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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*[1]. 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[2–4]. It is also known that *AMP* expression is highly sensitive to developmental stage in mammals as well as insects[5,6]. 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[7]. 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 *AMP*s[8]. 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[9]. These peptides are typically 23–25 amino acids long[10–17], and growth-blocking peptide (GBP) was the first member of this peptide family discovered[11]. Other known insect-specific cytokines include Unpaired-3[18], which was first identified in *Drosophila*, and hemocyte chemotactic peptide (HCP), identified in the armyworm *Pseudaletia separata*[19]. Stress-responsive

peptide (SRP) has also recently been identified in the common cutworm *Spodoptera litura*[20]. *Drosophila eda-like cell death trigger* (*eiger*)[21] and sex peptides[22] 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 wasp *Cotesia kariyai* and exposure to low temperature[23]. 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 ß-sheet (ß-hairpin)[24]. Structural comparisons indicated that the core ß-hairpin region adopts the C-terminal subdomain structure of human epidermal growth factor. Consistent with this structural similarity, GBP at concentrations of 10^{-1}–10^2 pmol/ml induced proliferation of human keratinocytes as well as insect Sf 9 cells[25].

At least 16 members of the insect ENF cytokine family have been identified. They have diverse functions such as growth retardation[11,12], paralysis induction[13,15,16], cardioacceleration[16], cell proliferation[25,26], embryogenic morphogenesis[27], and immune cell stimulation[14,28]. 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 region[15,28−30]. Because it has been demonstrated that ENF family peptides stimulate insect immune cells like plasmatocytes to spread on foreign surfaces[9,14,28], 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 *AMP*s. 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-infected 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[9]. 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 *GBP*s 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 *AMP*s 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 paralysis[17]. 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 transcriptional enhancement was observed in all the *AMP* genes, *Cecropin, Attacin,* and *Gloverin,* tested in the BmGBP-injected larvae. The elevation of *Cecropin* expression was the highest

