Noduler, A Novel Immune Up-Regulated Protein Mediates Nodulation Response in Insects[1]

Archana S. Gandhe, Serene H. John, and Javaregowda Nagaraju[2]

Insect immune system comprises of both humoral and cellular defenses. Nodulation is one of the major, yet very poorly understood cellular responses against microbial infections in insects. Through screening for novel immune genes from an Indian saturniid silkmoth *Antheraea mylitta*, we identified a protein up-regulated in hemolymph within minutes upon bacterial challenge. We have shown here, for first time, the involvement of this novel protein in mediating nodulation response against bacteria and hence designated it as Noduler. Noduler possessed a characteristic reeler domain found in several extracellular matrix vertebrate proteins. Noduler was shown in vitro to bind a wide range of bacteria, yeast, and also insect hemocytes. Furthermore, Noduler specifically bound LPS, lipotechoic acid, and β-1, 3 glucan components of microbial cell walls. RNA-interference mediated knock-down of the Noduler resulted in significant reduction in the number of nodules and consequent increase in bacterial load in larval hemolymph. The results suggest that the Noduler is widely conserved and is involved in very early clearance of bacteria by forming nodules of hemocytes and bacterial complexes in insects. The results would promote further studies for understanding of the crucial but hitherto overlooked nodulation mechanism in insects and also provide cues for the study of similar mammalian proteins whose function is not understood. ***The Journal of Immunology,*** **2007, 179: 6943–6951.**

Insect immunity has gained importance in the past decade as a model to study mammalian innate immunity due to several parallels between them (1). Insect immune system possesses only an innate immune arm that constitutes both humoral and cellular responses. Humoral response is characterized by a rapid activation of the phenoloxidase cascade (2) and synthesis of an array of antimicrobial proteins in the fat body within hours upon microbial challenge that are subsequently secreted in insect hemolymph (3–6). Cellular response, in contrast, is mediated by hemocytes and is an immediate response triggered within minutes of pathogen exposure (7). Cellular defense mechanisms constitute nodulation, encapsulation and phagocytosis.

Nodulation is quantitatively the most important defense mechanism against bacterial, fungal, and even viral infections in insects and other invertebrates (8, 9). Nodulation is mediated by hemocyte aggregate formation around bacteria and fungi (7). Encapsulation is similar to nodulation and refers to hemocyte aggregation around larger pathogens like parasitoids and nematodes. Although knowledge on antimicrobial peptide synthesis and phenoloxidase pathways is accumulating over the years, the cellular responses involved in phagocytosis and nodulation are relatively unknown. Only recently two transmembrane proteins namely Eater (10) and Nimrod (11) containing multiple epidermal growth factor-like repeats were identified on the surface of hemocytes in Drosophila. These proteins were shown to be major mediators of bacterial phagocytosis in Drosophila. However, no knowledge on players involved in nodulation is reported so far. Our initial analysis of *Antheraea mylitta* immune transcriptome identified a novel immune protein up-regulated in hemolymph upon bacterial infection referred to as defense protein 1 (12). In the present study, we assessed the functional role of defense protein 1 in immune response and named it as Noduler due to its involvement in nodule formation. Noduler was shown to bind several microorganisms and their ligands. Binding to microbial ligands is a criterion exhibited by insect pathogen recognition receptors that bind pathogen-associated molecular patterns (13, 14). In vivo RNA interference (RNAi)[3] mediated knock-down of Noduler especially affected the nodulation response thus suggesting a role for this protein in immunity. Similar proteins have been reported earlier from other lepidopterans but their role in immunity was not understood (15, 16).

Materials and Methods

Insects and bacteria

A. mylitta, fifth instar, day 3 larvae were procured from Regional Research Station (Warangal, India) and Central Tasar Research and Training Institute (Ranchi, India).

Isolation of Noduler cDNA sequence

A. mylitta Noduler cDNA sequence was identified from an *Escherichia coli* challenged fat body transcriptome (12). The protein sequence was derived from the cDNA sequence and signal peptide prediction was performed using SignalP program.

Multiple sequence alignment and phylogeny

Phylogenetic analysis was done by comparison of Noduler with similar protein sequences obtained by blastp (*e* value $\leq$1e-05). Reelin from *Homo sapiens* was used as an out-group. Only the reeler domains of the proteins

Centre of Excellence for Genetics and Genomics of Silkmoths, Laboratory of Molecular Genetics, Centre for DNA Fingerprinting and Diagnostics, ECIL Road, Nacharam, Hyderabad, India

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[2] Address correspondence and reprint requests to Dr. J. Nagaraju, Laboratory of Molecular Genetics, Centre for DNA Fingerprinting and Diagnostics, ECIL Road, Nacharam, Hyderabad, India. E-mail address: jnagaraju@cdfd.org.in

[3] Abbreviations used in this paper: RNAi, RNA interference; dsRNA, double stranded RNA; PTU, phenylthiourea; IBS, insect buffer saline; PGN, peptidoglycan; LTA, lipotechoic acid; GFP, green fluorescent protein.

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were used for the analysis. All the sequences were aligned using ClustalX 1.8 and manually edited using GeneDoc Version 2.6.002. A neighbor-joining tree was constructed using HYPHY and was used as a base tree to test the best-fit model of evolution in HYPHY. Akaike Information Criterion was used to select the best-fit model, which selected WAG model of amino acid substitution. A maximum likelihood tree was constructed with the program TREE-PUZZLE 5.2 that used 50,000 quartet-puzzling steps.

Recombinant expression of Noduler in insect cells

Noduler was expressed in Sf9 cells by constructing a recombinant baculovirus using the BAC-TO-BAC (Invitrogen Life Technologies) system. N-terminal 6-histidine tagged mature Noduler protein was expressed by amplification using the following primers: forward, 5′-CGGGATCC ATGCATCACCATCACCATCACTTCCCTACTGGTGCACCA-3′; reverse, 5′-CGCTCTAGATTAATGGTGACTTAAAATTTTTACGGGTG CCGAGGTTTG-3′. The PCR product was digested with *Bam*HI and *Xba*I and cloned into pFASTBAC vector (Invitrogen Life Technologies). The Noduler encoding recombinant baculoviruses were subsequently obtained using manufacturer's protocol (Invitrogen Life Technologies). Sf9 cells were harvested 72 h post infection (π) with recombinant baculovirus. The protein was purified under native conditions using BD TALON metal affinity resin (Clontech Laboratories) according to the manufacturer's instructions. The purified protein was analyzed by 15% reducing SDS-PAGE gels and Western blot with anti-histidine and Noduler-specific peptide Ab. Anti-Noduler Ab was generated in rabbits against an internal peptide sequence of Noduler - DKQTVSYVWTAPS DLEGDVVF. This peptide did not show similarity with any other protein in the NCBI database except Noduler and its homologues. The ability of this Ab to detect Noduler was confirmed against recombinant Noduler expressed in insect cells. However, the Noduler peptide exhibits 67 and 81% sequence identity with the corresponding peptides of Noduler homologue 1 and 2, respectively. Therefore, cross reactivity of anti-Noduler Ab against the two Noduler homologues in *A. mylitta* is possible.

