Supporting Information

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SI Results

Identification of SPR Ligands from *A. aegypti*. To search for SPR ligands from *A. aegypti*, we prepared whole-body extracts of *Aedes* (10 g) with acidified methanol, and subsequently subjected them to chromatographic fractionation using an analytical C4-reversed-phase HPLC (Fig. S4*A*). HPLC fractions were tested for the presence of SPR agonist activity using the Ca^{2+} assay with the *Aedes* SPR. Fifty HPLC fractions were analyzed and at least eight of these fractions showed significant agonist activity (Fig. S4*B*). As a negative control, the same fractions were tested with cells lacking SPR. This control tests for any SPR-independent activation, as might be caused for example by ATP acting on the endogenous P2-purinoreceptor of CHO cells (1). None of the tested fractions generated SPR-independent Ca^{2+} activation.

We wondered whether any of the fractions containing SPR agonists might contain members of the MIP family. As a first attempt to address this, we further purified the most active fractions by RP-HPLC using phenyl analytical and C18 narrow bore columns and analyzed fractions by MALDI-TOF mass spectrometry. No masses corresponding to known MIPs were identified, possibly due to the low abundance of these peptides in whole body extracts. To test whether the active fractions might nonetheless contain MIPs, we synthesized *Aedes* MIP peptides and analyzed them using the same HPLC protocol (Fig. S4*A*). *Aedes* MIPs 2 and 5, 1, and 4 had retention times corresponding to active fractions 17, 18, and 26, respectively (Fig. S4 *A* and *B*), suggesting that the activity in those HPLC fractions might be due to these peptides. In contrast, active fractions 21, 41, 45, 46, and 50 do not correspond to the elution positions of MIPs, raising the possibility of the third class of ligand for SPR, distinct from both SPs and MIPs. However, it is also equally possible that these SPR agonist activities come from partially processed peptides from the precursor and/or breakdown products of mature MIPs, because their molecular nature is not presently known.

The most potent fraction (#26), which corresponds to the elution position of MIP4, was subjected to further fractionation by RP-HPLC. After separation using an analytical phenyl column, biological activity eluted in a single fraction, which was then fractionated further using a narrow bore C_{18} column (Fig. S4*C*). Agonist activity eluted in fraction 41, which did not correspond to the elution position of MIP4 (Fig. S4*C*), nor any of the other known *Aedes* MIPs. This indicates that the most potent activity in *Aedes* whole body extracts may not be due to any of the known MIPs but possibly to another ligand.

SI Materials and Methods

Isolation of SPR Agonist Activities from *Aedes* Whole Body Extracts. Acidic methanol extracts of whole body *A. aegypti* were fractionated by RP-HPLC using a Jupiter C_4 10-μm 300-Å analytical column (250 mm × 4.6 mm i.d.; Phenomenex) with a linear gradient of 5–60% acetonitrile/0.1% TFA, over 55 min at a flow rate of 1 mL/min. Fractions (1 mL) were collected, and lyophilized for bioassay or mass analysis. Biological active fractions were further fractionated by RP-HPLC using a Brownlee aquapore phenyl 7-μm 300-Å analytical column (220 × 4.6 mm i.d.; Applied Biosystems), using the same condition as above, followed by separation on a Jupiter C_{18} 10-μm 300-Å narrow bore column (250 mm × 2.1 mm i.d.; Phenomenex) at a flow rate of 0.2 mL/min. Elution positions of synthetic MIPs were determined using similar columns and conditions.

1. Iredale PA, Hill SJ (1993) Increases in intracellular calcium via activation of an endogenous P2-purinoceptor in cultured CHO-K1 cells. *Br J Pharmacol* 110:1305–1310.

