Plasmatocyte-spreading peptide (PSP) is a 23-amino acid cytokine that activates a class of insect immune cells named plasmatocytes. The tertiary structure of PSP consists of an unstructured N terminus (residues 1–6) and a well structured core (residues 7–23). A prior study indicated that deletion of the N terminus from PSP eliminated all biological activity. Alanine substitution of the first three residues (Glu1-Asn2-Phe3) further indicated that only replacement of Phe3 resulted in a loss of activity equal to the N-terminal deletion mutant. Here, we characterized structural determinants of the N terminus. Adding a hydroxyl group to the aromatic ring of Phe3 (making a Tyr) greatly reduced activity, whereas the addition of a fluorine (p-fluoro) did not. Substitutions that changed the chirality or replaced the aromatic ring of Phe3 with a branched aliphatic chain (making a Val) also greatly decreased activity. The addition of a methylene group to Val (making a Leu) partially restored activity, whereas the removal of a methylene group from Phe (phenyl-Gly) eliminated all activity. These results indicated that a branched carbon chain with a methylene spacer at the third residue is the minimal structural motif required for activity. The deletion of Glu1 also eliminated activity. Additional experiments identified the charged N-terminal amine and backbone of Glu1 as key determinants for activity.

A key type of insect blood cell required for immune responses such as encapsulation and clotting is the plasmatocyte (1–3). Plasmatocytes normally circulate freely in the open hemocoel of lepidopteran insects (moths and butterflies), but when activated they gain the capacity to attach to foreign or damaged surfaces. Plasmatocyte-spreading peptide (PSP),1 a 23-amino acid cytokine identified from the moth Pseudoplusia includens, is the most potent known activator of plasmatocytes (4). In addition to inducing a change in the adhesive state of these immune cells, PSP has chemokinetic activity and also affects larval growth and movement (5, 6).2 These diverse activities may orchestrate inflammatory responses to infection as well as regulate other homeostatic functions during development. PSP homologs have been identified from a number of other moth species, and based on the consensus sequence of their N termini (Glu-Asn-Phe-X-Gly), these molecules are referred to collectively as the ENF peptide family (5). Other ENF peptides besides PSP are also reported to function as plasmatocyte activators, suggesting that these molecules may be of widespread importance as cytokines that regulate plasmatocyte–mediated immune responses in Lepidoptera (5, 6).

PSP is expressed as a propeptide of 142 residues with the PSP sequence located at the C terminus (7). This biologically inactive precursor is then cleaved by an unknown protease to release the mature peptide. The structure of PSP consists of a disordered N terminus (residues 1–6) and a well structured core (residues 7–22) stabilized by a disulfide bond, hydrophobic interactions, and a short β-hairpin turn (Fig. 1A) (8). Comparison with other proteins reveals that despite sequence identity at only four positions, the core region of PSP adopts a very similar structure to the C-terminal subdomain of human epidermal growth factor (hEGF) and the fifth domain of the anti-coagulant protein thrombomodulin (hTM5). In contrast, the N terminus of PSP is similar only to other members of the ENF peptide family (8). Previous alanine scanning mutagenesis experiments identified several residues critical for the plasmatocyte-spreading activity of PSP (9). In the structured core, these include Cys7 and Cys19, which form the disulfide bond required for the proper three-dimensional structure of PSP, and the charged residue Arg13 within the β-hairpin. The unstructured N terminus is also essential, because deletion of the first six residues of PSP eliminates all biological activity. Ala replacement of Phe3 abolished activity in a manner identical to deletion of the entire N terminus, whereas Ala replacement of Glu1 and Asn2 enhanced activity in comparison to wild-type PSP (9).

In this study we further characterized the structural features of the N terminus required for PSP activity by bioassaying mutants with specific alterations to the ENF sequence. Our results indicated that determinants of Phe3 required for activity include its chirality, aromatic ring, and the distance of this ring from the peptide backbone. We also determined that the primary amine and backbone of Glu1 are required for function.

