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A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response

Brad Davidson[1,2] and Billie J. Swalla[1,2,*]

[1]Box 351800, Zoology Department and Center for Developmental Biology, University of Washington, Seattle, WA 98195-1800, USA
[2]Friday Harbor Laboratories, 620 University Road, University of Washington, Friday Harbor, WA 98250-9299, USA
*Author for correspondence (e-mail: bjswalla@u.washington.edu)

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SUMMARY

Ascidian metamorphosis represents a powerful model for comparative work on chordate development that has remained largely unexplored. We isolated transcripts differentially expressed during metamorphosis in the ascidian *Boltenia villosa* by suppressive PCR subtractions of staged larval and juvenile cDNAs. We employed a series of three subtractions to dissect gene expression during metamorphosis. We have isolated 132 different protein coding sequences, and 65 of these transcripts show significant matches to GenBank proteins. Some of these genes have putative functions relevant to key metamorphic events including the differentiation of smooth muscle, blood cells, heart tissue and adult nervous system from larval rudiments. In addition, a significant fraction of the differentially expressed transcripts match identified genes from the innate immune system. Innate immunity confers a rapid response to pathogen-specific molecules and/or compromised self-tissues. The activation of innate immunity genes during metamorphosis may represent the programmed maturation of the adult immune system. In addition, this immune response may be necessary for phagocytosis and re-structuring of larval tissues. An innate immune-related inflammatory response may also underlie two waves of trans-epidermal blood cell migration that occur during the swimming larval period and immediately upon settlement. We characterized these trans-epidermal migrations and discovered that some migratory cells leave the animal entirely through an anterior tunnel in the tunic. We show that these cells are positioned to detect external settlement cues and hypothesize that the innate immune system may also be employed to detect and rapidly respond to environmental settlement cues.

Key words: Ascidian, Metamorphosis, Innate immunity, Subtractive hybridization

INTRODUCTION

Solitary ascidians have a long legacy as model organisms for investigating developmental mechanisms because of the simplicity of their embryogenesis and their basal phylogenetic position within the chordates (Berrill, 1929; Cameron et al., 2000; Conklin, 1905; Jeffery and Swalla, 1997; Kowalevsky, 1866; Satoh, 1994). The chordate traits that unite ascidians with the cephalochordates and vertebrates include the larval notochord and dorsal nerve cord (Fig. 1A), as well as the adult pharyngeal gill slits and endostyle (Fig. 1B) (Jeffery and Swalla, 1997). Recent investigations of ascidian development have focused almost exclusively on embryogenesis (Di Gregorio et al., 2001; Hotta et al., 2000; Jeffery and Swalla, 1997; Nishida and Sawada, 2001; Satoh, 1994). However, the majority of ascidian development, including the differentiation of the adult endoderm, body wall musculature, heart, blood cells and nervous system takes place during metamorphosis (Cloney, 1978; Hinman et al., 2000, Jeffery and Swalla, 1997; Satoh, 1994).

The metamorphosis of solitary ascidian tadpole larvae into sessile filter-feeding juveniles can be divided into two phases: a rapid settlement reaction followed by a prolonged period of juvenile differentiation. The initial settlement phase of ascidian metamorphosis has been thoroughly characterized by Cloney (Cloney, 1978; Cloney, 1982). After a rapid embryogenesis lasting 8-48 hours, solitary ascidian larvae hatch and swim within the plankton for a variable period of a few hours to five days. Solitary ascidian larvae have a well-developed tail including the notochord, dorsal neural tube as well as a larval CNS including an otolith and ocellus (Jeffery and Swalla, 1997) (Fig. 1A). However, they possess only rudimentary adult structures, including a single-layered endothelial rudiment of the gut and a pocket of trunk mesenchyme that will form the adult mesoderm (Hirano and Nishida, 1997) (Fig. 1B).

Initially, hatched larvae are unable to respond to either natural or artificial settlement cues. Within a discrete, species-specific number of hours after hatching, larvae become competent to respond to settlement cues and commence a series of rapid morphological changes (Degnan et al., 1997). Within the first 20 minutes of settlement, the papillae facilitate initial attachment, the tail is rapidly resorbed and the trunk

mesenchyme undergoes extensive migration both within the body and across the epidermis into the tunic (Fig. 1C-G). During the next few hours, the newly settled juvenile molts the outer larval tunic, extends epidermal ampullae, rotates the viscera to the adult feeding position, and begins to resorb the cerebral vesicle (Cloney, 1978) (summarized in Fig. 1H,I). The rudiments of adult structures including the heart, blood cells, musculature, gut and chordate pharyngeal slits then differentiate over a period ranging from days to weeks within the settled juvenile (Fig. 1B) (Hinman et al., 2000; Jeffery and Swalla, 1997).

Competent ascidian larvae can be induced to undergo settlement by a wide variety of both natural and artificial settlement cues (Cloney, 1990; Degnan et al., 1997). These include a variety of stressful stimuli such as trauma, crowding and osmotic changes. We have recently demonstrated that *Boltenia* larvae also settle in response to specific bacterial cues (B. D., B. J. S. and A. Aderem, unpublished). In addition, research has indicated that nitric oxide and Hsp-90 signaling may be involved in the initial settlement response (Bishop et al., 2001; Jackson and Swalla, 2001). Work in the Degnan lab on *Herdmania curvata* has established that settlement is triggered by a signal secreted at the anterior papillary region of the larvae (Degnan et al., 1997). They have further demonstrated a crucial role for EGF signaling in triggering settlement (Eri et al., 1999). The EGF-like molecule Hemps is secreted at the initiation of settlement by a group of anterior cells termed the papillae associated tissue (PAT) cells (Eri et al., 1999). They demonstrated that application of an antibody against Hemps specifically blocks settlement, whereas exposure of competent larvae to Hemps protein induces settlement (Eri et al., 1999). We have shown that a *Boltenia cornichon* homolog expressed in this anterior region during competency may potentiate the Hemps EGF signaling pathway (Davidson and Swalla, 2001). In this report, we demonstrate that these PAT cells are actually connected to the external environment by a tunnel through the anterior tunic and that they migrate out through this tunnel during metamorphosis (Davidson et al., 2001). Thus the PAT cells are positioned to respond to external signals and trigger settlement through the secretion of Hemps.

We employed suppressive subtractive hybridization in order to examine gene expression during metamorphosis in the solitary ascidian, *Boltenia villosa*. We conducted three screens focused on differential gene expression during: (1) the acquisition of larval competence, (2) the first hour of settlement and (3) the first two days of juvenile differentiation. These screens have led to the isolation of several transcripts whose putative proteins have been identified through matches in GenBank. These identified transcripts include genes with potentially interesting roles in the post-larval differentiation of adult rudiments (Nakayama et al., 2001) (B. D., S. Smith and B. J. S., unpublished). Intriguingly, a significant proportion of the identifiable transcripts putatively code for proteins involved in invertebrate innate immunity, including Mannose-specific lectin (MBL), MBL-associated serine protease (MASP), Hemocytin, four complement factors, two selectins, two von Willibrand factors, and Pentraxin.

Although it is established that ascidians are capable of innate immune-related reactions such as inflammation and cytotoxicity, it is only recently that the molecules involved in the ascidian innate immune system have been identified. It is now clear that ascidians possess a mature complement system, including both the lectin and alternative pathways (Nonaka, 2000). Research also indicates that ascidian immune responses include the use of an IL1-like cytokine that can activate and guide blood cells (Raftos et al., 1998). In addition, a variety of lectins have been identified in ascidians with putative roles in pathogen recognition (Abe et al., 1999; Kenjo et al., 2001; Matsumoto et al., 2001; Nair et al., 2001).

Our isolation and characterization of several innate-immunity-related transcripts expressed during *Boltenia* metamorphosis includes several novel ascidian immune-related genes but, more importantly, it is the first description of the developmental expression of innate immune-related genes in an ascidian. Our results detail the temporally discrete upregulation of innate immune-related genes during *B. villosa* post-larval development and metamorphosis. Such discrete upregulation suggests that innate immunity may have an unforeseen developmental role during ascidian metamorphosis. Innate immune-related genes have described roles in vertebrate developmental processes, particularly related to restructuring of differentiated tissues. For example, it has been shown that the complement component C3 is expressed during regeneration in both the axolotol limb (Del Rio-Tsonis et al., 1998) and during liver regeneration (Mastellos et al., 2001).