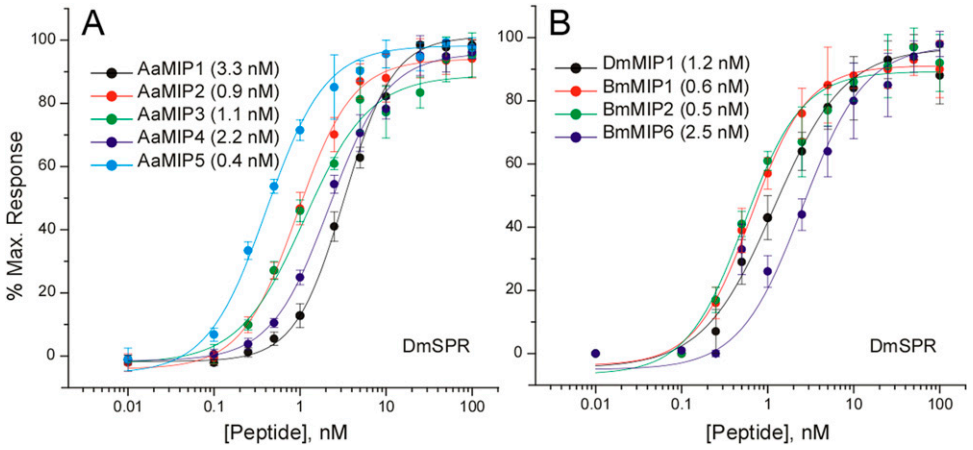

Fig. S1. Agonist activities of (*A*) *Aedes* and (*B*) *Bombyx* MIPs on *Drosophila* SPR. Dose-response curves were generated by treating varying concentrations of MIPs on CHO cells expressing *Drosophila* SPR, aequorin, and a chimeric G protein, $G\alpha_{qi}$. Numbers in parentheses indicate EC_{50} values. Each data point is mean ± SEM (n = 4).

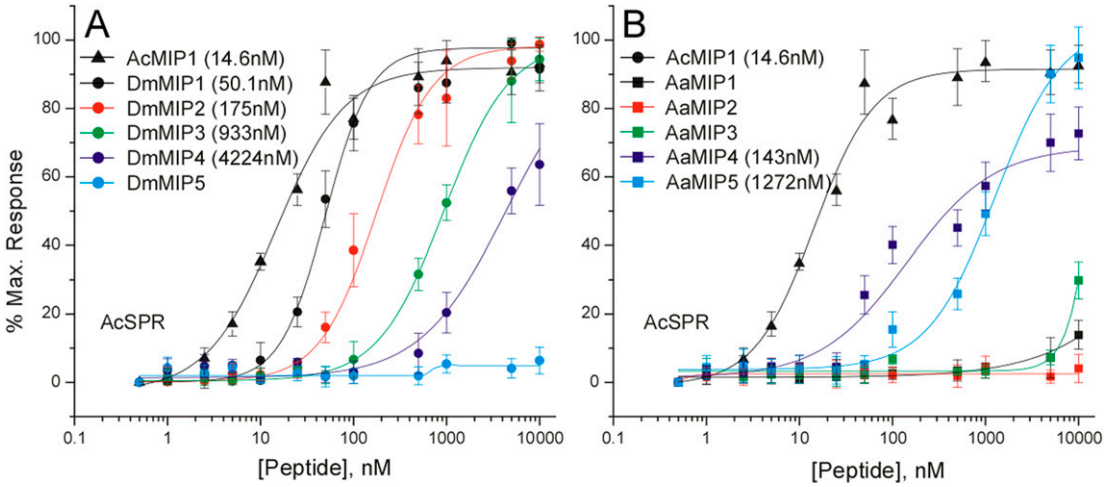

Fig. S2. Agonist activities of (*A*) *Drosophila* and (*B*) *Aedes* MIPs on *Aplysia* SPR. Dose-response curves were generated by treating varying concentrations of MIPs on CHO cells expressing *Aplysia* SPR, aequorin, and a chimeric G protein, $G\alpha_{qi}$. Numbers in parentheses indicate EC_{50} values. Each data point is mean ± SEM ($n = 4$).

