Activation of the Silkworm Cytokine by Bacterial and Fungal Cell Wall Components via a Reactive Oxygen Species-triggered Mechanism*

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Received for publication, July 5, 2007, and in revised form, August 17, 2007 Published, JBC Papers in Press, October 18, 2007, DOI 10.1074/jbc.M705480200

The insect cytokine paralytic peptide (PP) induces muscle contraction in silkworm larvae. Here we demonstrate that bacterial and fungal cell wall components peptidoglycan and glucan stimulate muscle contraction via activation of PP in the hemolymph. Anti-PP antibody suppressed the muscle contraction induced by PP, peptidoglycan, or glucan. The contraction was also inhibited by free radical scavengers and serine protease inhibitors. Moreover, injecting live silkworms with peptidoglycan or glucan generated the active form of PP. The active form of PP was also produced in vitro when peptidoglycan or glucan was incubated with hemolymph containing the PP precursor. Generation of the active form of PP was suppressed by free radical scavengers and serine protease inhibitors. Furthermore, PP activation in isolated hemolymph was inhibited by potassium cyanide, suggesting that cellular activity is involved. Stimulation by peptidoglycan promoted the generation of reactive oxygen species by silkworm hemocytes. The addition of either the active form of PP or anti-PP antibody to Staphylococcus aureus injected into silkworm larvae delayed or enhanced, respectively, the killing effect of S. aureus, suggesting that activated PP contributes to host resistance to infectious pathogens. These findings suggest that immunologic stimulants such as peptidoglycan or glucan induce reactive oxygen species production from larval hemocytes, followed by the activation of serine protease, which mediates the PP processing reaction and leads to defensive responses.

The immune system comprises a complex network of cells and molecules that protect the organism from infection. The system is divided into two categories: acquired immunity and innate immunity. Advances in immunologic research indicate that both types of immunity work closely together by inducing the secretion of cytokines (1–4). The cytokine network is indispensable for self-defense, but once the system goes out of control and an excessive amount of cytokine molecules is produced, serious damage, such as sepsis, occurs in the host animal (5–7). Because of the complicated nature of the cytokine network, the molecular mechanisms underlying such disorders are not yet clarified.

Many features of innate immunity are conserved from vertebrates through invertebrates (8, 9). Invertebrates lack acquired immunity, so they protect themselves against invaders such as bacteria and fungi by innate immunity alone. Therefore, invertebrates are simple and convenient models to use for investigating innate immunity (10).

Innate immunity in invertebrates consists of humoral and cellular components (11–14). The humoral component includes antimicrobial peptides (15–17), lectins (18), and melanin (19), and the cellular component includes phagocytosis by circulating hemocytes (20). Receptors that bind to various non-self components have been characterized (21); pattern recognition proteins, such as peptidoglycan recognition protein (PGRP)2 and β-1,3-glucan recognition protein (BGRP), recognize peptidoglycan and β-1,3-glucan, respectively (22). Downstream of these receptors, there are a number of protein cascades including kinases and proteases that transmit the information and induce various immunologic responses. For example, in insects, melanization begins after peptidoglycan binds to PGRP, subsequently activating the serine protease cascade, followed by the phenoloxidase-dependent synthesis of melanin and reactive quinones, which are toxic to microorganisms (23–25).

When the host insect is injured and invaded by microorganisms, free components circulating in the hemolymph are activated and transmit information among host cells (26). Paralytic peptide (PP) is one such molecule in the silkworm Bombyx mori. PP is a cytokine-like factor that has been purified from the hemolymph of silkworm larvae (27). PP belongs to the ENF peptide family based on its similarity with the primary sequence, especially at the C-terminal domain (28). PP is synthesized as an inactive precursor that is constitutively present in hemolymph. The active form of PP, the C-terminal 23-amino acid residue peptide, is generated from the precursor (29).