**EXPERIMENTAL PROCEDURES**

Insects—P. includens larvae were reared on an artificial diet at 27 °C and a 16-h light/8-h dark photoperiod (10). The moths were fed 20%...
sucrose in water and maintained under identical environmental conditions.

**Hemocyte Collection and Bioassays**—Plasmatocytes account for ~30% of the total circulating hemocyte population in fifth instar *P. includens* larvae (11, 12). For this study, total hemocytes were collected from *P. includens* larvae into anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM Na2EDTA, and 41 mM tricine acid, pH adjusted to 4.5) as described by Clark et al. (9). Plasmatocytes were then isolated to high purity from other hemocyte types using Percoll step gradients made in 12 × 75-mm plastic tubes (Falcon 352058). Gradients were made in Ex-cell 400 in two layers: a 2-ml bottom layer of 62.5% Percoll (100% Percoll equals 9 parts Percoll to 1 part 10–15% Pringle’s saline (150 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 20 mM dextrose)), and a 2-ml top layer of 52.5% Percoll. Plasmatocytes with an average purity of 93% were collected from the 52.5–62.5% Percoll interface, and the other hemocyte types (granular cells, spherule cells, and oenocytoids) banded elsewhere in the gradient. The number of plasmatocytes collected per gradient ranged from 1.0–1.4 × 10⁶ cells with the primary contaminant being granular cells.

The plasmatocyte-spreading activity of mutant and wild-type peptides was bioassayed in 96-well culture plates (Corning) using previously established methods (4). Peptides were first dissolved in Pringle’s saline. Plates were prepared by adding a given peptide solution (6 μl at 10× the desired final concentration) to each well. Wells were then filled with 54 μl of Ex-cell 400 medium that contained 1 × 10⁶ plasmatocytes. The percentage of plasmatocytes that spread in an assay was scored 1 h after adding peptide by counting 100 cells from a randomly selected field of view. Plasmatocytes were scored as spread if they assumed a flattened morphology and were >35 μm along their longest axis (4, 13). Unspread plasmatocytes remained spheroidal in shape. Each mutant peptide was bioassayed four times using an independently collected sample of plasmatocytes. Bioassays with each mutant peptide were always paired with bioassays of wild-type PSP to control for any variation in spreading response that might exist between plasmatocyte samples. The estimated maximum spreading response was defined as the percentage of plasmatocytes that spread when cultured in the highest concentration of peptide tested (100 μg/ml). The lowest concentration of peptide that induced ≥5% of plasmatocytes to spread was referred to as the threshold spreading response. Threshold spreading responses were determined empirically from dose-response curves.

**Peptide Synthesis and Purification**—All peptides were synthesized using Fmoc chemistry. The resin-peptide was cleaved and deprotected for 4 h in reagent K (14), a mixture containing 5% phenol, 1.25% water, 2.5% thioanisole, and 2.5% dithioethane in trifluoroacetic acid. After removing the resin from the reaction mixture by filtration, the peptide was precipitated in cold t-butylmethyl ether followed by repeated ether washes and air drying. Peptides were resuspended in 10 mM TRIS-HCl, pH 8.0, at a concentration of 1 mg/ml. Disulfide bond formation was periodically monitored by 5-μl injections onto an HPLC (Rheodyne 9725i manual injector, Hitachi L-6220 pump, Hitachi L-4500A photodiode array detector, and Hitachi D-7000 chromatography software), on which the reduced and oxidized peptides eluted in separate peaks on a C18 column (5-μm particle size, 4.6 mm × 25 cm, Supelco 58286) using HPLC-grade H2O (Sigma-Aldrich) and a linear gradient of acetonitrile (0–80 min, 20–60%) at 0.5 ml/min. Both the H2O and acetonitrile contained 0.05% trifluoroacetic acid. After the conversion was complete, the sample was purified by a series of 4–7 ml injections onto a preparatory HPLC column (10-μm particle size, 21.2 mm × 25 cm, Jupiter C18, Phenomenex, Inc., Torrance, CA) using HPLC-grade H2O and a linear gradient of acetonitrile (0–90 min, 10–90%) at 4 ml/min. The desired peak was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and disulfide bond formation was confirmed by NMR. After numerous purification runs, peaks were pooled, lyophilized, and resuspended in HPLC-grade H2O for the determination of amino acid composition and concentration.

