INCREASED LEVELS OF THE CELL CYCLE INHIBITOR PROTEIN, DACAPO, ACCOMPANY 20-HYDROXYECDYSONE-INDUCED G1 ARREST IN A MOSQUITO CELL LINE

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Abstract

When treated with the steroid hormone 20-hydroxyecdysone (20E), C7-10 cells from the mosquito, Aedes albopictus, arrest in the G1 phase of the cell cycle. To explore whether 20E-mediated cell cycle arrest proceeds through increased levels of cell cycle inhibitor (CKI) proteins, we cloned the Ae. albopictus homolog of dacapo, the single member of the Cip/Kip family of CKI proteins known from Drosophila melanogaster. The Ae. albopictus dacapo cDNA encoded a 261-amino acid homolog of the Aedes aegypti protein XP_001651102.1, which is encoded by an ~23 kb gene containing three exons. Like dacapo from D. melanogaster, the ~27 kDa protein from Aedes and Culex mosquitoes contained several S/TXXE/D motifs corresponding to potential protein kinase CK2 phosphorylation sites, and a binding site for proliferating cell nuclear antigen (PCNA). When extracts from cells treated with 20E were analyzed by western blotting, using a primary antibody to synthetic peptides from the mosquito dacapo protein, up-regulation of an ~27 kDa protein was observed within 24 h, and the abundance of the protein further increased by 48 h after hormone treatment. This is the first investigation of a cell cycle inhibitory protein in mosquitoes. The results reinforce growing evidence that 20E affects expression of proteins that regulate cell cycle progression.

Keywords

mosquito; insect cell line; cell cycle; dacapo; 20-hydroxyecdysone; G1 arrest

INTRODUCTION

Proliferation of 20-hydroxyecdysone (20E)-responsive insect cell lines is typically depressed in response to hormone treatment. In the Kc cell line from *Drosophila melanogaster*, and in some lepidopteran cells, 20E-treated cells arrest in the G2 phase of the cell cycle, after DNA synthesis has been completed (Besson et al., 1987; Auzoux-Bordenave et al., 2002; Siaussat et al., 2005, 2008). In contrast, 20E arrests proliferation of the C7-10 cell line from the mosquito, *Aedes albopictus*, in G1, and levels of cyclin A, which is required for progression into the S phase of the cycle, decrease in 20E-treated cells (Gerenday and Fallon, 2004). To better understand how 20E interacts with the cell cycle, we have begun to investigate proteins that participate in cell cycle control and are likely to be up-regulated in C7-10 cells after treatment with 20E.

In mammalian cells, G1 arrest is accompanied by accumulation of cyclin-dependent kinase inhibitor (CKI) proteins (Coats et al., 1996). CKIs that interact with cyclins D, E, A and B,

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and their associated cyclin dependent kinases (CDKs), belong to the Cip/Kip family, and include p21$^{Cip/Waf1/Sdi1}$ (p21), p27^{Kip1} (p27) and p57^{Kip2} (p57), which were initially thought to be tumor suppressors (Besson et al., 2008). Cip/Kip proteins share a conserved N-terminal domain that interacts with cyclins and CDKs that participate in the G1/S transition (Liu et al., 2002). In mammalian cells, p27 modulates cell cycle arrest/reentry in a tissue specific manner, and changes in abundance of p27 have been noted in cells treated with estrogen and progesterone (Musgrove et al., 1998). As a basis for this study, we hypothesized that mosquito homologs of the mammalian Cip/Kip proteins become more abundant as C7-10 cells arrest in response to 20E.

The *Drosophila melanogaster* genome encodes a single Cip/Kip protein known as dacapo (CG1772; synonyms include *dap*, CDI4, p27^{Dap}, and p21^{dacapo}), which has a predicted mass of ~27kDa. Absence of *dacapo* results in an embryonic-lethal phenotype, and loss-of-function mutants have an extra division cycle in embryonic epidermal cells. Conversely, premature expression of *dacapo* causes precocious G1 arrest, supporting the suggestion that dacapo coordinates exit from the cell cycle with terminal differentiation (De Nooij et al., 1996; Lane et al., 1996). In *Drosophila* oocytes, dacapo maintains a state of arrest in meiotic prophase II, and oscillation of dacapo abundance relative to levels of cyclin E regulates endocycling of the nurse cells (Hong et al., 2007).

We took advantage of limited amino acid sequence homologies between *D. melanogaster* dacapo and a hypothetical *Ae. aegypti* protein, to obtain cDNA encoding the *Ae. albopictus* dacapo. We used the deduced amino acid sequence of *Ae. albopictus* dacapo to identify short conserved peptides for production of polyclonal antibody, showed that the antibody reacts with a ~27 kDa protein in extracts from *Ae. albopictus* cells, and demonstrated that the abundance of this protein increases in cells treated with 20E.

