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Activated Phenoloxidase Interacts with a Novel Glycine-rich Protein on the Yeast Two-hybrid System

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One of the innate immune reactions in invertebrates is the pro-phenoloxidase (pro-PO) activation system that is involved in the generation of superoxide, melanin synthesis, and the subsequent sequestration of foreign matter entering the hemocoel of the invertebrates. However, the molecular mechanism of this biological reaction is still obscure. To expand our understanding of the biological roles of the pro-PO activation system in invertebrates, we performed a yeast two-hybrid screening by using three regions of pro-PO as bait and a yeast two-hybrid cDNA library from *Tenebrio molitor* larvae as prey. We isolated a novel partial cDNA clone that encodes a glycine-rich protein that interacted with the active phenoloxidase (termed phenoloxidase interacting protein, POIP). POIP consists of two domains: One is an N-terminal unique domain and the other is a C-terminal glycine-rich domain. The C-terminal glycine-rich domain showed sequential homology with those of insect antifungal proteins. Also, the yeast two-hybrid screen in a reverse orientation (using POIP as bait) yielded PO, suggesting that the PO-POIP interaction is specific. By using a 315 bp PCR fragment of the N-terminal unique region of POIP, we cloned the full-length cDNA of POIP from the *Tenebrio* cDNA library constructed by using *E. coli* injected larvae. The interaction analysis between PO, and a truncated fragment lacking the N-terminal unique region of POIP, indicated that the N-terminal unique region is necessary for interaction between PO and POIP. The expression level of the POIP mRNA is increased by bacterial injection into *T. molitor* larvae. This suggests that POIP might be engaged in the humoral defense reaction.

Keywords: Defense reaction, Melanin, Phenoloxidase, Prophenoloxidase, Yeast two-hybrid.

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Introduction

Invertebrates utilize two broad categories of defense responses against invading parasites and pathogens. They are cellular and humoral responses. The former includes encapsulation, phagocytosis and nodule formation (Rowley and Ratcliffe, 1981). The synthesis of a broad spectrum of potent antimicrobial proteins in insects (Boman, 1991; Natori, 1994; Hoffmann, 1996), the clotting system of crustaceans (Iwanaga, 1998; Hall *et al.*, 1999) and the prophenoloxidase activating system (pro-PO system) (Ashida and Brey, 1998; Söderhäll and Cerenius, 1998) belong to the latter immune response.

We have been studying the humoral and cellular defense response by using coleopteran insects, *T. molitor* and *H. diomphalia* larvae. We examined the humoral responses and reported the primary structures of antimicrobial proteins in the hemolymph of coleopteran insects, *Tenebrio molitor* and *Holotrichia diomphalia* larvae (Lee *et al.*, 1994; Moon *et al.*, 1994; Lee *et al.*, 1995; Lee *et al.*, 1998a). To clarify the mechanism of encapsulation at a molecular level, we also reported the purification and cDNA cloning of two early-stage encapsulation-related proteins (ERPs) from *T. molitor* larvae by injection of four kinds of non-self materials (Cho *et al.*, 1999a; Cho *et al.*, 1999b). We found that four proteins (86 kDa, 78 kDa, 56 kDa and 48 kDa) retrieved from the three different kinds of beads and a piece of suture, were enriched compared to the crude hemolymph. The deduced amino acid sequence of the 56 kDa and the 48 kDa ERP from their cDNA revealed that these two proteins are novel glutamine-rich proteins. Interestingly, the 48 kDa ERP was produced with the cleavage of Arg^{101}-Gly^{102} of 56 kDa ERP by a limited proteolysis. The pro-PO system is an important non-self recognition system in invertebrates. Non-self molecules, such as lipopolysaccharide (LPS), peptidoglycan and β-1,3-glucan, are recognized by non-self recognition proteins and their receptors causing activation of the pro-PO system. The activation of the pro-PO system is triggered by elicitors

derived from microbial cell wall components such as LPS, peptidoglycan and β-1,3-glucan (Ochiai and Ashida, 1998; Lee *et al.*, 2000). The active form of pro-PO, phenoloxidase (PO), is produced by serine proteinase(s) acting as pro-PO activating enzymes (Lee *et al.*, 1998b). Subsequently, PO oxidizes endogenous catechol, or catecholamine derivatives, to their quinone derivatives, which are converted to melanin through several non-enzymatic steps. The generated PO plays an important role as it can melanize pathogens (Richman and Kafatos, 1996), sclerotize the cuticle (Sugumaran, 1991) and play a part in the wound healing of invertebrates (Lai-Fook, 1966).