of all the *AMP*s 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 *Trichoplusia 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 proteinase 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 a 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). The
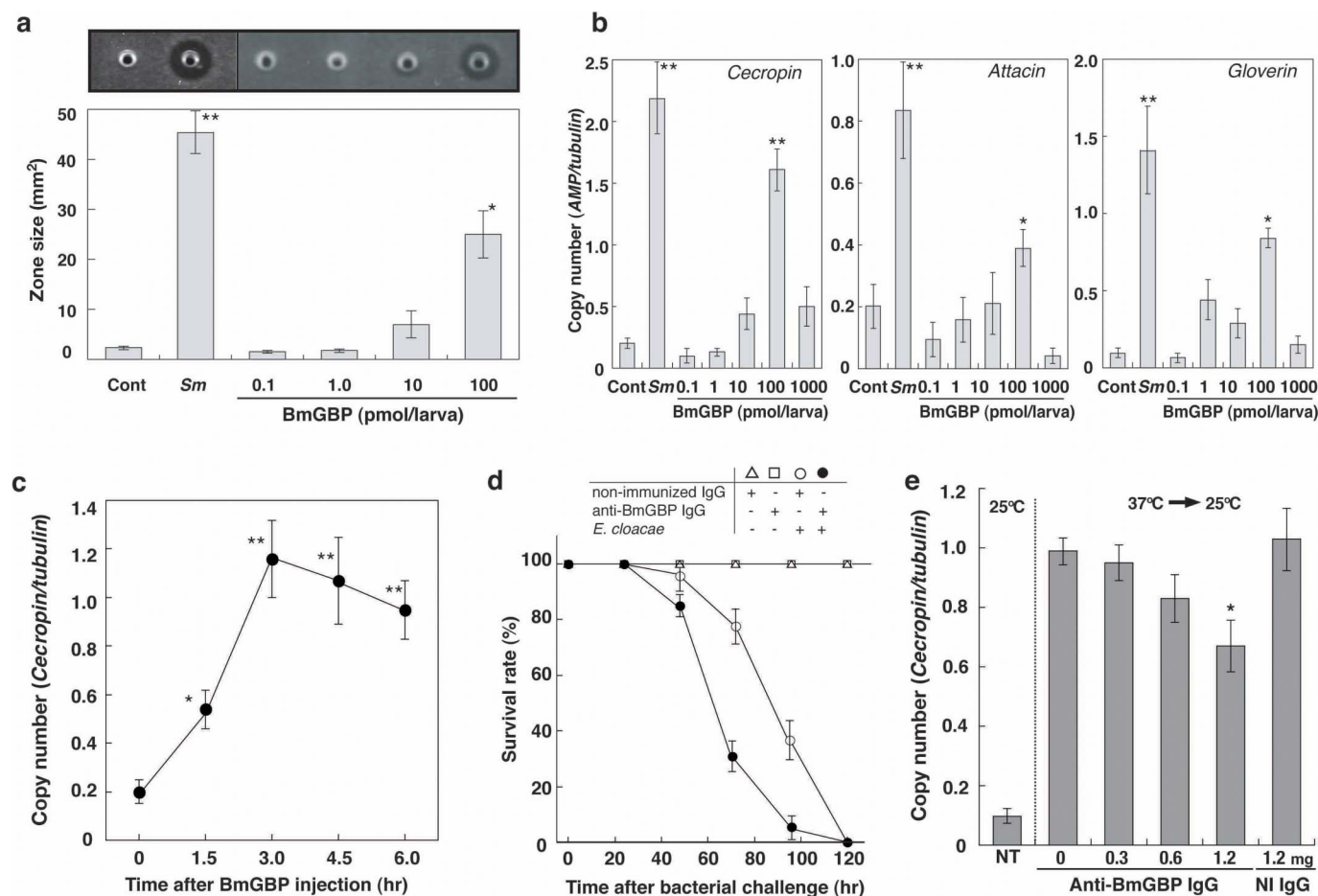
Figure 1 | Innate immune activity following bacterial challenge or BmGBP injection in silkworm larvae. (a) Antibacterial activities were measured in the hemolymph of silkworm larvae injected with bacteria or BmGBP by the agar well diffusion method. Hemolymph was collected from test last instar larvae 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).
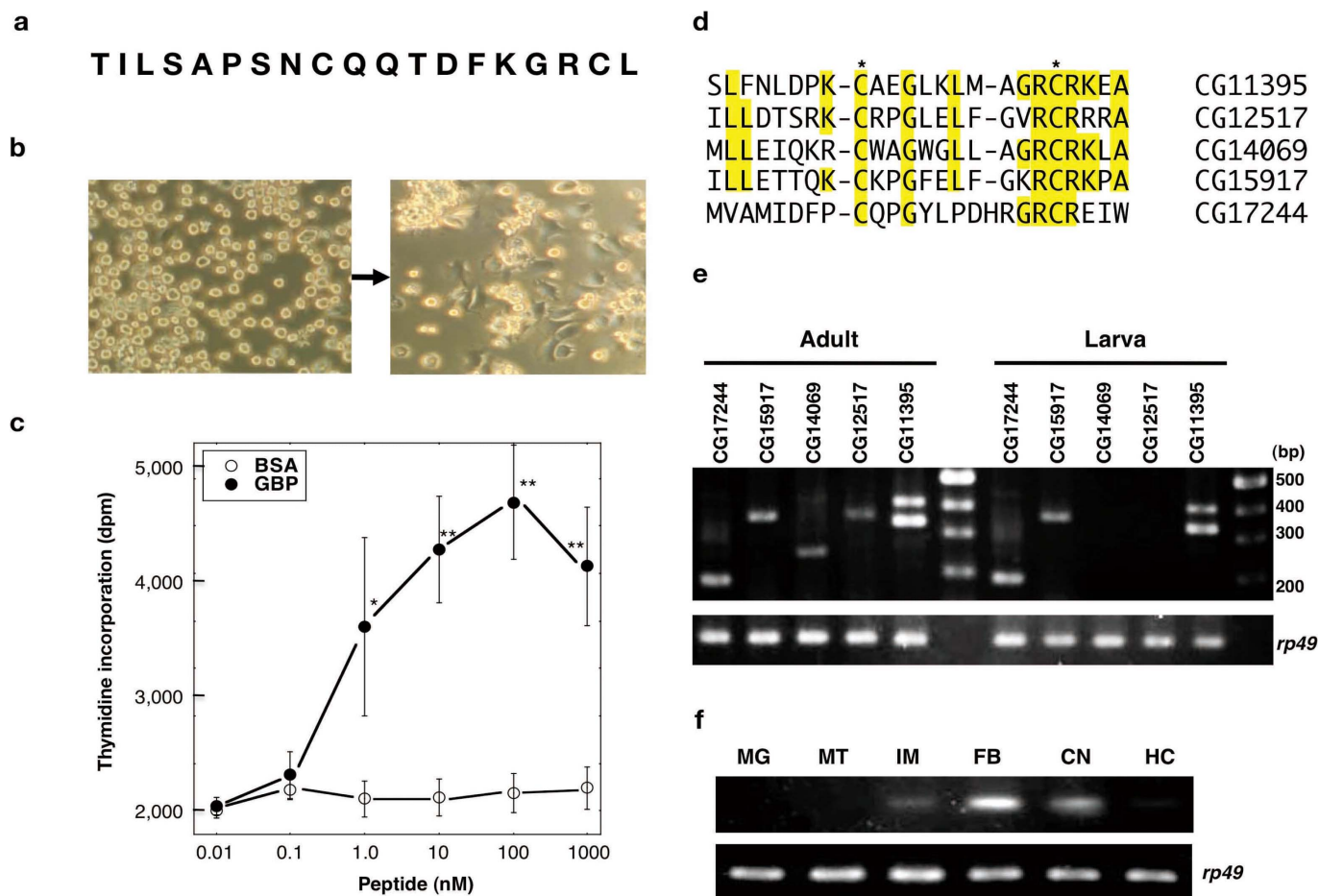
expression level was the highest in fat body, very faint in hemocytes, and the expression was undetectable in midgut and Malpighian tubules.