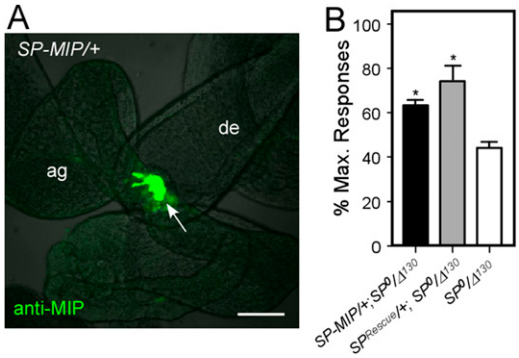

Fig. S3. Functional MIPs are expressed in the male reproductive organs of *SP-MIP* transgenics. (*A*) Anti-MIP staining of the genital tract of a *SP-MIP* male reveals strong immunoreactivity at the junction of the accessory glands and ejaculatory duct (arrow). (Scale bar, 100 μm.) Ag, accessory gland, de, ejaculatory duct. (*B*) SPR agonist activities of crude extracts prepared from the male genital tract (testes, accessory glands and ejaculatory duct) of genotypes tested in Fig. 6. Extracts from *SP* null mutants carrying SP-MIP or SP rescue transgenes have significantly higher levels of SPR agonist activities than extracts from *SP* null mutants. Each data point was generated by treating 0.5 fly-equivalents on CHO cells expressing *Drosophila* SPR, aequorin and a chimeric G protein, $G\alpha_{qi}$ The agonist activity in *SP* null mutant alone is due to SPR-dependent and SPR-independent activation of CHO cells caused by substances from the crude tissue extracts (for examples, Dup99B and ATP, respectively). Response from treatment of 100 nM synthetic SP was set as 100%. Each data point is mean ± SEM ($n = 3$). * $P < 0.05$ for comparison against SP^0/Δ^{130}, 1-way ANOVA.

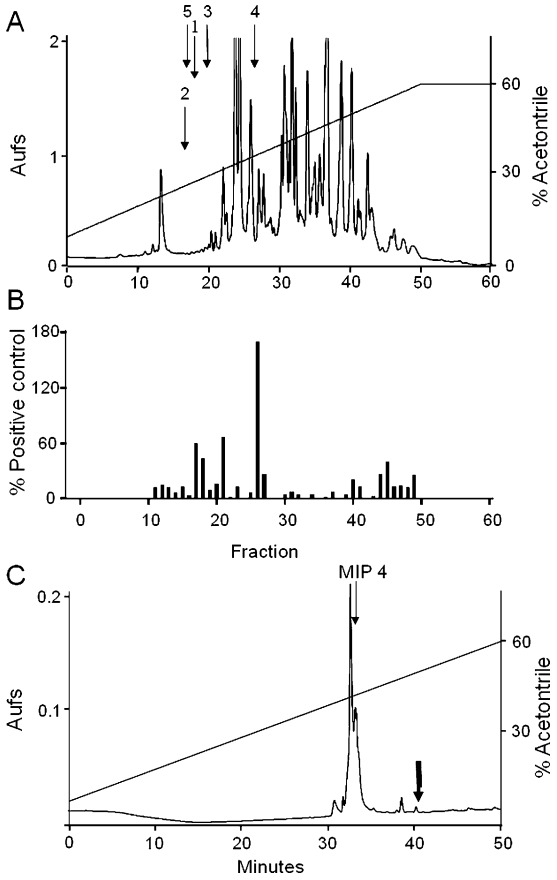

Fig. S4. Biochemical isolation of SP agonist activities from *A. aegypti* adults. (*A*) HPLC profile of an acidified methanol extract of 1 g of *A. aegypti* fractionated using an analytical C_4 reversed-phase column. Arrows indicate the elution positions of synthetic *Aedes* MIPs (AaMIP1-5) under the same HPLC conditions. (*B*) The activity of HPLC fractions on *Aedes* SPR represented as a percentage of positive controls (10 μM *Drosophila* SP). (*C*) Final fractionation, using a narrow bore C_{18} reversed-phase column, of the most potent HPLC fraction (fraction #26) from *A*. Elution position of MIP4 is shown. The thick arrow indicates the single biologically active fraction.

