Insect Immunity

CHARACTERIZATION OF A DROSOPHILA cDNA ENCODING A NOVEL MEMBER OF THE DIPTERICIN FAMILY OF IMMUNE PEPTIDES*

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Drosophila shows an immune response when challenged by injection of low doses of bacteria. To date, the molecules involved in this immune reaction have remained elusive, with the exception of cecropins (4-kDa antibacterial peptides initially isolated from the moth Hyalophora cecropia) for which three closely related genes have been characterized recently. We report the molecular cloning and sequencing of a cDNA from a library of immune Drosophila which encodes a novel member of the family of diptericins (9-kDa antibacterial peptides initially isolated from the fly Phormia terranovae). Transcripts for the Drosophila diptericin are detected 2 h after injection of bacteria. They are apparently derived from a single gene mapping at position 56 A on the right arm of the second chromosome.

We discuss the existence of a distant relationship between the diptericins and two other groups of antibacterial insect proteins, the attacins, and the sarcotoxins II.

Insect immunity is a rapidly expanding field of research. It is now well documented that members of the higher insect order Lepidoptera, Diptera, and Hymenoptera respond to septic or even aseptic injuries by the rapid synthesis of potent antibacterial peptides. These molecules, together with the long-known cellular reactions of phagocytosis and encapsulation, account for the remarkable resistance which these insects exhibit against numerous types of bacterial challenges. In recent years, several families of bactericidal peptides have been isolated from immune insects and their primary structures have been determined: cecropins (4 kDa) (1), attacins (>20 kDa) (2), diptericins (9 kDa) (3), insect defensins (4 kDa) (4), and apidaecins (2 kDa) (5) (and various homologues (~20 kDa) (2), diptericins (9 kDa) (3), insect defensins (4 kDa) (4), and apidaecins (2 kDa) (5) (and various homologues (6-8)). These bactericidal molecules are mostly cationic peptides with a broad spectrum of activity against Gram-positive and/or Gram-negative bacteria.

The predominant immune peptides in larvae of our laboratory insect Phormia terranovae (Diptera) are the anti-Gram-negative diptericins (three closely related isoforms (3)) and the anti-Gram-positive insect defensins (two isoforms (4)). In situ hybridization studies indicate that mRNAs encoding both peptide families appear in the same blood cells and adipocytes within 1 h after injection of the larvae (9). A challenging problem regards the control of the concomitant transcription of the genes coding for these immune peptides. While large insects, like Phormia, Sarcophaga, and Hyalophora, have proven extremely useful for biochemical studies on immune peptides, their genome sizes and the poor genetic background are relatively unfavorable for studies on the regulation of the transcription of these genes.

Drosophila, a dipteran distantly related to P. terranovae, obviously represents a better experimental model for such studies. Previous studies on the immune response in Drosophila (10-14) suggested the existence of many similarities with that of other Diptera and Lepidoptera, and the genes coding for a Drosophila homologue of cecropins, immune peptides that were initially isolated from the moth Hyalophora cecropia (1), have been recently characterized (15).

In this paper, we have addressed the question as to whether immunized Drosophila produce peptides structurally related to the diptericins of P. terranovae. We report the molecular cloning of a Drosophila cDNA encoding a peptide with significant homology to Phormia diptericins. We show that transcription of Drosophila diptericin occurs rapidly after injection of bacteria.

MATERIALS AND METHODS

Insects, Immunization, and Antibacterial Assays—Experiments were performed with the Canton S wild-type strain of Drosophila melanogaster, grown on standard medium at 20 °C.

Adult insects were immunized by injection of 0.1-0.2 μl of a diluted log-phase culture of Escherichia coli (500 live bacteria) and were returned to feeding tubes. One day after injection, groups of 20 flies were homogenized in 10% acetic acid to remove the larger proteins and the proteases from the medium. The homogenate was centrifuged for 5 min at 250 × g. Acetic acid extraction was repeated on the pellet, and the resulting supernatants were pooled. The acetic acid from the supernatant was removed by rotary vacuum evaporation. The residue was resuspended in 8 μl of distilled water and its activity tested on 2-μl aliquots against E. coli 111 and Micrococcus luteus. Antibacterial activities were recorded as growth inhibition produced in thin agar plates seeded with each of these strains, as previously described (2, 16).

