Specific Residues in Plasmatocyte-spreading Peptide Are Required for Receptor Binding and Functional Antagonism of Insect Immune Cells

Received for publication, February 2, 2004, and in revised form, June 8, 2004
Published, JBC Papers in Press, June 10, 2004, DOI 10.1074/jbc.M401157200

Kevin D. Clark‡, Stephen F. Garcynskiš, Aditi Arora¶, Joe W. Crim§, and Michael R. Strand¶¶
From the ‡Departments of Entomology and ¶¶Cellular Biology, University of Georgia, Athens, Georgia 30602 and the ¶Department of Entomology, University of Wisconsin-Madison, Madison, Wisconsin 53706

Plasmatocyte-spreading peptide (PSP) is a 23-amino acid cytokine that activates a class of insect immune cells called plasmatocytes. PSP consists of two regions: an unstructured N terminus (1–6) and a highly structured core (7–23). Prior studies identified specific residues in both the structured and unstructured regions required for biological activity. Most important for function were Arg¹, Phe³, Cys⁷, Cys¹⁹, and the N-terminal amine of Glu¹. Here we have built on these results by coexpressing cell binding and functional antagonism studies. Alanine replacement of Met¹² (M12A) resulted in a peptide with biological activity indistinguishable from PSP. Competitive binding experiments using unlabeled and¹²⁵I-M12A generated an IC₅₀ of 0.71 nM and indicated that unlabeled M12A, at concentrations ≥100 nM, completely blocked binding of label to hemocytes. We then tested the ability of other peptide mutants to displace¹²⁵I-M12A at a concentration of 100 nM. In the structured core, we found that Cys⁷ and Cys¹⁹ were essential for cell binding and functional antagonism, but these effects were likely because of the importance of these residues for maintaining the tertiary structure of PSP. Arg¹³, in contrast, was also essential for binding and activity but is not required for maintenance of structure. In the unstructured N-terminal region, deletion of the phenyl group from Phe³ yielded a peptide that reduced binding of¹²⁵I-M12A 326-fold. This and all other mutants of Phe³ we bioassayed were unable to antagonize PSP. Deletion of Glu¹ in contrast had almost no effect on binding and was a strong functional antagonist. Experiments using a photoaffinity analog indicated that PSP binds to a single 190-kDa protein.

The insect immune system consists of both cellular and humoral elements that innately recognize broad classes of foreign intruders (1–3). Insects usually kill multicellular parasites by encapsulation, which involves attachment of multiple layers of immune cells (hemocytes) to the foreign target. In Lepidoptera (moths and butterflies) like Pseudoplusia includens, many intruders are recognized as foreign by a class of hemocytes called granular cells, which release cytokines that induce the second class of hemocytes, plasmatocytes, to bind to the target and form a capsule (4, 5). The most potent known activator of plasmatocytes is plasmatocyte-spreading peptide (PSP) (6). Natural and synthetic PSP induce plasmatocytes to adhere and spread on foreign surfaces within minutes at concentrations ≥100 pM. PSP is expressed by granular cells and fat body 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 23-amino acid peptide (ENFNGCGLAGYMRTADGRCKPTF). 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 collectively referred to as the ENF peptide family (8). Other ENF peptides besides PSP also function as plasmatocyte activators, suggesting these molecules may be of widespread importance as regulators of the cellular immune response in Lepidoptera (8–10).