Here, we survey the initial results of our three subtractive hybridization screens. We then characterize the expression of several of the immune-related transcripts and discuss our hypotheses about the possible roles for innate immunity in ascidian metamorphosis. Characterization and discussion of other isolated transcripts with putative developmental roles will be published separately (B. D., S. Smith and B. J. S., unpublished). Research on the specific functions of these genes during ascidian metamorphosis is ongoing in the Swalla laboratory.

MATERIALS AND METHODS

Biological materials

Boltenia villosa were harvested at Friday Harbor Laboratories. Adults were sliced longitudinally and the gonads were pushed through 200 μm Nitex mesh. Fertilized eggs were washed and cultured in bag-filtered seawater (FSW). Larvae hatched after 24 hours at 12°C. Pre-competent larvae were collected within 1 hour after hatching (12°C) and tested for competence with 50 mM KCl FSW. Within 6-10 hours after hatching, larvae gain competence and begin to undergo metamorphosis in response to KCl treatment. Competent larvae were collected at 10-12 hours after hatching (12°C) and induced with 50 mM KCl FSW. Ten-hour old larvae respond rapidly, with >90% showing tail resorption within 1 hour. Some larvae were allowed to settle on petri dishes and the juveniles were maintained in 12°C FSW until they were harvested. Harvested embryos, larvae and juveniles were rinsed in Millipore-filtered SW (MFSW). They were then frozen in liquid nitrogen and stored at –80°C.

Subtractive hybridization

Total RNA was isolated from 50-100 mg samples of pre-competent larvae, competent larvae and 1 hour juveniles using RNAzol B (Tel-Test, Friendswood, TX, USA), a guanidinium thiocyanate-phenol-chloroform method. PolyA^+ RNA was collected from ~50 mg-100 mg samples of 1 hour and two day juveniles using magnetized oligo dT

beads (Dynal, Oslo, Norway). cDNA was then prepared by oligo-dT reverse transcription and amplified following the protocols of the Clontech Smart-PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA).

Differentially expressed transcripts were isolated using a series of three suppressive subtractive hybridization screens including: (1) Competent larval cDNA for the tester and pre-competent larval cDNA as the driver; (2) 1 hour juvenile cDNA for the tester and competent larval cDNA as the driver; and (3) two day juvenile cDNA for the tester and 1 hour juvenile cDNA as the driver following the protocols of the Clontech PCR-select cDNA subtraction kit. The resulting differentially expressed transcripts were ligated into the pT-Adv Vector (Clontech TA cloning; Clontech) and colonies were randomly selected, prepped and then sent off for sequencing by the UW Health Sciences Sequencing Facility. Additional sequencing was also conducted at the Institute for Systems Biology (Seattle, WA, USA). Protein homologies were detected through NCBI Blast (Altschul et al., 1997).

RT-PCR analysis

PolyA$^+$ RNA was collected from ~50-100 mg samples of 6 hour (gastrula), 11 hour (tailbud) and 16-hour-old embryos, larvae 2 and 11 hours after hatching (pre-competent and competent), and juveniles 1 hour, two days, four days and 10 days after induction of settlement using magnetized oligo dT beads (Dynal). These samples were then reverse transcribed using random primers. RT-PCR was performed on equalized amounts of these cDNA samples using the following protocol: 2 minutes at 95°C then 20-25 cycles: 95°C for 45 seconds, 50°C for 45 seconds and 72°C for 80 seconds, using primers specific for each gene of interest. Primers specific to *B. villosa* 16S ribosomal RNA were used as a control.

Isolation of full-length transcripts

Rapid amplification of cDNA ends was used to generate 5′ and 3′ PCR products. A pool of double stranded cDNA from larval and juvenile stages was initially isolated for subtractive hybridizations, amplified following the protocols of the Clontech Smart-PCR cDNA synthesis kit (Clontech Laboratories) (see above), and was then ligated to double stranded adaptors (Marathon cDNA Amplification Kit, Clontech). PCR was used to amplify 3′ and 5′ ends of specific transcripts using gene-specific primers and primers that anneal to the ligated adaptors as described in the Clontech protocol. Gel purified PCR products were cloned into the pT-Adv Vector (Clontech TA cloning) and sequenced at the Institute for Systems Biology.

Propidium iodide staining

Larvae and juveniles were fixed at 4°C in 4% (w/v) paraformaldehyde in 100 mM HEPES pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA for 24-48 hours. They were then washed three times for 10 minutes in phosphate-buffered saline (PBS) and stored at 4°C in PBS. Animals were then treated with 20 μg/ml RNase A for 2 hours at 37°C, followed by 2 μg/ml PI for 1 hour. Samples were washed four times in PBS and mounted in PBS:glycerol (1:1). Fluorescent images were obtained on a BioRad 600 laser scanning confocal microscope.

Whole-mount in situ hybridization

Larvae and juveniles were fixed at 4°C in 4% (w/v) paraformaldehyde in 100 mM HEPES pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA for 24-48 hours. They were then dehydrated in 50% ethanol, then 80% ethanol (30 minutes each) and stored at –20°C in 80% ethanol. Digoxigenin-labeled antisense probes were synthesized from linearized plasmids according to the protocols supplied with the DIG RNA Labeling kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). Whole mount in situ hybridizations were performed by a similar protocol to those of Swalla et al. (Swalla et al., 1994). Samples were washed with phosphate buffered saline with 0.1% Tween 20 (PBT) then treated with 10 μg/ml Proteinase K in PBT at 37°C for 10 minutes. The reaction was stopped in 2 mg/ml glycine in PBT, then washed with PBT. Samples were post-fixed in 4% paraformaldehyde in PBS, washed with PBT and treated with 0.25% anhydrous acetic acid in 0.1 M triethanolamine (pH 8.0) prepared just before use. Samples were hybridized overnight at 45°C, washed with 2XSSC at 45°C and treated with 20 μg/ml RNase at 37°C. Samples were blocked in 0.1% blocking reagent in PBT, then incubated in 1/2000 anti-DIG-AP in PBT, both from the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals). AP detection buffer contained levamisole and NBT/BCIP. After the desired staining was reached, samples were rinsed in PBS. Samples were then mounted in benzyl alcohol:benzyl benzoate after being dehydrated through a series of ethanol washes: 30%, 50%, 80%, 90%, 100% along with two washes in benzyl alcohol:benzyl benzoate 1:1.

RESULTS

Analysis of subtractive hybridization

We conducted a series of subtractive hybridizations to isolate genes that are differentially transcribed during metamorphic competence, settlement and adult differentiation. Suppressive PCR subtraction was used to differentially amplify low abundance transcripts (see Materials and Methods). The competence screen involved hybridization between amplified cDNA from pre-competent (1 hour after hatching) and competent larvae (10-12 hours after hatching). The settlement screen involved hybridization between amplified cDNA from competent larvae (10-12 hours after hatching) and juveniles one hour after induction of settlement. The juvenile differentiation screen involved hybridization between amplified cDNA from 1 hour juveniles and two-day old juveniles (it is between two and four days when the juveniles first show overt signs of differentiation).

An overview of the results for each subtraction is displayed in Table 1. Of the 195 transcripts sequenced, 132 are potential protein-coding genes. Only 37 transcripts were duplicates (representing 16 unique sequences), indicating that the isolated transcripts comprise only a fraction of the diverse genes transcribed during the targeted time periods. Only matches with less than a 1.0 e^{-5} probability of a chance occurrence were classified as significant. Of the 132 potential proteins, 65 showed a significant match to known proteins or protein

Table 1. Overall sequence distribution

Category	Total	A*	B*	C*
Significant match to known proteins	65	32	12	21
Significant match to unidentified proteins	8	6	0	2
Putative ‘housekeeping’ proteins	22	11	5	6
Putative ‘developmental’ proteins	27	13	5	9
Putative ‘innate immunity’ proteins	16	8	2	6
Unique protein coding sequences	73	38	12	23
3′ trailer sequence	15	6	3	6
No significant match to database	44	19	8	17
Possible unique protein coding sequences	132	63	23	46
Mitochondrial 16S sequences	26	0	14	12
Duplicated sequences	37	19	7	11
Total sequences	195	82	44	69

*Number of sequences in each category for each individual screen. A, competence screen; B, settlement screen; C, juvenile screen.

