Growth-blocking peptide (GBP) is a 25-amino acid cytokine isolated from the lepidopteran insect *Pseudaletia separata*. GBP exhibits various biological activities such as regulation of larval growth of insects, proliferation of a few kinds of cultured cells, and stimulation of a class of insect immune cells called plasmatocytes. The tertiary structure of GBP consists of a well structured core domain and unstructured N and C termini. Our previous studies revealed that, in addition to the structured core, specific residues in the unstructured N-terminal region (Glu and Phe) are also essential for the plasmatocyte-stimulating activity. In this study, a number of deletion, insertion, and site-directed mutants targeting the unstructured N-terminal residues of GBP were constructed to gain more detailed insight into the mode of interaction between the N-terminal region and GBP receptor. Alteration of the backbone length of the linker region between the core structure and N-terminal domain reduced plasmatocyte-stimulating activity. The substitutions of Gly or Gly in this linker region with more bulky residues, such as Phe and Pro, also remarkably reduced this activity. We conclude that the interaction of GBP with its receptor depends on the relative position of the N-terminal domain to the core structure, and therefore the backbone flexibility of Gly residues in the linker region is necessary for adoption of a proper conformation suited to receptor binding. Additionally, antagonistic experiments using deletion mutants confirmed that not only the core domain but also the N-terminal region of GBP are required for “receptor-binding,” and furthermore Phe is a binding determinant of the N-terminal domain.

Growth-blocking peptide (GBP) was initially identified as a peptidyl hormone that retards the larval development of the armyworm *Pseudaletia separata* (1–4). High concentrations of GBP induce significant reductions in larval growth, whereas low concentrations of GBP stimulate larval growth, thus suggesting that GBP acts as a growth factor in insects. Subsequent studies have shown that GBP has multiple functions, including stimulation of specific insect immune cells (plasmatocytes), proliferation of various types of cultured cells, and paralysis of larvae (5–7). Furthermore, our recent study showed that GBP stimulates cell proliferation of human keratinocyte cells through direct binding and activation of epidermal growth factor receptors (8).

GBP is a 25-amino acid peptide containing a disordered N terminus (residues 1–6) and a structured core (residues 7–22) defined by a disulfide bond and a short antiparallel β-sheet (9). Interestingly, both the structured core region of GBP and the disordered N-terminal region are required for biological activity (10). Previous studies demonstrated that the first three residues of the N terminus (Glu-Asn-Phe) of GBP and plasmatocyte spreading peptide (PSP) from *Pseudoplusia includens* play a critical role in plasmatocyte-stimulating activity (11–14). This Glu-Asn-Phe triad is conserved in the GBP homologues identified from other lepidopteran species. Because of this consensual ENF sequence, these homologues are now referred to as members of the ENF peptide family (Fig. 1) (14). Recent studies using mutants with specific point mutations or alterations to the ENF sequence in GBP as well as PSP showed the possibility that Phe interacts with putative receptors in plasmatocytes via its hydrophobic effect (11, 13, 14).

In the present study we focused on the two glycine residues (Gly and Gly) that are located in the linker region between the well structured core and the N-terminal ENF motif (Fig. 2A). Gly and Gly are highly conserved within the ENF peptide family. Because it has no side chain, glycine is more flexible than other residues and is often found in areas of the peptide backbone that need to move or hinge (15). We therefore predict that the flexibility of the Gly-Gly sequence in the linker region plays an important role in allowing the N-terminal activation site to interact with receptors. To confirm this idea, we constructed a number of GBP mutants in which various N-terminal residues, mainly Gly and Gly, are specifically altered by substitution or deletion. We then investigated the biological activity of these mutant peptides and their interaction with a putative receptor using bioassays and NMR analysis.

**EXPERIMENTAL PROCEDURES**

*Animals—* *P. separata* was reared on an artificial diet at 25 ± 1 °C with a photoperiod of 16 h of light and 8 h of dark (1). Penultimate
instar larvae undergoing ecdysis between 2 and 2.5 h after lights on were designated as day 0 last instar larvae (1).

