Sex-Peptides Bind to Two Molecularly Different Targets in *Drosophila melanogaster* Females

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ABSTRACT: Sex-Peptide (SP) and the peptide DUP99B elicit two postmating responses in *Drosophila melanogaster* females: receptivity is reduced and oviposition is increased. Both are synthesized in the male genital tract and transferred into the female during copulation. To elucidate their function, we characterized the binding properties of SP and DUP99B in females. Cryostat sections of adult females were incubated with alkaline phosphatase (AP)-tagged peptides. In virgin females, both peptides have specific target sites in the nervous system and in the genital tract. The binding pattern is almost identical for both peptides. Incubation of sections of mated females confirm that some of these target sites correspond to the in vivo targets of the two peptides. Neuronal binding is dependent on an intact C-terminal sequence of SP, binding in the genital tract is less demanding in terms of amino acid sequence requirement. On affinity blots the AP–SP probe binds to membrane proteins extracted from abdomen and head plus thorax, respectively. The binding proteins in the nervous system and the genital tract differ in their molecular properties. Calculation of dissociation constants (K<sub>d</sub>) and also determination of the minimal peptide concentrations necessary for binding, indicate that SP is the more important peptide inducing the postmating responses. Our results suggest that binding of SP in the nervous system is responsible for eliciting the postmating responses, whereas binding in the genital tract reflects the presence of a peptide transporter. © 2003 Wiley Periodicals, Inc. J Neurobiol 55: 372–384, 2003

Keywords: *Drosophila melanogaster*; sex-peptide; reproduction; nervous system; genital tract

INTRODUCTION

Mating in many insects drastically affects female reproductive physiology and behavior. The two most common and conspicuous postmating responses are increased egg laying and reduced receptivity (= reduced readiness to mate; Chen, 1984; Gillott, 1988, 2003; Kubli, 1996; Wolfner, 1997). In *D. melanogaster* two peptides, Sex-Peptide (SP) and DUP99B, are responsible for eliciting these two postmating responses (DUP99B = Ductus ejaculatorius peptide, cytological localization 99B; Chen et al., 1988; Aigaki et al., 1991; Saudan et al., 2002). Both are synthesized in the male genital tract and transferred to the female during copulation. Sex-Peptide (36 amino acids) is made in the accessory gland, DUP99B (31 amino acids) in the ejaculatory duct, respectively. A third peptide synthesized in the male accessory gland, Acp26Aa, stimulates oviposition on the first day after mating (Herndon and Wolfner, 1995). However, Acp26Aa has no influence on receptivity.

By comparing the sequences of the genes coding for SP and DUP99B, high similarities were found in the signal peptides of the peptide precursors and in the C-terminal domains of the mature peptides (Saudan et al., 2002). Therefore, we consider the two peptides as the first two members of the sex-peptide pheromone family (Saudan et al., 2002). In this article Sex-Peptide (SP) will designate the peptide isolated and characterized by Chen et al., (1988), the notion Sex-peptides (Sps) will refer to SP and DUP99B. "Sex-
sex-peptide response cascade” will refer to the biochemical processes elicited by the Sps from their entrance into the female body down to the two postmating responses.

Identification of the molecular targets of the two peptides is crucial for understanding the signalling cascades. Indirect evidence suggests that there might exist only one type of molecular receptor for both peptides with respect to the two postmating responses. First, injection of different fragments of SP elicits either both or none of the two postmating responses (Schmidt et al., 1993). Second, the same critical concentration of 0.6 pmol/female is needed to induce both postmating responses (Schmidt et al., 1993; Kubli, 1996). Third, ectopic expression of membrane-bound SP in different target tissues elicits either both or no response (Nakayama et al., 1997). However, analysis of gynandromorphs has revealed that two different parts of the central nervous system are involved in controlling female mating behaviour and oviposition, respectively (Szabad and Fajszl, 1982; Oettinger, 1983). But these regions may participate in the SP response cascade indirectly. Oettinger et al., (2000) have identified high affinity binding sites for the sex-peptide pheromones by incubating 125I-labeled peptides on cryostat sections of adult females. The Sps have widespread but specific target sites, including parts of the central and peripheral nervous system, and the genital tract. These binding sites may represent the localization of the receptor(s) for the Sp pheromones.

Besides the identification of the gene(s) coding for the proteins binding the Sps, several other important questions are still open. For example, where are the in vivo target sites of the two peptides localized? Are the two peptides functionally redundant? What are the biochemical properties of the binding protein(s)? Fusion proteins containing Alkaline phosphatase (AP) fused to ligands or receptors have been developed into an excellent tool to study ligand–receptor interactions in situ (Flanagan and Leder, 1990; Tartaglia et al., 1995; Flanagan et al., 2000; Flanagan and Cheng, 2000; Liu et al., 2002). The human placental enzyme used as an AP tag has several advantages. (1) Due to high heat stability the enzyme survives heat inactivation to eliminate background phosphatase activity of a sample. (2) A high turnover number (Kcat) allows sensitive detection. (3) Due to the intrinsic enzymatic activity it is neither necessary to purify the enzyme, nor to chemically label it or to use secondary reagents such as antibodies. (4) The AP gene has been modified into a secreted form to facilitate production. (5) A wide variety of substrates is available for AP detection and quantification.

