Cleavage of the *Drosophila* seminal protein Acp36DE in mated females enhances its sperm storage activity

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**A B S T R A C T**

Sperm storage in the mated female reproductive tract (RT) is required for optimal fertility in numerous species with internal fertilization. In *Drosophila melanogaster*, sperm storage is dependent on female receipt of seminal fluid proteins (SFPs) during mating. The seminal fluid protein Acp36DE is necessary for the accumulation of sperm into storage. In the female RT, Acp36DE localizes to the anterior mating plug and also to a site in the common oviduct, potentially “corraling” sperm near the entry sites into the storage organs. Genetic studies showed that Acp36DE is also required for a series of conformational changes of the uterus that begin at the onset of mating and are hypothesized to move sperm towards the entry sites of the sperm storage organs. After Acp36DE is transferred to the female RT, the protein is cleaved by the astacin-metalloprotease Semp1. However, the effect of this cleavage on Acp36DE’s function in sperm accumulation into storage is unknown. We used mass spectrometry to identify the single cleavage site in Acp36DE. We then mutated this site and tested the effects on sperm storage. Mutations of Acp36DE’s cleavage site that slowed or prevented cleavage of the protein slowed the accumulation of sperm into storage, although they did not affect uterine conformational changes in mated females. Moreover, the N-terminal cleavage product of Acp36DE was sufficient to mediate sperm accumulation in storage, and it did so faster than versions of Acp36DE that could not be cleaved or were only cleaved slowly. These results suggest that cleavage of Acp36DE may increase the number of bioactive molecules within the female RT, a mechanism similar to that hypothesized for Semp1’s other substrate, the seminal fluid protein ovalin.

**1. Introduction**

In a number of diverse species with internal fertilization, the storage of sperm in the mated female reproductive tract (RT) is often required for optimal fertility (Bloch Qazi et al., 2003; Avila et al., 2011; Schnakenberg et al., 2012). Much of our understanding of female sperm storage mechanisms derives from work in the genetic model *Drosophila melanogaster*, where studies have shown that seminal fluid proteins (SFPs) transferred to females during mating are integral for sperm storage to occur normally (reviewed in Avila et al., 2011). Further, the availability of GFP/RFP-labeled sperm have made it possible to follow storage dynamics in the female RT (Manier et al., 2010). To date, > 200 *D. melanogaster* SFPs have been identified (Findlay et al., 2008, 2009; Takemori and Yamamoto, 2009; Yamamoto and Takemori, 2010). Genetic ablation of the major site of SFP synthesis and secretion, the male accessory glands, impairs male fertility due to inefficient sperm storage and utilization (Kaib et al., 1993; Xue and Noll, 2000; Gligorov et al., 2013; Chow et al., 2015).

*Drosophila melanogaster* females possess two organs of the RT where sperm are stored: the single seminal receptacle and the paired spermathecae. After transfer to the female RT, sperm must travel to and enter these storage organs, be maintained viably within storage, and ultimately be released to fertilize eggs (Bloch Qazi et al., 2003). Individual SFPs act at each of these steps, working collectively to ensure that sperm storage occurs efficiently (Ravi Ram and Wolfner, 2007, 2009; Wong et al., 2008; Avila and Wolfner, 2009; Avila et al., 2010, 2015b; LaFlamme et al., 2012).

