FY 2006-2007 Report on Progress

Texas Imported Fire Ant Research And Management Project

Objective 1. Determine the Vitellogenin Receptor (VgR) localization in mated and virgin alate queens to understand Vg Receptor temporal/ spatial regulation

Aim 1: Production of a specific anti fire ant vitellogenin receptor (SiVgR) antibody

1.1.1 Selection of the *Si*VgR protein sequence for recombinant protein expression for antibody production

The sequences of low density lipoprotein receptor (LDLR), very low density lipoprotein receptor (VLDLR), LDLR-related proteins (LRP) and different regions of fire ant vitellogenin receptor (*Si*VgR) were aligned using the Clustal W computer program (http://www.ebi.ac.uk/clustalw) (Thompson *et al*., 1994). The simple modular architecture research tool (SMART) (http://smart.emblheidelberg.de) (Schultz *et al*., 1998; Letunic *et al*., 2002) was used for identification of *Si*VgR modular domains that were adjusted by eye gazing, when necessary. The accession numbers for the receptors used for multiple sequence alignment are as follows: *S. invicta* VgR (accession number AAP92450), Human LDLR (AAM56036), *Aedes aegypti* LDLR (EAT37281), Human VLDLR (BAA03946), *Drosophila melanogaster* LRP6 (AAF28358). The different modular domains of *Si*VgR, *D. melanogaster* yolk protein receptor (P98163) and *Blattella germanica* VgR (CAJ19121) were identified by SMART program and the pairwise alignment was performed with the EMBOSS Pairwise Alignment Algorithms of EBI (http://www.ebi.ac.uk/emboss/align). The DNASTAR software (DNASTAR Inc., Madison, WI) was used for assessment of the hydrophilicity and antigenicity of the fire ant Vg receptor amino acid sequence.

1.1.2 Construction of the expression vector: pET32a-VgR

A VgR fragment (encoding amino acid residues 648-887, corresponding to the second YWXD repeat region in the first EGF precursor homology domain) was amplified by PCR with the primer set SiVgR-f1 (5'-TGCGGATCCTGCCATCTGCAATTA-3') and SiVgR-r1 (5'-TGCGTCGACTTACTTTGGTA TATCATC-3'), which contain *BamH* I and *Sal* I restriction enzyme sites, respectively. PCR conditions were as follows: 400 nM of each primers; 376 ng pVgR2.3-4 plasmid (Chen, *et al*., 2004); 0.2 mM dNTPs mixture, Advantage 2™ TAQ Polymerase kit (Clontech, Mountain View, CA), and cycling conditions were: 94°C, 3 min; 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min; 72°C for 10 min. After electrophoresis analysis (1% agarose gel), a PCR product of about 740 bp was obtained, cleaned using QIAEX II Gel Extraction kit (QIAGEN, Valencia, CA) and cloned into pCR®2.1-TOPO® vector using TOPO TA cloning kit (Invitrogen™, Carlsbad, CA). Competent cells, Top10F' (Invitrogen™, Carlsbad, CA) were heat-shocked at 42°C to introduce the plasmid. Transformed colonies were selected based on blue and white screening and liquid cultured, and the plasmids were purified with a Qiaprep spin miniprep kit (Qiagen Inc., Chatsworth, CA). One µg plasmid DNA of each selected clone was digested with *EcoR* I restriction enzyme (Promega, Madison, WI) to verify the presence of insert and the plasmid was sequenced to confirm its identity. The DNA fragments were sequenced using ABI PRISM Big Dye Terminator Cycle sequencing Core kit and the sequences were obtained by the Gene Technology Laboratory (Texas A&M University, College Station, TX). The VgR fragments excised from the pCR2.1-TOPO® vector with *BamH* I and *Sal* I restriction enzymes (Promega, Madison, WI) were cloned into the *BamH* I and *Sal* I sites of the pET32a vector (Novagen, San Diego, CA) with T4 DNA ligase (Promega, Madison, WI) and transformed into Top10F′ to generated an expression plasmid **pET32a-VgR**. Colonies were selected and the plasmids were purified as above. Plasmid DNA (0.5 µg) of each selected clone was digested with *BamH* I and *Sal* I restriction enzymes to verify the

presence of insert and the plasmid was sequenced to confirm its identity.

1.1.3 Expression of the recombinant protein

E. coli strains BL21 (DE3) pLysS and BL21 (DE3) (Novagen, San Diego, CA) were heat-shocked at 42°C and transformed with the plasmid pET32a-VgR for expression of recombinant proteins following manufacturer's recommendations. Three colonies of each bacteria strain were selected and the plasmids were purified as above. Plasmid DNA (0.5 µg) of each selected clone was digested with *BamH* I and *Sal* I restriction enzymes to verify the presence of insert. The positive colonies were grown and 5 ml of overnight cultures were diluted in 100 ml LB medium containing 100 µg/ml of ampicillin, grown until OD_{600nm} reached 0.6 and split into two 50 ml cultures. IPTG (1mM) was added into one of the 50 ml cultures and the other culture was maintained as a non-induced control. After incubation at 25°C, bacterial cultures were centrifuged at 3,000 g (5,371 rpm) (C0650 rotor, BECKMAN Avanti^{TM} 30) and the pellets were sonicated in 2 ml phosphate-buffered saline (PBS: 140 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 20.4 mM Na_2HPO_4, pH 7.4) three times for 10 s each, with a 30 s pause on ice between each burst. Ten µl samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel). In a small-scale induction test, 5 ml bacterial cultures (the BL21 (DE3) strain transformed with the pET32a-VgR plasmid) were induced for 4 h with 0.1, 0.5, and 1 mM IPTG at 20°C and at 25°C and cultures were centrifuged at 10,397 g (10,000 rpm) for 20 min. After centrifugation, the pellets were re-suspended with 5 ml wash buffer and 10 µl of soluble protein in the supernatant (S) and insoluble proteins in the pellet (I) were analyzed by SDS-PAGE (10% gel).

1.1.4 Purification of the recombinant protein

For purification of the recombinant protein, bacterial culture (the BL21 (DE3) strain transformed with the pET32a-VgR) was induced with 1mM IPTG for 8 h at 20°C and 50 ml bacterial culture was centrifuged as above. The bacterial pellets were lyzed by sonication in Wash Buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) and centrifuged again at 10,397 g (10,000 rpm) for 20 min. The soluble proteins in the supernatant were purified with TALON® metal affinity resin (Clontech, Mountain View, CA) following the manufacturer's protocol and eluted with 150 mM imidazole. In order to reduce background binding to the affinity resin, bacterial cultures were lyzed with 6 M guanidine-HCl (Sigma, St. Louis, MO) in the Wash Buffer instead of Wash Buffer only. The lyzed bacterial culture was centrifuged at 10,397 g (10,000 rpm) for 20 min, and the soluble protein in the supernatant was purified in the same denaturing buffer following the manufacturer's protocol. The eluate was collected and further dialyzed at 4°C followed with decreasing concentration of guanidine-HCl from 5 to 4, 3, 2, 1.5, 1, 0.75, 0.5, and 0 M in PBS, each step for 2 h in a 10K MWCO SnakeSkin Dialysis Tubing (PIERCE, Rockford, IL). The dialyzed proteins were concentrated with a 30-kDa Amicon® Ultra-4 Centrifugal Filter (Millipore, Billerica, MA) by centrifugation at 4,000 g (SX4750 rotor, BECKMAN coulter) because the expected size was 44 kDa.

