Drosophila growth-blocking peptide-like factor mediates acute immune reactions during infectious and non-infectious stress

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Antimicrobial peptides (AMPs), major innate immune effectors, are induced to protect hosts against invading microorganisms. AMPs are also induced under non-infectious stress; however, the signaling pathways of non-infectious stress-induced AMP expression are yet unclear. We demonstrated that growth-blocking peptide (GBP) is a potent cytokine that regulates stressor-induced AMP expression in insects. GBP overexpression in Drosophila elevated expression of AMPs. GBP-induced AMP expression did not require Toll and immune deficiency (Imd) pathway-related genes, but imd and basket were essential, indicating that GBP signaling in Drosophila did not use the orthodox Toll or Imd pathway but used the JNK pathway after association with the adaptor protein Imd. The enhancement of AMP expression by non-infectious physical or environmental stressors was apparent in controls but not in GBP-knockdown larvae. These results indicate that the Drosophila GBP signaling pathway mediates acute innate immune reactions under various stresses, regardless of whether they are infectious or non-infectious.

The innate immune system of animals provides the first and most primitive line of defense against invading microorganisms. Antimicrobial peptides (AMPs) are produced as immune effector molecules to fight pathogenic infection, and the induction of AMPs is regulated through activation of the Toll and immune deficiency (Imd) pathways in Drosophila melanogaster. Although the activation of both signaling pathways in response to infection has been extensively investigated, we also know that changes in innate immune activities are sometimes unrelated to microbial infection. Various physical and physiological factors such as temperature, starvation, and diapause also elevate AMP expression levels. It is also known that AMP expression is highly sensitive to developmental stage in mammals as well as insects. Further, it has been recently reported that AMP expression in starved Drosophila is enhanced in response to the transcription factor FOXO, a key regulator of stress resistance, metabolism, and ageing, independently of the immunoregulatory pathways. Insulin signaling is currently the only known pathway for the induction of AMP expression by non-infectious stress. However, it is unlikely that animals cope with various non-infectious stressors by using the same signaling pathway that manages the regulation of innate immunity.

To investigate extracellular signaling in the innate immune regulation under non-infectious stresses, we focused on insect cytokines because cytokines in general regulate many physiological events including stress resistance through transmission of signals from outside the cell to the inside. While a large number of cytokines have been identified and their roles in mammals studied extensively, the number of known insect cytokines is quite limited. In Drosophila, spätzle is known as the cytokine that activates Toll signaling after microbial infection, which leads to expression of the target AMPs. In lepidopteran insects, structurally similar bioactive peptides have been reported in the last 20 years and are now recognized as the insect cytokine family referred to as the ENF peptides on the basis of their common N-terminal sequence, Glu-Asn-Phe.... These peptides are typically 23–25 amino acids long, and growth-blocking peptide (GBP) was the first member of this peptide family discovered. Other known insect-specific cytokines include Unpaired-3, which was first identified in Drosophila, and hemocyte chemotactic peptide (HCP), identified in the armyworm Pseudoletia separata. Stress-responsive
peptide (SRP) has also recently been identified in the common cutworm Spodoptera litura20. Drosophila eda-like cell death trigger (eiger)21 and sex peptides22 have been reported to modify AMP expression levels. Among these insect cytokines, we focused on characterizing the functional role of GBP in innate immunity because GBP was initially identified as the factor responsible for the reduced growth exhibited by armyworm P. separata larvae under stress conditions such as parasitization by the parasitoid wesp Cotesia kariyai and exposure to low temperature23. NMR analysis of GBP showed that it consists of flexible N- and C-termini, and a structured core stabilized by a disulfide bridge and a short antiparallel β-sheet (β-hairpin)24. Structural comparisons indicated that the core β-hairpin region adopts the C-terminal subdomain structure of human epidermal growth factor. Consistent with this structural similarity, GBP at concentrations of 10−1 to 10−9 pmol/ml induced proliferation of human keratinocytes as well as insect SF 9 cells25.