Peptide Preparation—All of the mutants used in this study were prepared by the Escherichia coli expression system. Expression and purification of peptide mutants was performed using our procedure previously established (11). cDNA encoding the entire GBP sequence of recombinant plasmids containing point-mutated GBP sequences. Mutations in the Unstructured N-terminal Region of GBP Do Not Alter the Structured Core of the Peptide—Prior studies have examined the relationship between GBP structure and function using a variety of mutants (10, 11). These experiments showed that GBP mutants whose secondary structures were disrupted relative to wild-type GBP had no activity, implying that the core structure was indispensable for the biological activity (10, 22). However, mutants in the unstructured N-terminal domain, such as a deletion of Glu1, also result in a complete loss of activity despite retaining the native core structure of GBP (10, 13). Experiments with mutants of GBP and PSP clearly showed that both the structure and hydrophobicity of the side chain of Phe3 are important for activity (11, 12). Extensive analyses of PSP mutants at Phe3 indicated that a branched carbon chain with a methylene spacer at the third residue is the minimal structural motif that is required for an active peptide (13). The fact that the three N-terminal residues (an ENF motif) of GBP and PSP are also completely conserved in all ENF family peptides suggests that these three residues may play a critical role in receptor activation. Thus, there has been great interest in the structural and functional relationship between the core domain and the N-terminal disordered region of these peptides. Based on these previous findings, we presumed that the highly conserved Gly residues (Gly-Gly sequence, fifth and sixth residues in GBP) in the "linker" region connecting the structured core and the ENF motif could perform a key role in this peptide as a flexible hinge-like element. To clarify this role, we produced a series of mutants modified in the region of GBP and all the mutants constructed in this study, except for (3–25)-GBP and (4–25)-GBP, are shown in Fig. 3. If specific mutations in the N-terminal region of GBP were to perturb the tertiary structure of the GBP core domain, some changes or loss of the chemical shift dispersion would be observed in the 1H NMR spectra due to their sensitivity to alterations of the secondary and tertiary structure of the peptide (10, 14). Our experiments showed no significant difference in chemical shift dispersion between wild-type GBP and all the

matocytes was counted after incubation for 20 min by the same procedure as that of the plasmatocyte-stimulating assay.

NMR Spectroscopy—All experiments were carried out on a Bruker DMX-500 spectrometer at 25 °C. The chemical shifts were measured from the internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Each mutant of GBP was dissolved at a final concentration of 0.3–0.7 mM in 350–500 μL of buffer containing 90% H2O/10% D2O, with 20 mM sodium phosphate at pH 6.5. All one-dimensional spectra were recorded during suppression of the water signal with WATER-GATE pulse (16). The NMR spectra of 15N-labeled samples were recorded under the same condition as unlabeled samples except for the peptide concentration range of 1.6–2.7 mM, using 15N-H single quantum coherence (HSQC) (17) and 15N-edited NOESY (18). Amido resonance assignments of the G5F and G6F mutants were made on the basis of the previously assigned 1H resonances of wild-type GBP using the measured 15N-edited spectra (9). 23N Relaxation Measurement—Measurements of the longitudinal (T1) and transverse (T2) relaxation times and the heteronuclear nuclear Overhauser effects (NOEs) of 15N nuclei were carried out as described previously (19). All spectra were processed and analyzed using NMR Pipe (20) and PIPP (21). The peak intensities of each cross-peak in a series of two-dimensional NMR data were extracted using NMR Pipe based on the peak positions defined by the contour averaging algorithm with 30% of peak integrals and a single exponential function. The 1H-15N cross-peak was used for the calculation of the T1 and T2 relaxation times, using a single-exponential model function. The steady-state 1H-15N NOE values were determined from the ratios of the intensities of the peaks with and without proton saturation.

