Mated *Drosophila melanogaster* Females Require a Seminal Fluid Protein, Acp36DE, to Store Sperm Efficiently

Deborah M. Neubaum[1] and Mariana F. Wolfner

Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853-2703

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ABSTRACT

Mated females of many animal species store sperm. Sperm storage profoundly influences the number, timing, and paternity of the female's progeny. To investigate mechanisms for sperm storage in *Drosophila melanogaster*, we generated and analyzed mutations in *Acp36DE*. Acp36DE is a male seminal fluid protein whose localization in mated females suggested a role in sperm storage. We report that male-derived Acp36DE is essential for efficient sperm storage by females. *Acp36DE[1]* (null) mutant males produced and transferred normal amounts of sperm and seminal fluid proteins. However, mates of *Acp36DE[1]* males stored only 15% as many sperm and produced 10% as many adult progeny as control-mated females. Moreover, without Acp36DE, mated females failed to maintain an elevated egg-laying rate and decreased receptivity, behaviors whose persistence (but not initiation) normally depends on the presence of stored sperm. Previous studies suggested that a barrier in the oviduct confines sperm and Acp36DE to a limited area near the storage organs. We show that Acp36DE is not required for barrier formation, but both Acp36DE and the barrier are required for maximal sperm storage. Acp36DE associates tightly with sperm. Our results indicate that Acp36DE is essential for the initial storage of sperm, and that it may also influence the arrangement and retention of stored sperm.

DROSOPHILA melanogaster females, like females of many other animal species, store the sperm they receive during mating (reviewed in Gilbert *et al.* 1981; Sander 1985; Birkhead and Møller 1993; Neubaum and Wolfner 1999). Drosophila females have three organs specialized for the retention of sperm, including a long, coiled tubule called the seminal receptacle and two sac-like organs termed spermathecae (Miller 1950). The female uses her stored sperm to fertilize several hundred eggs over the 2 wk after a mating (Lefevre and Jonsson 1962). The ability to store sperm is an integral part of the Drosophila pattern of reproduction, and it may increase fecundity (Cook 1970; Hihara 1981), minimize the biochemical and environmental hazards associated with copulation (Thornhill and Alcock 1983; Chapman *et al.* 1995), and provide a milieu for sperm competition (Harshman and Prout 1994; Clark *et al.* 1995, 1999; Clark and Begun 1998; Hughes 1997; Price 1997). Moreover, Drosophila females link the presence of stored sperm to postmating behaviors such as egg laying and receptivity to remating (Manning 1962, 1967; Hihara 1981; Scott 1987; Kalb *et al.* 1993; Tram and Wolfner 1998), which serves to further maximize progeny production.

Corresponding author: Mariana F. Wolfner, Department of Molecular Biology and Genetics, 423 Biotechnology Bldg., Cornell University, Ithaca, NY 14853-2703. E-mail: mfw5@cornell.edu

[1]*Present address:* Cardiovascular Research Center, Massachusetts General Hospital / Harvard Medical School, 149 13th St., Charlestown, MA 02129.

The mechanisms that govern sperm storage in Drosophila and other animals are likely to require a combination of anatomical features and the action of molecules contributed by males and females (DeVries 1964; Fowler 1973; Bertram *et al.* 1996; Arthur *et al.* 1998; Neubaum and Wolfner 1999). While previous studies have focused on the morphology of the male and female genitalia (Miller 1950; Bairati 1968), as well as the sperm storage organs and the arrangement of sperm within them (Filosi and Perotti 1975; Hihara and Hihara 1993), relatively little is known about the molecules furnished by males or females that are important for sperm storage.

Female *D. melanogaster* receive ~4000–6000 sperm in a single mating and store up to ~1100 of them (Gilbert 1981). They use up to 600–800 sperm to fertilize eggs over the subsequent 2 weeks (Kaplan *et al.* 1962; Gilbert *et al.* 1981). That male seminal fluid components play a role in the transfer or storage of sperm in females was first suggested by the low fertility of repeatedly mated males (Lefevre and Jonsson 1962; Hihara 1981). Kalb *et al.* (1993) obtained additional evidence favoring this idea when they observed that the mates of male flies deficient in Acps, seminal fluid proteins derived from male accessory glands, contained fewer sperm than normal at 6 hr postmating. In the accompanying article, Tram and Wolfner (1999) show that this phenotype results from a requirement for Acps for proper storage of sperm, rather than from insufficient sperm production or transfer by Acp-deficient males.

We wished to identify specific molecules that mediate

sperm storage. In particular, one Acp, Acp36DE, was a good candidate for involvement in sperm storage on the basis of its localization in the mated female genital tract (Bertram *et al.* 1996) and on data from Clark *et al.* (1995), which show a correlation of alleles at this locus and levels of sperm competition in chromosomes from natural populations. We show here that Acp36DE is a major player in the process of sperm storage.

Acp36DE is a large glycoprotein of 122 kD that is processed to a 68-kD form shortly after its transfer to females during mating (Bertram *et al.* 1996). In mated females, Acp36DE localizes to two specific areas: Acp-36DE binds to the lower oviduct on the ventral side, just above the openings to the three sperm storage organs. Acp36DE also associates with the mass of sperm and other seminal fluids in the uterus (the "sperm mass"). After mating, the sperm mass does not progress into the upper oviduct, but remains confined to the uterus, posterior to the site of Acp36DE localization. These observations led us to propose a model in which Acp-36DE marks the presence of a barrier in the mated female oviduct that blocks the movement of sperm into the upper oviduct (Bertram *et al.* 1996). The mating plug secretions, which occupy the lower portion of the uterus, are proposed to delimit the sperm at the posterior. In this manner, sperm are confined to a small region of the anterior uterus, which may facilitate their entry into the narrow openings of the storage organs. Whether Acp36DE serves only as a marker of the barrier in the oviduct or comprises part or all of the barrier was not possible to determine in the Bertram *et al.* (1996) study.

In mated eggless females, a proper barrier does not form in the oviduct, and sperm (as well as Acp36DE) become mislocalized into the anterior oviduct near the ovaries (Bertram *et al.* 1996). If the formation of an oviduct barrier (and the concomitant full localization of Acp36DE at the oviduct) is important for sperm storage, we predict that sperm storage will be compromised in eggless females mated to normal males.

Here, we report the isolation and characterization of a null mutation in *Acp36DE*. We show that this mutation severely compromises the ability of sperm of mutant males to be stored by their mates. To probe the mode of action of Acp36DE in sperm storage, we tested whether the presence of Acp36DE is necessary for sperm to be properly restricted from entry into the upper oviduct. In addition, we used biochemical and genetic means to perturb the association of Acp36DE with the sperm mass or with the posterior oviduct wall. We find that although Acp36DE is not necessary for the oviduct sperm barrier to form, the barrier is important in sperm storage, and Acp36DE's localization correlates with the efficiency of sperm storage. Furthermore, Acp36DE was found to tightly associate with sperm and to enter the storage organs.

MATERIALS AND METHODS

Fly strains and isolation of *Acp36DE* mutants: A strain of Oregon-R flies made isogenic for chromosome 2 was termed iso2. Three-day-old iso2 males were treated with 25 mm ethyl methanesulfonate (EMS; Lewis and Bacher 1968). A series of genetic crosses as in Herndon and Wolfner (1995) generated */*Df* males, where * represents an EMS-mutagenized chromosome and *Df* represents *Df(2L)H2O*, a deficiency that deletes 36A8-9; 36E1-2 (Simpson 1983). Individual */*Df* males were tested by dot blot or Western blotting (van Vactor *et al.* 1988; Herndon and Wolfner 1995) for loss or truncation of Acp36DE.

Acp36DE^{P}, kindly provided by A. Clark and C. Langley, contains a *P*-element insertion within the *Acp36DE* coding sequence (Clark *et al.* 1994; Bertram *et al.* 1996; D. M. Neubaum and M. F. Wolfner, unpublished results), which results in truncation of the protein. *Acp36DE^{P}* males appear to have normal fertility.

