Title

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Journal

Integrative and comparative biology, 56(5)

ISSN

1540-7063

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Publication Date

2016-11-01

DOI

10.1093/icb/icw076

Peer reviewed

Integrative and Comparative Biology

Integrative and Comparative Biology, volume 56, number 5, pp. 901–913
doi:10.1093/icb/icw076

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SYMPOSIUM

MULTIFUNCin: A Multifunctional Protein Cue Induces Habitat Selection by, and Predation on, Barnacles

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From the symposium "Neuroecology: Neural Mechanisms of Sensory and Motor Processes that Mediate Ecologically Relevant Behaviors" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2016 at Portland, Oregon.

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Synopsis Foundation species provide critical resources to ecological community members and are major determinants of biodiversity. The barnacle *Balanus glandula* is one such species and dominates space among the higher reaches on wave-swept shores. Here, we show that *B. glandula* produces a 199.6-kDa glycoprotein (named "MULTIFUNCin"), and following secretion, a 390-kDa homodimer in its native state. MULTIFUNCin expression is localized in the epidermis, cuticle, and new shell material. Consequently, this molecule can specify upon contact the immediate presence of a live barnacle. Shared, conserved domains place MULTIFUNCin in the α_2-macroglobulin (A2M) subgroup of the thioester-containing protein family. Although previously undescribed, MULTIFUNCin shares 78% nucleotide sequence homology with a settlement-inducing pheromone (SIP) of the barnacle, *Amphibalanus amphitrite*. Based on this and further evidence, we propose that the two proteins are orthologues and evolved ancestrally in structural and immunological roles. More recently, they became exploited as chemical cues for con- and heterospecific organisms, alike. MULTIFUNCin and SIP both induce habitat selection (settlement) by conspecific barnacle larvae. In addition, MULTIFUNCin acts as a potent feeding stimulant to major barnacle predators (sea stars and several whelk species). Promoting immigration via settlement on the one hand, and death via predation on the other, MULTIFUNCin simultaneously mediates opposing demographic processes toward structuring both predator and prey populations. As a multifunctional protein cue, MULTIFUNCin provides valuable sensory information, conveys different messages to different species, and drives complex biotic interactions.

Introduction

Proteins are an important class of signal molecules and a logical choice by organisms for several reasons. First, the machinery (enzymes), templates (DNA, through mRNA), and structural units (amino acids) for producing proteins are already available in every living cell (Lehninger et al. 1993). Second, using the 20 coded amino acids in eukaryotic systems, a vast array of information can be presented even in a short amino acid chain. With as few as six amino acids, there are $20^6 = 64$ million possible unmodified sequences. Third, intra- and extracellular proteases can rapidly degrade proteins to their constituent peptides or amino acids, thereby terminating signal initiation (but not necessarily propagation) (Hughes 1978). Finally, because of the hydrophilic or hydrophobic nature of their structural units, proteins can be either water-soluble or insoluble at neutral pH, respectively. These molecules thus transmit information both in dissolved and particulate forms.

Advanced Access publication July 1, 2016

Significant progress has been made toward defining the roles played by proteins in pheromone communication (Wyatt 2014). Mating in sea hares (*Aplysia californica*, *A. brasiliana*, *A. fasciata*, and *A. depilans*), for example, is mediated by a cocktail of water-soluble proteins (attractin, enticin, temptin, and seduction) (Painter et al. 1998, 2004; Cummins et al. 2004). The behavioral effect of attractin is abolished by altering any one of three charged amino acids in the heptapeptide sequence, IEECKTS. Even subtle changes in molecular structure thus have important consequences for bioactivity. Female squids (*Loligo pealeii*) embed a 10-kDa protein (*Loligo* β-microsmino-protein, *Loligo* β-MSP) in the outer tunic of egg capsules (Buresch et al. 2003; Cummins et al. 2011). Here, *Loligo* β-MSP functions in male exploitative competition for mates. Males are attracted visually to eggs, but upon touching them and contacting *Loligo* β-MSP, they escalate into intense physical combat with each other. A protein pheromone is also secreted by juvenile and adult barnacles (*Amphibalanus amphitrite*). This 169-kDa compound shares many structural similarities to the α_2-macroglobulins, contains three subunits in the native form, and is synthesized by epidermal cells that secrete the cuticle (Matsumura et al. 1998; Dreanno et al. 2006a, 2006b; Clare 2011). Whereas barnacle larvae may be passively transported by ocean currents, they settle actively on the sea bed among conspecifics in response to this particulate chemosensory cue. Group living by barnacles promotes individual reproductive success and fitness.