Immunoblot analysis

Hemolymph was collected from *A. mylitta* fifth instar, day 3, larvae in prechilled polypropylene tubes containing a few crystals of phenylthiourea (PTU) at 30 min, 1, 4, 8, and 24 h post *E. coli* challenge. The hemocytes were removed by centrifugation at 2000 rpm and equal amount of plasma samples from each time point were subjected to Western blot. The blots were probed with a 1000-fold dilution of Noduler-specific rabbit primary Ab. Detection was done with goat anti-rabbit IgG (1/2000 dilution) conjugated to HRP and ECL detection reagent (Amersham).

Binding assays with microorganisms, microbial cell wall components, and insect cells

Binding assays with bacteria, yeast, peptidoglycan (PGN), and curdlan were conducted using a protocol by Kang et al. (17) with a few modifications. Curdlan, *Staphylococcus aureus* PGN, and *Micrococcus luteus* PGN were purchased from Sigma-Aldrich. *E. coli* PGN was a gift from Dr. Bruno Lemaitre (Global Health Institute, Lausanne, Switzerland). We further repurified this *E. coli* PGN as described by Glauner et al. (18). In brief, *E. coli* PGN was treated with trypsin (200 μg) at 37°C for 30 min to remove any contaminating lipoprotein followed by extraction with 8% boiling SDS for 30 min. The pellet obtained was washed several times with water and used for binding assays. For the binding assays, log phase bacterial culture (3 ml) was pelleted down and resuspended in 20 μl PBS. Also, for curdlan and PGN binding experiments, 1 mg of each of the components was used and resuspended in 20 μl PBS. 1 μg of recombinant Noduler protein was added to the substrates. After 30-min incubation, the reaction components were pelleted down, the pellet was washed four times with PBS and the supernatant and wash fractions were collected. The pellets were resuspended in 40 μl PBS, boiled for 5 min after addition of 2× SDS-PAGE loading buffer and analyzed by Western blot. Anti-histidine primary Ab (Qiagen) at 1/2000 dilution and anti-mouse IgG secondary Ab conjugated to HRP (Amersham) at 1/2000 dilution was used. The detection was done with ECL Plus detection reagents (Amersham).

Binding of Noduler to LPS and lipotechoic acid (LTA) was determined by a competition assay wherein Noduler binding to *E. coli* and *S. aureus* was competed by adding 10 μl (50 μg) of soluble LPS (*E. coli* 0111:B4; Sigma-Aldrich) and LTA (*S. aureus*; Sigma-Aldrich), respectively. To remove possible PGN contamination from *E. coli* LPS (Sigma-Aldrich), we treated it with lysozyme for 12 h at 25°C according to a protocol by Werner et al. (19). Activity of lysozyme was confirmed on pure PGN and also it was not inhibited in the presence of LPS (data not shown). Furthermore, LPS was reextracted with phenol by a method described by Hirschfeld et al. (20). The binding assay samples were assayed by Western blot as described earlier. The band intensity of Noduler in the supernatant fraction in the presence or absence of competition (LPS and LTA) was estimated with ImageQuant 5.2 software. Amount of Noduler bound to soluble LPS or LTA was obtained by subtracting the band intensity of the supernatant fraction in the absence of LPS or LTA from that obtained in their presence. Noduler binding to LPS and LTA was expressed as a percentage value of the amount of bound Noduler as compared with the total protein (bound plus unbound fraction). The data shown is an average of four independent experiments and the error bars indicate the SDs. As a negative control, a histidine-tagged bacterial Hsp70 protein was used in all the binding assays.

Preparation of double stranded RNA (dsRNA)

The dsRNA specific to Noduler was synthesized corresponding to entire protein coding region (507 bp) using primers: forward primer, 5′-ATGA TGTTCGCGTACATAGTAGCTG-3′; reverse primer, 5′-TTAATGGTGA CTTAAAATTTTTACGGGTG-3′ (Fig. 1*A*); and cloned into pCRII-TOPO vector (Invitrogen Life Technologies) followed by amplification with M13 forward and reverse primers. This template with flanking T7 and SP6 promoters was used for in vitro transcription reaction and sense and antisense RNA strands were generated with the T7 and SP6 Megascript kits as prescribed by the manufacturer (Ambion). The DNA template was removed from the transcripts by DNase treatment and the RNA products were subsequently purified by Trizol extraction (Invitrogen Life Technologies) followed by isopropanol precipitation. The complementary single stranded RNAs were dissolved in DEPC treated water, combined in equimolar amounts in 1× insect buffer saline (IBS; composition: NaCl - 160 mM, KCl - 10 mM, $CaCl_2$ - 4 mM) and annealed by heating to 95°C and slow cooling overnight at room temperature. Similarly, dsRNA specific to full-length green fluorescent protein (GFP) was synthesized as a nonspecific control. The dsRNA formation was confirmed by agarose gel electrophoresis and the concentration was determined spectrophotometrically.

RNAi mediated knock-down and bacteria clearance assay

The in vivo functional analysis of Noduler was conducted by dsRNA mediated Noduler knock-down in fifth instar larvae. The ability of Noduler knock-down larvae to clear both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria that were injected systemically was subsequently assayed. Noduler-dsRNA (100 μg) was injected into a set of larvae (n = 8). GFP-dsRNA (nonspecific-dsRNA) or saline injected larvae (n = 8) were maintained as control. Twenty four hours following dsRNA injection, control and test larvae were injected with ~10^7 cells of log phase *E. coli* and *S. aureus* bacteria, separately, resuspended in 1× IBS. The larval hemolymph was collected aseptically; 30 min postbacterial injection in PTU and 100 μl of the 10-fold diluted hemolymph samples were plated on LB agar plates. The colony forming units were enumerated after overnight incubation of the LB plates. The data presented is an average of values obtained from 8 larvae in each set. The statistical significance was determined using t test.

