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Report

A Novel Peptide Mediates Aggregation and Migration of Hemocytes from an Insect

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Summary

Insect blood cells (hemocytes) comprise an essential arm of the immune system [1–7]. Several factors mediating recognition and phagocytosis of foreign intruders by hemocytes have been identified, but the mechanisms regulating hemocyte movement remain fragmentary. Embryonic hemocytes from *Drosophila* migrate along stereotypical routes in response to chemotactic signals from PVF ligands, members of the platelet-derived growth factor family [8–12]. Embryonic and larval hemocytes also accumulate at external wounds [11–13], but PVFs are not required for this response, suggesting involvement by other, unknown factors. Here we report the identification of hemocyte chemotactic peptide (HCP) from the moth *Pseudaletia separata* and present evidence that it stimulates aggregation and directed movement of phagocytic hemocytes. Spatiotemporal studies revealed that HCP is expressed in both epidermal cells and hemocytes, whereas structure-function studies identified post-translational modifications important for activity. HCP also shares similarities with another group of cytokines from moths called ENF peptides [14–17]. Taken together, our results identify HCP as a chemotactic cytokine that enhances clotting at wound sites in larvae.

Results and Discussion

P. separata is a common pest of rice in Asia. Circulating hemocytes in *P. separata* larvae, like other Lepidoptera, consist of four subpopulations: granulocytes, plasmatocytes, spherule cells, and oenocytoids, which are distinguished from one another by morphological, molecular, and functional characters [2–4, 18]. Granulocytes are the most abundant hemocyte type that upon activation rapidly adhere to foreign surfaces, are phagocytic, and secrete effector molecules including antimicrobial peptides. Plasmatocytes are larger adhesive cells that in cooperation with granulocytes form capsules around large intruders like parasitoid wasps. Granulocytes and plasmatocytes also aggregate at external wound sites in association with clotting. Oenocytoids are nonadhesive hemocytes that produce components of the phenoloxidase cascade whereas spherule cells are nonadhesive cells that are a potential source of cuticular proteins.

As observed in other insects [13, 18–20], hemocytes from last-stage (sixth instar) *P. separata* larvae rapidly aggregate at external wound sites (Figure S1 available online). As a first step in investigating the factors regulating this response, we prepared an integument extract and conducted two types of in vitro bioassays. An aggregation assay revealed integument extract dose dependently induced hemocyte aggregation, and a migration assay conducted in transwell chemotaxis chambers indicated that integument extract dose dependently stimulated the preferential migration of granulocytes (Figure S1). We also determined that boiling yielded a supernatant with biological activity identical to the crude extract, whereas treatment of the supernatant with trypsin eliminated activity (data not presented). Taken together, these results suggested that aggregation of hemocytes at external wounds in *P. separata* larvae involved release of a peptidyl factor(s) from the integument.

Identification of HCP

To identify this factor, four analytical high-performance liquid chromatography (HPLC) runs coupled with bioassays yielded isolation of a fraction we named HCP (Figure S2). N-terminal sequencing of purified HCP identified a 21 amino acid sequence but MALDI-TOF MS analysis indicated the molecular mass was 3982 Da ($M + H^+$), suggesting that this sequence was truncated. We therefore prepared cDNAs from total RNA isolated from *P. separata* larval integument and used RACE PCR methods to clone a 348 bp *hcp* cDNA (Figure 1A). The deduced protein encoded by *hcp* was 52 amino acids with an N-terminal signal sequence that SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/) predicted is cleaved after Ser20 followed by a 32 amino acid C-terminal domain (Figure 1A). The first 21 residues of the C-terminal domain matched identically the sequence obtained for HCP purified by HPLC (Figure 1A), whereas hybridization of *hcp* to a single mRNA of similar size on northern blots indicated that the clone was full-length (Figure S3). Overall, these results indicated that *hcp* encodes a small, secreted protein of 32 amino acids, but the predicted molecular mass for this peptide (3543 Da) remained 439 Da less than that determined for purified HCP.