Recently, we purified three novel pro-phenoloxidase (pro-PO) activating factors (PPAF-I, PPAF-II and PPAF-III) and examined the pro-PO activation mechanism *in vitro* (Lee *et al.*, 1998b; Lee *et al.*, 1998c; Kwon *et al.*, 2000). The overall structure of PPAF-I is highly similar to that of the *Drosophila* ester serine protease, an essential serine protease zymogen for pattern formation in normal embryonic development. A masquerade-like serine proteinase homologue (PPAF-III) is necessary for showing PO activity (Kwon *et al.*, 2000). A dopamine-like substance was induced in the hemolymph within 24 h when *E. coli* was injected into *T. molitor* larvae (Kim *et al.*, 2000). We demonstrated that the active PO of *Tenebrio* larvae is engaged in cell clump/cell adhesion as a cellular defense reaction (Lee *et al.*, 1999). Furthermore, the active PO of *Tenebrio* larvae used a vitellogenin-like protein during melanin syntheses (Lee *et al.*, 2000).

One of the biological functions of activated PO in arthropods is the synthesis of melanin around invaded foreign materials. However, little is known about how activated PO synthesizes melanin or what kinds of proteins are involved in melanin syntheses. To discover what kinds of proteins are involved in the multiprotein complex formed by active PO, we isolated the phenoloxidase-interacting protein (POIP) by a yeast two-hybrid screening to characterize the specific binding regions and expression of POIP by bacterial injection into *T. molitor* larvae.

Materials and Methods

Yeast two-hybrid screen and analysis of PO-POIP interaction Two-hybrid screening and assays were performed as described previously (Kim *et al.*, 1995) using L40 yeast strain harboring HIS3 and β-galactosidase (β-gal) as reporter genes. Using a *Tenebrio* pro-PO cDNA sequence data (Lee *et al.*, 1999), the complete coding region of the *Tenebrio* pro-PO gene (bait 1) was amplified by PCR using the 5' *primer* GC**GGATCC**GCATG GCCAGCAAGAAGAAC (*BamH1* site in bold) and the 3' primer GC**GGATCC**TAATTACGTGATTTCGGCCT (*BamH1* site in bold). The N-terminal pro-segment (bait 2) of *Tenebrio* pro-PO corresponding to amino acid residues 1-50 was amplified by 5'-primer GC**GAATTC**ATGGCCAGCAAGAAGAACATC (*Eco RI* site in bold) and the 3' primer GC**GGATCC**TAGCGGTTGGTG AGGGCCAC (*BamH1* site in bold). The active *Tenebrio* PO (bait 3) corresponding to amino acid residues 51-684 was amplified by using the 5' primer GC**GGATCC**GCT TCGGCGAAGACGCCG (*BamH1* site in bold) and 3' primer GC**GGATCC**TAATTACGTG ATTTCGGCCT (*BamH1* site in bold). The resulting PCR products were, after digestion, cloned in a frame into the pBHA vector (LexA fusion vector, Clontech, Palo Alto, USA). The synthesized Eco RI adapter with attached dsDNAs from the purified mRNA of *T. molitor* larvae were used for construction of a yeast two-hybrid cDNA library in pGAD10 (GAL4 activation domain vector, Clontech). The 2×10^6 clones of the cDNA library were screened with each bait. Deletion mutants of the POIP domain were created by PCR using specific primers and subcloned in frame into pBHA to generate LexA fusion. The active PO variant was created by PCR using specific primers and subcloned into pGAD10 to generate a GAL4 activation domain fusion. Deletion constructs were also tested for interaction in the yeast two-hybrid assay by using HIS3 and β-gal as reporter genes. All DNA constructs were confirmed by DNA sequencing.

