CLONING OF TROPONIN C AND OTHER GENE FRAGMENTS FROM THE RED IMPORTED FIRE ANT *Solenopsis invicta* BUREN (HYMENOPTERA, FORMICIDAE)

Patricia V. Pietrantonio[1], Steven P. Holmes[2], Christopher Jagge[3] and Sam K. Frazier[4]

ABSTRACT

During experiments aimed at cloning G protein-coupled receptors from worker red imported fire ants, several PCR products were obtained. One product of 685 bp encodes the full open reading frame of troponin C, a calcium-binding protein that is critical for muscle contraction. Other protein-encoding fragments were also identified, including PCR products corresponding to a putative glucose transporter protein (534 bp), elongation factor 2 (850 bp), filamin (600 bp), a cuticular protein (450 bp), dynein-associated polypeptide (652 bp) and dihydrolipoamide dehydrogenase (434 bp). These PCR products may be suitable for various experiments, such control probes for Southern and northern blots, *in situ* hybridization, semi-quantitative PCR or to design specific primers to amplify longer genomic DNA fragments to aid in phylogenetic analysis, or for cytogenetics and mapping.

INTRODUCTION

Most successful synthetic insecticides are nerve poisons. Except for organophosphates and carbamates that affect acetylcholinesterase, the majority of synthetic poisons and some natural insecticides affect either neurotransmitter receptors, voltage gated channels in nerves, or ion channels in muscles. Our research was initiated to search for new molecular targets that could be selectively exploited for control of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Invertebrate G protein-coupled receptors (GPCRs) are of interest to the pesticide industry (Gerhardt et al. 1997; Von Nickisch-Rosenegk et al. 1996). This family of receptors is characterized by a seven transmembrane (7TM) region structure. Formamidines, including the insecticides chlordimeform and amitraz, act on the receptor for the biogenic amine octopamine. However, few octopamine receptors have been cloned from insects. Other GPCRs may represent a currently unexploited and potentially suitable target for the development of insecticides with a novel mode of action. With a therapeutic purpose, the pharmaceutical industry concentrates drug development efforts in four key families of proteins: G-protein coupled receptors, ion channels, nuclear hormone receptors, and enzymes (Edvardsen and Kristiansen 1997; Lehmann 1996). In insects, however, these are targets for insecticides.

GPCRs could be used for management of ant populations by interfering with signal transduction pathways. For example, the identification of olfactory receptors and their

[1] Corresponding author, Department of Entomology, Texas A&M University, College Station, TX 77843-2475
[2, 3] Department of Entomology, Texas A&M University, College Station, TX 77843-2475
[4] Current address: Procter and Gamble, DE

expression *in vitro* could be used to screen combinatorial libraries for compounds that could be repellent, mating disrupters, or disrupters of searching mechanisms. For example, mosquito repellents such as DEET and experimental repellents such as KBR 3023 (Bayer AG) act via subsets of GPCRs and increase inositol triphosphate in sensory neurons (Boeckh et al. 1996). Importantly, invertebrate receptors often have a different pharmacological profile than mammalian receptors (Clark and Bradley 1997; references therein), a difference that can be exploited for ant control or behavioral modification, such as a reduction in aggressiveness. During our experiments aimed at obtaining DNA probes of these receptors, various PCR products were obtained. Here we report on the availability in our laboratory of several of these PCR products from worker fire ants. One of them, which we analyzed, encodes the full open reading frame for the fire ant troponin C.