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).

a

T I L S A P S N C Q Q T D F K G R C L

b

c

d


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SLFNLDPK-CAEGLKLM-AGRCRKEA    CG11395
ILLDTSRK-CRPGLELF-GVRCRRRA    CG12517
MLLEIQKR-CWAGWGLL-AGRCRKLA    CG14069
ILLETTQK-CKPGFELF-GKRCRKPA    CG15917
MVAMIDFP-CQPGYLPDHRGRCREIW    CG17244
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e

f

Figure 2 | Characterization of GBP homologs identified from *Lucilia* and *Drosophila* larvae. (a) Primary structure of GBP homolog purified from the larval hemolymph of the bluebottle fly *Lucilia cuprina*. (b) Hemocyte aggregation and spreading induced by synthetic *Lucilia* GBP (final concentration: 10 nM). (c) *Lucilia* GBP induced cell growth activities of insect High Five cells. Data are given as means±S.D. for five separate measurements. Asterisks indicate significant differences from each control value (*t*-test; *P<0.05, **P<0.01). (d) C-terminal peptide sequences of the ORFs of *Drosophila* genes encoding homologous peptides to *Lucilia* GBP and lepidopteran GBPs. Common amino acid residues (in more than 3 peptides) are emphasized by yellow bands and conserved cysteine residues are indicated by stars. (e) RT-PCR analysis using primer pairs specific to each *Drosophila* gene identified as *Drosophila* homologs of GBP. Note that all gene expression bands are visible in *Drosophila* adults, but *CG12517* and *CG14069* expression bands are not present in larvae. (f) RT-PCR analysis of *CG15917* expression in various tissues of *Drosophila* larvae: midgut (MG), Malpighian tubule (MT), integument (IM), fat body (FB), central nervous system (CN), and hemocytes (HC).