MATERIALS AND METHODS

Reagents

Deoxyribonucleotides and *Taq* DNA polymerase were from Promega (Madison, WI); Bio-Rad protein assay and Freeze "N" Squeeze DNA gel extraction spin columns were from Bio-Rad Laboratories (Hercules, CA), and Micro-Fast Track 2.0 kit, SuperScript® III First-Strand synthesis system, and pEXP5-NT/TOPO TA cloning vector and PCR primers were from Invitrogen Corporation (Carlsbad, CA). Primary antibody against *Ae. albopictus* dacapo was prepared by New England Peptide LLC (Gardner, MS) from rabbits immunized with a mixture of two synthetic peptides, Ac-VDKQESKKFIDRQLAC-amide and Ac-RITDFLKESKRLSPGS-amide. Western blots were probed with goat anti-rabbit IgG conjugated with horseradish peroxidase as the secondary antibody, and developed using Super Signal West Pico, from Thermo Scientific, Waltham, MA. 20E from Sigma-Aldrich (St. Louis, MO) was prepared in 10% ethanol as described previously (Gerenday and Fallon, 2004). Components for cell culture media were from Invitrogen, and fetal bovine serum from Atlanta Biologicals was heat-treated at 56°C.

Cells and Culture Conditions

Cells were maintained in Eagle's minimal essential medium supplemented with vitamins, glutamine, nonessential amino acids, antibiotics, and 5% heat-inactivated fetal bovine serum as described by Shih et al. (1998). Medium with 5% serum is called E-5 medium. For expression experiments, medium was aspirated from monolayer cultures, and replaced with fresh medium, with or without 20E.

cDNA Synthesis

Total RNA was prepared from C7-10 cells using guanidine isothiocyanate (Davis et al., 1986), and mRNA was prepared from total RNA using the Micro-Fast Track 2.0 kit. cDNA was prepared using the Superscript III First-Strand Synthesis system following the manufacturer's instructions. PCR products were recovered from agarose gels using Squeeze "N" freeze columns, and a portion of the flow through was sequenced at the University of Minnesota BioMedical Genomics center.

PCR Reactions

PCR reactions were carried out on a Techne TC-312 thermo cycler. Initial reactions with primer pairs F20/R23 and F23/Oligo(dT) included an initial denaturation at 94°C, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, and elongation at 72°C for 2 min, with a final extension at 72°C for 10 min. Full length dacapo cDNA was obtained using Platinum Taq DNA Polymerase High Fidelity with an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and elongation at 68°C for 2 min, with a final extension at 68°C for 10 min. The product was recovered from an agarose gel and cloned into pEXP5-NT/TOPO TA vector.

Western Blots

For detection of dacapo by Western blotting, cells (2×10^6 in 12 ml) were plated in E-5 medium in 100 mm diameter tissue culture dishes. After 48 h, the medium was replaced with fresh E-5 with or without 20E at a final concentration of 2×10^{-6} M. Cells were collected 24 and 48 h later and lysed in RIPA buffer containing proteinase inhibitors as described previously (Gerenday and Fallon, 2004). Cell pellets were disrupted by sonication, and total protein was assayed using the Bio-Rad Protein Assay. Approximately 40 µg/lane was electrophoresed on 12% polyacrylamide minigels with 1.5 mm spacers.

Gels were blotted onto nitrocellulose membrane using a Mini-Genie Blotter (Idea Scientific, Minneapolis, MN) at 12 volts for 1 h at room temperature using Tris-glycine transfer buffer containing 3.03 g of Tris base, 14.4 g glycine, and 20% methanol. The membrane was incubated in blocking solution made by dissolving 5% dry milk in TBST (20 mM Tris-HCl, pH 7.5, 0.5M NaCl, 0.05% Tween-20) for 1 h at room temperature, after which primary antibody (1:2,500 dilution) was added directly to the blocking solution. Incubation was continued overnight at 10°C with gentle rotation. The membrane was briefly rinsed three times with TBST at room temperature, and washed in fresh TBST for three consecutive 5 min periods, followed by a fourth wash in TBST for 15 min. The membrane was incubated with secondary antibody in blocking solution for 1 h at room temperature, and the membrane was washed as described above. Blots were developed using Super Signal West Pico (Thermo Scientific) according to the manufacturer's instructions. The image was visualized by exposure to Kodak Biomax Light Film for 3–5 min.

RESULTS

Ae. albopictus Dacapo cDNA

The *D. melanogaster* genome contains a single gene (CG1772) encoding a homolog of mammalian Cip/Kip CKI proteins. The CG1772 sequence spans 3.8 kb, and encodes a 2.4 kb mRNA, encoding a protein called dacapo. The *dacapo* gene has three exons, with a short 5′-intron separating exons 1 and 2, and an approximately 1.3 kb intron separating exons 2 and 3. Initial BLAST searches of the *An. gambiae* genome database with *dacapo*-based nucleotide or protein queries failed to reveal sufficient homology to support recovery of a mosquito homolog from C7-10 cells. After publication of the *Ae. aegypti* genome, a tblastn search recovered two separate peptides that allowed design of primers for PCR-based

recovery of the *Ae. albopictus* homolog. These peptides (boxed in Fig. 1) lie within hypothetical *Ae. aegypti* protein XP_001651102. With introduction of gaps, XP_001651102 was 36% identical to dacapo from *D. melanogaster*, with 51% positive amino acids over 237 residues, and an *E* value of 7*e*–21. Residues 40 to 90 corresponded to a conserved CDI (CKI) domain in NCBI CDD pfam 02234.