1.1.5 Removal of the protein tag by Enterokinase

In order to remove the protein tag, purified recombinant protein was digested with Tag off High Activity Enterokinase (Novagen, San Diego, CA) following the manufacturer's protocol with 0.1% Tween-20. After digestion of the purified protein (5 µg) with either 0.5U, 1U or 2U of enterokinase at 30°C for 16 h, the results were analyzed by SDS-PAGE (10% gel). This procedure failed because the tag could not be cleaved, therefore, a new construct was produced as indicated below.

1.1.6 Re-construction of the expression vector: pET28a-VgR

As the protein tag was not removed by treatment with enterokinase**,** the pET28a vector (Novagen, San Diego, CA) was used to replace the vector pET32a and re-construct the expression plasmid now

designated **pET28a-VgR**. The same VgR fragment (encoding residues 648-887) was excised from the pET32a-VgR plasmid with *BamH* I and *Sal* I restriction enzymes and cloned into the *BamH* I and *Sal* I sites of the pET28a vector with T4 DNA ligase, and transformed into Top10F′ to yield the plasmid pET28a-VgR as above. Three colonies were selected and the plasmids were purified as above. Plasmid DNA (0.5 µg) of each selected clone was digested with *BamH* I and *Sal* I restriction enzymes to verify the presence of insert and the plasmid was sequenced to confirm its identity.

1.1.7 Re-expression and purification of the recombinant protein

E. coli strain BL21 (DE3) was heat-shocked at 42°C and transformed with the plasmid pET28a-VgR for expression of the recombinant protein. Three colonies were selected and the plasmid DNA purified and digested with *BamH* I and *Sal* I restriction enzymes as above to verify the presence of insert. One of the positive colonies was grown and 5 ml of the overnight culture was diluted in 100 ml LB medium containing 30 µg/ml of kanamycin, grown until OD_{600nm} reached 0.6. After incubation at 20°C for 8 h with 1mM IPTG, cultures were centrifuged at 3,000 g (5,371 rpm) and the pellets were lyzed and purified by TALON® resin as described under section 1.1.4 for denaturing conditions. The denaturant was changed to 8 M urea in order to reduce protein precipitation during the dialysis step that followed (Gaberc-Porekar and Menart, 2001). Purified proteins were analyzed by SDS-PAGE (10% gel). The eluate was collected and further dialyzed at 4°C followed with decreasing concentration of urea from 8 to 7, 6, 4 and 2 M in PBS, each step for 2 h in a 10K MWCO SnakeSkin Dialysis Tubing (PIERCE, Rockford, IL). The dialyzed proteins were concentrated with a 10-kDa Amicon® Ultra-4 Centrifugal Filter (Millipore, Billerica, MA) by centrifugation at 4,000 g (SX4750 rotor, BECKMAN coulter).

1.1.8 Antibody production

Antibodies were produced by Robert Sargeant's Laboratory (Ramona, CA) and pre-immune sera was collected to be used as negative control. In the first injection, 0.2 mg of 30-kDa purified recombinant antigen was injected with Complete Freund's Adjuvant (CFA) into each of two New Zealand female white rabbits. The injections of antigen after first injection were with Incomplete Freund's Adjuvant (IFA) after every 25 days. Seven days after injections, rabbits were bled and sera was collected.

Aim2: Detection of VgR expression in fire ant queens

1.2.1 Insects

Seven polygyne (multiple-queens) colonies of *S. invicta* were obtained from the Fire Ant Research Laboratory in the Department of Entomology (Texas A&M University, College Station, TX). Queenright polygynous *S. invicta* were excavated from areas in Brazos County, Texas, and maintained in laboratory within plastic trays (27 x 40 x 9 cm) containing one nest in a 14 cm diameter Petri dish half-filled with damp Castone (Dentsply International Inc., York, PA). The colonies were reared at 27 ± 2°C (in a 16L:8D photoperiod) and fed daily with 20% honey-water and frozen crickets (Flukers, Port Allen, LA). Water was given *ad libitum*. Mated queens were dissected to verify their successful mating by observation of an inseminated spermatheca. Non-inseminated queens were not used in the experiments.

1.2.2 Detection of VgR specific expression in fire ant queens

The specificity of the antibodies in the queen ovary was verified by western blot methodology modified from Schonbaum *et al*. (1995) and Sappington *et al*. (1995). Ovaries of alate and dealate queens of different ages were dissected and homogenized in cold buffer A (25 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1mM Benzamidine, 1 µg/µl of pepstatin A, 1 µg/µl of leupeptin). The homogenates were centrifuged at 800g for 5 min and the supernatants were

collected and centrifuged at 100,000g (SW28 rotor, BACKMAN LE80K) for 1h at 4°C for membrane protein preparation. After ultra-centrifugation, the pellets were re-suspended in cold Buffer B (50 mM Tris HCl, pH 7.5, 2 mM $CaCl_2$, 1 mM PMSF, 1mM Benzamidine, 1 µg/µl of pepstatin A, 1 µg/µl of leupeptin). Proteins from 4 pairs of dealate ovaries and 16 pairs of alate ovaries were separated on SDS-PAGE (7.5% gel, Bio-Rad) and subsequently transferred to PVDF membrane (Millipore, MA). The membrane was blocked 1h at RT in 5% non-fat dry milk in TBST (10 mM Tris base, 140 mM NaCl, 0.1% Tween-20, pH 7.4) and incubated 1.5 hour at RT with 1 to 1000 dilution of rabbit anti-*Si*VgR antiserum (third bleed) in TBST. After 3 X 10 min washes with TBST, the membrane was then incubated with a 1:40,000 dilution of HRP-conjugated Goat anti-rabbit IgG antibody for 1 h at RT. After 3 X 10 min washes with TBST, the membrane was visualized by enhanced chemiluminescence, ECL (PIERCE, IL).

The specificity of the antibodies in the queen tissues was verified by western blot methodology as above. Head, fat body, gut and ovary of dealate queens and male abdomens were dissected and homogenized in cold buffer A. Membrane proteins of head, fat body, gut and ovary of dealate queens and male abdomens were ultra-centrifuged and re-suspended as above. Proteins from queen ovary, head, fat body, gut proteins (10µg) and male abdomen proteins (10µg) were separated on SDS-PAGE (7.5% gel, Bio-Rad) and subsequently transferred to PVDF membrane. The membrane was blocked 1h at RT in 5% non-fat dry milk in TBST and incubated 1.5 hour at RT with 1 to 1000 dilution of rabbit anti-*Si*VgR antiserum (third bleed) in TBST. After 3 X 10 min washes with TBST, the membrane was then incubated with a 1:40,000 dilution of HRP-conjugated Goat anti-rabbit IgG antibody for 1 h at RT. After 3 X 10 min washes with TBST, the membrane was visualized by enhanced chemiluminescence, ECL (PIERCE, IL).