A synthesis of research on protein pheromones is nonetheless incomplete. Previous investigations were limited to single species interactions and to lone behavioral or ecological functions. Pheromones can be eavesdropped and exploited by heterospecific organisms (Stowe et al. 1995; Lichtenberg et al. 2011; Powell et al. 2014). Consequently, these molecules may function simultaneously in multiple roles. Our recent investigation focused on the chemical identities and ecological consequences of bioactive materials in the barnacle, *Balanus glandula* (Ferrier 2010; Zimmer et al. in press). This animal is a foundation species, and whelk predation on barnacles impacts prey population stability, species coexistence, and species diversity (Connell 1961, 1970; Murdoch 1969; West 1986; Navarrete 1996). Through bioassay-guided fractionation, we found strong concordance between the active materials that cause whelks (*Acanthinucella spirata*, *Nucella emarginata*, *N. ostrina*, *N. lamellosa*, and *N. canaliculata*) to feed and barnacle (*B. glandula*) larvae to settle (Ferrier 2010; Zimmer et al. in press; Ferrier and Zimmer, unpublished data). The research yielded four glycoprotein candidates (199, 98, 88, and 78 kDa) from barnacle tissues for bioassay (Figure 1). Two (199 and 98 kDa) of these compounds were preferred significantly by whelks and conspecific larvae over organic enrichment and seawater controls, and chosen equally as often as a live adult barnacle or a crude barnacle extract (Ferrier 2010; Zimmer et al. in press).

Here, we identify the complete amino acid and nucleotide sequences for the bioactive barnacle (*Balanus glandula*) proteins and their encoding genes, respectively. Monoclonal antibodies and immunohistochemistry reveal sites of cue expression, and bioinformatics annotate nucleotide sequence information to construct a phylogenetic tree. Our findings indicate that the 98-kDa compound is a cleavage product of the 199-kDa protein and an artifact of the purification process. Consequently, the 199-kDa glycoprotein—alone—is necessary and sufficient as a cue to both whelk predation and barnacle larval settlement. As shown, a single compound is not restricted to a lone species interaction or sole ecological function. Complex biotic interactions therefore can be shaped by simple sensory systems and depend on the multifunctional properties of select bioactive proteins.

Materials and methods

Identification of barnacle protein cue(s)

Molecular weight and native state of the active glycoprotein

We used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) (Voyager DE-STR, Applied Biosystems, Foster City, CA) to determine the molecular weight of the single, bioactive compound. This protein was SDS-PAGE isolated from bulk extracts of adult barnacle tissues and gel purified via electroelution according to the established protocol (Ferrier 2010). Following dilution to a concentration of $1\,\mu g\ \mu l^{-1}$ with 50 mmol L^{-1} tris-HCl, five replicate samples ($1\,\mu l$ each) were spot mixed on a MALDI target plate with $1\,\mu l$ of sinapinic acid (SA) matrix ($20\,\mu g\ \mu l^{-1}$ SA in acetonitrile:water: trifluoroacetic acid [50:50:0.1]), and air dried. Mass spectra were analyzed after sample desorption/ionization using an N_2 laser ($\lambda = 337$ nm, 3 ns pulse, 20 Hz, 500–1000 shots per spectrum). Singly and doubly charged ions represented the intact protein.