Homozygous *bw sp tud^{1}* females (Boswell and Mahowald 1985) were mated to iso2-derived males to produce eggless daughters and spermless sons. Thus, the genetic background of eggless females is quite related to their control, iso2 females. Other balancers and markers are listed in Lindsley and Zimm (1992). All phenotypic analyses described in this article were carried out using *Acp36DE*/*Df* flies. Controls were Oregon-R (for Western blots) or *Acp36DE^{+}*/*Df* males, where the *Acp-36DE^{+}* chromosome was derived from a strain closely matched in genetic background to the mutants. This strain had been maintained in parallel to the *Acp36DE^{1}* and *Acp36DE^{2}* mutant strains (for sperm counts and phenotypic assays).

Genetic analysis: *Acp36DE* alleles were maintained by backcrossing *Acp36DE*/*CyO* males to *CyO*/*Df* females every generation. All phenotypes described here cosegregated. EMS-induced mutations at genomic locations other than chromosome 2 were segregated away from the genetic background of *Acp36DE** males as the number of backcrosses increased.

Nucleic acid and protein analysis: Recent EST sequences deposited in the databases by the Berkeley Drosophila Genome Project, in conjunction with our Northern blotting, have extended the Acp36DE sequence at the 5′ end of the gene (W. Swanson, personal communication; GenBank entry no. U85759 has been revised accordingly). The revised Acp36DE ORF spans 2736 bp, the equivalent of 912 amino acids. Amino acids 1–23 comprise a signal sequence conforming to the specifications of von Heijne (1983), leading us to believe that this is the likely start of Acp36DE's ORF. The *Acp36DE* mutant lesions were identified by PCR-amplifying DNA from */*Df* flies using two primers that include *Xba*I linkers and derive from 454 to 472 and 2774 to 2792 of the *Acp36DE* sequence (Wolfner *et al.* 1997). Amplified products were sequenced directly or cloned into pBluescript II SK +/− (Stratagene, La Jolla, CA), and the sequences of both strands were determined using automated cycle sequencing (Perkin-Elmer, Norwalk, CT/Applied Biosystems, Foster City, CA). *Acp36DE^{+}* cDNA sequence (Wolfner *et al.* 1997) or PCR-generated *Acp36DE* genomic sequence was used for comparison to mutant alleles.

Total poly(A)$^{+}$ RNA was isolated from adult male Drosophila using the Total RNA isolation kit (Promega, Madison, WI). Northern blots were prepared as described in Monsma and Wolfner (1988), with ~10 μg RNA per lane, and were probed with random-primed Acp36DE or, as a control, β-tubulin (Bialojan *et al.* 1984) DNA probes, followed by autoradiography.

Protein extracts from the genital tracts of 3-day-old */*Df (2L)H2O* males were prepared and immunoblotted as described in Monsma and Wolfner (1988). Blots were probed with affinity-purified Acp36DE (Bertram *et al.* 1996) or

Acp26Aa (Monsma and Wolfner 1988) primary antibodies diluted 1:1000 or 1:500, respectively, and with horseradish-peroxidase-conjugated secondary antibody (Sigma, St. Louis) diluted 1:2000. Proteins were visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Sperm motility, transfer, and quantitation: Mature sperm were dissected from the seminal vesicle and tested for motility by observing their sinuous tail undulations in physiological buffer (Cenci *et al.* 1994).

To evaluate sperm transfer by mutant males, the genital tracts of females mated to *Acp36DE*^{1}/*Df* or control (*Acp36DE*$^{+}$/*Df*) males were dissected at 30 min after the start of mating (~20 matings per genotype). After removal of the ovaries and oviduct, the mated female's uterus was squashed under a coverslip, causing the sperm mass to spread flat. In double blind experiments, we assigned sperm spreads a score of 1–5 on the basis of the size and density of the spread viewed at ×40 in a Zeiss (Thornwood, NY) Axioskop. Results were confirmed in duplicate experiments.

Sperm stored in female genital tracts were fixed and stained with 2% orcein in 60% acetic acid (Gilbert 1981; Gilbert *et al.* 1981) and counted at ×100 magnification using phase-contrast microscopy. Data were analyzed using the Statview 4.1 statistics program (Fisher's *t*-tests and ANOVA). Total sperm stored was calculated as the sum of those stored in the seminal receptacle and two spermathecae. Data for total sperm counts (see Table 2) were taken only from the subset of tracts that retained all three organs after squashing, although additional data for individual organs were taken from any sample that had intact and suitably squashed organs.

Egg-laying, fertility, receptivity, and life-span assays of female mates of *Acp36DE* mutant males: Control lines in all assays were overtly normal Acp36DE-positive lines retained from the mutagenesis experiment. Control and mutant lines were backcrossed every generation to *Df*/*CyO* stocks to isogenize genetic background and to avoid selection of modifiers. For all assays, Oregon-R females and hemizygous *Df* males (mutant or control) were collected a few hours after eclosion and aged 3–5 days. Flies were maintained at constant temperature (24° ± 0.5°), humidity, and day/night cycle (12:12 hr light:dark).

Egg-laying and receptivity responses were examined as described in Kalb *et al.* (1993) and Herndon and Wolfner (1995).

The effect of *Acp36DE* on life span was assayed in experiments like those of Chapman *et al.* (1995), with the exception that microcauterized flies were not included. Each Oregon-R female was placed with a *Acp36DE*^{1}/*Df* or control (*Acp36DE*$^{+}$/*Df*) male for 2 days, but every third day was housed with a control male to control for nonseminal fluid costs of mating, such as egg production (Partridge *et al.* 1987). A total of 30 females were used per experimental group.

Using the Statview 4.1 statistics program, we performed ANOVA and Fisher's *t*-tests on data from all assays.

Sperm-binding assays: *In vivo assay:* For all assays described in this paper, males and females were held in isolation for 3–5 days before mating. Female genital tracts were dissected 30 min after the start of mating, and the ovaries were removed. The mass of sperm present in the anterior uterus was then squeezed out of the torn oviduct by applying pressure to the uterus. Sperm masses were transferred to a microfuge tube, where 500 μl (>1000× volume) of buffer was added. Buffers contained 1× PBS ± 0.1% Tween 20 + 0.0, 0.1, 0.5, or 1 m NaCl, and were adjusted to pH 5, 7, or 10 with 1 n HCl or 1 n NaOH. In some experiments, samples were vortexed vigorously for 10 sec. All samples were incubated for 1 hr and then spun in a microfuge for 5 min to pellet the sperm. Supernatants were spun in a SpeedVac to reduce volume. Pellets and supernatants were resuspended in 2× SDS-PAGE loading buffer, run on 10% SDS-PAGE gels, and Western blotted (Monsma and Wolfner 1988). After probing with anti-Acp36DE, blots were stripped and reprobed with anti-Acp26Aa (Park and Wolfner 1995). To determine whether soluble Acp36DE was precipitated by any of the buffers, a parallel experiment was performed using accessory gland homogenate (spun free of particulate matter) in each of the buffers under similar conditions.

In vitro assay: Using 40 males per treatment, mature sperm were dissected from the seminal vesicle, while the accessory glands (AG) were homogenized in protease-inhibitory buffer (Park and Wolfner 1995) and centrifuged briefly to remove particulate matter. AG extracts (100 μl) were mixed with sperm (or not, as a control) for 1 hr at 4° with gentle shaking. Samples were then layered onto 30–70% sucrose gradients and spun for 20 min at 5000 × *g* in a swinging bucket rotor (SW50.1) at 4°. Fractions (250 μl) were collected beginning at the top of the gradient and examined under a microscope for the presence of sperm. Samples were reduced in volume, loading buffer was added, and samples were run on gels and immunoblotted.

RESULTS

Isolation of *Acp36DE* mutants: Since the nature and severity of the *Acp36DE* mutant phenotype was not predictable, we searched for mutations in *Acp36DE* with a screen based on protein expression rather than function. To identify mutant lines with decreased or absent *Acp36DE* expression, affinity-purified Acp36DE polyclonal antibodies (Bertram *et al.* 1996) were used to screen dot or Western blots (van Vactor *et al.* 1988; Herndon and Wolfner 1995) containing extracts of males heterozygous for a mutagenized chromosome 2 and a deficiency that uncovers *Acp36DE* (*/*Df* males).