At least 16 members of the insect ENF cytokine family have been identified. They have diverse functions such as growth retardation14−16, paralysis induction14,15,16, cardioacceleration16, cell proliferation25,26, embryogenic morphogenesis27, and immune cell stimulation14,28. Characterization of some of these peptide cDNAs demonstrated that the ENF peptides are synthesized as a precursor form in which the active peptide is located at the C-terminal region15,28. Because it has been demonstrated that ENF family peptides stimulate insect immune cells like plasmatocytes to spread on foreign surfaces22−24,28, we first examined whether GBP affects humoral immune activity in a lepidopteran insect, the silkworm Bombyx mori. As expected, injection of B. mori GBP into B. mori larvae elevated the expression of AMPS. Further, GBP-induced elevation of AMP expression was demonstrated in silkworm larvae exposed to heat stress. Although this result demonstrated GBP-dependent induction of AMP expression in non-injected stressed silkworm larvae, elucidating in detail the pathway of GBP signaling in the immune system required analysis in Drosophila because little is known about the signaling pathways that activate AMP gene expression in non-Drosophila insects like B. mori. However, none of the ENF family cytokines have been identified in insect orders outside the Lepidoptera, making it necessary to identify the Drosophila GBP homolog. Database searches did not reveal any obvious homologs in the fly genome, which suggested either that the Diptera lack ENF genes or that members of this gene family might have diverged too much to be identified on a sequence level in Diptera. Therefore, we first purified a peptidergic factor with GBP-like activity from the bluebottle fly Lucilia cuprina. Using the sequence of the bluebottle fly GBP homolog for motif and FASTA searches, we identified five Drosophila melanogaster homologs, among which CG15917 was most similar to lepidopteran GBPs in terms of the primary structure of its ORF. Overexpression and RNAi knockdown of GBP in the Drosophila larvae indicated that GBP regulates the expression of AMPS through a novel pathway associated with Imd and JNK. The Drosophila GBP signaling pathway stimulated AMP expression in Drosophila larvae in response to external stressors, whether they were infectious or non-infectious.

**Results**

**Innate immune activity of B. mori GBP.** The B. mori GBP homolog (BmGBP) is referred to as the ‘Bm paralytic peptide’ because it was initially isolated following induction of paralysis27. First, to examine whether BmGBP is involved in the humoral immune response of the silkworm, the antibacterial activity of the serum (hemolymph devoid of hemocytes) of B. mori larvae injected with BmGBP was measured. The antibacterial activity of the serum was clearly increased by injection of BmGBP as well as by injection of Serratia marcescens (Fig. 1a). The transcripational enhancement was observed in all of the AMP genes, Cecropin, Attacin, and Gloverin, tested in the BmGBP-injected larvae. The elevation of Cecropin expression was the highest of all the AMPS tested here (Fig. 1b), and the Cecropin expression increased linearly for 3 h following injection with 100 pmol/larva BmGBP and then decreased slightly (Fig. 1c). To confirm the contribution of BmGBP to protection of the larvae from bacterial infection, we compared the survival rates after S. marcescens injection of silkworm larvae pre-injected with anti-BmGBP IgG and those treated with non-immunized IgG. The larvae pre-injected with anti-BmGBP IgG were significantly more susceptible to bacterial infection than control larvae treated with non-immunized IgG (Fig. 1d), suggesting that BmGBP plays a vital role in defending silkworm larvae against bacterial infection. Further, heat treatment clearly elevated Cecropin expression, but the heat-induced elevation of its gene expression was slightly but significantly repressed by pretreatment with anti-BmGBP IgG (Fig. 1e).

**Bluebottle fly and Drosophila GBP.** D. melanogaster is an ideal insect with which to study the innate humoral immune response induced by GBP-like cytokines because much is known about its immune system; however, no Drosophila homolog of GBP has been identified. Because sequence searches did not reveal obvious homologs, we speculated that members of this gene family might have diverged too far to be identified on a sequence level. Therefore, we first tried to purify a peptidergic factor with GBP-like activity from Drosophila larvae, but they proved too small to yield enough peptidergic factor from their hemolymph. Thus, we started with the bluebottle fly L. cuprina larvae and used sequences from it to find GBP-like molecules in Drosophila. We isolated a bluebottle fly cytokine that induced strong aggregation of hemocytes (Supplementary Fig. 1), and sequenced the responsible peptide. This peptide is only 19 amino acids and shares a partial sequence similarity with lepidopteran GBPs (Fig. 2 and Supplementary Fig. 2); in particular, two cysteine residues are similarly located. It showed GBP-like activities such as hemocyte aggregation activity and mitogenic activity on High Five cells, an insect cell line derived from Trichophasia ni (Fig. 2b, c). 5' and 3'-RACE reactions with degenerate primers prepared based on the peptide sequence revealed a 435-bp cDNA with a 333-bp ORF (Supplementary Fig. 2) that encodes a precursor protein whose size is similar to those of lepidopteran GBP precursors (proGBPs). Further, the overall structure is consistent with the fact that lepidopteran GBPs are synthesized as pre-pro-peptides and are generated by cleavage of the C-termini of the proGBPs. Based on the structural features together with its biological activities, we concluded that the isolated peptide is the bluebottle fly homolog of lepidopteran GBPs.