Nodule formation

Noduler-dsRNA or GFP-dsRNA or saline was injected to fifth instar, day 3 larvae followed by an *E. coli* MG1655 or *S. aureus* injection (~10^7 cells/larva) 6 h later. Eighteen hours following *E. coli* challenge, the larvae were dissected under a saturated solution of PTU prepared in 1× IBS and nodule formation was assessed as described by Eleftherianos et al. (21). The nodules were counted manually using a stereomicroscope. The data presented is an average of eight larvae in each group and statistical analysis was done using t test.

Noduler protein expression in nodule extract and tissues

Nodules and different tissues (fat body, mid gut, silk gland, and epidermis) were collected from *A. mylitta* larvae challenged with *E. coli* MG1655 at 18 h π. A total of 5 mg of the nodules and various tissues were washed with 1× IBS twice to remove hemolymph traces and then were crushed in 1× IBS. Protein estimation was done by Bradford's method and equal amount of protein was loaded on a SDS-PAGE gel followed by immunoblot analysis with Noduler-specific Ab. The band intensity was measured by densitometric analysis. As a loading control the blot was probed with α-tubulin Ab. Also, the presence of *E. coli* MG1655 in the nodules was confirmed by

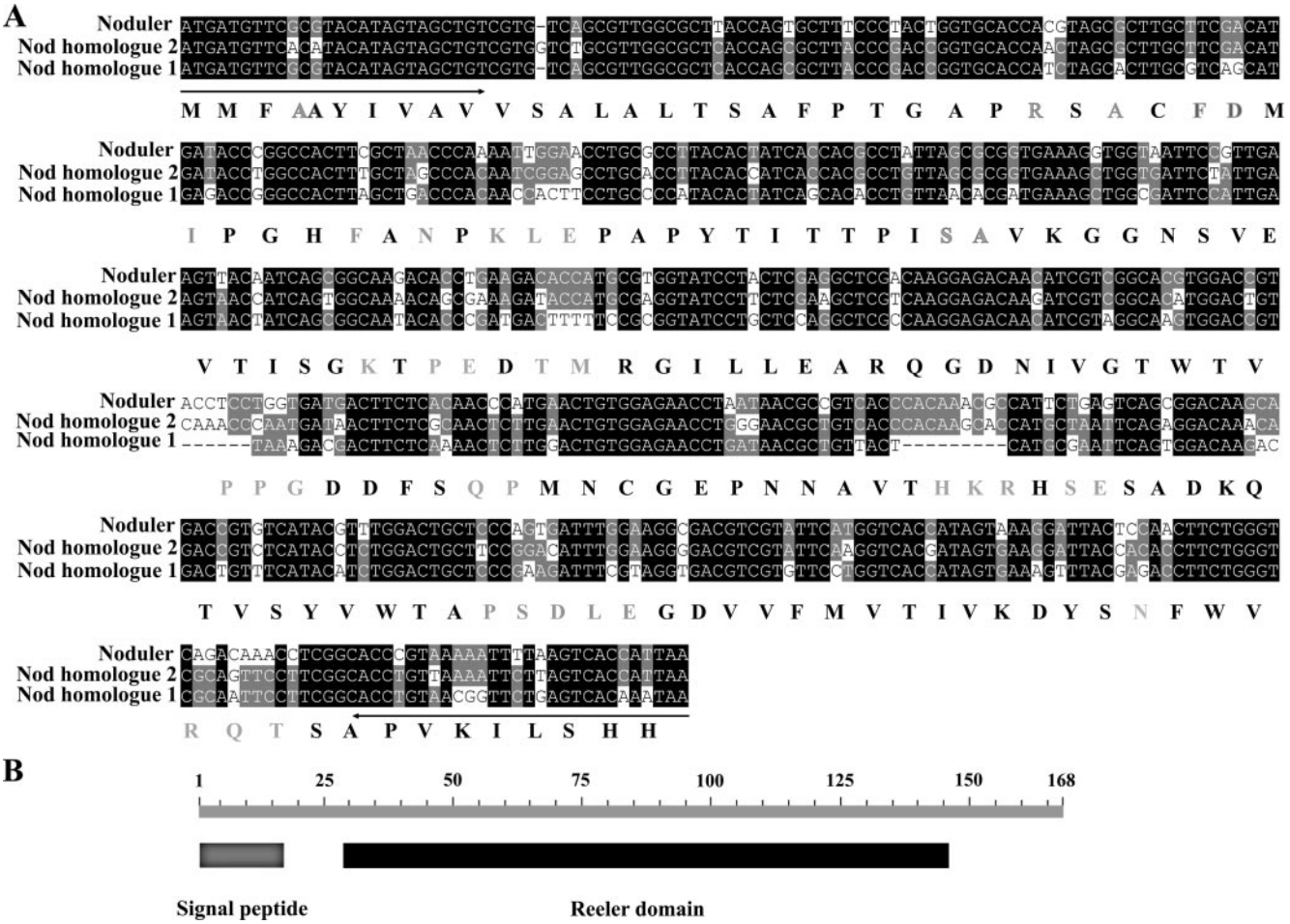

FIGURE 1. Noduler and its two homologues in *A. mylitta* (*A*) The cDNA sequence comparison of Noduler and its two homologues, Nod homologue 1 and Nod homologue 2. The forward and backward arrows indicate the regions used for designing forward and reverse primers, respectively for preparation of dsRNA for RNAi experiments. The full-length protein sequence of Noduler is shown below the cDNA sequence alignment. The amino acid residues completely conserved among all the three proteins are indicated in black color while the partially conserved or nonconserved residues are shown in gray color. *B*, Schematic representation of the organization of the Noduler from *A. mylitta*. The scale represents numbered amino acid residues.

streaking the nodule extract on LB agar plates followed by overnight incubation at 37°C. The bacterial colonies were confirmed to be *E. coli* MG1655 by morphological and biochemical analysis.