From 8805 mutagenized lines tested, we recovered two in which full-length Acp36DE was undetectable on Western blots of extracts of */*Df* males (Figure 1A). In contrast to wild-type males, which had a prominent 122-kD Acp36DE band (lane 1), *Acp36DE*^{1}/*Df* males had no detectable Acp36DE (lane 2), even when lanes were loaded with extracts of 10-fold more flies than control lanes. *Acp36DE*^{1}/*Df* males did not make a detectable Acp36DE RNA transcript (Figure 1B). A second *Acp36DE* mutant, *Acp36DE*^{2}, gave rise to a 56-kD Acp36DE (Figure 1A, lane 3), whose signal on Western blots was less intense than that of the wild-type Acp36DE. Acp36DE^{2} is likely truncated and less abundant or less well recognized by the polyclonal Acp36DE antibody. *Acp36DE*^{2} tested normal in all functional assays and will not be discussed further in this article.

Sequence analysis of *Acp36DE*^{1} showed that a nonsense mutation, created by a C-to-T transition at position 823 in the ORF sequence, caused Gln^{274} to be replaced with a stop codon (Figure 1C). The shortened open reading frame could encode a secreted protein of ~28 kD, but no novel Acp36DE cross-reactive band of this or any other size was detectable on immunoblots, presumably because *Acp36DE*^{1} mRNA was destabilized by

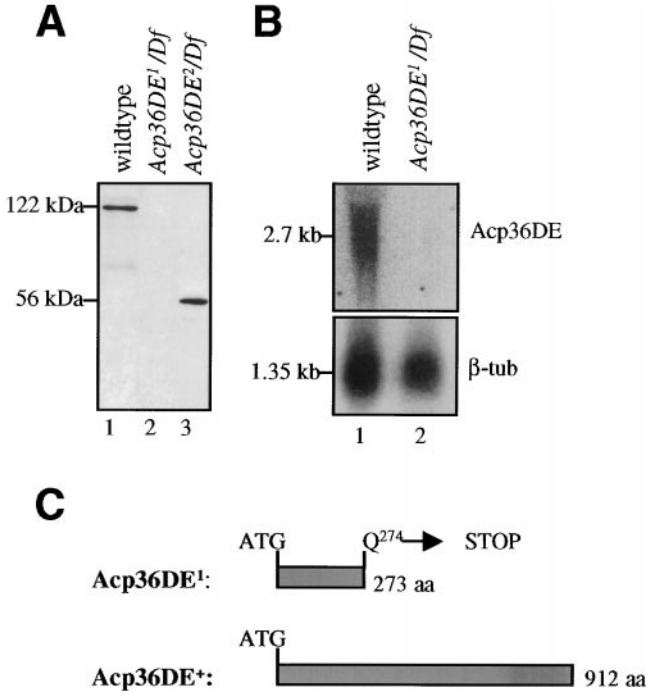

Figure 1.—Molecular characterization of Acp36DE mutants. (A) A Western blot probed with anti-Acp36DE. Lanes contain extracts of accessory glands from 3-day-old virgin Oregon-R (wild-type) males (lane 1, one pair of accessory glands), *Acp36DE*^{1}/*Df* males (lane 2, 10 pairs), or *Acp36DE*^{2}/*Df* males (lane 3, 10 pairs). (B) Northern blot of total RNA isolated from control or *Acp36DE*^{1}/*Df* males probed for *Acp36DE* mRNA (top) or, as a control, β-*tubulin* mRNA (bottom). (C) Diagram of proteins encoded by the *Acp36DE*$^{+}$ and *Acp36DE*^{1} alleles. Acp36DE^{1} is predicted to be truncated by a stop codon at amino acid 274 (CAA → TAA).

the mutation (Sachs 1993; Herndon and Wolfner 1995). On the basis of the lack of detectable *Acp36DE* mRNA or protein, we conclude that *Acp36DE*^{1} is a null allele.

We believe the phenotypes described in the remainder of this article are the result of mutations in the *Acp36DE* gene for the following reasons. The phenotypes we describe are observed in *Acp36DE*^{1}/*Df* males, but not in *Acp36DE*^{1}/*Acp36DE*$^{+}$ males, which have normal fertility. The fully recessive nature of the *Acp36DE*^{1} mutation indicates that the gene responsible for the phenotypes we report below must lie in the region uncovered by *Df(2L)H2O*. The only known gene within this region with a mutation affecting male fertility is *blanks* (36A8; 36B6); the *bln*^{1} allele causes a postmeiotic differentiation defect so that *bln* mutants do not make sperm (Castrillon *et al.* 1993). Since the mutant males described in this article do make motile sperm (see below), they are unlikely to have a mutation in *bln*. Male sterile mutants that make motile sperm are rare (10 in 400, Fitch *et al.* 1998). Thus, the likelihood is very low that we induced simultaneously, in the small genetic region uncovered by *Df(2L)H2O*, two independent mutations, one that abolished production of Acp36DE and another that resulted in semisterility despite production of normal amounts of motile sperm.

***Acp36DE* mutant males had normal viability and produced normal amounts of sperm and seminal fluid proteins other than Acp36DE:** *Acp36DE* mutant flies were fully viable and displayed no abnormal visible morphological phenotypes. The accessory glands of mutants were normal in morphology and size, and produced normal amounts of other Acps (including Acps 26Aa, 26Ab, 32CD, 62F, and 63F/64A, Monsma and Wolfner 1988; Wolfner *et al.* 1997; data not shown), as well as the ejaculatory duct protein Est-6 (Gilbert 1981; data not shown).

Acp36DE mutant males produced abundant, morphologically normal, motile sperm. Sperm of mutant males are functional, since eggs fertilized by sperm of *Acp36DE* mutant males survived to adulthood, and the number of progeny produced by *Acp36DE* mutant males was proportional to the number of sperm stored by their mates (see below). The time (± SE) *Acp36DE*^{1}/*Df* or control males spent in copulation was not significantly different (t = 20.86 ± 0.533 and 23.29 ± 1.155 min, respectively; for *Acp36DE*^{1}/*Df* and *Acp36DE*$^{+}$/*Df*, P = 0.1964). An opaque mass was observed in the uteri of females recently mated to *Acp36DE*^{1}/*Df* males (Figure 2A), but not in virgin females. When dissected, the opaque mass was found to contain hundreds of sperm tangled together. To evaluate the efficiency of sperm transfer, we dissected and squashed uteri of mated females and scored the sperm spreads using phase-contrast microscopy. In a double-blind experiment, the mass of sperm transferred by mutant males (n = 20) was indistinguishable in size, area, and sperm density from the mass of sperm observed in females mated to control males (n = 20). This suggests that *Acp36DE*^{1} mutant males consistently transferred a number of sperm similar to control males. Tram and Wolfner (1999) observed variability in sperm transfer by males lacking Acps. Our observation that *Acp36DE*^{1} males appear to transfer sperm normally suggests that other Acps underlie effects on the uniformity of sperm transfer.

