Isolation and Identification of a Plasmatocyte-spreading Peptide from the Hemolymph of the Lepidopteran Insect Pseudoplusia includens*

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Insect blood cells (hemocytes) play an essential role in defense against parasites and other pathogenic organisms that infect insects. A key class of hemocytes involved in insect cellular immunity is plasmatocytes. Here we describe the isolation and identification of a peptide from the moth Pseudoplusia includens that mediates the spreading of plasmatocytes to foreign surfaces. This peptide, designated plasmatocyte-spreading peptide (PSP1), contains 23 amino acid residues in the following sequence: H-ENFNGGCLAGYMRTADGRCK-PTF-OH. In vitro assays using the synthetic peptide at concentrations ≥2 nM induced plasmatocytes from P. includens to spread on the surface of culture dishes. Injection of this peptide into P. includens larvae caused a transient depletion of plasmatocytes from circulation. Labeling studies indicated that this peptide induced 75% of plasmatocytes that were double-labeled by the monoclonal antibodies 49G3A3 and 43E9A8 to spread, whereas plasma induced significantly more plasmatocytes to spread. This suggests that only a certain subpopulation of plasmatocytes responds to the peptide and that other peptidyl factors mediate plasmatocyte adhesion responses.

The immune system of insects is the most important internal defense against parasites and pathogens. Elimination of organisms that enter the insect hemocoel requires that blood cells (hemocytes) be able to recognize and respond to the invading species (1–3). Large metazoan parasites are usually killed by encapsulation, a process in which certain classes of hemocytes recognize, adhere to, and spread across the surface of the foreign target to form a multilayered sheath of cells. In contrast, smaller pathogens are either phagocytized or enveloped by small aggregations of hemocytes that result in the formation of nodules. Hemocytes also participate in clotting and formation of aggregations at wound sites if the integument is damaged (1, 3).

Insect hemocytes are divided into several classes on the basis of morphology, antigenic properties, and function (2, 4–6). In insects like moths and butterflies (Lepidoptera), granular cells and plasmatocytes are the primary classes of hemocytes involved in cellular defense responses (2, 3, 7). However, little is known about the molecules mediating the function and trafficking of these cells (3, 8). The activity of hemocytes is affected in some insect species by endocrine factors, eicosanoids, and biogenic amines (9–11). Epidermal tissue, fat body, and hemolymph also contain unidentified peptidyl factors that affect hemocyte function or the abundance of specific subpopulations in circulation (12–15). While these and other factors from invertebrates have the biochemical and functional properties of vertebrate cytokines (16), no specific cytokine-like molecules have yet been purified and characterized from insects.

Identification of molecules mediating hemocyte function has proved difficult for two reasons. First, the small size of most insects limits the amount of material available for isolation and purification of active factors. Second, in vitro bioassays are required to characterize the response of hemocytes to specific molecules, yet insect blood cells are notoriously difficult to manipulate in culture due to their tendency to aggregate or lyse spontaneously upon removal from the hemocoel (2). In our studies of the moth Pseudoplusia includens, we have developed assay methods that allow us to manipulate hemocytes in vitro for extended periods of time (17–20). Using these methods, we observed that cell-free hemolymph (plasma) from P. includens stimulated the adhesion and spreading of plasmatocytes to foreign surfaces (17). Herein we report the purification and sequence of a 23-amino acid peptide from P. includens hemolymph that induces plasmatocytes to rapidly spread on foreign surfaces.

EXPERIMENTAL PROCEDURES

Insects—P. includens larvae were reared on an artificial diet at 27 °C and with a 16-h light/8-h dark photoperiod (21). Moths were fed 20% sucrose in water and maintained under identical environmental conditions.

Hemocyte Collection and in Vitro Bioassays—Hemocytes were collected from P. includens larvae by established methods (17, 20). Briefly, 36–48-h fifth instar larvae were anesthetized with CO₂ and bled from a cut across the last abdominal segment directly into anticoagulant solution (98 mM NaOH, 186 mM NaCl, 17 mM Na₂EDTA, and 41 mM citric acid, pH 4.5) (22). After a 30-min incubation at 4 °C, hemocytes were washed twice by centrifugation in Ex-cell 400 medium (JRH Biosciences). Plasmatocytes and other classes of hemocytes were identified using previously established morphological and antigenic criteria (6, 17, 19, 23). Plasmatocytes and granular cells account for ~30 and 65%, respectively, of the total hemocyte population in P. includens and are the only classes of hemocytes that spread on foreign surfaces. The other nonadhesive classes of hemocytes in P. includens are spherule cells, oenocytoids, and prohemocytes (17, 23).