The male accessory gland glycoprotein, Acp36DE, is required for sperm accumulation into the female storage organs (Neubaum and Wolfner, 1999; Bloch Qazi and Wolfner, 2003). When Acp36DE is absent from the ejaculate, the number of sperm stored in mated females is significantly reduced, resulting in decreased fertility (Neubaum and Wolfner, 1999; Bloch Qazi and Wolfner, 2003). Acp36DE has a number of features consistent with its role in sperm storage. First, the protein is part of the anterior mating plug (Bertram et al., 1996; Avila et al., 2015a), which forms in the uterus and is thought to help trap sperm close to the openings of the storage organs in the upper uterus. Second, the protein accumulates at a site in the lower common oviduct, just
above the openings to sperm storage organs. In wildtype matings, few if any sperm are seen in the oviducts above this position, but in matings to Acp36DE null males, sperm move into the oviducts (Bertram et al., 1996). These results suggest that Acp36DE may form part of a physical structure that "corrals" sperm between the mating plug and the oviduct site, concentrating them near the openings to the sperm storage organs. Finally, Acp36DE mediates a series of morphological changes of the female uterus that ‘open’ the uterus from a tightly closed, pre-mated conformation. The mating-induced opening of the uterus is proposed to move sperm towards the sites of storage and allow access to the storage organs (Adams and Wolfer, 2007; Avila and Wolfer, 2009). All three phenomena could, separately or together, facilitate sperm entry into storage.

Acp36DE is proteolytically cleaved within the RT of the mated female (Bertram et al., 1996), due to the action of Seminal metallocproteinase-1 (Semp1; LaFlamme et al., 2014). Cleavage of Acp36DE is first detected ~8 min after the start of mating (ASM), similar to the timing of cleavage of another seminal protein, ovulin, by Semp1 (Monsma et al., 1996; LaFlamme et al., 2014). Cleavage of seminal proteins by seminal proteases is observed in a number of taxa (Pampalakis and Sotiropoulou, 2007; LaFlamme and Wolfer, 2013) and is often associated with events that allow sperm to move from the ejaculate to the sites of storage or fertilization. For example, in humans, cleavage of semenogelins by kallikreins such as the Prostate-specific (PSA) results in liquefaction of the semen clot, liberating sperm to be stored or used for fertilization (Lilja et al., 1989).

The role of Acp36DE’s cleavage in the protein’s sperm storage activity is not known. Its cleavage might be to activate the protein or to liberate active sub-regions from an inactive (or poorly active) protein. It is also possible that cleavage eliminates activity of the protein. The cleavage of ovulin, the other identified target of Semp1 (LaFlamme et al., 2014), appears to increase the number of active molecules in the female RT, as two of its cleavage products can each, independently, induce ovulation (Heifetz et al., 2005). That cleavage of Acp36DE occurs in a stereotypic manner, suggests that its cleavage might also work to increase the proteins bioactivity. However, this hypothesis has not been tested.

To determine whether cleavage of Acp36DE plays a role in facilitating sperm storage or fertility, we used mass spectrometry to identify the cleavage site in Acp36DE. Then, using those results to guide our mutagenesis, we created transgenes of multiple versions of Acp36DE: the full-length wild-type protein (which served as a positive control), a mutated version that slowed cleavage of Acp36DE, a mutated version that prevented cleavage of Acp36DE, and the N-terminal cleavage product of Acp36DE. We then measured the effects of each of these versions on accumulation of sperm into the primary sperm storage organ, the seminal receptacle (Pitnick et al., 1999; Manier et al., 2010).

We found that cleavage of Acp36DE increases its ability to facilitate sperm accumulation in storage, and that its N-terminal cleavage product is sufficient for this function. Interestingly, receipt of any version of Acp36DE, even an uncleavable one, was sufficient to mediate uterine conformational changes, suggesting that this aspect of Acp36DE action does not require cleavage of the protein.

