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Rapid Communication

The house fly aliesterase gene (*MdαE7*) is not associated with insecticide resistance or P450 expression in three strains of house fly

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Abstract

It was recently proposed that a mutation (G137D) in the *MdαE7* gene was responsible for increasing transcription of a P450 (*CYP6A1*) resulting in resistance to diazinon. To examine if *MdαE7* had a role in resistance in other strains we sequenced a fragment (~700 bp) of the *MdαE7* gene from individual flies of two insecticide susceptible and three insecticide resistant (due to increased monooxygenase-mediated detoxification) strains. Five unique alleles were discovered. While all of the susceptible strains had Gly137, so did the resistant LPR and NG98 strains. Of the two alleles in the YPER strain one had the G137D substitution and the other did not. Based on the lack of correlation between the presence of the 'mutant' *MdαE7* and resistance (or P450 levels), we conclude that the G137D mutation in *MdαE7* is not involved in transcriptional control of the P450s involved in resistance in the LPR, NG98 or YPER strains. The relationship between *MdαE7* alleles and insecticide resistance is discussed in light of these findings.

Keywords: Gene regulation; Cytochrome P450; Insecticide metabolism; Mutant aliesterase

1. Introduction

Insecticide resistance is a significant problem, both in terms of control of pests of economic and health importance, and as a valuable phenomenon for investigating basic scientific questions such as evolution, toxicology, and gene regulation. Thus, understanding of the molecular mechanisms involved is of fundamental importance to advances in both the applied and basic aspects of resistance studies.

The cytochrome P450 monooxygenases are an important metabolic system involved in the detoxification/activation of xenobiotics (as well as in the anabolism and catabolism of endogenous compounds including hormones and pheromones). P450 monooxygenases have a large array of substrates they metabolize because of the presence of numerous P450s in each species (genome sequencing revealed 80 putative P450 genes in *Caenorhabditis elegans* (Consortium, 1998), 57 in humans (Nelson, 2002), 90 (including four to seven pseudogenes) in *Drosophila melanogaster* (Adams et al., 2000; Tijet et al., 2001)) and the broad substrate specificity of some P450s (Rendic and Di Carlo, 1997). For these reasons, P450 monooxygenases play an important role in insecticide metabolism. For some classes of insecticides (e.g. pyrethroids), P450s are almost exclusively involved in detoxification. For other classes of insecticides, such as many organophosphates (OPs), P450s are involved both in the activation of the parent compound, such as diazinon (a phosphorothionate) into insecticidal diazoxon (a phosphate), as well as being involved in detoxification. P450 monooxygenases are thus critical for the insecticidal activity of OPs to be manifest. While a given P450 may be readily able to convert the non-toxic OP into the activated compound, the same P450 may have little or no ability to metabolize the activated phosphate (Hatano and Scott, 1993). Thus, cases of insecticide resistance can logically be tied to overexpression of a P450 involved in detoxification of

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compounds such as pyrethroids. However, the role of P450s in OP resistance will be more complex; being determined by the balance of activation and detoxification reactions (Shono, 1974a; Shono, 1974b).

Hydrolases are a large group of heterogeneous enzymes present in most organisms. Included in this category are phosphatases, carboxylesterases and others. Hydrolases (specifically carboxylesterases) have been implicated as a mechanism of resistance to OPs and carbamates in numerous species (Hemingway and Karunarantne, 1998). Some of the first studies on hydrolase-mediated resistance noted that OP resistant strains had a decreased ability to hydrolyze model substrates such as α-naphthyl acetate, leading to the so called 'mutant aliesterase' theory (Oppenoorth, 1985). Subsequent studies showed that OP-resistant strains may have equal, greater or lower rates of α-naphthyl acetate hydrolysis compared to susceptible strains. Thus, the mutant aliesterase hypothesis lay dormant for many years. In 1997, new insight was provided with studies on OP resistant *Lucilia cuprina* by Newcomb et al who found that a single amino acid (G137D) mutation caused a carboxylesterase (E3) to lose its catalytic activity toward model substrates, but gain activity toward the OP chlorfenvinphos (Newcomb et al., 1997). Because chlorfenvinphos is the active form of the insecticide (i.e. it does not require activation), metabolism can logically be equated to resistance. Thus, a mutation resulting in a single amino acid change was sufficient to cause resistance. Recently, the homologous gene (*MdαE7*) was isolated from house fly and a similar substitution was identified in the OP resistant Rutgers strain which overexpresses CYP6A1 and CYP6D1 (Carino et al., 1992). In the Rutgers strain there was a strong correlation between resistance (or CYP6A1 expression) and the lack of the Gly137 *MdαE7* allele, leading the authors to propose that *MdαE7* controlled transcription of the P450s involved in resistance (Sabourault et al., 2001).