cDNA cloning of the full length POIP from the *Tenebrio* cDNA library As we obtained a partial cDNA clone of POIP by the yeast two-hybrid screening, we tried to screen the full length cDNA of POIP from a cDNA library prepared by using *E. coli* that was injected into *Tenebrio* larvae. A cDNA library of *T. molitor* larvae was constructed by a previously published method (Moon *et al.*, 1994). The 316 bp PCR fragment of the N-terminal unique region of POIP was labeled with [α-^{32}P]dCTP using a Ready-To-Go™ DNA-labeling kit (Pharmacia Biotech Inc., Uppsala, Sweden). After secondary screening from a *Tenebrio* cDNA library, the plasmids containing the cDNA insert were prepared from the positive plaques and their DNA sequences determined.

Northern Blot Analysis Total RNA was extracted by the guanidine-thiocyanate method (Chomezynski and Sacchi, 1987). Approximately 25 μg of total RNA taken at various times after *E. coli* injection into *T. molitor* larvae, was electrophoresed onto a 2.2 M formaldehyde gel and transferred to a nitrocellulose filter using the previously described method (Thomas, 1980), and hybridized with a radiolabeled DNA probe. The DNA used as a probe was the N-terminal unique region of POIP labeled with [α-^{32}P] dCTP using a random primer labeling kit (Takara, Tokyo, Japan).

Electrophoresis SDS-PAGE was performed according to Laemmli (Laemmli, 1970) with 3.75% stacking gel and 20% separating gel. Proteins were stained with Coomassie brilliant blue R-250. Samples for SDS-PAGE were prepared by heating at 75°C for 20 min in a medium (pH 6.8) containing 1.6% SDS, 4.2% 2-mercaptoethanol, 0.17 M Tris, 0.047 M HCl, 11.2% glycerol and 0.04% bromophenol blue.

Results

Isolation of a novel glycine-rich gene by interaction with PO Recently, we determined the cDNA sequence and biological functions of *Tenebrio* pro-PO and activated PO (Lee *et al.*, 1999). To identify PO-interacting proteins (POIP)

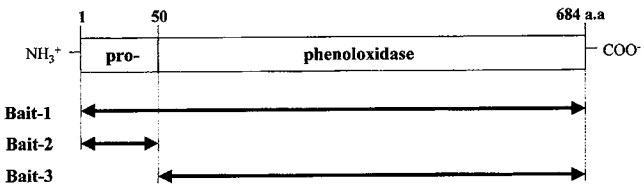

Fig. 1. Schematic representation of the constructed baits. The full-length pro-PO (residue 1-684, bait 1) or pro-segment of pro-PO (residue 1-50, bait 2) or active PO (residue 51-684, bait 3) are as depicted. The amino acid numbers for each construct are shown.

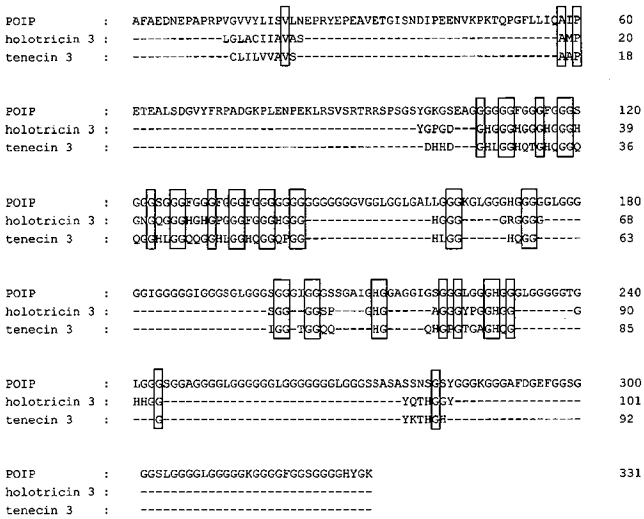

Fig. 2. Amino acid similarity between the C-terminal glycine rich-domain and insect antifungal proteins, tenecin 3 and holotricin ,3. Numbers refer to the predicted protein sequence. Gaps (-) are inserted to maximize the sequence alignment. Identical residues are *boxed.*