The abbreviations used are: PGRP, peptidoglycan recognition protein; BGRP, β-1,3-glucan recognition protein; PP, paralytic peptide; ROS, reactive oxygen species; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NBT, nitro blue tetrazolium; p-APMSF, p-(amidinophenyl)mercuri-anesulfonyl fluoride.

* This work was supported by funds from Genome Pharmaceuticals Institute Co., Ltd. (to K. S.) and from the Bio-oriented Technology Research Advancement Institution of Japan (to M. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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addition to paralysis, PP induces morphologic changes of the plasmatocytes, a key hemocyte subtype in cellular defensive reactions (30). Generation of the active form of PP is suggested to be important for cessation of bleeding in the host and for eliminating the non-self substance. The mechanism by which PP is activated in the presence of foreign invaders, however, remains to be elucidated.

It was previously reported in another lepidopteran insect, Manduca sexta, that the activation of PP is mediated by serine proteases (31). When hemolymph is exposed to atmospheric oxygen, reactive oxygen species (ROS) might be produced, resulting in the activation of serine proteases. In this study, we examined whether the stimulation of innate immunity leads to the production of ROS followed by the activation of serine proteases, which produce the active form of PP. The results demonstrated that PP processing is induced by peptidoglycan or glucan in the silkworm hemolymph and that this reaction is mediated by ROS and serine proteases.

**EXPERIMENTAL PROCEDURES**

**Insects**—Silkworm eggs (B. mori, Hu-Yo × Tukuba-Ne) were purchased from Ehime Sanshu (Ehime, Japan). Silkworm larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27 °C.

**Muscle Contraction Assay**—The measurement of muscle contraction activity using silkworm is described previously (32). Briefly, the heads of fifth instar silkworm larvae (3–4 g) were cut off, and the peritrophic membranes were removed. Each specimen was tied and attached to a transducer to measure isometric contraction with a load of 27 g. Test samples were dissolved or suspended in 0.9% NaCl and injected into the body fluid of a specimen with a 1-ml syringe attached to a 27-gauge needle (Terumo, Japan). The intensity of the muscle contraction was expressed as the contraction value, calculated by measuring the maximum length of each specimen before (x cm) and after (y cm) the injection using the formula \((x – y)/x\). Immune stimulants, peptidoglycan from Staphylococcus aureus, and glucan from Saccharomyces cerevisiae, were purchased from Sigma-Aldrich. The inhibitors, N-acetyl-L-cysteine, edaravone, benzamidine chloride, and \(p\)-(amidinophenyl)methanesulfonyl fluoride were purchased from Sigma-Aldrich, Mitsubishi Pharma Corporation, Nacalai tesque, and Wako, respectively.

**Detection of ROS Generation from Silkworm Hemocytes**—Hemolymph collected from cutting the abdominal legs of fifth instar silkworm larvae was incubated with test samples at 25 °C for 3 min and then boiled for 5 min. The samples were centrifuged at 10,000 \(\times\) g for 10 min and subjected to Western blot analysis.

**Detection of the Active Form of PP in Vitro in Isolated Hemolymph**—Hemolymph collected from cutting the abdominal legs of fifth instar silkworm larvae was incubated with test samples at 25 °C for 3 min and then boiled for 5 min. The resulting supernatant was subjected to Western blot analysis.

**Detection of the Active Form of PP and anti-PP Antiserum on Host Resistance to Bacterial Infection**—Saline, synthetic PP, anti-PP antiserum, or normal rabbit serum was filtered through a sterile 0.22-\(\mu m\) polyvinylidene difluoride filter (Millipore) and mixed with S. aureus MSSA1 (cultured for 18 h in LB10 medium) or saline, respectively. Test samples (50 \(\mu l\)) were injected into the body fluid of fifth instar silkworm larvae (day 1). Infected silkworms were incubated at 27 °C, and the number of viable larvae was counted.