In this article, we have taken advantage of this sensitive probe to expand and deepen our previous approach with 125I-labeled peptides. We study the in vivo targets sites of the Sps and compare their binding properties. We provide evidence that SP is probably the key player in eliciting the two postmating responses. Furthermore, we show that SP interacts with two different molecular targets, very likely corresponding to the receptor of SP in the nervous system and a peptide transporter in the genital tract of the female, respectively.

MATERIALS AND METHODS

Fly Stocks and Maintenance

Flies were reared on standard yeast-cornmeal-agar medium in a 12-h/12-h light/dark photoperiod at 25°C. To obtain large numbers of virgins, females of the genotype y cm SxpM1; virD1 bw/SM5 were crossed to Oregon R wild-type males. This cross generates only females (Hilfiker et al., 1995). Females lacking the SM5 balancer were collected on ice to immobilize the flies and avoid the ether shock. Fifty to 60 females per bottle were kept at 25°C for 5 days. Fresh fly food was provided every 2 days.

Injection and Ovulation Test

Peptide injections were done according to Soller et al. (1997). Flies were anesthesized with CO2, chilled on ice, and injected with 50 nL Ringer solution containing 3 pmol peptide. For the ovulation test, 20–30 flies were kept at 25°C after injection. The ovulation rates were measured at different time points after the injection of the peptides. To check for ovulation the females were chilled on ice. Most of the females with an egg in the uterus extrude it spontaneously. Females that did not spontaneously extrude an egg were examined for the presence of an egg by gently squeezing the tip of the abdomen with forceps. Ovulation was expressed as percentage of females extruding an egg from the uterus.

Construction and Expression of Fusion Proteins

To produce different AP–SP, AP–DUP99B, SP–AP, and AP–Acp26Aa fusion constructs, cDNA sequences of SP (Styger, 1992), fragments thereof, DUP99B (Staudan et al., 2002), and Acp26Aa were amplified by PCR (Ding, 2002). To eliminate the S–S disulfide bridge of SP, the Cys codon (36th amino acid) was changed into an Ala codon. In the AP–SP and the AP–DUP99B fusion constructs, BglII and XbaI restriction sites were introduced at the ends of the inserts by PCR. After enzyme digestion, the PCR products were inserted into the BglIII and XbaI sites of APtag-4 (Flanagan et al., 2000). For the SP–AP fusion construct,
HindIII and BgIII restriction sites were added at the end of the insert by PCR. After enzyme digestion, the PCR product was ligated into the HindIII and BgIII sites of APtag-2 (Flanagan et al., 2000). Correct insertion was confirmed by sequencing.

Each plasmid was transiently transfected into COS-7 cells (4 μg per 100 mm plate) using Lipofectamine (Gibco BRL). After transfection cells were grown to confluence in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum and after another medium change grown further for 4 days. Then the cells were sedimented by centrifugation, the supernatant filtered through a 0.45-μm filter (Schleicher and Schuell), and stored at 4°C with 20 mM HEPES (pH 7.0) and 0.05% sodium azide. Alkaline phosphatase activity in conditioned medium was assayed as described by Flanagan and Leder (1990).

**Incubation of Cryostat Sections with Fusion Proteins**

Cryostat sections and incubations with the probes were carried out according to Ottiger et al. (2000). Briefly, after preincubation with incubation buffer (50 mM Tris-HCl pH 7.4, 120 mM NaCl, 5 mg/mL BSA) for 20 min at room temperature, excess buffer was removed, then the slides were incubated with probes of different concentrations (in incubation buffer) for 2 h. The sections were fixed in 3% formaldehyde for 1 min and washed twice in incubation buffer. The sections were then heated in a 65°C water bath for 20 min to inactivate endogenous alkaline phosphatase activity. After rinsing briefly with AP staining buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂), the sections were stained for about 30–40 min in the same buffer containing 0.17 mg/mL BCIP, 0.33 mg/mL NBT, and 10 mM homoarginine.

**Calculation of the Dissociation Constant Kd**

The signal intensities of the sections of the antennal nerve (as representative for the labeling of the nervous system) and the uterus (as representative for the labelling of the genital tract), respectively, were measured using a NIH image program on a Macintosh computer according to Ottiger et al. (2000). The value of the signal intensity is calculated as average signal intensity per pixel. To eliminate background interference, an average value of the background intensity was deduced from the signal intensities. At least three different samples were measured for each concentration. The saturation curves and the Scatchard-plots were done with the PRISM program (www.graphpad.com). This Web site contains a tutorial for GraphPad Prism. The instructions given for the analysis of data obtained from saturation binding experiments, and the transformation of the data into a Scatchard-plots were followed in detail.