in the *Tenebrio* pro-PO activating system, we used the yeast two-hybrid screening method. Three independent screens of *Tenebrio* larval cDNA library were performed using, as bait, either full length pro-PO (residue 1-684, bait 1), a pro-segment of pro-PO (residue 1-50, bait 2), or an active PO (residue 51-684, bait 3) as shown in Fig. 1. The yeast strain L40 was cotransformed with the plasmid containing the bait DNA sequence and *Tenebrio* cDNA library created for use in the two-hybrid system. Yeast strain L40 contains two reporter genes whose transcription indicates an interaction between the two GAL4 fusion proteins. We first selected transformants for their ability to activate the HIS3 reporter gene by plating on the media lacking histidine. Histidine prototrophs were selected and subsequently tested for activation of the lacZ reporter gene. After screening 2×10^6 transformants, five independent cDNA clones from bait 3, specifically showing positive activity in a β-galactosidase assay, were obtained and fully sequenced in order to discover all of DNA sequences that were the same. However, we found no initiation methionine residue when we translated the five partial cDNAs that were obtained. A partial DNA sequence and amino acid

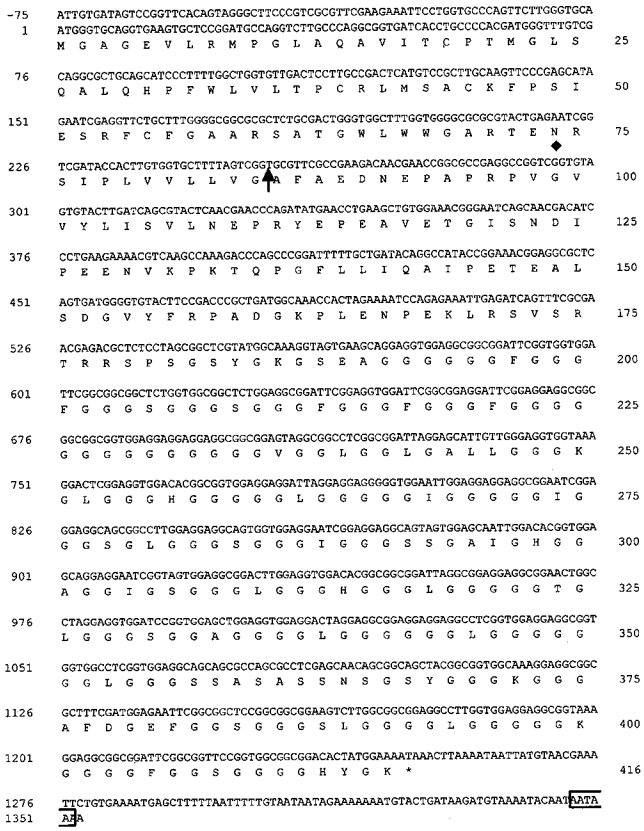

Fig. 3. Nucleotide and deduced amino acid sequence of the full-length cDNA encoding POIP. Numbers of nucleotides starting from the first base at the 5'-end are shown on the *left* of each line; the deduced amino acid sequence is numbered from the initiating Met residue on the *right* of each line. The *arrow* indicates the first amino acid residue of a partially obtained amino acid sequence by the yeast two-hybrid screening. The potential attachment sites for the *N*-linked carbohydrate chain indicated by *diamond.* A poly (A) additional signal is *boxed.*

sequence was determined (data not shown). This cDNA encoded a novel glycine-rich protein, termed POIP, consisted of two domains: The N-terminal unique region and C-terminal glycine-rich domain. The N-terminal unique region showed no homology to any known polypeptides on a Blast search of the GenBank database. The C-terminal glycine-rich domain showed homology with those of insect antifungal proteins as shown in Fig. 2.

To confirm the interaction between PO and the cloned glycine-rich protein POIP, we performed reverse screening using POIP as a bait and the *Tenebrio* cDNA library as a prey. When we determined DNA sequences of 14 positive clones, they all showed the same DNA sequences as *Tenebrio* PO (data not shown).