RESULTS AND DISCUSSION

Mutations in the Unstructured N-terminal Region of GBP Do Not Alter the Structured Core of the Peptide—Prior studies have examined the relationship between GBP structure and function using a variety of mutants (10, 11). These experiments showed that GBP mutants whose secondary structures were disrupted relative to wild-type GBP had no activity, implying that the core structure was indispensable for the biological activity (10, 22). However, mutants in the unstructured N-terminal domain, such as a deletion of Glu1, also result in a complete loss of activity despite retaining the native core structure of GBP (10, 13). Experiments with mutants of GBP and PSP clearly showed that both the structure and hydrophobicity of the side chain of Phe3 are important for activity (11, 12). Extensive analyses of PSP mutants at Phe3 indicated that a branched carbon chain with a methylene spacer at the third residue is the minimal structural motif that is required for an active peptide (13). The fact that the three N-terminal residues (an ENF motif) of GBP and PSP are also completely conserved in all ENF family peptides suggests that these three residues may play a critical role in receptor activation. Thus, there has been great interest in the structural and functional relationship between the core domain and the N-terminal disordered region of these peptides. Based on these previous findings, we presumed that the highly conserved Gly residues (Gly-Gly sequence, fifth and sixth residues in GBP) in the "linker" region connecting the structured core and the ENF motif could perform a key role in this peptide as a flexible hinge-like element. To clarify this role, we produced a series of mutants modified in this linker region (Fig. 3B). Before assaying for activity, we first determined using 1H NMR spectroscopy whether these mutations had any effects on the conformation of the core domain. One-dimensional NMR spectra of the amide proton region of GBP and all the mutants constructed in this study, except for (3–25)-GBP and (4–25)-GBP, are shown in Fig. 3. If specific mutations in the N-terminal region of GBP were to perturb the tertiary structure of the GBP core domain, some changes or loss of the chemical shift dispersion would be observed in the 1H NMR spectra due to their sensitivity to alterations of the secondary and tertiary structure of the peptide (10, 14). Our experiments showed no significant difference in chemical shift dispersion between wild-type GBP and all the
mutants tested. In particular, signals for the amide resonances of Met^{12}, Thr^{14}, and Lys^{20}, all components of the rigid core structure of native GBP, remain unaffected, suggesting that the native core structure of wild-type GBP is conserved. These results confirmed that any effects these mutants may have on activity would be due to local effects in the N-terminal domain rather than conformational changes of the core. These results also suggested that the N-terminal domain of GBP does not contribute to maintaining the tertiary structure of the core domain. Although the 1H NMR spectra of (3–25)-GBP and (4–25)-GBP was not measured, it is reasonable to assume that (3–25)-GBP and (4–25)-GBP also retained the native core structure, because even the (7–25)-GBP mutant without the entire N-terminal region retained the native structure of wild-type GBP.

The Length of the Linker Region between the Core Domain and the ENF Motif Is Critical for Activity—As a first step toward examining the role of the distinctive Gly^5-Gly^6 sequence, we constructed a set of GBP mutants that altered the length of the peptide backbone between the ENF motif and the core domain. These included the deletion mutant (−1G)-GBP and the insertion mutant (+1G)-GBP, in which the ENF motif was positioned, respectively, either closer to or farther from the core domain by addition or subtraction of a Gly residue (Fig. 4A). The (−1G)-GBP lost almost all activity, revealing that a certain minimum length of the backbone “linker” region is required for activity. The (+1G)-GBP also had no activity up to 10 nM and only about one-half of wild-type GBP even at a concentration of 100 nM. These results show that the activity of GBP is affected by the distance of the ENF motif from the core domain. We also prepared a hexapeptide (ENFSGG) that consisted of the first six residues of GBP and a deletion mutant (7–25)-GBP lacking the segment corresponding to the hexapeptide. These mutants mimicked a normal GBP molecule in sequence, yet lacked any covalent attachment between the core domain and the N-terminal region (Fig. 4B). As expected, (7–25)-GBP alone did not possess any activity without the critical ENF motif. Moreover, (7–25)-GBP exhibited no activity even when mixed with the N-terminal hexapeptide, indicating that the peptide bond between the N-terminal and core region of GBP is indispensable for activity. Based on these data, we concluded that the proper length of the backbone linker that connects the ENF motif to the core domain is essential in activating GBP receptors.