The three-dimensional structure of PSP consists of a disordered N terminus (residues 1–6) and a well defined core (residues 7–22) stabilized by a disulfide bond between Cys⁷ and Cys¹⁹, a hydrophobic interactions, and a short β-hairpin turn (11). Comparison with other proteins reveals that the core region of PSP adopts a very similar structure to the C-terminal subdomain of human epidermal growth factor and the anticoagulant protein thrombomodulin. No consensus binding site has yet been identified for EGF domains and their receptors, but several studies have implicated the C-loop as a critical region for binding (12–15). In contrast, the consensus sequence for the N terminus of PSP is found solely in the ENF peptide family (11). Using alanine replacement and deletion mutants, we also have identified residues in both the structured and unstructured regions of PSP that are essential for plasmatocyte-spreading activity (16, 17). 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 turn. Deletion mutants in the unstructured N terminus also eliminate all biological activity. Ala replacement of Phe³ (F3A) abolishes activity because of the specific requirement of the phenyl side chain (16). In contrast, Ala replacement of Glu¹ (E1A) actually enhances activity (17). This is, in part, because the activity of PSP requires the presence of the N-terminal primary amine but not the side chain of Glu¹ (17). Recent studies of the PSP homolog growth-blocking peptide likewise identify Phe³ as critical for plasmatocyte activation in Pseudaletia separata (10).

The preceding studies provide important insight about structure-function relationships but do not indicate whether muta-
tions in PSP that reduce biological activity affect receptor binding. In the current study we addressed this question by conducting competitive binding and functional antagonism experiments. Our results indicate that in most instances mutant peptides that strongly bound to hemocytes also functionally antagonized plasmaocyte spreading. However, we also identified a few mutants that bound to hemocytes but were poor functional antagonists. Receptor binding experiments showed that a photoaffinity analog of PSP bound to a single 190-kDa protein in a manner consistent with our binding and antagonism results.

**EXPERIMENTAL PROCEDURES**

**Insects—**P. includens larvae were reared on an artificial diet at 27 °C with a 16-h light/8-h dark photoperiod (18). Moths were fed 20% sucrose in water and maintained under identical environmental conditions. All experiments were conducted with hemocytes collected from 36-48-h fifth instar larvae.

**Hemocyte Collection and Bioassays—**Hemocytes were collected by anesthetizing larvae with CO₂ and bleeding them from an incision across the last abdominal segment. Hemolymph was collected in a microtube containing anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM Na₃EDTA, and 41 mM citric acid, pH adjusted to 4.5). The ratio of hemolymph to buffer was 1:1–1:2. Hemolymph was centrifuged at 14,000 × g, and the plasma-buffer supernatant was then removed. Hemocytes were resuspended in 1 ml of fresh anticoagulant. After a 40-min incubation at 4 °C, hemocytes were washed twice by centrifugation in Excell 400 insect cell culture medium (JRH Biosciences). These unseparated hemocytes were then used in binding assays (see below). Plasmaocytes and granular cells account for 30 and 65%, respectively, of the total hemocyte population in *P. includens* (19–20). Plasmaocytes were isolated to high purity on Percoll step gradients as previously described (16). On average, 1.2 × 10⁶ plasmaocytes were collected per gradient. Purity was 95%, with the primary contaminant being granular cells. These plasmaocytes were washed once in Excell 400 before use in antagonism assays (see below).

**Peptide Synthesis and Purification—**All peptides were synthesized on an Applied Biosystems 433 synthesizer using standard Fmoc chemistry as previously described (16, 17). The resin-peptide was cleaved and deprotected for 4 h in reagent K (21), a mixture containing 5% phenol, 1.25% water, 2.5% thioanisole, and 2.5% diethylether in trifluoroacetic acid. After removing the resin from the reaction mixture by filtration, the peptide was precipitated in cold methanol and washed twice with cold methanol to remove the resin. The desired peak was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry; disulfide bond formation was confirmed by NMRR. After numerous purification runs, peaks were pooled, lyophilized, and resuspended in HPLC-grade H₂O for the determination of amino acid composition and concentration. Mutant peptides were named according to the residue that was modified. For determination of amino acid composition and concentration, mutant peptides were purified by a preparatory HPLC column (10-μm particle size, 4.6 mm × 21.2 mm) using HPLC-grade H₂O containing 0.05% trifluoroacetic acid. The radiolabeled mixture was fractionated by high performance liquid chromatography on a reverse phase C8 column (Vydac, 300 Å, 4.6 × 150 mm): solvent A, water with 0.1% trifluoroacetic acid, and solvent B, 80% acetonitrile and 20% water with 0.1% trifluoroacetic acid (gradient program: 0–29% B, 29 min, 29–31% B, 40 min, 1 ml/min).