Results

A reeler domain spans a major part of Noduler protein

Noduler transcript (GenBank no. ABG72705) was recently identified as one of the most abundant ESTs in *A. mylitta* immune transcriptome and its immune up-regulation was confirmed by semiquantitative RT-PCR (12). Two more highly similar Noduler homologues, Nod homologue 1 (80% similarity at nucleotide and amino acid level) and Nod homologue 2 (83 and 85% similarity at nucleotide and amino acid level, respectively) were also identified in immune transcriptome of *A. mylitta* (Fig. 1*A*). Noduler is a 168 aa protein with a predicted secretory signal at the N-terminal end (Fig. 1*B*). A reeler domain spans almost entire length (aa residues 28–155) of the protein (Fig. 1*B*). Noduler homologues have been reported to be immune up-regulated in other lepidopterans (one homologue identified in each of *Samia cynthia ricini*, *Lonomia obliqua*, *Bombyx mori*, *Manduca sexta* and *Hyphantria cunea*) (15, 16, 22, 23). Among the other insects, three Noduler homologues were found in *Anopheles gambiae*, four in *Drosophila melanogaster*, two in *D. pseudoobscura*, three in *Aedes aegypti*, three in *Tribolium castaneum,* and one in *Apis mellifera* (24, 25). However, no functional analysis has been done to assign a role for these proteins in immunity. Noduler also showed similarity with many vertebrate extracellular matrix proteins that include stromal cell derived factor receptor 2, ferric chelate reductase, reelin and many predicted or putative protein sequences. The common feature of all these vertebrate proteins is the presence of an N-terminal reeler domain. The vertebrate proteins showing similarity to Noduler were much larger in size and possessed other domains in addition to reeler unlike insect homologues, which were of similar size with no additional protein domains. Recently, mindin, an ECM protein from mice, has been shown to function as a pattern recognition receptor (26). However, mindin possesses an N-terminal spondin domain unlike the reeler domain present in Noduler. A phylogenetic analysis of all the reeler domains from Noduler and its insect and vertebrate homologues was done (Fig. 2). All the lepidopteran sequences clustered together, whereas the remaining insect sequences formed three different groups. All the mammalian sequences grouped into a separate cluster. A mammalian protein of unknown function, stromal cell derived factor receptor 2, showed highest similarity with Noduler among all the vertebrate proteins. Thus, proteins similar to Noduler seem to be widespread in animal kingdom but their function remains unknown till date.

Noduler is up-regulated upon infection in hemolymph

Immunoblot analysis of bacteria challenged hemolymph samples at different time points π was done with anti-Noduler Ab (Fig. 3*A*).

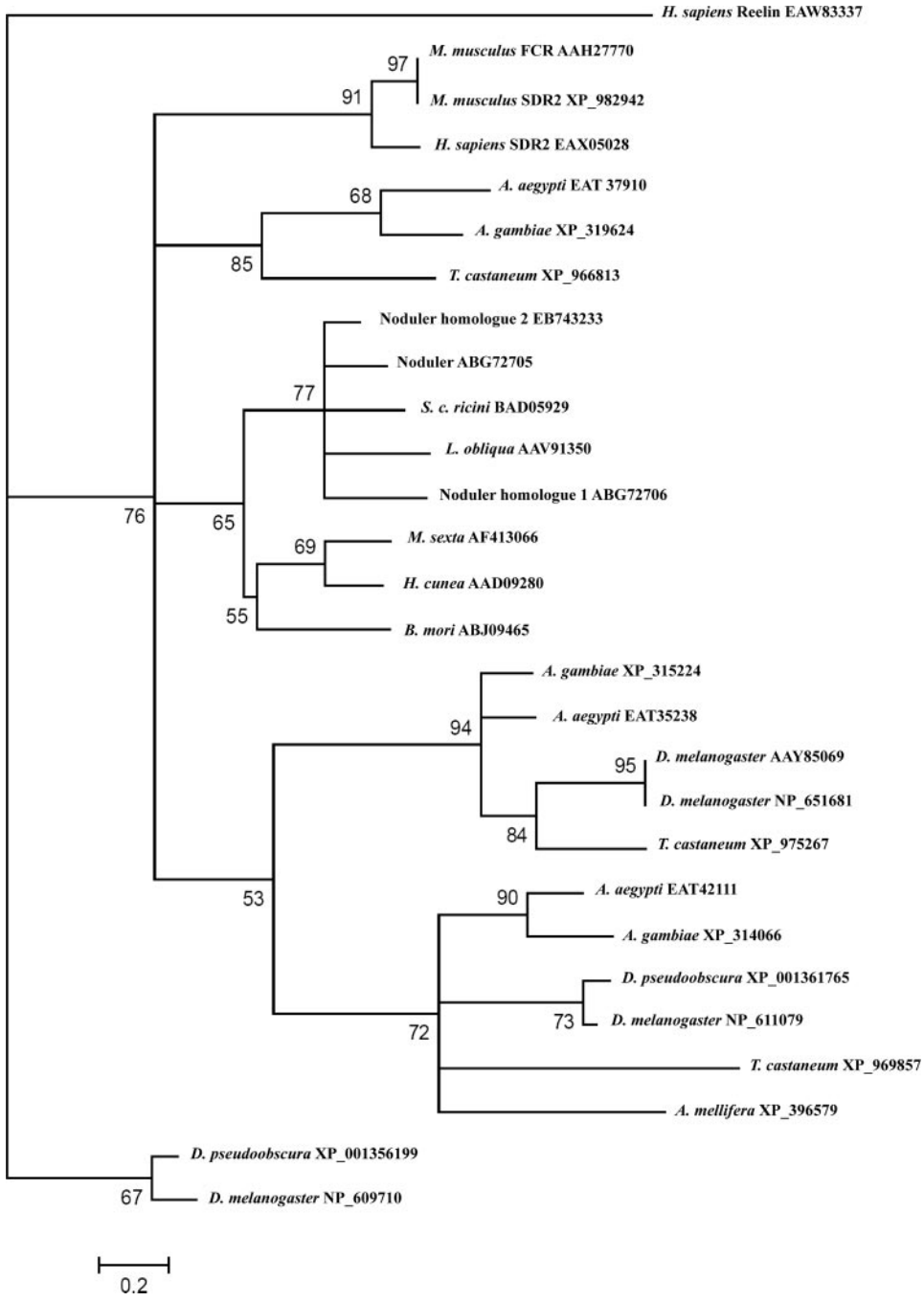

FIGURE 2. The phylogenetic tree of Noduler and its homologues. Phylogenetic comparison of Noduler with similar protein sequences obtained by blastp (*e* value ≤1e-05). Reelin from *Homo sapiens* was used as an outgroup. Only the reeler domains of the proteins were used for analysis. The bootstrap values are represented as a percentage at the branch nodes. The branch lengths are proportional to the amino acid substitutions. Scale, 0.2 amino acid substitutions/site. FCR, Ferric chelate reductase; SDR2, Stromal cell derived factor receptor 2.