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 *AMP*s 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*
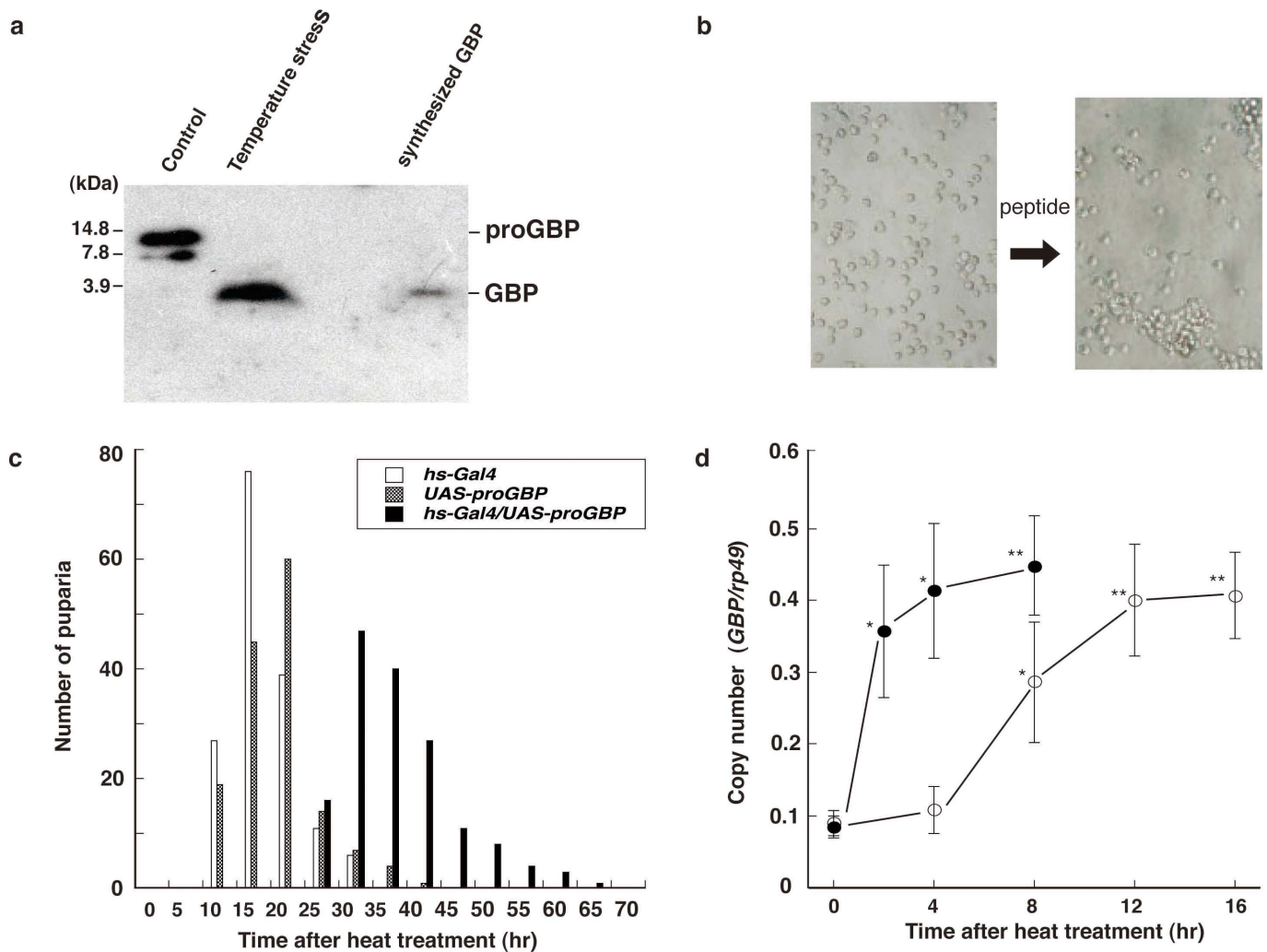
Figure 3 | Characterization of structure and activities of *Drosophila* GBP. (a) Western blot analysis of proGBP processing using anti-GBP IgG. Whole bodies of test *Drosophila* larvae placed at 4°C for 2 h were frozen immediately in liquid nitrogen 30 min after transferring them from 4°C to 25°C. Frozen bodies were homogenized as described in Materials and Methods and used for this analysis. Control larvae were constantly kept at 25°C. (b) Hemocytes isolated from *Drosophila y w* larvae were incubated with 100 nM GBP (or 100 nM BSA as the control) for 10 min. (c) Timing of pupariation of third instar larvae of transgenic *Drosophila* larvae. Same-staged larvae of control lines (*hs-Gal4* and *UAS-proGBP*) and *proGBP* overexpression line (*hs-Gal4/UAS-proGBP*) were heated at 33°C for 45 min. (d) Elevation of *GBP* expression by *S. marcescens* injection (●) or by cold stress at 4°C (○). Data are given as means±S.D. for four separate measurements. Asterisks indicate significant differences from control (*t*-test; *P<0.05, **P<0.01).