Determination of the full length POIP cDNA sequence

Using the 0.3 kbp POIP fragment obtained from the yeast two-hybrid system, the *Tenebrio* cDNA library was

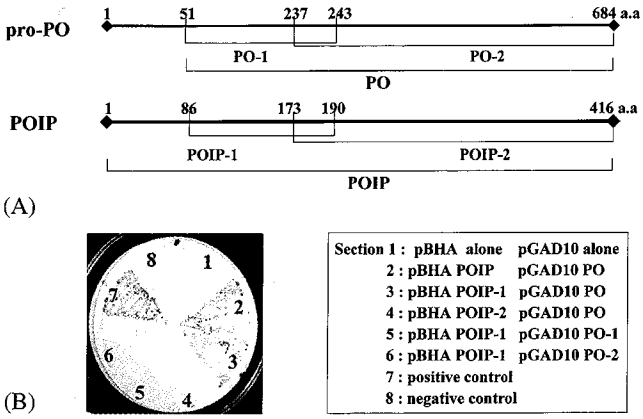

Fig. 4. Schematic representation of the deletion mutants for determination of the minimal binding region required for the interaction between pro-PO and POIP (A), and the results of interaction between baits and preys (B). (A), The full-length PO (residue 51-684) or PO-1 (residue 51-243) or PO-2 (residue 237-684) are cloned into the pGAD 10 vector as prey. The full-length POIP (residue 1-416) or POIP-1 (residue 86-190) or POIP-2 (residue 173-416) are cloned into the pBHA vector as bait. The amino acid numbers for each construct are shown. (B), The indicated baits in (A) and preys were transformed into a yeast strain containing HIS3 and β-gal as reported genes. The reproducible results were obtained using colonies from a separate transformation. The positive activity of β-gal activities were shown in section 2, 3 and 7. As a positive control, we used two kinds of vectors (pVA3-1 vector containing DNA binding domain and pTD1-1 vector containing activating domain) provided by the manufacturer. As a negative control, the pBHA vector containing POIP and the pGAD10 vector alone were used.

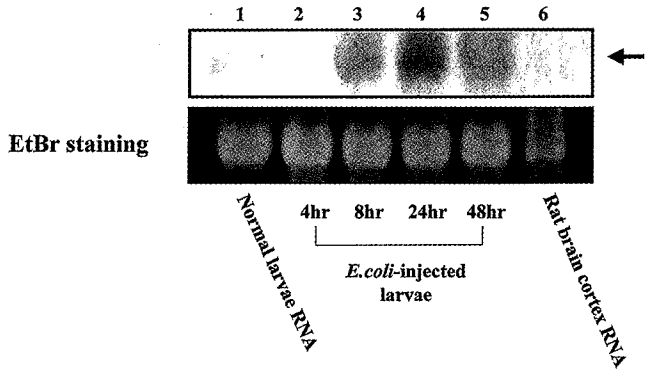

Fig. 5. Northern blot analysis of the POIP gene expression. Total RNAs extracted from native larvae and *E. coli* injected larvae at various times were subjected to Northern blot hybridization using the N-terminal unique region of POIP as a probe. The recovery and integrity of each RNA were assessed from the 18 S ribosomal RNA pattern. Each lane contained 25 μg total RNA. *Lanes*: 1, RNA from the naive larvae; 2, 4 h after *E. coli* injection; 3, 4, 5, and 6 show 8h, 24 h, 48 h after *E. coli* injection, and rat brain cortex RNA, respectively. The *arrow* indicates the position of the POIP mRNA.

constructed from *E. coli*-injected *Tenebrio* larvae. Also, three positive clones with a 1.4 kbp pair insert were obtained and their DNA sequences determined. The nucleotide and deduced amino acid sequences are shown in Fig. 3. The cDNA included 1430 nucleotides with an open reading frame of 1248 nucleotides. The open reading frame of the cDNA encoded for a mature protein of 416 amino acid residues with the calculated molecular weight of 42876. The candidate for an initiation codon ATG was found at nucleotide position 76. The stop codon at position 1249 was followed by a polyadenylation signal AATAAA, starting at position 1347. The amino acid sequence determined by the yeast two-hybrid screen started exactly from the position 86 (indicated by an arrow in Fig. 3), clearly indicating that the isolated cDNA clone corresponded to POIP. The deduced protein sequence contains one potential N-linked glycosylation site at Asn-74. A search of the N-terminal unique region on SWISS-PROT showed no sequence similarity of POIP to other known proteins. The C-terminal glycine region showed a sequence homology with insect antifungal proteins as described above.