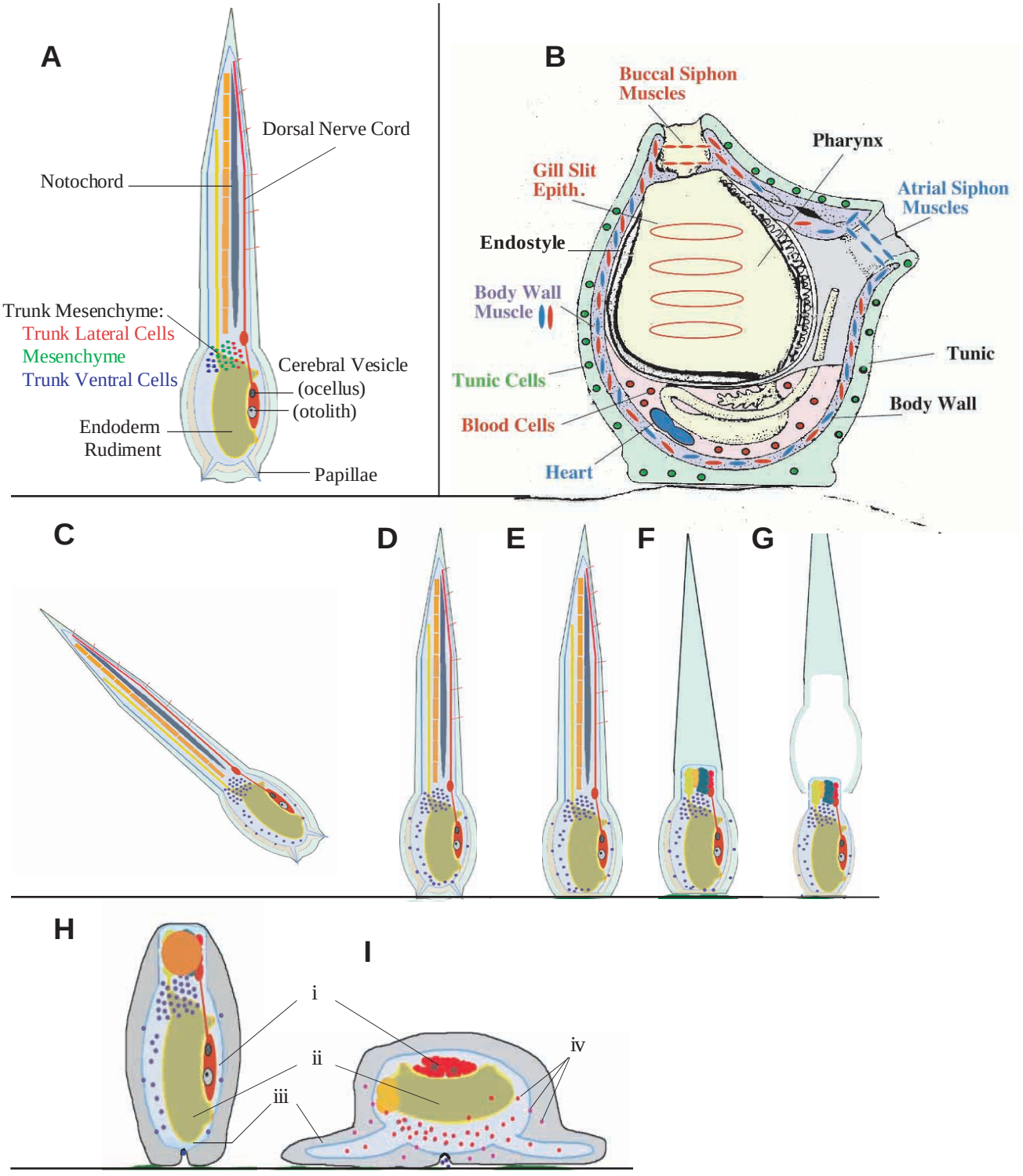

Fig. 1. Ascidian metamorphosis (Cloney, 1990; Hirano and Nishida, 1997; Satoh, 1994). (A) Pre-competent ascidian larvae, showing the chordate dorsal nerve cord and notochord. The three cell lineages within the trunk mesenchyme form distinct mesodermal structures, as in B. (B) An adult ascidian showing the chordate endostyle and pharyngeal slits. The trunk lateral cells form structures (colored in red) including the blood cells, parts of the body wall musculature and the pharyngeal gill slit endothelia. The mesenchyme cells (colored green) migrate into the tunic. The trunk ventral cells form structures (colored in blue) including the heart and parts of the body wall musculature. (C-D) Upon detection of the appropriate settlement cue, competent larvae adhere to the substrate through adhesives secreted by the papillae. (E) The papillae retract, pulling the larval head against the substrate. (F) Over the next 20 minutes the tail is resorbed. (G) Within an hour, the outer larval tunic is molted. (H,I) Close up diagrams of the newly settled juvenile during the first few hours of metamorphosis. During this time (i) the cerebral vesicle is resorbed; (ii) the viscera rotates 90 degrees; (iii) ampullae extend from the anterior epidermis, pushing the juvenile tunic along the substrate; and (iv) there is extensive migration of trunk mesenchyme cells both within the body, across the epidermis into the tunic and through a tube to the exterior.

domains. In addition there were 26 sequences matching mitochondrial 16S rRNA.

Overall, the identified transcripts can be sorted into three categories based on the functions of the matching proteins. (1) A notable fraction (16/65) of the identified transcripts are potentially involved in innate immunity; (2) There was also a large fraction (27/65) of transcripts with potential developmental roles such as signaling factors and transcriptional regulators; and (3) The remaining identified transcripts (22/65) have housekeeping functions (cytoskeleton, cell metabolism, etc), so their differential transcription may be an artifact of an overall increase in transcriptional activity. This proportion of housekeeping transcripts (34%) is relatively low in comparison to larger EST analyses that often consist of 50-60% housekeeping proteins (Lee et al., 1999; Makabe et al., 2001). This result is probably because of the successful use of suppressive subtractive hybridization, which eliminates many housekeeping genes present at both time points and serves to amplify low abundance transcripts.

A complete list of identified transcripts is displayed in Table 2. Transcripts are sub-divided into the three screens by which they were identified. In addition each transcript has been assigned a provisional functional identity based on its match to known proteins. These seven functional categories include housekeeping, extracellular matrix, cytoskeletal, muscle-related, stress proteins, developmental (signaling and transcription) and immune-related proteins (as described in the Table legend). Some proteins are assigned to more than one potential category, as they have overlapping functions.

One conspicuous functional group of transcripts are those that putatively encode proteins with roles in immunity. This includes six transcripts that match proteins involved in complement signaling (four putative complement factors, *Bv-MBL* and *Bv-MASP*), along with three von Willebrand factors (*Bv-VWa1-3*), Pentraxin (*Bv-Ptx*), Hemocytin (*Bv-Hmct*), two Selectins (*Bv-Sccp1-2*) and a leucine-rich repeat protein resembling Toll (*Bv-LRR*). The complement system plays an integral role in the detection of and response to pathogens or compromised self-tissues. The complement pathway includes a wide array of proteins, many of which contain CCP (complement control) repeats. Three of the putative complement factors (*Bv-Ccp1-3*) were isolated in separate screens. They all contain multiple CCP domains. Two of them show high similarity to complement receptors (see Table 3), the other is a smaller fragment and may either be a complement receptor, ligand or regulatory factor. MBL and MASP are key proteins in the lectin pathway. MBL (mannonse-specific lectin) binds to pathogen-specific molecules, leading to the activation of MASP (MBL-activated serine protease). MASP then activates further complement signaling (Matsushita et al., 1998; Sekine et al., 2001). We also classified a short fragment containing a trypsin-like serine protease domain (*Bv-Trsp*) as a complement factor. Complement proteins often contain trypsin-like serine protease domains which are involved in proteolytic regulatory cascades (Goldsby et al., 2000), however it is also possible that this fragment represents a different protein. Selectins are lectin-containing adhesion proteins central to blood cell migration during inflammation (see below) (Ley, 2001; Vestweber and Blanks, 1999). Von Willebrand A (VWa) domains are present in proteins with functions in cell adhesion, particularly during blood clotting, and are also found in complement factors (Tuckwell, 1999). *Bv-VWa1* also contains a CCP domain and therefore is likely to act as a complement factor. *Bv-LRR* matches the cytoplasmic leucine-rich repeat region of *Drosophila* Toll. Toll receptors are involved in cell-cell signaling during development and also have a key role in innate immune signaling (Aderem and Ulevitch, 2000; Imler and Hoffman, 2000; van Eaden and St Johnston, 1999) (see below). Hemocytin is a lectin with VWa and coagulation factor domains isolated in the silkworm where it may have roles in both innate immunity and metamorphosis (Yamakawa and Tanaka, 1999). Pentraxins are a family of proteins that are often upregulated during infection or trauma (as part of the acute phase response) and participate in complement signaling as well as potentiating phagocytosis by blood cells (Goodman et al., 1996; Rovere et al., 2000).