A cysteine-based motif search together with a FASTA search of the D. melanogaster genome database for the bluebottle fly GBP identified five genes encoding homologous peptides in the C-terminal regions of the ORFs (Fig. 2d). To determine which genes might be expressed in Drosophila larvae, we performed RT-PCR with gene-specific primer pairs using larval fat body mRNAs as templates, and confirmed expression of three genes (CG11395, CG15917, and CG17244) among the five (Fig. 2e). RT-PCR using adult fat body mRNAs showed a striking contrast to the pattern of the larval gene expression: all five genes including CG12517 and CG14069 were expressed in adult fat body (Fig. 2e). To select the Drosophila GBP homolog most similar to lepidopteran GBPs, we applied two criteria: the ORF should consist of 100–200 amino acid residues and contain an arginine residue at an appropriate position (7–9 residues upstream of the first cysteine) for the preferential cleavage by a serine proteasine that is presumed to be a proGBP processing enzyme. Among the three candidate genes expressing in the larval fat body, only CG15917 fulfilled the criteria because it encoded an 118 amino acid protein that included an arginine residue (R94) located nine residues upstream of the first cysteine (Supplementary Fig. 3). RT-PCR with CG15917 gene-specific primers showed its expression in three organs: fat body, integuments, and hemocytes (Fig. 2f).
expression level was the highest in fat body, very faint in hemocytes, and the expression was undetectable in midgut and Malpighian tubules.

Characterization of *Drosophila* GBP (CG15917). To confirm the structural and functional similarities of *Drosophila* GBP and lepidopteran GBP, we first examined whether *Drosophila* proGBP is processed as expected at the arginine residue (R94) in vivo. Western blotting of the extract prepared from control *Drosophila* larvae using the anti-*Drosophila* GBP antibody showed one main positive band of the predicted size (approximately 13 kDa) for proGBP together with one minor band that might be artificially derived from the main band protein. Exposure to temperature stress eliminated positive bands and produced one low molecular weight band of the predicted size (approximately 3 kDa) for GBP (Fig. 3A). N-terminal sequencing of the positive 3-kDa band peptide resulted in the predicted sequence of the active GBP, Ile-Leu-Leu-.

In addition, a synthetic active GBP peptide induced aggregation of *Drosophila* y w larval hemocytes (Fig. 3B). Further, overexpression of CG15917 retarded larval development: pupariation of GBP-overexpressing larvae (hs-Gal4/UAS-proGBP or hs-Gal4/UAS-GBP) was delayed about 10–15 h as compared with control larvae (Fig. 3C, Supplementary Fig. 4). These results clearly demonstrated that the processing pattern of *Drosophila* proGBP was the same as that of lepidopteran proGBP and that *Drosophila* GBP also showed lepidopteran GBP-like activities, such as cellular immune function and larval growth retardation.

GBP expression levels were examined in *Drosophila* larvae injected with *S. marcescens* or exposed to low temperature (4°C). The transcriptional enhancement was observed in both cases: GBP expression increased and reached a maximum level 4 h after *S. marcescens* injection while it reached a maximum 12 h after transfer from 25°C to 4°C (Fig. 3D).

