Roles of Aromatic Residues in the Structure and Biological Activity of the Small Cytokine, Growth-blocking Peptide (GBP)*

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Growth-blocking peptide (GBP) is a small (25 amino acids) insect cytokine with a variety of functions: controlling the larval development of lepidopteran insects, acting as a mitogen for various types of cultured cells, and stimulating insect blood cells. The aromatic residues of GBP (Phe-3, Tyr-11, and Phe-23) are highly conserved in the ENF peptide family found in lepidopteran insects. We investigated the relationship between the biological activities and structural properties of a series of GBP mutants, in which each of the three aromatic residues is replaced by a different residue. The results of the hemocytes-stimulating assays of GBP mutants indicated that Phe-3 is the key residue in this activity: Ala or Tyr replacement resulted in significant loss of the activity, but Leu replacement did not. The replacements of other aromatic residues hardly affected the activity. On the other hand, NMR analysis of the mutants suggested that Tyr-11 is a key residue for maintaining the core structure of GBP. Surprisingly, the Y11A mutant maintained its biological activity, although its native-like structure of GBP. Detailed analyses of the 15N-labeled Y11A mutant by heteronuclear NMR spectroscopy showed that the native-like β-sheet structure of Y11A was induced by the addition of 2,2,2-trifluoroethanol. The results suggest that Y11A has a tendency to form a native-like structure, and this property may give the Y11A mutant native-like activity.

Growth-blocking peptide (GBP) was first identified as a growth factor that retards the larval development of the armyworm, Pseudaletia separata, parasitized by the parasitoid wasp, Cotesia kariyai (1, 2). Injection of GBP into non-parasitized armyworm larvae during the last larval instar retards larval growth and causes a delay in pupation (3, 4). Recently, GBP has been shown to demonstrate multifunctionality: cell proliferation, stimulation of immune cells (plasmatocytes), and paralysis (5). GBP homologous peptides have been identified from other lepidopteran species and, based on their consensus N-terminal sequences (Glu-Asn-Phe), have been expediently designated the ENF peptide family (Fig. 1) (6). Although each of these peptide family members was originally identified as a unique biological active peptide (1, 8, 11, 12), it has since been demonstrated that some of them exert multiple activities, such as larval retardation, plasmatocyte stimulation, and paralysis (6, 13, 14). Therefore, it seems reasonable to categorize the ENF family peptides as a cytokine family primarily found in insects.

GBP exhibits a strong mitogenic activity on cultured cells, such as insect Sf9 and human keratinocytes. Interestingly, GBP and epidermal growth factor (EGF) are more or less equivalent with respect to their cell proliferation activity in keratinocytes (5). Recent studies have indicated that GBP binds directly to EGF receptors in keratinocytes and phosphorylates their tyrosine residues as EGF upon stimulating mitogenesis of keratinocyte cells (15). Structural analyses of GBP as well as other ENF peptides using NMR spectroscopy have revealed that they consist of a core region with a striking similarity to the C-terminal β-loop domain of EGF and short disordered N and C termini (16–18). A previous study demonstrated that the minimal structure of GBP-maintaining mitogenic activity is 2–23 GBP, whereas that with plasmatocyte-stimulating activity is 1–22 GBP (16). Further, the GBP mutants, having a Glu, Leu, or Asn residue at Asp-16, lost their mitogenic activity but retained about 50% of their plasmatocyte-stimulating activity. Based on these results, we conclude that specific residues in the structured and unstructured domains of GBP differently affect the biological activities of GBP.

The present studies focused on the three aromatic amino acid residues (Phe-3, Tyr-11, and Phe-23) in the GBP molecule (Fig. 2A) because these residues are highly conserved among the ENF family peptides. We constructed eight kinds of point-mutated GBP variants at the aromatic residues and analyzed the relationship between their biological activities and structures.