1.2.3 Immunofluorescence of fire ant Vg receptor in the ovary

Ovaries were dissected under PBS, fixed 4 h in 4% paraformaldehyde (Sigma, St. Louis, MO) in PBS at 4°C and serially dehydrated in 50, 70, 95, 100% ethanol for 2 X 30 min each. Ethanol was replaced with xylene for 3 X 20 min and tissues were penetrated in Paraplast-Xtra (Fisher Scientific, Pittsburgh, PA) at 60°C for 4 h and embedded blocks were stored at 4°C. Using a rotatory microtome, sections (12 µm thick) were cut and placed on Superfrost Plus™ slides (Fisher) and dried for 2 days at 39°C. Sections were de-waxed 2 X 5 min in xylene and then rehydrated serially for 10 min each in 100%, 95% and 70% ethanol and equilibrated in water for 30 min at RT. After rinsing 2 X 5 min with PBS containing 0.05% Triton X-100 (PBST), the slides were blocked in blocking solution (5% normal goat serum (Sigma) and 0.5% BSA (Sigma) in PBST) for 1 h at RT. Slides were incubated overnight in a wet chamber at 4°C with the anti-SiVgR antiserum as primary antibody diluted 1:2500 in blocking solution. The anti-SiVgR antiserum was preabsorbed at 4°C, overnight, with 100µg VgR antigen and diluted 1:2500 in blocking solution was used as a negative control. Pre-immune sera was diluted 1:2500 in blocking solution to be used as a negative control. After rinsing 3 X 10 min with PBST, the slides were incubated in 1:200 diluted of biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch™) in blocking solution for 1 h. Sections were rinsed 3 X 10 min in PBST and incubated in a 1:200 dilution of Alexa Fluor 546 Streptavidin (Invitrogen™) for 1 h and rinsed 3 X 10 min in PBST again. Sections were mounted in Vectashield Mounting medium with DAPI for nuclear staining (Vector Laboratories) and observed under a Carl Zeiss Axioimger A1 microscope with filters for DAPI (G 365 nm, FT 395 nm, BP 445 nm) and Alexa Fluor 546 (BP 546 nm, FT 560 nm, BP 575-640 nm). Sections were analysed with 20X objective. Images were obtained with an AxioCam MRc color camera (Carl Zeiss) and analyzed with AxioVision image program (Carl Zeiss).

Objective 3. Determine the ligand of sNPF receptor by *in vitro* functional studies

3.1 Construction of the expression vector

The cDNA coding region of the fire ant sNPF receptor was amplified by PCR. The sense primer

is sNPFRf5: 5'-AGA*CTCGAG*GCC**ACCATGG**AGAGAGATAAC-3', which contained an *Xho* I restriction site for directional cloning (italics), Kozak sequence (Kozak, 1986) with an initiation codon (bold) and a partial sequence of the sNPF receptor sequence (underlined). The anti-sense primer is sNPFR-r3: 5'-CGT*GAATTC***TTA**CAAAGTGTCGTCAGAATC-3', which included an *EcoR* I restriction site for directional cloning (italics) a stop codon (bold), and the sNPF receptor sequence (underlined). PCR conditions were as follows: 1 μM of primers; 200 ng plasmid p*Si*sNPFR 16 (pCR 2.1 containing the full-length cDNA clone of the fire ant sNPF receptor; Chen and Pietrantonio, 2006); 0.2 mM dNTPs mixture, Advantage 2™ TAQ Polymerase kit (Clontech, Mountain View, CA). Parameters were: 94, 3 min; then 94°C, 30 s; 65°C, 1 min; 72°C, 90 s; forty cycles; 72°C, 5 min. After electrophoresis analysis (1% agarose), the PCR product of about 1200 bp was cleaned and cloned into pCR®2.1-TOPO® vector (Invitrogen™, Carlsbad, CA), as previously described in section 1.1.2. Competent cells, Top10F′ (Invitrogen™, Carlsbad, CA), were heat-shocked at 42°C to introduce the plasmid. Constructed plasmids were purified and 1 μg DNA of each was digested with *EcoR* I (Promega, Madison, WI) to verify the presence of insert and the plasmid was sequenced to confirm its identity as previously described in section 1.1.2. The fragments excised from the pCR2.1-TOPO® vector with *Xho* I and *EcoR* I (Promega, Madison, WI) were cloned into an expression vector pcDNA3.1(-) (Invitrogen™, Carlsbad, CA) with T4 DNA ligase (Promega, Madison, WI) and transformed into Top10F′ to generated an expression plasmid **pcDNA3.1(-)-*Si*sNPFR**. The colonies were selected and the plasmid DNAs were purified and digested with *Xho* I and *EcoR* I restriction enzymes and sequenced to confirm their identity. This plasmid was introduced into CHO-K1 cells for stable expression of fire ant short NPF receptor.

3.2 Transfection of the pcDNA3.1(-)-*Si*sNPFR into mammalian cells and confirmation of the ligand selectivity for sNPF of the putative sNPF receptor by a Ca^{2+} bioluminescence plate assay

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown as described (Holmes *et al*., 2003) in a 5% CO_2 humidified incubator at 37°C. CHO-K1 cells were maintained in F-12K (Invitrogen™, Carlsbad, CA) medium plus 10% fetal bovine serum (EquiTech Bio, Kerrville, TX) with antibiotic antimycotic solution (Gibco, Invitrogen™, Carlsbad, CA). For transfection with pcDNA3.1(-)-*Si*sNPFR, CHO-K1 cells were seeded into T-25 tissue culture flasks and allowed to grow overnight until 50% confluent. Cells were transfected in serum-free Opti-MEM medium (Life Technologies, Gaithersburg, MD, USA) with 6 μl cationic lipid reagent Lipofectin® (Life Technologies) and 2 μg of the expression plasmid pcDNA3.1(-)-*Si*sNPFR according to the manufacturer's protocol. After 6 h, the lipofectin-containing medium was removed and replaced with F-12K (Invitrogen™, Carlsbad, CA) medium plus 10% fetal bovine serum (EquiTech Bio, Kerrville, TX) without antibiotic. After 48 h of growth, the medium were replaced with the selection medium (F-12K medium with 800 μg/ml GENETICIN®, Life Technologies) and selection continued for 3 weeks. Single clonal cell lines were screened in 96 well plates. A calcium bioluminescence plate assay as previously described (Pietrantonio *et al*., 2005) will be applied to test several short NPF peptides from fruit fly, mosquito and honeybee for verification of short NPF receptor functionality and determination of the most active peptide.

