al.* 2001) might have made it disadvantageous to make the apparently non-sequence-selective transport system (Lung and Wolfner 1999) more selective. Instead, post-transport mechanisms might have evolved to mitigate the negative consequences of Acps in the hemolymph, for example, a protein in adult hemolymph that could bind to and restrain Acp62F. It is interesting in this light that adult flies are more resistant than preadult flies to the toxicity of ectopic Acp62F, since normally only adults encounter Acps.

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