Noduler was confirmed to be secreted into the hemolymph as predicted from the signal peptide analysis. Noduler was present constitutively in the insect hemolymph and was up-regulated several fold upon infection. Up-regulation of Noduler was detected as early as 30 min π and maximum expression was seen at around 4 h π. A loading control (Fig. 3*B*) of the immunoblot analysis samples indicates intensely stained protein bands around 85 kDa in size that are differentially expressed at different stages of infection. These bands most likely represent the storage proteins (MW ~85 kDa) that are accumulated in hemolymph of holometabolous insects like silkworm in the final instar larvae (27, 28). Thus, the increase in quantity seen in this protein band post infection is rather due to the advancement in developmental stages of the larvae. Other possible candidates present in this region could also be the stress proteins that are synthesized in larval hemolymph post infection (29).

Recombinant expression of Noduler in Sf9 cells

Noduler was expressed in Sf9 cells by baculovirus mediated expression system as a recombinant protein with an N-terminal 6-histidine tag (Fig. 4*A*). The recombinant Noduler was purified as a native protein by affinity chromatography. The homogeneity and specificity of the purified Noduler was confirmed by SDS-PAGE (Fig. 4*B*) and Western blot analysis (Fig. 4*C*). The observed m.w. of the recombinant Noduler was consistent with the expected one (19 kDa).

Recombinant Noduler binds to a wide range of microorganisms as well as hemocytes

Several antimicrobial proteins are secreted into hemolymph upon infection. However, Noduler was distinct in that it did not possess

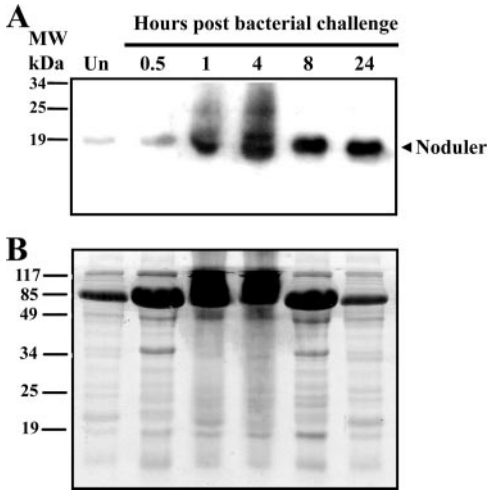

FIGURE 3. Immune up-regulation of Noduler in hemolymph. *A*, Immunoblot analysis of *E. coli* challenged *A. mylitta* hemolymph samples at different time points post infection using the Noduler-specific Ab. Un, Unchallenged. *B*, Coomassie stained gel of the above samples as a loading control for immunoblot analysis. Protein m.w. (MW) standards are indicated.

antibacterial activity as revealed by radial diffusion and growth inhibition assays (data not shown). To discern its possible role in bacterial recognition, we analyzed the binding affinity of Noduler to Gram-positive and Gram-negative bacteria. Noduler was found to bind Gram-positive (*Bacillus subtilis*, *Bacillus megaterium*, *M. luteus*, and *S. aureus*) and Gram-negative (*E. coli*, *Klebsiella pneumoniae*) bacteria tested (Fig. 5*A*). Noduler also bound strongly to yeast (*Saccharomyces cerevisiae*) suggesting that it binds a wide variety of microorganisms (Fig. 5*A*). We further identified the specific microbial cell wall components that bind to Noduler. Noduler was found to strongly bind β-1, 3 glucans of yeast (Fig. 5*B*) and LPS and LTA (Fig. 5*C*) cell wall components in Gram-negative and Gram-positive bacteria, respectively. However, Noduler did not bind to either Gram-positive (lysine-type) or Gram-negative

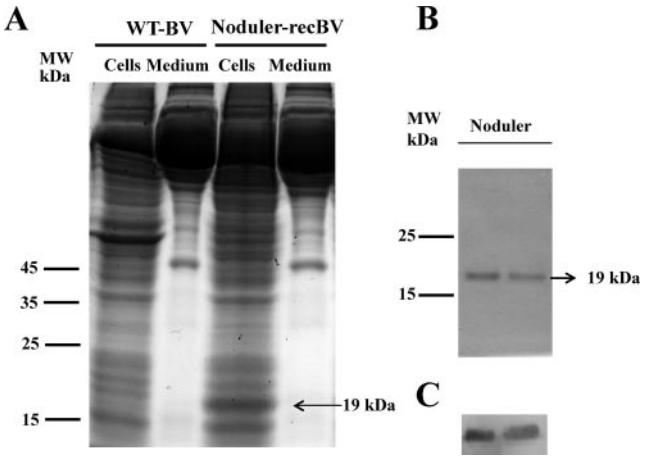

FIGURE 4. Expression and purification of recombinant Noduler expressed in Sf9 cells. *A*, Expression of Noduler in Sf9 cells as analyzed by SDS-PAGE. Recombinant Noduler expressed as a 19 kDa protein in Sf9 cells infected with recombinant baculoviruses (Noduler-recBV). No expression was seen in Sf9 cells incubated with wild type baculoviruses (WT-BV), which was used as control. *B*, SDS-PAGE analysis of recombinant Noduler purified by affinity chromatography. *C*, Western blot analysis of purified recombinant Noduler protein with Noduler-specific Ab.