**RESULTS**

Induction of Muscle Contraction in Silkworm Larvae by Bacterial and Fungal Cell Wall Components—Previously, we reported a sepsis model using silkworm larvae by intrahemolymph injection of pathogenic bacteria and fungi (34, 35). During the course of the study, we noticed that paralysis occurred after injecting the larvae with a highly concentrated culture of bacteria or fungi. Paralysis was observed even if heat-killed bacteria or fungi were injected. Because the paralysis was accompanied by shrinkage of the silkworm body, we suspected that the bacteria or fungi induced the contraction of the silkworm muscles. We focused on this observation and examined it further using a method we previously reported for quantitative measurement of muscle contraction of the silkworm muscles.

**Induction of Muscle Contraction in Silkworm Larvae by Bacterial and Fungal Cell Wall Components**—Previously, we reported a sepsis model using silkworm larvae by intrahemolymph injection of pathogenic bacteria and fungi (34, 35). During the course of the study, we noticed that paralysis occurred after injecting the larvae with a highly concentrated culture of bacteria or fungi. Paralysis was observed even if heat-killed bacteria or fungi were injected. Because the paralysis was accompanied by shrinkage of the silkworm body, we suspected that the bacteria or fungi induced the contraction of the silkworm muscles. We focused on this observation and examined it further using a method we previously reported for quantitative measurement of muscle contraction of the silkworm muscles.

**Silkworm Cytokine Activation by Cell Wall Components**

Protein samples were resolved by Tricine-SDS polyacrylamide gel electrophoresis on a 16.5% gel and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore). The membrane was probed with anti-PP antiserum diluted 1:6000 in blocking solution. For detection, the membrane was secondarily probed with horseradish peroxidase-linked antirabbit Ig from donkey (Amersham Biosciences), followed by reaction with Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences), and then exposed to Hyperfilm-ECL (Amersham Biosciences, UK).
contraction of silkworm larvae (32). Injection of heat-killed \textit{S. aureus} into larval muscle specimens induced muscle contraction (Fig. 1A). Peptidoglycan, a major component of the Gram-positive bacterial cell wall, is responsible for this reaction, because purified peptidoglycan from \textit{S. aureus} also induced muscle contraction (Fig. 1B). In addition, glucan from \textit{S. cerevisiae} induced muscle contraction (Fig. 1C). We previously reported that kainic acid mediates muscle contraction in silkworms (32). The process proceeds rapidly, suggesting that kainic acid acts as a receptor agonist at the nerve-muscle junction. The contraction induced by peptidoglycan or glucan described above, however, differed from that mediated by kainic acid in two points. First, the reaction was very slow; the time required to reach maximum contraction by peptidoglycan or glucan was \(\sim 10\) min, whereas kainic acid induced a contraction within 2 s (data not shown). Second, muscle contraction by kainic acid was severely inhibited by L-glutamic acid (32), whereas the reaction by peptidoglycan or glucan was not (data not shown). Therefore, we concluded that muscle contraction induced by peptidoglycan or glucan was a different process of innate immunity (36). Moreover, in hemolymph exposed to air, active PP is processed from precursors by proteases (29). Together with our present results, we speculated that ROS are involved in activating the protease that generates the active form of PP when larvae are injected with peptidoglycan or glucan. To test this hypothesis, we examined the effect of \(\text{H}_2\text{O}_2\), a source of ROS, on silkworm muscle contraction. \(\text{H}_2\text{O}_2\) slowly induced larval muscle contraction in a dose-dependent manner (data not shown). This reaction was repressed by the prior injection of \(N\)-acetyl-L-cysteine or edaravone (37), free radical scavengers (data not shown). We then tested whether these compounds inhibit contraction caused by peptidoglycan or glucan. These radical scavengers repressed peptidoglycan- and glucan-induced muscle contraction but did not inhibit PP-induced muscle contraction (Fig. 2A). These findings suggest that peptidoglycan and glucan induce ROS production, resulting in PP activation followed by muscle contraction.