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 non-infectious 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
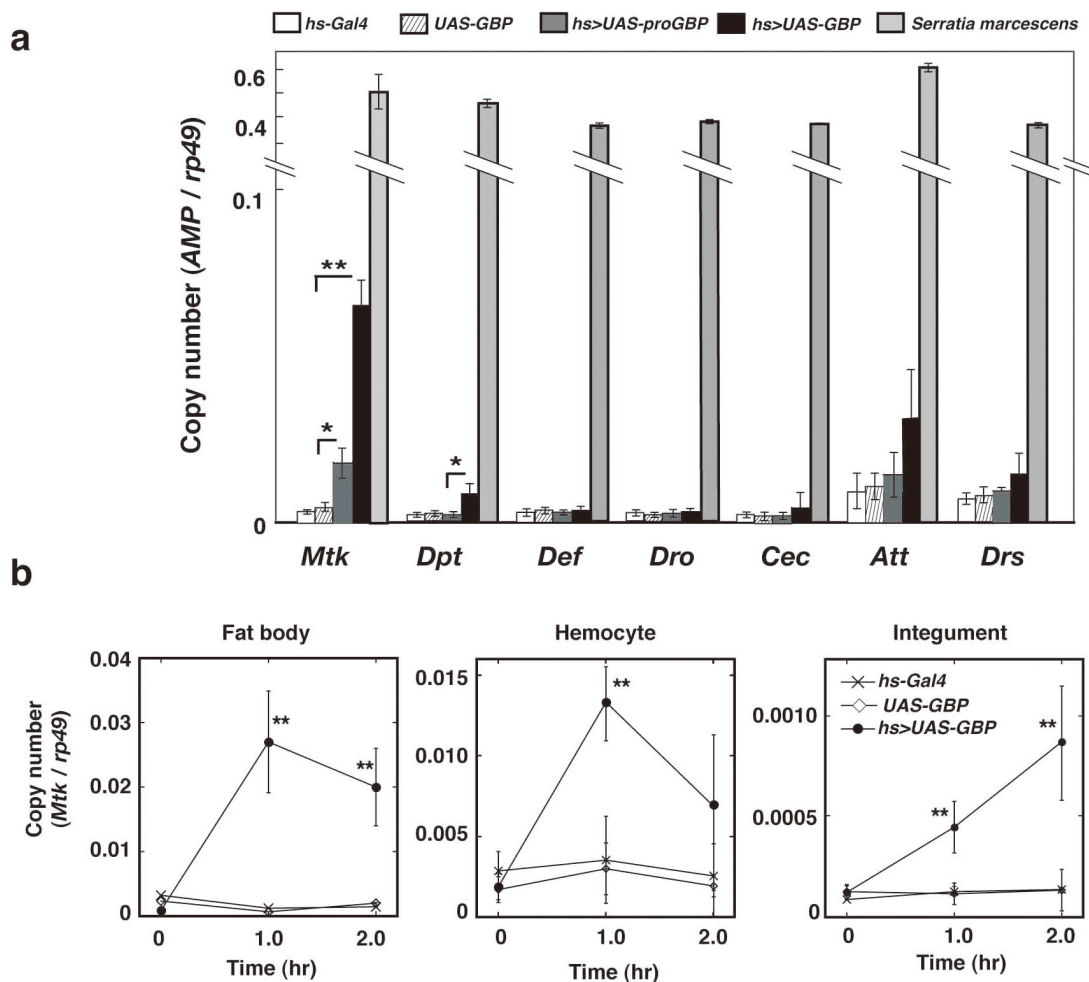
Figure 4 | Induction of *AMP* expression by GBP in *Drosophila* larvae. (a) Induction of *AMP* expression by forced expression of *proGBP* or *GBP* under the direction of an *hs-Gal4* driver in *Drosophila* larvae at 1 h (for *GBP*) or 3 h (for *proGBP*) after heat treatment or bacterial challenge (3 h after injection of heat-killed *Serratia marcescens*, $OD_{600}=1\times10^{-2}$). The amount of mRNA was quantified by real-time quantitative PCR, and *rp49* was used to normalize the expression levels. Significant transcriptional enhancement of *GBP* (or *proGBP*) was observed in *hs>UAS-GBP* (or *hs>UAS-proGBP*) larvae within 30 min after heat treatment. Data are given as means±S.D. for four separate measurements. Asterisks indicate significant differences from control (*t*-test; *P<0.05, **P<0.01). (*hs>UAS-GBP* or *hs>UAS-proGBP*) *hs-Gal4/UAS-GBP* or *hs-Gal4/UAS-proGBP*. (b) Time course of *Mtk* expression after forced expression of *GBP* in fat body, hemocytes, and integuments of *Drosophila* larvae. Other explanations are as in (a).

