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Binding properties of the regulatory domains in *Manduca sexta* hemolymph proteinase-14, an initiation enzyme of the prophenoloxidase activation system

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ABSTRACT

Pathogen recognition and rapid initiation of defense responses are essential for the survival of host insects. In *Manduca sexta*, hemolymph proteinase-14 precursor (proHP14) senses non-self presence and triggers a branched serine proteinase pathway which leads to prophenoloxidase activation and melanin formation around the invading organisms. To understand functions of individual domains in HP14, we have produced a series of HP14 domains and truncation mutants and studied their interactions with microbial polysaccharides and β-1,3-glucan recognition protein-1 (βGRP1)—a biosensor for fungal and bacterial infection. These include: the low-density lipoprotein receptor class A repeats 1–5 (LDL_{1-5}), Sushi domain, Wonton domain, and proteinase catalytic domain of HP14, as well as proHP14 missing 1–4 LDL repeats (ΔLDL_1, ΔLDL_{12}, ΔLDL_{1-3} and ΔLDL_{1-4}). LDL_{1-5}, Sushi, and Wonton domains specifically recognized Lys-type PG, whereas the latter two also bound βGRP1. Wonton in addition bound to lipopolysaccharide (LPS), lipoteichoic acid (LTA), and *meso*-diaminopimelic acid (DAP)-type peptidoglycan (PG). The four N-terminally truncated proHP14 (ΔL_x) further confirmed specific interactions with LPS, LTA, DAP-PG, Lys-PG, laminarin, and βGRP1. These binding data suggest a broad specificity of proHP14 in pattern recognition. Its role in mediating immune responses is anticipated to be influenced by other plasma factors and surface structures of invading pathogens.

1. Introduction

Innate immunity is critically important for the survival and well being of insects in environments abundant in pathogenic microorganisms. This defense system is composed of factors that recognize/immobilize microbes, relay signals outside and inside immune tissues/cells, kill the invading organisms, and regulate immune mechanisms [1–4]. Some of the cellular and humoral mechanisms are mediated by an extracellular serine proteinase network which generates spätzle, phenoloxidase (PO), and plasmatocyte spreading peptide via limited proteolysis [5,6]. Among the network components discovered so far, *Manduca sexta* hemolymph proteinase-14 (HP14) and its orthologs in other insects are unique in three ways: (1) they contain 4–5 low-density lipoprotein receptor class A (LDLA or simply LDL) repeats, a Sushi domain, a Wonton domain, and a carboxyl-terminal serine proteinase domain (PD) (Fig. 1), (2) they directly or indirectly recognize pathogen surface molecules such as bacterial peptidoglycans (PGs) and fungal β-1,3-glucan, and (3) they autoactivate and trigger the serine proteinase network [7–10]. Interaction of recombinant *M. sexta* proHP14 with Lys-type PG resulted in its proteolytic processing, and supplementing hemolymph with the proHP14 greatly enhanced prophenoloxidase (proPO) activation in response to *M. luteus* [7]. ProHP14 purified from hemolymph of *M. sexta* larvae injected with bacteria was converted to a two-chain active form after incubation with β-1,3-glucan and *M. sexta* βGRP2 [8]. Such autoactivated HP14 greatly elevated PO activity in the larval plasma. HP14 activates proHP21 to HP21, HP21 converts proPAP2/3 to PAP2/3, and PAP2/3 generates active PO in the presence of a high M_r complex of clip-domain serine proteinase homolog-1 and -2 (SPH1 and SPH2) [11,12].

In order to investigate roles of the amino-terminal putative regulatory domains in *M. sexta* HP14, we produced different regions of the zymogen and studied interactions of the recombinant proteins with microbial cell wall components. In this paper, we report binding properties of individual domains or regions in proHP14 and their associations with *M. sexta* βGRP1 [13], a protein similar to βGRP2 which recognizes fungi and bacteria [14]. Implications of the broad binding spectrum of binding are also discussed.