Trypsinization of synthetic and purified HCP followed by comparison of the resulting fragments by LC-ESI-MS/MS resolved this discrepancy by revealing Met11 and Met13 from purified HCP were oxidized whereas Thr22 and Thr23 were O-glycosylated with N-acetylhexosamines (Figure S4). These modifications increased the predicted molecular mass of HCP to 3981 Da, which was identical to the mass determined for the purified peptide. Characterization of the solution structure of recombinant HCP by NMR further indicated that the peptide consists of a well-structured central domain stabilized by a disulfide bond between Cys7 and Cys19 and two

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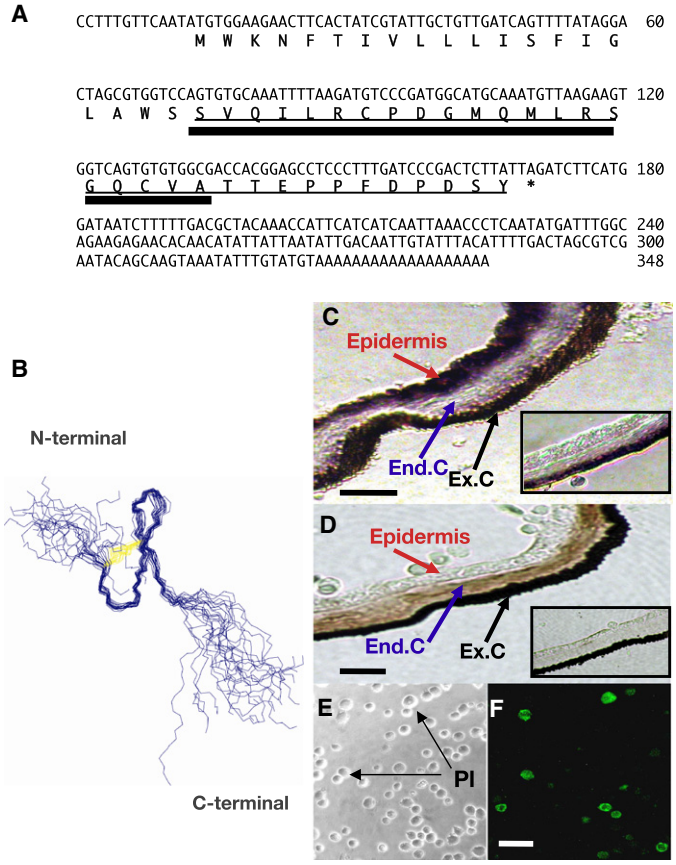

Figure 1. A 348 bp cDNA Encodes HCP, which Is Expressed in Integument and Hemocytes

(A) Nucleotide sequence of *hcp* with the deduced amino acid sequence for the predicted protein shown below the corresponding codons. The underlined sequence indicates the predicted sequence of HCP whereas the bold line indicates the amino acid sequence obtained from N-terminal sequencing of HCP purified from the integument.

(B) Ensemble of 20 HCP conformers after alignment of the backbone atoms of residues 7–22.

(C) In situ section of integument from sixth instar *P. separata* probed with digoxigenin-labeled antisense *hcp*. The hybridization signal (purple) localizes to the epidermis with little or no signal detected in the endocuticle (End.C) or exocuticle (Ex.C). Inset shows integument hybridized with sense *hcp*. Note the absence of signal in the epidermis.

(D) Immunocytochemical section of integument probed with anti-HCP and visualized with an HRP-conjugated secondary antibody. HCP is detected in the endocuticle (brown) (scale bar represents 300 μm). Inset shows integument probed with HCP preimmune serum.