Electrospray-ionization gas-phase electrophoretic-mobility molecular analysis (ESI-GEMMA) was employed to identify the native quaternary structure of

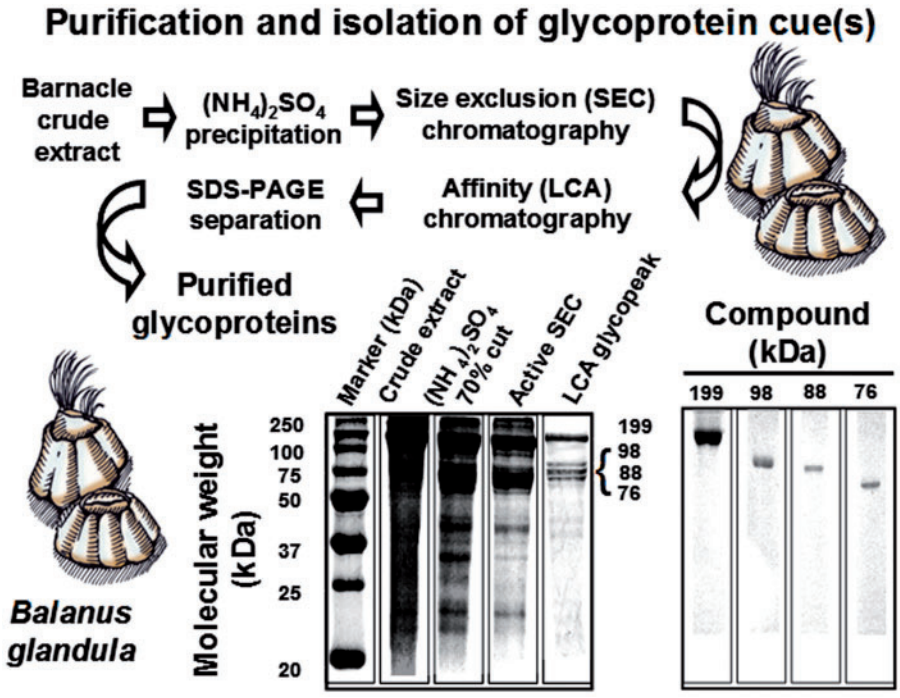

Fig. 1 SDS-PAGE visualization showing protein composition at each fractionation and purification step (from Ferrier 2010). Separation methods were based on studies with the SIP of *Amphibalanus amphitrite* (also known as settlement inducting protein complex, SIPC; Matsumura et al. 1998; Dreanno et al. 2006a). All products (5 μg protein lane^{-1}) were analyzed on 10% SDS-PAGE gels in the presence of β-mercaptoethanol. Separated proteins were stained with Coomassie Brilliant Blue, and their molecular weights estimated using Precision Plus Protein StandardsTM (Bio-Rad Laboratories, Hercules, CA). Lanes (left-right): molecular weight markers, barnacle crude tissue extract in 50 mmol L^{-1} Tris-HCl at pH 7.5 (Crude extract), 35–70% ammonium sulfate ($[NH_4]_2SO_4$) precipitation (70% cut), 170–530 kDa peak of size exclusion chromatography (Active SEC), glycopeak of lentil-lectin affinity chromatography (LCA glycopeak), and purified 199-kDa, 98-kDa, 88-kDa, and 76-kDa glycoproteins. Each visualized fraction induced significant settlement by barnacle larvae and feeding on faux prey by whelks. Only the 199-kDa and 98-kDa proteins were bioactive.

the bioactive protein. Here, we substituted the active LCA (*Lens culinaris* lectin) glyco-fraction for the fully-purified natural product. Because MULTIFUNCin is denatured during SDS-PAGE isolation, its native assembly could not be elucidated from the purified compound. Three replicate samples (1 mg ml^{-1}) of the glyco-fraction were diluted in ammonium acetate (20 mmol L^{-1}) at pH 8.0 (native conditions). From GEMMA spectra, the electrophoretic mobility diameter (EMD) and corresponding molecular weight were ascertained for the native protein oligomer (Kaddis et al. 2007).

Monosaccharide composition of the active glycoprotein

Monosaccharide composition of the bioactive compound was determined by the Glycotechnology Core Resource Facility at the University of California, San Diego. To cleave glycosidic linkages, three replicate samples (0.1 mg each) were reacted for 4 h with 200 µl of 2 mol L^{-1} trifluoroacetic acid (TFA) at 100 °C. The TFA was removed with a N_2 flush, followed by co-evaporation with 200 µl of 1:1 isopropanol:water. Each TFA-free sample was dissolved in 1-ml Nanopure-grade DI water and applied to a SepPak C18 column. Monosaccharides were eluted from SepPaks by flushing with water (peptides remain bound to the resin) and dried via speedvac. Dried materials from each sample were resuspended in 1-ml Nanopure-grade DI water prior to chemical analysis. Identification and concentration of each dissolved monosaccharide were determined using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex Model 3000, Sunnyvale, CA) on a CarboPac PA-1 column (Dionex) with isocratic elution at 1 ml min^{-1} using a mixture of 100 mmol L^{-1} NaOH and 5 mmol L^{-1} NaOHAc.