**NMR Spectroscopy**—NMR samples were prepared by dissolving lyophilized peptide in 0.5 ml of suspension buffer (80% H2O/10% D2O and 20 mM sodium phosphate, pH adjusted to 6.0). Peptide concentrations ranged from 0.5 to 1.0 mM. All NMR spectra were recorded at 10°C on Bruker DMX 600 or DMX 750 spectrometers equipped with triple-resonance (1H/13C/15N) probes and three-axis pulsed-field gradient capabilities. One-dimensional 1H spectra were acquired with 4096 complex points and a spectral width of 12.5 ppm. All Fourier transformations of NMR data were performed with nmrPipe (15), and chemical shift assignments were obtained using the program XEASY (8). All 1H dimensions were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate.
N-terminal Determinants Required for Activity of PSP

FIG. 2. In vitro spreading responses of plasmatocytes to mutant Phe3 peptides in relation to wild-type PSP. A, plasmatocyte-spreading response to the b-enantiomer of Phe (F3F(D)), the para-fluoro derivative of Phe (F3F(p-fluoro)), and the phenylglycine (F3phenylG). B, plasmatocyte-spreading response to replacement of Phe3 with valine (F3V), tyrosine (F3Y), leucine (F3L), and alanine (F3A). Spreading was assayed by observing the cells after 1 h in culture with each peptide. Each data point is the mean percentage ± S. D. of plasmatocytes spread from four independent collections of plasmatocytes.

members (Fig. 1, A and B). Prior studies indicated that Ala substitution at either Glu1 or Asn2 resulted in mutant peptides with higher plasmatocyte-spreading activity than wild-type PSP, whereas Ala substitution at Phe3 eliminated all spreading activity (9). To further characterize the importance of Phe3 for PSP function, we synthesized mutants with specific modifications to this residue (Fig. 1C). Because the α-carbon of Phe is chiral, the first mutant we synthesized substituted the normal L-Phe with b-Phe. This enantiomer (F3F(D)) had no plasmatocyte-spreading activity (Fig. 2A), which suggests that the binding site for the putative PSP receptor depends on the L-chirality of this residue. We next tested mutants that altered the aromatic ring of Phe3 at the para position (Fig. 1C). The addition of a fluorine (p-fluoro) did not alter activity appreciably, yet the addition of a hydroxyl group, converting Phe3 to a tyrosine residue (F3Y), destroyed activity (Fig. 2A). This loss of activity for the F3Y mutant could be caused by the larger van der Waals radius of the hydroxyl group compared with fluorine, which in turn could prevent the F3Y mutant from fitting into the putative receptor binding pocket. Alternatively, a hydroxyl group by virtue of its dipole moment is more hydrophilic than a fluorine, which could reduce binding of the aromatic ring to a hydrophobic pocket. We next synthesized a peptide that preserved the aromatic ring of Phe3 but that moved its position closer to the peptide backbone by removing the methylene (β-CH2) spacer (Fig. 1C). This mutant (phenyl-Gly) also had little activity, suggesting that interaction of PSP with its receptor depends on the aromatic ring of Phe3 being at an appropriate distance from the peptide backbone (Fig. 2A). Our final manipulations replaced Phe3 with a valine or leucine to determine whether the branched portion of the aromatic ring structure was sufficient for biological activity (Fig. 1C). The F3V mutant had no spreading activity up to 100 μM, whereas F3L had only 50-fold less activity than wild-type PSP (Fig. 2B). Because leucine only differs from valine by an additional methylene group (β-CH2), we speculate that F3L maintains activity because it preserves in part the branched structure of the aromatic ring and has a long enough spacer for the side chain to insert into its binding pocket.

FIG. 3. In vitro spreading responses of plasmatocytes to mutant Glu1 and Asn1 peptides in relation to wild-type PSP. The mutants assayed were Ala replacement of Glu1 and Asn1 (AA-PSP), deletion of Glu1 (PSP(2–23)), and deletion of Glu1 and Asn2 (PSP(3–23)). Peptides were bioassayed as described in the Fig. 2 legend.