The aequorin plasmid mtAEQ/pcDNA1 (a kind gift from Drs. C.J.P. Grimmelikhuijzen and Michael Williamson, University of Copenhagen, Denmark) was grown in *E. coli* cells MC1061/P3 (Invitrogen™, Carlsbad, CA) and the plasmid DNA was purified as previously described in section 1.1.2. Transient transfection with this plasmid was as described by Staubli *et al.* (2002). For this, the cells expressing the sNPF receptor were grown in F12K media containing 10% fetal bovine serum and 400 mg/ml GENETICIN® to about 90% confluency in T-25 flasks. Cells were trypsinized with 0.05% Trypsin-EDTA (Gibco, Invitrogen™, Carlsbad, CA) and $2X10^5$ cells in 2 ml of media were seeded in each well of 6 well tissue culture plates. Cells were allowed to grow for 24 h in the incubator to 60% confluent. The media was removed and replaced with OPTI-MEM media (Gibco,

Invitrogen™, Carlsbad, CA). For transfection of cells in each well, 96 µl of OPTI-MEM media was mixed with 4 µl of the transfection reagent Fugene 6 (Roche Biochemicals) in a microfuge tube. The mixture was incubated for 5 min at room temperature after which 1 µg of mtAEQ/pcDNA1 plasmid DNA (in 10 mM Tris buffer, pH 8.5) was added and incubated for another 15 min at room temperature and added drop wise to each well with gentle manual shaking; plates were incubated for 6 h and then the media was changed to F12K media containing 10% fetal bovine serum without antibiotic. After 24 h, cells were trypsinized and transferred to 96-well plate (Costar 3610, Cambridge, MA) at a density of 40,000 cells/100 ml per well and incubated for 24 h after which they reached a confluency of 80%, optimal for performing the bioluminescence assay.

To reconstitute the aequorin complex, cells were incubated in 90 ml/well of calcium-free DMEM media (GIBCO, Invitrogen™, Carlsbad, CA) containing 5 mM coelenterazine (Molecular Probes, Eugene, OR) for 3 h (Stables *et al*., 1997) in the dark at 37°C and 5% CO_2. Cells were then challenged with 1 µM of short NPF peptides in a volume of 10µl (10X) solubilized in calcium-free DMEM media. A bioluminescence assay with the mosquito kinin receptor cell line was used as positive control by testing the FFFSWGa peptide, a potent kinin agonist (Pietrantonio *et al*., 2005). The assay was performed using the NOVOstar (BMG Labtechnologies) plate reader in bioluminescence mode at room temperature. Light emission (469 nm) was recorded every 2 s over a period of 50 s per well.

RESULTS

1.1.1 Selection of the *Si*VgR protein sequence for recombinant protein expression for antibody production

The Vg receptor belongs to the LDLR superfamily. Proteins of this superfamily share some common structural elements. In order to determine a specific sequence region for recombinant protein expression for antibody production, the amino acid sequences of known members of the LDLR superfamily were aligned and compared (Table 1). The alignment showed that the second YWXD repeat region in the first epidermal growth factor (EGF) precursor homology domain is a highly variable region among LDLR family members, exhibiting the lowest similar scores from range 7 to 16 (Table 1, marked in bold).

*Si*VgR regions	Length (a. a.)	LDLR members	Length (a. a.)	Similarity score
YWXD-1 (Residues 283-552)	270	*Hs*LDLR	860	30
		*Aa*LDLR	1847	42
		*Hs*VLDLR	873	31
		*Dm*LRP6	1678	26
YWXD-2 (Residues 588-889)	**302**	***Hs*LDLR**	**860**	**12**
		***Aa*LDLR**	**1847**	**16**
		***Hs*VLDLR**	**873**	**7**
		***Dm*LRP6**	**1678**	**14**
YWXD-3 (Residues 1337-1610)	274	*Hs*LDLR	860	21
		*Aa*LDLR	1847	27
		*Hs*VLDLR	873	22
		*Dm*LRP6	1678	21
LBD-1 (Residues 36-204)	169	*Hs*LDLR	860	37
		*Aa*LDLR	1847	42
		*Hs*VLDLR	873	36
		*Dm*LRP6	1678	31
LBD-2 (Residues 932-1263)	332	*Hs*LDLR	860	34
		*Aa*LDLR	1847	40
		*Hs*VLDLR	873	35
		*Dm*LRP6	1678	23

Table 1. An alignment of amino acid sequences of three YWXD repeat regions (YWXD-1, -2, -3) and two ligand binding domains (LBD-1, -2) of *Si*VgR with the LDLR superfamily members from insects and human was performed with Clustal W program. The highly variable region is marked in bold. The alignment showed that the YWXD-2 region has the lowest similarity scores ranging from 7 to 16 indicating this is a highly variable region among LDLR superfamily members. *Si*VgR: *S. invicta* VgR; *Hs*LDLR: Human LDLR; *Aa*LDLR: *A. aegypti* LDLR; *Hs*VLDLR: Human VLDLR; *Dm*LRP6: *D. melanogaster* LRP6.

Among VgR in insects, the fruit fly *Drosophila melanogaster* YPR (*Dm*YPR) and the cockroach *Blattella germanica* VgR (*Bg*VgR) are two Vg receptors towards which anti-Vg receptor antibodies

have been produced against a recombinant protein fragment of VgR (Schonbaum *et al*., 1995; Ciudad *et al*., 2006). The pairwise alignment of different modular domains of *Si*VgR, *Dm*YPR and *Bg*VgR also showed that the second YWXD repeat region in the first EGF precursor homology domain is highly variable (Fig. 1A).

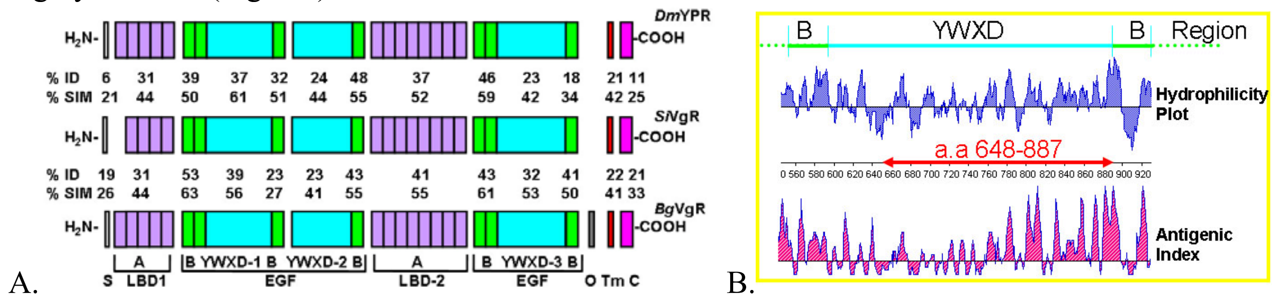


Fig. 1. A. The arrangement of modular domains for insect VgRs and the schematic alignment of *Si*VgR modular domains with those of *Bg*VgR and *Dm*YPR. Pairwise alignment was performed with EMBOSS Pairwise Alignment Algorithms of EBI and the percent identity (% ID) and percent similarity (% SIM) showed that the YWXD-2 region has the lowest identity and similarity scores among three VgR. Signal peptide (SP); Class A cysteine-rich repeat (A); Class B cysteine-rich repeat (B); ligand binding domain (LBD); EGF precursor homology domain (EGF) contains Class B cysteine-rich repeats (B) and YWXD repeats (YWXD); O-linked sugar domain (O) (*Bg*VgR only); transmembrane domain (TM); cytoplasmic tail (C). B. The DNASTAR software was used to analyze the hydrophilicity and antigenicity of fire ant Vg receptor amino acid sequence. A region from amino acid residues 648 to 887 was chosen as antigen to develop a polyclonal antibody.