**Iodination of the PSP Mutant M12A—**Preliminary studies indicated that a photoaffinity analog of PSP bound to a single 190-kDa protein in a manner consistent with our binding and antagonism results. Our results indicate that in most instances mutant peptides that strongly bound to hemocytes also functionally antagonized plasmaocyte spreading. However, we also identified a few mutants that bound to hemocytes but were poor functional antagonists. Receptor binding experiments showed that a photoaffinity analog of PSP bound to a single 190-kDa protein in a manner consistent with our binding and antagonism results.
RESULTS

Binding Constants of Representative Peptides—Prior analysis indicated that several residues are invariant among ENF peptide family members (8, 16). These include Cys7 and Cys19, Thr14 and Thr22, all of the charged amino acids in the structured C terminus (Arg13, Asp16, Arg18, and Lys20), and the three N-terminal residues Glu1, Asn2, and Phe3. As previously noted, mutagenesis studies indicate that several of these residues are essential for biological activity (16, 17). The first step of the current study was to conduct full dose-response, competitive binding experiments using M12A and a subset of these mutant peptides. This allowed us to establish parameters for M12A binding that could then be used to optimize experiments with other mutants. For these studies, we used the alanine replacement mutants R13A and F3A, and AcPSP-(2–23), which lacked Glu1 and is acetylated at Asn2 to block the terminal amine. Each of these peptides has little or no biological activity and targets essential residues in the structured and unstructured regions of PSP. Our results indicated that M12A had an IC50 value of 0.71 nM and fully inhibited binding of 125I-M12A to hemocytes at concentrations $\leq$100 nM (Fig. 1). IC50 values for AcPSP-(2–23) (4 nM) and F3A (232.8 nM) were 5- and 326-fold higher, respectively, than M12A. R13A had an IC50 of 289.4 nM and did not fully inhibit binding of M12A at any concentration tested (Fig. 1).

PSP Mutants of the Core Region Exhibit Congruent Patterns of Competitive Binding and Functional Antagonism—We next conducted competitive binding and functional antagonism experiments using R13A and other alanine replacement mutants targeting the structured core region of PSP. Antagonism experiments were conducted over a range of concentrations (100 px100 µM). Binding experiments, in contrast, were conducted using only 100 nM of the unlabeled competitor because the data in Fig. 1 clearly indicate that unlabeled M12A at this concentration fully displaced binding of 100 px100 µM 125I-M12A. Alanine replacement of the two cysteine residues (C7A,C19A) results in a linearized peptide lacking the β-hairpin turn and $>$1000-fold loss of spreading activity (16). Commensurately, we found in the current study that C7A,C19A was an even poorer binding competitor than R13A (Fig. 2A). C7A,C19A and R13A were also poor functional antagonists that reduced the percentage of spread plasmatocytes only slightly at the highest concentrations tested (Fig. 2B). The other mutants we tested (D16A, R18A, and K20A) each strongly inhibited binding of 125I-M12A relative to R13A or C7A,C19A (Fig. 2A). Functional antagonism experiments were not conducted with these mutants because each has plasmatocyte-spreading activity only 10–100 times lower than PSP (16).

FIG. 1. Displacement of 125I-M12A by unlabeled M12A, R13A, AcPSP-(2–23), and F3A. Hemocytes were incubated with 100 px100 125I-M12A for 3 h along with unlabeled peptides ranging from 10 px100 to 10 µM. Values indicate means + S.E. (n = 6). IC50 values are presented in the legend to the right of each competitor tested. Cell binding assays are described under “Experimental Procedures.”