(E and F) Immunocytochemical visualization of HCP in hemocytes from sixth instar larvae with anti-HCP visualized via an Alexa 488-conjugated secondary antibody. A phase-contrast micrograph (E) and the corresponding fluorescent image (F) are shown (scale bar represents 100 μm). HCP is detected predominantly in plasmatocytes (PI).

flanking domains at the N and C termini that are disordered (Figure 1B). Database searches failed to identify significant similarities in primary structure between HCP and known proteins. However, a partially matching nucleotide sequence was identified in the genome of the moth *Bombyx mori* (BM scaf45 contig24645) with KAIKOBLAST suggesting that other Lepidoptera encode HCP homologs. We also noted that the tertiary structure and position of selected residues in HCP share features with previously identified ENF peptides from Lepidoptera, which function as plasmatocyte activators (Figure S6, see Supplemental Results and Discussion).

HCP Is Expressed in Epidermal Cells and Hemocytes

RT-PCR analysis indicated that *hcp* is constitutively expressed in *P. separata* larvae and pupae but is spatially restricted to the integument, hemocytes, and nervous system (Figure S3). In situ hybridization localized *hcp* expression to the epidermis (Figure 1C), whereas immunocytochemistry with anti-HCP localized the peptide to the endocuticle (Figure 1D). In hemocytes, we detected HCP in the cytoplasm of primarily plasmatocytes (Figures 1E and 1F) although some plasmatocytes were unlabeled. We also detected HCP in a small proportion of granulocytes but never detected HCP in spherule cells or oenocytoids. Wounding did not alter *hcp* transcript or protein abundance in the integument as assessed by real-time PCR analysis and immunoblotting.

Purified HCP Exhibits Enhanced Aggregation and Chemotactic Activity

To evaluate whether the post-translational modifications identified in purified HCP are important for biological activity, we compared the aggregation response of hemocytes to purified and synthetic HCP. Purified HCP induced a significantly greater aggregation response than did synthetic HCP at all concentrations tested (Figure 2A). Transwell assays indicated that purified HCP also induced a stronger migration response than synthetic HCP over a concentration range of 20 and 200 nM (Figure 2B). The majority of hemocytes (81%) that moved to the lower chamber were also granulocytes as found for crude integument extract (see Figure S1).

We recognized that hemocyte migration to the lower chamber could reflect either a chemokinetic or chemotactic response to HCP. To distinguish between these possibilities, we added 0.2 μM of purified HCP to both the upper and lower chamber, which would be predicted to reduce hemocyte movement to the lower chamber if HCP is chemotactic but not reduce movement if chemokinetic. Our results indicated that HCP in both chambers reduced hemocyte movement to near control levels (Figure 2B). Additional evidence for the chemotactic activity of HCP derived from assays with the EZ-TAXIScan platform, which measures single-cell movement to a concentration gradient. These experiments indicated that purified HCP stimulated directional movement of hemocytes and that the number of responding cells increased with HCP concentrations (Figure 2C). We also conducted antibody neutralization experiments with our transwell assay to assess whether other factors in integument besides HCP stimulated hemocyte migration. Addition of integument extract plus preimmune serum to the lower chamber resulted in a hemocyte migration response similar to purified HCP alone, whereas the combination of integument extract plus anti-HCP or purified HCP + anti-HCP reduced hemocyte migration to similar levels as observed when no HCP was present (Figure 2D). These results indicated that anti-HCP had strong neutralizing activity and also suggested that HCP is the primary factor in integument mediating hemocyte movement.