Determination of the binding domain of POIP To determine the minimal sequence requirements for POIP interaction to PO, we constructed deletion mutants as shown in Fig 4A. PO-I and PO-2 mutants of pro-PO were constructed to contain the N-terminal region and the C-terminal region of PO, respectively. Also, POIP-1 and POIP-2 mutants contained the N-terminal unique region and the C-terminal glycine-rich domain of POIP, respectively. Deletions into the C-terminal glycine-rich domain of POIP or the N-terminal, C-terminal domain of PO resulted in the loss of interaction (Fig. 4B). This suggests that the entire PO domain is required for binding to POIP. However, the N-terminal unique region of POIP showed an interaction with the entire PO. The C-terminal glycine-rich domain of POIP was not required for PO binding.

Bacterial injection increased expression of 45 kDa mRNA in hemocyte lysate It is well known that invertebrate pro-PO systems are activated by microbial cell wall components such LPS, peptidoglycan, and β-1,3-glucan. As the N-terminal unique region of POIP is necessary for interaction with PO, possibly it may be induced by bacterial injection into *Tenebrio* larvae. To investigate the expression of the mRNA of POIP at various times after *E. coli* injection, we performed Northern blot analysis. As shown in Fig. 5, mRNA was undetected without an *E. coli* injection (lane 1), but mRNA was increased 8 h after the *E. coli* injection (lane 3) and sustained for 48 h (lane 5). These results suggest that the POIP might be an inducible protein by a bacterial injection. We recently reported that the expression of mRNA for pro-PO activating factor-III (45 kDa masquerade-like serine proteinase homologue) was also induced by bacterial injection (Kwon *et al.*, 2000). It has been consistently reported that the *Anopheles* serine proteinase homologue, ispl5, was induced by bacterial injection and malarial parasite infection (Dimopoulos *et al.*,

1997), suggesting that ispl5 might be engaged in an immune-related signaling process.

Discussion

Innate immunity plays an important role in protecting invertebrates from the attack of non-self foreign substances, such as fungi and bacteria. Non-self recognition proteins serve as a biosensor for detection of invading microbial pathogens in innate immune systems of vertebrate and invertebrate animals (Janeway, 1989). They also play a crucial role in regulating the adaptive immune reactions carried out by vertebrate lymphocytes (Medzhitov and Janeway, 1997a, 1997b). Pattern recognition molecules bind to certain pathogen-associated molecular patterns that are not found in the host, such as LPS or peptidoglycan from bacterial cell walls and β-1,3-glucan from fungal cell walls. Upon binding to the foreign invaders, pattern recognition proteins trigger defense pathways, such as the complement system in vertebrates (Medzhitov and Janeway, 1997a) and the pro-PO activation system in insects and other arthropods (Ashida and Brey, 1998). The pro-PO activation system, like the complement system, involves a protease cascade. Determining the molecular mechanism of the pattern recognition proteins that differentiate non-self from self is the key in understanding how to regulate the innate immune systems. Furthermore, it is essential to determine the identity of the pro-PO interacting protein (POIP) to expand our knowledge of the biological functions of the activated PO *in vivo*. Until now, several groups have carried out intensive studies on the pro-PO activation mechanism (Aspan *et al.*, 1995; Hall *et al.*, 1995; Kawabata *et al.*, 1995). However, the molecular mechanism of melanin synthesis induced by activated PO is not understood well.

In this study, we cloned POIP that interacted with PO by using the yeast two-hybrid screening method. The amino acid sequence of the cloned cDNA showed that POIP consisted of an N-terminal unique region and a C-terminal glycine-rich domain. The C-terminal region has a homology with insect antifungal proteins. However, the N-terminal unique region showed no homology with known proteins.

The biological function of this POIP is not yet known. Perhaps binding to some defense molecules is thought to induce some other defense reaction in *Tenebrio* larvae. Further study of this POIP in arthropods should lead to a better understanding of the function and evolution of innate immunity related proteins that can be induced by microbial cell wall components.

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