Alanine-scanning Mutagenesis of Plasmatocyte Spreading Peptide Identifies Critical Residues for Biological Activity*  

Received for publication, January 22, 2001  
Published, JBC Papers in Press, February 21, 2001, DOI 10.1074/jbc.M100579200  

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Plasmatocyte spreading peptide (PSP) is a 23-amino acid cytokine that induces a class of insect immune cells called plasmatocytes to spread on foreign surfaces. The structure of PSP consists of a disordered N terminus (residues 1–6) and a well-defined core (residues 7–23) stabilized by a disulfide bridge between Cys7 and Cys19, hydrophobic interactions, and a short β-hairpin. Structural comparisons also indicate that the core region of PSP adopts an epidermal growth factor (EGF)-like fold very similar to the C-terminal subdomain of EGF-like module 5 of thrombomodulin. To identify residues important for plasmatocyte spreading activity, we bioassayed PSP mutants in which amino acids were either replaced with alanine or deleted. Within the well-defined core of PSP, alanine replacement of Cys2 and Cys19 (C7.19A) eliminated all activity. Alanine replacement of Arg13 reduced activity 1,000-fold in comparison to wild-type PSP, whereas replacement of the other charged residues (Asp16, Arg18, Lys29) surrounding Cys19 diminished activity to a lesser degree. The point mutants Y11A, T14A, T22A, and F23A had activity identical or only slightly reduced to that of wild-type PSP. The mutant PSP-(7–23) lacked the entire unstructured domain of PSP and was found to have no plasmatocyte spreading activity. Surprisingly, E1A and N2A had higher activity than wild-type PSP, but F3A had almost no activity. We thus concluded that the lack of activity for PSP-(7–23) was largely due to the critical importance of Phe3. To determine whether reductions in activity correlated with alterations in tertiary structure, we compared the C7.19A, R13A, R18A, and F3A mutants to wild-type PSP by NMR spectroscopy. As expected, the simultaneous replacement of Cys7 and Cys19 profoundly affected tertiary structure, but the R13A, R18A, and F3A mutants did not differ from wild-type PSP. Collectively, these results indicate that residues in both the unstructured and structured domains of PSP are required for plasmatocyte-spreading activity.

In insects, the primary immune response toward parasites and other large foreign targets that enter the hemocoel is encapsulation (1, 2). During an encapsulation response, circulating blood cells (hemocytes) attach to the target and one another to form a smooth capsule comprised of overlapping layers of cells. The two types of hemocytes most often observed in capsules formed by Lepidoptera (moths and butterflies) are granular cells and plasmatocytes. In the moth Pseuodoplusia includens, some foreign entities are recognized by factors in plasma, but most are recognized by surface receptors on granular cells (3–5). After attaching to the target, granular cells and/or humoral opsonins then induce plasmatocytes to change from non-adhesive to strongly adhesive cells that form the capsule. Previously, we determined that plasmatocyte activation is mediated by a 23-amino acid cytokine named plasmatocyte spreading peptide (PSP)1,2 (6). Both purified and synthetic PSP induce plasmatocytes to rapidly adhere and spread across foreign surfaces at concentrations ≥ 1 nm (6, 7). PSP homologs occur in other Lepidoptera and in some cases have been shown to have plasmatocyte spreading activity (8, 9). Based on the consensus sequence of their N termini (Glu-Asn-Phe-X-X-Gly-Cys), these peptides are now referred to as the ENP peptide family (9).

PSP is expressed by granular cells and fat body as a propeptide of 142 residues with the PSP sequence located at the C terminus (10). This biologically inactive precursor is then cleaved by an unknown protease to release the mature peptide. 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 and a short β-hairpin turn (11). 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 anticoagulant protein thrombomodulin (hTM5). 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 known only among members of the ENF peptide family (9). In this study, we used an alanine scanning mutagenesis approach to identify residues critical for the plasmatocyte spreading activity of PSP. Our results indicate that selected residues in both the core and unstructured domains of PSP are required for plasmatocyte spreading activity.

