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Role of the transcriptional repressor *mdGfi-1* in *CYP6D1v1*-mediated insecticide resistance in the house fly, *Musca domestica*

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Abstract

Gfi-1 is a C_2H_2-type zinc finger protein that is a transcriptional repressor in vertebrates and has been implicated in control of CYP6D1 expression in house flies (*Musca domestica*). A 15 bp insert, which disrupts a putative *mdGfi-1* binding site in the *CYP6D1v1* promoter has been implicated as a cause of increased expression of CYP6D1, and thus insecticide resistance. Using electrophoretic mobility shift assays we demonstrate that the CYP6D1 promoter from susceptible strains binds *mdGfi-1*. The 15 bp insert that interrupts the *mdGfi-1*-binding site in insecticide-resistant strains reduces the amount of *mdGfi-1* binding by 9- to 20-fold, consistent with the role of *mdGfi-1* in resistance. Partial sequences of *mdGfi-1* (spanning the first intron) from individual houseflies from 11 different strains revealed the presence of 23 alleles. There was no consistent difference in the *mdGfi-1* alleles between susceptible and CYP6D1-mediated insecticide-resistant strains, indicating that *mdGfi-1* alleles were not likely involved in resistance. Polymorphisms were used to map *mdGfi-1* to autosome 1. Quantitative real time PCR (qRT-PCR) revealed Gfi-1 expression was higher in the thorax compared to the head and abdomen, and varied between life stages and between strains. However, similar levels of *mdGfi-1* were detected in susceptible and resistant adults suggesting that altered levels of *mdGfi-1* were not likely a cause of insecticide resistance. The significance of these results to understanding insecticide resistance is discussed.

Keywords: Gfi-1; Cytochrome P450 CYP6D1; Electrophoretic mobility shift assays; Linkage analysis; Polymorphism; Quantitative real-time PCR; Insecta

1. Introduction

Gfi-1 is a C_2H_2-type zinc finger protein that is a transcriptional repressor. The proto-oncogene Gfi-1 was first cloned from rats, where it was shown to be expressed at high levels in Moloney murine leukemia virus-induced T cell lymphomas selected for growth on interleukin-2-free media (Zweidler-McKay et al., 1996). Subsequently Gfi-1 has been shown to regulate several genes involved in cell proliferation, inhibition of apoptosis, T-cell development, granulopoiesis and expression of the innate immune response (Doan et al., 2004; Duan and Horwitz, 2003; Grimes et al., 1996; Hock et al., 2004; Person et al., 2003; Yucel et al., 2004). The Gfi-1 consensus sequence has been identified in the promoter of several vertebrate genes (Zweidler-McKay et al., 1996). Recently, Gfi-1 has been shown to be a repressor of the human P450 CYP27B1 (Dwivedi et al., 2005). Sequencing of *mdGfi-1* from housefly (*Musca domestica*) revealed a high degree of sequence conservation (especially across the zinc finger region of the protein) between insects and mammals, leading to the suggestion that Gfi-1 may be an important transcriptional repressor in many taxa (Kasai and Scott, 2001). However, little is know about the functions of Gfi-1 in insects.

Cytochrome P450 CYP6D1 carries out detoxification of phenoxybenzyl pyrethroids in housefly (Scott, 1999). The LPR (pyrethroid-resistant) strain of housefly expresses a nine-fold higher level of CYP6D1 mRNA and protein relative to susceptible strains. This elevated expression is due to increased transcription of *CYP6D1v1*, and is not due to gene amplification or mRNA stabilization (Liu and Scott, 1996; Liu and Scott, 1998; Scott, 1999; Tomita and Scott, 1995). CYP6D1 was mapped to chromosome 1 and the factors responsible for increased transcription of

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CYP6D1 were mapped to chromosomes 1 and 2 (Liu and Scott, 1996; Liu and Scott, 1998; Liu et al., 1995). To identify transcription regulatory elements involved in CYP6D1 expression, the 5′-flanking region of CYP6D1 was sequenced from pyrethroid-resistant and susceptible strains of housefly (Scott et al., 1999). The most notable difference between resistant ($n = 2$) and susceptible ($n = 8$) strains was the presence of a 15 bp insert found only in the resistant strains (Scott et al., 1999; Seifert and Scott, 2002). This 15 bp insert interrupted a putative Gfi-1 binding site, leading to the suggestion that the *CYP6D1v1* promoter (from resistant strains) would bind less Gfi-1 (i.e. repressor) and this could be responsible for increased transcription of *CYP6D1v1* in resistant strains (Scott et al., 1999).

In this study, we employed electrophoretic mobility shift assays (EMSAs or gel-shifts) to demonstrate that the CYP6D1 promoters from susceptible strains bind more *mdGfi-1* than do the same promoter regions from resistant houseflies. Partial *mdGfi-1* genomic sequences were amplified from several strains of housefly and revealed numerous polymorphisms that were used to map *mdGfi-1* to autosome 1. Quantitative real time PCR (qRT-PCR) revealed Gfi-1 expression varied between life stages, strains and body regions in adults. However, similar levels of mnGfi-1 were detected in susceptible and resistant adults suggesting that altered levels of *mdGfi-1* were not likely involved in resistance. The significance of these findings to understanding insecticide resistance and the role of *Gfi-1* in insects is discussed.