**EXPERIMENTAL PROCEDURES**

Preparation and Identification of Site-direct Mutants of GBP—The series of GBP mutants prepared by the bacterial expression system are listed in Fig. 2B. The construction of the expression system and purification of GBP were performed according to our procedure (2, 8) eDNA encoding the entire GBP sequence was used as a template, and poly-
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P. s. GBP ENESGCGCAVGRMPRTDKGRSPFTYQ
M. b. GBP ENAGGGCTCTFGMRPRTDKGRSPFTYQ
S. l. GBP ENAGAGCTCTFGMRPRTDKGRSPFTYQ
T. n. PP I ENESGCGCAVGRMPRTDKGRSPFTYQ
T. n. PP II ENAGAGCGCTCTFGMRPRTDKGRSPFTYQ
H. v. PP I ENAGAGCGCTCTFGMRPRTDKGRSPFTYQ
H. v. PP II ENESGCGCAVGRMPRTDKGRSPFTYQ
M. s. PP I ENAGAGCGCTCTFGMRPRTDKGRSPFTYQ
M. s. PP II ENESGCGCAVGRMPRTDKGRSPFTYQ
S. e. PP I ENESGCGCAVGRMPRTDKGRSPFTYQ
S. e. PP II ENAGAGCGCTCTFGMRPRTDKGRSPFTYQ
S. e. PP III ENESGCGCAVGRMPRTDKGRSPFTYQ
A. y. PP ENAGAGCGCTCTFGMRPRTDKGRSPFTYQ
B. m. PP ENAGAGCGCTCTFGMRPRTDKGRSPFTYQ
P. i. PSP ENAGAGCGCTCTFGMRPRTDKGRSPFTYQ
S. e. CAP ENESGCGCAVGRMPRTDKGRSPFTYQ

FIG. 1. Sequence alignment of GBP and other ENF peptides. GBP from *P. separata* (P. s. GBP), GBP from *Mamestra brassicae* (M. b. GBP), *Spodoptera litura* (S. l. GBP), *Trichoplusia ni* (T. n. PP), *Manduca sexta* (M. s. PP), *Spodoptera exigua* (S. e. PP), *Bombyx mori* (B. m. PP) (9), and GBP from *Pseudopulalia includens* (P. i. PSP) were aligned. Residues within the boxes are identical between the peptides. Phe-3, Tyr-11, and Phe-23 are shaded in gray.

FIG. 2. Three-dimensional structures of GBP and sequences of GBP mutants. A, the ensemble of NMR solution structures of GBP determined by Aizawa et al. (16). The backbone atoms are shown in the figure as blue sticks and the side chains of aromatic residues are drawn in black. The 15 NMR structures of GBP are superimposed. B, the amino acid sequence of GBP mutants used in this study. Three aromatic residues (Phe-3, Tyr-11, and Phe-23) are underlined.

FIG. 3. In vitro spreading response of plasmatocytes to wild-type or mutant GBPs. Spreading was assayed by scoring 200 randomly selected cells after 20 min in culture with 0.1 nM to 1 μM wild-type or mutant GBP. Plasmatocytes were scored as spread if they assumed flattened morphologies and were 35 μm along their longest axis. Each bar represents the mean ± S.D. from four independent collections of hemocytes. Abbreviations of the peptides are as in Fig. 2B.

Each mutated GBP was dissolved at a final concentration of 1.5–3.0 mM in 350–500 μl of buffer containing 90% H2O/10% D2O, 50% H2O/50% 2,2,2-trifluoroethanol (TFE)-D3, and 0.1% n-octyl-β-D-glucopyranoside. Double quantum filtered correlation spectroscopy (DQF-COSY) spectra (20), total correlation spectroscopy (TOSY) spectra (21), and nuclear Overhauser effect spectroscopy (NOESY) spectra with a mixing time of 100–300 ms (22) were recorded during suppression of the water signal by WATER-GATE pulse (23). The NMR spectra of 15N-labeled samples were recorded under the same condition with non-labeled samples using [15N-1H] heteronuclear single quantum coherence (HSQC) (24), 13N-edited TOCSY (25), and 15N-edited NOESY (25).

Cell Growth Assay—The cell growth assay was performed according to the previously described method (14). Insect High Five™ cells (In...
vitrogen) were maintained in modified Grace’s medium (MGM; Grace’s medium containing 0.33% tissue culture lactoalbumin hydrolysate and 0.33% tissue culture yeastolate (DIFCO)) with 10% fetal bovine serum. After culturing the cells in MGM without fetal bovine serum or any growth factor for 2 days, the cells were cultured in medium containing GBP or an analog plus 1 µCi of [3H]thymidine for 32 h. Cells were washed three times with 0.15 M NaCl, lysed with 100 µl of 0.3 N NaOH, and then the incorporated thymidine was counted using a liquid scintillation counter (LSC-5100; Aloka). Cells were in their exponential growth phase at the time of labeling.

Fig. 4. NMR spectra of GBP and Y11A mutant. A, fingerprint region of the COSY spectra of the native GBP and Y11A mutant. B, 15N-HSQC spectrum of the 15N-labeled Y11A mutant.