Figure 1 | Innate immune activity following bacterial challenge or BmGBP injection in silkworm larvae. (a) Antibacterial activities were measured in the hemolymph of silkworm larvae injected with bacteria or BmGBP by the agar well diffusion method. Hemolymph was collected from test last instar larvae 12 h after injection of 100 ng/larva *Serratia marcescens* (Sm) or indicated doses of BmGBP. The supernatant after removing hemocytes by centrifugation was heated at 60°C for 10 min and then applied to a LB plate containing *E. coli*. BSA (10 nM) was used as the Control. Data are given as means±S.D. for five separate measurements. Asterisks indicate significant differences from control (t-test; *P<0.05, **P<0.01). (b) The bacterial challenge and BmGBP injection elevated all tested AMP expression levels. Total RNA was prepared from the isolated integument attached to the fat body of test larvae 3 h after injection of *S. marcescens* or indicated doses of BmGBP, and AMP expression was measured by real-time quantitative PCR. BSA (10 nM) was used as the Control. Other explanations are as in (a). (c) Effect of time after injection of 100 pmol/larva BmGBP on Cecropin expression. Data are given as means±S.D. for five separate measurements. Asterisks indicate significant differences from value at 0 h (t-test; *P<0.05, **P<0.01). (d) Survival rates of silkworm larvae preinjected with anti-BmGBP antibody after infection with *S. marcescens*. Silkworm larvae pretreated with non-immunized antibody were used as control animals. Data are given as means±S.D. for four separate measurements. A significant difference between control (○) and test (●) larva slopes was determined using ANOVA with the general linear model procedures of Minitab (release 14, Minitab Inc., USA): P=0.01. (e) Heat stress-induced elevation of Cecropin expression. Each silkworm larva preinjected with indicated dose of anti-BmGBP antibody was heated at 37°C for 9 h and its Cecropin expression level was measured 4 h after transferring them from 37°C to 25°C. NT: control larvae without heat treatment. NI IgG: Non-immunized IgG. Other explanations are as in (a).
Innate immune function of Drosophila GBP. To determine whether the effects of Drosophila GBP on the Drosophila immune response were similar to those of BmGBP, we established transgenic lines carrying UAS-GBP and UAS-proGBP. Although overexpression of both Drosophila GBP and proGBP under the direction of an hs-Gal4 driver enhanced expression of most AMPs in the whole bodies of Drosophila larvae, the elevation was not as high as after bacterial infection (Fig. 4a). However, it was apparent that the AMP expression levels were much higher in the GBP-expressing larvae than in the proGBP-expressing larvae, indicating that the processing of proGBP to active GBP is prerequisite for the GBP-dependent elevation of AMP expression. Further, Metchnikowin (Mtk) expression showed the highest elevation of seven tested AMP genes. We then determined which tissue was the most sensitive to GBP in terms of elevation of Mtk expression by RT-PCR. Similar enhancements of Mtk expression were observed from 1 h after overexpression of GBP in all tissues tested: fat body, hemocytes, and integument (Fig. 4b). To confirm the GBP-dependent enhancement of Mtk expression, transgenic larvae expressing green fluorescence protein (GFP) under the control of the Mtk promoter were used to visualize the spatial localization of the GBP-induced gene expression (Supplementary Fig. 5). Overexpression of GBP caused significant fluorescent signals in fat body. Further, injection of GBP peptide produced strong fluorescent signals in fat body and faint signals in epithelia, indicating that Mtk expression is activated by GBP in various tissues. Further, forced expression of GBP in adult males caused a significant elevation of Diptericin (Dpt) expression as well as Mtk expression. However, the elevation of Mtk expression was not as prominent as that in larvae with overexpression of GBP (Supplementary Fig. 6), implying the possibility that, although GBP elevates AMP expression in adults, there are some differences between adults and larvae in their immune responses to GBP.

GBP enhances AMP gene expression in response to aseptic as well as septic wounding. To confirm the contribution of GBP to the regulation of Drosophila humoral immunity, Mtk and Dpt expression levels were examined in GBP RNAi knockdown larvae. Because we had verified a greater than 90% reduction of GBP expression in UAS-dsGBP;hs-Gal4 larvae without any accompanying morphological change in fat body 36 h after heat treatment (Supplementary Fig. 7), larvae of the RNAi knockdown line were subjected to a challenge with the Gram-negative bacterium S. marcescens. Although challenged control larvae exhibited robust expressions of Mtk and Dpt, targeting of GBP by RNAi significantly reduced the AMP expression levels.
expression (Fig. 5a). Measurement of the susceptibilities of GBP RNAi larvae to bacterial infection after injection of three different doses of *S. marcescens* showed that the GBP knockdown significantly increased sensitivity to the bacteria (Fig. 5b). Furthermore, counting the number of bacteria present in the hemolymph clearly demonstrated slower clearance in the GBP RNAi larvae compared with control larvae at 6 h after the challenge (Supplementary Fig. 8). Together, these data provide strong evidence that the GBP signaling pathway plays an essential role in the pathogen-induced innate immune response.