The N-terminal Amine and Backbone of Glu1 Are Also Essential for Activity—Because Ala substitution at Glu1 (E1A) or Asn2 (N2A) increases activity over wild-type PSP, we assessed whether PSP activity could be enhanced further by Ala substitution at both Glu1 and Asn2. This mutant (AA-PSP) exhibited activity that was 100–1000-fold greater than wild-type PSP at all concentrations, making it the most active PSP analog identified to date (Fig. 3). We then synthesized truncated PSPs lacking Glu1 and Asn2. Neither the Glu1 mutant (PSP(2-23)) nor Glu-Asn2 deletion mutants (PSP(3-23)) had any spreading activity despite the presence of Phe3 (Fig. 3). These results suggested first that Glu1 is potentially more essential than Asn2. They also suggested that the backbones of the first two residues are more important for function than their specific side chains given that AA-PSP has higher activity than the wild-type peptide. We tested this idea by replacing Glu1 with a glycine (E1G), which resulted in a mutant with only the backbone of Glu1 (Fig. 1B), and which also lacked the methyl group side chain present when Glu1 was replaced by Ala (E1A). Like the E1A mutant (9), E1G had higher activity than wild-type PSP (Fig. 4). This confirmed the importance of the Glu1 backbone and also implicated the primary amine at the N terminus as the most likely functional group required for activity. The necessity of the primary amine was tested in bioassays using an E1G-like mutant lacking the N-terminal amine. This acetylated PSP mutant, designated AcPSP-(2-23) (see Fig. 1B), had no activity (Fig. 4). An acetylated version of wild-type PSP (AcPSP) (Fig. 1B) also lacked activity (Fig. 4). The loss of function for these mutants could be caused by acetylation either blocking interaction of the amine with its binding target via steric hindrance or elimination of the positive charge of the primary amine (ammonium). In this regard, we also note that the 142-amino acid pro-PSP peptide lacks plasmatocyte-spreading activity (7). Many factors could account for the lack of activity of the
PSP precursor protein, but based on these results it is tempting to speculate that activity depends on cleavage of the precursor protein to form the N-terminal amine at Glu\(^1\). What remains unclear is why all ENF peptide family members have a glutamic acid and asparagine residue in the first two positions when other residues such as alanine or glycine clearly result in peptides with much higher biological activity.

The ENF Sequence Requires Covalent Linkage to the Structured C Terminus of PSP for Activity—Although the ENF sequence is critical for PSP function, the tripeptide ENF had no plasmatocyte-spreading activity (Fig. 5). The ENF tripeptide also had no activity when bioassayed in combination with the N-terminal deletion mutant that lacked the first six residues of wild-type PSP (PSP-(7-23)) (Fig. 5). Because we had determined previously that PSP-(7-23) alone lacks plasmatocyte-spreading activity (9), we conclude that a covalent connection between the unstructured and structured domains of PSP is required for function. To assess whether the position of the ENF motif is important for activity, we placed an additional glycine residue in the “linker” region between ENF and the C terminus (ENFNGC\(^3\)). This pep tide had similar activity to wild-type PSP (data not shown).

Mutations in the Unstructured N Terminus of PSP Do Not Alter Tertiary Structure—Most of the mutant peptides tested here had reduced plasmatocyte-spreading activity in comparison to wild-type PSP. The complete loss of activity of PSP-(2-23) and PSP-(3-23) was particularly surprising given that Ala substitution for Glu\(^1\) and Asn\(^5\) increased activity. Although these mutants all targeted the unstructured N-terminal domain of PSP, we compared them to wild-type PSP by NMR spectroscopy to ensure that altered activity was not caused by a change in overall tertiary structure. Because amide proton shifts are very sensitive to secondary and tertiary protein structure, we found in previous studies that changes in the \(\beta\)-hairpin or hydrophobic core of PSP mutants relative to wild-type could be detected by changes in chemical shift (>0.5 ppm) (9). Here, we found no difference in the \(^1\)H NMR spectra of PSP-(2-23) and PSP-(3-23) to that of wild-type, indicating that the loss of biological activity was not caused by significant alterations in structure (data not shown).