Fig. 5. Chemical shift differences of α-protons of amino acid residues in native GBP and GBP mutants. The differences in chemical shift defined as δ(GBP or mutant)−δ(random coil) are plotted. Arrows indicate β-sheet regions.
Experimental Injection of GBP and GBP Analogue into Larva—The experimental injection method was a modification of the previously published method of Hayakawa and Ohnishi (5). Day 0 last instar larvae (0.17–0.18 g) of armyworm were injected with the indicated amounts of GBP and its analog early in the last larval instar and weighed at 16 h after injection.

RESULTS
Plasmatocyte-stimulating Activity of GBP Mutants—The assay for the plasmatocyte-stimulating activity of the GBP mutants indicated that GBP mutants of Phe-23 (F23A, F23L, and F23Y) and of Tyr-11 (Y11A and Y11F) showed the highest rates of plasmatocyte stimulation; for both mutants, the rates of stimulation were almost equivalent to that of the wild-type GBP (Fig. 3). The mutation at Phe-3 had a significant effect on this activity: F3A and F3Y lost the activity, but F3L did not. These results clearly indicated that Phe-3 is an essential residue for GBP to exert the plasmatocyte-stimulating activity.

NMR Spectra of GBP Mutants—The chemical shift assignments of the GBP mutants were performed using their NOESY and TOCSY spectra to compare the structural characters between native GBP and its mutants. DQF-COSY spectra of most GBP mutants are almost similar to the spectrum of wild-type GBP. However, Y11A showed a completely different DQF-COSY spectrum in the fingerprint regions (Fig. 4A). 15N-labeled Y11A was prepared, and its 15N-edited spectra were analyzed to assign its NMR signals completely (Fig. 4B).

Chemical Shift Deviations of GBP Mutants—The chemical shifts of C-H protons are very sensitive to backbone conformation and secondary structure (26). The chemical shift deviations of C-H in amino acid residues of native GBP and GBP mutants are shown in Fig. 5. A positive value of chemical shift deviation suggests a β-sheet structure, and a negative value of chemical shift deviation suggests an α-helix structure (26). The patterns of chemical shift deviation in GBP mutants resemble that of native GBP, with the exception of the deviation for Y11A. In particular, the chemical shift deviations of the β-sheet
region in wild-type GBP (Tyr-11–Arg-13, Cys-19–Pro-21) also showed positive values in these mutants. In contrast, Y11A showed a different chemical shift deviation pattern around the β-sheet region. The chemical shift deviations of the β-sheet region of Y11A are smaller than that of native GBP. These results showed that the native-like β-sheet structure is disordered in Y11A mutants.

Biological Activities of Y11A—To further clarify the character of Y11A, we investigated two of its other biological activities, i.e. its mitogenic and larval growth activities. Y11A showed a strong mitogenic activity, almost equivalent to that of the wild-type GBP, for insect High Five cells (Fig. 6A). The growth-inhibitory effect of Y11A on P. separata larvae was also examined (Fig. 6B). Larvae injected with over 4 pmol of GBP gained significantly less weight than bovine serum albumin-injected larvae. Y11A was effective when over 40 pmol were injected to larvae. Therefore, the growth-inhibitory activity of Y11A on larvae was less effective than that of native GBP, but the Y11A mutant did retain some degree of this activity.

NMR Analysis of Y11A in TFE Solvent—The Y11A mutant maintained almost the same activities as the wild type, although the secondary structure was disordered. These results raised the possibility that Y11A GBP might have a tendency to form the native-like structure easily. To investigate this possibility, we examined the structural alteration of the Y11A mutant in TFE solvent. TFE has been shown to induce and stabilize intrinsic secondary structures of the peptides, such as α-helices, β-turns, or β-sheets (27). Chemical shift deviations of C/H in amino acid residues of native GBP and Y11A in 50% TFE solvent are shown in Fig. 7. Compared with the pattern of the chemical shift deviation of Y11A in a water solution (Fig. 5), that of Y11A in 50% TFE solvent more closely resembles the pattern of native GBP. In particular, the chemical shifts of the residues, which form a β-sheet structure in native GBP (Tyr-11–Arg-13, Cys-19–Pro-21), showed remarkable change and the feature of a β-sheet structure in Y11A dissolved in TFE solution. These results seem to suggest that Y11A GBP has the potential to hold the native-like core β-sheet structure.