Gly-Gly Linker Substitutions That Restrict the Conformation of the N Terminus Greatly Decrease Activity—To characterize the functional significance of Gly^5 and Gly^6 in more detail, additional mutants were constructed at these positions using site-directed mutagenesis. If the Gly side chains (hydrogens) are directly involved in receptor activation, then the addition of methyl groups to these Gly residues may have some effect on activity. However, Ala substitutions at either Gly^5 or Gly^6 (G5A, G6A) had no effect on the activity (Fig. 5), suggesting that the side chains (hydrogens) of these Gly residues are not essential for receptor binding and that the relatively small volume of the linker region and its flexibility may be more important. This prediction was confirmed by using substitution mutants in which Gly^5 and Gly^6 were substituted with Phe or Pro (G5F, G5P, G6F, and G6P), producing peptides that were much more restricted in their N-terminal polypeptide backbone conformation (Fig. 5). Interestingly, substitutions at these two positions showed strikingly different results. A Phe substitution at Gly^5 (G5F) slightly reduced activity: its activity was less than 10 nM than that of the wild-type GBP but was almost same at 100 nM. In contrast, G5F had almost no activity and only exhibited a low level of response at 1 μM, the highest concentration tested. In the case of Pro substitutions, although G5P had no activity even at a concentration of 1 μM (Fig. 5A), the
activity of G6P was only reduced about one order of magnitude relative to that of wild-type GBP (Fig. 5B). Thus, the presence of a Gly residue at position 6 in GBP seems to be less important for activity than that at position 5. In addition, the effect of Pro substitutions was more significant than that of Phe substitution in both cases at Gly5 and Gly6 in terms of interfering with the activation of plasmatocytes. This difference is thought to be due to the fact that Pro possesses the strongest stereochemical constraints of any amino acid residue by potentially restricting the degree of freedom of the backbone dihedral angles (15). These results suggest that the high flexibility of the linker residues, especially Gly5, is crucial for interaction with the putative GBP receptor.

**Phe** Is Required for Efficient Binding of GBP to Plasmatocytes—Our previous studies identified several residues crucial for the plasmatocyte-stimulating activity of GBP (10, 11), but it has been impossible to distinguish between residues necessary for receptor binding and those involved in receptor activation. To distinguish between binding and activation, we measured the ability of the inactive N-terminal GBP mutants to antagonize the plasmatocyte-stimulating activity of intact GBP. The N-terminal deletion mutants, all of which are inactive analogues of GBP, should be capable of antagonizing GBP if they retain their ability to bind. As expected, the deletion mutant lacking the first two residues of wild-type GBP (3–25)-GBP efficiently antagonized the plasmatocyte-stimulating activity of wild-type GBP, suggesting that (3–25)-GBP still retains critical determinants for receptor binding (Fig. 6). However, the additional deletion of Phe3 (4–25)-GBP from the N terminus, or all of the residues in the N-terminal disordered region (7–25)-GBP led to dramatic reductions in their ability to antagonize relative to (3–25)-GBP. These data clearly indicated that Phe3 is a critical residue for receptor binding of GBP. Additionally, the (7–25)-GBP did not antagonize the stimulating activity of wild-type GBP at all, implying an inability of the core domain of GBP to bind to the putative receptor without the disordered N-terminal region. The N-terminal hexapeptide ENFSGG alone also exhibited no antagonistic activity (data not shown). These results suggest that when the N terminus and the core domain of GBP are separated that neither has the capacity for a stable peptide-receptor interaction. Based on these findings, it seems reasonable that the linker region between the two domains makes it possible for them to synergistically bind to the putative receptor by anchoring the N-terminal domain to the core domain. Our data clearly demonstrate that Phe3 is the most important source of the binding energy in the N-terminal domain. Furthermore, (4–25)-GBP slightly exhibited antagonistic activity only at a concentration of 100 μM, whereas (7–25)-GBP almost completely lacked the activity, suggesting that the linker region, containing Gly5 and Gly6, is also involved to some extent in receptor binding. We also examined whether the (3–25)-GBP mutant antagonizes the mi-
togenic activity of wild-type GBP. Interestingly, in contrast to its ability to antagonize plasmatocyte-stimulating activity, the mitogenic activity was not antagonized by (3–25)-GBP (data not shown). These results support our previous proposal that mitogenic and plasmatocyte-stimulating activities may be mediated by different types of GBP receptors (10).