Because we found that BmGBP regulates noninfectious stress-induced elevation of *Cecropin* expression, we tested whether stressors associated with noninfectious stimuli induce GBP-dependent innate immune responses in *Drosophila* larvae. First, mortality rates after aseptic wounding were enhanced in GBP RNAi larvae compared to control larvae (Supplementary Fig. 9). The AMP expression patterns of the GBP RNAi and control larvae were compared after pinching them with forceps without breaching the barrier function of the overlying cuticle. When test larvae were pinched, *Mtk* and *Dpt* expression levels in the control larvae were much higher than in the GBP RNAi larvae, while no significant difference was observed between *Drosomycin* (*Drs*) expression levels in the control and GBP RNAi larvae (Fig. 5c). The patterns of stressor-dependent AMP expression in both control and GBP RNAi larvae were similarly reproduced by temperature change stress: *Mtk* and *Dpt* expression levels were elevated in control larvae but not in GBP RNAi larvae 2 h after transfer from 4°C to 25°C (Fig. 5d). Further, constant cold (4°C) stress-induced elevation of *Mtk* expression in larvae was also significantly repressed by GBP RNAi (Fig. 5e). These results strongly demonstrated that the GBP pathway quickly mediates AMP expression when larvae are physically or environmentally stimulated, regardless of infection. Therefore, it is supposed that the rapid induction of GBP-dependent AMP expression in *Drosophila* larvae contributes to protection from both infectious and noninfectious stressors.

GBP enhances AMP gene expression through IMD/JNK pathway. To characterize the GBP signaling pathway in the immune system of *Drosophila* larvae, the transcription levels of *Mtk* were analyzed in transgenic *Drosophila* larvae with disturbed expression of genes related to the innate immune system. Normal GBP-dependent enhancement of *Mtk* expression was observed despite loss-of-function
disruption of the Toll pathway-related genes, spätzle, dorsal, and dorsal-related immune factor (Dif), indicating that GBP controls Mtk expression independently of the Toll pathway (Fig. 6a). By contrast, GBP did not induce Mtk expression inimd loss-of-function background larvae, but it did enhance Mtk expression in peptidoglycan recognition protein-LC (PGRP-LC), PGRP-LE, and Relish (Rel) loss-of-function background larvae (Fig. 6a, b). We interpreted these results as indicating that Imd is essential for the GBP signaling pathway to enhance the AMP gene expression, but that its pathway is different from the orthodox Imd pathway.

Immune activation of the Imd pathway has been reported to stimulate the JNK pathway due to a bifurcation at transforming growth factor-activated kinase 1 (TAK1)31,32. We therefore addressed whether JNK is involved in the GBP-dependent induction of Mtk expression. GBP-mediated induction of Mtk expression was not observed at all inbasket (bsk) knockdown larvae (Fig. 6c, Supplementary Fig. 10), supporting our hypothesis that JNK signaling is required for proper activation of the GBP pathway for induction of Mtk expression. This interpretation was confirmed by an in vitro study indicating that the GBP peptide did not elevate Mtk expression in fat body isolated from bsk knockdown larvae, while the peptide clearly did elevate Mtk expression in fat body from control larvae (Fig. 6d). Further, we found that JNK was phosphorylated in the fat body of Drosophila larvae with overexpression of GBP. GBP-induced phosphorylation of JNK was also observed in Drosophila S2 cells 10 min after adding synthetic GBP to the culture medium (Supplementary Fig. 11).

Discussion

The present study revealed the innate immune activity of GBP in insects by demonstrating that it elevated expression levels of some AMPs in Drosophila larvae as well as in Bombyx larvae. In order to clarify the role and mechanism of GBP in inducing AMP expression in insects, we analyzed its signaling pathway in Drosophila because no detailed signaling pathway of an innate immune system had been recognized in non-Drosophila species. To perform the analyses, we first isolated a GBP-like cytokine from bluebottle fly larvae, and then identified 46Drosophila genes encoding its homologous peptide by a cysteine-based motif search (http://www.genome.jp/tools/motif/MOTIF2.html) using the C-(X except for C)6-8-C-(X except for C)1-4 pattern. Among these genes, CG14069 and CG17244 were selected as candidate Drosophila GBP genes whose ORFs consist of 100-200 amino acid residues. Subsequent FASTA searches (http://www.genome.jp/tools/fasta/) for the C-terminal 25-amino acid peptide
sequence of CG14069 identified CG11395, CG15917, and CG12517. These five genes were found to encode GBP-like peptide sequences at the C-terminal ends. Among the five genes, we further selected the peptide gene most homologous to the bluebottle fly GBP and lepidopteran GBP genes based on the characteristics common to those GBP genes: the size (100–200 amino acids) of the ORF and the presence of an arginine residue at an appropriate position (7–9 residues upstream of the first cysteine). Further, after confirming the gene expression in the larval fat body, CG15917 remained as the most plausible candidate. The functional GBP peptide located in the C-terminal region of the CG15917 ORF stimulated aggregation of hemocytes from Drosophila y w larvae. Further, overexpression of CG15917 retarded larval development, creating a phenotype similar to that seen following injection of GBP into lepidopteran larvae10–12. We interpreted these results as an indication that the CG15917 product has a physiological role identical to that of lepidopteran GBPs.