We used primers based on *Ae. aegypti* nucleotide sequences and oligo(dT) to obtain the dacapo cDNA from *Ae. albopictus* (Fig. 2). Initially, PCR products from primer pairs F20/R23 and F23/oligo(dT) were excised from agarose gels, sequenced, and compared with the genomic DNA sequence from *Ae. aegypti*. To synthesize the full length cDNA from *Ae. albopictus*, we used primer F20 with the specific reverse primer R20, based on nucleotide sequence downstream of the stop codon (Fig. 2).

A search with the full-length *Ae. albopictus* cDNA against sequences in the insect nr database (taxid 50557) by discontinuous megablast (Altschul et al., 1997) gave matches to partial mRNA XM_001651052.1 from *Ae. aegypti* (*E* value 0.0), and XM_001847110.1 from *Culex quinquefasciatus* (9*e*–56), but failed to find matches with nucleotide sequence data from *Anopheles* mosquitoes. As expected, nucleotide identities were higher between the two *Aedes* mosquitoes, relative to *Cx. quinquefasciatus*, and most of the short insertions in the *Culex* sequence were multiples of three nucleotides. Two stretches of *Culex* sequence (nt 1–403 and nt 571–658) are shown in Figure 2. With TBLASTX we identified XM_001651052.1 from *Ae. aegypti* (2*e*–84), and XM_001847110.1 from *Cx. quinquefasciatus* (5*e*–48), as well as several matches with members of the Drosophilidae, and with the human body louse (4*e*–5).

Aedes albopictus Dacapo Protein

The conceptual *Ae. albopictus* dacapo protein contained 261 amino acids, with 76% identity, 84% similarity to its *Ae. aegypti* homolog; 55% identity, 72% similarity to the *Cx. quinquefasciatus* homolog; 37% identity, 53% similarity to *D. melanogaster*, and 36% identity, 52% similarity to *Chymomyza costata* protein sequences. Note that *C. costata* is in the family Drosophilidae, and that several dacapo homologs in various species of *Drosophila* are not included in the alignment.

Our search for Dipteran homologs of *Ae. albopictus* dacapo uncovered hypothetical protein AND-15659 from *Anopheles darlingi*. Although this protein has N-terminal sequence indicative of a member of the CDI superfamily, its limited similarity to dacapo proteins from either the Culicidae or the Drosophilidae (Fig. 3) suggest that it is not the *Anopheles* homolog of the *Ae. albopictus* dacapo described here. Likewise, generation of a tree from all dacapo sequences described for Insecta (taxid 50557) with the NCBI multiple alignment tool, COBALT, showed that the *An. darlingi* protein had less homology to the other mosquito proteins than the multiple dacapo proteins in various *Drosophila* species (Fig. 4). This inconsistency with the known phylogeny of the Diptera raises the possibility that mosquitoes express an additional CKI protein reminiscent of the multiple Cip/Kip proteins in mammals. Outside the diptera, putative dacapo homologs from the beetle, *Tribolium castaneum* (XP_976393.1) and from *An. gambiae* (XP_307826.3) were too short to align accurately.

In contrast to mammalian p27 proteins, which share a single strict consensus CD2 phosphorylation site (S/TXXE/D) at serine-83 (Tapia et al., 2004), *Ae. albopictus* dacapo contains four S/TXXE/D motifs that are potentially phosphorylated by protein kinase CK2. These lie at residues 10–13 and 116–119; two tandem motifs occur at residues 160–163; 164–168. With the exception of *An. gambiae* and *An. darlingi*, the tandem motifs are conserved among the Diptera, and in the Drosophilidae, a shared motif is displaced by only

three amino acids from the 116–119 motif in *Aedes* and *Culex* mosquitoes (Fig. 3). In general, however, the positions of additional S/TXXE/D boxes in *Drosophila* and *Chymomyza* differ from those of the mosquito homologs. The abundance of potential CK2 phosphorylation sites in the Dipteran proteins, relative to mammalian p27, may reflect a multiplicity of functions in a single Cip/Kip protein, relative to three Cip/Kip proteins in mammals. For example, in *Drosophila*, dacapo is involved in embryonic functions that resemble those of mammalian p57.