2. Methods and materials

2.1. Construction of expression plasmids for producing proHP14 domain regions in Escherichia coli

Full-length cDNA for *M. sexta* HP14 was used as template for amplification of the four segments with the primer pairs listed in

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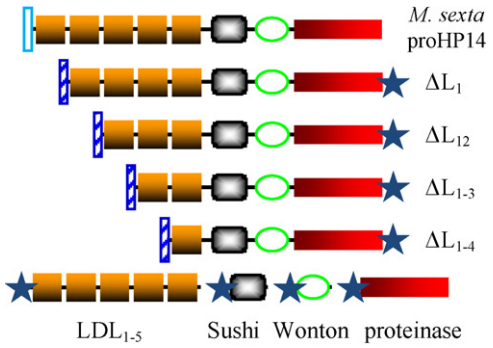

Fig. 1. Domain structure of *M. sexta* proHP14 (*top*), its deletion mutants (*middle*) and domain regions (*bottom*). The vertical bars (*open* and *hatched*) represent *M. sexta* proHP14 and honeybee melittin signal peptides, respectively. The hexahistidine tag is denoted by a star.

Table 1. The 25 μl reaction contained 2 ng template, 10 pmol of each primer, and 2.5 U Advantage cDNA polymerase mix (Clontech). The thermal cycling conditions were 35 cycles of 94 °C for 30 s; 50 °C for 30 s; 68 °C for 60 s, followed by 3 min of incubation at 68 °C. Following gel purification, the PCR products were cloned into pGEM-T (Promega) and the transformants were examined for correct restriction digestion patterns, insert sizes, and nucleotide sequences. The cDNA segments, retrieved by digestion with NcoI and BamHI/SphI, were inserted to the same sites of plasmid H6pQE60 [15] to generate plasmids LDL_{1-5}/H6pQE60, Sushi/H6pQE60, Wonton/H6pQE60, and PD/H6pQE60. The transformants were examined for induced expression of recombinant proteins at expected sizes by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblot analysis using 1:2000 diluted anti-HP14 serum as the first antibody.

2.2. Prokaryotic expression, purification, antibody raising, and renaturation of the four domain regions

The domain regions of *M. sexta* proHP14 were individually produced using *E. coli* JM109 harboring the recombinant plasmids according to Wang et al. [16]. The hexahistidine-tagged proteins from 500 ml of the cultures were purified on a nickel-nitrilotriacetic acid (NTA) agarose column under denaturing condition. The affinity-purified proteins were resolved by 10% SDS–PAGE, and the gel slices containing LDL_{1-5}, Sushi, Wonton, and PD (0.4 mg each) were used as antigens to generate four region-specific rabbit polyclonal antisera (Cocalico Biologicals Inc.). An aliquot of each protein was renatured by dialysis against 50 mM Tris–HCl (pH 8.0), 3 mM reduced glutathione, 1 mM oxidized glutathione, and 0.5 M arginine for 16 h at 4 °C and then 20 mM Tris–HCl (pH 7.5), 50 mM NaCl for 8 h at 4 °C, and then centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatants (10 μl) were analyzed by 15% SDS–PAGE and visualized by Coomassie Blue staining.

2.3. Preparation of expression constructs for producing N-terminally truncated proHP14 in baculovirus–insect cell system

For PCR amplification of the cDNA fragments, each 50 μl-reaction contained 5 ng full-length proHP14 cDNA, 20 pmol of each primer, and 5 U Advantage cDNA polymerase mix (Clontech). The thermal cycling conditions were 35 cycles of 94 °C for 20 s; 50 °C for 30 s; 68 °C for 90 s, followed by 10 min of incubation at 68 °C. The gel-purified PCR products were cloned into pGEM-T and plasmids from the resulting transformants were sequenced entirely to ensure error-free inserts. The cDNA segments, retrieved by digestion with EcoRI and XhoI, were inserted to the same sites of plasmid pMFH6 [17] to generate plasmids ΔL_1/pMFH6, ΔL_{12}/pMFH6, ΔL_{1-3}/pMFH6, and ΔL_{1-4}/pMFH6. The modified Bac-to-Bac vector allowed the recombinant proteins to be synthesized under the control of polyhedrin promoter, secreted into the medium using the honeybee mellitin signal peptide, and purified on a Ni-NTA column via the carboxyl-terminal hexahistidine tag.