Overexpression of proGBP as well as GBP significantly elevated Mtk and Dpt expression with the aid of the adaptor protein Imd in Drosophila larvae. The results are partially consistent with previously published data on the Drosophila gene CG15917: the gene was detected, along with many other genes, by microarray analysis as being transcriptionally activated three hours after septic injury of Drosophila adults13. We further revealed that the GBP-dependent induction of Mtk expression was abolished in larvae whose bsk expression was reduced by RNAi, indicating that the GBP pathway stimulated AMP expression through JNK signaling after recruitment of Imd to the activated GBP receptor (GBPR). This interpretation was partially confirmed by showing that GBP induced Mtk expression independently of PGRP-LC, PGRP-LE, and Relish (Fig. 6a, b). The biological importance of GBP was demonstrated by the finding that RNAi targeting of GBP significantly repressed AMP expression during the initial phase of bacterial infection and consequently made the larvae more susceptible to the bacteria than control larvae. Moreover, GBP RNAi repression of AMP expression was found to occur when test larvae were subcutaneously damaged without bleeding by pinching. The GBP RNAi-induced repression of AMP expression was also observed in Drosophila larvae exposed to temperature stresses: AMP expression was significantly elevated in control larvae by transferring them from 4 °C to 25 °C.

Figure 5 | Disturbance of antimicrobial response in Drosophila GBP RNAi mutant larvae. (a) Suppression of AMP expression in GBP RNAi larvae. AMP expression was measured at 0.5 h after Serratia marcescens challenge (48 h post-heat treatment, OD<sub>600</sub>=1×10<sup>-3</sup>) to transgenic larvae. Data are given as means±S.D. for four separate measurements. Asterisks indicate significant differences from controls (t-test; *P<0.01). (b) Survival rates of GBP RNAi larvae after bacterial challenge. Control and GBP RNAi larvae were stabbed with a very thin tungsten needle (diameter: approximately 0.05 mm) coated with indicated concentrations of Serratia marcescens, and the survival rates were measured at indicated times. Significant differences between control and test larval slopes were determined using ANOVA with the general linear model procedures of Minitab (release 14, Minitab Inc., USA): P=0.001 for 10<sup>-3</sup> and 5×10<sup>-3</sup>; P=0.01 for 10<sup>-2</sup>. (c) AMP expression in control and GBP RNAi larvae after pinching them with forceps, preserving the barrier function of the overlying cuticle. Data are given as means±S.D. for five separate measurements. Asterisks indicate significant differences from one of the controls (results of hs-Gal4 larvae) (t-test; *P<0.05, **P<0.01). (d) AMP expression in control and GBP RNAi larvae 2 h after transferring them from 4 °C to 25 °C. Other explanations are as in (c). (e) AMP expression in control and GBP RNAi larvae after exposing them to 4 °C. Other explanations are as in (c).
GBP signaling pathway for AMP expression. (a) Analysis of Mtk expression with various mutant backgrounds under the forced expression of GBP in Drosophila larvae. Induction of Mtk expression by overexpression of GBP under the direction of an hs-Gal4 driver in Drosophila larvae at 3 h after heat treatment. The J4 deletion represents a mutation of both Dif and dorsal. Data are given as means±S.D. for five separate measurements. Asterisks indicate significant differences from control (t-test; *P<0.05, **P<0.01). (b) Analysis of Mtk expression with PGRP-LC and PGRP-LE mutant backgrounds under forced expression of GBP in Drosophila larvae (hs-Gal4 UAS-GBP;LC and hs-Gal4 UAS-GBP). Other explanations are as in (a). (c) Analysis of Mtk expression with bsk RNAi backgrounds under forced expression of GBP in Drosophila larvae. Induction of Mtk expression by forced expression of GBP was measured in larvae whose bsk expression was almost completely repressed as shown in Supplementary Fig. 10. Other explanations are as in (a). (d) In vitro analysis of Mtk expression with bsk knockdown backgrounds by Drosophila GBP peptide in fat body of Drosophila larvae. Fat body isolated from bsk knockdown larvae (actin-Gal4/UAS-dsbsk) was divided longitudinally into halves, and each half was then incubated with 100 nM BSA or 100 nM GBP. The capacity of GBP to enhance Mtk expression was compared with its own control value (that in the other fat body half incubated with BSA). Other explanations are as in (a). (e) Model of AMP expression through the Drosophila GBP signaling pathway. GBP produced by processing of proGBP by various stressors may activate the GBP signaling pathway. Recruitment of adaptor protein Imd to the activated GBP receptor (?)-P77 adaptor protein complex may initiate the intracellular signaling to the induction of AMP expression via the JNK signaling. Although we have not yet isolated the GBP receptor, we have demonstrated its presence together with its adaptor protein (P77)\(^{30,34}\). The putative GBP receptor is indicated as ‘?’. 4°C, but it was not seen in GBP RNAi larvae. The results clearly show that induction of GBP-dependent AMP expression does not require a pathogen-associated molecular pattern, and that such non-infectious or non-injurious stimuli-dependent AMP expression is probably mediated by the GBP signaling pathway.