2. Materials and methods

2.1. Flies

We reared flies under standard laboratory conditions on yeast/glucose media at 23 ± 2 °C on a 12:12 light/dark cycle. Flies of the Canton S strain were used to determine Acp36DE’s cleavage site and Canton S females were used in our sperm storage and uterine conformation assays. We recombined each of our Acp36DE ectopic expression transgenes (UAS-Acp36DESTOP) onto the second chromosome that contained the Df(2L)H2O deficiency (Bloomington stock #3180: Df(2L)H2O, b1 pr1 cn1 sca1/CyO) to generate the UAS-Acp36DESTOP, Df(2L)H2O/CyO stock. The Df(2L)H2O deletion uncovers Acp36DE. In addition, we used the Acp36DE null allele Acp36DESTOP (Bertram et al., 1996) and paired-GAL4/TM3, Sb (Bloomington stock #1947) to generate the Acp36DESTOP/G; prd-GAL4/TM3, Sb stock. In our experiments, we crossed these two stocks to generate experimental males that ectopically expressed Acp36DE in the Acp36DE null background (UAS-Acp36DESTOP, Df(2L)H2O/Acp36DESTOP; prd-GAL4/+ ) and Acp36DE null males (UAS-Acp36DESTOP, Df(2L)H2O/Acp36DESTOP; TM3, Sb/+ ), which acted as a negative control.

2.2. Identification of Acp36DE’s cleavage site

We mated Canton S males and females and flash froze mated females in liquid nitrogen at 1 h after the initiation of mating. Mated female uteri were dissected in 1 × PBS under a dissecting microscope. Dissected tissues were placed in 20 µL of 1 × PBS in a 0.5-mL Eppendorf tube and briefly ground with a pestle (25 female RTs in total were dissected). Next, 20 µL of 2 × SDS sample buffer containing 10% 2-mercaptoethanol was added and the sample was subsequently boiled for 5 min.

We separated samples using SDS/PAGE on a 12% polyacrylamide gel and visualized total protein with Coomassie Brilliant Blue R-250 dye. Because of the presence of cross-reactive bands with the affinity-purified antibodies, to aid in the identification of full-length Acp36DE and its two cleavage products, we ran the mated female sample side-by-side with the same number of non-reproductive (gut) tissues (collected during the same dissections) as well as uteri from unmated females (Fig. S1). Bands that corresponded to full-length Acp36DE (122 kDa), its first cleavage product (CP1; runs at 68 kDa) and the second cleavage product (CP2; runs at 50 kDa) were excised from the gel using a sterile razor blade. Proteins were eluted from the gel, protelozied with trypsin and subjected to mass spectrometry (LC-MS/MS) at the Cornell University Proteomics & Mass Spectrometry facility.

2.3. Cloning and transgenic fly creation

We used the Gateway (Invitrogen)-compatible pbU-UASc-G vector (Pfriffer et al., 2008) to clone the full-length coding region of Acp36DE (Acp36DESTOP2) and the first cleavage product (Acp36DESTOP2P). pbU-UASc-G is compatible with the PhiC31 site-specific integrase system in Drosophila (Groth et al., 2004; Bateman et al., 2006; Dietzl et al., 2007). Full-length Acp36DE and the first cleavage product were amplified from Canton S genomic DNA using the following primers: FL-Fwd: 5’ – CAC GTG AAC TAC GAC TAC GCT GAA ACA A – 3’; FL-Rev: 5’ – TTA ACC TGA AAA ATT TAG CAG CGC – 3’, CP1: FL-Fwd primer (see above); CP1-Rev: 5’ – CTA TAG CGT ATT CGT CTG – 3’. In addition to the wild-type Acp36DE sequences, we cloned two constructs with mutations in Acp36DE at residue 516 (Acp36DESTOP366A) and 517 (Acp36DESTOP367A) using the QuikChange Lightning kit (Agilent) with the following primers: SS16A-Fwd: 5’ – CAG CAG CAA CTA AAA GGA GAT GAA CAA ACC CAG – 3’; SS16AF-Rev: 5’ – CTT GGT TGT ATC TGG TTG TTG ATC TGG TTG TTG CTG CTG – 3’; D517A-Fwd: 5’ – CAG CAG CAA CTA AAA GGA GAT GAA CAA ACC CAG – 3’; D517A-Rev: 5’ – CTA TAG CGT ATT CGT CTG – 3’. Genetic Services, Inc. (Cambridge, MA) injected constructs into embryos expressing the Phil31 integrase and with an Attp40 site at cytogenetic location 25C7 of chromosome 2L (Bloomington stock #36304; Markstein et al., 2008).