Finally, dacapo proteins from both Culicidae and Drosophilidae contain a proliferating cell nuclear antigen (PCNA) binding motif toward the C-terminal end of the protein (Fig. 3; Warbrick et al., 1988). Among the mammalian CKIs, PCNA binding has been observed only with p21, but not with p27 and p57 (Parekh et al., 1997). The presence of this site in dacapo proteins supports the possibility that it has multiple functions, relative to mammalian p27.

Dacapo Gene Structure in Ae. aegypti

We used the *Ae. albopictus* cDNA sequence to deduce the exon–intron organization of the 23 kb gene encoding *Ae. aegypti* XP_001651102.1. Because annotation of the *Ae. aegypti* gene is available in the VB-2011-02 release of VectorBase, under *Ae. aegypti* transcript AAEL005580-RA, the details of our analysis will not be described here. Note, however, that the introns in the *Ae. aegypti* gene measure ~6 and 16 kb, and are substantially longer than the corresponding introns in the *D. melanogaster* gene. As would be expected based on the cDNA alignment (Fig. 2), the highest level of conservation occurs within the first exon and 5′-half of second exon, whereas the third exon shows more divergence. *Ae. albopictus* nt 1–152 also recovered a 72% identity in the *Culex pipiens* genome, corresponding to a hypothetical mRNA with an estimated size greater than 5 kb, encoding hypothetical protein XP_001847162, shown in Figure 3. Matches to other Dipteran sequences, including those of *An. gambiae*, were short, with only 3–8% coverage.

Dacapo Expression in 20E-Treated Cells

We used Western blots to examine whether treatment with 20E affected levels of Ae. albopictus dacapo protein. In preliminary experiments, we established that antibody to *Ae. albopictus* dacapo synthetic peptides detected a band at ~27 kDa, consistent with the calculated mass (27,061 kDa) for the translation product. In pilot studies (not shown), we verified that signal intensity increased with protein amount in the range of 40–70 μg of total protein per lane on mini-gels. With 40 μg samples, dacapo signal increased in normally growing cells as they approached confluency, and also increased when cells were incubated in nutrient-depleted medium. Likewise, dacapo signal decreased when nutrient-starved cells were refed. Thus, in the absence of 20E, dacapo signal intensity reflected growth conditions, increased with conditions associated with cell cycle inhibition (age of the culture), and decreased with cell cycle reentry (nutrient replenishment).

We evaluated decapo expression using cells grown in hormone-free medium for 48 h, then treated with 2×10^{-6}M 20E. Hormone was added directly to the original culture medium (Fig. 5, lanes 2 and 6), and control cells were maintained without hormone (Fig. 5, lanes 1 and 5). To ensure that dacapo expression represented a bona fide response to 20E, rather than nutrient depletion, a second set of cells was refed with fresh medium containing 20E (Fig. 5, lanes 4 and 8) or lacking 20E (Fig. 5, lanes 3 and 7). Cells were harvested and assayed for dacapo expression by western blot after 24 (Fig. 5, lanes 1–4) or 48 h (Fig. 5, lanes 5–8). Dacapo levels were low but detectable in the cells without 20E, with a slight increase between 24 and 48 h. In the presence of 20E, dacapo levels showed a consistent increase, which was enhanced after 48, relative to 24 h of treatment. The amount of dacapo

protein was similar regardless of medium replacement, indicating that the cells were growing exponentially, in the presence of adequate nutrients.

DISCUSSION

Ecdysone Responses in Mosquito Cells

Although their responses differ in detail, proliferation of 20E-responsive insect cell lines is invariably inhibited by hormone treatment. Consistent with its negative effect on the cell cycle, only a small number of proteins have been shown to be up-regulated after 20E treatment (for a review, see Echalier, 1997), and among different cell lines, identities of ecdysone-inducible proteins (EIPs) fail to converge on consensus regulatory molecules/pathways that provide a paradigm for understanding how the 20E response engages the cell cycle machinery.

Pathways that link steroid hormone receptor to cell cycle machinery are best understood in mammalian cells that respond to estrogen and progestin in the context of breast cancer, where detailed exploration of regulatory protein expression has potential therapeutic implications. Insofar as 20E-treated C7-10 mosquito cells complete the ongoing cycle and divide before arresting in G1 (Gerenday and Fallon, 2004; Fallon and Gerenday, 2010), their response to 20E resembles the biphasic effects of progesterone (Musgrove et al., 1991) more closely than the stimulatory effects of estrogen (for a review, see Butt et al., 2008). The diversity of phenotypes exhibited by steroid-responsive mammalian cell lines underscores the potential complexity of 20E-response pathways in insects and provides a context for understanding differences between the few insect cell lines in which these responses have been investigated.

Among 11 EIPs detected in our earlier analysis of *Ae. albopictus* cells (Lan et al., 1993), a ~26 kDa protein from total and cytoplasmic extracts migrated to a position consistent with the pI (9.8) calculated from the *Ae. albopictus* dacapo cDNA described in this study. Because we excluded the possibility that this protein was one of the small heat shock proteins that are induced in some 20E-responsive *Drosophila* cell lines (Ireland and Berger, 1982), it will be of interest to reexamine whether this protein is dacapo. Likewise, given that *Ae. albopictus* dacapo has a PCNA binding site, a ~29 kDa protein recovered by co-immunoprecipitation with PCNA (Ma et al., 2006) also merits re-investigation using antibodies to dacapo.