MATERIALS AND METHODS

PCR with Degenerate Primers for Arthropod Octopamine Receptors. Worker ants from a monogyne colony were starved for 24 h and frozen at -80°C. Whole ants were ground under liquid nitrogen, and poly-A$^+$ RNA was isolated using a Poly(A) Pure™ kit (Ambion, Austin, TX) following the manufacturer's protocol. First strand cDNA was synthesized from 0.75 µg of poly-A$^+$ RNA with oligo(dT) primer using Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 37°C for 1 h. This single stranded cDNA (1 µl) was used as the template for PCR with two degenerate oligonucleotide primers designed to correspond to transmembrane regions III and VI, of highly conserved nucleotide sequence, based on the alignment of octopamine or tyramine receptor open reading frames from *Boophilus microplus* (Baxter and Barker 1999) (AJ010743), *Bombyx mori* (X95607), *Heliothis virescens* (Von Nickisch-Rosenegk et al. 1996) (X95606), and *Locusta migratoria* (Vanden Broeck et al. 1995)(Q25321). The sense primer Oct-D1F 5'-TG TGG CTI ACI TG(T/C) GAC (A/G)TI (A/C)T(C/G) TG(T/C) TG-3' and antisense Oct-D3R 5'-AA GAA IGG IA(A/G) CCA (A/G)CA IA(C/G) (C/G)AC GAA-3', (I= inosine), correspond to residues 132-141 (LWLTCDVLCC) and 350-358 (FVLCWLPFF) in the *B. microplus* octopamine-like receptor protein (AJ010743), respectively.

The reaction was carried out in a 50 µl volume containing TAQ Polymerase (0.5 µl) and PCR reaction buffer 10x (5 µl) (Boehringer Mannheim), 0.2 mM each of four dNTPs, and primers to final concentrations of 1 µM. PCR conditions were: initial denaturation at 94°C for 1 min followed by 8 cycles of 94°C for 20 sec, an annealing step of 63°C for 30 sec that was decreased by 0.5°C per cycle, and an extension step of 72 °C for 1 min. This was followed by 40 cycles of 94°C for 20 sec (denaturation), 59°C for 30 sec, and 72°C for 1 min. This initial PCR product was diluted 1:50,000 (final concentration in reaction) and reamplified in a second PCR with the same reagents and the following conditions: initial denaturation at 94°C for 1 min followed by 40 cycles of 94 °C for 20 sec (denaturation), 60°C for 30 sec, and 72 °C for 30 sec, and a final extension step of 72 °C for 4 min.

Agarose electrophoresis revealed a product at approximately 700 bp. DNA from this band was extracted using the Qiagen Quickgel extraction kit and cloned into pCR-TOPO plasmid vectors using a TOPO-cloning kit and its accompanying protocol (Invitrogen, Carlsbad, CA) and heat-shocked into Electrocomp Topo 10F' cells. Positive colonies were selected with blue-white screening and plasmids were purified using Wizard Plus Minipreps (Promega Corp., Madison, WI). Insertion was confirmed by restriction with *Eco*RI which cut the 700 bp product in two fragments. Plasmid inserts were

sequenced with the PE-Applied Biosystems International (Foster City, CA) Big Dye Cycle sequencing kit according to the manufacturer's protocol (PE Applied Biosystems 1998). The reaction products were electrophoresed and analyzed on an automated DNA sequencer (Applied Biosystems). The insert was sequenced in both directions with vector primers. Analysis of the sequence was by DNASTAR™ software and ExPASy: ScanProsite (http://ca.expasy.org).

PCR with Degenerate Primers for Class A GPCRs. Worker ants from a polygyne colony were frozen at -80°C and ground under liquid nitrogen. Total RNA was purified using TRIzol Reagent (Gibco BRL) following the manufacturer's protocol. Poly-A^+ RNA was purified from the total RNA using an Oligotex mRNA Kit (Qiagen, Santa Clarita, CA). First strand cDNA was synthesized from the Poly-A^+ RNA at 42°C for 50 min with an antisense primer for GPCRs (Pietrantonio et al. 2001; Holmes et al. 2000; Cox et al. 1997) using Superscript II reverse transcriptase (Superscript Preamplification System, Life Technologies). This cDNA (5 μl) was used as the template for PCR with two degenerate oligonucleotide primers: sense 5'-CCG GAT CCG (CT)(GC)A T(CT)(GA) (GC)I(GC) TGG AC(CA)G (GC)TA C-3' and antisense 5'-ACG AAT TCG G(GC) (CA) ICC A(GA)C AGA I(GC)(GA) (CT)(GA)A A-3' (Cox et al., 1997).