2.4. Western blot analysis

Lower female reproductive tracts (uterus, sperm storage organs and common oviduct) were dissected in 1 × PBS under a dissecting microscope. We placed tissues in 10 µL of 1 × PBS in a 0.5-mL Eppendorf tube, briefly ground them with a pestle, added 10 µL of 2 × SDS sample buffer containing 10% 2-mercaptoethanol and subsequently boiled
them for 5 min.

We separated samples using SDS/PAGE on 5–15% polyacrylamide gradient gels. Western blotting was carried out as in Ravi Ram and Wolfner (2009). anti-Acp36DE (Bertram et al., 1996) was used at a concentration of 1:10,000 in PBST (1X PBS, 0.1% Triton X-100) + 5% non-fat dry milk blocking solution and anti-alpha-Tubulin (Sigma TS168, clone B-5-1-2) at 1:10,000. Blots were treated with Amersham ECL Plus (GE Healthcare Life Sciences) and imaged using a Typhoon 8600 imager (GE Healthcare Life Sciences).

2.5. Uterine conformation assays

Uterine conformation assays were performed as in Avila and Wolfner (2009). Females were individually mated to experimental or control males and frozen in liquid nitrogen at 35 min after mating began. All matings were observed. Uteri were dissected in 1X PBS, visualized with an Olympus SZ61 dissection microscope and staged as in Adams and Wolfner (2007). The distribution of stages in females mated to experimental males compared to females mated to control males was analyzed using a Wilcoxon test (rank sums) in JMP 13.

2.6. Sperm counts

Canton S females were individually mated to experimental males ecotopically expressing an Acp36E construct (Acp36DEfl, Acp36DECP1, Acp36DE516A or Acp36DE517A) or Acp36DE null males. All matings were observed. Mated females were flash frozen in liquid nitrogen at 35 min after mating. Sperm were stained as in Avila et al. (2010). In brief, lower female RTs were dissected in 50% acetic acid and stained with 2% orcein in 50% acetic acid. Sperm in the seminal receptacle were counted under 1000x magnification using a Zeiss 47 30 9901 stereo microscope. Samples were blind-coded before counting to avoid bias. We had a repeatability of > 93% for each experiment based on duplicate counts of a subset of samples; experimental data sets that failed to meet this criterion were not considered for analysis. Counts were analyzed using Wilcoxon tests (rank sums) in JMP 13.

3. Results and discussion

3.1. Identification of Acp36DE’s cleavage site

We individually subjected full-length Acp36DE and its two cleavage products to mass spectrometry (LC–MS/MS) in order to identify the terminal amino acids of peptides generated after trypsin digestion. Analysis of Acp36DE’s first cleavage product (CP1) showed that this fragment covered residues 24 to 515 or 516. Analysis of Acp36DE’s second cleavage product (CP2) showed that this fragment began at residue 517 and ended at the C-terminus of Acp36DE (residue 911; see Fig. S2A and S2B).

Because the molecular weights of CP1 and CP2 predicted that cleavage occurred between aa 476 and aa 547, we examined the termini of peptides that contained amino acids from this region. We searched for peptides from CP1 or CP2 that differed from those generated from the full-length protein, as their extent would identify the cleavage site. Relative quantification of the targeted peptides across our samples (i.e. in the range of residues 476–547) identified 6 peptides in each sample (CP1 and CP2; Fig. 1A and 1B, respectively). Examination of the peptide termini (C-termini for CP1 and N-termini for CP2) suggested that cleavage occurred between residues S516 and D517. However, we also detected peptide fragments that ended after residue K515. A possible reason for the ambiguity of the terminal residue of CP1 fragments is that K515 cleavage would also occur with the trypsin that was used to prepare the samples for mass spectrometry. Thus, our results indicated that cleavage of Acp36DE in Drosophila female reproductive tracts occurs immediately after residues K515 and/or S516.