Gel filtration and strong anion-exchange column fractions were assayed for their ability to induce spreading of plasmatocytes by conducting bioassays in 96-well culture dishes (Corning, tissue culture-treated polystyrene, 25861). Into each well was placed 27 μl of the fraction, 3 μl of 10× Pringle’s saline (24), 30 μl of Ex-cell 400 medium, and 5 μl of the mixed hemocyte suspension producing a final density of 1 × 10⁴ hemocytes/well. Hemocytes were monitored by phase-contrast microscopy using a Nikon TMS inverted microscope. Due to the limited amount of
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bioactive material in HPLC fractions, bioassays with these fractions were carried out in a 5-μl volume. Each experiment consisted of placing 1.8 μl of the HPLC fraction, 0.2 μl of 10 × Pringle’s saline, 2 μl of Ex-cell 400 medium, and 1 μl of the mixed hemocyte suspension onto a tissue culture dish (60 × 15 mm, Falcon 3002, Becton Dickinson). The final hemocyte plate was a 50 μl of Ex-cell 400 medium plus cell number. A 0.2 μl solution of the synthesized peptide diluted in 5 μl of PBS, pH 7.2. Larvae injected with PBS only served as a control. Larvae were anesthetized with carbon dioxide and injected through a proboscis using a glass needle mounted on a micromanipulator. At selected intervals, post-injection larvae were bled and the total hemocyte counts were made using a Neubauer hemocytometer. Differential hemocyte counts were made by taking a 1-μl aliquot of hemolymph from each larva and pipetting it into individual wells of a 96-well culture plate containing 50 μl of Ex-cell 400 medium. After 1 h, hemocytes were fixed and processed for immunocytochemical staining of plasmatocytes. Two-hundred cells from a randomly selected field of view were counted and identified by subclass. Immunocytochemistry—Plasmatocyte subpopulations were labeled with the plasmatocyte-specific mAbs 43E9A8 and 49G3A3 (6). Briefly, hemocytes were fixed in 10% Formalin for 10 min, rinsed with PBS, and permeabilized for 15 min in PBS plus 0.1% Triton X-100 (PBT). Cells were blocked for 1 h in 3% bovine serum albumin fraction (V, Boehring-Mannheim) in PBT, followed by incubation for 1 h with the primary antibody. After rinsing four times in PBT, hemocytes were incubated with fluorescein isothiocyanate- or Texas Red-conjugated goat anti-mouse secondary antibody (IgG; Jackson ImmunoResearch Laboratories, Inc.). Double labeling of hemocytes with mAbs 43E9A8 and 49G3A3 was conducted as described elsewhere (20). Samples were examined, and the number of labeled hemocytes was counted using the Nikon Diaphot microscope.

Preparation of Plasma—Purification of a plasmatocyte-spreading factor began by collecting hemolymph from a minimum of 500 fifth instar P. includens larvae, which yielded 20 ml of hemolymph. Immediately after the hemolymph was collected, glutathione (100 mM in H2O) was added to each sample to inhibit melanization (17). Hemocytes were pelleted in a benchtop centrifuge (Eppendorf 5415 C) for 5 min at 200 × g and discarded. The resulting cell-free hemolymph, referred to as plasma, was stored at −80 °C before analysis.

Purification of a Plasmatocyte-spreading Factor from Hemolymph—Preliminary experiments on the size and chemical nature of spreading activity were first performed by fractionating plasma using a 10-kDa cutoff Centricon filter (Amicon, Inc., Beverly, MA). Boiled plasma was also treated with carboxypeptidase Y (Pierce), aminopeptidase M (Pierce), trypsin (Sigma), or proteinase K (Life Technologies, Inc.) according to the manufacturer’s instructions and then passed through a 10-kDa filter. Other samples of plasma were boiled for 4 min in 1.5 ml Eppendorf centrifuge tubes, cooled on ice for 20 min, and centrifuged for 20 min at 16,000 × g using an Eppendorf 5415 C centrifuge to remove the precipitate. The supernatant was then passed through a 10-kDa cutoff filter as described above.