Table S1. SP and DUP99B precursor sequences of *Drosophila* species

Drosophila species	Precursor Sequence
pseudoobscura_SP	MKVATSAMLLLMLVEAAVGVPAWGRMTSRRP.....TPKQSQAQFQKWCRLNFGPAWGGRGC..
persimilis_SP	MKVATSAMLLLMLVEAAVGVPAWGRMTSRRP.....TPKQSQAQFQKWCRLNFGPAWGGRGC..
erecta_SP	MKAVS...LLLVLVCIVGLVQSWTWPWQKKPPV....KPPIPSPNPRDKWCRLNLGPGWGGR.C..
yakuba_SP	MNTVA...LLLVLLCIVSLVQSWTWPWQKKKP.....KPPIPSPNPRDKWCRLNLGPGWGGR.C..
simulans_SP	MKTLS...LPLVLVCLLGLVQSWEWPWNRKP.....TKYPIPSPNPRDKWCRLNLGPAWGGR.C..
mauritiana_SP	MKTLS...LPLVLVCLLGLVQSWEWPWNRKP.....TKYPIPSPNPRDKWCRLNLGPAWGGR.C..
sechellia_SP	MKTLS...VPLVLVCLLGLVQSWEWPWNRQP.....TRYPIPSPNPRDKWCRLNLGPAWGGR.C..
melanogaster_SP	MKTLA...LPLVLVCVLGLVQAWEWPWNRKP.....TKPPIPSPNPRDKWCRLNLGPAWGGR.C..
suzukii_SP	MKALI...LLLVLVWIVGLVNSWEWPWNQKKPWERPRPIPNPNPRDKWCRLNLGPAWGGR.C..
willistoni_SP	MQAPIS..ILLLVLAIVS..QSMANPNPERG.............GDKCKWCRLNLGPAYGGR.C..
virilis_SP	MQA.TFS.IIFILLSILCCSRGEYKT.TPWPR..........YPN...KWCRLNYGPYLGGR.C..
melanogaster_Dup99B	MKTPLFL.LLVVIASLLGLALSQERNDTEWIQ..........SQKDREKWCRLNLGPYLGGR.GRK
sechellia_Dup99B	MKTPLFL.LLVVIASLLGLAVSQLRNSTEWMK..........SQKDREKWCRLNLGPYLGGR.C..
simulans_Dup99B	MKTPLFL.LLVVIASLLGLAVSQLRNSPEWKK..........SQMERDKWCRLNLGPYLGGR.C..
yakuba_Dup99B	MKTQVFL.LLVVIASLLGLAVSQNRTEAERQK..........TQEEFEKWCRLGHG..EGGK.GRK

SP sequences from *D. suzukii*, *D. mauritiana*, *D. simulans*, *D. sechellia* are previously reported (1, 2). Other sequences are curated from BLAST results of fly base TBLASTN searches against *Drosophila* species genome database.

1. Schmidt T, et al. (1993) Drosophila suzukii contains a peptide homologous to the Drosophila melanogaster sex-peptide and functional in both species. *Insect Biochem Mol Biol* 23: 571–579.
2. Cirera S, Aguadé M (1997) Evolutionary history of the sex-peptide (Acp70A) gene region in Drosophila melanogaster. *Genetics* 147:189–197.

Table S2. Amino acid sequence of SP, Dup99B, and MIPs

SP and MIPs (PTSPs)	Amino acid Sequence
DmSP	WEWPWNRKPTKFPIPSPNPRDKWCRLNLGPAWGGRC
DmDUP99B	*QDRNDTEWIQSQKDREKWCRLNLGPYLGGRC
DmMIP1	AWQSLQSSW-a
DmMIP2	AWKSMNVAW-a
DmMIP3	EAQGWNKFRGAW-a
DmMIP4	EPTWNNLKGMW-a
DmMIP5	DQWQKLHGGW-a
AaMIP1	TWKNLQGGW-a
AaMIP2	AWNKINGGW-a
AaMIP3	VNAGPAQWNKFRGSW-a
AaMIP4	EPGWNNLKGLW-a
AaMIP5	SEKWNKLSSSW-a
AgMIP1	SWSKMNAAW-a
AgMIP2	LSGGNRNSGWTKFGAAW-a
AgMIP3	EPGWNNLKGLW-a
AgMIP4	ADKWDKLASAW-a
BmPTSP1	WQDLNSAW-a
BmPTSP2	GWQDLNSAW-a
BmPTSP3	APEKWAAFHGSW-a
BmPTSP4	GWNDISSVW-a
BmPTSP5	AWQDMSSAW-a
BmPTSP6	AWSALHGTW-a
BmPTSP7	SWQDLNSVW-a
BmPTSP8	WSSLHSGWA
AcMIP1	WKQMSVW-a

*Q, underlined P and underlined Cs indicate pyroglutamic acid, hydroxyproline, and disulfide bonds between Cs respectively. Note all insect MIPs share a highly conserved sequence, $W(X)_6W$-amide. Sequences of MIP (or PTSP) peptides are predicted from cDNA sequences (*D. melanogaster*, GenBank access no. CAC17604; *A. agypti*, EAT35715; *Anopheles gambiae*, EAA12136.3; *B. mori* BAB70681; *A. californica* EB246635.1).