Acp36DE is cleaved by Seminal metalloprotease 1 (Semp1), a member of the astacin family of metalloproteases (LaFlamme et al., 2014). Although metalloproteases do not have consensus cleavage sites, Becker-Pauly et al. (2011) found a strong preference for negatively charged residues at the P1’ position (the immediate residue on the prime side of the proteolytic cleavage event) when examining physiological substrates of astacin metalloproteases. Our results are in agreement with that study; a negatively charged aspartic acid lies at aa 517 of Acp36DE, in the P1’ position of the Semp1 cleavage site.

To confirm that Acp36DE is cleaved in females at position 516/517, we generated two mutant forms of Acp36DE by changing, individually, the residues at positions 516 and 517 to alanine, generating Acp36DE516A and Acp36DE517A. In parallel, we cloned a full-length version of Acp36DE (Acp36DEfl) and Acp36DE’s first cleavage product (aa1-516: Acp36DECP1). We cloned these versions of Acp36DE behind a UAS-promoter and generated transgenic fly lines. We ecotopically expressed each form of Acp36DE by introducing these transgenes into flies that carried a prd-GAL4 driver and were also homozygous for an Acp36DE null mutation (see Materials and Methods). prd-GAL4 drives expression in the male accessory glands (Xue and Noll, 2002), the normal site of Acp36DE expression.

We then examined the sizes of the transgene-encoded Acp36DE after its transfer to the female RT during mating. Cleavage of our control transgenic Acp36DEfl occurred normally. However, mutations of both S516 and D517 affected cleavage of Acp36DE: cleavage of Acp36DE516A occurred at a slower rate than Acp36DEfl and cleavage of Acp36DE517A was undetectable (Fig. 2). These results confirm that in vivo cleavage of Acp36DE occurs after the serine residue at position 516.

3.2. Cleavage of Acp36DE enhances its ability to accumulate sperm into storage

Acp36DE is required for sperm to accumulate normally into storage. When females do not receive Acp36DE during mating, the number of sperm stored is significantly reduced (Neubam and Wolfner, 1999; Bloch Qazi and Wolfner, 2003). Using males ecotopically expressing different versions of Acp36DE in an Acp36DE null background (see Section 2)—the full-length version of Acp36DE (Acp36DEfl), the first cleavage product (Acp36DECP1), the slow-cleaving mutant (Acp36DE516A), and the mutant from that is not cleaved (Acp36DE517A)—we examined sperm accumulation into storage when females received one of these forms of Acp36DE relative to females mated to Acp36DE null males. We focused on sperm accumulation into the seminal receptacle at 35 min ASM. At this time, sperm are rapidly entering the seminal receptacle (Manier et al., 2010) and defects in sperm storage there are readily detected (Avila et al., 2015b).

Females that received any transgenic version of Acp36DE stored more sperm than females that did not receive Acp36DE (Fig. 3), while receipt of Acp36DEfl, Acp36DECP1 and Acp36DE516A led to significantly more sperm stored in the seminal receptacle than females that did not receive any version of the protein (Fig. 3). Further, the number of sperm in the seminal receptacle at 35 min when females received Acp36DEfl was similar to females who received endogenous Acp36DE at the same time point (Avila et al., 2015b). Females mated to Acp36DECP1 males stored the most sperm, although they did not contain significantly more sperm than those mated to Acp36DEfl males. Slowing the rate of cleavage of Acp36DE, or preventing cleavage, decreased the number of sperm stored, suggesting that rapid cleavage of Acp36DE in the female RT is required for the protein’s sperm storage activity. When examining females that received any version of Acp36DE, we observed that receipt of Acp36DECP1 led to significantly more sperm in storage when compared to receipt of Acp36DE516A (Fig. 3).