**Affinity Blots**

The procedure is similar to the Far-Western approach for detecting protein–protein interactions (Blackwood and Eisenman, 1991). Briefly, the following steps were followed. (1) Solubilization of membrane proteins. The whole fly, or different parts of the body, were homogenized in a glass homogenizer in 50 nM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM PMSF, protease inhibitor cocktail (Roche; =homogenization buffer), and centrifuged at high speed (>10,000 × g) at 4°C for 1 h. The supernatant (=cytosolic extract) was collected. One percent Triton X-100 in homogenization buffer was added to the pellet (=crude membrane extract) and left on ice for 20–30 min to solubilize the membrane extract. Afterwards the sample was centrifuged again at low speed (1000 × g) to clarify the membrane extract from debris or particles. (2) Native-PAGE. Native PAGE sample buffer (without SDS and mercaptoethanol) was added to the extracts. Six percent native-PAGE gels were prepared and the samples loaded onto the gel. To make sure that the amounts of extracts loaded were the same, only samples from the same preparation were used on the same gel (approximately 20–30 flies per well). (3) Blotting. After running the gel in a Mini-PROTEAN II (BIO-RAD) system, the proteins were transferred onto Nitrocellulose membranes using the semidry method (LKB, NOVABLOT). (4) Incubation and staining. After blocking the membranes with 5% nonfat milk overnight at 4°C, the membranes were incubated with the AP–SP probe for about 1.5 h. Then the membranes were briefly washed once in Tris-HCl buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl), followed by another washing in the same buffer for 2 min. The substrates NBT/BCIP were added to the membranes to develop the color reaction, with positive staining appearing after about 30 min. False positives appeared only several hours later.

**RESULTS**

**Biological Activity of the Alkaline Phosphatase Tagged Probes**

The first set of experiments was performed to demonstrate (1) the biological activity of the alkaline phosphatase tagged probes, and (2) to show that the nature of the tags (125I or alkaline phosphatase, respectively) did not affect the labeling pattern obtained on cryostat sections. The binding patterns of iodinated SP and DUP99B to cryostat sections of females are almost identical (Ottiger et al., 2000). Competition experiments with full-size peptides and peptide fragments showed that the peptides bind with their homologous C-terminal regions. Thus, it is not surprising that both peptides interact with the same putative targets. The C-terminal part of SP is also essential for biological activity (Schmidt et al., 1993). Even when SP is fused to a big
molecular tag (protein A, GST, or GFP) it still keeps its biological activity as long as the C-terminus is intact and left free to interact with binding proteins (Schmidt et al., 1993; Peyre, 2001; P. Saudan, Y. Choffat, and E. Kubli, unpublished). Partial biological activity of AP–SP and AP–DUP99B fusion proteins was also observed after injection into virgin females (Ding, 2002). The fact that we obtain only partial activity is very likely due to the partial insolubility of the purified fusion proteins used for these assays (incubations on cryostat sections are done with the supernatants containing unpurified fusion proteins). Thus, addition of an AP tag to the peptides does not interfere with biological activity, a prerequisite to obtain meaningful binding data.

Therefore, we assumed that the binding sites of the AP–SP or AP–DUP99B fusion proteins would be the same as those characterized in incubations with 125I-labeled peptides (Ottiger et al., 2000). This is indeed the case (Ding, 2002). Hence, below we will only document selected results. Alkaline phosphatase fused to full-size SP (AP–SP1–36) and to two different fragments of SP (AP–SP8–36, and AP–SP11–36) used for incubation of cryostat sections yielded the same results as 125I-labeled peptides (results not shown; Ding, 2002). AP–SP1–36 (full-length SP) produced a higher background, probably due to the hydrophobic part of the N-terminal sequence of SP (as also observed with the full-size 125I-labeled SP, Ottiger et al., 2000). The AP tag itself does not bind to any of these sites [results not shown; Ding, 2002; see also Fig. 5(B)]. We also repeated the experiments at different developmental stages. Figure 1(A)–(H) shows that the fusion protein AP–SP8–36 reproduces exactly the results obtained with 125I-labeled DUP99B (Ottiger et al., 2000). Labeling of the antennal nerve [Fig. 1(A)–(D)] starts only after hatching from the pupa and is fully established 72 h after eclosion. Labeling of the genital tract [Fig. 1(E)–(H)] starts already in the last pupal stage P15. These results not only confirm that the labeling pattern is the same, but also that the relative intensity of the labeling with AP fusion proteins is identical to the labeling with iodinated peptides. For a summary of the binding sites of the two peptides we therefore refer to Ottiger et al. (2000). These results show that the presence of an AP tag does not influence the activity and binding properties of SP and its fragments.