This is the same antigen region cloned to produce the anti-*Dm*YPR antibody and anti-*Bg*VgR antibodies. After analyzed the hydrophilicity and antigenicity of the fire ant Vg receptor amino acid sequence, a region from amino acid residues 648 to 887 within the YWXD-2 variable region was chosen to develop a recombinant antigen to be injected into rabbits for polyclonal antibody production (Fig. 1B).

1.1.2 Construction of the expression vector: pET32a-VgR

A VgR fragment (a. a. 648-887, corresponding to the second YWXD repeat region in the first EGF precursor homology domain) was amplified by PCR (Fig. 2A).

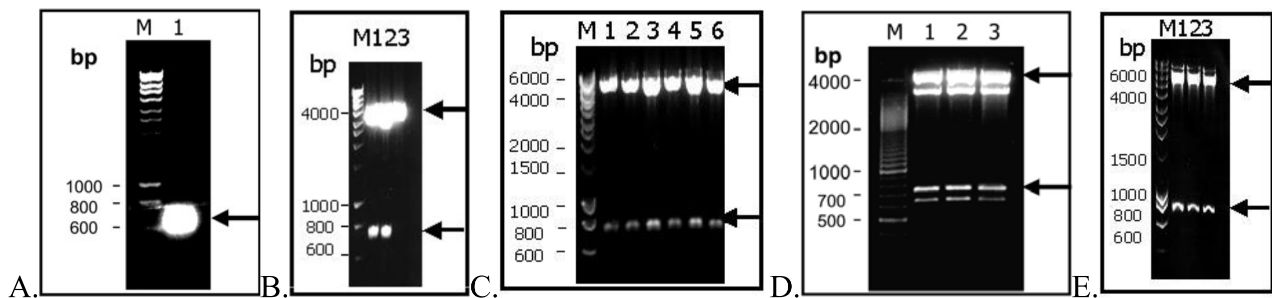


Fig. 2. Construction of the expression vector pET32a-*SiVgR*. A. The PCR product of about 740 bp was amplified by PCR. B. TA cloning of the PCR product. Plasmid DNA from three colonies was digested with *EcoR* I restriction enzyme to verify the presence of insert. Plasmids from two colonies contained the correct insert and were sequenced (lanes 1 and 2). C. The plasmid pET32a-VgR was constructed. Colonies were selected and their plasmid DNA was digested and analyzed with *BamH* I and *Sal* I restriction enzymes. D. *E. coli* strain BL21 (DE3) pLysS was transformed with the plasmid pET32a-VgR and three colonies were selected and plasmid DNA was analyzed with *BamH* I and *Sal* I restriction enzymes. E. *E. coli* strain BL21 (DE3) was transformed with the plasmid pET32a-VgR and three colonies were selected and plasmid DNA was analyzed with *BamH* I and *Sal* I restriction

enzymes. M: marker.

A PCR product of about 740 bp was cloned into pCR®2.1-TOPO® vector and three colonies were selected and in two of the colonies the presence of insert was verified (Fig. 2B, lanes 1 and 2). The VgR PCR product was sub-cloned into the pET32a vector to yield the plasmid pET32a-VgR, resulting in a VgR fragment (a. a. 648-887) with a N-terminal extension of Trx-, His-, and S-tag recombinant protein. Six colonies were selected and the presence of the plasmid pET32a-VgR was verified by restriction analysis (Fig. 2C). *E. coli* strains BL21 (DE3) pLysS (Fig. 2D) and BL21 (DE3) (Fig. 2E) were transformed with the plasmid pET32a-VgR for expression of recombinant proteins and three colonies of each bacteria strain were selected and the presence of insert was verified by restriction analysis. The pLysS plasmid in the BL21 (DE3) pLysS strain also has a *BamH* I restriction enzyme site therefore created the lower band in the gel when DNA was treated with *BamH* I and *Sal* I restriction enzymes.

1.1.3 Expression of recombinant protein

E. coli strain BL21 (DE3) pLysS transformed with the plasmid pET32a-VgR failed to induce the 44-kDa recombinant protein expression with 1 mM IPTG at 25°C (Fig. 3, lane 6). However, the control culture (the BL21 (DE3) pLysS strain transformed with the vector pET32a) was induced a vector protein about 20-kDa as expected (Fig. 3, lane 3). This is in agreement with previous findings in which the pLysS plasmid in the BL21 (DE3) pLysS strain over represses the expression of the larger recombinant protein (Clontech).

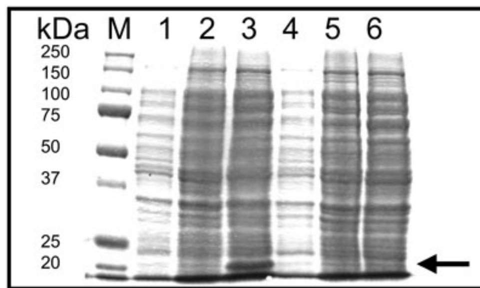


Fig. 3 Analysis of the expression of recombinant protein. Bacterial cultures of the BL21 (DE3) pLysS cells transformed with the pET32a vector (lanes 1 to 3) and transformed with the plasmid pET32a-VgR (lanes 4 to 6) were centrifuged at 3,000 g (5,371 rpm) after incubation at 25°C. The pellets were sonicated and 10 µl samples were analyzed by SDS-PAGE (10% gel). The results showed that after induction, the BL21 (DE3) pLysS strain expressed a 20-kDa vector protein (lane 3, arrow) but failed to induce the expression of VgR protein with 1 mM IPTG (lane 6). Lane 1 and lane 4 showed the proteins before induction. Lane 2 and lane 5 showed the proteins in the un-induced cultures. Lane 3 and lane 6 showed the cultures induced with 1mM IPTG at 25°C for 6 h. M: marker.

In contrast, the BL21 (DE3) transformed with the pET32a-VgR plasmid was successfully induced with IPTG and expressed the 44-kDa recombinant protein (Fig. 4). In order to determine the best conditions for maximum recombinant protein yield, a small-scale induction test was performed. One of the positive colonies of BL21 (DE3) strain transformed with the pET32a-VgR plasmid was grown at different induction temperatures for various induction times with different concentrations of promoter inducer, isopropyl-D-thiogalactopyranoside (IPTG). After analyzed by SDS-PAGE (10% gel), the results showed that at 20°C, more soluble protein was produced than at 25°C, and 1mM IPTG induced more soluble protein than 0.1 and 0.5 mM IPTG (Fig. 4).