Sperm stored in mates of *Acp36DE*^{1}/*Df* males were few in number and were arranged in a disorganized fashion: On the basis of its localization in mated females (Bertram 1996), we hypothesized that lack of Acp36DE would affect the number of sperm stored in females. Accordingly, we counted sperm in the storage organs of mated females at 6 hr after mating, by which time sperm storage is complete (Gilbert 1981). In seminal receptacles, on average, 17% as many sperm were stored by mates of *Acp36DE*^{1}/*Df* males as by mates of controls ($P < 0.0001$, Table 1). The few sperm that were present in the seminal receptacles of mates of mutant males were in disorganized clumps, with the sperm heads in twisted disarray (Figure 2D), in contrast to the more parallel arrangement of sperm from control matings

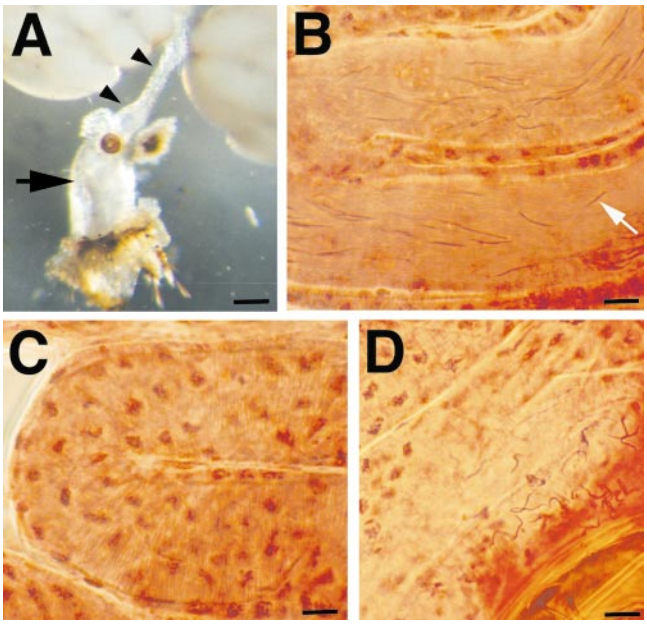

Figure 2.—Sperm in the genital tract of mated females. (A) A large mass of sperm (black arrow) is observed in the uterus of a female immediately after mating to a *Acp36DE*^{1}/*Df* male. Sperm are not mislocalized to the upper oviduct (arrowheads), but, as in wild-type matings, remain largely posterior to a boundary in the lower oviduct. Bar, 0.128 mm. (B) Inside the seminal receptacles of females mated to control males, the staining of sperm heads (*e.g.*, white arrow) reveals numerous sperm arrayed in a roughly parallel fashion. (C) In females mated to *Acp36DE*^{1}/*Df* males, the seminal receptacle (shown) and spermathecae are mostly empty. (D) The few sperm that are present in mates of *Acp36DE*^{1}/*Df* males tend to clump in a nonparallel arrangement. Diffusely staining round nuclei are female somatic nuclei. (B–D) Bar, 0.009 mm.

(Figure 2B). Similarly, at this time, spermathecae of *Acp36DE*^{1}/*Df* males had only 11% as many sperm on average as mates of controls. A summation of total sperm stored in the seminal receptacle and two spermathecae indicates that mates of *Acp36DE*^{1}/*Df* males stored overall ~15% as many sperm as mates of control males.

To determine whether mates of *Acp36DE*^{1}/*Df* males lost (or used) sperm from the storage organs at the same rate as mates of controls, we compared the numbers of sperm stored by females at 6, 24, and 48 hr after mating (Figure 3, Table 1). Although females mated to *Acp-36DE*^{1}/*Df* males stored far fewer sperm than females mated to controls, both types of female lost a similar proportion of stored sperm from their spermathecae over the first 48 hr after mating and from their seminal receptacle over the first 24 hr after mating. On the second day after mating, however, females mated to mutant males rapidly lost sperm from their seminal receptacles, reducing the number stored to almost zero. In contrast, females mated to controls still retained ~50% of the initially stored sperm at 48 hr after mating. Thus, though the primary effect of Acp36DE appears to be on the number of sperm that initially get stored by females, absence of Acp36DE also has an effect on the retention of sperm in the seminal receptacle by day 2 after mating.

Mates of *Acp36DE* mutant males produced 10% as many progeny as mates of controls: To address the role of Acp36DE in fertility, we determined the number of progeny from single matings of *Acp36DE*^{1}/*Df* or control males mated to wild-type females (Figure 4A). While *Acp36DE*^{1}/*Df* males were not sterile, their mates produced over the mating period only 10% as many progeny as females mated to control males ($P < 0.001$).

Moreover, progeny production by mates of *Acp36DE*^{1}/*Df* males showed a different pattern and kinetics from that of the controls (Figure 4A). Although female mates of *Acp36DE*^{1}/*Df* males laid a nearly normal number of eggs on the first day after mating (see below), only 33% on average of those eggs ultimately gave rise to adults, suggesting that the remaining eggs were unfertilized. The majority of progeny from *Acp36DE*^{1}/*Df* matings came from eggs laid on days 1 and 2 after mating. Very few progeny resulted from eggs laid three or more days after mating, suggesting that those eggs were nearly all unfertilized. In contrast, on average, >80% of eggs laid by control mates on each of the first through ninth day after mating gave rise to adults. These data are consistent with the compromised storage and retention of sperm in mates of *Acp36DE*^{1}/*Df* males.

Mates of *Acp36DE* mutant males fail to maintain at

TABLE 1

Mean number of sperm stored over time in mated females

		Mean ± SE, (*n*), %[a]		
	Male mate	6 hr[b]	24 hr[b]	48 hr[b]
SR	*Acp36DE*^{1}/*Df*	73.1 ± 20.0, (31), 17	51.5 ± 22.2, (21), 16	3.1 ± 2.9, (11), 1.4
	Control	424.5 ± 42.2, (18)	327.2 ± 17.0, (24)	223.3 ± 26.5, (10)
Sp	*Acp36DE*^{1}/*Df*	14.7 ± 3.8, (31), 11	10.6 ± 2.5, (31), 9	15.6 ± 42.0, (19), 12
	Control	135.6 ± 13.3, (22)	117.0 ± 14.6, (20)	133.9 ± 10.0, (19)

[a] Percent relative to the mean number stored by controls.
[b] Hours after mating.
SR, seminal receptacle; Sp, spermatheca (single).

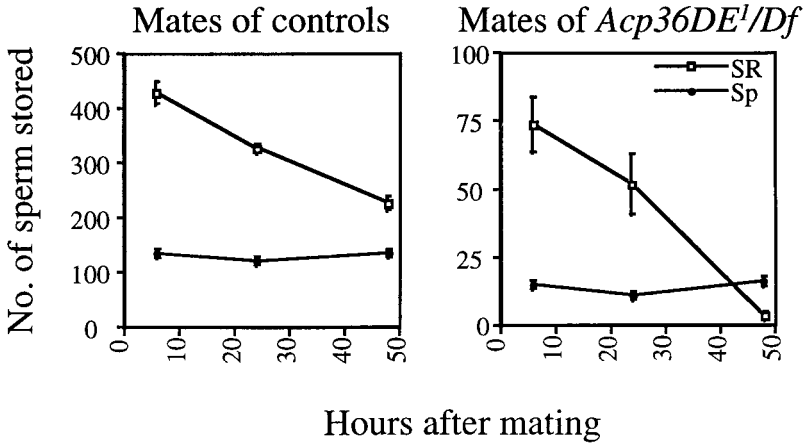

Figure 3.—Sperm depletion from the seminal receptacle (SR) or spermathecae (Sp) of mates of *Acp36DE*^{1}/*Df* or control males. Numbers are from Table 1. Note that in the first 48 hr after mating, few sperm are lost from the spermathecae of mates of *Acp36DE*^{1}/*Df* or control males, whereas in the seminal receptacle, mates of *Acp-36DE*^{1}/*Df* males show a proportionally more rapid sperm depletion than mates of controls.

least two sperm-storage-dependent behaviors: Persistence of increased egg-laying rate and decreased receptivity to courtship for more than 1 day after mating depends on stored sperm in the female (Manning 1962, 1967; Garcia-Bellido 1964; Kalb *et al.* 1993). Since lack of Acp36DE decreases sperm storage, we tested whether females mated to *Acp36DE* mutant males can maintain these behavioral responses.