**In Vivo Target Sites of Sex-Peptides**

Binding patterns of Sps resulting from incubations of cryostat sections of sexually mature virgin females does not necessarily reflect the in vivo situation, i.e., the target sites available after a normal copulation in a mated female. Sex-peptides enter the female via the genital tract and have to enter the hemolymph to attain putative targets of the nervous system. Although the two postmating responses can be elicited by either injecting SP into the hemolymph (Schmidt et al., 1993), or by expressing SP ectopically in the fat body of transgenic females (Aigaki et al., 1991), SP is barely detectable in the hemolymph of mated females (Ottiger et al., 2000; M. Soller and E. Kubli, unpublished results). To address the question of in vivo targets, flies were sectioned at defined time points after mating. The sections were then incubated with AP–SP probes (Fig. 2). If SP binds to the nervous system and the genital tract in vivo, these target sites should be blocked by the peptides transferred during copulation. Hence, signals should not be detectable at these sites by subsequent incubation with AP–SP probes. No signal was observed 2 and 4 h after mating in the uterus [Fig. 2(F) and (G)]. Seven hours after mating, the signal in the uterus was again at the level of the signal on sections of virgin females [Fig. 2(H); compare with Fig. 1(H)]. In the oviduct and the nervous system the pattern and the intensity of the signals corresponds to the signals obtained from virgin females [Fig. 2(B)–(D), (F)–(H)]. We conclude that at least in the uterus the binding sites determined by our approach reflect the in vivo binding sites of the Sps. We can not exclude, however, that other sites may bind Sps in vivo as well.

**The Binding Properties of Sex-Peptide and DUP99B Suggest That Sex-Peptide Is More Important In Vivo**

Sex-Peptide and DUP99B differ in their N-terminal sequences and show high similarity in the C-terminal parts of the mature peptides (Saudan et al., 2002). Only SP induces enhanced juvenile hormone synthesis in isolated corpora cardiaca/corpora allata complexes (Fan et al., 2000). But both peptides elicit both postmating responses when injected into virgin females. Hence, some functions in vivo may be redundant, others may not. To investigate whether SP and DUP99B play an equal role in eliciting the two postmating responses in vivo, the binding properties of AP–SP and AP–DUP99B to their targets on cryostat sections were compared. For this purpose we determined the dissociation constants (Kd) of the two peptides. Cryostat sections of the head and abdomen were incubated with a concentration series of AP–SP or AP–DUP99B probes. Signal intensities in the antennal nerve (reflecting binding to the nervous system) and the uterus (reflecting binding to the genital tract)
were measured and used to establish saturation curves. The $K_d$s were calculated from Scatchard plots (Fig. 3).

For AP–SP, the $K_d$ in the antennal nerve is 1.07 nM, the $K_d$ in the genital tract 0.64 nM, respectively [Fig. 3(A) and (B); Table 1]. For AP–DUP99B, the

Figure 1 Development of Sex-Peptide target sites. Cryostat sections of different developmental stages were incubated with 20 nM AP–SP$_{8-36}$ fusion protein. Panel (H) is magnified by a factor of 2. (A–D) Frontal sections of the head. Binding of AP–SP to the antennal nerve (arrow) at different developmental stages after eclosion from the pupa. Nonspecific binding is found in the compound eyes. (A) Eighteen hours after adult eclosion; (B) 24 h after adult eclosion; (C) 48 h after adult eclosion; (D) 72 h after adult eclosion. (E–H) Sagittal sections through the abdomen. Binding to the uterus (arrow) at different developmental stages. Anterior is left, top is dorsal. (E) Early pupa, uterus is not formed at this stage; (F) pupal stage 15; (G) 0 h after adult eclosion; (H) 5 h after adult eclosion.
**Figure 2** *In vivo* target sites of Sex-Peptide. Sagittal sections through mated females. (A) and (E) Scheme of the sagittal sections through head and thorax (A) and abdomen (E). Arrows point to brain and subesophageal ganglion (B), thoracic ganglion (T), oviduct (O), and uterus (U). (B) and (F) Incubation of female sections prepared 2 h after copulation with 20 nM AP–SP₈–₃₆. (C) and (G) Incubation of female sections prepared 4 h after copulation with 20 nM AP–SP₈–₃₆. (D) and (H) Incubation of female sections prepared 7 h after copulation with 20 nM AP–SP₈–₃₆.
$K_d$ in the genital tract is 14.65 nM [Fig. 3(C), Table 1]. We could not determine the $K_d$ of DUP99B with the AP–DUP99B probe in the nervous system, because we did not obtain the highest concentrations of DUP99B fusion protein necessary for this experiment with the transfection technique. Thus, the $K_{d,SP}$ for AP–SP in the two examined tissues differ by a factor of about 2. The $K_{d,SP}$ of AP–SP and AP–DUP99B in the genital tract differ by a factor of about 20.