DISCUSSION

GBP mutants at the aromatic residues, Phe-3, Tyr-11, and Phe-23, show significant differences in their biological activities and structural characteristics. Phe-3 is important for the plasmatocyte-stimulating activity because the mutants at Phe-3 (F3A and F3Y) significantly reduced the activity or lost it (Fig. 3). The fact that the activity of F3L was stronger than that of F3Y suggests that the hydrophobicity of the side chain at the position of Phe-3 promotes this activity. In contrast with the plasmatocyte-stimulating activity, Phe-3 does not play an important role in maintaining the structure of GBP since the point mutations at Phe-3 had little effect on the chemical shift deviation patterns of the backbone structure (Fig. 5).

The GBP mutations at Phe-23 did not affect either the plasmatocyte-stimulating activity or the backbone structure. Deletion of Tyr-24 and Gln-25 from the Phe-23 mutants also had no effect on their characters. Prior studies have shown that the deletion mutation of 1–22 GBP resulted in a loss of the mitogenic activity, although the plasmatocyte-stimulating activity was maintained in this mutant (14). From these data, it is reasonable to assume that Phe-23 plays an essential role only in the mitogenic activity.

Recent NMR studies of GBP as well as other ENF peptides have suggested that the aromatic residue at position 11 is a key residue for maintenance of the β-sheet structure (16–18). Many NOEs have been observed between the side chain of Tyr-11 and other hydrophobic residues, suggesting the existence of hydrophobic interactions between them. In the present study, we were unable to prepare the Y11L mutant, probably due to the lack of stability of this mutant.
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due to proteolysis. On the other hand, Y11F has almost the same NMR spectra and biological activity as the wild-type GBP. Therefore, it may be possible to replace Tyr-11 with a phenylalanine residue and thereby maintain the secondary structure of GBP.

Interestingly, the NMR spectra of Y11A are very different from those of native GBP (Fig. 4), despite the fact that Y11A maintains almost the full amount of plasmacytoma-stimulating activity. This mutant is quite attractive as a model of induced fitting because another mutant, D16β (GBP with a β-Asp bond at Asp-16), has been shown to have a completely unordered structure and to exert no biological activities (14, 28). It was difficult to assign the signals of the 1H-two-dimensional NMR spectra of Y11A because of the extreme overlap of the signals (Fig. 4A). However, we were able to execute a complete assignment by using 15N-labeled Y11A (Fig. 4B). Complete assignment of Y11A enabled us to characterize the detailed structural features of this mutant. The considerably small values of the chemical shift deviations of CαH around Tyr-11–Arg-13 and Cys-19–Pro-21, corresponding to the β-sheet region in wild-type GBP, suggested the disorder of the native-like β-sheet structure in the Y11A mutant (Fig. 5). Furthermore, NOE data also suggest that the tertiary structure of Y11A is completely different from that of the native peptide. A number of NOE signals of Y11A were considerably decreased, and the unique NOEs for the β-sheet structure, such as the interstrand NOE between CαH of Arg-13 and HN of Lys-20, had completely disappeared (Fig. 8A). We thus concluded that the native β-sheet structure was disordered in the Y11A mutant.

Although the tertiary structure of Y11A was disordered, this mutant retained the plasmacytoma-stimulating, mitogenic, and growth-retardation activities (Figs. 3 and 6). It seems likely that Y11A easily forms a β-sheet structure upon interaction with target molecules such as GBP receptor proteins. The decrease in the larval growth inhibitory activity of Y11A could be explained by the disappearance of its secondary structure since Y11A is thought to be more susceptible to proteolysis than the wild-type peptide in vivo. These hypotheses were strongly supported by NMR analyses of Y11A in TFE solvent (Fig. 7). Based on the pattern of the chemical shift deviation of Y11A in TFE, it is most likely that Y11A folds the native-like core β-sheet structure in this solvent. Furthermore, some unique NOEs that suggest formation of a β-sheet, such as the interstrand NOE between CαH of Arg-13 and HN of Lys-20, were clearly observed in the spectra of Y11A in TFE solvent (Fig. 8). Investigations of the effects of TFE on peptides have demonstrated that TFE can stabilize secondary structures (27, 29). In those studies, the peptides had a tendency to fold into a more stabilized, low-energy level structure by forming hydrogen bonds in TFE solvent. Thus, the enhancement of the secondary structure of Y11A in TFE implies the ease of folding of this mutant into the native structure in receptor binding, and the considerably high activity of this mutant could be attributed to this induced-fitting model.

In conclusion, we have shown the roles of each aromatic residue in GBP; in particular, Phe-3 had significant roles for the biological activity, and Tyr-11 maintained the structural properties of GBP. The interesting properties of the Y11A mutant, which maintains its activities despite its loss of the secondary structure, may be useful clues for a further investigation of the interaction between ENF family peptides and their receptors.

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