Egg laying: On the first day after mating, when the egg-laying rate is largely independent of sperm (Kalb *et al.* 1993), mates of *Acp36DE*^{1}/*Df* males showed a robust response, laying a number of eggs equivalent to controls ($P = 0.1082$, Figure 4B). The high rate of egg laying was not maintained in mates of *Acp36DE*^{1}/*Df* males. By the third day after mating, the number of eggs laid by *Acp36DE*^{1} mates was intermediate between and significantly different from the number laid by mates of controls ($P = 0.0014$) and that of virgins ($P = 0.0022$). On the fourth and subsequent days after mating, the number of eggs laid by *Acp36DE*^{1} mates was indistinguishable from that of virgins ($P = 0.1622$) and significantly less than that of the controls ($P = 0.0003$, day 4; $P < 0.0001$, days 5 and 6). By day 7 after mating, the number of eggs laid by controls had also declined, and the number of eggs laid by *Acp36DE*^{1} mates was indistinguishable from that of controls ($P = 0.3236$).

Receptivity: On the first day after mating, females mated to all classes of males remained unreceptive to remating (Figure 4C). Mates of *Acp36DE*^{1}/*Df* males became receptive starting on the second day after mating and remated at frequencies similar to those of virgin females by the third day after mating. This pattern of remating is similar to the behavior of mates of spermless males (Kalb *et al.* 1993; Figure 4C).

Thus, in both egg-laying and receptivity assays, mates of *Acp36DE*^{1} mutants responded normally on the first day after mating, a time when these behaviors can be driven by Acps, without stored sperm (Kalb *et al.* 1993). A response on day 1 is consistent with the receipt of Acps other than Acp36DE by mates of *Acp36DE*^{1} mutants. Acp26Aa (Herndon and Wolfner 1995) and sex peptide (Acp70A, Chen *et al.* 1988) stimulate egg laying, and sex peptide depresses receptivity on the first day after mating. Mates of *Acp36DE*^{1}/*Df* males did not maintain an elevated egg-laying rate or a depressed receptivity on the second and subsequent days after mating, when these behaviors depend on the presence of stored sperm. The fall-off in egg laying and in mating recalcitrance on day 2 and later are not likely to result directly from Acp36DE, since this protein was not detectable in extracts of whole female genital tracts after 6 hr post-mating (Bertram *et al.* 1996).

Life span of mated females was not affected by lack of Acp36DE from their mates: Chapman *et al.* (1995) demonstrated that the life span of females was decreased in part by receipt of Acps during mating. In tests of the effects of the *Acp36DE*^{1} mutation on the mates of mutant males, we found no significant differences in the life span of females mated to *Acp36DE*^{1}/*Df* relative to control males ($P = 0.1566$, Figure 4D). Thus, Acp36DE does not account to any significant degree for the toxic effect of seminal fluid.

The barrier in the oviduct is important for sperm storage, but Acp36DE is not required for formation of this barrier: The preceding experiments established a function for Acp36DE in sperm storage in females. We next addressed how Acp36DE might execute this function. Upon noting the presence of Acp36DE in the oviduct, we had hypothesized that Acp36DE marked or was part of a barrier that prevented sperm entry into the upper oviduct and corralled sperm to a region near the sperm storage organs, thus facilitating their storage (Bertram *et al.* 1996). To investigate whether Acp36DE was required in females for barrier formation, we mated normal females to *Acp36DE*^{1}/*Df* or *Acp36DE*$^+$/*Df* males. The localization of sperm in females mated to control males ($n = 20$) or to *Acp36DE*^{1}/*Df* males ($n = 20$) did not differ. Although a large mass of sperm was observed in the anterior uterus of females mated to null males, sperm did not travel beyond this boundary to the upper genital tract (Figure 2A, arrowheads). Genital tracts of females mated to control males appeared identical to

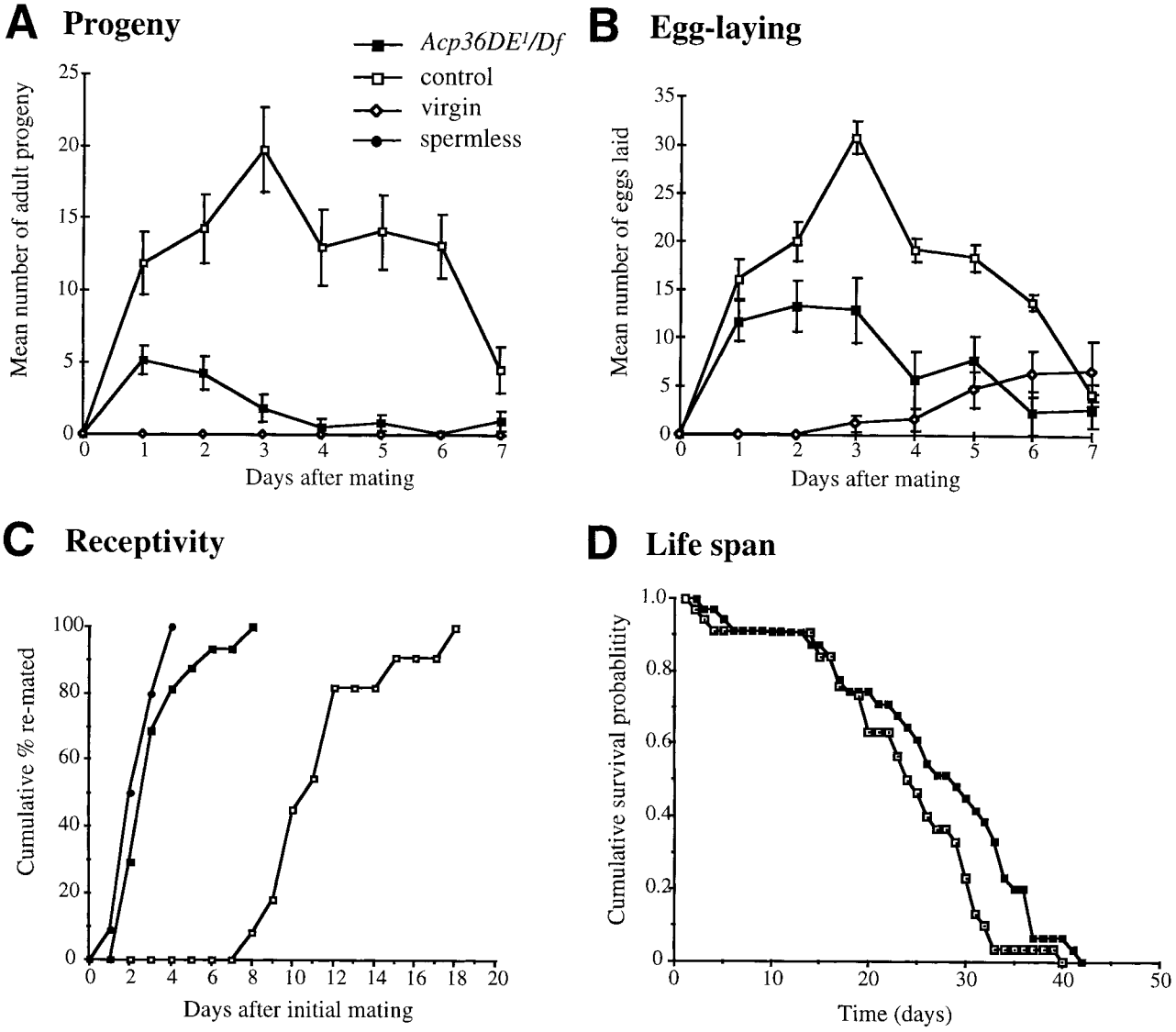

Figure 4.—Behavioral responses in females mated to *Acp36DE* mutant males. Data from a typical experiment are shown in each panel. In all cases, experiments were replicated three times, with similar results each time. Oregon-R females were tested as virgins (open diamonds) or after mating to a *Acp36DE*^{1}/*Df* male (solid squares), control male (open squares), or a spermless son of a *bw sp tud*^{1} mother (solid circles). (A) Fertility: The mean number (± SE) of adult progeny obtained from virgin or mated females is shown for each day after mating. (B) Egg laying: The mean number (± SE) of eggs laid by virgin or mated females is shown for each day after mating. The numbers of females tested per group were 48 mates of *Acp36DE*^{1}/*Df* males, 23 mates of control males, and 17 virgins. (C) Receptivity: Oregon-R females were initially mated to a *Acp36DE*^{1}/*Df* male, a control male, or a spermless male. The cumulative percentage of females that were willing to mate again with a wild-type control male during a 1-hr challenge period is shown for each day after the initial mating. The numbers of females tested per group were 17 mates of *Acp36DE*^{1}/*Df*, 19 mates of control, and 11 mates of spermless. (D) Life span: Survival curves for females mated to *Acp36DE* mutant (solid squares) or control males (open squares) are shown. A total of 30 females were tested per group.

that shown in Figure 2A. Therefore, the presence of Acp36DE in the oviduct was not necessary to keep sperm from mislocalizing to the upper genital tract.