In lepidopteran larvae, GBP is abundantly present in a precursor form (proGBP) in hemolymph\(^{30,34}\). Our present experiments showed that this is also true for Drosophila. Non-injurious stimuli instantly triggered activation of GBP-processing enzyme(s) in hemolymph, by which the proGBP was proteolytically activated to GBP. The active GBP that resulted from the proteolytic processing under various stresses should trigger GBP-dependent AMP expression. It is reasonable to expect that such processing of proGBP enables the production of AMPs for the swift supply of active GBP under stress conditions. This interpretation is consistent with the fact that AMP expression was enhanced more by overexpression of active GBP than that of proGBP. Therefore, it is reasonable to propose that GBP serves as a key cytokine in the enhancement of AMP gene expression through the Imd/JNK pathway in Drosophila larvae exposed to various stressors, regardless of whether they are infectious or non-infectious (Fig. 6c).

Although there have been reports of the cross-regulation or cross-modification of two signaling pathways, Toll and Imd or JNK (or JAK/STAT) and NF-κB, our current knowledge is not still adequate to fully understand all mechanisms controlling the regulatory system\(^{8,11}\). Further, recent studies suggest the existence of an evolutionarily conserved mechanism of cross-regulation of metabolism and innate immunity\(^{26,39}\). It might be worth emphasizing that GBP was initially identified as a growth inhibitory factor in armyworm larvae. Although we confirmed that GBP overexpression retards normal larval development in Drosophila, we confirmed that this GBP-induced growth retardation did not affect AMP expression levels in test larvae under the present experimental conditions (Materials and Methods in Supplementary Information). Further, GBP-induced AMP expression was observed in Drosophila adults. Therefore, it is reasonable to propose that the insect cytokine GBP contributes to regulation of both growth and innate immunity. It may be possible to solve important problems concerning the adaptation of organismal defense to environmental stresses if we take the contributions of the novel signaling pathway through GBP-JNK into account there.

**Methods**

**Animals.** The silkworm Bombyx mori larvae were reared on an artificial diet at 25±1°C with a photoperiod of 16 h light:8 h dark. Larvae of the bluebottle fly, Lucilia cuprina, were reared on pork liver at 25±1°C, and adults were fed dry milk, sugar, and fresh water. Drosophila larvae and adults were reared on artificial food containing 8.7% (w/w) cornmeal, 5.2% (w/w) glucose, 3.5% (w/w) dried yeast, 0.3% antiseptic reagents, and 0.8% (w/w) agar at 25±1°C. The transgenic strains UAS-GBP, UAS-proGBP, and UAS-dsGBP were generated as described below, and Mtk-GFP was
gift from Jean-Luc Imler (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France). UAS-dsh-R was supplied by NIG-FLY (National Institute of Genetics, Mishima, Japan). UAS-GFP, hs-GaLa, tnmd, Releish24, spätzle47, 14, PGRP-LEc95, and PGRP-LCe96 were described elsewhere44,45.

**Peptides and antibody.** The Lucilia homologous peptide of GBP was purified by HPLC with reversed phase columns, C18, C4, and CN, as described in the Supplementary Information. Bombyx GBP (paralytic peptide)46, Lucilia GBP, and Drosophila GBP (CG15917, 24-amino acid peptide in Supplementary Fig. 3) were synthesized by the solid phase method using standard Fmoc (N-(9-fluorenlymethyl)oxycarbonyl) chemistry. Keyhole limpet hemocyanin-conjugated Drosophila GBP, emulsified in TiterMax Gold (TiterMax USA Inc.), was injected to immunize a rabbit. Anti GBP IgG was prepared as described previously45.