Conformational Dynamics of GBP and the GBP Mutants G5F and G6F—

15N NMR relaxation measurements can be used to characterize the intramolecular conformational dynamics of proteins and peptides (23). To elucidate the dynamics of the N-terminal domain of GBP, a set of 15N relaxation data, including \( R_1 \) and \( R_2 \) rates and \( \{1H\}^{15}N \) heteronuclear NOEs were measured for wild-type GBP and the Gly replacement mutants G5F and G6F at 500 MHz and 25 °C (Fig. 7). Because calculation of Model-free parameters using the Model-free formalism would be impractical due to the lower molecular mass and rotational correlation time of GBP (2.8kDa), we did not perform a Model-free analysis of the data and extracted only the general trends for dynamic behavior (24–26). In general, it is believed that 15N transverse relaxation rates \( R_2 \) lower than the average \( R_2 \) value for the molecule indicate internal motion. As expected, low \( R_2 \) values for the disordered N and C termini of GBP were observed, suggesting an increased mobility due to flexibility in these regions. Additionally, much lower NOE intensities were exhibited by both ends of GBP, showing that they are less ordered than the
internal domain of the molecule. These results are collectively indicative of a lack of structural definition in these regions and are in agreement with the solution structure of GBP previously determined by 1H NMR spectroscopy (Fig. 2A). Of particular interest is the fact that the G5F mutant exhibited almost the same R2 value as wild-type GBP despite its greater loss of stimulating activity than the G6F mutant, suggesting that Phe substitution at Gly5 has little effect on the mobility of the N-terminal region of GBP. This also implies that the greatly reduced activity of the G5F mutant may be mainly due to steric hindrance between the aromatic side chain of the Phe and the putative binding site of the linker region on the receptor.

Receptor Binding Model of GBP—Together with our previous published data, the results presented here suggest a mechanism by which GBP binds to its putative receptor. First, the initial binding of GBP is most likely mediated by the well-structured core domain of GBP. In support of this, mutant peptides that result in a loss of structure, such as the D16/H9252 mutant, which contained the -aspartyl bond between Asp16 and Gly, and double mutant C7.19A, which deleted the disulfide bridge in the core domain, are completely inactive (11, 12). Binding of the core domain to the receptor is then followed by binding of the unstructured N terminus, an interaction mediated primarily by Phe3, as has been demonstrated in both GBP and PSP (11, 13). Because the hexapeptide ENFSGG is both inactive and unable to antagonize, we assume that the interaction of the core domain of GBP with its receptor induces the interaction between Phe3 and a specific binding pocket on the receptor, presumably a hydrophobic cavity. Consequently, the N-terminal activating residue, Glu1, is presented to its complimentary site on the receptor and is ultimately responsible for activating it.

We show here that the linker region of GBP is also essential for its activity. The inherent flexibility of the linker, which permits a high degree of freedom of the conformation of the N terminus, may be required to allow the ENF motif to assume the proper conformation necessary to contact and activate the GBP receptor. The different activities exhibited by the Gly2 and Gly3 mutants may be due to their putative binding site in the receptor, which is likely to adopt a groove-like structure. The higher antagonistic activity of the (4–25)-GBP mutant relative to (7–25)-GBP is also consistent with this model, because it appears that the residues in the linker region also bind with some affinity to the putative binding cavity. This is presumably the reason why (4–25)-GBP had antagonistic activity higher than (7–25)-GBP. In addition to this requirement for flexibility, we show that the length of the linker region is critical for activity. A single Gly addition to the linker region still allows for some receptor activation, but removing a Gly results in a large loss of activity. The decreased length of the N terminus presumably precludes the N-terminal amine from gaining access to its activation site and suggests a highly precise fit between peptide and receptor.

Although the structure of GBP receptor has not yet been reported, recent studies suggesting that the dissociating constant for 125I-labeled GBP in plasmatocytes was 1.26 nM. Interestingly, this Kd values for GBP in plasmatocyte were different from that in insect Sf-9 cells, 0.25 nM (8). In accordance with this result, we have previously reported that GBP has multiple activities, and experiments using the GBP mutants suggest that a different type of GBP receptor may mediate each of these activities (10). In the case of mitogenic activity of GBP toward Sf-9 cells, it is likely that the tyrosine phosphorylation of the receptor triggers a series of intracellular signaling events (8). In the case of plasmatocyte stimulation by GBP, however, the signaling mechanism is still unknown. In the present study, we have proposed a model of GBP receptor binding in

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2 Y. Oda and Y. Hayakawa, unpublished results.
plasmatocytes that relies on the extensive data provided by the recombinant GBP mutants. The data reported here confirm the significance of the flexible N-terminal region of GBP and are also expected to provide useful clues as to the mechanism of receptor activation in other activities that GBP exhibits such as mitogenic activity in cultured cells.

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