FIG. 2. Displacement and functional antagonism experiments using alanine replacement mutants of the PSP structured core. A, displacement of 125I-M12A by C7A,C19A, R13A, D16A, R18A, and R20A. Hemocytes were incubated with 100 px100 125I-M12A for 3 h along with 100 µM unlabeled peptides. Values indicate means + S.E. (n = 6). B, functional antagonism of PSP by C7A,C19A and R13A. Plasmatocytes were incubated with 3 µM PSP along with 100 px100 µM of indicated competitors and then assayed for spreading after 30 min in culture. Each data point is the mean percentage ± S.E. of spread plasmatocytes from four independent collections of cells. Cell binding and functional antagonism assays are described under “Experimental Procedures.”

N-terminal-truncated and -acetylated Peptides Also Exhibit Congruent Patterns of Binding and Functional Antagonism—No PSP mutant with an N-terminal truncation has any plasmatocyte-spreading activity (17). However, deletion mutants varied considerably in their ability to compete for binding
Receptor Binding and Functional Antagonism of PSP

Fig. 3. Displacement and functional antagonism experiments using deletion and acetylated mutants of the PSP N terminus. A, displacement of 125I-M12A by PSP-(7–23), PSP-(3–23), PSP-(2–23), AcPSP, and AcPSP-(2–23). Hemocytes were incubated with 100 µM 125I-M12A for 3 h along with 100 nM unlabeled peptides. Values indicate means ± S.E. (n = 6). B, functional antagonism of PSP by PSP-(2–23), PSP-(3–23), and PSP-(7–23). C, functional antagonism of PSP by AcPSP and AcPSP-(2–23). Plasmatocytes were incubated with 3 µM PSP along with 100 µM-100 µM of indicated competitors and then assayed for spreading after 30 min in culture. Each data point is the mean percentage ± S.E. of spread plasmatocytes from four independent collections of cells. Cell binding and functional antagonism assays are described under “Experimental Procedures.”

Some Phe3 Mutants Bind to Hemocytes but None Functionally Antagonizes PSP—Most alterations to Phe3 eliminate or greatly reduce plasmatocyte-spreading activity (17). These include substituting the D-isomer (F3F(D)), removing the phenyl group (F3A), and substituting a branched chain aliphatic (valine) that lacks a methylene spacer (F3V). Inserting a methylene spacer into F3V, making leucine (F3L), restores some activity. Additions at the paraposition of the phenyl ring have varying effects on activity. Adding a hydroxyl group, making tyrosine (F3Y), results in almost complete loss of activity, but replacing the hydroxyl group with a fluoride (F3F-fluoro) restores activity to levels almost identical to PSP. In the current study, we found that the F3A, F3F(D), F3V, and F3phenylG mutants each reduced binding of 125I-M12A to hemocytes by 40–50% (Fig. 4A) but none antagonized the plasmatocyte-spreading activity of PSP (Fig. 4B). F3Y reduced binding of 125I-M12A by 70%, whereas F3L and F3F-fluoro reduced binding more than 95% (Fig. 4A). However, in bioassays using F3V, F3Y, F3A, F3F(D), and F3phenylG, no functional antagonism of PSP was observed at any concentration tested (Fig. 4, B and C).

ENFN neither Binds to Hemocytes nor Functionally Antagonizes PSP—Despite the presence of the primary amine of Glu1 and phenyl group of Phe3, bioassays using the tripeptide ENF (Fig. 4B) and functional antagonism of PSP by AcPSP-(2–23) reduced binding of 125I-M12A by 40–50% (Fig. 4A) but none antagonized the plasmatocyte-spreading activity of PSP (Fig. 4B). F3Y reduced binding of 125I-M12A by 70%, whereas F3L and F3phenylG reduced binding more than 95% (Fig. 4A). However, in bioassays using F3V, F3Y, F3A, F3F(D), and F3phenylG, no functional antagonism of PSP was observed at any concentration tested (Fig. 4, B and C).
PSP (Fig. 5). We also previously reported that alanine replacement of both Glu\(^1\) and Asn\(^2\) resulted in a mutant peptide 100-fold more active than PSP (16). However, the tetrapeptide AAFN was as poor a functional antagonist of PSP as ENFN (Fig. 5A).