Cloning Procedure—We screened 30,000 plaques from a Drosophila melanogaster grown on standard medium at 20 °C. Adult insects were immunized by injection of 0.1-0.2 μl of a diluted log-phase culture of Escherichia coli (500 live bacteria) and were returned to feeding tubes. One day after injection, groups of 20 flies were homogenized in 10% acetic acid to remove the larger proteins and the proteases from the medium. The homogenate was centrifuged for 5 min at 250 × g. Acetic acid extraction was repeated on the pellet, and the resulting supernatants were pooled. The acetic acid from the supernatant was removed by rotary vacuum evaporation. The residue was resuspended in 8 μl of distilled water and its activity tested on 2-μl aliquots against E. coli 111 and Micrococcus luteus. Antibacterial activities were recorded as growth inhibition produced in thin agar plates seeded with each of these strains, as previously described (2, 16).

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performed as described (17). After baking for 2 h at 80 °C under vacuum, the filters were prehybridized at 42 °C for 3 h in 5 × SSC (standard sodium citrate), 50 mM phosphate buffer, pH 6.5, 5 × Denhardt’s solution, 100 µg/ml denatured salmon sperm DNA, 0.1% sodium dodecyl sulfate. Hybridization was performed in the same solution at 42 °C overnight. The filters were washed three times for 15 min at room temperature in 2 × SSC, 0.1% sodium dodecyl sulfate and subjected to autoradiography.

**RESULTS**

**Antibacterial Activities in Normal and Immune Drosophila**—We first confirmed that in our conditions *Drosophila* responds to a bacterial challenge by building up an immune response. We injected third instar larvae and adults with a low dose of bacteria and monitored the presence of an antibacterial activity after 24 h in whole body extracts. The activity was measured by the growth inhibition assay on agar plates seeded either with the gram negative *E. coli* D 31 or the Gram-positive *M. luteus*. As is apparent from Table I, both larvae and adults showed a significant antibacterial activity against the Gram-negative and the Gram-positive test organisms. Similar assays performed on normal insects revealed no constitutive antibacterial activity in larvae or adults. However, a marked activity was observed in normal early pupae (day 1–2) and was directed against both types of bacteria. In contrast, late (day 4–5) pupae were devoid of any antibacterial activity after 24 h in whole body extracts. The test organisms, similar assays performed on normal insects, revealed no constitutive antibacterial activity in larvae or adults. Similar assays performed on normal insects showed similar results.

**TABLE I**

**Antibacterial activities in extracts of Drosophila**

For each assay, 20 insects were subjected to whole body extraction in acetate acid and after desiccation, the residue was resuspended in 8 µl of distilled water. Aliquots of 2 µl were tested in the growth inhibition assay. Results are given as +, clearly detectable inhibition and −, no detectable inhibition. This experiment was repeated several times yielding similar results.

| Bacterial strains | E. coli D 31 | M. luteus |
|------------------|------------|-----------|
| Larvae (3rd instar) | 0 | 0 |
| Pupae (early, 1–2 days) | + | + |
| (late, 4–5 days) | 0 | 0 |
| Adults (4 h) | 0 | 0 |
| (7 days) | 0 | 0 |
| Experimental insectsa | + | + |
| Larvae (5th instar) | 0 | 0 |
| Adults (2 days) | + | + |

1 Insects were challenged with an injection of ~800 log-phase cells of *E. coli*, 24 h before extraction as in 1.

**Isolation of cDNA Clones**—The radioactive probe pool was used to screen a cDNA library which had been prepared from immune adults (18-h post inoculation of bacteria) of *Drosophila* (19) and 9 hybridization-positive clones were isolated which contained cDNA inserts between approximately 0.1 and 0.5 kilobase pairs. We have focused our attention on the clone containing the largest insert. The sequence complementary to the probe in the diptericin coding region is also underlined. The pentagyneic region is boxed. Note the presence of a HindIII restriction site (AAGCTT) beginning at the second nucleotide of the stop codon.

**Fig. 1.** Nucleotide sequence of a cDNA encoding *Drosophila* prediptericin. The clone consists of 486 nucleotides and shows an open reading frame, beginning with an ATG triplet, which allows us to deduce a 106-residue peptide as shown below the nucleotide sequence. The sequence complementary to the probe in the diptericin coding region is also underlined. The pentagyneic region is boxed. Note the presence of a HindIII restriction site (AAGCTT) beginning at the second nucleotide of the stop codon.
that the *Drosophila* clone codes for a diptericin homologue. In particular, the pentaglycine stretch characteristic of the *Phormia* diptericin family is present in the *Drosophila* peptide in a similar position. Two regions of perfect homology extending over 10 and 12 amino acids are also evident as well as several minor stretches with identical sequence. A weak homology is also seen to the much larger antibacterial proteins attacin and sarcotoxin II (see “Discussion”).