The supernatant from boiled plasma was lyophilized, resuspended in HPLC-grade H2O at a 5-fold concentration, and fractionated by gravity flow through a Kontes ( Fisher ) 50 × 2.5-cm glass column packed with Bio-Gel P-4 (M, range of 800–4000; Bio-Rad) at a flow rate of 0.5 ml/min. Three-ml fractions were eluted with 1 ml MOPS ( Fisher ), pH 7.2. bovine serum albumin was added to each fraction to achieve protein density of 5–10 mg/ml. The column was monitored for absorbance at 280 nm and biologically active fractions were pooled into three major peaks: shallow 15–30% acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid achieved by rechromatographing on the same HPLC column using a 15–30% acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid over 60 min. Fractions were lyophilized overnight, resuspended in 100 μl of HPLC-grade H2O, and bioassayed. The biologically active peak was dried in a Speed-Vac, yielding 1.1 μg of peptide, which was submitted for sequence analysis. This represents a 55% yield from the 20 ml of plasma that served as starting material. Our determination of yield was based on a quantitative immunoaassay using a recently developed PSF-specific antibody, which indicates that the concentration of PSF1 in wandering stage (fifth instar) P. includens larvae is 180 ng/ml of plasma.

N-terminal sequences were determined by Edman degradation using an Applied Biosystems Model 477A pulsed liquid-phase protein sequencer in the Protein/Nucleic Acid Shared Laboratory of the Medical College of Wisconsin. Amino acid composition analysis was performed in vacuo by vapor from 5.7 M HCl and 0.02% β-mercaptoethanol at 110 °C for 20 h. Identity of cysteine residues was determined by performic acid oxidation. Hydrolysates were analyzed using a Beckman 6300 post-column ninhydrin system analyzer. The peptide was synthesized in the same laboratory on a MilliGen 9050 peptide synthesizer using Fmoc chemistry and Fmoc-Phe-NovaSyn KA100 resin. The peptide was dried from the resin, and the protecting groups were removed with standard King’s reagent. To ensure that the desired structure was obtained, the synthesized peptide was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Mass spectral data were generated on a Bruker REFLEX II mass spectrometer operating in a reflection and delayed extraction mode using a 337-nm nitrogen laser and external calibration. The matrix was 2,5-dihydroxybenzoic acid. Mass spectral data for the synthetic peptide were compared with mass spectral data for the purified peptide to assess whether any post-translational modifications existed in the peptide isolated from P. includens plasma. Sequence analysis was performed using the Genetics Computer Group package (25).

RESULTS

A Factor in Plasma Induces Plasmatocytes to Spread on Foreign Surfaces—A previous study revealed that plasmatocytes from P. includens spread much more rapidly on the surface of culture plates when incubated in Ex-cell 400 medium supplemented with plasma than when cultured in medium alone or medium supplemented with vertebrate serum (19). When plasma was fractionated using a 10-kDa cutoff filter, we found that >80% of plasmatocytes spread within 1 h when cultured in the <10-kDa fraction, whereas almost no plasmatocytes spread when cultured in Ex-cell 400 medium (Fig. 1). Serial dilution of the <10-kDa fraction resulted in a linear decrease in the proportion of spread plasmatocytes, indicating that the factor(s) was not present at a response-saturating concentration. Similar results were obtained using the flow-through from plasma fractionated on a 3-kDa Centricon cutoff filter (data not shown).

Purification of a Plasmatocyte-spreading Factor—To determine whether the plasmatocyte spreading activity was sensitive to heat, plasma from P. includens was boiled and then centrifuged to remove the very large precipitate that formed (40% of the original volume). Few plasmatocytes spread on the

1 The abbreviations used are: HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; mAb, monoclonal antibody; MOPS, 3-(N-morpholino)propanesulfonic acid; PSF1, plasmatocyte-spreading peptide 1; Fmoc, N-(9-fluorenyl)methoxycarbonyl

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FIG. 1. Spreading and aggregation of plasmatocytes from P. includens. A, effect of plasma concentration (v/v) on spreading of plasmatocytes in vitro. Spreading was assayed by observing cells after 1 h in culture with plasma. Each data point is the mean percentage ± S.E. of plasmatocytes spread from three independent collections of hemocytes. B, Hoffman modulation-contrast micrograph of unspread (U) plasmatocytes 1 h after culture in Ex-cell 400 medium alone. Bar = 15 μm. C, micrograph of spread (S) plasmatocytes 1 h after culture in medium supplemented with 80% plasma from P. includens. Bar = 15 μm. D, micrograph of a hemocyte aggregation (A) formed 1 h after culture in the >10-kDa fraction from boiled plasma (see “Results”). Bar = 150 μm.