In this study, we sequenced *MdαE7* from two susceptible and three insecticide resistant (monooxygenase-mediated) strains. There was no correlation between the presence of the 'mutant' *MdαE7* and resistance. The relationship between *MdαE7* alleles and insecticide resistance is discussed in light of these findings.

2. Materials and methods

Five house fly strains were used. The aabys strain is insecticide-susceptible and bears the recessive visible mutant markers *ali-curve* (*ac*), *aristapedia* (*ar*), *brown body* (*bwb*), *yellow eyes* (*ye*) and *snip wings* (*snp*) on chromosomes 1 to 5, respectively. This inbred strain was originally obtained from T. Hiroyoshi (Osaka University, Japan) and has been reared in our laboratory for 16 years. CS (Cornell-susceptible) is a wild-type susceptible strain (Scott et al., 1996) maintained in our laboratory for >10 years. LPR is a multi-resistant strain (originally from New York) with high levels of resistance to pyrethroid insecticides due to *kdr*, *pen* and increased oxidative metabolism mediated by *CYP6D1v1* (Scott and Georghiou, 1986; Seifert and Scott, 2002; Shono et al., 2002; Wheelock and Scott, 1992). LPR has 13- to 29-fold resistance to the three OPs it has been evaluated against (Scott and Georghiou, 1986), and has been reared in our laboratory for 16 years. NG98 is a multi-resistant strain (originally collected in Georgia in 1998) with 3660-fold resistance to permethrin due to monooxygenase-mediated detoxification and *kdr* (Kasai and Scott, 2000) (Scott and Kasai, unpublished). YPER is a multi-resistant strain (originally collected in Japan in 1997), with >18 000-fold resistance to permethrin due to monooxygenase-mediated detoxification and *super-kdr* (Shono et al., 2002). Although there are no reports investigating OP resistance in YPER, the site where this strain was collected (3rd Yumenoshima Island) has a long history of OP use. Previous collections of flies from 3rd Yumenoshima Island showed high levels of resistance to OPs such as malathion, fenitrothion and diazinon (Yasutomi et al., 1988).

Genomic DNA was extracted from single flies as described previously (Seifert and Scott, 2002) with some modifications. Individual flies were homogenized in 0.5 ml lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 0.15 mM spermine and 0.5 mM spermidine) with 1% SDS and 2.5 μl of proteinase K (>400 U/ml, Gibco BRL) and incubated at 37°C for 1–2 h. The mixture was then extracted three times with equal volumes of phenol:chloroform (1:1). After precipitation with ethanol, DNA was dissolved in 0.2 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 0.1 mg/ml of RNase and incubated at 37°C for 30 min. The solution was extracted with an equal volume of phenol:chloroform (1:1) and precipitated again with ethanol. The pelleted genomic DNA was dissolved in H_2O and used as the template for the polymerase chain reaction (PCR). PCR was performed with *MdαE7* specific primers based on the sequence in GenBank (accession No. AF133341): Md_aliest_F, ACAGACAGATTTCATAAGTGG; Md_aliest_R, TTTGCATTTCTTCGGGTGTCA. Taq polymerase was from Gibco/BRL. PCR conditions were the same as in Sabourault et al., 2001. The PCR products were purified using Prep-A-Gene DNA purification kit (Bio-Rad) and sequenced by the Cornell Biotechnology Facility.

Sequences were aligned and compared using the MegAlign (3.06 b) program from DNASTAR (Madison, WI, USA). The sequence of *MdαE7* was determined from a minimum of three individual flies in both the forward and reverse direction for each strain, and then compared with the *MdαE7* allele from the Rutgers strain (GenBank AF133341) (Claudianos et al., 1999).