The differential expression of these immune-related transcripts was an unexpected result with profound implications as to the possible functions of an immune response during ascidian metamorphosis. In addition, the expression of genes coding for ECM modifying proteins such as Tenascin c, Tenascin x, Arylsulfatase and Thrombospondin may reflect immune-related tissue remodeling (see Discussion).

Isolation of full-length transcripts

The majority of the transcripts isolated in our screens represented fragments of the full-length cDNAs ranging in size from ~200 bp to 2 kb. Many of these fragments contained the 3′ end of the transcript. We employed RACE to isolate the 5′ and 3′ ends of these incomplete fragments, focusing specifically on some of the immune-related transcripts, whose identities were initially unclear. A summary of our results is displayed in Table 3, which describes the length and match of the fuller length fragments that we obtained.

Temporal expression of isolated transcripts

In order to determine when the immune-related transcripts were first expressed and how their expression changed over time, we conducted a series of RT-PCR reactions. This series included all the stages used in our screens (pre-competent larvae, competent larvae, 1 hour post-settlement, and two-day old juveniles). We also extended the series to include stages representing embryogenesis and juvenile differentiation: fertilized eggs, gastrulae, tailbud and 16 hours post-fertilization (midway between tailbud and hatching), four-day old juveniles (when differentiation of organ rudiments is just beginning), and 10-day old juveniles (which have completed differentiation of the organs necessary to begin feeding). Therefore, our series represents a complete range of developmental stages from fertilized egg to feeding juvenile.

We have conducted RT-PCR reactions for seven immune-related transcripts (shown in Fig. 2) along with 10 transcripts with putative developmental roles (*Bv-Crn* is included in Fig. 2 and the rest will be published separately). 16S mitochondrial ribosomal transcript levels were used as controls.

Most ‘developmental’ transcripts were initially expressed during embryogenesis, as shown for *Bv-Crn* (*Coronin)*. In contrast, all of the immune-related transcripts were initially expressed during larval or post-larval stages. *Bv-LRR*, *Bv-VWa1* (*von Willebrand factor*), *Bv-Sccp2* (*Selectin 2*) and *Bv-Masp* were first detected in pre-competent larvae and were

Table 2. Identification and categorization of transcripts

Category	Name	Group*	Gene identity†	*P*‡	Accession number
(A) Competence screen					
Immunity	Bv-Ccp1	I	NP 073725 Complement (CCP domains) 1	1e-06	AF483032
Immunity	Bv-Hmct	I	AF070482 Coagulation factor, hemocytin	2e-15	AF483027
Immunity	Bv-Lrr1	D,I	L25390 Toll (leucine-rich repeat domain)	1e-08	AF483022
Immunity	Bv-Sccp1	I	NP 035477 Selectin 1 (Sushi repeats)	2e-07	AF483007
Immunity	Bv-Vwa1	E,I	XP004249 von Willebrand factor type A (VWa) domain	5e-21	AF483010
Immunity	Bv-Vwa2	E,C,I	CAA50063 VWa domain/Actin (has matching domain to each)	1e-10,e-14	AF483015
Immunity	Bv-Vwa3	I,E	P32018 VWa domain	2e-09	AF483028
Development	Bv-Aip	D	AAF08220 Program cell death 6 interacting protein	4e-30	AF483018
Development	Bv-Ars	E,D	P14000 Arylsulfatase	3e-45	AF483013
Development	Bv-Cni	D	O35372 Cornichon	6e-58	AF329820
Development	Bv-Crn	D,C, I	92176 Coronin	1e-27	AF483004
Development	Bv-DRG	D	P32233 DRG, Developmentally regulated G protein	1e-28	AF483034
Development	Bv-IAP	D	90660 Inhibitor of apoptosis	1e-13	AF483030
Development	Bv-Lin41	D	AF195610 Lin-41	1e-19	AF483040
Development	Bv-Meta1	D	BAB40596 Ci-Meta1 (Fibullin)	2e-11	AF483021
Development	Bv-Naca	M, D	AAB18733 α-NAC	1e-25	AF483005
Development	Bv-Tnx	D,E	P22105 Tenascin x	3e-11	AF483029
Development	Bv-Tsp1	E,D	NP001695.1 3 Thrombospondin 1 domains 1	5e-21	AF483011
Other	Bv-CA2	C	AF076518 Cytoskeletal actin 2	7e-34	AF483031
Other	Bv-NoA	C	BAA96545 Notochord actin	3e-08	AF483033
Other	Bv-Muc1	E	CAC40991 Oikosin 1 (Mucin repeats)	9e-15	AF483008
Other	Bv-Trnp	H	AE002414 Transposase	2e-05	AF483024
Other	Bv-G3P	H	Q00301 GADPH	4e-15	AF483035
Other	Bv-Asml	H	NP_055289 Sphingomyelinase like phosphodiesterase	2e-38	AF483036
Other	Bv-GSt	H,I	AF071160 Glutathione S-transferase	5e-44	AF483037
Other	Bv-MA2/4	C, M	P27130 Muscle actin 2/4/4a	3e-102	AF483014
Other	Bv-Serca	H, M	CAA51262 Ca ATPase	1e-81	AF483025
Other	Bv-Tbc	D, M	s24403 Tropomyosin	5e-26	AF483023
Other	Bv-HsBP2	S	AAF35833 HSP 70 binding protein	4e-21	AF483019
Other	Bv-Post	U	AB005753 Hr-Post-1	4e-49	AF483038
(B) Settlement screen					
Immunity	Bv-Ccp2	I	AAG32160 Complement (CCP domains) 2	2e-15	AF483043
Immunity	Bv-Trsp	I,D	Smart00020 Trypsin-like serine protease	2e-12	AF483050
Development	Bv-Hasp	D,H	AAB07135 PDGFA-associated protein 1	1e-12	AF483052
Development	Bv-Pop	D	AAD51779 BVES, Popeye	1e-21	AF483041
Development	Bv-Rp8	D	P47816 Programmed cell death protein 2	8e-8	AF483051
Development	Bv-Tsp 2	E, D	AAC32224.1 Thrombospondin domains 2	1e-16	AF483049
Other	Bv-Acy	H,D	NP__000657 aminoacylase 1	2e-19	AF483053
Other	Bv-CA1	C,H	P07461 Cytoplasmic actin	4e-82	AF483048
Other	Bv-Sulf	H	BAA83029 Sulfatase	4e-10	AF483044
Other	Bv-Tba1	C, H	P06603 Tubulin, α1	4e-77	AF483042
Other	Bv-Mlc	M	O60241 Myosin regulatory light chain	2e-24	AF483047
Other	Bv-Bip	S	O91883 BIP	2e-49	AF483045
(C) Juvenile screen					
Immunity	Bv-Ccp3	I	CAA25077 Complement (CCP domains) 3	2e-14	AF483056
Immunity	Bv-Masp	I	BAA19763 AsMASPb, mannan-binding lectin serine protease	2e-46	AF483072
Immunity	Bv-MBL	I, D	BAB20045 Mannose-specific lectin	2e-09	AF483075
Immunity	Bv-ProtY	H, I	BAA19760 Proteasome subunit Y	2e-17	AF483068
Immunity	Bv-Ptx	I	NP 02843 Pentraxin 3	2e-06	AF483054
Immunity	Bv-Sccp2	I	P16109 Selectin 2 (sushi repeats)	3e-22	AF483065
Development	Bv-1433	D	AAC41251 14-3-3 (suppressor of RAS)	6e-23	AF483069
Development	Bv-7tm	D	AF374376 Similar to transmembrane receptor	3e-15	AF483077
Development	Bv-Apeg	D,E	A37331 Mucin (growth factor) trefoil domain	2e-15	AF483060
Development	Bv-Notch	D	A24420 Notch (EGF repeats)	9e-28	AF483064
Development	Bv-Obrgr	D	O89013 OB-RGRB (leptin receptor-related protein)	5e-10	AF483070
Development	Bv-PP2aB	D	O9Z176 Protein phosphatase 2A regulatory subunit B''	2e-19	AF483061
Development	Bv-Rab	D	AAF02485 Rab 36	4e-06	AF483063
Development	Bv-Set	D	AAB62936 SET	1e-13	AF483062
Development	Bv-Strp	D	NP 033383 Steroid acute regulatory factor (StAR)	4e-27	AF483067
Development	Bv-Tnc	D, E	NP 035737 Tenascin C	6e-09	AF483055
Other	Bv-EF1	H	AAF70832 EF-1a	2e-13	AF483073
Other	Bv-His2a	H, D	P08991 Histone 2A Variant	3e-29	AF483074
Other	Bv-Smd	D,H	NM_032881 U7 snRNP specific sm-like protein LSM10	2e-10	AF483079
Other	Bv-Umps	H	DCMSOP UMP synthase	6e-17	AF483059
Other	Bv-Vatl	H	P23956 Vacuolar ATP synthase 16 kDa proteolipid subunit	2e-6	AF483078

*Provisional functional groups, including H (housekeeping), S (stress), E (extracellular matrix component or modifier), C (cytoskeletal), D (potential developmental roles, including cell-cell signaling and transcription), M (muscle structure and/or development), I (immune-related functions).