Immunodepletion of HCP Increases Blood Loss after External Wounding

Adult insects possess a rigid integument and their hemocoels usually contain low volumes of hemolymph where hemocytes are predominantly sessile on the surface of tissues [21]. In

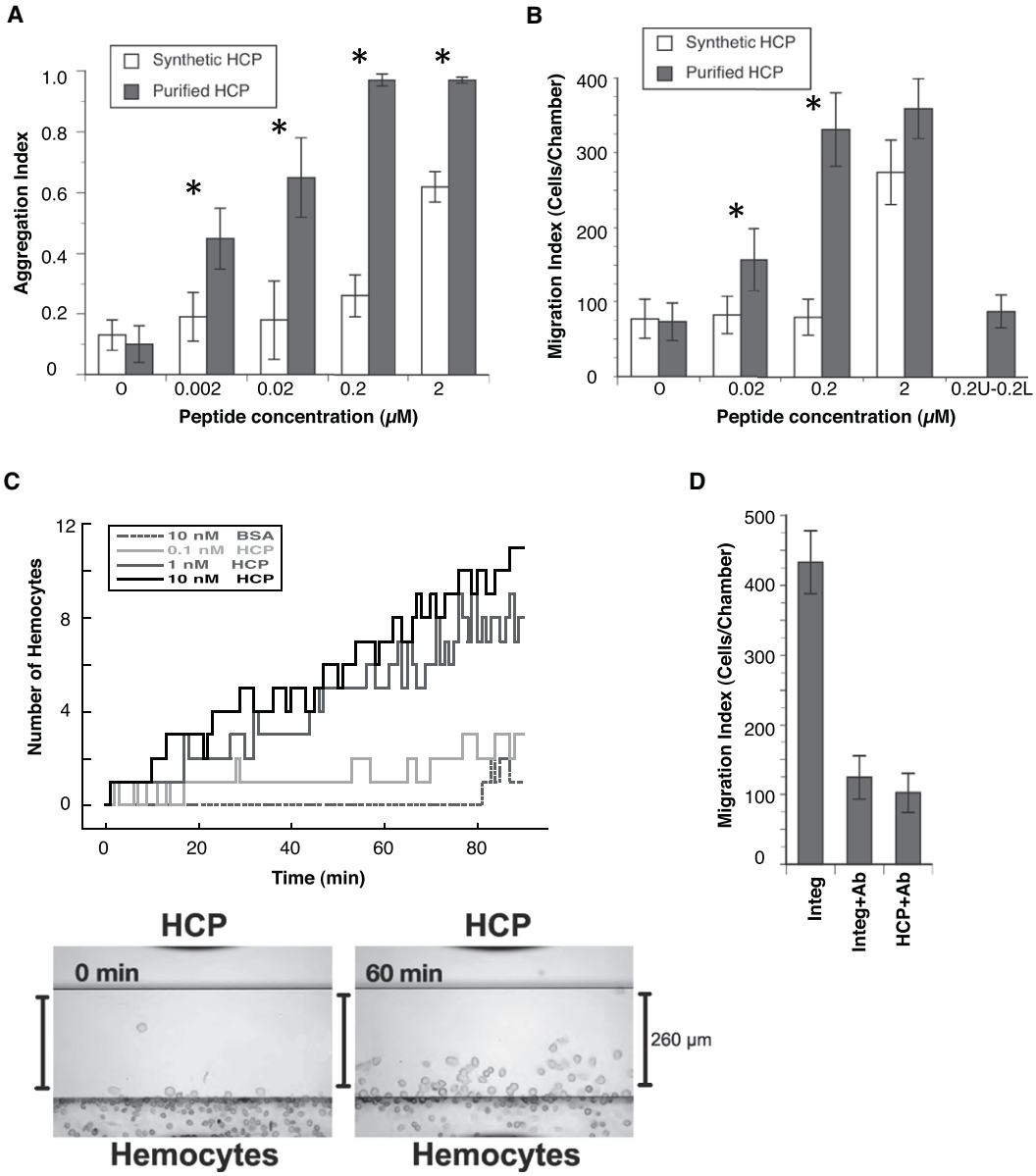

Figure 2. Purified HCP Exhibits Greater Biological Activity than Does Synthetic HCP

(A) Dose-dependent effects of synthetic and purified HCP on hemocyte aggregation in vitro (n = 6 per treatment). Aggregation indexes were determined as described in Figure S1. Asterisks above bars for a given peptide concentration indicate that purified HCP induced a significantly stronger response than did synthetic HCP (t test; $p < 0.05$).