3. Results and discussion

A fragment (~700 bp) of the *MdαE7* gene was amplified from individual flies of two insecticide susceptible and three insecticide resistant strains. Five unique alleles were discovered (Table 1). The CS and aabys strains had identical alleles. The NG98 and YPER stains were both found to contain two different alleles. One of the alleles in YPER was identical to that found in CS and aabys (YPER-2, Table 1). All sequences indicated the presence of two introns (II and III) with sizes of 63–64 bp and 62 bp, respectively which agrees with a previous study (Claudianos et al., 1999). Both introns follow the GT AG rule (Lewin, 1997). These alleles were 95.5-99.7% identical (after removal of the introns) to the previously published cDNA sequence (Claudianos et al., 1999) from the Rutgers strain (Table 1). The sequence of *MdαE7* from aabys, CS and LPR was determined using five, seven and four individual flies, respectively. There was no polymorphism detected in these three strains. The NG98-1 and NG98-2 *MdαE7* alleles were each detected (apparently homozygous) in two individual flies. A fifth NG98 fly was polymorphic for both alleles. The YPER-2 allele was detected in three YPER flies (apparently homozygous). The YPER-1 allele was found in two flies that were heterozygous for the YPER-1 and YPER-2 alleles. In only one case was there a polymorphism corresponding to the G137D substitution (i.e. all alleles had G137 except for YPER-1).

If the G137D mutation in *MdαE7* was responsible for resistance by increasing transcription of P450 genes as was previously proposed (Sabourault et al., 2001), we would hypothesize that the monooxygenase-mediated resistant strains (which overexpress P450s) would have the G137D substitution and the susceptible strains would not. This was not what we found. While all of the susceptible strains had Gly137, so did the resistant LPR and NG98 strains (both alleles in NG98). Of the two alleles in the YPER strain, one had the G137D mutation and the other did not. An alignment of the predicted amino acid sequences of the different alleles is given in Fig. 1. Although there are increased levels of P450s in the LPR, NG98 and YPER resistant strains, and in at least one case (*CYP6D1* in LPR) this has been directly shown to be due to in increased rate of transcription (Liu and Scott, 1998), there is no association between the G137D substitution in *MdαE7* and insecticide resistance, total P450 levels or levels of *CYP6D1*. Additionally, LPR has been shown to overexpress CYP6A1 mRNA (levels are greater than in Rutgers) (Carino et al., 1992), and CYP6D1 (which is overexpressed in LPR) is capable of metabolizing chlorpyrifos to chlorpyrifos oxon (Hatano and Scott, 1993). These results indicate there is no clear correlation between the *MdαE7* allele and CYP6A1 expression, or the *MdαE7* allele and expression of P450s involved in OP metabolism. Thus, we conclude that the G137D mutation in *MdαE7* is not involved in transcriptional control of the P450s involved in resistance in the LPR, NG98 or YPER strains. Therefore, it is doubtful if *MdαE7* is the 'master gene' (Plapp, 1984) responsible for resistance on autosome 2.

Given that *MdαE7* is not associated with resistance in the LPR, NG98 or YPER strains, a re-examination of its role in diazinon resistance in the Rutgers strain (Sabourault et al., 2001) appears necessary. In the Rutgers strain there was a strong correlation between resistance (or CYP6A1 expression) and the lack of the Gly137 *MdαE7* allele, leading the authors to propose that *MdαE7* controlled transcription of *CYP6A1* (and thus resistance). There are some potential problems with this proposed model for the Rutgers strain. First, diazinon is not a potent inhibitor of acetylcholinesterase, the target site for OP insecticides. It requires activation (by P450s) to diazoxon to exert its highly toxic effects. Because CYP6A1 activates diazinon to diazoxon, it is difficult to reconcile how overexpression of this P450 (in the absence of other factors) could cause resistance. The role of P450s in diazinon/diazoxon resistance is quite complex; being determined by the balance of activation and detoxification reactions as well as being influenced by