A second up-regulated EIP in *Ae. albopictus* cells is a 52 kDa protein homolog of *D. melanogaster* gene CG17337, which is conserved in *An. gambiae,* as well as in more distantly related organisms (Eccleston et al., 2002). Although little more has been learned of this protein's function, its putative role in proteolysis reduces the likelihood that 52 kDa EIP is a second mosquito CKI or a core component of the cell cycle machinery.

Dacapo in Anopheles Mosquitoes

It remains unclear whether a homolog of *Ae. albopictus dacapo* has been annotated in *Anopheles* mosquitoes. Based on evolutionary considerations, we expected to uncover evidence for an *Anopheles* protein similar to dacapo from *D. melanogaster*, but with a sequence more closely related to the *Aedes* and *Culex* proteins, relative to the *Drosophila* homolog. The closest *Anopheles gambiae* candidate was a partial, 81 amino acid peptide (XP_307826.3) with good homology with the dacapo PCNA binding domain near the C-terminal end of the protein (Fig. 3). However, this protein fragment did not include upstream sequence corresponding to the better-conserved N-terminal domain of CKI proteins. The closest full-length match to dacapo from *Aedes* and *Culex* was hypothetical protein AND_15659 from *An. darlingi* (Fig. 3), a 366 amino acid protein with ~40% amino acid

identity in a CDI domain spanning residues 40–90. Inspection of the alignment in Figure 3 indicates that this *An. darlingi* protein is ~100 residues longer than *Ae. albopictus* dacapo, and lacks the PCNA binding motif. This poor alignment between the *An. darlingi* protein and the other dacapo proteins (Figs. 3 and 4) raises the possibility that there may be multiple CKIs in mosquitoes.

A tblastn search with this *An. darlingi* query further uncovered a 1,410-nucleotide mRNA sequence in *An. funestris* (Afun003500). Using this mRNA sequence as query in a discontiguous megablast against Est_others, we pulled out short sequences with ~85% identity to *An stephensi* (Accession FL 484363) and *An. gambiae* (BM635733, corresponding to XM_310511.2), which encodes a 61 amino acid protein with two ~15 amino acid stretches that align (with gaps) to the *Ae. albopictus* dacapo upstream of the 81 amino acid *An. gambiae* sequence (XP_307826.3) containing the PCNA binding domain shown in Figure 3. Although the *An. gambiae* database assigns these short homologies to different proteins on chromosomes X (XM_310511.2) and 3L (XP_307826.3) respectively, these short matches support the likelihood that *Anopheles* genomes encode a *dacapo* homolog, perhaps in a region of genomic DNA for which annotation is incomplete. Finally, we note that the gene *roughex*, which encodes a putative cyclin A inhibitor protein with limited structural similarity to dacapo, is evolving rapidly in *Drosophila* (Avedisov et al., 2001), and no clear homologies to the roughex protein have yet been described in mosquitoes.

Dacapo and the Cell Cycle

Aside from efforts to elucidate the structure and evolution of *dacapo* genes in insects, the involvement of dacapo and other cell cycle regulatory proteins in insect growth and metamorphosis remain to be explored. Before embryogenesis, dacapo is involved in mitotic to endocycle transitions in ovarian follicle cells (Shcherbata et al., 2004) as well as in the oocyte and nurse cells (Hong et al., 2003, 2007). During *Drosophila* embryogenesis, regulation of *dacapo* involves a complex assembly of *cis*-acting factors, and its expression in the embryo is not necessarily coupled with cell cycle progression (Meyer et al., 2002). Because maternal effects may influence embryogenesis, it will be of interest to examine expression of cell cycle regulatory proteins in imaginal discs or larval epidermis, particularly during their response to 20E. Insofar as cell cycling is influenced by nutrient availability, we note that Terashima et al. (2005) describe a correlation between nutritional deprivation and 20E-mediated apoptosis in *Drosophila* ovaries, and Gu and Lin (2009) provide evidence for DNA synthesis preceding an increase in ecdysteroidogenesis in *Bombyx mori* prothoracic glands. As additional systems are developed, we anticipate that a wide range of cell cycle regulatory proteins will be found to participate in hormone-mediated events essential to insect growth and development.