Surface of our culture wells after 1 h when incubated in the supernatant from boiled plasma. However, this material did promote hemocytes to aggregate and form nodules (Fig. 1D). The boiled plasma was then passed through a 10-kDa cutoff filter to estimate the size of the aggregating factor(s). Incubation of hemocytes in the >10-kDa fraction induced hemocytes to aggregate, whereas the <10-kDa flow-through induced >70% of plasmatocytes in assay wells to spread on the surface of the culture plate. No further characterization of the factor(s) in the >10-kDa fraction was pursued during this study. However, because boiling removed most of the protein from plasma, we used this procedure as the first step in purifying a plasmatocyte-spreading factor. As a final preliminary experiment, we treated boiled plasma with trypsin, proteinase K, aminopeptidase M, and carboxypeptidase Y and then passed this material through a <10-kDa filter. Less than 5% of plasmatocytes spread after 1 h in vitro if cultured with material pre-treated with any of these proteases. Based on these results, we concluded that the spreading activity in boiled plasma was associated at least in part with a peptide unblocked at either its N or C terminus. Because of the peptidyl nature of the spreading factor, subsequent purification steps were monitored at an absorbance of 210 nm.

When boiled plasma was loaded onto a gel filtration column, three main peaks were eluted: one, at the void volume (fractions 22–27), corresponded to compounds of at least 4000 Da, whereas the second (fractions 51–58) and third (fractions 59–69) peaks were eluted at the low molecular mass range of the column (Fig. 2A). Spreading activity usually eluted as a single peak (fractions 42–51), although occasionally an earlier eluting peak containing a lesser amount of spreading activity was observed (data not shown). This earlier eluting peak was not included in the subsequent purification steps. Bioassays using fractions 42–51 induced plasmatocytes to spread in a manner identical to what we observed in bioassays using the flow-through from the 10-kDa cutoff filter.

When the partially purified spreading activity was analyzed by reverse-phase chromatography on an HPLC apparatus, the elution profile was so complex that an intermediate purification step became necessary. Active material from the gel filtration column was loaded onto a strong anion-exchange column and eluted with 0.1 M NaMOPS, pH 7.2. Monitoring at A210 showed one major peak at the void volume of the column (fractions 13–17), a second closely eluting peak (fractions 17–21), a third large peak (fractions 27–33), and a fourth small peak (fractions 47–49) (Fig. 2B). A minor amount of spreading activity appeared within the first major peak (fraction 15–16), whereas the majority of the activity coeluted with the second A210 peak (fractions 17–21). While two separate active peaks may have been present, they could not be completely resolved, so all marked fractions were pooled. These results also suggested that the active compound(s) must be only weakly negatively charged since no salt gradient was necessary to elute these peaks. Active fractions from the strong anion-exchange column were further purified by HPLC (Fig. 3A). The region from 52 to 54 min contained several small but relatively well resolved peaks that possessed spreading activity when bioassayed. These fractions were therefore rechromatographed on the same HPLC column using a shallower acetonitrile gradient (Fig. 3B). Although not completely base line-resolved, the spreading activity from this region was associated predominantly with a peak that eluted at 35.5 min. This peak was collected and lyophilized for further analysis.

Sequencing—Sequence and amino acid composition analyses of the isolated HPLC peak identified a 23-amino acid peptide (Fig. 4). While 2 internal amino acids (residues 7 and 19) could not be assigned by sequencing, performic acid oxidation analysis using material from the same sample indicated that these residues were cysteines. We repeated our isolation of this HPLC peak twice and obtained identical sequence and amino acid composition data. On the basis of the number of moles present in the sequenced sample divided by the original volume of plasma, we estimated the concentration of this peptide in plasma to be 40 nm. Comparison with sequences in the SwissProt and GenBank™/EMBL Data Banks revealed that the identified peptide shared significant sequence homology...
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Fig. 2. A. Gel filtration chromatography of boiled plasma from *P. includens*. Two ml of 5 × concentrated boiled plasma was loaded onto a Bio-Rad Bio-Gel P-4 column and monitored at A210. Sixty drop fractions were collected (open circles) and bioassayed for the ability to induce spreading of plasmatocytes. B. Strong anion-exchange chromatography of pooled active fractions from the gel filtration column. Fractions 41–51 from the gel filtration column were pooled, lyophilized, and resuspended at a 10× concentration in H2O. The sample was then loaded onto a Bio-Rad anion-exchange AG 1-×2 resin column and monitored at A210. The columns were run sequentially. Horizontal bars indicate fractions that induced >50% of plasmatocytes to spread after 1 h in culture. Spreading of plasmatocytes in response to each fraction was assayed as described for Fig. 1A.