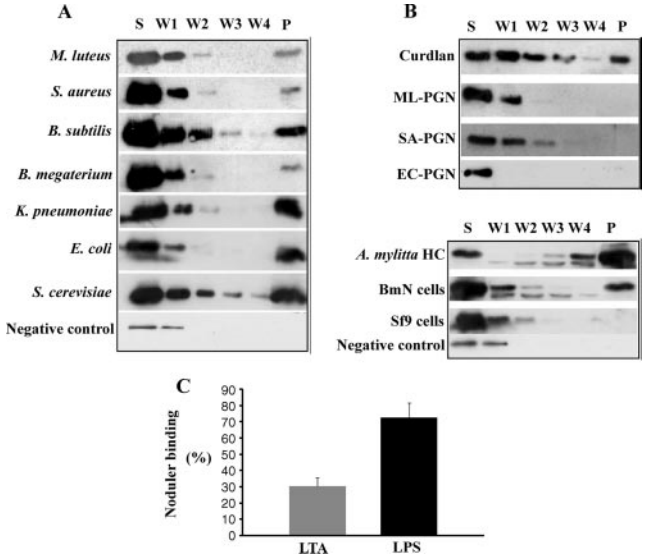

FIGURE 5. Binding assays. *A*, Binding of recombinant Noduler to Gram-positive bacteria (*Bacillus* sp., *M. luteus*, and S. *aureus*), Gram-negative bacteria (*E. coli*, *K. pneumoniae*), and yeast (*S. cerevesiae*) as analyzed by Western blot as mentioned in text. W1, W2, W3, and W4 are wash fractions 1, 2, 3, and 4, respectively; S, supernatant; P, pellet. As a negative control, prokaryotic Hsp70 protein was assayed in a similar manner and is indicated in the lowest panel. *B*, Binding assay of the recombinant Noduler with curdlan, PGN, and insect cells. ML-PGN, PGN from *M. luteus*; SA-PGN, PGN from *S. aureus;* EC-PGN, PGN from *E. coli*, curdlan was from *Alcaligenes faecalis*. *A. mylitta* HC, *A. mylitta* hemocytes. A histidine tagged Hsp70 protein was used as a negative control. *C*, LPS and LTA binding assay of Noduler. LPS (LPS from *E. coli* 0111:4) was purified to remove possible PGN contamination and Noduler binding was determined by a competition assay as described in the text. *y*-axis, The extent of Noduler binding to LPS or LTA as a percentage of the total amount of the Noduler. Error bars, SD of four independent experiments. LTA (LTA from *S. aureus*) binding assay of Noduler was conducted similar to LPS binding assay. A histidine tagged Hsp70 protein was used as a negative control.

(diaminopimelic acid-type) PGN (Fig. 5*B*) indicating its specificity in binding bacterial ligands. We also demonstrated binding of Noduler to *A. mylitta* hemocytes and a *B. mori* cell line (Fig. 5*B*). However, Noduler did not bind to Sf9 cell line derived from *Spodoptera frugiperda*. The prokaryotic heat shock protein used as a negative control in all the binding assays did not bind to any of the microbial ligands or insect cells.

In vivo knock-down of Noduler by RNAi results in increased bacterial load

The in vivo role of Noduler in immunity was assessed by this experiment. Noduler protein was knocked down in *A. mylitta* fifth instar, day 3 larvae by injection of Noduler-dsRNA followed by Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria independently. Hemolymph bacterial load was estimated 30 min π. Around 10-fold higher staphylococcal load (p value <0.001) was observed in hemolymph of Noduler knock-down larvae in comparison with the control (Fig. 6*A*). *E. coli* load in hemolymph was found to be 4- to 5-fold higher (p value <0.001) in Noduler knock-down larvae (Fig. 6*B*) as compared with control larvae (saline injected or GFP-dsRNA injected). These results established the in vivo immune related function of Noduler protein and suggested its involvement in early immune mechanisms activated as early as 30 min π. Western blot analysis of hemolymph from Noduler-dsRNA injected larvae showed a complete depletion of

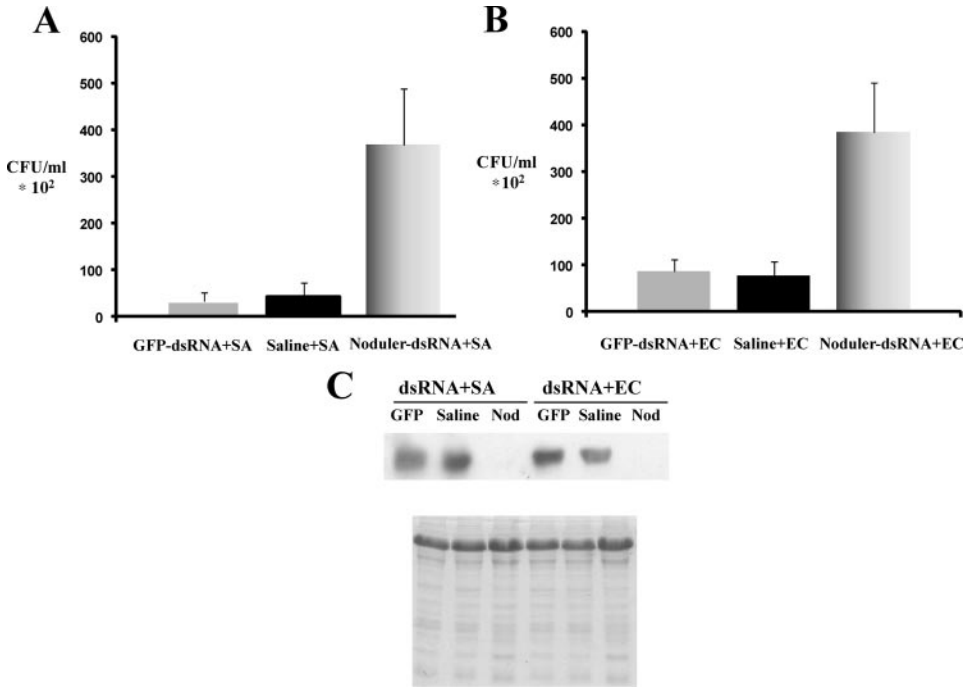

FIGURE 6. Bacteria clearance assay. RNAi mediated Noduler knock-down larvae exhibited decreased clearance of *S. aureus* (*A*) and *E. coli* (*B*) injected into fifth instar larval hemocoel compared with GFP-dsRNA or saline injected larvae. *y*-axis shows the average of colony forming U/ml hemolymph ($n = 8$) and error bars indicate SD of three independent experiments. The statistical significance was estimated by *t* test and the *p* value was <0.001. SA, *S. aureus*; EC, *E. coli*. *C*, Molecular analysis of RNAi mediated knock-down of Noduler. Western analysis of hemolymph samples from Noduler-dsRNA injected larvae showed complete knock-down of Noduler protein as compared with GFP-dsRNA injected or saline injected larvae. Lower panel shows the Coomassie stained SDS-PAGE gel of hemolymph samples used for Western blot as a loading control. Nod, Noduler.

Noduler as compared with GFP-dsRNA injected or saline-injected larvae (Fig. 6*C*) indicating that dsRNA mediated knock-down of Noduler can be achieved successfully in silkmoths. To our knowledge, other than phenoloxidase, so far no other molecules related to immune response is identified to be actively involved at very early stage of bacterial infection.