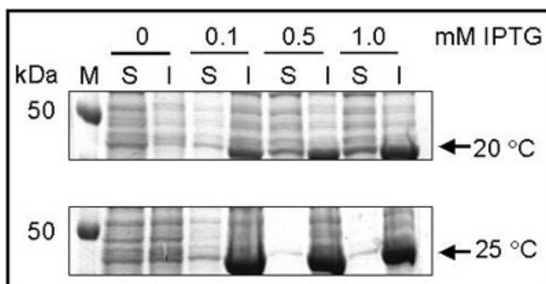


Fig. 4. Small-scale induction test of the BL21 (DE3) cells transformed with the pET32a-VgR plasmid. The bacterial cultures were induced for 4 h with 0.1, 0.5, and 1 mM IPTG at 20 (top panel) and at 25°C (bottom panel) and cultures were centrifuged at 10,397 g (10,000 rpm) for 20 min to separate the soluble protein in the supernatant (S) and insoluble protein in the pellet (I). Ten µl samples were analyzed by SDS-PAGE (10% gel) and the results indicated that at 20°C, more soluble

protein was produced than at 25°C, and 1mM IPTG induced more soluble protein than 0.1 and 0.5 mM IPTG. S: soluble proteins in the supernatant; I: insoluble proteins in the pellet; M: marker.

Therefore, bacterial cultures were induced with 1 mM IPTG at 20°C for 8 h in the following experiments.

1.1.4 Purification of the recombinant protein

After induced with 1mM IPTG at 20°C for 8 h, the recombinant protein of the bacterial culture (the BL21 (DE3) strain transformed with the pET32a-VgR plasmid) was purified under native condition using TALON® metal affinity resin. As observed in Fig. 5A, lanes 5 and 6, the resin appears to bind non-specifically to other bacterial proteins. In order to attempt to reduce this background binding, bacterial culture were lyzed with 6 M guanidine-HCl. This procedure successfully reduced the TALON® resin background binding (Fig. 5B, lanes 5 to 8). The eluate protein was collected, dialyzed, and concentrated for further digestion (Fig. 5C).

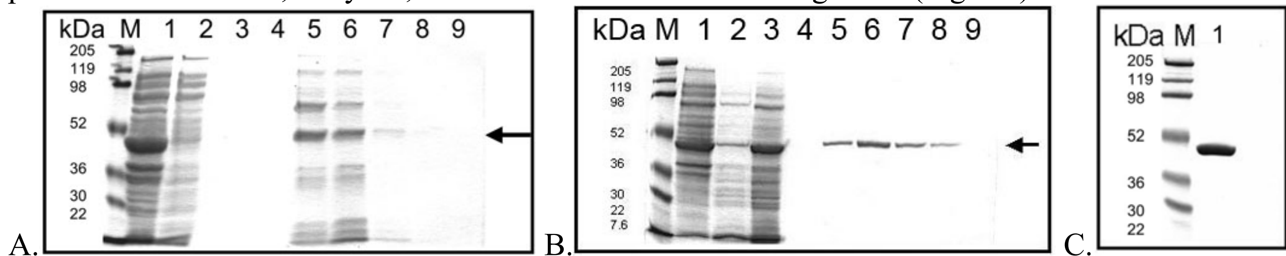


Fig. 5. Purification of the His-Tag recombinant protein by TALON® metal affinity chromatography analyzed by SDS-PAGE (10% gel). A. The recombinant protein was purified with TALON® resin under native conditions and eluted with 150 mM imidazole (lanes 4 to 9). The eluate was serially collected as 1 ml fractions. The resin appears to bind non-specifically to other bacterial proteins (lanes 5 and 6). Lane 1, Insoluble proteins (5 µl); Lane 2, Soluble proteins (5 µl); Lane 3, Protein washed out from final wash step (20 µl); Lanes 4 to 9, Proteins from eluate (20 µl). B: The recombinant protein was purified with TALON® resin under denaturing conditions with 6 M guanidine-HCl and eluted with 150 mM imidazole (lanes 5 to 9). Purification of the recombinant protein with 6 M guanidine-HCl in the Wash buffer successfully reduced the TALON® resin background binding. Lane 1, Insoluble proteins after centrifuged the lyzed culture at 10,397 g (10,000 rpm) for 20 min (5 µl); Lane 2, Soluble proteins after centrifugation (5 µl); Lane 3, Soluble proteins after binding with resin (5 µl); Lane 4, Protein washed out in final wash step (20 µl); Lanes 5 to 9, Proteins from eluate (20 µl). C. The recombinant protein purified under denaturing conditions was further dialyzed and concentrated in PBS (Lane 1, 5 µg proteins). M: Marker.

1.1.5 Removal of the protein tag by Enterokinase

In order to remove the protein tag, purified 44-kDa recombinant protein (solid arrow) was digested with Tag off High Activity Enterokinase, and one band of approximately the expected molecular weight of 28-kDa was detected (Fig. 6, lanes 2 to 4, open arrow). However, the enzyme efficiency was very low and increasing the enzyme concentration up to 2 Units did not improve the efficiency.

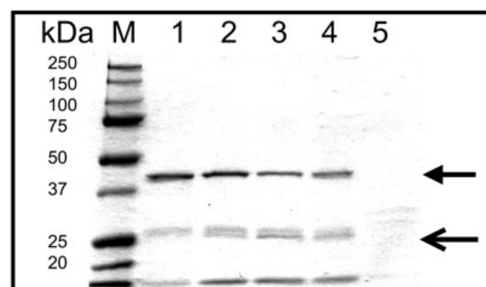


Fig. 6. Digestion of the 44-kDa recombinant protein with Tag off High Activity Enterokinase. In order to remove the protein tag, purified 44-kDa recombinant protein (5 µg) (solid arrow) was digested with 0.5U (lane 2), 1U (lane 3), and 2U (lane 4) enterokinase in the enterokinase buffer with 0.1% Tween-20 at 30°C for 16 h and analyzed by SDS-PAGE (10% gel). The enzyme efficiency is low and one band of approximately the expected molecular weight of

28-kDa was detected after digestion (open arrow). No enzyme was added to the recombinant protein in lane 1 as negative control, and 0.5U enzyme was added to the control protein as positive control for enterokinase activity (lane 5). Notice that the control protein was totally digested indicating that the enzyme was active. M: Marker.

1.1.6 Re-construction of the expression vector: pET28a-VgR

The previous expression of the plasmid pET32a-VgR in the BL21 (DE3) strain resulted in a large protein that was difficult to cleave by enterokinase to remove the protein tag. In order to solve this problem, the pET28a vector was used to replace the vector pET32a and a new expression plasmid was constructed now designated pET28a-VgR. For this, the pET32a-VgR was digested to obtain the coding region of the VgR (Fig. A, lane 1) to be cloned into *BamH* I and *Sal* I sites of the pET28a vector (Fig. A, lane 2).