†GenBank reference number and description of matching protein.

‡Probability of similarity by chance occurrence.

Table 3. RACE products

Name	Length (bp)	Section	Gene identity	*P**
Bv-VWa1	2171	3′	VWa domain/CCP domain	5e-35/7e-08
Bv-LRR	1395	Full	L25390 Toll (leucine-rich repeat domains)	2e-08
Bv-CCP2	2963	3′	P20023 Complement receptor type 2	7e-56
Bv-Ptx	1931	3′	NM 014293 neuronal pentraxin receptor	1e-09
Bv-Ccp3	1047	3′	X98171 Complement receptor 2	3e-23
Bv-Sccp2	1030	3′	P16581 E-Selectin	4e-28

*Probability of similarity by chance occurrence.

distinctly upregulated during larval competence (Fig. 2). *Bv-Ccp2* (*complement factor 2*) is first detected in competent larvae and is then highly upregulated within one hour after settlement (Fig. 2). *Bv-Ptx* (*Pentraxin*) and *Bv-Ccp3* (*complement factor 3*) are first detected shortly after settlement and are upregulated during the first two days after settlement (Fig. 2).

The majority of the immune-related transcripts display dynamic patterns of temporal expression (Fig. 2). *Bv-VWa1*, *Bv-Ptx* and *Bv-Ccp3* show distinct peaks of expression followed by declining levels of expression. *Bv-LRR* and *Bv-Ccp2* had the most dynamic expression patterns, both displaying two peaks of expression during larval or post-larval development. Only *Bv-Masp* and possibly *Bv-Sccp2* showed more linear patterns of temporal expression, both of them increasing during larval competence and then remaining relatively stable.

Of the 17 transcripts analyzed, 13 are distinctly upregulated at the time points at which they were isolated by subtractive hybridization (‘developmental’ transcript data not shown). This pattern is demonstrated in Fig. 2, in which arrowheads indicate the time points of the subtractive screens. *Bv-Lrr*, *Bv-Vwa2*, *Bv-Ccp2*, *Bv-Ptx* and *Bv-Ccp3* all show dramatic upregulation at the expected time points. *Bv-Crn* displays a more gradual rise in expression. *Bv-Sccp2* and *Bv-Masp* both show the greatest increase in expression at the acquisition of competence, prior to the post-settlement time point at which they were isolated, indicating that they were probably isolated because of a gradual increase in expression rather than a dramatic upregulation.

Expression patterns of immune-related transcripts

We have conducted whole mount in situ hybridizations for several of the immune-related transcripts, including *Bv-MASP*, *Bv-Sccp2* (*Selectin*), *Bv-Ptx* (*Pentraxin*), *Bv-Ccp3* (*Complement factor 3*) and *Bv-Ccp2* (*Complement factor 2*). An overview of the expression patterns of these five transcripts is displayed in Fig. 3. We have also included the expression patterns from a non-immune-related transcript, *Bv-HspBP2* (Hsp-70 binding protein), as a control (Fig. 3U-W). We have examined a range of different stages but show only the four stages included in our screens; namely, pre-competent larvae (Fig. 3A-E), competent larvae (Fig. 3F-U), 1 hour after settlement (Fig. 3K-V) and two days after settlement (Fig. 3P-W). In addition, Fig. 4 displays some more detailed photos of particular expression patterns. In the pre-competent larvae (Fig. 3A-E), none of the immune-related transcripts displayed detectable expression. In contrast, ‘developmental’ transcripts are often expressed in the mesenchyme of pre-competent larvae, as shown for *Bv-Crn* (Fig. 4A). In competent larvae (Fig. 3F-U), *Bv-Ptx* is expressed at low levels in the anterior papillary region (Fig. 3H), whereas *Bv-Sccp2*, *Bv-MASP* and *Bv-Ccp2* all display stronger expression in this same region (Fig. 3F,G,J). *Bv-Ccp3* is expressed in the anterior trunk epidermis as well as in a subset of cells along the mid-trunk region where they appear to have migrated into the tunic (Fig. 3I). In contrast, ‘non-immune’ transcripts show either no significant expression at this time (*Bv-HspBP2*; Fig. 3U) or show distinct patterns of expression (*Bv-Crn*; Fig. 4B). One hour after settlement, *Bv-Ccp2* shows strong expression throughout the trunk epidermis (Fig. 3O). The other four immune genes are expressed in the anterior papillary region (Fig. 3K-N). *Bv-Ptx* is also expressed in the area of the resorbing cerebral vesicle (Fig. 3M; Fig. 4C). The control (*Bv-HspBP2*) shows no significant expression (Fig. 3V). We have conducted in situ hybridizations for *Tenascin* (*Bv-Tenc*) expression and have included a photo of *Bv-Tenc* expression 1 hour after settlement to show that this putative ECM

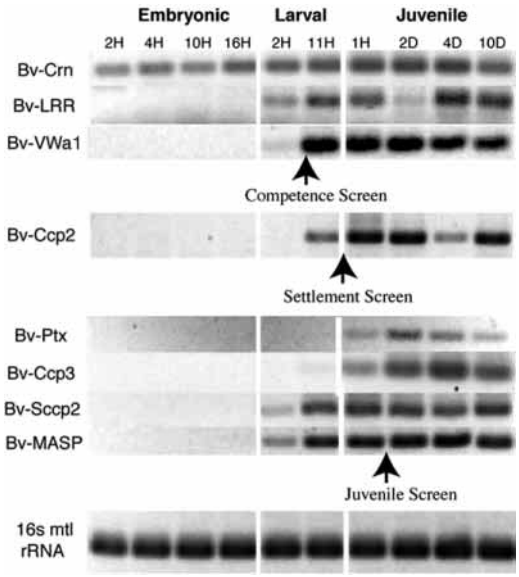

Fig. 2. Temporal expression of immune-related transcripts visualized through RT-PCR analysis. Results for nine transcripts over 10 developmental stages are shown. Stages include four embryonic stages (in hours after fertilization), two larval stages (pre-competent larvae two hours after hatching and competent larvae 10 hours after hatching), and four juvenile stages (time after settlement). The 16S mtl rRNA bands at the bottom were used as a loading control. Transcripts are grouped according to the screens by which they were isolated. The title of each screen is labeled above the relevant set of transcripts and the timing of the subtractive hybridization is indicated by an arrow.