Amino acid and nucleotide sequences for the active glycoprotein and its encoding gene

The complete amino acid sequence of the bioactive protein was established through a combination of mass spectrometry-based peptide fingerprinting, cDNA construction, and translation of the full

cDNA transcript. To start, the active molecule was cleaved at each methionine residue with cyanogen bromide (CNBr) at the carboxy terminus (see "Methods" section in Andrews et al. 1992). The resulting CNBr peptide fragments were then Tricine gel-separated (Life Technologies, Grand Island, NY) and digested, in gel, with trypsin (see "Methods" section in Thevis et al. 2003). Subsequently, all tryptic digest products were extracted from the Tricine gels, pooled, and concentrated. Each product was isolated by reversed phase high-performance liquid chromatography (Hewlett-Packard Model 1090; Agilent Technologies, Santa Clara, CA; eluting at 1 ml min^{-1} with 100% acetonitrile, 0.1% TFA over 30 min), detected using an electrospray quadrupole Time-of-Flight mass spectrometer (ESI/Q-ToF MS) (QSTAR XL; Applied Biosystems/Sciex, Foster City, CA), and *de novo* sequenced from the resulting tandem mass spectra (Ma et al. 2003). From these peptide sequences, we designed degenerate primers for use in cDNA amplification.

Qualitative examination, using SDS-PAGE, identified barnacle cuticle as the location in which the active protein was expressed in greatest quantity. Consequently, the cuticle was targeted for RNA isolation. The cuticle and epicuticle of 10 adult *Balanus glandula* were dissected on site at Broad Beach (Malibu, CA; 34°02′ 04.37″N, 118°51′ 42.74″W) and immediately submerged in RNAlater™ stabilization buffer (Qiagen, Valencia, CA). Back in the lab, stabilized tissues were ground into a powder under LN_2 and total RNA was purified according to the specifications of an RNeasy™ kit (Qiagen). Poly (A+) mRNA was then isolated from total RNA using an Oligotex™ kit (Qiagen). All intact mRNA was reverse transcribed into first strand cDNA via Rapid Amplification of cDNA Ends (RACE) (SMARTer IIA™ RACE kit) (Clontech, Mountain View, CA). Specific transcripts from the first strand cDNA population were selected and further amplified by polymerase chain reaction (PCR) using forward and reverse degenerate primers as designed from MS-derived peptide sequences. The resulting cDNA fragments were partially sequenced, thus enabling construction of gene-specific forward and reverse primers. These primers were used to complete the nucleotide sequencing through both 5′ and 3′ ends (including the untranslated region [UTR] and poly-A tail).

Seven replicate cDNA sequences were obtained, providing clear consensus on the full-length gene transcript. A DNA translation tool of the Swiss Institute of Bioinformatics (SIB) Expert Protein Analysis System (ExPASy) was employed to determine the complete amino acid (protein) sequence. This tool also yielded information on length and location of the 5′ UTR, start codon, open reading frame (ORF), stop codon, and 3′ UTR. The cDNA/amino acid translation contained accurate sequences for all of the identified peptide fingerprints, indicating successful identification and isolation of the targeted encoding gene.

Bioinformatics and structure-function relationships

The full amino acid sequence was uploaded to the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) for species comparisons, protein matches, and conserved domain identifications. Complete sequence alignments to BLAST matches were performed using Clustal-W (European Bioinformatics Institute), and signal peptide cleavage locations mapped via SignalP (v 3.1; Center for Biological Sequence Analysis). Domain structure and disulfide bridging patterns of the bioactive protein were predicted using the simple modular architecture research tool (SMART; European Molecular Biology Laboratory) in conjunction with the diaminoacid neural network application (DiANNA v 1.1) (Ferrè and Clote 2005), respectively. Conserved domain comparisons and phylogenetic tree constructions by the neighbor-joining method were done with Molecular Evolution Genetic Analysis (MEGA v 5.0) (Kumar et al. 2008). From these sequence analyses and comparisons, we annotated structure-function relationships for the identified protein.