As an alternative, semiquantitative approach to study the binding properties of the two Sps, we determined the minimal concentrations of fusion proteins needed to obtain a visible signal (Table 1). For AP–SP, we obtained values of 0.5–1 nM for the genital tract and 1–2 nM for the nervous system, respectively. The corresponding values for AP–DUP99B were 5–10 nM for the genital tract, and 30–40 nM for the nervous system, respectively. Thus, the minimal concentrations needed to obtain a signal in the genital tract are lower than for the nervous system. This correlates well with the data obtained for the $K_d$ of the two peptides (Table 1). These findings indicate that the targets of the Sps may be different. Furthermore, the figures obtained for binding of AP–SP and DUP99B to the genital tract and of the two peptides to the nervous tissue differ by a factor of 10 and 20, respectively. This correlates with the finding that in the genital tract the $K_{d,SP}$ of the two peptides differ by a factor of about 20. Hence, the two methods yield
comparable results in terms of the relative binding properties of the fusion proteins.

To further investigate the in vivo function of the two peptides, we compared time-dependent ovulation after a normal mating with the response obtained after injection of SP or DUP99B, respectively (Fig. 4). Comparison of the form of the three response curves together shows that they differ significantly (Probit-analysis. Chi-squared parallelism test $H_1 = 163.474; df = 2; p < .001$). An analogous comparison of curves A or B with C, respectively, also reveals statistically significant differences (A with C: chi-squared $= 69.113; df = 1; p < .001$; B with C: chi-squared $= 103.567; df = 1; p < .001$). In contrast, no statistical difference is found if the form of curve A is compared with that of curve B (chi-squared $= 0.000; df = 1; p > .2$). However, the time points of initiation of ovulation, as measured by the time position of the sigmoid inflexion points, are significantly different for A and B, and B and C, respectively (analysis of variance. A and B: $p = .0004, t = 3.11$; B and C: $p = .0032, t = 2.48$).

Visual inspection of the response curves of a normal mating and a SP injection reveals that both reach their maximum in a short time interval, between 50 to 90 min after the response starts. However, for DUP99B it takes almost 3 h to reach the full response. Thus, when injected, SP seems to be more efficient than DUP99B in eliciting the ovulation response. Furthermore, injection seems to shift the initiation of ovulation by about 1 h. This latter finding may reflect the fact that in vivo the peptides are first transferred into the genital tract. Initiation of ovulation may only be possible once the peptides reach their putative targets in the nervous system, and they might be accessible only via hemolymph. Based on this bioassay we conclude that Sex-Peptide is the more efficient peptide, and, thus, very likely also the more important peptide in vivo.

### Table 1 $K_d$s and Minimal Concentrations of Fusion Proteins

<table>
<thead>
<tr>
<th>Fusion Proteins</th>
<th>Genital Tract</th>
<th>Nervous System</th>
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<tbody>
<tr>
<td>$K_d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-SP$_{1-36}$</td>
<td>0.64 nmol</td>
<td>1.07 nmol</td>
</tr>
<tr>
<td>AP-DUP99B</td>
<td>14.65 nmol</td>
<td>(6.4 nmol)</td>
</tr>
<tr>
<td>Minimal conc. for binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP-SP$_{1-36}$</td>
<td>0.5–1 nmol/l</td>
<td>1–2 nmol/l</td>
</tr>
<tr>
<td>AP-DUP99B</td>
<td>5–10 nmol/l</td>
<td>30–40 nmol/l</td>
</tr>
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</table>

First two lines: dissociation constants ($K_d$) for binding of AP-SP to antennal nerve and genital tract, of AP-DUP99B to the genital tract, and, in brackets, of $^{125}$-labeled DUP99B to the antennal nerve (Ottiger et al., 2000).

Third and forth lines: Minimal concentrations needed for binding of AP-SP$_{1-36}$ or AP-DUP99B, respectively, to the genital tract and the nervous system. Minimal concentrations were determined by calculation of the signal intensities in the genital tract and the nervous system. The minimal concentrations refer to the average signal intensity below 50 as calculated with the NIH image program.

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**Figure 4** Ovulation at different time points after mating, SP injection, and native DUP99B injection, respectively (native DUP99B is glycosylated, synthetic is not). Data represent mean value ± S.D. calculated from three experiments (20 flies per experiment). Three pmol peptide was injected per fly. Left arrows indicate the start of the response. Right arrows indicate maximal response. Grey bar = time to build up the response. Statistical analysis: sigmoid curve fitted by SPSS (SPSS-Incorporation, 1988, 1990a, 1990b). (A) Ovulation response curve after mating. (B) Ovulation response curve after injection of SP. The data points at 120’ and 130’ are each based on one experiment. Hence, they have not been incorporated into the curve fitting. (C) Ovulation response curve after injection of native DUP99B.
SP Interacts with Two Different Molecular Targets

The differential binding of the Sex-peptides to the nervous system and to the genital tract, respectively, suggests that the two target sites may be different (Fig. 3, Table 1). The following results support this hypothesis.