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The Synthetic Peptide Induces Plasmatocytes to Spread on Foreign Surfaces—To determine whether the synthetic peptide induced spreading of plasmatocytes, we conducted dose-response studies. A 200 μM stock solution of the peptide was made in PBS containing 10 mM dithiothreitol to ensure that the cysteines were in a reduced state. From this, dilutions were made into Ex-cell 400 medium and tested from 1 nM to 100 μM. Hemocytes incubated in 1 or 10 μM synthetic peptide induced 70% of plasmatocytes to spread after 1 h (Fig. 5). The percentage of spread plasmatocytes decreased linearly in the presence of 1 μM to 1 nM peptide. Less than 2% of plasmatocytes spread after 1 h in lower concentrations of the peptide or in control wells that contained medium without peptide. Concurrent observations revealed that PSP1 did not stimulate the spreading of granular cells (data not shown). On the basis of these results, we hereafter refer to this peptide as plasmatocyte-spreading peptide (PSP1).

We further characterized the spreading response of plasmatocytes to PSP1 by conducting immunocytochemical studies. Previous studies revealed that 28–36% of the total hemocyte population in fifth instar *P. includens* larvae are plasmatocytes (17, 23). As shown in Fig. 1, plasmatocytes first placed into culture are rounded and unspread, yet in the presence of plasma, plasmatocytes rapidly spread and usually assume a fibroblastic morphology. However, the use of two monoclonal antibodies raised against *P. includens* hemocytes suggests that plasmatocytes consist of at least two subpopulations (6). While both mAbs stain virtually all plasmatocytes that spread on the surface of culture plates, ~20% of the hemocytes labeled by mAb 49G3A3 never spread in vitro despite morphologically looking like the other plasmatocytes that rapidly spread in the presence of plasma. These unspread plasmatocytes labeled by mAb 49G3A3 are not labeled by mAb 43E9A8.

To determine whether all plasmatocytes labeled by mAb 43E9A8 spread in response to PSP1, we collected hemocytes from individual larvae as described previously. Aliquots of hemocytes from each larva were cultured for 1 h in Ex-cell 400 medium (1 × 10^7 hemocytes/well) containing 10 nM, 100 nM, or 1 μM PSP1 or 80% plasma or in Ex-cell 400 medium alone. Note that 1 μM PSP1 and 80% plasma induced the highest response of plasmatocyte spreading in earlier experiments (see Figs. 1 and 5). At the end of the incubation period, hemocytes were double-labeled with mAbs 49G3A3 and 43E9A8. From a randomly selected field of view, 200 plasmatocytes labeled by mAb 49G3A3 were counted. Concurrently, we also scored each mAb 49G3A3-positive cell for whether or not it had spread on the surface of the culture plate and whether or not it was labeled by mAb 43E9A8.

As expected, the number of plasmatocytes that spread in vitro increased with increasing concentrations of PSP1 (Fig. 5B). Comparing hemocytes cultured in 1 μM PSP1 and 80% plasma revealed no significant difference in the number of cells stained by mAb 43E9A8 (paired t test; t = 0.8; p > 0.5). However, significantly fewer mAb 43E9A8-positive plasmatocytes had spread after 1 h in 1 μM PSP1 than in plasma (t = 5.2; p < 0.0001) (Fig. 5B). Increasing the concentration of PSP1 did not alter this trend (data not shown). To determine whether mAb 43E9A8-positive plasmatocytes that did not spread in the presence of PSP1 could be induced to spread by plasma, we monitored individual plasmatocytes in cultures that contained 1 μM PSP1. We then added plasma to each culture to a final concentration of 50%. As expected, while many plasmatocytes spread in the presence of PSP1, some plasmatocytes did not (Fig. 6, A and B). However, most of these unspread cells rapidly spread when plasma was added to the culture medium (Fig. 6C).

PSP1 Induces Depletion of Plasmatocytes from Circulation When Injected into *P. includens*—Injection of PSP1 into the

3 S. Loret, E. Gardiner, and M. R. Strand, unpublished results.
hemocoel of *P. includens* larvae induced a dose-dependent but transient decrease in the total number of hemocytes in circulation (Fig. 7A). Differential hemocyte counts revealed that the decrease in total hemocyte counts was due to a decline in the number of plasmatocytes in circulation. This was clearly evident when larvae injected with 5 µl of 200 µM PSP1 were compared with control larvae injected with medium only (Fig. 7B). The proportion of plasmatocytes in circulation dropped markedly 30 min after injection of this quantity of PSP1, but thereafter increased to control levels by 2 h post-injection. The estimated working concentration of the synthetic peptide in this experiment was 20 µM since fifth instar *P. includens* larvae contain ~50 µl of hemolymph. As little as 5 µl of 2 nm peptide induced a transient depletion of plasmatocytes from circulation (data not shown).