**EXPERIMENTAL PROCEDURES**

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

**Hemocyte Collection and Bioassays—P. includens** hemolymph contains four hemocyte types (plasmatocytes, granular cells, spherule cells, and oenocytoids) with plasmatocytes and granular cells accounting for 30 and 65%, respectively, of the total hemocyte population (3, 18). Hemocytes were collected by anesthetizing 36- to 48-h fifth instar larvae with CO2 and bleeding them from an incision across the last abdominal segment. Hemolymph was collected in a microcentrifuge tube containing anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM Na3EDTA, and 41 mM citric acid, with pH adjusted to 4.5). The ratio of hemolymph to buffer was approximately 1:5. Hemocytes were pelleted for 1 min at 200 × g, and the plasma-buffer supernatant was then removed. Hemocytes were then resuspended in 1 ml of fresh anticoagulant. After a 40-min incubation at 4 °C, hemocytes were washed twice by centrifugation in Ex-cell 400 medium (URH Biosciences). Plasmatocytes were isolated to high purity by loading hemocytes from 8–10 larvae onto 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× Pringle’s saline (150 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 20 mM Dextrose), and a 2 ml top layer of 47.5% Percoll. Plasmatocytes with an average purity of 93% were collected from the 47.5–62.5% Percoll interface whereas 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 to 1.4 × 108 cells with the primary contaminant being granular cells.

The plasmatocyte spreading activity of wild-type and mutant peptides was assayed in 96-well culture plates (Corning) as described by Clark et al. (6). Plates were prepared by first adding the indicated 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 containing 1 × 105 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 (3, 9). Unspread plasmatocytes were removed from the 7–22) stabilized by a combination of the covalent disulfide linkage between Cys9 and Cys21, hydrogen bonding within the β-hairpin, and hydrophobic packing (Fig. 1A). Fourteen other ENF peptides have also been identified (6, 21–26). Comparing the primary sequences of these peptides to PSP indicated that the most variable positions were positions 4, 5, 8, 9, and 12 (Fig. 1B). Some variation also existed at positions 11, 15, 21, and 23 (Fig. 1B). Prolines were present at positions 9, 15, and 21 in some family members, which could potentially alter their backbone structure in comparison to PSP. Several family members also differed from PSP at positions 11 and 23, but these were conservative aromatic ring substitutions. The remaining 14 residues were invariant among family members. These included Cys3 and Cys19, Thr14 and Thr22, all of the charged amino acids in the structured C terminus (Arg2, Asp16, Arg18, and Lys20), and the three N-terminal residues Glu1, Asn2, and Phe1. In addition to being identical at these positions, three ENF peptide family members (PSP, Pss GBP, and Mas PP1) are also known to have plasmatocyte spreading activity (see legend, Fig. 1B). We therefore hypothesized that residues important for plasmatocyte spreading activity most likely reside among these 14 conserved residues and the aromatic amino acids at positions 11 and 23. To test this hypothesis, we synthesized PSP mutants in which amino acids were either replaced with alanine or deleted. Alanine was selected as the replacement residue because of its lack of charge, functional side groups, and because it was least likely to alter the backbone structure of the peptide (17). Cys3 and Cys19 were simultaneously replaced with alanine in one mutant (C7.19A), whereas the first six residues of wild-type PSP were deleted for another mutant designated PSP(7–23). Three of the 11 constructs we synthesized were all alanine point mutants. Although Gly6, Gly10, and Gly17 are conserved among all ENF peptide family members (Fig. 1A), we did not synthesize alanine mutants at these positions, because we assumed the role of these residues was primarily for proper folding of the peptide.
Selected Residues of the PSP Core Domain Are Critical for Biological Activity—The most important element for maintaining the three-dimensional structure of PSP is the disulfide bond between Cys7 and Cys19. Plasmatocyte spreading assays indicated that C7.19A had no biological activity except at the highest concentration tested (10 μM). In contrast, wild-type PSP showed a normal dose-response with a threshold at 1 nM and saturation at 1 μM (Fig. 2). We next considered the uncharged hydrophilic threonines at positions 14 and 22. Thr14 resides in the β-hairpin of PSP but faces away from the charged residues, whereas Thr22 resides in the less-ordered C terminus (see Fig. 1A). The T14A mutant exhibited slightly higher plasmatocyte spreading activity than wild-type PSP at all concentrations tested, whereas T22A exhibited activity that was reduced by one-half to one-third at concentrations from 1 nM to 1 μM (Fig. 3A).