Previous studies demonstrated that when bacteria were added to insect hemocyte culture medium, certain species of
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FIGURE 2. Involvement of ROS in muscle contraction induced by bacterial and fungal cell wall components. A, effect of radical scavengers on muscle contraction of silkworm larval specimen induced by peptidoglycan, glucan, or synthesized PP. Silkworm larval specimen was preinjected with 100 mM N-acetyl-L-cysteine (NAC) or 3 mg/ml edaravone, and muscle contraction induced by 1 mg/ml peptidoglycan, 1 mg/ml glucan, and 4 µg/ml PP was tested. B, ROS generation from silkworm larvae stimulated by peptidoglycan. Silkworm hemocytes (10⁶ cells/ml) were incubated with peptidoglycan (10 mg/ml) or phorbol myristate acetate (PMA; 10 µg/ml) for 60 min, and ROS production was measured by NBT reduction assay. The values represent the means ± S.D., n = 3. PBS, phosphate-buffered saline.

ROS were secreted from the cells (38). We tested whether silkworm hemocytes produce ROS when stimulated with peptidoglycan. We performed the NBT reduction assay to measure ROS, and ROS production significantly increased in hemocytes incubated with peptidoglycan (Fig. 2B). Microscopic observation revealed that insoluble NBT formazan particles, a reaction product of NBT and ROS, appeared inside the hemocytes (data not shown). These results support the notion that hemocytes recognize the outer components of microorganisms and produce ROS, which then mediate PP processing.

Involvement of Serine Proteases in Muscle Contraction Induced by Bacterial and Fungal Cell Wall Components—Soluble receptors for peptidoglycan or glucan have been identified in silkworm hemolymph (39, 40). These receptors activate a serine protease cascade and induce melanization (24). Moreover, the formation of active PP in hemolymph exposed to air is suppressed by a serine protease inhibitor in another moth, M. sexta (31). Therefore, we hypothesized that serine proteases are involved in the activation of silkworm PP triggered by peptidoglycan or glucan. Pretreatment of larval muscle specimens with a serine protease inhibitor, benzamidine or p-(amidino-phenyl)methanesulfonyl fluoride (p-APMSF), significantly inhibited peptidoglycan or glucan-induced muscle contraction (Fig. 3). On the other hand, these serine protease inhibitors did not suppress PP-induced muscle contraction (Fig. 3). Thus, serine proteases activate PP when stimulated by peptidoglycan or glucan.

Generation of Mature PP in Hemolymph of Live Silkworm Larvae by Injection of Bacterial and Fungal Cell Wall Components—PP exists in an inactive form (proPP) in silkworm hemolymph. When hemolymph bled from the body is exposed to air, proPP is cleaved by serine proteases, resulting in the formation of active PP (31). We examined whether the active form of PP is generated when muscle contraction is induced by peptidoglycan or glucan. After injecting live silkworm larvae with peptidoglycan or glucan, hemolymph was collected and subjected to Western blot analysis probing with an anti-PP antibody. Mature PP appeared in response to these stimulants in a dose-dependent manner (Fig. 4A). The generation of mature PP was also observed when H₂O₂ was injected into the hemolymph of live larvae (Fig. 4B). Therefore, ROS generated from H₂O₂ in larval hemolymph might stimulate PP processing. We further examined the effect of serine protease inhibitors on PP processing induced by H₂O₂ in the hemolymph of live silkworms. The injection of benzamidine or p-APMSF together with H₂O₂ into the hemolymph of live larvae inhibited the formation of mature PP (Fig. 4B). These results suggest that proPP in the hemolymph is cleaved by serine proteases that are activated by ROS.

Generation of the Active Form of PP in Vitro in Isolated Hemolymph—We then examined the activation of PP in vitro using hemolymph collected from intact larvae. Isolated hemolymph was incubated with peptidoglycan or glucan, and the samples were subjected to Western blot analysis probing with an anti-PP antibody. The active form of PP was generated in hemolymph incubated with peptidoglycan or glucan in vitro (Fig. 5A). N-Acetyl-L-cysteine (Fig. 5B), benzamidine, and p-APMSF (Fig. 5C) inhibited the reaction, suggesting that ROS and serine proteases mediate PP processing induced by the components of bacteria or fungi.