(B) Dose-dependent effects of synthetic and purified HCP in the lower chamber of transwell chemotaxis chambers on hemocyte migration (n = 6 per treatment). Migration indexes were determined as described in Figure S1. Asterisks indicate that purified HCP induced a significantly stronger migration response than did synthetic HCP (t test; $p < 0.05$). Addition of purified HCP (0.2 μM) to both the upper and lower chambers is indicated by the bar labeled 0.2 U–0.2L.

(C) Dose-dependent migration of hemocytes in medium containing 0.1–10 nM purified HCP or 10 nM BSA (negative control) with the EX-TAXIScan assay. The EZ-TAXIScan apparatus counted the total number of hemocytes that migrated ≥50 μm in one half of the microchannel. The micrographs below the graph show hemocyte chemotaxis toward 10 nM purified HCP in the 260 μm microchannel of the apparatus. The left image shows hemocytes (1×10^3) at the beginning of the assay (0 min) aligned on the bottom edge of the microchannel. Medium containing 0.1 nM HCP was injected into the opposite compartment (top) resulting in formation of a concentration gradient. The single cell in the middle of the channel at 0 min is due to the drawing of medium when aligning the cells. The right image shows the same microchannel at 60 min with numerous hemocytes (primarily granulocytes) migrating toward HCP.

(D) Hemocyte migration in transwell chemotaxis chambers to integument extract (0.1 μg/ml) plus preimmune serum (1:100 dilution) (Integ), integument extract (0.1 μg/ml) plus anti-HCP antibody (1:100 dilution) (Integ+Ab), or HCP (0.2 μM) plus anti-HCP antibody (1:100) (HCP + Ab) (n = 6 per treatment). Anti-HCP significantly reduced migration relative to preimmune serum (F-test; $p < 0.001$).

contrast, most larval stage insects including Lepidoptera have a flexible integument and hemocoels that contain large volumes of hemolymph in which most hemocytes circulate [13, 22]. A soft, pliable cuticle is likely an adaptation for accommodating the rapid growth that occurs during the larval stage but it also renders larvae more vulnerable to significant blood loss if wounded. We thus hypothesized that HCP functions as a component of the basal immune response with