Acknowledgments

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```
DACAPO    2   VSARVLNPVMISEFCKMSSSPAVSRNLACGRQLNRIKRDLFGSSKSAEGTANKTPFNSEL   61
              +SARV NPV +SE K+ S   V + +      L R+KRDLFG      E   +K   + +L
Aedes     1   MSARVCNPVALSEIAKLRSPAVVRKPMTNSISLARVKRDLFGPVDKQE---SKNFIDRQL   57

DACAPO   62   ERHQELATQKWGFDFRAGCPLAEKSPYIWERVSFQESSFAPE----MYTLTRAAHVRPSA  117
                +  ++KWGFDF AG PL      Y WERV     + AP     M TLTR AH  P +
Aedes    58   AAQNDALSKKWGFDFTAGEPLQNHEQYQWERVP---PTSAPACFTGMVTLTRGAHRVPQS  114

DACAPO  118   DASPSDMDILVNERSEREN-----FGSNLVNSSLESNTDNES-CYDSQDESLAMRLSSSS  171
                     D  L+++R+EREN         S++ + +  S +D+ES C     +  + L S +
Aedes   115   STISED---LLDQRAERENASLYRHPSSISSPASVSGSDSESDCSFETVRTHPLVLRSET  171

DACAPO  172   TTST----------------SSIVLRKRQPKITEFMKERKRLAQAPKKLSPAKRMR      211
                 S                    +++    KRQ +IT+++KERKRL+    K + AK+ R
Aedes   172   IVSINTASTTTITSSSTPSFPATVNRAKRQQRITDYLKERKRLSTGAPKSTAAKKAR    228
```

Figure 1.
Alignment of dacapo from *D. melanogaster* and the hypothetical homolog XP_001651102 from *Ae. aegypti*. Boxes indicate conserved peptides used to design PCR primers based on the *Ae. aegypti* nucleotide sequence (see Fig. 2).

```
                              F20
                         ┌──────────────────────►
albopictus     1   ATGAGTGCCAGAGTGTGTAATCCGGTGTCGCTGACGGAGATAGCAAAGCTGCGGTCTCCG   60
aegypti        1   ..........................G.....T..........C...........C...   60
Culex          1   ..........AG....AC..C..A..ATA...T.......T.G.C...C..T..G...   60

albopictus    61   GTCGTGATCCGGCGGCAGATGTCGGCCGGTAGCTCCCTGGCCAGGGTGAAGAGGGACCTG  120
aegypti       61   .C.....G.T...AA..C....A.CAA.A...T...G...............C.......T  120
Culex         61   .C.A.CGGT.......C..C...C..G.CCC....A.G....C....C....C........  129
                                       \        \    |     \
                                        |        |    |      |
                                    GCGCCG              A    CC

albopictus   121   TTCGGACCGGTGGATAAGCAGGAGTCGAAGAAATTCATCGACCGGCAGCTGGCTGCCCAA  180
aegypti      121   .....C........C................C..T.....T...........C......  180
Culex        130   ..............C...G....C................T..G..CG.......C.....G  189

albopictus   181   AGCGCTGCTCTCAGCCAAAAGTGGGGCTTCGATTTCGCCGCAGGCAAACCGCTACTGAAC  240
aegypti      181   .A..A..C......A.G.................A....C..TG.G....G.A...T  240
Culex        190   .A..AC.TC......A.G...........CG.T.C...G.G..C..C.A....  249

albopictus   241   CACGATCAGTACAAGTGGGAACGGGTTCCACCGACGTCGGCTCCGGCCTGTTTCGTCGAA  300
aegypti      241   .....A......C.......G...........................AC..G.  300
Culex        250   .....G......C.......G..C..A..C.....T..C..C...T.....C.......G.  312
                                                                        \
                                                                         |
                                                                        CGG

albopictus   301   ATGGTGACCCTCACCCGAACGGCTCACCTGGTGCCCCAAAGCTCCACCAGCGCCGAGGAT  360
aegypti      301   ..........G.....GGC..C..T.GT.....A.....T.....A.T.T.......C  360
Culex        313   .....TG....G..G..GG.C..C...G.C..-.GG...CG.--...GC.A....A...  369
                        ◄──F23──         ─────R23────►
albopictus   361   CTGCTGGATCAGCGTGCCGAACGAGAGAACGGAAGCCTCTACCGCCACCACCAATCGTCG  420
aegypti      361   ...................G........T.CC.....G....T..T.------...  414
Culex        370   ...A....C....C..G.....C..A......  403

albopictus   421   CCAATCATTGGATCTTCTCCTGCATCCGTTTCCGGATCGGATTCCGATTCGGACTGTAGC  480
aegypti      415   T.C....------G...G............C..........C..G..A...........  468

albopictus   481   TTCGGATCGGTGGTCAGGACATACCCGTTGGCGCTGCAAAGTGAGGCGATCGCTTCCGTT  540
aegypti      469   ....A.A.--...T.....GC.T.......T.....G.......A.C..T.TA...A.C  525

albopictus   541   ACCACCGCATCTACCACCACCGGCAAATCGCCTTCCTCCTCCGGAGCGACGATGAACCGA  600
aegypti      526   .A.........C.........A...GCAGCA.AC.G..T.T.CC.......G.C......  591
                                     \
                                      |
                                  GACCAT