As noted above, all ENF peptides have either a phenylalanine or tyrosine residue at positions 11 and 23. Tyr11 appears important for proper conformation of PSP as the side chains of Cys5, Leu7, Ala9, Cys19, Pro21, and Phe23 form a well defined hydrophobic core centered around the side chain of this residue (11). Comparison of PSP to other EGF-like subdomains also revealed the presence of an aromatic residue at a position equivalent to Tyr11 in transforming growth factor-alpha (TGF-α (Tyr25)), the fifth subdomain of human thrombomodulin (hTM5 (Tyr11)), and human epidermal growth factor (hEGF (Tyr7)) (Fig. 1C). Mutagenesis experiments indicate these Tyr11 equivalents are essential for activity of TGF-α and TM, but not EGF (12, 15). Our bioassays indicated that plasmatocyte spreading activity of the Y11A mutant was only slightly reduced to that of wild-type PSP (Fig. 3B). The activity of F23A was virtually the same as wild-type PSP (Fig. 3B).

We next considered the four charged residues (Arg13, Asp16, Arg18, Lys20) surrounding Cys19. Each of these amino acids residues within the β-hairpin turn with Asp16, Arg18, and Lys20 in opposite orientation to Arg13 (Fig. 1A). Alanine replacements at each of these positions reduced plasmatocyte spreading activity compared with wild-type PSP (Fig. 4). The mutants that least affected activity were K20A and R18A, which showed a threshold response at 10 nM and saturation in the 1–10 μM range. D16A showed a greater loss of activity with a response threshold that was two orders of magnitude lower than wild-type PSP. However, the maximal response of D16A did not differ from intact PSP. R13A had the lowest plasmatocyte spreading activity of the charged residue replacements (Fig. 4).

The threshold response for this mutant was 10 μM, making it 1000-fold less active than wild-type PSP. Although Arg13 is of prime importance for the activity of PSP, data base searches revealed no homologous residue among other EGF-like domains.

The Unstructured N-terminal domain of PSP Is Also Critical for Biological Activity—The lack of defined structure over the first six residues of PSP originally suggested to us that this domain was likely less important for receptor binding and
plasmatocyte activation than the highly structured C-terminal domain (Fig. 1A). However, the conservation of the first three residues (Glu-Asn-Phe) among all ENF peptide family members (Fig. 1B) circumstantially argued that the unstructured N terminus may be more important for activity than the tertiary structure of PSP suggested. Bioassays with PSP-(7–23) clearly supported a role for the unstructured N terminus in plasmatocyte activation as this mutant had no activity at any concentration tested (Fig. 5A). PSP-(7–23) also lacked spreading activity at a concentration of 100 μM (data not presented). We then tested alanine point mutants in the unstructured N terminus. Surprisingly, both E1A and N2A had higher activity than wild-type PSP with threshold responses shifted to approximately a 100-fold lower level than the native peptide (Fig. 5B).

In contrast, F3A had no plasmatocyte spreading activity from 1 nM to 10 μM and only a low level of activity at 100 μM. We thus concluded that the lack of activity for PSP-(7–23) is largely due to the critical importance of Phe3.

Structural Analysis of PSP mutants—The mutant peptides C7.19A, F3A, R13A, and R18A all had reduced plasmatocyte spreading activity when compared with wild-type PSP. To determine whether this reduction in activity correlated with alterations in tertiary structure, we compared these mutants to wild-type PSP by NMR spectroscopy. Because amide proton shifts are quite sensitive to secondary and tertiary protein structure, changes in the β-hairpin or hydrophobic core of PSP can be expected to produce dramatic changes in chemical shift (>0.5 ppm). The 1H NMR spectra of PSP and the aforementioned mutants are presented in Fig. 6. As expected, the simultaneous replacement of Cys7 and Cys19 with alanine profoundly affected tertiary structure as evidenced by the collapse in chemical shift dispersion for this peptide in comparison to wild-type PSP (Fig. 6A and B). This collapse is consistent with the absence of a unique folded peptide structure. Signals from Lys20 (K20) and Met12 (M12) at −9.5 ppm in wild-type PSP were also shifted far upfield in the C7.19A mutant (Fig. 6B), indicating that the PSP β-hairpin structure is not formed in the mutant. In contrast, the R13A, R18A, and F3A replacements had no significant affect on the 1H NMR spectrum, indicating that the reduced biological activity of these peptides is not due to significant alterations in structure (Fig. 6C–E). NMR spectra were also obtained on all of the other single amino acid replacements we synthesized, and they too had no significant effect on the tertiary structure of the peptide (data not presented). As a further check on the structural integrity of the PSP mutants, two-dimensional NMR spectra were used to obtain complete backbone 1H chemical shift assignments for the PSP-(7–23), R13A, D16A, R18A, and K20A mutants. 1H–1H N and 1H N–1H N regions of NOESY spectra were also compared with wild-type PSP to identify the patterns of NOEs characteristic of
Mutagenesis of Plasmatocyte Spreading Peptide