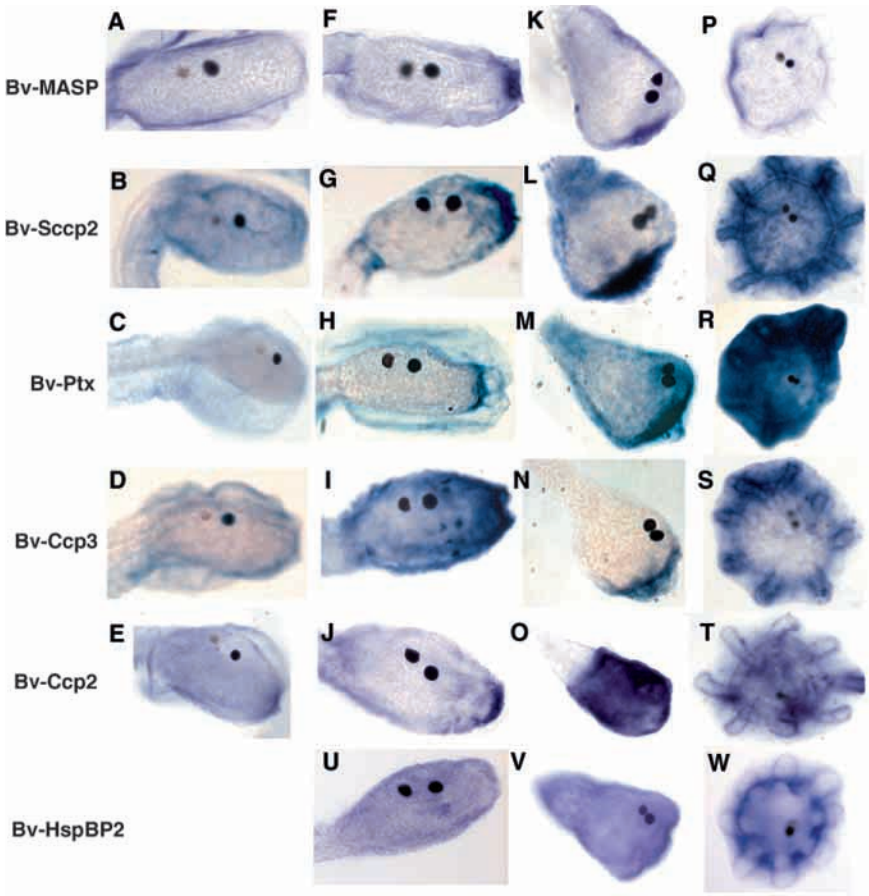

Fig. 3. Expression patterns of isolated transcripts visualized through in situ hybridization. Results for five immune-related transcripts and one control transcript over the four developmental stages included in our screens are displayed. Transcripts are identified to the left of each row of images displaying their expression pattern over time. (A-E) Pre-competent larva, 1-2 hours after hatching. (F-U) Competent larvae, 10-12 hours after hatching. For all larval pictures we have chosen to display only the head, because this allows the details of expression to be discerned and there was no significant expression in the tail region for any of the genes examined. (K-V) Juveniles 1 hour after settlement. (P-W) Juveniles two days after settlement.

remodeling gene is expressed in the resorbing cerebral vesicle, tail and muscle granules as well as in the papillary region (Fig. 4D). In juveniles, two days after settlement, *Bv-MASP* expression is limited to faint staining of the body wall epidermis (Fig. 3P). The other four immune-related transcripts are expressed strongly in the epidermis of the ampullae as well as body wall epidermis. (Fig. 3Q-T). At higher magnification of these two-day juveniles, expression of *Bv-Sccp2* (Fig. 4E) and *Bv-Ccp3* (Fig. 4F) can be discerned in both the epidermis and in nearby blood cells. In contrast, expression of *Bv-HspBP2* is not observed in the ampullae, and displays a distinctive pattern concentrated around the bases of the ampullae (Fig. 3W).

Cell migrations across the epidermis during *Boltenia* metamorphosis

Innate immune-related proteins may play a role in the adhesion and migration of blood cells that occur during *B. villosa* metamorphosis. We used propidium iodide staining to analyze the timing of cell migrations across the epidermis. As shown in Fig. 5, propidium iodide staining demonstrates that during the swimming larval stage, prior to metamorphosis, cells have already begun to migrate across the epidermis into the juvenile tunic. This early migration is evident when comparing the 2-hour larvae, in which there are no cells in the juvenile tunic (Fig. 5A), to the 15-hour larvae in which cells are visible in a regularly spaced pattern closely apposed to the epidermis (Fig. 5D). Intriguingly, cells first migrate into the tunic at 5-7 hours after hatching, corresponding to

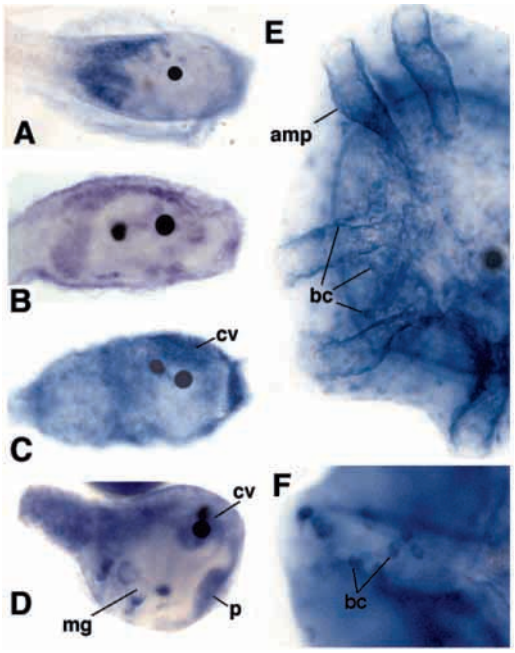

Fig. 4. Close up views of expression patterns. (A) *Bv-Crn* (*Coronin*) expression in a pre-competent larva. Note expression in the mesenchymal cells at the base of the larval trunk. (B) *Bv-Crn* expression in a competent larva. Note expression in scattered mesenchyme cells that have migrated anteriorly along the trunk; also note the lack of strong staining in the papillae region, characteristic of the immune-related transcripts. (C) *Bv-Ptx* (*Pentraxin*) expression in newly settled juveniles. Note expression in the area of the resorbing cerebral vesicle (cv). (D) *Bv-Tenc* (*Tenascin*) expression in a juvenile shortly after settlement. Note expression in the papillae region (p), in the resorbing cerebral vesicle, in the tail and in the muscle granules (mg) which are undergoing phagocytosis. (E) Bv-Sccp2 (*Selectin*) expression in a juvenile two days after settlement. Note expression in the epidermis, particularly in the ampullae (amp), as well as in blood cells (bc) in and around the ampullae. (F) *Bv-Ccp3* (*Complement receptor*) expression in a juvenile two days after settlement. This is a close up view of a single ampullae. Note expression in the ampullae epidermis and in the cytoplasm of blood cells around the ampullae.

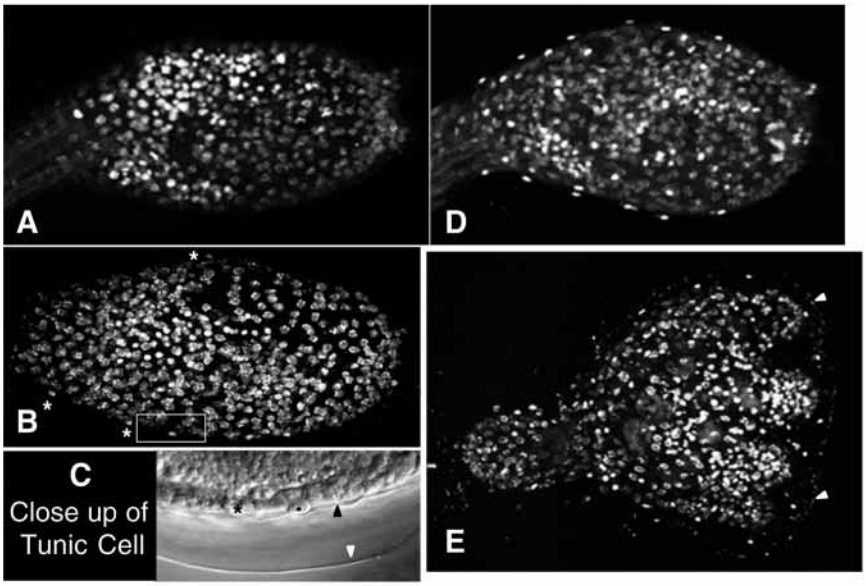

Fig. 5. Propidium iodide staining reveals timing of trans-epidermal migrations. (A,B,D,E) Projected compilations of confocal z-series, C is a DIC image (anterior is to the right). (A) Pre-competent larvae two hours after hatching; note the lack of any cells outside of the epidermis. Pre-competent larva at five hours after hatching also lack cells within the juvenile tunic (data not shown). (B) A competent larva, seven hours after hatching. Cells that have migrated into the tunic are labeled with an asterisk. (C) Close up DIC image of a cell that has migrated into the tunic in a 7-hour old larva (indicated in B by the white box). Note that the cell clearly lies on the outside of the epidermis but still within the juvenile tunic. The migrated cell is marked by a black dot, an asterisk indicates the epidermis, a black arrowhead indicates the juvenile tunic and a white arrowhead indicates the larval tunic. (D) A competent larva 15 hours after hatching; a regular array of cells can clearly be seen within the juvenile tunic, closely apposed to the outside of the epidermis. (E) Newly settled juvenile, 17 hours after settlement; migrated cells are now embedded within the expanding juvenile tunic, away from the epidermis. The juvenile tunic is indicated by the white arrowheads (at this point the larval tunic has been molted).

the time when the larvae first become competent (Fig. 5B). After settlement, these cells migrate away from the epidermis within the expanding tunic and there is an additional migration of cells across the epidermis in both the trunk as well as the anterior papillary region (Fig. 5E, and see Fig. 6D). Although the migration of cells across the epidermis has been previously described, this had been characterized as occurring only after settlement (Cloney and Grimm, 1970). The initial migration of these cells into the tunic during the swimming larval period may have implications for the acquisition of larval competence, as discussed below.