We then reexamined our initial hypothesis and asked whether the establishment of a barrier and the presence of Acp36DE near the barrier was important for Acp-36DE's function in sperm storage. To test this, we evaluated sperm storage in eggless females mated to normal (Oregon-R) males. In eggless females, a greatly reduced amount of Acp36DE localizes to the oviduct, and a sperm barrier does not form (Bertram *et al.* 1996). We counted the number of sperm present in the storage organs of mated eggless females at 6 hr after mating, the time of maximal sperm storage (Gilbert 1981). Significantly fewer ($P = 0.0086$) sperm were stored in the seminal receptacles of eggless females relative to Oregon-R females (Table 2). Likewise, significantly fewer ($P < 0.0001$) sperm were found in the spermathecae at this time (Table 2). On average, eggless females stored 19% fewer sperm than control females

TABLE 2

Mean number of sperm stored in Oregon-R or eggless females mated to Oregon-R males at 6 hr after the start of mating

	Mean ± SE, (*n*), %[a]		
Female	Seminal receptacle	Spermathecae	All storage organs
Eggless	363.5 ± 17.9, (37), 82.4	87.5 ± 5.7, (71), 65.1	552.6 ± 25.4, (28), 81.1
Ore-R	441.1 ± 23.1, (36)	134.5 ± 8.8, (51)	681.3 ± 25.4, (26)

[a] Percentage relative to the mean number of sperm stored by Ore-R controls.

mated to normal males. However, note that eggless females with their reduced oviductal localization of Acp36DE still stored far more sperm than females mated to *Acp36DE*^{1}/*Df* males, who received no Acp36DE at all. The two experiments described above demonstrate that although Acp36DE is not necessary to form the barrier, the presence of the barrier and/or the full and normal localization of Acp36DE in the female is necessary for maximal sperm storage.

Acp36DE binds to sperm: The association of Acp36DE with the sperm mass in the mated female genital tract (Bertram *et al.* 1996) could be important for its function in sperm storage. Alternatively, Acp36DE may be present in the sperm mass without interacting specifically with sperm. To assess the strength of the association of Acp36DE and sperm, we tested whether Acp36DE could be dissociated from the sperm mass in the presence of high salt, detergent, or extreme pH. Sperm masses were removed from mated female genital tracts and placed in one of seven buffers (see materials and methods; Figure 5 legend). After incubation, sperm were pelleted by centrifugation. The sperm fraction (pellet) and soluble fraction (supernatant) were assayed by Western blotting. Both the full-length (122 kD) and processed (68 kD) Acp36DE products remained associated with sperm. In all treatments, >80% of both forms of Acp36DE remained associated with the sperm pellet (Figure 5, lanes 1–14). Vortexing the sperm samples at the start of incubation did not release more Acp36DE to the supernatant (data not shown). Except for buffer 1, the presence of Acp36DE in the pellet was not caused by precipitation (buffers 2–7 in Figure 5), since Acp36DE present in male accessory gland homogenates did not pellet when incubated alone (Figure 5, lanes 15 and 16), or when mixed with the torn-open testes and seminal vesicles of spermless males (not shown). Moreover, a different seminal fluid protein, Acp26Aa (Monsma and Wolfner 1988; Park and Wolfner 1995), readily dissociated from sperm upon the addition of NaCl (Figure 5, lanes 5–14). This result suggests that the association of Acp36DE with sperm is caused by biochemical features of Acp36DE rather than by any nonspecific "stickiness" of sperm. We also tested the association of a functional though truncated form of Acp36DE (Acp36DE^{p}, Clark *et al.* 1994) with the sperm mass. The 56-kD Acp36DE^{p} interacted tightly with the sperm mass in a manner similar to full-length Acp36DE (data not shown). Thus, both Acp36DE and Acp36DE^{p} interact with the sperm mass *in vivo*, and this interaction is refractory to biochemical dissociation.

As a tool for further examination of Acp36DE-sperm interactions, we developed a sperm-binding assay to test

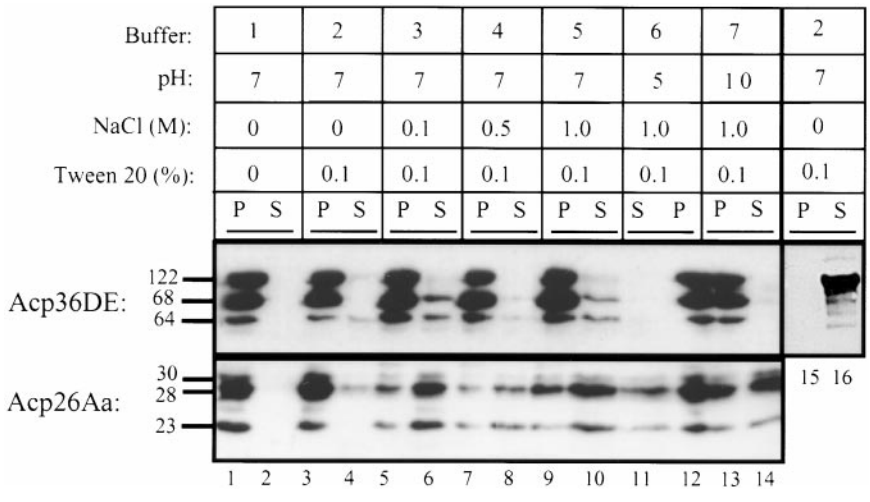

Figure 5.—The association of Acp36DE or Acp26Aa with the sperm mass *in vivo*. Western blot of sperm mass preparations probed with anti-Acp36DE or anti-Acp26Aa, showing proteins present in the pellet (P), which contains sperm, or the supernatant (S), which contains proteins that have dissociated from the sperm mass (lanes 1–14). Since the sperm masses were removed from mated females at 30 min after the start of mating, they show the processed products of Acp36DE (68 kD; and the female-specific, cross-reactive 64-kD band; Bertram *et al.* 1996) or Acp26Aa (30, 28, and 23 kD; Park and Wolfner 1995) normally present at that time. The components of seven PBS-based buffers are shown. As a control, accessory gland extract containing Acp36DE was incubated in each buffer without sperm (lanes 15 and 16 for buffer 2, data not shown for the other buffers). No precipitation of Acp36DE was observed for buffers 2–7, though slight precipitation occurred in buffer 1.