We observed that these two target sites appear at different developmental stages (Ottiger et al., 2000, and this article). Incubation of AP–SP probes on sections prepared from different developmental stages showed that the target site in the uterus appears at the last pupal stage P15, i.e., before hatching [Fig. 1(E)–(H)]. At this time the development of the uterus is completed (Demerc, 1965). However, the target sites in the nervous system develop only after hatching, and are fully developed only on the second day after adult eclosion [Fig. 1(A)–(D); Ottiger et al., 2000]. Thus, the signal in the nervous system appears between 24 and 48 h after adult eclosion. This is the time when females become sexually mature, i.e., there exists an exact correlation between the appearance of the binding sites in the nervous system and the full responsiveness of the female to SP injection (Fleischmann, 1991; Moshitzky et al., 1996).

Furthermore, fragments of SP bind differentially to the targets in the nervous system and the genital tract. However, AP–SP$_{1–36}$A (C-terminal Cys replaced by Ala), AP–SP$_{21–36}$, AP–SP$_{25–36}$, AP–SP$_{8–23}$, and SP–AP fusion proteins bind only to the genital tract and not to the nervous system (Table 2). The first series of fusion proteins contains full size SP or SP fragments that are either active as synthetic SP fragments (Schmidt et al., 1993) or as fusion proteins (see above). The second series contains SP fragments that have been shown to be inactive as synthetic SP fragments (Schmidt et al., 1993) or as fusion proteins (see above). Taken together, these results suggest that the targets in the nervous system and in the genital tract are different.

This interpretation was further confirmed by a biochemical approach. Membrane and cytosolic extracts were prepared from head plus thorax and abdomen, respectively, of adult females. The extracts were separated on nondenaturing PAGE, transferred to nitrocellulose filters, and probed with AP–SP. On the affinity blots, two different bands show up in the membrane extracts prepared from the head plus thorax and the abdomen fractions, respectively (Fig. 5). The two binding proteins are molecularly different, but because the gel was nondenaturing we cannot tell whether this is based on different protein sequences or on protein modification, or both. Under denaturing conditions this approach does not yield any signals (results not shown). This latter finding indicates that an appropriate conformation of the binding proteins is needed to bind the ligand.

Taken together, these results demonstrate that in the adult fly SP binds to two molecularly different proteins. Because head and thorax show labeling of

### Table 2 Binding of AP-SP, SP-AP, and SP-Acp 26Aa Fusion Proteins to the Genital Tract and Nervous System

<table>
<thead>
<tr>
<th>Fusion Proteins</th>
<th>Binding on Genital Tract</th>
<th>Binding on Nervous System</th>
<th>Biological Activity of Synthetic Peptide Fragments</th>
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<tbody>
<tr>
<td>AP-SP$_{1–36}$</td>
<td>++ + + +</td>
<td>++ + + +</td>
<td>Schmidt et al. (1993)</td>
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<tr>
<td>AP-SP$_{8–36}$</td>
<td>++ + + +</td>
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<tr>
<td>AP-SP$_{11–36}$</td>
<td>++ + + +</td>
<td>++ + + +</td>
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<tr>
<td>AP-SP$_{1–36A}$</td>
<td>++ + + +</td>
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<tr>
<td>AP-SP$_{21–36}$</td>
<td>++ + + +</td>
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<tr>
<td>AP-SP$_{8–23}$</td>
<td>++ +</td>
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<tr>
<td>AP-SP$_{25–36}$</td>
<td>++ +</td>
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<tr>
<td>SP$_{1–36}-AP$</td>
<td>++ +</td>
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<tr>
<td>AP-Acp26Aa</td>
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</table>

The rightmost lane indicates the biological activity of synthetic SP and fragments thereof (data taken from Schmidt et al., 1993). Binding to the nervous system is only possible with biologically active peptides.

++ + + + = strongest signal, signal intensity is over 200. ++ = signal intensity between 100–150. + = signal intensity below 50. Signal intensities were calculated with the NIH program. SP$_{1–36A}$: 36th amino acid Cysteine is changed into an Alanine. SP-AP: C-terminal part of SP has been fused to the N-terminal part of AP. Two different fragments of Acp26Aa were used. They generated the same result; hence, Acp26Aa refers to both fragments: 1–264 (full length), and 118–264.
the nervous tissue exclusively, and the abdomen mainly labeling of the oviduct and uterus (Ottiger et al., 2000; Fig. 2(B)–(D) and Fig. 1(E)–(H)), we conclude that the binding protein of the nervous system is different from the binding protein of the genital tract. Furthermore, binding to the former is more stringent in terms of sequence requirements than binding to the latter.