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 in *imd* 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 in *basket* (*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 46 *Drosophila* 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
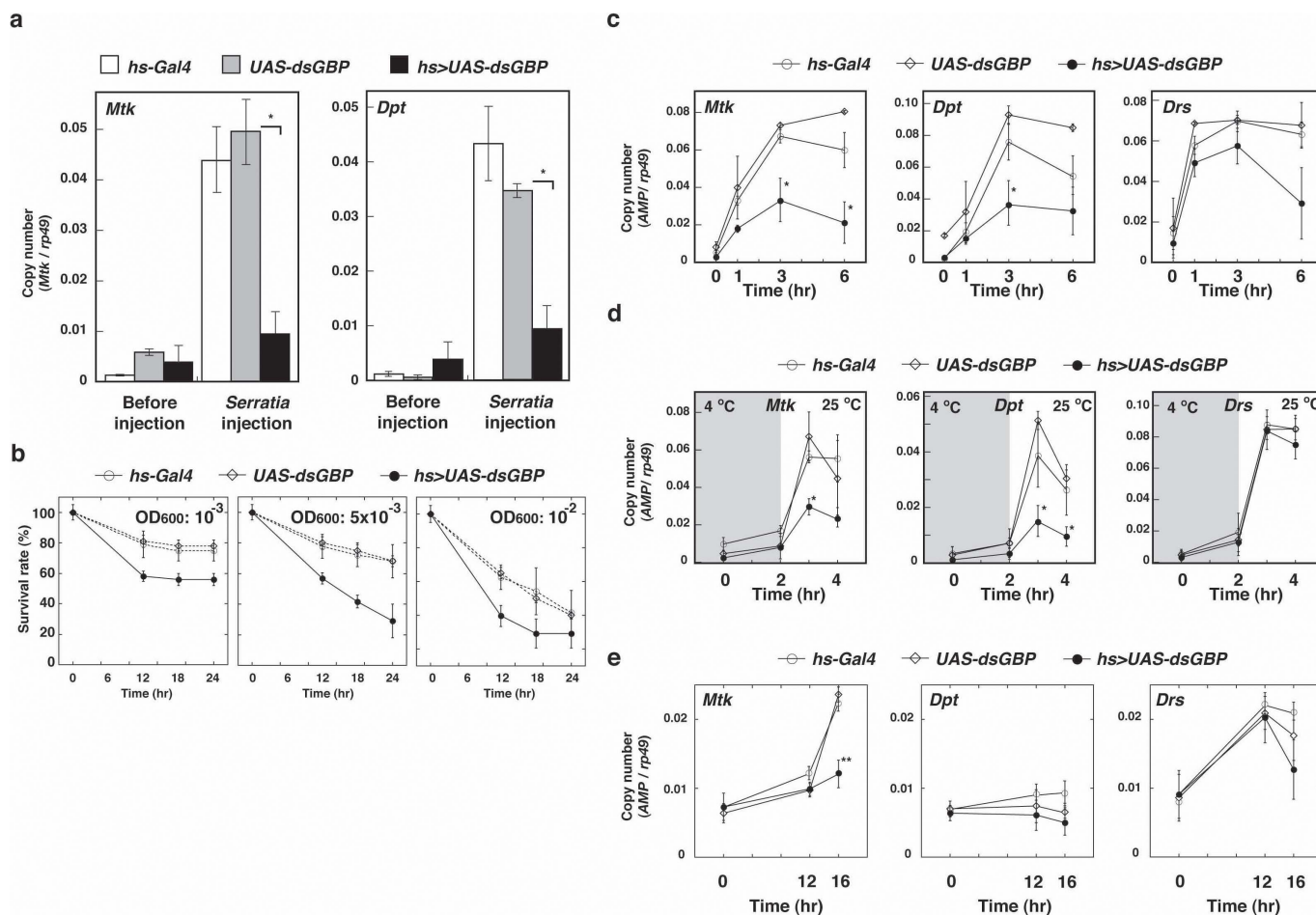
Figure 5 | Disturbance of antimicrobial response in *Drosophila GBP* RNAi mutant larvae. (a) Suppression of *AMP* expression in *GBP* RNAi larvae. *AMP* expression was measured at 0.5 h after *Serratia marcescens* challenge (48 h post-heat treatment, $OD_{600}=1\times10^{-3}$) to transgenic larvae. Data 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^{-3} and 5×10^{-3}, P=0.01 for 10^{-2}. (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).