In mammalian innate immune systems, macrophages and lymphocytes secrete ROS in response to bacteria (41). We
examined whether the activation of PP in silkworm hemolymph involves cellular processes. Potassium cyanide, an inhibitor of cellular ATP synthesis, inhibited the generation of the active form of PP in the hemolymph (Fig. 5D). This finding suggests that, different from the melanization that occurs in cell-free silkworm plasma, PP activation induced by peptidoglycan or glucan involves cellular process mediated by hemocytes.

Effect of the Active Form of PP and Anti-PP Antiserum on Host Resistance to Bacterial Infection—Although PP is considered to be involved in innate immunity, it has been unclear whether the active form of PP contributes to host resistance to bacterial infection. We examined the effect of the active form of PP and anti-PP antiserum on the survival of silkworms infected with S. aureus. Silkworms injected with S. aureus died within 100 h, whereas 50% of silkworms injected with a mixture of S. aureus and active PP survived for more than 110 h (Fig. 6). On the other hand, 50% of silkworms co-injected with S. aureus and anti-PP antiserum died within 70 h (Fig. 6). Analysis using the Kaplan-Meier method indicated that the effects of active PP and anti-PP antiserum on the survival of silkworms were statistically significant (p < 0.01), whereas the effect of normal rabbit serum was not (p > 0.05). These results suggest that PP activation is critical for host protection in the presence of infectious bacteria.

DISCUSSION

Activation of Cytokine-like Factor PP by Bacterial and Fungal Cell Wall Components—The cytokine-like factor PP from silkworm B. mori was originally characterized as a peptide that causes paralysis of silkworms when injected into the hemolymph (27). Homologous peptides were identified from lepidopteran insects such as Trichoplusia ni, Heliothis virescens, M. sexta, Spodoptera exigua, and Antheraea yamamai (42, 43). Later, active PP was reported to induce the hemocyte spreading activity that is responsible for insect innate immunity (30). Nevertheless, the mechanism of PP activation by physiologic factors has remained unknown. Our present results suggest that (i) peptidoglycan and glucan, well known stimulants of innate immunity, induce PP activation in silkworm hemolymph, (ii) the process is mediated by ROS and serine proteases, and (iii) a cellular process is involved (Fig. 7).

In invertebrates, there are several receptors that recognize peptidoglycan or glucan. For example, PGRP is well characterized, and it mediates the melanization and production of antimicrobial peptides (39, 44). βGRP, Gram-negative bacteria-binding protein, and apolipoprotein bind to glucan (40, 45–47). These receptors are candidates that might be involved in the first step of PP activation. Silkworm PGRP and βGRP have two isoforms: a free type and a membrane-bound type (48). Recently, the presence of an intracellular type was demonstrated in Drosophila melanogaster (49). The free forms of βGRP and PGRP are suggested to mediate melanization, whereas the roles of cellular receptors in the immune network...
Silkworm Cytokine Activation by Cell Wall Components

Peptidoglycan, Glucan

ROS production by hemocytes

Serine proteases activation

PP processing

Defensive responses

FIGURE 7. Defensive reactions of silkworm larvae induced by bacterial and fungal cell wall components.

are not known. Our results suggest that hemocyte receptors contribute to the processing of PP, an insect cytokine-like factor.

ROS Mediate PP Processing Induced by Bacterial and Fungal Cell Wall Components—ROS are produced by stimulated immune cells and show various activities in innate immunity. ROS not only kill microorganisms directly by oxidizing the membrane lipids, but they also act as intercellular messengers. ROS induce nuclear factor-κB-mediated inflammatory cytokine production, antimicrobial peptide synthesis, and apoptosis of severely infected cells (36, 50). ROS generation occurs both inside and outside of immune cells. When the cells recognize foreign substances, they undergo a process called oxidative burst in which NADPH oxidase at the cell surface is hyperactivated and ROS generation occurs (51). This phenomena is well characterized in mammalian neutrophils and is also reported in some insect hemocytes such as Bladerus discoidalis (52) and Galleria mellonella (53).