The reaction was carried out in a 50 μl volume containing TAQ Polymerase (1 μl) and PCR reaction buffer (Boehringer Mannheim), 10x (5 μl), 0.2 mM each of four dNTPs, and primers to final concentrations of 0.4 μM. PCR conditions were: initial denaturation at 94°C for 1 min followed by 50 cycles of 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72 °C for 45 sec.

Products were electrophoresed and bands of about 850, 600, 450, and 350 bp were cut. DNA was extracted using the Qiagen Quickgel extraction kit. DNA from the 850 bp band was cloned and DNA from the 350, 450, and 600 bp bands was reamplified by a second PCR with initial denaturation at 94°C for 1 min followed by 40 cycles of 94°C for 20 sec, annealing at 65°C for 45 sec, and extension at 72°C for 45 sec. The 350-, 450-, and 600-bp products were isolated from the reamplification PCR, and cloned into pCR-TOPO plasmid vector and heatshocked into Electrocomp Topo 10F' cells as above. Positive colonies were selected and plasmids were purified and sequenced as above. Sequences in GenBank with significant alignment were searched using the NCBI Blast 2.2.1 Program.

RESULTS AND DISCUSSION

A 685 bp PCR product (700 bp apparent) from monogyne worker fire ant cDNA was obtained while using degenerate primers for arthropod octopamine/tyramine GPCRs. Both primers were used in the PCR, but this product resulted from amplification by the 3R primer only since the sequence corresponding to the reverse primer was found at both ends of the sequence. The production of such spurious products is somewhat expected because the primers are degenerate. Blast X searches were used to compare the sequence obtained with all available sequences on GenBank. The results of this search indicated that the product encodes a troponin C from the fire ant (Fig. 1). Troponin C is a calcium binding protein that is critical for contraction of muscles. In vertebrate skeletal muscle calcium regulates the affinity of myosin for actin. Calcium binding to troponin C results in the release of the tropomyosin and troponin I inhibition of the actin-myosin ATPase activity, and results in activation of muscle contraction, as the myosin heads can interact with actin (Darnell et al. 1986). Regulation of insect contraction differs from that of

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 AAGAAGGGGAGCCAACAGACGACGAATAGCGCGTATAGTTACCATCGCT  -1
ATGGATGATTTAACCAAGGATCAGCAGGCCCTGTTGAAGAAAGCCTTTGAT  51
 M  D  D  L  T  K  D  Q  Q  A  L  L  K  K  A  F  D   17