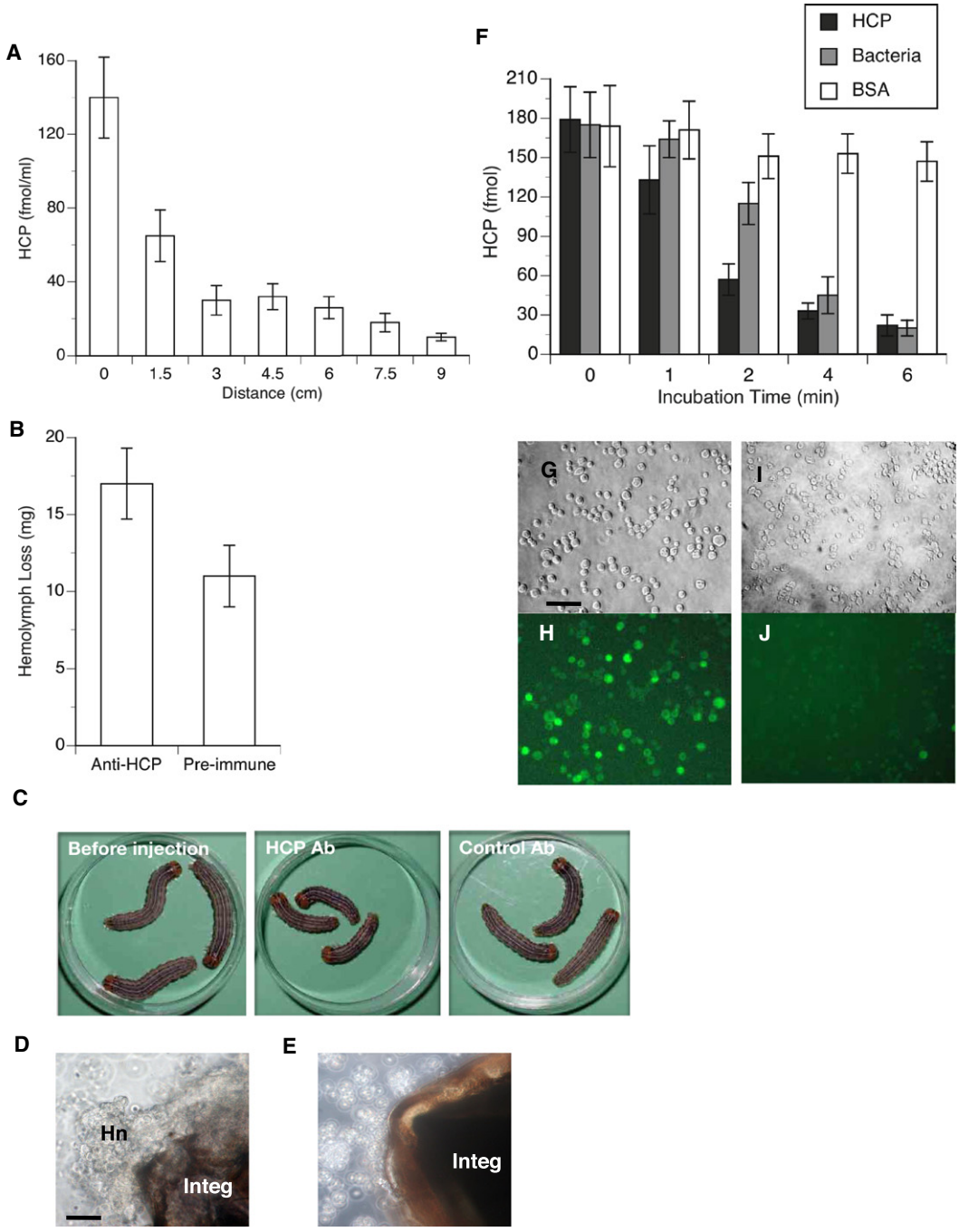

Figure 3. HCP Reduces Blood Loss and Stimulates Release of HCP from Hemocytes

(A) HCP from integument rapidly forms a concentration gradient. Fragments of integument (ca. 1 × 1 mm) were placed in one end of a primary culture chamber (9 cm long × 3 mm wide) in 3 ml of ExCell 420 medium. HCP concentration was then measured by ELISA at 1.5 cm intervals by removing an aliquot of medium after 20 min (n = 3 replicates).

(B–E) Immunodepletion of HCP increases blood loss and decreases accumulation of hemocytes at wound sites.

(B) Two hours after injection of anti-HCP or preimmune serum (10 mg/larva), a single proleg was severed (n = 12 larvae for each treatment) and the amount of blood loss was determined after 20 min. Larvae injected with anti-HCP lost significantly more hemolymph than did larvae injected with preimmune serum (t test; $p \leq 0.01$).

(C) Photographs of larvae before injection and wounding and at the end of the assay. Larvae are clearly larger before antibody injection and wounding (before injection) than after. At the end of the assay, larvae treated with anti-HCP are also smaller than larvae treated with preimmune serum (Control Ab) resulting from greater loss of hemolymph.

(D and E) Phase-contrast micrographs of wounded integument from larvae treated with preimmune serum (D) versus anti-HCP (E). Large numbers of aggregated hemocytes (Hn) are bound to the damaged integument (Integ) in the preimmune serum treatment, whereas few hemocytes are attached to the integument in the anti-HCP treatment. Scale bar in (D) represents 200 μM.