Although we observed ROS production inside the peptidoglycan-stimulated hemocytes, we failed to detect ROS in hemocytes incubated with glucan using the NBT assay. This result might reflect the specificity of the NBT and ROS reaction. Because different receptors recognize peptidoglycan or glucan, it seems reasonable to speculate that the type of ROS produced by these two immunity stimulants might also be different. In the PP activation process, more detailed studies are required to determine the type of cells that produce ROS and the type of ROS produced in response to peptidoglycan or glucan stimulation.

Our findings indicating the cellular production of ROS by peptidoglycan might support our hypothesis, but we could not exclude other possible interpretations; for example, ROS generated extracellularly might permeate the immune cells and activate subcellular proteases. Further studies are needed to determine the compartments in which ROS production and protease activation actually occur.

Serine Proteases Mediate PP Processing Induced by Bacterial and Fungal Cell Wall Components—By using inhibitors, we showed that serine proteases are involved in PP processing induced by peptidoglycan or glucan. Serine proteases generally possess high substrate specificities and regulate various processes in self-defense systems. In the invertebrate innate immune response, serine proteases mediate hemolymph coagulation, antimicrobial peptide production, and melanization (54, 55). The primary structure of mature PP is highly homologous to that of other ENF family peptides such as growth block peptide, plasmocyte spreading peptide, and cardioactive protein (27). All of these mature peptides have 23–25 amino acid residues and are generated from larger precursors, but none of the cleaving enzymes have been identified. Four amino acid residues near the cleavage site are highly conserved among ENF family peptides. Therefore, serine proteases that participate in generating the active form of PP in silkworms might be involved in activating other ENF family peptides. Biochemical characterization of these serine proteases is required for further understanding of the production mechanism of ENF family peptides, including PP.

Some enzymes are activated by ROS-mediated oxidation. For example, the activity of a typical metalloprotease that degrades collagen is stimulated by H_2O_2 or xanthine/xanthine oxidase, which produces ROS (56). Here, we demonstrated that both PP processing and muscle contraction induced by H_2O_2 were suppressed by serine protease inhibitors. The results suggest that ROS activate a serine protease that cleaves the PP precursor, resulting in muscle contraction. In general, ROS act on cysteine and methionine residues of proteins and oxidize them (57). Therefore, we considered the possibility that the oxidation of amino acid residues in the serine protease by ROS might stimulate the enzyme activity to generate mature PP.

The Biologic Relevance of the Activation of Insect Cytokine PP Induced by Bacterial and Fungal Cell Wall Components—We demonstrated that peptidoglycan and glucan, innate immunity stimulants, induce the activation of the insect cytokine-like factor PP, followed by muscle contraction of silkworm larvae. A coupling between innate immunity and muscle contraction was previously demonstrated in mammals; the cytokine interleukin-4 induces the contraction of smooth muscle in the digestive tract (58, 59). This process is regarded as an immune response against pathogenic parasites. We speculate that PP activated in response to the invasion of microorganisms might provide similar defensive effects in the host insect. PP activation in infected silkworms was critical for the resistance to infectious pathogens; therefore, we propose that cytokine-like factor PP be redefined as a true “cytokine” that contributes to self-defense in the host animals.

Although cytokines are important for the host to fight pathogenic invaders under regulated conditions, an exaggerated response by activated cytokines causes serious problems in the host (7). For example, excess cytokines produced in the mammalian digestive tract cause intestinal disorders (60). Moreover, excessive production of cytokines in sepsis patients causes multiple organ failure (61). Intense muscle contraction by activated PP might be an outcome of such an exaggerated response. We previously reported that an overdose of PP killed silkworms (62). Our present finding of PP activation by innate immunity stimuli resulting in muscle contraction will provide clues to
elucidate the molecular mechanisms of disorders caused by excessive cytokine production in body fluid.

Acknowledgment—We thank Dr. N. Higashi (The University of Tokyo) for useful discussions.

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