GCTTTCGATCACGAAAAGAAAGGCAGTATCGGTACTAACATGGTAGGCACG 102
 A  F  D  H  E  K  K  G  S  I  G  T  N  M  V  G  T   34

ATATTGACCATGTTGGGTTACGAGCTTAGCGAAAAGACACTGCAGGAAATC 153
 I  L  T  M  L  G  Y  E  L  S  E  K  T  L  Q  E  I   51

ATTAAGGAAGTTGACGAAGACGGTTCCGGAGAACTCGAGCTCGAAGAATTC 204
 I  K  E  V  D  E  D  G  S  G  E  L  E  L  E  E  F   68

TGCACGCTGGCTGCTAGATTCTTACTAGAAGAGGATTCGGAAGCGATGCAG 255
 C  T  L  A  A  R  F  L  L  E  E  D  S  E  A  M  Q   85

CAAGAATTACGTGAGGCATTCCGGTTGTATGACAAAGAGGGCAACGGCTAC 306
 Q  E  L  R  E  A  F  R  L  Y  D  K  E  G  N  G  Y  102

ATAACTACCGCTGTTTTTCGCGATATCCTTCACGAACTCGACGACAAGCTA 357
 I  T  T  A  V  F  R  D  I  L  H  E  L  D  D  K  L  119

TCACCGGAAGAACTCGATCTAATGATCGAAGAGATCGACGCTGACGGCTCA 408
 S  P  E  E  L  D  L  M  I  E  E  I  D  A  D  G  S  136

GGAACGCTCGATTTTGACGAGTTCATGGAAGTCATGACGGGAGGCGACGAC 459
 G  T  L  D  F  D  E  F  M  E  V  M  T  G  G  D  D  153

TAAGGCAAGTTGGTAATGAAGATGGGCATTCGGAGGCTGGTTGTTAACGCA 510
ATTGGCAACGCCCGTCAGAGAGAGCGTTCTTTCTTCATCATTCAGGCACGC 561
ACTTCTGCAAACCACTAAACTACGGCCAAGCGGGAAATTGGACGTTTATGA 612
TCATTCGTGGTCTGCTGGCTCCCT                            636
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Fig. 1. Nucleotide Sequence and Deduced Amino Acid Sequence from the Red Imported Fire Ant 685 bp PCR Product. The fragment encodes troponin C. Underlined with solid lines are the two probable calcium-binding (EF-hand) domains (residues 56-67 and 132-144). Underlined with a dash-dot line are the two ancestral calcium-binding domains. (See Swiss-Prot P47947, analysis of *Drosophila melanogaster* troponin C and Fyrberg et al., 1994).

vertebrates. In insect muscles, calcium directly activates the thin and the thick filaments by binding to, respectively, troponin-C and myosin light chains (Fyrberg et al., 1994).

The 685 bp clone shows the highest similarity to the fruit fly, *Drosophila melanogaster*, troponin C 41C gene product (E value = 7.10^{-29}) the protein being 73.9% identical, followed by *D. melanogaster* troponin C isoform 1 (73.1% identity), troponin C2 from the barnacle *Balanus nubilus* (64.9%), other troponin C isoforms from the fruit fly (isoform 3, 61.9%; isoform 73F, 61.3%, isoform 2, 61%). The fire ant protein is also similar to the troponin C isoform 2a from the American lobster *Homarus americanus* and the horseshoe crab, *Tachypleus tridentatus* (Fig. 2). Analysis of the amino acid sequence against PROSITE identified two EF-hand calcium-binding domains. These correspond to residues 56-67 and 132-144, respectively, and are underlined with a solid line in Fig. 1. The dotted line shows the extent of the only EF-hand domain (residues 123-151) identified by a search using the Conserved Domain Browser (CD) from the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd). As defined at this URL, the EF-hand motif consists on a 12-residue loop, flanked on either side by a 12-residue alpha helix. Two other potential ancestral calcium binding sites are underlined with a dash-dotted line (Fig. 2). In the fruit fly *D. melanogaster*, the isoform troponin 41 C is the principal isoform of adult muscles and the isoforms 47D, 73F and 41 C may correspond to the complete complement of *Drosophila* troponin genes (Fyrberg et al., 1994). In addition to the tree troponin genes, a gene product less similar to the fire ant troponin C, CG12408 (E value = 1.10^{-24}) has been reported recently by the *Drosophila* genome project, but it is a putative calcium-binding protein similar to calmodulin (www.flybase.org).

Several other PCR products were obtained while attempting reactions with degenerate primers for transmembrane regions III and VI of class A GPCRs. These yielded the partial cloning of fire ant elongation factor 2 (EF2), filamin, and one cuticle protein. The EF2 fire ant product (850 bp) corresponds to the C-terminal region of the most similar *Drosophila* EF2 protein (P13060), and is 98% identical to the fruit fly corresponding fragment (E value = 6e-59). Many other EF2 proteins from other organisms were similar as resulted from a BlastX search. The fire ant filamin fragment (600 bp) corresponds to a region of the C-terminus of the fruit fly filamin, which is a high molecular weight protein of about 250kDa. In this homologous *D. melanogaster* sequence (accession number AF174492) it corresponds to residues 1860-2060; the full coding region being 2200 residues. In *Drosophila*, filamin is required for ring canal assembly during oogenesis (Li et al. 1999). Several other filamins from humans and chicken were also similar. The putative cuticle protein (450 bp) fragment corresponds approximately to the N-terminal half (85 residues) of the cDNA if we consider that the homologous protein in *Drosophila* has 135 residues (AAF58694.1; CG9079 gene product). It is also similar to an endocuticular protein SgAbd-3 from the desert locust (S78093), two gene products from *D. melanogaster* (CG18349 and CG7941) and a larval cuticle protein from the silk worm *Bombyx mori*, LCP18 (AB012081).