albopictus   601   GCGAAGCTGCAACCACGAATCACAGACTTCCTGAAGGAACGCAAGCGCCTCTCCACCGGA  660
aegypti      592   .......GA..G.A..............A...A.........A..T..T.A......  651
Culex        571   ..............A......A..G.....A........G.G.A.C  615

albopictus   661   TCTCCAAAGCCCACCGCCGCCAAGAAGGCCCGTCATATGCTGATGCCCTCCTCCTCTGCT  720
aegypti      652   G.C......T..........................G......C...A.......C.AT..  714
                                                                       \
                                                                        |
                                                                       GCT
Culex        616   G.C..G...GT.G..CTG......CGA.....C..G.......  658

albopictus   721   TCATCCTCCAATATTTCGTTGTCTTCGCCATCGAATGCCCAGCCACAACAACAACAGGAC  780
aegypti      715   G.CG......G..C....C.......T..G.......A.....--------..G......  768
                                                             \
                                                              |
                                                              A
                                              R20
                              ◄─────────────────
albopictus   781   CAC[TAG]GTGTGCGCGAGCTCAACCACTCGCCAAAGAAGGGTGATCCGG
aegypti      769   ...|...|
```

Figure 2.
Alignment of dacapo cDNAs from *Ae. Albopictus*, *Ae. aegypti*, and *Culex pipiens*. Arrows indicate PCR primers used to obtain the full-length *Ae. albopictus* cDNA. The boxed TAG is the stop codon.

```
Ae.albopictus    -MSARVCNPVSLTEIAKLRSPVVIRRQMSAGSS---LARVKRDLFGPVDKQESKKFIDRQ
Ae.aegypti       -.........A.S........A.V.KP.TNSI.---..................N.....
Cx.quinque       -..K.Y..MI.S..ST....AIG..PAPTSAARITA.............ED.....E.E
D.mel            MV....L...MIS.FC.MS.SPAVS.NLAC.RQLNRIK.DLFGSSKSAEGTAN.TPFNSE
C.costata        ------------------------------------------------------------
An.gambiae       ------------------------------------------------------------
An.darlingi      -MG.Q.Y.RMIEQRLHHTPP..IG.SAANRPI..ATNT..I..N...R..STSV.L.VEE.


Ae.albopictus    LAAQSAALSQKWGFDFAAGKPLLNHDQYKWERVPPTSAPACFVEMVTLTRTAHLVPQ---
Ae.aegypti       ....ND...K......T..E..Q..E..Q.............TG......G..R...---
Cx.quinque       ....NDV..K......RS.E..Q..E..Q..........S...GGMVALTR.AH.VG---
D.mel            .ERHQELAT.......R..C..AEKSP.I...S-FQESTFAP..Y....A..VR.S---
C.costata        -----ELAT.......R..N..GSNSP.I...S-FQESSFAPAVY....A..VR.----
An.gambiae       ------------------------------------------------------------
An.darlingi      I.S.NEEKRK..N...ST.R.DE.-GS......ANPLTVSPSSA......A..TFSAGRR


Ae.albopictus    -----SSTSAEDLLDQRAERENGSLYRHHQSSPIIGSSPASVSGSDSDSDCSFGSVVRTY
Ae.aegypti       ------..IS.............A.....PS.----I............E.....ET.-..H
Cx.quinque       -----TA.AT...M.........TCFS---------TTA.TA....T.M.TPVLTHP---
D.mel            -----ADA.PS.MDILVN..SERENFGSNLVNSSLE.NTDNE.CY..QDESLAMRLSS--
C.costata        -----MPAEPT.MDTLLN..ATRENG--NL..SSLE.HTDNE.CY..HDETLLRPFNLP-
An.gambiae       ------------------------------------------------------------
An.darlingi      RGPMVDEQEPLPMENLMD..AEKANLEQGDV..VVPEV.EAIRTYPNLRST.TAT.TSAT


Ae.albopictus    PLALQSEAIASVTTASTTTGKSPSSS--GATMNRAKLQPRITDFLKERKRLSTGSPKPTA
Ae.aegypti       ..V.R..T.V.IN......IT.S.TPSFP..V...R.Q....Y.........A..S..
Cx.quinque       --------L.KIS..T.VVCL.S...SFP.SAT..RR.Q....Y........SSA..VAL
D.mel            ----------------------..TTSTSSIVLR.R..K..E.M......AQAPK.LSP
C.costata        --------------SCSNSCP.S.TTAPTLSCTLR.R..K..E.M.--------------
An.gambiae       --------------KKKSAARSQ.PKPSSSKGSKLR..Q..E.......RLSSTVAVEK
An.darlingi      IE..PTTPPS.P..I.RSSNS.S...SSSSSSRVTRSRQMKMTAERTE.GV.VPLFDCWK