**DISCUSSION**

We describe here the purification and initial characterization of PSP1, a peptide in the hemolymph of *P. includens* larvae. The peptide purified from *P. includens* plasma rapidly induced
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plasmatocytes to spread in our in vitro bioassay. Synthetic PSP1 induced an identical dose-dependent spreading response and also caused a transient depletion of plasmatocytes from circulation when injected into the hemocoe of *P. includens* larvae. Since PSP1 does not affect the spreading activity or abundance of other hemocyte classes in circulation, we conclude that PSP1 mediates the spreading behavior of plasmatocytes. Plasmatocytes could be induced to spread in *vitro* by PSP1 at concentrations ≥1 nM, which is well within the concentration range (40–70 nM) we estimate to be present in the hemolymph of last stadium *P. includens* larvae. Higher concentrations of the peptide were required to induce high levels of plasmatocyte depletion in *vitro*, but this could be due to rapid metabolism of the peptide in the hemolymph or other factors that might influence biological activity. Quantification studies using an anti-PSP1 antibody indicate that the concentration of the peptide in *P. includens* plasma varies within and between instars of normal larvae and can also fluctuate in response to wounding or infection. We hypothesize that these changes in peptide concentration may be important in mediating the function of plasmatocytes.

PSP1 shares no significant sequence similarity with any peptide isolated from vertebrates, including identified chemokines implicated in leukocyte trafficking and inflammation responses. However, this peptide is similar to two classes of peptides identified in insects: growth-blocking peptide in the hemolymph of *Pseudoletia separata* (26, 28, 29) and a series of related molecules from *Manduca sexta*, *Heliothis virescens*, and *Spodoptera exigua* designated as paralytic peptides (27). Injection of growth-blocking peptide into last instar *P. separata* larvae transiently represses plasma juvenile hormone esterase activity and elevates dopamine levels. These effects are hypothesized to account for the delays in pupation that occur when last instar larvae are injected with this peptide (26, 30). In contrast, paralytic peptides induce a temporary paralysis when injected into larvae (27). Since our interest was in identifying factors that mediate the spreading of hemocytes, the isolation of PSP1 was coupled to a plasmatocyte spreading assay, and we employed purification methods that differed in many ways from the approaches used to isolate growth-blocking and paralytic peptides. We were thus surprised when the sequence for PSP1 shared substantial similarity with these molecules, including conservation in location of the cysteine residues. In comparing growth-blocking peptide with the seven paralytic peptides, we note that residues 4 and 8 are the most hyper-variable positions. Consistent with this hypervariability, the asparagine and leucine residues at these positions in PSP1 differ from any of the residues reported at these positions in the homologous peptides. Thus, out of a total of nine isolated peptides (growth-blocking peptide, seven paralytic peptides, and PSP1), 4 different amino acids are found at position 4, and 5 different amino acids are found at position 8.

At this time, we can only speculate about the significance of these structural differences and the divergent biological activities associated with this putative family of peptides. One possibility is that several related peptides may exist in Lepidoptera that act through a common signal transduction pathway. However, differences in sequence between PSP1, growth-blocking peptide, and the paralytic peptides, for example, may be significant in cell targeting and biological activity. Supporting this suggestion, injection of PSP1 over a range of concentrations did not induce significant delays in pupation or any paralysis of *P. includens* larvae. PSP1 also had no paralytic activity when injected into the larval stage of *M. sexta,* the species Skinner et al. (27) reported to be the most sensitive to the effects of the paralytic peptides they identified. We have not assessed whether PSP1 has any effect on juvenile hormone esterase activity or biogenic amines in *P. includens*. However, the estimated concentration of PSP1 that we found in *P. includens* plasma is broadly consistent with the concentration range of growth-blocking peptide in the hemolymph of *P. separata* larvae (~5–40 nM) (29). Alternatively, the differences observed between the sequence of PSP1 and those of the peptides described from other insects may reflect distinct interspecific differences in both structure and function. Comparative studies are currently underway to resolve the relationship between these molecules and their function in insect physiology.