Additional products were obtained using a pair of degenerate primers for class A GPCRs in a separate reaction. A 434 bp PCR product showed the highest similarity to mitochondrial dihydrolipoamide dehydrogenase from the pig *Sus scrofa* (E value = 6.10^{-51}) (P09623) and other organisms. The seventh most similar sequence was the *D. melanogaster* gene product CG7430, accession AAF49294.1. A 652 bp product designated FA700 bp #5 is most similar to the *D. melanogaster* (E value =10^{-14}) CG9279 gene product (which is similar to the "Glued" gene), dynein-associated polypeptide from *Drosophila*, and dynactin from the mouse (E value = 10^{-9}) and other organisms. "Glued" produces a protein that is possibly an architectural filamentous component in cells of the fruit fly while dynactin plays a part in axonal transport of vesicles and organelles in the cell (Swaroop et al. 1987). The clone FA330 yielded an insert of about 300bp that has low

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Troponin C,  S.invicta           M - - - - D D L T K D Q Q A L L K K A F D A F D H E K K G S I G T N M V G T I L T M L G Y E L S E K T L Q E I I K E V D  56
TpnC41C  D.mel                   M S - - - D E L T K E Q T A L L R N A F N A F D P E K N G Y I N T A M V G T I L S M L G H Q L D D A T L A D I I A E V D  57
Troponin C, Iso 1  D.mel         M S - - - D E L T K E Q T A L L R N A F N A F D P E K N G Y I N T A M V G T I L S M L G H Q L D D A T L A D I I A E V D  57
Troponin C2,  B.nubilus          M M - - - D E L D K D Q I A M L K K A F D G F D H E K K G A I N C D V V A T I L R M M G Q A Y N A Q T L K E L I D E V D  57
Troponin C, Iso 3  D.mel         M S S V D E D L T P E Q I A V L Q K A F N S F D H Q K T G S I P T E M V A D I L R L M G Q P F D K K I L E E L I E E V D  60
Troponin C, Iso 73F  D.mel       M S S V D E D L T P E Q I A V L Q K A F N S F D H Q K T G S I P T E M V A D I L R L M G Q P F D K K I L E E L I E E V D  60
Troponin C, Iso 2a H.americanus  M D S L D E E - - - - Q I G A L Q K A F D S F D T D S K G F I T P E T V G V I L R M M G V K I S E K N L Q E V I A E T D  56
Troponin C, Iso 2  D.mel         M D N I D E D L T P E Q I A V L Q K A F N S F D H Q K T G S I P T E M V A D I L R L M G Q P F D R Q I L D E L M H E V D  60
Troponin C, T.tridentatus        M D N I D E D L T P E Q I A V L Q K A F N S F D H Q K T G S I P T E M V A D I L R L M G Q P F D R Q I L D E L M H E V D  60