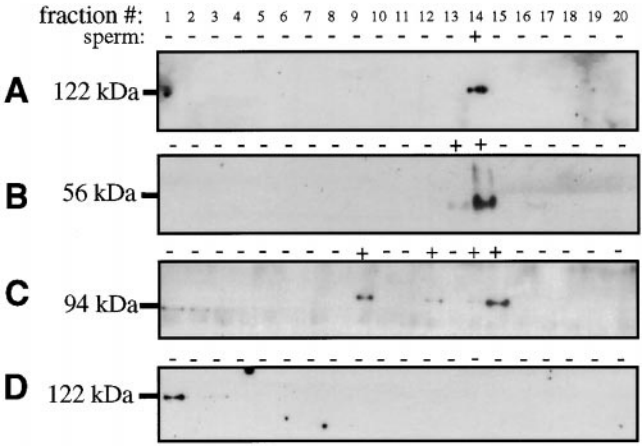

Figure 6.—The cosedimentation of endogenous Acp36DE and *E. coli*-made Acp36DE with sperm *in vitro*. Western blots of sucrose gradient fractions probed with anti-Acp36DE and the corresponding fractions that contain sperm. After spinning to equilibrium, the gradient was divided into 20 equally sized fractions ranging from 30% sucrose (fraction 1) to 70% sucrose (fraction 20). Sperm was mixed with accessory gland extracts of (A) wild-type males, (B) *Acp36DE*^{p} males, or (C) Acp36DE-GST fusion protein made in *E. coli*. As a control, (D) accessory gland extracts were tested without sperm. The increasing amount of sucrose in the deeper fractions of the gradient slightly retards mobility of the proteins in the gels. +, sperm seen in the fraction upon microscopic examination; −, no sperm detected in the fraction.

whether Acp36DE could associate with sperm *in vitro*. Mature sperm dissected from the seminal vesicle were incubated for 1 hr with accessory gland homogenates containing Acp36DE. Samples were then spun through sucrose gradients until equilibrium was reached for the sample components. Fractions taken from the gradients were assayed for colocalization of Acp36DE and sperm. As shown in Figure 6, Acp36DE and sperm were both present in fractions ~70% into the gradient (around fraction 14, Figure 6A). In control assays without sperm, Acp36DE remained in the top 10–15% of the gradient (Figure 6D). In parallel experiments, Acp36DE^{p} was found in the sperm-containing fractions in a manner similar to the full-length protein (Figure 6B; data not shown). Acp36DE made as a GST fusion protein in *Escherichia coli* also migrated with sperm in the gradient (Figure 6C), suggesting that posttranslational modifications such as glycosylation are not required for binding of sperm by Acp36DE, and that the presence of other Acps is dispensable for Acp36DE binding to sperm. A control protein, Acp26Aa (Monsma and Wolfner 1988; Park and Wolfner 1995), did not bind to sperm in parallel assays (data not shown). Thus, Acp36DE can interact with sperm in the absence of other Acps.

Acp36DE enters the sperm storage organs in a normal mating: Although large amounts of Acp36DE were not detected inside the storage organs of females in previous immunohistochemical experiments (Bertram *et al.* 1996), the impermeability of these chitin-lined organs to the staining reagents, and possibly the brown color of the spermathecae, prevented detection of small amounts of Acp36DE in these tissues. The tight association of Acp36DE with sperm in the uterus suggests that the protein must either accompany the sperm into the organs or rapidly dissociate from the sperm as they are stored in the first hours after mating. To distinguish between these explanations, we assayed Acp36DE entry into the storage organs by a method independent of immunohistochemistry. Using fine tweezers, we cleanly separated spermathecae and seminal receptacles from the rest of the genital tract of mated females. Spermathecal and seminal receptacle homogenates from wild-type matings (Ore-R × Ore-R) were tested for Acp36DE on Western blots. Both organs contained full-length (122

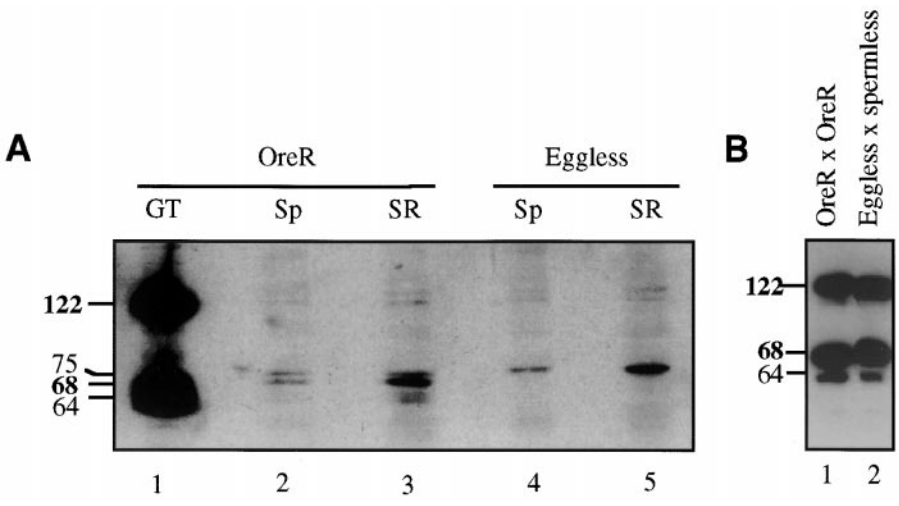

Figure 7.—Entry of Acp36DE into the storage organs and processing of Acp-36DE in the absence of sperm or eggs. (A) Western blot of female sperm storage organ extracts probed with anti-Acp36DE. Each lane contains the spermathecae (Sp) or seminal receptacles (SR) of 62 mated females of Oregon-R (OreR, lanes 2 and 3) or eggless females (lanes 4 and 5), all dissected at 2 hr after the start of mating. Lane 1 contains an extract of one female genital tract (GT) with the ovaries removed and dissected at 30 min after the start of mating. 122 kD, Acp36DE (full length); 68 kD, Acp36DE (processed); 64- and 75-kD, female-specific, cross-reactive proteins (Neubaum 1999). (*Acp36DE* is expressed only in males.) (B) Western blot of 10 mated female genital tracts from OreR × OreR (lane 1) or spermless × eggless (lane 2) matings probed with anti-Acp36DE.

kD) and processed (68 kD) Acp36DE (Figure 7A, lanes 1 and 2) at 2 hr after the start of mating, though slightly more Acp36DE was observed in the seminal receptacle than in the spermathecae. The signal obtained from ~120 spermathecae and ~60 seminal receptacles was ~10–50 times fainter than the signal of a single female genital tract extract (uterus plus sperm storage organs). Thus, a small fraction of the total Acp36DE transferred to a female does enter each of the sperm storage organs.

Since eggless females fail to localize Acp36DE properly in the lower oviduct, we tested whether Acp36DE could enter the storage organs of eggless females. Eggless females mated to Ore-R males stored an equivalent amount of full-length Acp36DE, but its 68-kD processing product could not be detected in either the spermathecae or the seminal receptacles (Figure 7A, lanes 4 and 5). The lack of detectable 68-kD Acp36DE in the sperm storage organs of mated eggless females does not reflect a lack of processing in these females. Processing of Acp36DE in the female genital tracts, which normally requires ≥20 min after mating (Bertram *et al.* 1996), is independent of the presence of eggs or sperm (Figure 7B). Thus, we believe the lack of processed Acp36DE in the sperm storage organs of mated eggless females may indicate that storage can occur properly for only a brief time immediately after mating, when full-length but not processed Acp36DE is present in the genital tract.

DISCUSSION

Storage of sperm by mated females is an important aspect of the reproductive strategy of many animals; yet its molecular basis is poorly understood. In Drosophila, molecules required for sperm storage are made by the male's accessory gland (Tram and Wolfner 1999). Here, we have identified the first molecule essential for sperm storage, and we have shown that this protein, the Drosophila accessory gland protein Acp36DE, is a major contributor to the process of sperm storage in females.

Acp36DE^{1} null mutant males made abundant, motile, morphologically normal sperm and transferred them in large numbers to females during mating (Figure 2A). However, sperm failed to accumulate properly in the storage organs of females mated to *Acp36DE*^{1} mutant males (Figure 2, C and D). A small number of sperm, roughly 15% relative to wild-type controls, did enter the storage organs and were stored. Some of these sperm successfully fertilized eggs (confirming that the sperm are functional), while others were lost from the storage organs at a greater rate than wild-type controls (Figure 3). As a result of storing few sperm, females mated to males lacking Acp36DE produced few progeny, only 10% of the number produced by females mated to normal males (Figure 4A). Moreover, the egg-laying and receptivity behaviors of females mated to Acp36DE-deficient mates reverted to a premated state within 2 or 3 days after mating instead of after the normal 10–14 days (Figure 4, B and C). Thus, the lack of Acp36DE indirectly affected egg-laying and receptivity behaviors, and served to minimize the number of progeny produced. While mutations in *Acp36DE* did not result in complete sterility, the fecundity of mutant males was severely compromised. The direct and indirect phenotypes of *Acp36DE* mutants emphasize the importance that female sperm storage has for the reproductive strategy of *D. melanogaster* and the role that male-derived proteins play in this process.