2. Experimental

2.1. Electrophoretic mobility shift assays (EMSA or gel-shift)

The *mdGfi-1* protein was synthesized by a coupled transcription and translation rabbit reticulocyte lysate kit (TNT®, Promega, Madison, WI). RT-PCR amplification (Section 2.2)(forward primer: 5′-CTCGAGATGCATCA CCATCACCATCACATGTTTCAAAGACCCTTTCTA TCGCCTGCG-3′; reverse primer: 5′-TCTAGATTATAC CAACAATGAT ACCTTTTGGC-3) of *mdGfi-1* (Kasai and Scott, 2001)(accession no. AF339860) from CS house flies generated a product that was inserted into *Xho*I/*Xba*I digested pTNT® vector (Promega, Madison, WI) to produce the pTNT™-6 × His-*mdGfi-1* vector. The sequence was confirmed by direct sequencing at Cornell's Biotechnology Resource Center.

To confirm expression of *mdGfi-1*, reticulocyte lysate was separated by SDS/PAGE and electroblotted onto nitrocellulose using a BioRad (Hercules, CA) mini transblot apparatus. The membrane was developed using anti-His antibody:peroxidase conjugated ECL anti-mouse IgG (1:10000) using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Probes used for EMSA were based on the promoter sequences of CYP6D1 in the pyrethroid-susceptible (CS) and the pyrethroid-resistant (LPR) strain of housefly (Fig. 1). Probe 13 (5′-AATTTGTAAATAAAATTAA TGCTAGC-3′) was from a region of the CYP6D1 promoter that had no homology to the putative Gfi-1 binding site (i.e. used as a negative control). The sense and antisense probes were 5′-end labeled with the following reaction mixture: 1 ul oligonucleotide solution (20 ng/ul), 2 ul of 10 × T4-kinase buffer, 0.5 ul of T4-polynucleotide kinase (10 U/ul)(Invitrogen, Carlsbad, CA) and 2 ul of [γ-^{32}P] ATP (aqueous solution, 10 mCi/ml, Perkin-Elmer, Boston, MA). The mixture was incubated for 30 min at 37 °C. The labeled probe was purified with a QIAquick nucleotide removal kit (Qiagen, Valencia, CA). Probes were eluted from the column with 100 ul 10 mM Tris (pH 8.0). The purified sense and antisense probes (both 100 ul) were combined and incubated for 10 min at RT for annealing. Binding reactions (12 ul) contained 1 ul labeled double-stranded oligonucleotide probe (0.1 ng/ul), 20 mM HEPES, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 0.1 ul poly(dI-dC)(10 ug/ul, Roche Applied Science, Indianapolis, IN), and 0.05–1 ul reticulocyte lysate containing *mdGfi-1* protein. The mixture was incubated for 15 min at 37 °C and analyzed on a 5% polyacrylamide gel (16 × 14 cm). The gel was run for 1.5–2 h at 100 V, dried and analyzed using a Storm® gel and blot imaging system (Amersham Biosciences, Piscataway, NJ). Quantitation was performed using ImageQuant TL (Amersham Biosciences, Piscataway, NJ) software. The experiment using probes I and II (Fig. 3) was replicated three times, and the experiment using probes 13, 15 and 16 (Fig. 4) was replicated six times.

2.2. Polymorphisms of mdGfi-1

Eleven laboratory strains of housefly were used. Cornell susceptible (CS) is an insecticide-susceptible strain reared without exposure to insecticides (Hamm et al., 2005), SRS is

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  Probe I  (oligo)                      5′-TTTACATGCATTCGAATCATTCTGTTTCACAAAATGACCGGCAACTATTCAGTTG-3′
  Probe II (oligo)                      5′-TTTACATGCATTCGAATCAT---------------GACCGGCAACTATTCAGTTG-3′
  Probe 15 (Oligo)                      5′-TTTACATGCATTCGAATCAT---------------GACCGGCAACTATTCA-3′
  Probe 16 (Oligo)                            5′-CATTCGAATCATTCTGTTTCACAAAATGACCGGCAA-3′
LPR CYP6D1 Promoter 5′-ATGATAAGAAATGTGCAAAGTTTTACATGCATTCGAATCATTCTGTTTCACAAAATGACCGGCAACTATTCAGTTGTTAATGTAACA-3′
CS  CYP6D1 Promoter 5′-ATGATAAGAAATATGCAAAGTTTTACATGCATTCGAATCAT---------------GACCGGCAACTATTCAGTTGTTAATGTAACA-3′
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Fig. 1. Probes used in the electrophoretic mobility-shift assay and their positions in the *CYP6D1* promoter of susceptible (CS) and resistant (LPR) strains. Probes I and 16 contained the 15 bp-insert (5′-TCTGTTTCACAAAAT-3′) characteristic of resistant strains. Probes II and 15 contain the putative Gfi-1-binding site (shaded) without the 15 bp-insert.