Localization and distribution of protein cue(s) in adult barnacle tissues

Two surveys were combined to establish the distribution and expression patterns of the bioactive protein. This molecule was hypothesized to act as a contact cue, given its high molecular weight and low solubility in water. Here, we determined whether, or not, the substance was expressed on exposed surfaces, that is cuticle, feeding appendages (cirri), and shell, of live adult barnacles. As a product of epidermal origin, the bioactive protein might also find expression within the cuticular linings of the hindgut and penis, among other organs.

SDS-PAGE characterization of protein cue expression

An initial survey was used to probe the external surfaces of live (cirri, cuticle, and shell) adult barnacles and of empty shells (no longer inhabited). Theoretically, a contact protein cue would have greatest value for conspecific larvae and whelk predators if its distribution was limited to live barnacles.

A total of 30 live (adult) *Balanus glandula* (0.4–0.6 cm height) and 30 empty shells (same height) were collected from a single plot (0.25 m long × 0.25 m wide) in the high intertidal at Broad Beach. All materials were placed on ice and brought immediately back to the laboratory for dissection and tissue preparation. Each live barnacle was separated from the shell, and cirri removed using a sterile scalpel and forceps under a dissecting microscope (Leica S6E at 10× magnification). Any remaining cirri stumps were excised and discarded before slicing the barnacle body open along a sagittal plane. Internal organs were extricated and cuticle flushed vigorously (five rinses, each of 25 ml Nanopure-grade DI water) to eliminate tissue fragments. For each "live" shell, we removed all visible plant and animal material before shaving the external surface to a depth of 0.5–1 mm. Shavings were collected from each shell and placed in individual (sterile, organic-free) polyethylene vials. These procedures were then repeated for "dead" shells. No attempt was made to remove proteinaceous microbial films from exposed surfaces through the use of chemical oxidation, combustion, or detergent. Invasive cleaning treatments would have compromised any native protein residue of barnacle origin. Because the targeted bioactive protein is unique to barnacles, interference by microbial natural products was unlikely.

Residues of the bioactive protein, if present, might occur only at low to trace levels within dead shells. For this reason, intact cirri, cuticles, and scrapings from live and dead shells were each isolated, but pooled separately, across all 30 barnacles. Combining samples boosted the likelihood of protein detection in subsequent analysis using SDS-PAGE. All other separation and purification procedures (ammonium sulfate precipitation, SEC and affinity chromatographies, and protein isolation) followed those described in Ferrier (2010) and in Figure 1.

Immunohistochemistry

A second survey broadened the search for tissues and cell types containing the bioactive protein. Localized expression was explored through immunohistochemistry (IHC) using a monoclonal antibody (mAb) raised against the targeted protein antigen. To begin, proprietary software (Epishot^{TM}, Abgent; San Diego, CA) was used to select the "best" putative epitope (KVHDFYRPEERNI (positions 1542–1554). An antigen matching this epitope was fabricated through proprietary solid phase peptide synthesis (Abgent). Antibody production was then induced in three Balb/c mice with a primary inoculation of the synthesized antigen at day 0, followed by boosters at days 14 and 21. B cells, containing antibodies, were harvested at day 28 from the spleens of inoculated mice, and hybridomas generated by fusing B cells with myeloma cells. After growing in culture (Serum-Free Hybridoma Growth Medium; Life Technologies Corp., Carlsbad, CA), each hybridoma line was screened for mAb production using an enzyme-linked immunosorbent assay (ELISA). The immunizing epitope (KVHDFYRPEERNI) provided substrate in each ELISA test. Individual cells of hybridoma colonies reacting positively to epitope were isolated, cloned, and again grown in culture prior to final antibody harvest. Purification of anti-MULTIFUNCin mAb was achieved via affinity chromatography using agarose beads bound with the immunizing epitope. Immunoblots were used to establish monoclonal binding affinity. No recognition by mAb was found for any protein in crude extracts of mussel (*Mytilus californianus*) or black turban snail (*Chlorostoma funebralis*) origin, or in antigen-free mouse serum. The mAb did react, but only with the single bioactive protein (MULTIFUNCin) in barnacle crude extract. Monoclonal affinity thus was stringent for the specific, targeted, protein antigen.