DISCUSSION

In Vivo Binding Sites

In D. melanogaster females Sex-peptides elicit two major responses: receptivity is reduced and egg laying increases. Control of receptivity and egg laying are very likely achieved via the nervous system. These results are in accord with our findings that $^{125}$I-iodinated Sps and alkaline phosphatase-tagged peptides label specific parts of the nervous system and the female genital tract (Ottiger et al., 2000; this article; for a discussion of binding sites and function of Sps, see Ottiger et al., 2000). We believe that our approach has identified the presence of Sp-binding proteins in adult females for the following reasons. (1) Strong binding is only observed with fusion proteins containing a SP sequence that has been shown to be biologically active as a peptide fragment or as a fusion protein (Table 2; Schmidt et al., 1993; Peyre, 2001; P. Saudan, Y. Choffat, and E. Kubli, unpublished results). (2) The calculated dissociation constants ($K_d$, Table 1) are in the order of magnitude expected for hormone–receptor interactions (Merckaert and Vandesande, 1996; Cicutti et al., 1999). (3) Both probes label the same sites (Figs. 1 and 2; Ottiger et al., 2000; Ding, 2002). (4) The appearance of the binding proteins during development in the nervous system and the genital tract is independent of the labeling method of the probe.

The binding sites observed on cryostat tissue sections of virgin females do not necessarily reflect the sites of interaction of the ligand with functional proteins in vivo. By incubating sections of mated females with the AP–SP probe, we have tried to identify the in vivo targets of Sps after mating (Fig. 2). We found that only the uterus was blocked by previously transferred peptides [Fig. 2(F) and (G)], Neither the target sites in the upper part of the genital tract nor those in the nervous system were blocked [Fig. 2(B)–(D), (F)–(H)]. Thus, from these results one might conclude that only the uterus is an in vivo target. As explained below, we believe, at least for the labeling of the nervous system, that this is not the case. Both SP and DUP99B are transferred in picomolar amounts to the female during copulation (Chen et al., 1988; A. Rexhepaj, J. Peng, and E. Kubli, unpublished results). However, the amount of SP in the hemolymph is very small and barely detectable by Western blotting (in the femtomolar range; M. Soller and E. Kubli, unpublished results). This is very likely due to rapid uptake of SP by the pericardial cells (Peyre, 2001) resulting in a low concentration of SP in the hemolymph. It is reasonable to assume that these small amounts of peptide can only partially block the many target sites accessible via hemolymph. Hence, most binding sites in the nervous system may still be available for binding. Only the first contact site of the peptides after mating, the uterus, was blocked by the transferred peptides, and this only for the first few hours [Fig. 2(F) and (G)]. Most of the transferred peptides are lost from the genital tract by the expulsion of the first egg. The concentration of the peptides in the female decreases drastically within the first 2 h after mating (M. Soller and E. Kubli, unpublished results). Because
binding of the two peptides occurs with the almost identical C-terminal parts, we cannot determine the proportion of SP and/or DUP99B binding to the uterus with this approach.

Using $^{125}$I-DUP99B, Ottiger et al. (2000) had found no differences in the binding patterns of virgin and mated females. This discrepancy is very likely due to the different time points chosen for analysis. Seven hours after mating the uterus may not be saturated any more by the previously transferred peptides, because, as shown by the experiments reported in this article, the uterus is fully labeled again [Fig. 2(H)]. Another possible reason may be that a radioactively labeled DUP99B probe was used for the experiments of Ottiger et al. (2000), whereas in our experiments we used AP–SP fusion protein. Alkaline phosphatase-tagged SP and DUP99B peptides show differences in their binding affinities (Fig. 3 and Table 1). The higher affinity of SP may block the binding of DUP99B.

Are Sex-Peptide and DUP99B Functionally Redundant?

The two Sex-peptides differ partially in their amino acid sequence. Whereas the amino acid sequences encoded by the first exons are different, the sequences encoded by the second exons are almost identical (Saudan et al., 2002). Thus, one would expect functional redundancy for the C-terminal parts of the peptides, but not for the N-terminal parts. Indeed Sex-Peptide stimulates juvenile hormone synthesis in isolated corpora cardiaca/corpora allata complexes, whereas DUP99B does not (Moshitzky et al., 1996; Fan et al., 2000). It has been shown that the N-terminal amino acids of SP are responsible for this stimulation (Fan et al., 2000). The almost identical C-terminal parts are known to be responsible for eliciting the two postmating responses (Schmidt et al., 1993; this article). Thus, they seem to be functionally redundant. Nevertheless, the import of the two peptides in inducing the two responses in vivo may not be the same.