Table 1
Percent identity of eight different *MdαE7* alleles from different strains of housefly. Values in upper right portion of table are for genomic sequences and values in lower left are for cDNA sequences (i.e. genomic sequences with the introns removed)

	aabys	CS	LPR	NG98-1	NG98-2	YPER-1	YPER-2
aabys	-----	100.0	97.3	97.9	96.6	96.3	100.0
CS	100	-----	97.3	97.9	96.6	96.3	100.0
LPR	97.8	97.8	-----	96.7	96.5	97.9	96.7
NG98-1	98.3	98.3	97.6	-----	97.5	96.1	95.8
NG98-2	97.0	97.0	96.2	97.4	-----	99.9	96.6
YPER-1	96.7	96.7	95.9	97.1	99.8	-----	96.3
YPER-2	100	100	97.7	98.3	97.0	96.7	-----
Rutgers[a]	96.4	96.4	95.5	96.9	99.5	99.7	96.3

[a] From (Claudianos et al., 1999).

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aabys     GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGGFIFGEANRNWYGPDYFMKK   50
CS        GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGGFIFGEANRNWYGPDYFMKK   50
LPR       GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGGFIFGEANRNWYGPDYFMKK   50
NG98-1    GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGGFIFGEANRNWFGPDYFMKK   50
NG98-2    GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGGFIFGEANRNWFGPDYFMKK   50
YPER-1    GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGDFIFGEANRNWFGPDYFMKK   50
YPER-2    GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGGFIFGEANRNWYGPDYFMKK   50
Rutgers   GSEDCLYLNVYTNDLNPDKKRPVMVFIHGGDFIFGEANRNWFGPDYFMKK   50

aabys     PVVLVTVQYRLGVLGFLSLKSENLNVPGNAGLKDQVMALRWVKSNIANFG  100
CS        PVVLVTVQYRLGVLGFLSLKSENLNVPGNAGLKDQVMALRWVKSNIANFG  100
LPR       PVVLVTVQYRLGVLGFLSLKSENLNVPGNAGLKDQVMALRWVKSNIANFG  100
NG98-1    PVVLVTVQYRLGVLGFLSLISENLNVPGNAGLKDQVMALRWVKSNIANFG  100
NG98-2    PVVLVTVQYRLGVLGFLSLKSENLNVPGNAGLKDQVMALRWVKSNIANFG  100
YPER-1    PVVLVTVQYRLGVLGFLSLKSENLNVPGNAGLKDQVMALRWVKSNIANFG  100
YPER-2    PVVLVTVQYRLGVLGFLSLKSENLNVPGNAGLKDQVMALRWVKSNIANFG  100
Rutgers   PVVLVTVQYRLGVLGFLSLKSENLNVPGNAGLKDQVMALRWVKSNIANFG  100

aabys     GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSWASTEC  150
CS        GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSWASTEC  150
LPR       GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSSASTEC  150
NG98-1    GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSLASTEC  150
NG98-2    GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSWASTEC  150
YPER-1    GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSWASTEC  150
YPER-2    GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSWASTEC  150
Rutgers   GDVDNITVFGESAGGASTHYMMITEQTRGLFHRGIMMSGNSMCSWASTEC  150

aabys     QSRALTMAKRVGYKGEENEKDILEFLMKANPYDLIKEEPQ            190
CS        QSRALTMAKRVGYKGEENEKDILEFLMKANPYDLIKEEPQ            190
LPR       QSRALTMAKRVGYKGEENEKDILEFLMKANPYDLIKEEPQ            190
NG98-1    QSRALTMAKRVGYKGEDNEKDILEFLMKANPYDLIKEEPQ            190
NG98-2    QSRALTMAKRVGYKGEDNEKDILEFLMKANPYDLIKEEPQ            190
YPER-1    QSRALTMAKRVGYKGEDNEKDILEFLMKANPYDLIKEEPQ            190
YPER-2    QSRALTMAKRVGYKGEENEKDILEFLMKANPYDLIKEEPQ            190
Rutgers   QSRALTMAKRVGYKGEDNEKDILEFLMKANPYDLIKEEPQ            190
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Fig. 1. Alignment of the deduced amino acid sequences of *MdαE7* alleles from susceptible (aabys and CS) and monooxygenase-mediated insecticide resistant strains (LPR, NG98 (two alleles) and YPER (two alleles)) of house fly in comparison to the previously published sequence from the Rutgers strain (Claudianos et al., 1999). Amino acids differing from the Rutgers sequence are boxed. The position corresponding to Gly137 of *MdαE7* and *LcαE7* (Claudianos et al., 1999) is indicated by an asterisk.