Careful observation of settling larvae has led to the detection of a group of anterior most cells in the papillary region which migrate through a tunnel in the juvenile tunic to the external environment. Fig. 6 shows these cells at various stages during this migration. We have also conducted time-lapse movies clearly demonstrating this peculiar migration (Davidson et al., 2001). The position and morphology of these cells indicates that they are the same PAT cells identified by Eri et al. (Eri et al., 1999) as having a key role in metamorphic signaling. Eri et al. described these cells as being confined within a pocket of juvenile tunic but did not note any migration out of the tunic. Further work in the Degnan lab, simultaneous to our own, has confirmed that the PAT cells in *Herdmania curvata* also migrate out through the tunic (Bernie Degnan, personal communication), as we have described here for *Boltenia villosa*.

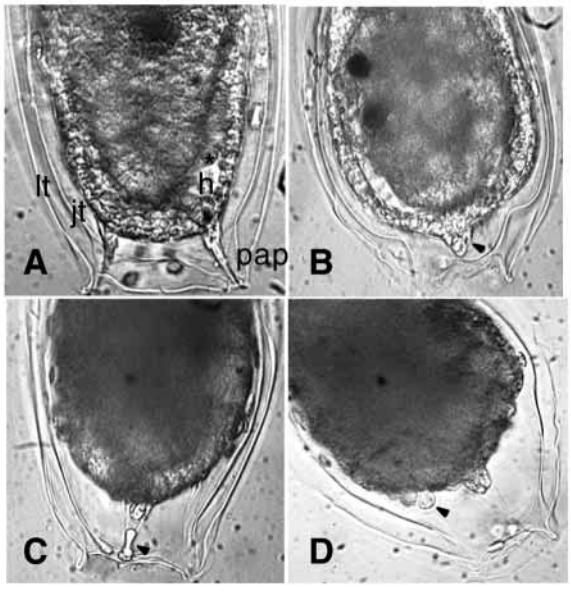

Fig. 6. Observations of PAT cell migrations (anterior is downward). (A) A competent larva viewed ventrally. Note extended papillae (pap), two layers of tunic (lt, larval tunic; jt, juvenile tunic), rounded blood cells (*) within the hemocoel (h). (B) 15 minutes after induction of settlement with 50 mM KCl filtered sea water. Note retraction of the papillae, an anterior cone of PAT cells has extended into the tunic space (black arrowhead), the anterior region has flattened and the tunic has begun to expand anteriorly. (C) 25 minutes after induction, note the anterior tunnel through the tunic and the PAT cell clearly migrating through the tunnel (black arrowhead). (D) 45 minutes after induction, the migrating PAT cell is now outside of the juvenile tunic. Under natural conditions the larval tunic is molted, leaving the migrating PAT completely exposed to the external environment. Also note the continued migration of rounded blood cells across the epidermis in the anterior (black arrowhead).

DISCUSSION

An ascidian immune response during metamorphosis

Many of the genes that were isolated in our screens match those with described roles in the innate immune system and vertebrate inflammatory responses, including two Selectins, three von Willebrand factors, *Hemocytin*, *Pentraxin*, three complement factors, a trypsin-like serine protease, and two

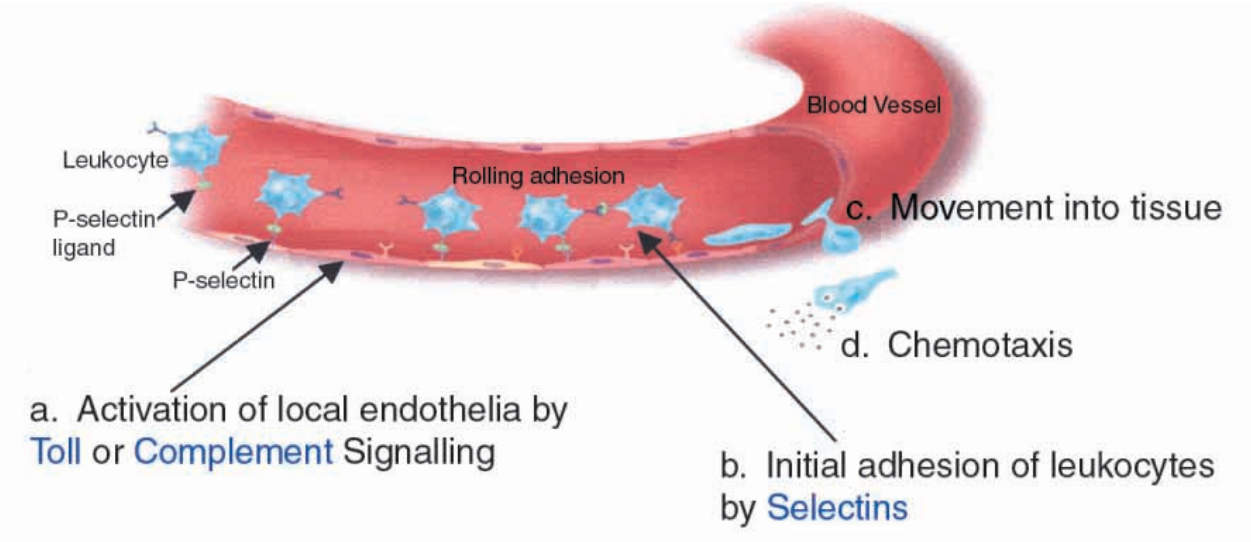

Fig. 7. Diagram of blood cell extravasation across the endothelia during vertebrate inflammation. Modified from Alper (Alper, J., 2001). Our results indicate that a similar process may underlie cell migrations across the epidermis and ampullae during ascidian metamorphosis.

key genes of the lectin pathway, mannose-specific lectin and MASP. Innate immunity represents a primitive form of immunity conserved within all bilaterians as opposed to the vertebrate-specific adaptive immune system (Nonaka, 2000). In the innate immune system, lectins, toll receptors and complement factors are used in place of antibodies for the recognition of pathogen-specific classes of molecules and/or compromised self-tissue (Goldsby et al., 2000; Imler and Hoffman 2000). Signaling downstream of this initial detection leads to a rapid immune response including cytokine secretion and inflammation. In vertebrates and ascidians, the inflammatory reaction includes targeted blood cell migration across epithelia (extravasation) into areas of trauma or infection (Di Bella and De Leo, 2000) (Fig. 7). There is also evidence that ascidians may use some of the same cytokines (such as IL1) as inflammatory signals (Raftos et al., 1998).

The upregulation of innate-immune-related genes during metamorphosis in *Boltenia villosa* was an unexpected result with provocative implications about the role of innate immunity during ascidian metamorphosis. Our analyses of the temporal and spatial expression patterns of these genes (Figs 2-4) demonstrates that their expression is strictly limited to larval and post-larval stages. Our analysis also demonstrates discrete and dynamic spatial and temporal expression patterns indicative of specific developmental roles for these immune transcripts during *Boltenia* metamorphosis (Figs 2-4). Innate immune signaling during *Boltenia* metamorphosis may be involved in coordinating the resorption of larval tissues, the maturation of the tunic or the adhesion and migration of mesenchyme cells. In addition, innate immune signaling during larval competence may underlie the ability of ascidian larvae to detect and respond to bacterial settlement cues. Alternatively, this may simply represent the developmental maturation of the innate immune system in solitary ascidians. These various hypotheses are discussed more fully below.

Innate immune signaling may coordinate the resorption of larval tissues

The comprehensive resorption of larval tissues may require a highly coordinated immune response. The differential transcription of genes involved in coagulation and targeting of tissues for phagocytosis, including complement factors, von Willebrand factors, *Hemocytin* and *Pentraxin* support this hypothesis. In addition, the differential transcription of putative extracellular matrix-modifying genes such as *Tenascin c*, *Thrombospondin*, *Arylsulfatase* and *Tenascin-x* indicate the possible involvement of immune-related cells, such as macrophages, in the restructuring and repairing of transforming tissues, in parallel with the function of these genes in similar vertebrate processes (Jones and Jones, 2000a; Jones and Jones, 2000b; Murphy-Ullrich, 2001). The complement system has also been implicated as playing roles in liver regeneration (Mastellos et al., 2001) and in the regeneration of urodele limbs (Del Rio-Tsonis et al., 1998). Our results demonstrating expression of *Bv-Ptx* (*Pentraxin*) and *Bv-Tenc* (*Tenascin*) in and around resorbing *Boltenia* larval tissues lends further support to these putative functions (Fig. 5C,D). A related hypothesis is that innate immunity is upregulated during metamorphosis not only to coordinate larval tissue resorption, but also as a response to the extraordinary levels of stress entailed in the death and reorganization of larval tissues. Such a hypothesis emerges from Matzinger's danger model of immune activation (Gallucci and Matzinger, 2001; Matzinger, 2002). According to the danger model, immune activation is more responsive to damaged self-tissue than to non-self antigens. Matzinger describes how signals produced by stressed or damaged cells, including heat shock proteins and cell surface fragments, are integral activators of the immune system. Thus immune activation during *Boltenia* metamorphosis would not require the detection of a foreign antigen, as in classic immune responses, but only the detection of endogenous signals indicative of tissue damage.