Acp36DE appears to be one of the primary male proteins responsible for eliciting female sperm storage. *Acp36DE*^{1}/*Df* mutant males elicited approximately the same level of sperm storage as males with genetically ablated accessory glands, who produce almost no Acps at all (Tram and Wolfner 1999). In contrast, the stimulation of egg laying, another postmating effect of males upon females, results from the cumulative but apparently independent effects of at least two gene products, Acp26Aa (Herndon and Wolfner 1995) and sex peptide (Chen *et al.* 1988; Moshitzky *et al.* 1996).

A protein that is needed for sperm storage might act at any of several levels: helping sperm enter the storage organs, helping sperm arrange themselves appropriately inside the organs, maintaining sperm viability over time, or controlling the release of sperm over time. Our data favor the primary role of Acp36DE as being in the entry of sperm into storage, though we describe observations that suggest a secondary effect of the protein in sperm arrangement in and release from storage. A possible role for *Acp36DE* in promoting the entry of sperm into storage was suggested initially by the localization of Acp36DE protein in the female (Bertram *et al.* 1996). Since Acp36DE localization in the oviduct of wild-type mated females was coincident with the upper limit at which sperm were observed in the genital tract, we suggested that Acp36DE marks or forms a barrier in the oviduct that confines sperm within an area from which they can be easily channeled into the storage organs (Bertram *et al.* 1996). Here, we present four observations important for understanding the mode of action of Acp36DE in sperm storage. (1) The presence of Acp36DE was not necessary for sperm to be restrained from entering the upper oviduct of mated females (*i.e.*, for "barrier formation"). (2) In eggless females, inefficient barrier formation and incomplete binding of Acp36DE at the lower oviduct correlated with fewer sperm stored. (3) Acp36DE tightly associated with sperm *in vivo*, and sperm and Acp36DE cosedimented *in vitro*. (4) Acp36DE was found in the sperm storage organs of mated females in both its full-length and processed forms. These points will be discussed in turn.

While the nature of the mechanism (or "barrier") that holds sperm in the posterior genital tract is unknown, it appears to be intact in females mated to *Acp36DE*^{1} null mutant males. Females mated to males lacking Acp36DE

retained a large mass of sperm in the uterus immediately after mating, and few, if any, sperm were observed in the upper oviduct near the ovaries. Despite proper barrier formation, 85% fewer sperm were stored than when Acp36DE was present. Thus, the presence of the barrier was not sufficient alone for normal efficient sperm storage.

The reverse experiment, in which normal amounts of Acp36DE are supplied but formation of the barrier is faulty, was performed by mating normal males to eggless females. In these matings, Acp36DE localization in the lower oviduct was weakened but not eliminated (Bertram *et al.* 1996). Eggless females stored significantly fewer sperm (averaging 19% less overall) relative to wild-type matings. These data show that without an oviduct barrier, a full aliquot of Acp36DE is not sufficient for full sperm storage. Therefore, the barrier must play some role in sperm storage, even if it is indirect. The barrier may be important simply because it traps Acp36DE at a critical location within the genital tract, from which Acp36DE exerts its action upon sperm storage. Alternatively, the activity of the barrier in sperm storage may be independent of Acp36DE. However, both the barrier and Acp36DE are required for optimal sperm storage in *D. melanogaster*.

What sort of action might Acp36DE exert from its location at the lower oviduct near the sperm storage organs? From the lower oviduct, Acp36DE could act on muscles or on the female nervous system to stimulate the storage organs to open or to begin contractions that pump sperm into storage. This type of mechanism is used in *Rhodnius prolixus*, where accessory gland secretions are reported to stimulate the genital ducts of females to contract (Davey 1960, 1965). Drosophila females might require ductile contractions to move sperm into storage, since the openings to the storage organs are extremely narrow and may impose limitations on the undulatory movements of sperm (see Linley and Simmons 1981). Interestingly, Arthur *et al.* (1998) report that *D. melanogaster* flies with female bodies but masculinized central nervous systems store dramatically fewer sperm than control females, indicating that the female nervous system plays an important role in Drosophila sperm storage. Alternatively, Acp36DE might complement the action of a physical barrier by providing a chemical signal to sperm to deflect them away from the upper regions of the genital tract and into the storage organs. Acp36DE and the sperm barrier may work together to ensure the efficient storage of sperm before the advent of egg laying results in the unstored sperm being pushed out of the uterus.

In addition to associating with the oviduct wall, Acp36DE also associates *in vivo* and *in vitro* with the sperm mass. The Acp36DE-sperm interaction was strong enough to withstand vortexing and incubation in high salt, detergent, and extreme pH (Figure 5). Such strong interactions are reminiscent of carbohydrate interactions of lectins and the proteins that bind to them (Koehler 1978; Nicholson and Yanagimachi 1979). As a glycoprotein, Acp36DE may interact with moieties found on the surface of sperm. The *in vitro* association of recombinant Acp36DE produced by *E. coli* suggests that the proteinaceous component of Acp36DE is sufficient to bind to sperm (Figure 6), but this result does not preclude the possible interaction of Drosophila Acp36DE's carbohydrate with sperm as well.

Since Acp36DE associated tightly with sperm in the uterus, we hypothesized that Acp36DE might enter the storage organs bound to sperm. Sperm enter the seminal receptacle earlier than the spermathecae and are stored there in greater numbers (Gilbert 1981; Tram and Wolfner 1999). Consistent with our hypothesis, Acp36DE was detected inside the storage organs by 2 hr after mating (Figure 7), with slightly more Acp36DE observed in the seminal receptacle than in the spermathecae at this time. Thus, Acp36DE was present at a time and place from which it might influence the sperm once they are inside the organs, possibly helping sperm to assume or retain an orderly parallel arrangement, or preventing their premature loss from the organs. In this context, it is interesting that sperm in the storage organs of females mated to males lacking Acp36DE failed to assume their normal parallel configuration (Figure 2D). In addition to Acp36DE, another *D. melanogaster* seminal fluid molecule influences sperm storage: the ejaculatory product esterase-6, a carboxylesterase (Gilbert 1981; Gilbert *et al.* 1981). In contrast to *Acp36DE*, null mutations in *esterase-6* decrease rather than increase the rate of sperm loss (Gilbert 1981), suggesting that this protein operates differently from Acp36DE.

The processed (68 kD) form of Acp36DE was detected in the storage organs of wild-type but not eggless females, although full-length Acp36DE entered the storage organs of both types of females in approximately equivalent amounts (Figure 7). These observations may be explained if sperm stop entering storage earlier in eggless females, perhaps because of the impaired localization of Acp36DE at the oviduct. For 15–20 min after the start of mating, sperm and full-length Acp36DE (the only form available during that interval since processed products are not detected immediately after mating, Bertram *et al.* 1996) begin entering the storage organs, with Acp36DE possibly bound to the sperm. As the 68-kD form of Acp36DE begins to appear, it also enters the storage organs. In eggless females, the mislocalization of sperm up the oviduct may divert sperm (and Acp36DE) from entering the storage organs.

Acp36DE is one of several Acp genes for which polymorphisms in natural populations were reported to correlate with levels of sperm competition, specifically with the ability of stored sperm to resist displacement by sperm from a subsequent mate (Clark *et al.* 1994). Given the role we demonstrate here for Acp36DE in the storage and retention of sperm, we propose that

the effect of the wild-caught *Acp36DE* polymorphic alleles on sperm competition may reflect the action of the protein in storing and retaining sperm rather than an active participation in resistance to competition.

In summary, Acp36DE plays a critical role in the entry of sperm into storage and influences the maintenance or release of stored sperm. To date, most mechanisms proposed to facilitate sperm storage derive from morphological and behavioral criteria (for review see Birkhead and Møller 1993; Eberhard 1996; Neubaum and Wolfner 1999). Our identification of Acp36DE as essential for sperm storage now permits a molecular genetic dissection of this essential reproductive process.

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