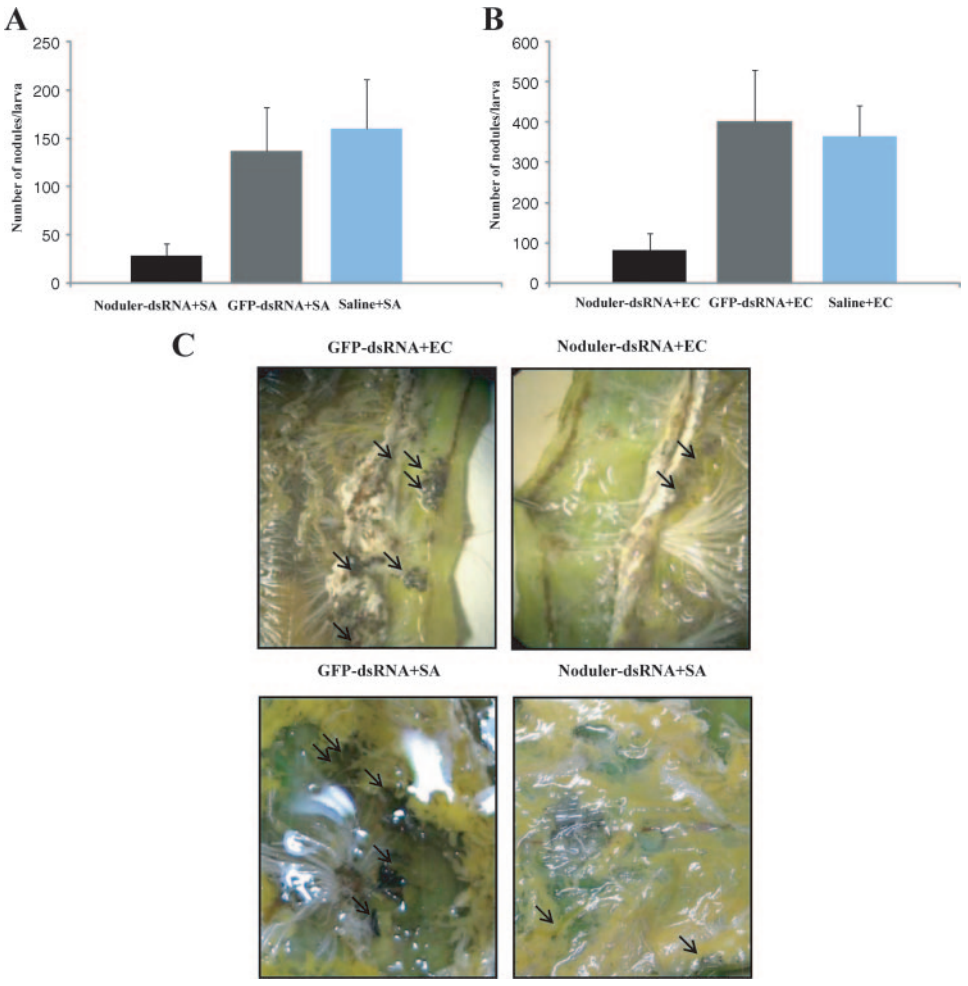

FIGURE 7. Effect of RNAi mediated Noduler depletion on nodulation response. Nodulation was reduced significantly ($p < 0.001$) in larvae injected with Noduler-dsRNA before *S. aureus* (*A*) or *E. coli* (*B*) injection as compared with GFP-dsRNA or saline injected larvae before bacterial injection. The average number of nodules per larva ($n = 8$) in each group is plotted on *y*-axis and the error bars represent SD of three independent experiments. *C*, Formation of melanised nodules in Noduler knock-down (Noduler-dsRNA + *S. aureus*/*E. coli*) and control larvae (GFP-dsRNA + *S. aureus*/*E. coli*). The black arrows indicate the nodules. SA, *S. aureus*; EC, *E. coli*.

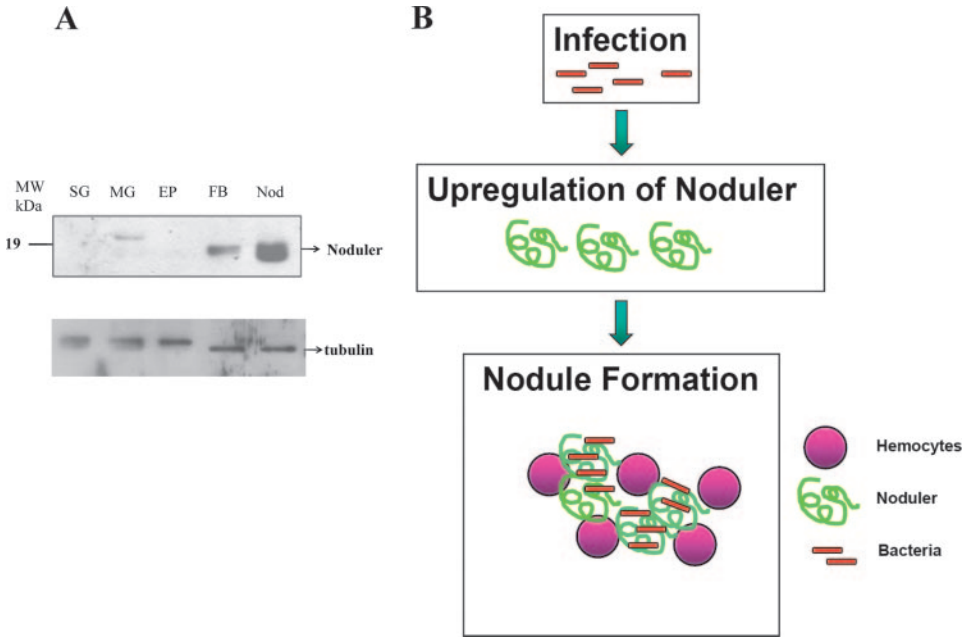

FIGURE 8. Noduler protein is concentrated in nodules. *A*, Immunoblot analysis of nodule and other tissue extracts with Noduler-specific Ab. Nodules and various tissues were collected 18 h post *E. coli* injection and the extracts were prepared as indicated in text. Equal amount of total protein (21 μg) from each tissue was loaded. The loading control (α-tubulin) is shown in lower panel. SG, Silk gland; MG, mid gut; EP, epidermis; FB, fat body; NOD, nodules. *B*, The suggested role for Noduler in the formation of nodules.

Noduler is involved in nodulation responses against bacteria

Furthermore, we analyzed the effect of Noduler knock-down on immediate early immune mechanisms of phagocytosis, phenoloxidase cascade, and nodulation. Noduler knock-down larvae showed a drastically reduced number of nodules as compared with the control larvae (p value <0.001). A 3- to 5-fold reduction in the number of nodules per insect upon infection with *S. aureus* (Fig. 7, *A* and *C*) or *E. coli* (Fig. 7, *B* and *C*) was observed in the Noduler depleted larvae as compared with saline injected or GFP-dsRNA injected larvae. We also analyzed the role of Noduler-depletion on the phenoloxidase pathway as it is involved in the nodulation phenomenon mediating melanisation of nodules. Phenoloxidase activity was found to be marginally lower in Noduler knock-down larvae as compared with control however the difference was not significant (data not shown). Noduler knock-down larvae did not show any difference in phagocytosis activity as compared with the control larvae ruling out its involvement in phagocytosis (data not shown).