Our results suggest that cleavage of Acp36DE improves its ability to move sperm into storage, and that CP1 is sufficient for this effect. That mates of Acp36DEfl males stored as many sperm as mates of...
Acp36DEFL indicates that CP1 is sufficient to promote sperm entry into storage; the slight though nonsignificant increase in sperm storage driven by Acp36DECP1 and the apparent lack of a need for CP2 suggests that the cleavage of Acp36DE may liberate a more active form of the protein. Interestingly, cleavage of the other Semp1 target, ovulin, has also been proposed to increase the rate of its function (ovulation-promotion), suggesting that the cleaved products are more active than the full-length precursors (Heifetz et al., 2000, 2005; LaFlamme et al., 2014).
3.3. Cleavage of Acp36DE is not required to mediate the conformational changes of the mated female uterus

Acp36DE mediates a series of conformational changes of the uterus that initiate during mating and open the lumen of the uterus, potentially facilitating access to the sperm to storage sites (Adams and Wolfner, 2007; Avila and Wolfner, 2009). To determine whether cleavage of Acp36DE affects its role in mediating uterine conformation, we examined uterine conformation at 35 min ASM, the time by which we saw differences in sperm accumulation in storage in the mutants. At this time, the uterus is typically nearing completion of the uterine shape changes (Adams and Wolfner, 2007; Avila and Wolfner, 2009).

Using males ectopically expressing the different versions of Acp36DE in an Acp36DE null background, we saw no significant differences in the progression of the uterine changes when females received Acp36DECP1, Acp36DESS16A or Acp36DEDS17A compared to uterine progression when they received Acp36DEFL (Fig. 4); effects of the latter are like those of receipt of endogenous Acp36DE (Avila and Wolfner, 2009). Further, receipt of any of the constructs we tested significantly differed from females who did not receive Acp36DE (Fig. 4), suggesting that receiving of any of these versions of Acp36E is sufficient to mediate progression of the uterine shape changes and allow access to the sites of storage.

4. Conclusions

Our results identify the cleavage site within Acp36DE and show that this cleavage plays a role in the protein’s efficiency in moving sperm into storage. Slowing or preventing cleavage of Acp36DE reduced the protein’s ability to move sperm into storage. Further, solely providing the N-terminal cleavage product (CP1) was sufficient to get sperm into storage with normal efficiency. These results suggest that cleavage of Acp36DE’s cleavage site results in fewer sperm in the SR at 35 min relative to the number stored in the presence of full-length Acp36DE or its N-terminal-cleavage product. Females that received Acp36DECP1, Acp36DESS16A and Acp36DEDS17A each stored significantly more sperm than females mated to Acp36DE null males at 35 min ASM: Acp36DEFL (NCP1 = 20, NCont = 17, p = 0.0002), Acp36DECP1 (NSS16A = 18, NS17 = 17, p = 0.0013), Acp36DESS16A (NSS17A = 12, NCont = 17, p = 0.138). Females that received Acp36DECP1 stored significantly more sperm than females that received Acp36DEDS16A, the slow-cleaving mutant (NCP1 = 20, NSS16A = 18, p = 0.0366), unpublished data.
Acp36DE may be essential to liberate its active region from the full-length precursor, allowing it to function to facilitate sperm storage. Interestingly, the same mutations that decreased sperm accumulation into storage did not impact Acp36DE’s function in mediating changes in uterine conformation, at least by 35 min ASM. This suggests that other aspects of Acp36DE’s function may also be important in facilitating sperm entry into storage. For example, cleavage of Acp36DE may be necessary for the protein’s role in the mating plug or the oviduct block that “corral” the sperm near the site of the sperm storage organs or, analogous to the effects of cleavage on semenogelin in mammals (LaFlamme and Wolfner, 2013), may liberate sperm from an Acp36DE-containing matrix in the mating plug, allowing them to move into storage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.06.015.

References