Maturation of immune competence during metamorphosis

The upregulation of immune-related transcripts during metamorphosis may represent the programmed maturation of the adult immune system. The timing of this immune maturation may simply be tied to an overall maturation of adult tissues. Alternatively, settling ascidians may initiate immune competence to deal with the new set of threats posed by the benthic habitat they are about to occupy. Clearly, there is a much higher concentration of bacteria, fungus and other pathogens on the substrate than in the open ocean, and therefore ascidians may not need a mature immune system until settlement. The migration of blood cells into the tunic before and during settlement may be considered part of this immune maturation, converting the newly synthesized tunic into an immune-ready tissue containing immune-competent blood cells. Temporal expression patterns of some of the isolated immune transcripts (*Bv-MASP* and *Bv-Sccp2*) support this hypothesis as they are upregulated in larvae and then maintained at steady levels throughout juvenile differentiation (Fig. 2). However, the more dynamic temporal expression patterns of the other immune transcripts, particularly *Bv-LRR* and *Bv-Ccp2*, suggest a more specific developmental role.

Pan-epithelial migration of trunk mesenchyme during metamorphosis as an inflammatory process

During metamorphosis in both colonial and solitary ascidians, blood cells and mesenchymal stem cells undergo a variety of targeted migrations (Cloney, 1982; Cloney and Grimm, 1970). Some migrate within the body, eventually forming the adult mesoderm, others are observed to migrate across the epidermis into the tunic. The discovery that inflammation-related genes are expressed during metamorphosis leads us to the novel hypothesis that this blood cell migration represents an inflammatory reaction akin to the extravasation of leukocytes across endothelia. The differential transcription of putative complement factors and selectins support this hypothesis (Fig. 7). Complement signaling is involved in initiating an inflammatory response (Goldsby et al., 2000), whereas selectins are specifically involved in the initial adherence of migrating blood cells to the endothelia during extravasation (Vestweber and Blanks, 1999). Furthermore, the expression of these same transcripts in the migrating blood cells and the areas of the epidermis (the anterior papillary region and ampullae) across which they migrate reinforce this hypothesis (Figs 3, 4). A similar pattern of cross-epithelia blood cell migration also occurs during inflammatory responses in colonial ascidians (Magor et al., 1999; Rinkevich and Weissman, 1992). A well-described inflammatory response occurs during rejection reactions between two unrelated ascidian colonies which contact each other. In this case, inflammation is mediated by blood cells that migrate across the ampullae epidermis bordering the colony, mirroring the similar migration of blood cells across the ampullae epidermis during metamorphosis in *Boltenia*.

Innate immune-related transcripts may have purely developmental functions

The upregulation of innate-immune-related genes during *Boltenia* metamorphosis does not necessarily involve an immune response per se. It may be that in *B. villosa* these immune-related proteins function in the developmental regulation of cell adhesion and migration. Some innate immunity genes, such as selectins and tolls, have overlapping immune and non-immune functions. Functional characterizations of upregulated genes will help to clarify if they actually represent an immune response or are part of more general developmental processes. Presently, our research into the response of *B. villosa* larvae to specific bacterial cues, discussed below, strongly supports our hypothesis that the upregulation of immune-related genes during metamorphosis is indicative of an immune response.

Innate immune signaling may underlie ascidian larval competence

Another novel hypothesis is that ascidians may use their innate immune system to initiate metamorphosis in response to specific bacterial cues. There is a growing body of research indicating the prevalent use of specific bacterial cues and/or host-produced sugar molecules by settling marine invertebrates to detect appropriate microhabitats and initiate settlement (Beckmann et al., 1999; Chia and Bickell, 1978; Johnson and Sutton, 1994; Maki and Mitchell, 1985; Orlov, 1996; Strathmann, 1978; Unabia and Hadfield, 1999). However, such responses have generally been assumed to involve detection by sensory neurons. Some researchers have previously hypothesized that marine invertebrate larvae may employ lectins to detect bacterial settlement cues, however, this was proposed in a non-immune context (Maki and Mitchell, 1985; Orlov, 1996).

Propidium iodide staining of *Boltenia villosa* larvae demonstrates that cell migration across the epidermis is occurring before metamorphosis (Fig. 5). This migration leads to a set of regularly spaced cells along the outside of the epidermis in competent larvae. These cells were previously undescribed and their function is still unknown. Thus, there are at least two waves of trans-epidermal cell migration, one, described here for the first time, occurs during the acquisition of competence and the other occurs soon after settlement (Cloney and Grimm, 1970). In addition, we have detected a group of cells, presumed to be the PAT cells (Eri et al., 1999), that migrate through an anterior tunnel in the tunic into the external environment (Davidson et al., 2001). This tunnel forms during the acquisition of larval competence (B. D., unpublished). Our *Bv-Crn* in situ hybridizations (Fig. 4A,B) along with the cell lineage work of Hirano and Nishida (Hirano and Nishida, 1997) indicate that all of these migrating cells originate from the trunk mesenchyme. Together, these observations indicate that correct positioning of trunk mesenchyme cells through targeted migrations may have an important role in establishing competence to undergo metamorphosis.

Our in situ hybridization results indicate that migrating mesenchyme cells in the papillae and trunk region express immune-related transcripts including a putative *Selectin* (*Bv-Sccp2*) and two complement factors (*Bv-Ccp2* and *Bv-Ccp3*). This may indicate that these immune-related genes mediate migration across an epithelial layer as they do in vertebrate inflammation (Fig. 7) (see above). Alternatively, expression of these genes may be involved in regulating adhesion and migration of mesenchyme cells in a manner unrelated to immunity (see above). A third possibility is that immune

responsiveness of cells within the tunic, and those with access to the external environment through an anterior tunnel, may mediate the detection of and response to bacterial settlement cues.

We have recently completed a set of experiments demonstrating that *Boltenia* larvae will undergo metamorphosis in response to the presence of specific types of marine bacteria (B. D., B. J. S. and A. Aderem, unpublished). Our results indicate that this settlement response is mediated through the detection of peptidoglycans from the bacterial cell wall and can be inhibited through the application of immunosuppressant drugs (in preparation). Thus, our results strongly support our hypothesis that detection of bacterial settlement cues in *Boltenia* is mediated by the innate immune system. Furthermore, our data suggests that this immune reception of bacterial cues probably occurs in the PAT or tunic cells. The exposure of the PAT cells to the external environment through an anterior opening in the tunic, the central role of these cells in initiating metamorphic signaling (Eri et al., 1999), and the expression of putative innate immune mediators such as complement factors in the PATs and tunic cells together lend strong support to the idea that the PAT or tunic cells are employed to detect and respond to specific bacterial cues. This hypothesis has important implications for settlement of marine invertebrate larvae. The use of bacterial settlement cues is widespread among marine invertebrates, although the nature of the receptors and signaling systems mediating this response has remained largely unexplored. Conserved components of the innate immune system are ideally suited to coordinate the rapid physiological response to bacterial and/or host-specific cues involved in larval settlement.

Perspectives

We have begun to characterize some of the molecular signals involved in coordinating the complex developmental events of ascidian metamorphosis. Our results indicate that ascidian metamorphosis represents a valuable resource for exploring the origins of innate immune function in chordates. It is conjectured that the lack of an adaptive immune system in ancestral chordates and jawless vertebrates may be compensated for by a highly developed innate immune system (Nonaka, 2000; Smith et al., 2001). The differential expression of a wide variety of innate immune-related factors during ascidian metamorphosis represents an excellent model for investigating innate immune function in ascidians, and raises intriguing possibilities of an overlap between developmental and immune signaling during invertebrate metamorphosis. In order to discover the function of this metamorphic immune response, we are attempting to specifically disrupt single genes or components of the complement pathway and examine potential effects on metamorphosis.

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