First, the $K_\text{d}$ of the two peptides indicate that SP has an about 20 times higher binding affinity than DUP99B in the genital tract (Fig. 3 and Table 1). For the nervous system we can only compare $K_\text{d}$ values obtained with two different labelling procedures. Hence, the data have to be interpreted with caution. For AP–SP, the $K_\text{d}$ in the nervous system is 1.07 nM, for $^{125}$I-labeled DUP99B 6.4 nM, respectively (Fig. 3 and Table 1; Ottiger et al., 2000). We also compared the minimum concentrations needed to obtain a signal (Table 1). They are about 10 times higher for DUP99B in the genital tract, and 20 times higher in the nervous system, respectively. Hence, by comparing the $K_\text{d}$ data obtained with the different labeling procedures, we may underestimate the differences in the affinity of the two peptides to the binding protein of the nervous system. Thus, our data show that SP has a higher binding affinity than DUP99B in the nervous system and in the genital tract.

This interpretation is also supported by the ovulation response measured at different time points after mating, SP, or DUP99B injections, respectively (Fig. 4). For this experiment we used native, i.e., glycosylated DUP99B. In comparison with synthetic, unglycosylated DUP99B, less native DUP99B is needed to elicit the postmating responses (Saudan et al., 2002). The glycosyl group could increase the stability and/or the affinity of the native peptide for its receptor. Nevertheless, with native DUP99B the time span needed to obtain a maximal response of ovulation is about twice the time span observed after a normal mating or a SP injection (Fig. 4). These results suggest that the efficiency of DUP99B in inducing the postmating responses is lower than that of SP. Taken together, our results suggest that SP is the key player in eliciting the two postmating responses.

Two Molecular Targets

Although the differences in the binding properties to the nervous system and the genital tract of each of both peptides are only two- to fourfold (Table 1), this is a first indication that there may exist two molecular types of binding proteins. The other results reported in this article confirm this hypothesis. The sequence requirements for binding to the two tissues are also different (Table 2). Whereas an intact C-terminal part is needed for binding to the nervous system, binding to the genital tract is less stringent. Thus, the properties for binding to the nervous system are identical to the properties for eliciting the two postmating responses (Schmidt et al., 1993). Furthermore, these results indicate that the $K_\text{d}$s and the minimal concentrations needed for binding as determined for the antennal nerve and the uterus, reflect the binding properties for the whole nervous system or the whole genital tract, respectively.

Previous experiments had suggested that SP might act via one type of molecular receptor located at one, or at several, target sites to elicit the two postmating responses (Kubli, 1992, 1996; Schmidt et al., 1993; Nakayama et al., 1997). The results discussed above suggest that this putative receptor may be localized in the nervous system. The binding sites in the nervous system appear between 24 and 48 h after eclosion.
This is consistent with the finding that the SP response cascade is also established after eclosion (Moshitzky et al., 1996). Ovulation can only be induced by SP injection 38 h after eclosion, although mature eggs are present and are eventually laid after one day. Sex-peptide injections reduce receptivity fully only 72 h after eclosion. Although binding in the genital tract can be detected already at the pupal stage P15, these proteins are either nonfunctional in terms of eliciting the postmating responses immediately after hatching, or serve another function, for example, transport of the peptides into the hemolymph. Seminal fluid proteins can enter the hemolymph by crossing the posterior vaginal wall (Lung and Wolfner, 1999). Thus, binding of the peptides to the genital tract may reflect binding to a transport protein. The fact that peptides that are unable to elicit the postmating responses nevertheless bind to the genital tract, further supports this conclusion. However, we cannot exclude that these proteins might be involved in the postmating responses, for example, via a myotropic effect stimulating egg release from the ovary (Yi and Gillott, 1999; Heifetz et al., 2000). In our bioassays the peptides are injected into the abdomen and, thus, the peptides may not reach the binding proteins in the uterus and the oviduct (injections into the vagina are not feasible for technical reasons).

Affinity blotting indeed demonstrates that SP binds to two molecularly different membrane proteins located in the abdomen and head plus thorax extracts (Fig. 5). Efforts have been made to use stage P15 pupae and newly eclosed adults as sources for extracts for affinity blotting. However, the high alkaline phosphatase background in the samples did not allow the detection of specific bands (data not shown). Because all the binding sites in the head and thorax are localized either on the central or peripheral nervous system, we conclude that nervous tissue contains only one molecular type of binding protein. In the abdomen, most of the binding is on the oviduct and the uterus. Thus, these reproductive tissues contain very likely the other molecular type of binding protein. In sum, we suggest that the binding protein of the genital tract is a transporter responsible for an efficient transport of the peptides into the hemolymph from where they can reach their targets (Kubli, 1996). The binding in the nervous system, however, reflects the localization of the receptor for the two peptides residing at the top of a signalling cascade eventually leading to the two postmating responses.

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