The sequence analysis of the mRNA of *Phormia* diptericin had shown previously that this antibacterial peptide is synthesized as a prepeptide with a putative signal sequence of 18 amino acid residues preceding a hydrophilic stretch of several minor stretches with identical sequence. A weak homology is also seen to the much larger antibacterial proteins attacin and sarcotoxin II (see “Discussion”).

**FIG. 2.** Sequence comparison between the amino acid sequences of *Drosophila* diptericin (deduced from the cDNA sequence in Fig. 2), *Phormia* diptericin 1 (cDNA clone D153) (26), and parts of *Sarcophaga* sarcotoxin IIA (7) and *Hyalophora* acidic attacin (27). Sequence identities with one or the other diptericins are shown with bold typeface; identities between *G. domoicus* domains of sarcotoxin II or attacin and any of the other domains are underlined. Further identities exist between attacin and sarcotoxin II (7), but are not enhanced. Secondary amino acid modifications are indicated with asterisks: the NH₂-terminal residue in sarcotoxin II is pyroglutamate, and the COOH-terminal glycine residues in *Phormia* diptericin and in sarcotoxin II are lost in the formation of COOH-terminal amide groups. The signal peptides are aligned with the most likely cleavage site for signal peptidase (29) in the same position; an alternative possible cleavage site is indicated for *Phormia* diptericin. The inset illustrates the suggested domain structures of *Drosophila* diptericin, *Sarcophaga* sarcotoxin, and *Hyalophora* attacin (see “Discussion”). The structure of attacin pre- and propeptides is from Ref. 28.
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pae, pg of total RNA was used for each hybridization and the gel was exposed for 2 h.

RNase protection. Early and late third instar larvae refers to feeding and wandering stages, respectively; white, white prepupae; early pupae, up to 49 h after pupariation; late pupae, 49 h until eclosion. Ten μg of total RNA was used for each hybridization and the gel was exposed for 2 h.

The presence of a HindIII restriction site in the diptericin cDNA (see HindIII (H3) digested genomic Drosophila DNA hybridized at high stringency with the diptericin cDNA probe. Fig. 1) generates two bands hybridizing with the diptericin probe (lane H3).

Insects regardless of the developmental stages investigated, with the exception of early pupae which responded only faintly. Interestingly, unchallenged early pupae and adults appeared to contain low but significant amounts of diptericin transcripts which were, however, not detected in embryos and larvae. In comparison to cecropin transcripts (15), the diptericin transcripts were found to be very abundant in induced insects. Equal loading and integrity of the RNA were tested in Northern blots probed with actin (data not shown).

Existence of a Single Diptericin Gene and Cytological Localization—High stringency hybridization of EcoRI or BamHI digested genomic DNA on Southern blots with the cDNA insert probe gave a single signal. In the case of digestion with HindIII, two bands hybridized with the diptericin probe (Fig. 5), since a HindIII restriction site is present in the cDNA as apparent in Fig. 1. The chromosomal position of the diptericin locus was mapped by hybridization to salivary gland polytene chromosomes (Fig. 6). A single signal was observed at position 56 A, on the right arm of the second chromosome.

FIG. 6. Localization of the diptericin gene by in situ hybridization of the labeled cDNA probe with polytene chromosomes. The diptericin cDNA probe maps to the 56A region in the middle portion of the right arm of the second chromosome (indicated by the arrowhead).

DISCUSSION

Our results demonstrate that Drosophila synthesizes a mRNA encoding a homologue of the diptericins recently described in the Dipteran P. teranovae. The synthesis of this mRNA can be induced by the inoculation of bacteria, indicating that it is part of the immune response of this insect. Other molecules are obviously involved in this response in Drosophila, as the transcription of several cecropin genes has also been observed in similar conditions (15).

So far, no structural information has been published on diptericins or diptericin-related molecules from insects other than Phormia. The homologies between Phormia and Drosophila diptericins are particularly striking as regards the following criteria: (i) the molecular masses (~9 kDa) of the two peptides; (ii) the hydrophilicity profiles; (iii) the amino acid compositions. Both peptides are remarkably rich in Gly (18, 22%, respectively) and Pro (11%, 10%). This richness has undoubtedly a major influence on the tertiary structure of both peptides and hence on their mode of action. (iv) The primary sequence, which shows an overall homology of approximately 60%, with several regions (of 4 residues and more) of full homology between positions 19 and 69. As already noted, the characteristic pentaglycine stretch of the Phormia diptericins is present in an equivalent relative position in Drosophila. It has been suggested that this sequence confers extreme flexibility to the peptidic chain (9).