More important, we note that the biological activity we found for PSP1 parallels the response induced by unidentified mediators of hemocyte responses in other species of Lepidoptera. Partial purification of a protease-sensitive factor from plasma of *H. virescens* resulted in the designation of encapsulation-promoting factor (19). This factor has an estimated molecular

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Fig. 6. Hoffman modulation-contrast micrographs of plasmatocyte spreading in response to synthetic PSP1 and *P. includens* plasma. *A*, hemocytes (1 × 10⁶ cells/well) 10 min after placement in a culture well containing Ex-cell 400 medium. Four selected plasmatocytes are indicated by arrows. Each of these plasmatocytes is unspread (U). *B*, hemocytes 1 h after the addition of PSP1 to the culture well at a final concentration of 1 μM. Two of the plasmatocytes that were unspread in *A* are now spread (S), whereas the other two plasmatocytes remain unspread. *C*, hemocytes 1 h after the addition of 50% (v/v) plasma to the culture. The two plasmatocytes that were unspread in *B* are now spread.

Fig. 7. Hemocytes in circulation following direct injection of PSP1 into fifth instar *P. includens* larvae. *A*, total hemocyte counts were made 2–120 min post-injection of 5 μl of PBS (control; □) or of 2 mM (×), 200 nM (■), and 200 μM (○) PSP1 in PBS. Each data point is the mean number of hemocytes (10⁶) per ml ± S.D. from five different larvae. *B*, mean percentage ± S.D. of hemocytes in circulation that are plasmatocytes following injection of PSP1 into fifth instar *P. includens* larvae. Five μl of PBS (control; □) or of PSP1 (200 μM) in PBS (○) was injected per larva. Larvae were then bled, and the percentage of plasmatocytes in the sample was determined by morphology and labeling with mAb 49G3A3. Each data point is the mean percentage of plasmatocytes from five larvae.

mass of <3500 Da and specifically promotes the spreading of plasmatocytes in a manner very similar to PSP1. A second putative class of regulatory molecules reported in the hemolymph of *Galleria mellonella* and *M. sexta* have been referred to as plasmatocyte depletion factors (14, 15). Injection of bacteria into these species results in a rapid but transient depletion of plasmatocytes from circulation. Injection of plasma collected from larvae previously challenged with bacteria had a similar effect, suggesting that a humoral factor is released into hemolymph that induces circulating plasmatocytes to transiently adhere to the hemocoel lining or internal organs. In vitro experiments suggested hemocytes as a source of plasmatocyte depletion factor in *G. mellonella* (14), whereas the source of encapsulation promoting activity in *H. virescens* was not identified (13).

Tissue-specific sources of PSP1 remain uncharacterized, but we have found PSP1-like activity in both hemocyte-conditioned medium (17, 19) and epidermal tissue, which suggests these cells as potential sources of synthesis or as sites of sequestration for this factor. Expression of growth-blocking propeptide mRNA in fat body and nervous tissue of *P. separata* also suggests these tissues as candidate sources for PSP1 (28). The methods we used for collecting plasma during the current study undoubtedly resulted in lysis of hemocytes and damage to the integument and fat body, which may have resulted in release of PSP1 into the hemolymph. Finally, we note that other peptides, possibly related to PSP1, likely serve as mediators of hemocyte activity. This is supported first by the observation that only a portion of plasmatocytes labeled by mAb 43E9A8 spread in response to PSP1, whereas the addition of plasma induced spreading of additional plasmatocytes recognized by this antibody. Second, we have partially purified other peptides that induce plasmatocyte spreading or aggregation. These peptides are clearly related to one another by size, charge, and hydrophobicity. Although some of these peaks may be breakdown products of native peptides, partial sequencing indicates that they are not breakdown products of PSP1.

Chadwick and Aston (8) suggested “hemokine” as a generic term for regulatory peptides that mediate the function of invertebrate hemocytes. Our results clearly indicate that PSP1 affects the function of a specific subpopulation of insect hemocytes and as such would be the first identified molecule from insects with the physiochemical properties of a hemokine. Many basic questions remain about the cells and/or tissues that produce PSP1 and how it affects the adhesive properties of hemocytes. We hypothesize that PSP1 is involved in regulating plasmatocyte adhesion in response to wounding of the integument or capsule formation. If so, PSP1 may be released from damaged tissues in close proximity to wound sites or foreign entities in the hemocoel. Other factors regulating adhesion may then come into play that result in strong attachment of plasmatocytes to a foreign surface or one another. The concept

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that PSP1 acts as a hemokine mediating plasmatocyte adhesion is not without precedent since studies of the mammalian immune system suggest an interdependence between proteoglycans, cell adhesion molecules, and cytokines (31–33). Future studies will undoubtedly clarify the role of PSP1 in insect cellular defense responses.