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 larvae[10–12]. 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* adults[33]. 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 or exposing to
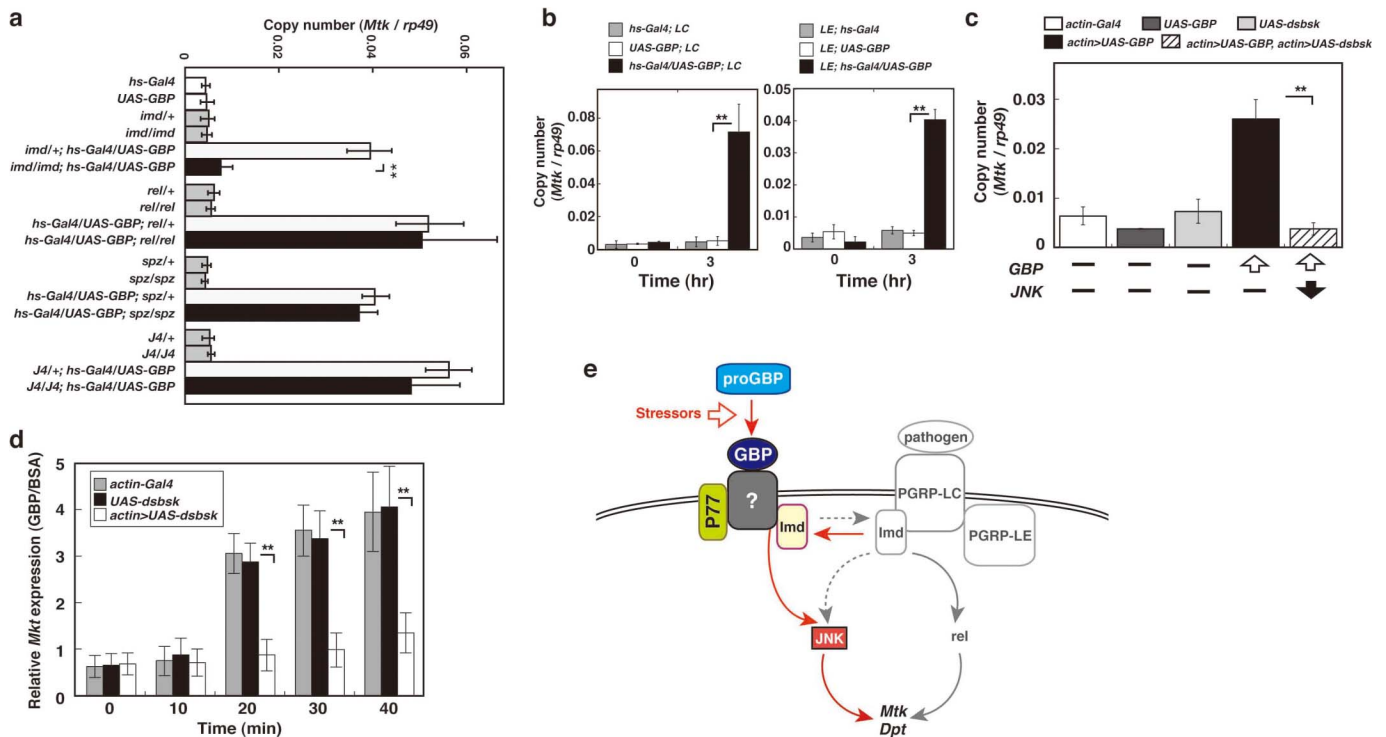
Figure 6 | GBP signaling pathway for AMP expression. (a) Analysis of *Mtk* expression with various mutant backgrounds under the forced expression of *GBP* in *Drosophila* larvae. Induction of *Mtk* expression by overexpression of *GBP* under the direction of an *hs-Gal4* driver in *Drosophila* larvae 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 *LE*;*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)[26,34]. 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. 6e).