Ae.albopictus    AKKARHMLMPSSSASSSNISLSSPSNAQPQQQQDH-----------------------
Ae.aegypti       .....Q...T.A.P.AASSIS..S.ANATA..DH------------------------
Cx.quinque       ..R..Q..SG.PT....SSQ-------------------------------------
D.mel            ..RM.TSSPS..AT...SS.GGFGLG.HFGAMLKRPRHN-------------------
C.costata        ---------------------------------------------------------
An.gambiae       ISAKKVRM.MGA.S...ENGDGAAGGP.EASASTAN----------------------
An.darlingi      SIS.PGEQQEGATSVCCRLEEKAKGDNCTIGR.QEHEPAAQRIVFLAPKDRDHVTMLRDV


An.darlingi      RSYFLKERKRPSTGASVGRTQVKKVRVLATMSSNGADPKKAITSPVTRSHSVPSTSSG
```
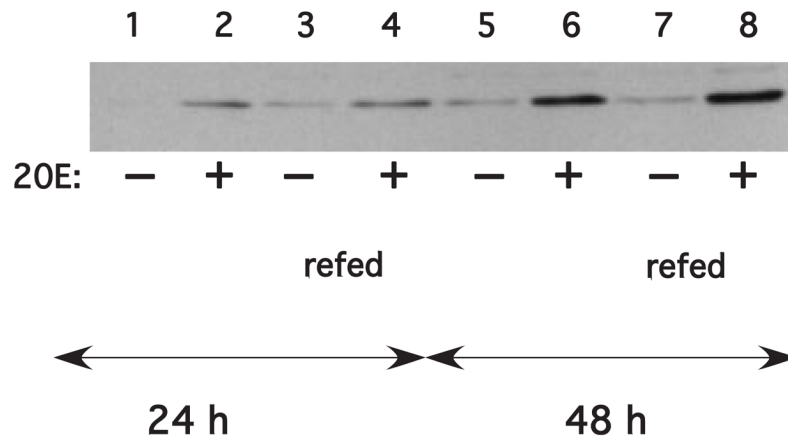
Figure 3.
Alignment of dacapo protein sequences from *Ae. albopictus*, *Ae. aegypti* (XP_001651102.1), *Culex quinquefasciatus* (XP_001847162.1), *D. melanogaster* (gbAAB36557.1), *Chymomyza costata* (gbACT79565.1) and putative dacapo homologs from *An. gambiae* (XP_307826.3) and *An. darlingi* (AND_15659). The alignment was done with ClustalW (1.81) at the KEGG (Kyoto University Bioinformatics Center) website using the slow/ accurate method and the following parameters: Pairwise settings: Gap open penalty 35; gap extension penalty 0.3; Multiple alignment settings: gap open penalty 15; gap extension penalty 0.3 with weight transition and hydrophilic gaps off. Dots were substituted to represent identities to the *Ae. albopictus* sequence at top and dashes indicate gaps. Open boxes designate S/TXXE/D CD2 motifs. The shaded box indicates a PCNA binding domain.
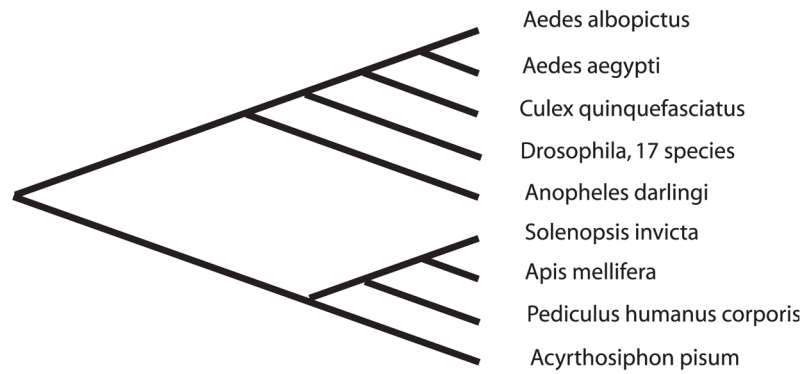
Figure 4.
Relationships among decapo proteins from the Insecta (Taxid 50577). Proteins were obtained by BLASTP at the NCBI website and the tree was produced using COBALT (Papadopoulos and Agarwala, 2007). Accessions for the Diptera are given in the legend to Figure 3; the *An. gambiae* sequence was not retrieved in this search and 17 *Drosophila* sequences show as a single branch on the tree. Accessions were EFZ13437 for the fire ant *Solenopsis invicta*, XP_003251288 for the honeybee *Apis mellifera*, XP_002424234 for the body louse *Pediculus humanus corporis*, and XP_001947107 for the aphid *Acyrthosiphon pisum*.

Figure 5.
Effect of 20E on dacapo abundance. Cells were seeded as detailed in the *Materials and Methods* and incubated in the presence (+) or absence (−) of 2×10^{-6}M 20E for 24 or 48 h. For cells in lanes 1, 2, 5, and 6 20E was added to the growth medium; in lanes 3, 4, 7, and 8 the medium was replaced with fresh E-5 medium containing 2×10^{-6} M 20E.