**PSP Binds with High Affinity to a 190-kDa Protein**—To determine whether PSP binds to a specific receptor on hemocytes, we used data presented both here and in previous studies (17) to design a high affinity analog that could be photochemically cross-linked. This ligand, AAFNGGCLAGYARTADGRCPSP-(7–23), was found to completely compete away labeled PSP (residues 1–4) does not inhibit binding of M12A or functionally antagonize PSP, whereas PSP-(7–23) binds as well as M12A whereas reducing the length two residues (PSP-(3–23)) reduces binding 20% and is a functional antagonist. The key residue in the C-terminal region required for binding appears to be the charged residue Arg\(^{13}\). In contrast, alanine replacement of the other charged residues surrounding Cys\(^{19}\) on the \(\beta\)-hairpin (Asp\(^{16}\), Arg\(^{18}\), and Lys\(^{20}\)) have little effect on binding of \(125^I\)-M12A. These data collectively suggest that initial binding of the C-terminal region to the receptor allows the unstructured region (residues 7–23 of PSP) to bind. Our results strongly suggest that the phenyl side chain of Phe\(^{2}\) is essential for binding of the N terminus and that this interaction “locks” PSP into place. The peptide backbone of the N terminus also likely contributes to binding energy, given that decreasing the length of PSP by one residue (PSP-(2–23)) binds as well as M12A whereas reducing the length two residues (PSP-(3–23)) reduces binding 35%. In contrast, deletion of the side chain from Asn\(^2\) (N2A) has no deleterious effect on either activity (17) or binding (data not presented). These results are also consistent with the ability of N2A to completely compete with \(125^I\)-F23Bpa in the receptor labeling experiments.

Prior studies indicated that the N-terminal amine of Glu\(^1\) is essential for biological activity (7), yet our current results reveal it is relatively unimportant for binding. This conclusion derives from our experiments with AcPSP and AcPSP-(2–23). AcPSP increased the overall length of the N terminus and removed the positive charge normally present at neutral pH,
whereas AcPSP-(2–23) also removed the positive charge and shortened the peptide. Although AcPSP-(2–23) binds strongly to hemocytes, AcPSP does not, indicating that increasing the length of the peptide backbone hinders attachment to its activating binding pocket whereas charge removal has little effect on binding. The inability of ENFN and PSP-(7–23) together to activate the receptor further suggests the possibility that binding sites for the N- and C-terminal regions of PSP may reside on different domains or subunits of the receptor that must be “cross-linked” by full-length PSP for hemocyte activation to occur.

The relationship between the phenyl ring of Phe3 and terminal amine of Glu1 is particularly intriguing when compared with the thrombin receptor involved in platelet activation (24, 25). The thrombin receptor is activated when thrombin cleaves the amino-terminal extension of its receptor to reveal a new N terminus with 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.

FIG. 6. Photo cross-linking of 125I-F23Bpa to PSPR. Mixed hemocytes from P. includens (400,000 cells/lane) were incubated with 50 pM–5 nM 125I-F23Bpa for 2 h at room temperature. After washing and UV cross-linking, cells were lysed and prepared for SDS-PAGE. After separation, proteins were transferred to nitrocellulose and exposed to film for 48 h. 125I-F23Bpa concentrations are indicated above each set of three lanes. –, no addition of unlabeled peptide; +N, addition of 1 μM N2A; +R, addition of 1 μM R13A.

Matz’s 190-kDa receptor model suggests the sensitivity of plasmatocytes to PSP varies with the larval stage of the insect cells collected. It also suggests that receptor abundance may be an important factor in regulating PSP function. Molecular identification of the putative 190-kDa PSP receptor is obviously key to testing the validity of these ideas.