Troponin C,  S.invicta           E D G S G E L E L E E F C T L A A R F L L E E D S E A M Q Q E L R E A F R L Y D K E G N G Y I T T A V F R D I L H E L D  116
TpnC41C  D.mel                   E D G S G Q I E F E E F T T L A A R F L V E E D A E A M M A E L K E A F R L Y D K E G N G Y I T T G V L R E I L R E L D  117
Troponin C, Iso 1  D.mel         E D G S G Q I E F E E F T T L A A R F L V E E D A E R M M A E L K E A F R L Y D K E G N G Y I T T G V L R E I L R E L D  117
Troponin C2,  B.nubilus          A D G S G M L E F E E F V T L A A K F L I D D D A E A M A K E L K E A F R L Y D K A G K G Y I P T S A L K D I L K E L D  117
Troponin C, Iso 3  D.mel         E D K S G R L E F G E F V Q L A A K F I V E E D A E A M Q K E L R E A F R L Y D K Q G N G F I P T T C L K E I L K E L D  120
Troponin C, Iso 73F  D.mel       E D K S G R L E F G E F V Q L A A K F I V E E D A E A M Q K E L A E A F R L Y D K Q G N G F I P T T C L K E I L K E L D  120
Troponin C, Iso 2a H.americanus  E D G S G E L E F E E F V E L A A K F L I E E D E E A L K T E L R E A F R V Y D K E G N G Y I T T D V L K E I L R E L D  116
Troponin C, Iso 2  D.mel         E D K S G R L E F E E F V Q L A A K F I V E E D D E A M Q K D V R E A F R L Y D K Q G N G Y I P T S C L K E I L K E L D  120
Troponin C, T.tridentatus        E D K S G R L E F E E F V Q L A A K F I V E E D D E A M Q K D V R E A F R L Y D K Q G N G Y I P T S C L K E I L K E L D  120

Troponin C,  S.invicta           D K L S P E E L D L M I E E I D A D G S G T L D F D E F M E V M T G G D D  154
TpnC41C  D.mel                   D K L T N D D L D M M I E E I D S D G S G T V D F D E F M E V M T G G D D  154
Troponin C, Iso 1  D.mel         D K L T N D D L D M M I E E I D S D G S G T V D F D E F M E V M T G G D    153
Troponin C2,  B.nubilus          E T L N A E D L D N I I G E I D T D G S G T V D F D E F M E M M T G        151
Troponin C, Iso 3  D.mel         D Q L T E Q E L D I M I E E I D S D G S G T V D F D E F M E M M T G E      155
Troponin C, Iso 73F  D.mel       D Q L T E Q E L D I M I E E I D S D G S G T V D F D E F M E M M T G E      155
Troponin C, Iso 2a H.americanus  N R L T E E D L D S I I E E V D E D G S G T L D F N E F M E M M N G        150
Troponin C, Iso 2  D.mel         D Q L T E Q E L D I M I E E I D S D G S G T V D F D E F M E M M T G E      155
Troponin C, T.tridentatus        D Q L T E Q E L D I M I E E I D S D G S G T V D F D E F M E M M T G E      155
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Fig. 2. Amino Acid Sequence Alignment of the Putative Fire Ant Troponin C with Troponin C Proteins from other Organisms. Identical amino acid residues are boxed. Abbreviations as follows: *S. invicta*, red imported fire ant; *D. mel.*, fruit fly; *Balanus nubilus*, barnacle; *Homarus americanus*, American lobster; *Tachypleus tridentatus*, horse shoe crab.

similarity to several different genes: translation initiation factor from bacteria, elastin precursor from rat, fruit fly *D. melanogaster* oocyte-specific regulatory protein K10, beta-3 adrenergic receptor from cat, retinoid X receptor from human, and methyl aspartate receptor from rat.

A product of 534 bp encodes for a fragment of a putative glucose transporter. Sequence analysis comparisons and hydropathy plots revealed that the isolated probe encompasses transmembrane regions 9-12 and includes the C-terminus and part of the 3' untranslated region. It is most similar (E value = 2.10^{-52}) to the *D. melanogaster* CG7801 gene product that encodes for a glucose transporter, followed by CG8234, CG10960, CG6484, all from the fruit fly, and the human glucose transporter 8 (Doege et al. 2000).

With the exception of the troponin C-encoding product, all of these PCR products encode partial open reading frames. Although we did not obtain their full sequences, these fragments can be very useful to the community of scientists studying the fire ant or other ants since they have originated from transcribed genes. These PCR products may be suitable for various experiments, such as control probes for Southern or northern blots, *in situ* hybridization, semi-quantitative PCR, or to design specific primers to amplify genomic regions to aid in phylogenetic analysis or cytogenetics and mapping.

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