hydrolases and glutathione *S*-transferases (Motoyama and Dauterman, 1975; Shono, 1974a; Shono, 1974b). Second, CYP6A1 mRNA is expressed at higher levels in the LPR strain than in the Rutgers strain (Carino et al., 1992), even though the LPR strain has the wild-type *MdαE7* allele (Fig. 1). Third, the increased level of CYP6A1 mRNA found in the Rutgers strain is not necessarily due to increased transcription, as P450 mRNA levels can also be elevated by mRNA stabilization (Porter and Coon, 1991).

It was suggested (Sabourault et al., 2001) that some strains of house fly may have a *MdαE7* null allele based on the absence of PCR product (using a single pair of primers). Our results suggest an alternative reason why no PCR product could have been formed from some strains. *MdαE7* is reasonably polymorphic based on our results (Table 1). This observation, combined with the fact that the reverse primer used for detection (i.e. lack of PCR product formation) of the null allele was not an exact match (to the sequence in GenBank), suggests a polymorphism could have reduced annealing sufficiently to prevent PCR amplification.

If *MdαE7* is not responsible for increased transcription of *CYP6A1* then why is it linked with diazinon resistance in Rutgers? *MdαE7* is a carboxylesterase capable of metabolizing substrates such as methyl butyrate or naphthyl acetate (Newcomb et al., 1997). The Gly137Asp mutation results in an enzyme with a decreased ability to hydrolyze these substrates and an increased ability to hydrolyze chlorfenvinphos (Newcomb et al., 1997). This would logically lead to resistance to some OPs. It was suggested (Sabourault et al., 2001) that the protection (i.e. detoxification of diazoxon) afforded by the mutant *MdαE7* in the Rutgers strain was not important relative to the rates at which CYP6A1 and CYP12A1 metabolized diazoxon. However, these calculations appear to be based on an invalid comparison. The catalytic activities of CYP6A1 and CYP12A1 were evaluated only against diazinon. P450 metabolism of OPs such as diazinon leads to an intermediate

(Nakatsugawa and Morelli, 1976) which then forms either diazoxon (highly toxic) or 2-isopropyl-4-methyl-6-hydroxypyrimidine (practically non-toxic)(Shono, 1974a; Shono, 1974b). Thus, formation of 2-isopropyl-4-methyl-6-hydroxypyrimidine from diazinon does not indicate anything about the rate at which diazoxon is detoxified. The only way that the catalytic activity toward diazoxon can be addressed is by using it as the substrate. Since this was not done, the actual rates of diazoxon detoxification by CYP6A1 and CYP12A1 are unknown. Because the mutant *MdαE7* was evaluated only against chlorfenvinphos (a phosphate, i.e. the active form of the insecticide (Claudianos et al., 1999)) it may be reasonably linked to diazinon/diazoxon resistance (i.e. the mutant form in the resistant strain detoxifies chlorfenvinphos, while the form in the susceptible strain does not). Unfortunately, the catalytic activity towards diazoxon has not been reported. Thus, we suggest that the mutant esterase found in Rutgers is actually the major mechanism of diazinon/diazoxon resistance because of its likely ability to detoxify the activated insecticide (but not because of its ability to control the transcription of P450s) and that the role of CYP6A1 in diazinon resistance remains uncertain. Although CYP6A1 plays a role in diazinon metabolism, simultaneously generating the highly toxic diazoxon and converting some of the diazinon into inactive 2-isopropyl-4-methyl-6-hydroxypyrimidine, its role (if any) in metabolism of diazoxon (and thus resistance) remains unknown. One possible explanation for the strong correlation between the increased levels of CYP6A1 and the mutant *MdαE7* gene could be that they work in concert to give OP resistance (CYP6A1 for conversion of diazinon to diazoxon (and 2-isopropyl-4-methyl-6-hydroxypyrimidine) plus *MdαE7* for detoxification of diazoxon). This is an intriguing possibility that deserves further study.

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