(F) Purified HCP and bacteria stimulate release of HCP from hemocytes. Ten fmol of purified HCP or 1×10^4 heat-killed *E. cloacae* cells were added to hemocytes in 100 μl of ExCell medium. Addition of 10 fmol BSA to cells served as the control. The amount of HCP (fmol) in hemocytes was then determined at 1 min intervals after treatment by ELISA.

wounding of the integument resulting in exposure of the endocuticle to the hemocoel. As a result, HCP is released into hemolymph, which activates and recruits circulating hemocytes to the site of injury. Because our data indicate that HCP is expressed in hemocytes, we further hypothesized that hemocytes also release HCP in response to wounding.

To test these hypotheses, we first confirmed by enzyme-linked immunosorbant assay (ELISA) that damaged integument rapidly releases HCP, which forms a concentration gradient (Figure 3A). We then reasoned that depletion of HCP after wounding would result in increased blood loss because of compromised recruitment of hemocytes. We tested this by a neutralization approach founded on our in vitro data (Figure 2D) and the broader literature in which immunodepletion is often used to selectively remove coagulation factors from vertebrate plasma. These experiments indicated that larvae preinjected with anti-HCP lost significantly more hemolymph than did control larvae injected with preimmune serum (Figures 3B and 3C). This effect also correlated with differential accumulation of hemocytes at the wound site (Figures 3D and 3E). We then examined whether hemocytes release HCP in response to two factors that are introduced into the hemocoel upon wounding: bacteria from the surface of the larva and HCP itself. Our results indicated that both caused hemocytes in primary culture to rapidly release HCP as evidenced by decreased HCP levels in hemocytes themselves (Figures 3F–3J) and a concomitant rise in HCP concentration in the medium (data not shown).

Unlike embryos and presumably adults [8–12, 21], live imaging studies in *Drosophila* larvae indicate that hemocytes arrive at sites of injury by direct capture from circulation of individual cells or clusters (= aggregations) [13]. Our results indicate that localization to the endocuticle isolates HCP from hemocytes under homeostatic conditions but that wounding stimulates rapid release of HCP into circulation. This response initially induces aggregation of circulating hemocytes in proximity to the wound, but is also reinforced through HCP and/or bacteria-induced release of HCP from hemocytes themselves. Upon binding to the damaged integument, we speculate that HCP mediates chemotactic movement of individual hemocytes to fill the wound. This model is diagrammed in Figure 4.

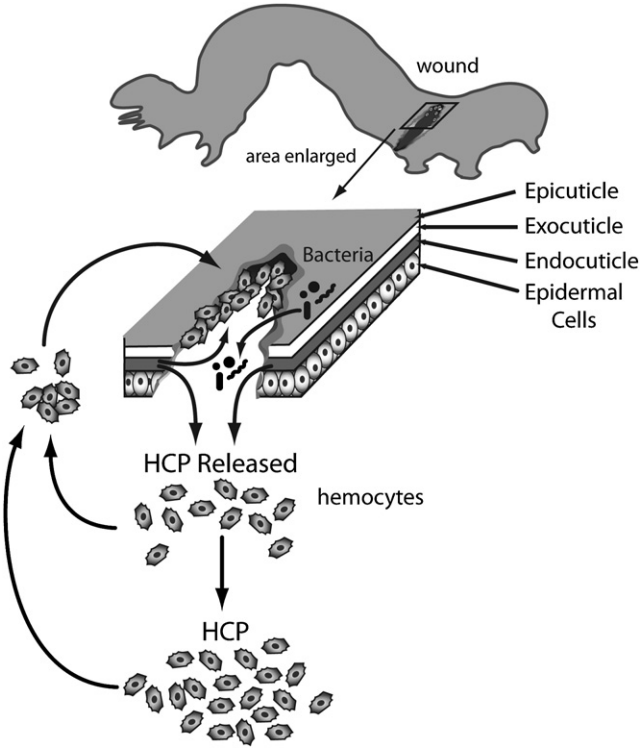

Figure 4. Model of HCP Activity after External Wounding of *P. separata*

The top of the figure shows a wounded larva. Below the larva is shown an enlargement of the wound site with HCP from the endocuticle and bacteria on the surface of the larva entering the hemolymph at the wound site. HCP from the endocuticle stimulates aggregation of circulating hemocytes in proximity to the wound and release of HCP from hemocytes themselves, which accelerates clotting (see Results and Discussion).