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REFERENCES

1. Ratcliffe, N. A., Rowley, A. F., Fitzgerald, S. W., and Rhodes, C. P. (1985) Int. Rev. Cytol. 97, 186–350
2. Strand, M. R., and Pech, L. L. (1995) Annu. Rev. Entomol. 40, 31–56
3. Gillespie, J. P., Kanost, M. R., and Trenczek, T. (1997) Annu. Rev. Entomol. 42, 611–643
4. Chain, B. M., Leyshon-Sorland, K., and Siva-Jothy, M. T. (1992) J. Cell Sci. 103, 1261–1267
5. Willot, E., Trenczek, T., Thrower, L. W., and Kanost, M. R. (1994) Eur. J. Cell Biol. 65, 417–423
6. Strand, M. R., and Johnson J. A. (1996) J. Insect Physiol. 42, 21–31
7. Ratcliffe, N. A. (1995) in Parasites and Pathogens of Insects (Beckage, N., Thompson, S., and Federici, B., eds) Vol. 1, pp. 267–304, Academic Press, Inc., San Diego, CA
8. Chadwick, J. S., and Aston, W. P. (1992) in Immunology of Insects and Other Arthropods (Gupta, A., ed) pp. 387–395, CRC Press, Inc., Boca Raton, FL
9. Stanley-Samuelson, D. W. (1994) J. Insect Physiol. 40, 3–11
10. Baines, D., and Downer, R. G. H. (1992) Arch. Insect Biochem. Physiol. 21, 303–316
11. Diehl-Jones, W., Mandato, C. A., Whent, G., and Downer, R. G. H. (1996) J. Insect Physiol. 42, 13–19
12. Cherbas, L. (1973) J. Insect Physiol. 19, 2011–2023
13. Davies, D. H., Hayes, T. K., and Vinson, S. B. (1988) Dev. Comp. Immunol. 12, 241–253
14. Chain, B. M., and Anderson, R. S. (1983) J. Insect Physiol. 29, 1–4
15. Geng, C., and Dunn P. E. (1989) Dev. Comp. Immunol. 13, 17–23
16. Habicht, G. S., and Beck, G. (1994) in Phylogenetic Perspectives in Immunity: The Insect Host Defense (Hoffman, J., Janeway, C., and Natori, S., eds) pp. 159–166, CRC Press, Inc., Boca Raton, FL
17. Pech, L. L., Trudeau, D., and Strand, M. R. (1994) Cell Tissue Res. 277, 159–167
18. Pech, L. L., Trudeau, D., and Strand, M. R. (1995) J. Insect Physiol. 41, 801–807
19. Pech, L. L., and Strand, M. R. (1995) J. Insect Physiol. 41, 481–488
20. Pech, L. L., and Strand, M. R. (1996) J. Cell Sci. 109, 2053–2060
21. Strand, M. R. (1990) Annu. Entomol. Soc. Am. 83, 538–544
22. Mead, G. P., Ratcliffe, N. A., and Renwrantz, L. R. (1986) J. Insect Physiol. 25, 795–803
23. Strand, M. R., and Noda, T. (1991) J. Insect. Physiol. 37, 839–850
24. Pringle, J. H. (1988) J. Exp. Biol. 15, 101–103
25. Devereux, J. D., Haerberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395
26. Hayakawa, Y. (1990) J. Biol. Chem. 265, 10813–10816
27. Skinner, W. S., Dennis, P. A., Li, J. P., Summerfelt, R. M., Carney, R. L., and Quistad, G. B. (1991) J. Biol. Chem. 266, 12873–12877
28. Hayakawa, Y., Ohnishi, A., Yamanaka, A., Izumi, S., and Tomizo, S. (1995) FEBS Lett. 376, 185–189
29. Ohnishi, A., Hayakawa, Y., Matsuda, Y., Kwon, K. W., Takahashi, T. A., and Sekiguchi, S. (1996) Insect Biochem. Mol. Biol. 25, 1121–1127
30. Noguchi, H., Hayakawa, Y., and Downer, R. G. H. (1996) J. Insect Physiol. 25, 197–201
31. Kincade, P. W. (1993) Nature 361, 15–16
32. Schall, T. J., and Rosen, K. B. (1994) Curr. Opin. Immunol. 6, 865–873
33. Butcher, E. C., and Picker, L. J. (1996) Science 272, 69–66