Acknowledgments—We thank L. Kapa for assistance with the functional antagonism experiments, J. A. Johnson for Fig. 6, D. King for assistance and advice in peptide synthesis, and D. Phillips of the University of Georgia mass spectrometry center.

REFERENCES
1. Hoffmann, J. A., Reichhart, J.-M., and Hetru, C. (1996) Curr. Opin. Immunol. 8, 8–13
2. Gillespie, J. P., Kanost, M. R., and Trenzcek, T. (1997) Ann. Rev. Entomol. 42, 611–643
3. Lavine, M. L., and Strand, M. R. (2001) J. Insect. Physiol. 47, 965–974
4. Clark, K., Pech, L. L., and Strand, M. R. (1997) J. Biol. Chem. 272, 23440–23447
5. Clark, K., Witherell, A., and Strand, M. R. (1998) Biochem. Biophys. Res. Comm. 250, 479–485
6. Strand, M. R., Hayakawa, Y., and Clark, K. D. (2000) J. Insect. Physiol. 46, 817–824
7. Wang, Y., Jiang, H., and Kanost, M. R. (1999) Insect. Biochem. Mol. Biol. 29, 1075–1086
8. Tada, M., Aizawa, T., Shinohara, Y., Matsubara, K., Miura, K., Yoshida, M., Shitara, K., Kouno, T., Mizuguchi, M., Nitta, K., Hayakawa, Y., and Kawano, K. (2003) J. Biol. Chem. 278, 10778–10783
9. Volkman, B. J., Anderson, M. E., Clark, K. D., Hayakawa, Y., Strand, M. R., and Markley, J. L. (1999) J. Biol. Chem. 274, 4493–4496
10. Nagashima, M., Lundh, K., Leonard, J. C., Morser, J., and Parkinson, J. F. (1995) J. Biol. Chem. 270, 28888–28892
11. Mathews, I. I., Padmanabhan, K. P., Tulinsky, A., and Sadler, J. E. (1994) Biochemistry 33, 13547–13552
12. Hrabal, R., Komives, E. A., and Ni, F. (1996) Protein Sci. 5, 195–203

3 K. D. Clark, L. Kapa, and M. R. Strand, unpublished results.
15. McInnes, C., and Sykes, B. D. (1998) *Biopolymers* 43, 339–366
16. Clark, K. D., Volkman, B. F., Thoetiatical, H., King, H. B., Hayakawa, Y., and Strand, M. R. (2001) *J. Biol. Chem.* 276, 18491–18496
17. Clark, K. D., Volkman, B. F., Thoetiatical, H., Hayakawa, Y., and Strand, M. R. (2001) *J. Biol. Chem.* 276, 37431–37435
18. Strand, M. R. (1990) *Ann. Entomol. Soc. Am.* 83, 538–544
19. Pech, L. L., Trudeau, D., and Strand, M. R. (1994) *Cell Tissue Res.* 277, 159–167
20. Gardiner, E. M. M., and Strand, M. R. (1999) *J. Insect. Physiol.* 45, 113–126
21. King, D. S., Fields, C. G., and Fields, G. B. (1990) *J. Pep. Res.* 36, 255–266
22. Crim, J. W., Garceynski, S. F., and Brown, M. R. (2002) *Peptides* 23, 2045–2051
23. Strand, M. R., and Clark, K. D. (1999) *Arch. Insect. Biochem. Physiol.* 42, 213–223
24. Scarborough, R. M., Naughton, M. A., Teng, W., Hung, D. T., Rose, J., Vu, T.-K. H., Wheaton, Y. I., Turck, C. W., and Coughlin, S. R. (1992) *J. Biol. Chem.* 267, 13146–13149
25. Vergnolle, N., Ferazzini, M., D'Andrea, M. R., Boddenkotte, J., and Steinhoff, M. (2003) *Trends Neurosci.* 26, 496–500
26. Breslav, M., Becker, J., and Naider, F. (1997) *Tetrahedron Lett.* 38, 2219, 2222