Conclusions

Chemokines are functionally defined as small, secreted cytokines that mediate immune cell activation and migration, whereas vertebrate chemokines are structurally classified on the basis of cysteine residue spacing patterns [23, 24]. No homologs of vertebrate chemokine family members have been identified in invertebrates including insects. However, other factors likely function as invertebrate chemokines given that hemocytes exhibit migratory behavior [9, 11–13]. Our results support the conclusion that HCP functions as an invertebrate chemokine. That the primary responding hemocytes are granulocytes is also fully consistent with their role as the professional phagocytes in Lepidoptera [3] and the need for rapid clearance of microbes, which otherwise would enter the insect's hemocoel after wounding. Release of HCP from hemocytes after exposure to bacteria or HCP itself further suggests an amplification mechanism for enhancing clotting.

HCP adopts a tertiary structure comprised of a disordered N terminus of six amino acids, a structured domain distinguished by a type II β-turn and disulfide bond for stabilizing overall topology, and a disordered C terminus in which Thr22 and Thr23 are glycosylated. Despite low sequence homology, these features strongly resemble ENF peptides that induce adhesion and chemokinetic movement of plasmatocytes [16, 25–29]. In addition to the importance of the structured core of ENF peptides for biological activity, their conserved six amino acid N termini serve as an essential signaling domain, which is highly sensitive to length and specific functional groups including a charged N-terminal amine [30–32]. Whether the N-terminal domain is similarly essential for the biological activity of HCP is unknown, although we think it likely given these structural similarities. We thus conclude that HCP and ENF peptides share sufficient features with one another to consider them members of a family of insect cytokines.

Although clearly not homologs of vertebrate chemokines, HCP and other ENF peptides do exhibit structural determinants shared with some vertebrate chemokine family members. Similar to ENF peptides, for example, the activity of the CC subfamily chemokines CCL2 (MCP-1) and CCL5 (Rantes) critically depends on the length of their N termini

(G and H) Light and epifluorescent micrographs of hemocytes before treatment with purified HCP. HCP in hemocytes (green) was visualized with anti-HCP and an Alexa fluor 488 secondary antibody. Scale bar in (G) represents 120 μm.

(I and J) Light and epifluorescent micrographs of hemocytes 5 min after treatment with purified HCP. Note that almost no HCP is detectable.

and the presence of a free N-terminal amine [33–35]. Like HCP, glycosylation of specific C-terminal domain residues also significantly enhances biological activity including chemotaxis of the C subfamily member XCL1 (lymphotactin) [36]. Far less clear is whether parallels also exist between HCP or other ENF peptides and vertebrate chemokines in the downstream signaling factors required for chemotaxis. All known vertebrate chemokine receptors with signaling activity are also G protein-coupled receptors (GPCRs) with chemotaxis requiring activation of phosphatidylinositol 3-kinases (PI3Ks), the serine/threonine kinase Akt/PK, and one or more Rho GTPases that modulate polarization of the cytoskeleton [23, 24]. In contrast, little is known of the signaling pathways that operate in chemotaxing and aggregating hemocytes, although studies with embryonic hemocytes from *Drosophila* do demonstrate a requirement for PI3K in chemotaxis toward wound sites [12].

Supplemental Data

Supplemental Data include Supplemental Results and Discussion, Supplemental Experimental Procedures, one table, and seven figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)00904-X.

Acknowledgments

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