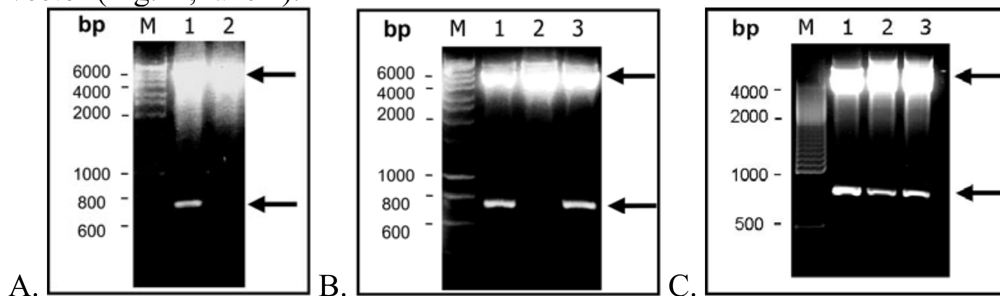
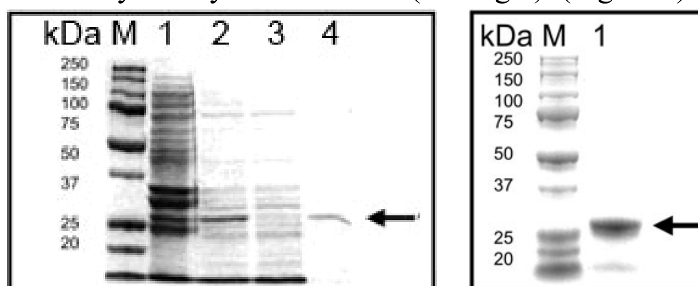


Fig. 7. Re-construction of the expression plasmid pET28a-VgR. The pET28a vector was used to replace the pET32a vector and re-construct the expression plasmid designated pET28a-VgR. A. Digestion of the pET32a-VgR plasmid (lane 1) and the pET28a vector (lane 2) with *BamH* I and *Sal* I restriction enzymes. B. After ligation of the VgR fragment digested from the pET32a-VgR plasmid with the pET28a vector and transformation into competent cells, Top10F', the pET28a-VgR plasmid DNA from three colonies was digested with *BamH* I and *Sal* I restriction enzymes to verify the presence of insert. Plasmids from two colonies contained the correct insert and were sequenced (lanes 1 and 3). C. The plasmid pET28a-VgR was transformed into the BL21 (DE3) strain. Three colonies were selected and plasmid DNA was analyzed with *BamH* I and *Sal* I restriction enzymes. M: marker.

The plasmid, pET28a-VgR, expressed the amino acid sequences 648-887 of Vg receptor with only an additional N-terminal extension of 32 amino acid residues including the His-tag and T7-tag sequences. Three colonies were selected and two of the colonies had plasmid with insert (Fig. 7B). The plasmid was transformed into BL21 (DE3) strain and three colonies were selected and plasmid DNA was analyzed with *BamH* I and *Sal* I restriction enzymes. One of the positive colonies was used for recombinant protein production (Fig. 7C).

1.1.7 Re-expression and purification of the recombinant protein

E. coli strain BL21 (DE3) was transformed with the plasmid pET28a-VgR for expression of the recombinant protein. Three colonies were selected and the presence of insert was verified and one of the positive colonies was grown and induced with 1mM IPTG at 20°C for 8 h. The bacterial pellets were lyzed and purified by TALON® resin with denaturant changed to 8 M urea. Purified proteins were analyzed by SDS-PAGE (10% gel) (Fig. 8A) and the analysis showed that the plasmid pET28a-VgR expressed a 30-kDa recombinant protein after induction by 1mM IPTG, as expected. The eluate was collected, dialyzed and concentrated for the anti-*Si*VgR antibody production (Fig. 8B). Fig. 8. Purification of the His-Tag



recombinant protein from the BL21 (DE3) strain (transformed with the pET28a-VgR plasmid) under denaturing conditions (8 M urea) by TALON® metal affinity chromatography. Proteins were analyzed by SDS-PAGE (10% gel). A. Bacterial culture was induced with 1mM IPTG for 8 h at 20°C. The recombinant protein was purified and eluted with 150 mM imidazole (lane 4). Lane 1, Insoluble proteins in the pellet after centrifugation of the lyzed culture at 10,397 g (10,000 rpm) for 20 min (5 µl); Lane 2, Soluble proteins in the supernatant after centrifugation (5 µl); Lane 3, Soluble proteins after binding lane 2 protein with resin (5 µl); Lane 4, Proteins from eluate (20 µl). B. The purified recombinant protein (30-kDa) was further dialyzed and concentrated in PBS (lane 1, 5 µg protein). M: Marker.

1.2.2 Detection of VgR expression in fire ant queens

The specific fire ant VgR protein expression profiles in ovaries of dealates and alates were detected using the anti-*Si*VgR antibodies (Fig. 9 and 10). *Si*VgR recombinant protein was detected as expected molecular weight of 30kDa (Fig. 9, lane 1, open arrow). Bands at expected size around 202-kDa (solid arrow) was recognized in ovaries of dealate queens (Fig. 9, lane 2) and alate queens (Fig. 9, lane 3). No signal was detected using pre-immune serum (Fig. 9, lanes 4 to 6). Bands at expected size around 202-kDa was recognized in ovaries of dealate queens (Fig. 10, lane 1) and no signal was detected with male abdomen (Fig. 10, lane 2), dealate queen head (Fig. 10, lane 3), fat body (Fig. 10, lane 4) and gut (Fig. 10, lane 5) membrane proteins (10 µg).

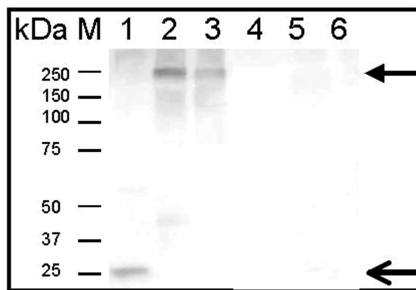


Fig. 9. Verification of the specificity of the anti-SiVgR antibodies in the ovary of fire ant queens. VgR recombinant protein 0.05 µg (lanes 1 and 4), membrane protein of ovaries of dealate queens (lanes 2 and 5, from protein equal to 4 pairs of ovaries) and alate queens (lanes 3 and 6, from protein equal to 16 pairs of ovaries) were analyzed by western blot. The anti-SiVgR antibody was used as primary antibody in lane 1 to 3 (1 to 1000 dilution). Pre-immune serum was used as primary antibody in lane 4 to 6 (1 to 1000 dilution). As expected, a band at 202-kDa was recognized by the anti-SiVgR antibody in ovaries of dealate queens (lane 2) and alate queens (lane 3, solid arrow). M: Marker.

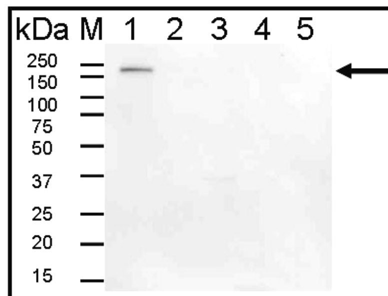


Fig. 10. Verification of the specificity of the anti-SiVgR antibodies in the different tissues of fire ant queens. Membrane protein (10 µg) of dealate queen ovary (lane 1), male abdomen (lane 2), dealate queen head (lane 3), fat body (lane 4) and gut (lane 5) were analyzed by western blot. The anti-SiVgR antibody was used as primary antibody. Pre-immune serum was used as primary antibody as negative control (data not show). A band at 202-kDa was detected by the anti-SiVgR antibody in ovaries of dealate queens (lane 1). No signal was detected in other tissues (lane 2-5). M: Marker.