Conceptually, the diptericin molecules can be divided in two major domains. At the NH2-terminal there is a short, very proline-rich "P domain." Five of the first 15 residues are Pro in Drosophila, 6 of 17 in Phormia. This remarkable richness in Pro residues is reminiscent of another family of antibacterial peptides, the apidaecins, that were recently isolated from honeybees (5). In these small (2.1 kDa) peptides, Pro accounts for 33% of the total amino acid content. The remaining part of the diptericin molecule constitutes a long Gly-rich "G domain," where the pentaglycine stretch is situated near the junction to the P domain.

Interestingly, the Drosophila diptericin, like its Phormia counterparts, appears to be distantly related to two classes of much larger insect antibacterial peptides, the attacins and the attacin-related sarcotoxins II. Both classes of molecules have two G domain like sequences adjacent to the COOH-terminal (Fig. 2), although they are less Gly rich. The similarities in pairwise comparisons of the different G domains range between 11% (sarcotoxin II G1-Phormia diptericin) and 34%
identity (sarcotoxin II G2-Phormia diptericin), and in most cases they are better than 20%. Many of the differences represent conservative amino acid replacements. Furthermore, a P domain can be identified at the NH2-terminal of sarcotoxin II, but not in the attacins. The absence of a P domain in the attacins may be related to the additional processing step that occurs in these proteins where an NH2-terminal propeptide is proteolytically removed (27). Further support for the suggested domain structure comes from Sun et al., who recently found that each G domain in the attacin genes is encoded by a separate exon. The conservation of the P and G domains implies that they may correspond to functional units, and that the diptericin, attacin, and sarcotoxin II families are phylogenetically related. Thus the diptericins, with just a P and a single G domain, appear to define the minimal member of a new superfamily of related bactericidal proteins.

By analogy with Phormia, we assume that the mature NH2-terminal of Drosophila diptericin is at the Asp(+1) residue (Fig. 2). However, the most likely site for signal peptidase cleavage is between Ala(−5) and Tyr(−4), as predicted by the algorithm of von Heijne (29). The mature end of diptericin may then be created by the further removal of two successive X-Pro dipeptides by dipeptidyl peptidase, similar to the processing of the proforms of the inducible antibacterial peptides cecropins A and B in Hyalophora cecropia (30) and of many other small peptides where X-Pro or X-Ala dipeptides are trimmed off (31). A similar scheme has also been suggested for the processing of sarcotoxin IIA (7). The Phormia diptericin is more problematic, but in this case the signal peptidase may in fact cut directly at the NH2-terminal of the mature peptide. This position is the second most likely cleavage site (probability score 8.57 versus 9.38 for the position at Ala(−4), on a scale where most sites get negative scores).

For Phormia diptericin, peptide sequencing data indicated that the COOH-terminal residue is a Phe (3). Given that the mRNA coding for this peptide shows a triplet for Gln between the Phe triplet and the STOP codon, it was inferred that the mature Phormia diptericin is amidated (26). COOH-terminal amidation appears to be relatively common among the antibacterial peptides involved in the immune response of insects (cecropins (32, 33), sarcotoxin II (7)), and is also observed in other low molecular weight antibacterial peptides such as melittin (34). It has been demonstrated in the case of cecropins that COOH-terminal amidation enhances the antibacterial activity of the peptide (35, 36). In contrast, the present data suggest that the Drosophila diptericin is not amidated. Indeed, when the sequence for Phormia and Drosophila diptricins are aligned as shown in Fig. 2, it is apparent that the Drosophila peptide has an additional COOH-terminal stretch of three amino acids, when compared to that of Phormia, and that the STOP codon is not preceded by a Gly, excluding the possible action of a peptidyl-glycine-α-amidating monooxygenase.

Our data on the hybridization of the Drosophila cDNA to genomic DNA and to polytene chromosomes from salivary glands indicate that transcription occurs from a single gene located in a relatively poorly investigated region of the second chromosome. This differs from the situation observed for cecropin in Drosophila for which a cluster of three active genes (plus two pseudogenes) at locus 99E has been found (15).

Of great potential interest is the observation that constitutive expression of the diptericin gene occurs in early pupae and in adults. A similar result has been observed for cecropins in Drosophila (37). Constitutive expression of another inducible antibacterial peptide, insect defensin, has also been observed in pupae of Sarcophaga (38) and Phormia (9). These results suggest that at least some of the inducible immune peptides of insects might play specific roles in normal development. The constitutive expression of the diptericin and cecropin genes in early pupae of Drosophila probably accounts for the strong antibacterial activity which we have monitored in whole extracts of such animals in this study (Table I).

We are now extending our studies to the control of the expression of the Drosophila diptericin gene in normal and challenged insects. The role(s) of diptericin in normal development will also be of center of interest.

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