Implications of PSP Structure/Activity Data for Receptor Activation—For larger proteins, mutagenesis studies alone often cannot distinguish between residues critical for proper folding and residues required for receptor binding and/or biological activity. In contrast, the small size of PSP makes it especially well suited to a mutagenesis approach because of the relative ease of synthesizing peptides with precise alterations in individual residues and testing their effects on structure and biological activity. This and a previous study (9) together identify several residues important for the structure and plasmatocyte-spreading activity of PSP. Within the core domain of PSP (residues 7–23), Cys\(^7\) and Cys\(^19\) are required for maintenance of a proper structure but likely have no direct role in interaction with the receptor. The core domain is also distinguished by four charged residues (Arg\(^{13}\), Asp\(^{16}\), Arg\(^{18}\), and Lys\(^{20}\) within the \(\beta\)-hairpin (8)). Individually replacing these residues with Ala does not affect tertiary structure, but each does reduce activity with Ala substitution of Arg\(^{13}\) having the greatest effect (9). Ala replacement of residues in the unstructured N terminus similarly does not affect tertiary structure, but replacement of Phe\(^3\) or deletion of Glu\(^1\) both render PSP biologically inactive. Recently, we initiated studies to determine whether the R13A, F3A, and PSP-(2-23) mutants antagonize the plasmatocyte-spreading activity of wild-type PSP.\(^3\)

Our preliminary results suggest that PSP-(2-23) blocks the spreading activity of wild-type PSP when each peptide is present in bioassays at equal concentrations. The F3A mutant also antagonizes wild-type peptide activity but must be present at a higher concentration to do so. In contrast, R13A does not antagonize the activity of wild-type PSP at any concentration.

Although we have not yet measured receptor binding, the very similar tertiary structure of the aforementioned mutants to wild-type PSP makes it likely that they would function as competitive inhibitors for receptor binding. If so, the antagonism experiments described above suggest that PSP-(2-23) and wild-type PSP have equivalent binding characteristics, whereas the lack of antagonistic activity for the R13A mutant is indicative of Arg\(^{13}\) being required for receptor binding. We also suggest that these results in combination with our other data offer clues as to how PSP might activate its receptor. The most reasonable scenario would be that the PSP receptor first recognizes the structured domain of PSP and interacts with the charged residues in the \(\beta\)-hairpin. As noted above, Ala replacement of these residues individually reduces activity, but together they present a charged motif (+ + + +) to the receptor that is likely very important for binding. This interaction “locks” the peptide into place, yet clearly does not activate the receptor given that deletion of the N terminus eliminates all

\(^3\) K. D. Clark and M. R. Strand, unpublished results.
biological activity. We thus hypothesize that binding of the structured domain to the receptor stabilizes the N terminus in a conformation that allows it to fit into its binding pocket. The binding energy for the N terminus derives from the structural features of Phe3, and only after Phe3 attaches is the charged N-terminal amine brought into place to activate the receptor. Identification of the PSP receptor is obviously key to testing these ideas, but previous studies of the platelet activator thrombin offers interesting parallels (16). The thrombin receptor is activated when thrombin cleaves the N-terminal extension of its receptor to reveal a new N terminus that has a serine residue at position one and a phenylalanine at position two. This new extracellular domain then functions as a tethered ligand that self-activates the receptor. Of particular interest to this study, mutagenesis of the thrombin receptor indicates that both the N-terminal amine of Ser1 but not the side chain and the phenyl ring of Phe2 are required for receptor activation. Although the particular N-terminal residue and distance to the phenylalanine residue differ between this ligand and PSP, the overall similarities suggest a general mechanism for receptor activation that requires binding of an aromatic ring and a closely spaced primary amine with receptor-activating properties.

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N-terminal Residues of Plasmatocyte-spreading Peptide Possess Specific Determinants Required for Biological Activity
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