**Western blot analysis.** Drosophila third instar larvae were frozen in liquid nitrogen immediately after temperature stress. Frozen bodies were quickly homogenized in 20 mM Tris-HCl (pH 7.0) containing 1% SDS, and after centrifuging at 20,000 g for 10 min at 4 °C the supernatant was separated by SDS/PAGE, then transferred onto an Immobilon-P PVDF membrane (Millipore). Western blot analysis with anti-Drosophila GBP IgG was carried out as described previously.

Western blot analysis of JNK and phosphorylated JNK were carried out using the specific antibodies, anti-JNK (full length) (SC-572, Santa Cruz) and anti-active JNK (V7931, Promega), according to the method of Igaki et al.44.

**In vitro assay of GBPs in cultured cells and fat body.** Insect High Five cells were purchased from Invitrogen and maintained in modified Grace’s medium (MMG; Grace’s medium containing 0.33% lactalbumin hydrolysate and 0.33% yeastolate) with 5% fetal bovine serum. After culturing the cells in MG4 medium without fetal bovine serum or any growth factor at 25 °C for 2 d, the cells were cultured in medium containing GBP plus 1 μg/l of (H)-thymidine for 32 h. The cells were washed three times with 0.15 M NaCl, and then lysed with 100 mM N NaOH, and the incorporated thymidine was counted using a liquid scintillation counter (Alrosa LSC-5109). The cells were in their exponential growth phase at the time of labeling.

Third instar Drosophila larvae were cut longitudinally, and the fat body was isolated. After washing with Grace’s medium, the isolated fat body was separated into two almost equal halves, and then one of two halves was incubated in Grace’s insect medium containing BSA (control) and the other half incubated in the medium containing GBP. After incubation for indicated periods, total RNA was prepared from each fat body fragment as described previously.

**Establishment and analysis of transgenic fly.** The GBP cDNA was obtained by PCR using fat body cDNA prepared from y w strain larvae. EcoRI and XbaI sites were introduced at the 5’ and 3’ ends, respectively, of the proGBP cDNA by using the following primer set: 5’-CGGAAATCCAGCTGGAAGAGCTCAG-3’ and 5’-GGCTCTAGACAAATACTGCTTATG-3’. Similarly, EcoRI and XbaI sites were introduced at the 5’ and 3’ ends, respectively, of the GBP cDNA by using the following primer set: 5’-AGAGTCAGAGAATCGATGAGGAGAGACCC-3’ and 5’-GGCTCTAGAAGAACCAATCAATCTATTG-3’. Construction of the inverted repeat transgene of GBP was carried out according to the protocol of Lee and Carter45 using the following primer set: 5’-CGGAATTTACGATGAGGAGAGACCC-3’ and 5’-GGCTCTAGACAAATACTGCTTATG-3’. The triple HAT-resistant colonies were selected with the HAT selection medium using the triple HAT-resistant colonies. After checking the constructs, they were injected into embryos. The Gal4-dependent overexpression or knockdown of GBP in Drosophila was induced by heat treatment at 35 °C for 30 min.

**Infection and aseptic wound experiments.** Direct bacterial infection was induced by stabbing third instar larvae with a thin tungsten needle (diameter: approximately 0.05 mm) previously dipped into a concentrated culture of Serratia marcescens. The survival rates were measured at indicated times after the treatment. The survival rates of third instar larvae aseptically injured with a thin sterilized needle and then incubated in Grace’s medium containing BSA (control) and the other half incubated in Grace’s medium containing GBP. After incubation for indicated periods, total RNA was prepared from each fat body fragment as described previously.

**Sequence analysis.** To identify Drosophila homologs of Lucilia GBP, we first performed a motif search for C-X (except for Cys) or C-X-C (except for Cys) in the protein sequence database (KEGG GENES) on the MOTIF search web site (http://www.genome.jp/tools/motif/MOTIF2.html). Among 4,500 hit sequences, 46 were derived from Drosophila melanogaster genes, and two candidate genes, CG11395 and CG14069, were selected by the criterion that the GBP-like sequence is located at the C-terminus of the ORF with 100–200 amino acid residues like lepidopteran GBPs. Subsequent FASTA analyses (http://www.genome.jp/tools/fasta/) using the C-terminal peptide sequence of CG14069 was used to identify CG11395 and CG14069. Sequence similarities were also analyzed using the BLAST program against databases on the flybase (http://flybase.bio.indiana.edu/) web site. For more details, see Supplementary Information (Materials and Methods).
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Author contributions
ST and MO carried out most experiments, HM and AO performed biochemical experiments and analysis, SK contributed to the development of transgenic flies by providing scientific inputs and suggestions, and YH planned the study and wrote the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

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