Noduler protein promotes the assembly of bacteria and hemocytes to form nodules

To ascertain that Noduler is indeed involved in the formation of nodules, Noduler protein in nodules was analyzed by SDS-PAGE of nodule extract along with other tissues (silk gland, mid gut, epidermis, and fat body) from bacteria challenged larvae and probed with Noduler-specific Ab (Fig. 8*A*). Noduler was indeed found to be concentrated in the nodules and was at 4-fold higher concentration as compared with fat body, which is a major organ of immune peptide synthesis (Fig. 8*A*). Among the other tissues analyzed, Noduler was not detected in silk gland or epidermis though a faint band of slightly higher m.w. was observed in mid gut, which could be the pro-Noduler with intact signal peptide. The presence of the injected bacterial strain (*E. coli* MG1655) was also confirmed in the nodules by streaking the nodule extract on a LB plate. The bacterial colonies obtained on the LB plate after overnight incubation were confirmed to be *E. coli* MG1655 by gram staining, morphology, and biochemical methods (data not shown).

Noduler probably traps bacteria and hemocytes during nodulation

Our experimental data suggest that Noduler protein is a facilitator of nodulation. We propose that Noduler initiates nodulation by binding both microorganisms as well as host hemocytes. The enhanced concentration of Noduler in the nodules, sequestration of bacteria in nodules, binding of Noduler to bacteria and hemocytes and significant reduction in number of nodules upon Noduler knock-down all of them suggest that Noduler is a key molecule in nodulation. Its co-occurrence in nodules along with hemocytes and bacteria further strengthens this proposal. A schematic representation of the proposed mechanism of Noduler action is shown in Fig. 8*B*.

Discussion

Nodulation is a predominant cellular defense mechanism in insects and other invertebrates against diverse pathogens (30, 31). However, it is very poorly understood in comparison with other immune responses. The only information available so far is from eicosanoids that are shown to mediate nodulation in many insect species (8, 32, 33). Role of eicosanoids is well documented in mammalian immunity and they are shown to have profound effects on macrophage locomotion, phagocytosis and cell shape changes (8) and probably exert similar effects on insect hemocytes. However, eicosanoids are signaling molecules not specific to nodulation alone but mediate other cellular and humoral responses in insects (34). In the present study, we have identified a key player that distinctively mediates nodulation responses in insects.

Hemocyte aggregation, the first step in nodulation, results in entrapment of the invading microorganisms (7). Such an event requires recognition molecules that interact with both hemocytes as well as invading microorganisms. We hypothesize that Noduler mediates nodulation by virtue of its binding property to bacteria as well as hemocytes. Our hypothesis stems from the following experimental observations: Up-regulation of Noduler within minutes of bacterial infection, reduction in number of nodules upon Noduler silencing and subsequent increase in bacterial load, binding of

Noduler to bacteria, yeast and insect hemocytes, and the co-occurrence of bacteria, Noduler and hemocytes in the nodules. However, we did not observe hemocyte aggregation under in vitro conditions upon addition of exogenous Noduler and bacteria to hemocytes (data not shown) suggesting that additional players are probably involved in this mechanism. Also, because nodules are generally encountered adhering to the tissues, the phenomenon probably requires an in vivo milieu in the form of a tissue support. Nodules generally appear dark in color due to phenoloxidase activity at site of nodule formation (7). We did find a slight effect of Noduler silencing on the overall phenoloxidase activity in the hemolymph, which needs to be confirmed in future. As shown in our study, Noduler did not affect phagocytosis and thus seems to be specific in mediating only the nodulation response of cellular immunity although its role in phenoloxidase cascade cannot be ruled out. The specificity of Noduler action can probably be explained in the light of three types of hemocytes identified in Drosophila namely, plasmatocytes, lamellocytes, and crystal cells that mediate phagocytosis, nodulation, and phenoloxidase responses, respectively (10). It remains to be investigated whether Noduler specifically binds to a particular hemocyte subtype to bring about nodulation. Immune response of encapsulation, which refers to aggregation of hemocytes around large size invaders like parasites, is in principle similar to nodulation (7). Thus, it is quite possible that Noduler is involved in encapsulation reactions and thus merits further study.

Two additional homologues of Noduler were also identified in *A. mylitta*, which is consistent with previous observations of gene expansion of immune gene families in insects, potentially enabling diversified pathogen defense (35, 36). Both the Noduler homologues are highly similar to Noduler suggesting that they are involved in a similar function. Furthermore, high level of sequence similarity at both nucleotide and amino acid level makes the detection of effect of knock-down of each of the three Noduler family proteins in *A. mylitta* very difficult at molecular level. Thus it seems most likely that the RNAi mediated phenotype of Noduler in the present study is a result of knock down of all the three Noduler family proteins. Noduler-like proteins have been identified to be immune up-regulated in other insects but their function was unknown (15). Kanost et al. observed that the Noduler-like protein from locust copurified with apolipohorin-III, a plasma protein identical in size and charge (16). He further adds that the immune role attributed to apolipophorin-III (37) might be due to the copurified Noduler-like proteins. Our experimental results have indeed shown Noduler to have an immune function and hence the immune role of apolipophorin-III needs to be revisited. In the present study, Noduler was purified from a recombinant expression system thus ruling out the possibility of a contaminating hemolymph protein mediating the effects. Also, we have shown the functional role of Noduler by in vivo RNAi based experiments, which further confirms its role in nodulation.

A detailed understanding of insect immune system assumes importance for designing novel disease control strategies against insect-vector borne diseases. Toward this goal, several large-scale genome and transcriptome projects have been initiated in insects in the past decade (36, 38). Noduler was found to be evolutionarily conserved among insects and it would be worthwhile to analyze its role in other insects. Several parallels have been observed between insect and mammalian innate immune pathways recently. However, nodulation like response is not reminiscent of any of the vertebrate innate immune mechanisms. Nevertheless, several mammalian extra cellular matrix proteins of unknown function show resemblance with Noduler. Thus, it will be interesting to explore their role in vertebrate immune pathways in view of the results presented here.

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Disclosures

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