2.4. Baculovirus generation, insect cell infection, and protein isolation

In vivo transposition of the expression cassette in ΔL_x/pMFH6, selection of bacterial colonies carrying recombinant bacmids, and isolation of bacmid DNA were performed according to the manufacturer's protocols (Invitrogen Life Technologies). The initial viral stocks were separately obtained by transfecting *Spodoptera frugiperda Sf*21 cells with a bacmid DNA–CellFECTIN mixture, and their titers were improved through serial infections [18]. The V_5 viral stocks, containing the highest levels of baculoviruses ($1–2 \times 10^8$), were stored at −70 °C for further experiments. *Sf*21 cells (at 2.4×10^6 cells/ml) in 1.0 L of Sf-900™ III serum-free medium (Invitrogen Life Technologies) were separately infected with the baculovirus stocks at a multiplicity of infection of 10 and grown at 27 °C for 84 h with gentle agitation (100 rpm). After the cells were removed by centrifugation at 5000 × *g* for 10 min, a 50 ml-aliquot of the supernatant was diluted with an equal volume of 1 mM benzamidine and gently mixed with 6.0 ml dextran sulfate (DS)–Sepharose CL-6B beads equilibrated in buffer A (0.01% Tween-20, 1 mM benzamidine, 10 mM potassium phosphate, pH 6.4) on ice for 1 h. The suspension was loaded into a column, washed with 30 ml buffer A, and eluted with a linear gradient of 0–1 M NaCl in

Table 1
Oligonucleotides for generating expression constructs of *M. sexta* proHP14 mutants.

Construct name	Primer name and sequence (5′ to 3′)	Vector/host cells	Product size (bp)
LDL_{1-5}	J494 (*forward*): CCCC*CATGG*CAGTGTTGAAAG J532 (*reverse*): ATG*AAGCTT*ACTTAACCGGTTG	H6pQE60/*E. coli*	729
Sushi	J533 (*forward*): CTGC*CATGG*ATACAAGCGTCCA J534 (*reverse*): GATA*AGCTT*AACGTGTACATTTCG	H6pQE60/*E. coli*	243
Wonton	J535 (*forward*): ACC*CATGG*GTACACGTTTCTGC J536 (*reverse*): CAA*AAGCTT*ACTCTGTGCCATTTG	H6pQE60/*E. coli*	272
PD	J537 (*forward*): CAG*CCATGG*TGCTTGGCGGGGA J538 (*reverse*): GCA*GCATGC*AGTAACCTGAATC	H6pQE60/*E. coli*	835
ΔL_1	J558 (*forward*): C*GAATTC*CTAATTGTCGGATTAGTC J503 (*reverse*): ACT*CTCGAG*ATACTCGTCGGTCCA	pMFH6/*Sf*21	1773
ΔL_{12}	J559 (*forward*): T*GAATTC*AGCGACAATGTCAGTAC J503 (*reverse*): ACT*CTCGAG*ATACTCGTCGGTCCA	pMFH6/*Sf*21	1653
ΔL_{1-3}	J560 (*forward*): T*GAATTC*ACGAGACCGAGGAGATC J503 (*reverse*): ACT*CTCGAG*ATACTCGTCGGTCCA	pMFH6/*Sf*21	1530
ΔL_{1-4}	J561 (*forward*): G*GAATTC*CCTGCCTGTCCTATTTG J503 (*reverse*): ACT*CTCGAG*ATACTCGTCGGTCCA	pMFH6/*Sf*21	1398