FIG. 6. 1H NMR spectra of wild-type PSP and the mutant peptides C7.19A, R13A, R18A, and F3A. A, backbone amide resonances are shown and labeled by residue for wild-type PSP. B, the reduction in chemical shift dispersion for the C7.19A spectrum reflects the lack of tertiary structure as a result of removing the disulfide bridge. C–E, substitution of alanine for Arg13, Arg18, and Phe3 results in only minor perturbations to the amide 1H shifts, and no apparent change to the structure determined for wild-type PSP. Other variations in baseline noise or line widths are due to differences in experimental acquisition parameters.

its tertiary structure. Overall, analysis of the one- and two-dimensional NMR data indicated that, with the exception of C7–19A, all of the other mutants retained the tertiary fold determined for wild-type PSP (data not presented).

Implications for Receptor Binding and Activation—The PSP receptor has not been identified, but the strong similarity in the structure of PSP and the C-terminal subdomains of other EGF-like molecules suggest that PSP-receptor interactions may be analogous to other EGF domain-receptor complexes (15). As such, we initially hypothesized that the ordered portion of PSP (residues 7–21) would be the region most essential for biological activity. The distinction between residues critical for proper folding and those directly involved in target cell binding (and biological activity) are not always clear from mutagenesis experiments alone. However, the small size of PSP makes this molecule especially amenable for assessing how specific mutations affect activity and structure. Our results with C7.19A clearly indicate that the disulfide bond is essential for maintenance of both the structure and activity of PSP. In contrast, the F3A and R13A mutants exhibit greatly reduced activity without a concomitant change in tertiary structure. Arg13 and Phe3 therefore may be especially critical residues for receptor binding. The loss of activity associated with F3A and R13A also indicates that both the ordered and disordered domains of PSP are important for plasmatocyte activation.

The three charged amino acids surrounding Cys19 on the 73–81 helix (Asp16, Arg18, and Lys20) all align on one side of PSP, presenting a structural motif that may be important for receptor binding. Of these three charged residues, we originally were most interested in Arg18, because arginines exist in analogous positions in the receptor binding domains of both EGF (Arg41) and TGF-α (Arg22) that are essential for binding and activity. However, R18A had only slightly reduced activity compared with wild-type PSP. K20A similarly exhibited a small loss, whereas D20A had a more moderate loss of activity. Based on these results, we anticipate that PSP requires the complete charge sequence (− + +) provided by these residues for normal activity, but we hypothesize that removal of the positive charge at either Arg18 or Lys20 is partially compensated by the other residue, resulting in only small changes in activity. Although replacement of Arg13 greatly reduced activity of PSP, no analogous residue exists in EGF, TGF-α, or TM (Fig. 1C). The similarity in structure of R13A and wild-type PSP suggests that Arg13 may be a third residue important for receptor binding. Also supporting this suggestion are the results of competition experiments showing that R13A does not antagonize the plasmatocyte spreading activity of wild-type PSP.3 We view PSP-(7–23) and F3A as the most intriguing mutants, because they clearly indicate that the disordered N terminus of PSP is required for biological activity. The N terminus does not appear to be essential for receptor binding, because both PSP-(7–23) and F3A antagonize the plasmatocyte spreading activity of PSP.3 However, binding of the PSP core may promote a conformational stabilization of the normally unstructured N terminus so that Phe3 interacts with and activates the receptor. Identification of the PSP receptor is obviously essential to testing these hypotheses and identifying binding determinants in this novel family of insect cytokines.

Acknowledgments—We thank Martha Vestling for assistance in acquiring and processing of mass spectral data and members of the University of Wisconsin Biotechnology facility for assistance in synthesis of mutant peptides. We also thank the National Magnetic Resonance Facility at Madison and the University of Texas at Galveston Protein Chemistry laboratory for help during the study.

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