1.2.3 Immunofluorescence of fire ant Vg receptor in the ovary

*Si*VgR localization in ovaries was examined by immunofluorescence in the ovary of mated queen. *Si*VgR protein was evenly distributed in the oocyte cytoplasm at the earlier stage oocyte (Fig. 11 A, arrow). At later stage oocyte, *Si*VgR became clearly visible accumulating in the oocyte membrane (Fig. 11. A and B, arrowhead). No signal was detected as expected with pre-immune serum and antigen subtracted antiserum (Fig. 11. C and D). The cockroach, *Blattella germanica*, Vg receptor (*Bg*VgR) antiserum, (a generous gift from Dr. M. Dolors, Spain) was examined by immunohistochemistry in the ovary of mated queen and no signal was detected (data not show). This result indicated that the specific antibody against fire ant VgR is important to be developed.

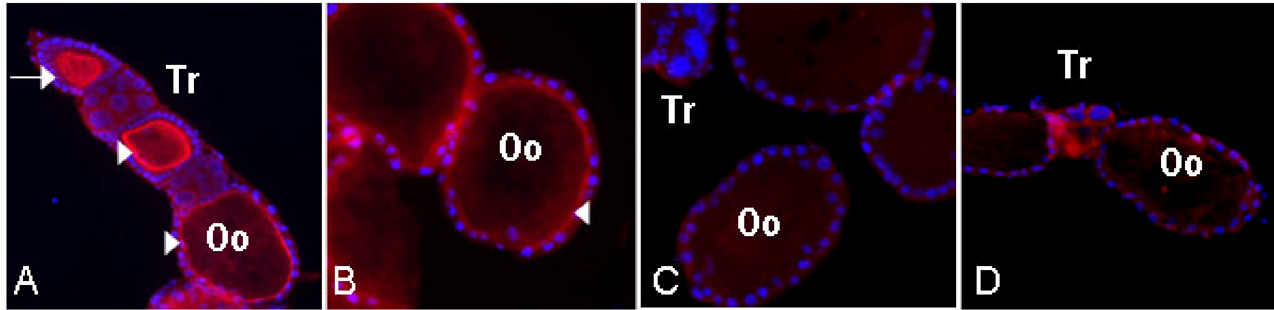


Fig. 11. Immunolocalization of fire ant Vg receptor in ovaries (one ovariole) of fire ant mated queen (A. and B.). Fire ant Vg receptor accumulated in the cytoplasm of early stage oocytes (Oo) (A, top arrow, red), and in the membrane of later stage oocytes (A, arrowhead, red). B. Cross section of a mature oocyte showing Vg receptor signal in the plasma membrane, as expected. C. No signal is present in pre-immune control. D. No signal is present in antiserum subtracted with 100 μg VgR recombinant protein control. Nuclei (in blue) of trophocytes (Tr) are bigger and grouped as in the drawing.

Objective 3. Determine the ligand of sNPF receptor by *in vitro* functional studies

3.1 Construction of the expression vector

The cDNA coding region of the fire ant sNPF receptor was amplified by PCR (Fig. 12A). The PCR product of about 1200 bp was cleaned and cloned into pCR®2.1-TOPO® vector (Fig. 12B). The sNPF receptor PCR product excised from the pCR2.1-TOPO® vector was sub-cloned into the pcDNA3.1(-) vector to generate an expression plasmid pcDNA3.1(-)-*Si*sNPFR (Fig. 12C). The plasmid pcDNA3.1(-)-*Si*sNPFR was transformed into competent cells and four colonies were selected and the presence of insert was verified by restriction analysis and the plasmid was sequenced to confirm its identity.

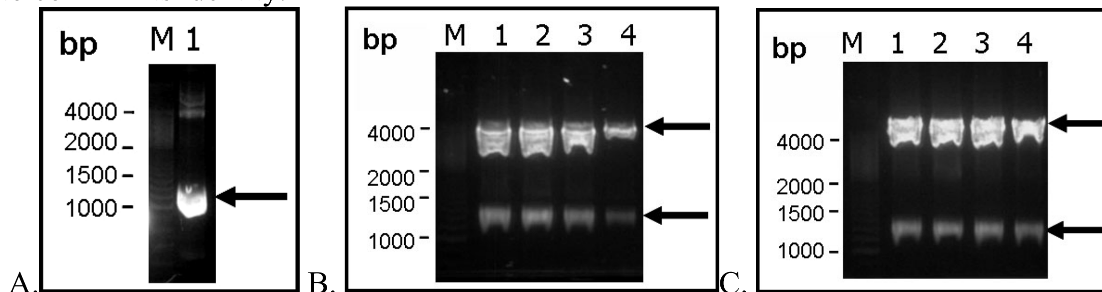


Fig. 12. Construction of the expression vector pcDNA3.1(-)-*Si*sNPFR. A. A product of about 1200 bp was amplified by PCR. B. TA cloning of the PCR product. Four colonies were selected and plasmid DNA was digested only with *EcoR* I restriction enzyme to verify the presence of insert. C. Fragments were excised from the pCR2.1-TOPO® vector with *Xho* I and *EcoR* I restriction enzymes for cloning into the pcDNA3.1(-) vector. Four colonies were selected and the plasmid DNA was digested with *Xho* I and *EcoR* I restriction enzymes to verify the presence of insert (lanes 1 to 4) and the plasmid was sequenced to confirm its identity. This pcDNA3.1(-)-*Si*sNPFR was introduced into CHO-K1 cells with Lipofectin® as described.

3.2 Transfection of the pcDNA3.1(-)-*Si*sNPFR into mammalian cells and confirmation the ligand selectivity for sNPF of the putative sNPF receptor by a Ca^{2+} bioluminescence plate assay

We have recently obtained several fruit fly and mosquito short NPF peptides to be tested (Table 2). Most of the peptides were a generous gift from Dr. J. W. Crim (University of Georgia, Athens) and Dr. L. Schoofs (Belgium). A calcium bioluminescence plate assay will be applied to test several

Insect	Peptide Name	short NPF peptide sequence
Fruit fly, *D. melanogaster*	Drm-sNPF-1	AQRSPSLRLRFa
	Drm-sNPF-2	WFGDVNQKPIRSPSLRLRFa
	Drm-sNPF-$2_{9\text{-}19}$	PIRSPSLRLRFa
	Drm-sNPF-$2_{12\text{-}19}$**	SPSLRLRFa
	Drm-sNPF-3	PQRLRWa
	Drm-sNPF-4	PMRLRWa
Locust, *S. gregaria*	Scg-NPF	YSQVARPRFa
Mosquito, *An. gambiae*	Ang-sNPF1(Aea-sNPF1)	AVRSPSLRLRFa
	Ang-sNPF2 (Aea-sNPF4)	AIRAPQLRLRFa
Mosquito, *Ae. aegypti*	Aea-sNPF2	SIRAPQLRLRFa
	Aea-sNPF$2_{4\text{-}12}$	APQLRLRFa

short NPF peptides from fruit fly, mosquito and honeybee for verification of short NPF receptor functionality and determination of the most active peptide. **Table 2.** Amino acid sequences of insect short neuropeptide F (short NPF) peptides available in our laboratory. ** Same sequence as honeybee short NPF (Hummon et al., 2006).

We are currently re-selecting clonal cell lines because the originally selected lines were contaminated and were discarded.

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