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[35–37]. Further, recent studies suggest the existence of an evolutionarily conserved mechanism of cross-regulation of metabolism and innate immunity[7,38,39]. 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

gift from Jean-Luc Imler (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France)[40]. *UAS-dsbsk* was supplied by NIG-FLY (National Institute of Genetics, Mishima, Japan). *UAS-GFP, hs-Gal4, imd¹, Relish^{E20}, spätzle^{rm7},* J4, *PGRP-LE^{112},* and *PGRP-LC^{7454}* were described elsewhere[41,42].

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)[17], *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-fluorenyl)methoxycarbonyl) 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 previously[43].

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[34].

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 (MGM; Grace's medium containing 0.33% lactalbumin hydrolysate and 0.33% yeastolate) with 5% fetal bovine serum. After culturing the cells in MGM without fetal bovine serum or any growth factor at 25°C for 2 d, the cells were cultured in medium containing GBP plus 1 μCi of (³H)-thymidine for 32 h. The cells were washed three times with 0.15 M NaCl and lysed with 100 μl 0.3 N NaOH, and the incorporated thymidine was counted using a liquid scintillation counter (Aloka LSC-5100)[26]. 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[42].

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′-CGGAATTCAGTCATCGAAGCTTCATCGT-3′ and 5′-CGTCTAGAACACAAATATCACTTTATTGG-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′-AGAGTACAGAATTCATGATATTGCTGGAGACGACC-3′ and 5′-CGTCTAGAACACAAATATCACTTTATTGG-3′. Construction of the inverted repeat transgene of GBP was carried out according to the protocol of Lee and Carthe[45] using the following primer sets: 5′-CGGAATTCATGTTGATACGTATT-AATCC-3′, 5′-CGACTAGTTTACGCCGGCTTTCTGCATC-3′, 5′-CCACTA-GTAGGTGAGTTTCTATTCGCGGT-3′, 5′-CCGCTAGCCTGAGTTTCAAA-TTGGTAAT-3′, 5′-CGGCTAGCTTACGCCGGCTTTCTGCATC-3′, and 5′-CGTCTAGAATGTTGATACGTATTAAATCC-3′. Each PCR product was subcloned into a *pUAST* vector. After checking the sequences of these 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 tungsten needle (diameter: approximately 0.23 mm) were also measured by the same procedures as described above.

Sequence analysis. To identify *Drosophila* homologs of *Lucilia* GBP, we first performed a motif search for 'C-(X except for C)₆₋₈-C-(X except for C)₁₋₄' in the protein sequence database (KEGG GENES) on the MOTIF search web site (http://www.genome.jp/tools/motif/MOTIF2.html). Among 3,500 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* enabled us to identify *CG12517, CG15917*, and *CG17244*. 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

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