Immunohistochemical (IHC) probing was performed on the cirri, thoracic cuticles/epicuticles, hindguts, penises, and shells of three live barnacles (0.4–0.6 cm height), on barnacle larvae developing in three egg cases, and on three uninhabited (dead) barnacle shells (same height). All materials were collected simultaneously from a 0.25 m × 0.25 m plot in the high intertidal at Broad Beach. Transport from field and delivery to the lab, dissection, and tissue preparation followed procedures cited above (see preceding sub-section, "SDS-PAGE characterization of protein cue expression"). After each item was fixed in 10% (v/v) formalin for 12 h at 4 °C, it was whole mounted in paraffin wax; shells were prepared identically to soft tissues and cuticles. Five thin sections (7 μm thick) then were sliced, as replicates, in a transverse plane through the center of each embedded item using a Leica Model RM2255 fully automated rotary microtome. One thin section was mounted per microscope slide and paraffin removed with 100% xylene. All slide preparations were rehydrated for 1 h in an alcohol bath (18:1:1; 100% ethanol, 100% methanol, 100% isopropanol) at 37°C. Slides, next, were bathed for 1 h at 37 °C in soaking buffer (90% methanol, 3% H_2O_2), and bathed, again, for another 1 h at the same temperature in blocking buffer (2% mouse serum, 1% BSA, 0.1% cold fish skin gelatin, 0.1% Triton X-200, 0.05% Tween, 0.05% sodium azide).

Blocked slides were ready for conjugation with mAb, additional staining, and microscope analysis of protein expression patterns. Following incubation for 1 h at 37 °C in a 1:500 mAb:blocking buffer solution, each slide preparation was rinsed with 500 ml phosphate buffered (10 mmol L^{-1}) saline. It then was bathed for 30 min at 37 °C in a solution of 50:1 (v/v) phosphate buffered saline:anti-mouse universal secondary antibody in conjugation with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). Bioactive protein antigen:mAb complexes were visualized with 3′3′-diaminobenzidine stain (applied at 1 mg ml^{-1}). Localized expression appeared as a red/brown color. The slides also were counterstained with hematoxylin (for cell nuclei, as applied at 1 mg ml^{-1}), producing a blue color in all regions lacking the protein antigen. Digital images of all thin sections were visualized at 10× and 40× magnification on an Olympus (Model BH2) compound microscope and recorded using a *Canon Powershot* camera.

Results

Identification and basic properties of barnacle protein cue

A combination of proteomic and genetic evidence confirmed the molecular weight and established the native state of the active material. Initial MALDI-ToF mass spectrometry analysis of the larger, bioactive SDS-PAGE product showed both singly- and doubly-charged ions with peaks corresponding to 199.6 kDa (Figure 2a). Eight putative N-linked glycosylation sites were distributed throughout the amino acid sequence and mannose (58.5% of total oligosaccharide composition) was the dominant sugar moiety, a finding supported by the strong affinity to lentil-lectin (Table 1). A signal peptide characterized the first 17 amino acids at the amino-terminus as expected for a secreted product (Figure 3). In addition, disulfide bridging was predicted to connect 8 of the 20 cysteines and to produce significant three-dimensional folding (Ferrè and Clote 2005).

The findings indicated further that the 98-kDa compound was a cleavage product of the 199-kDa molecule and an artifact of the purification process. First, the same partial sequence (ETFLTLVQTDK) was found both in the 98- and in the 199-kDa proteins, and this sequence was used to make a degenerate primer (Table 2). A single gene encoding a protein of 172.5 kDa and 1567 amino acids ultimately was amplified, isolated, and sequenced (Table 3). The total molecular mass closely approximated 199 kDa after incorporation of post-translational glycosylation. Second, all peptide fragments for the CNBr/trypsin digest of the 98-KDa compound (Table 2) were identified within the complete amino acid sequence of the 199-kDa protein

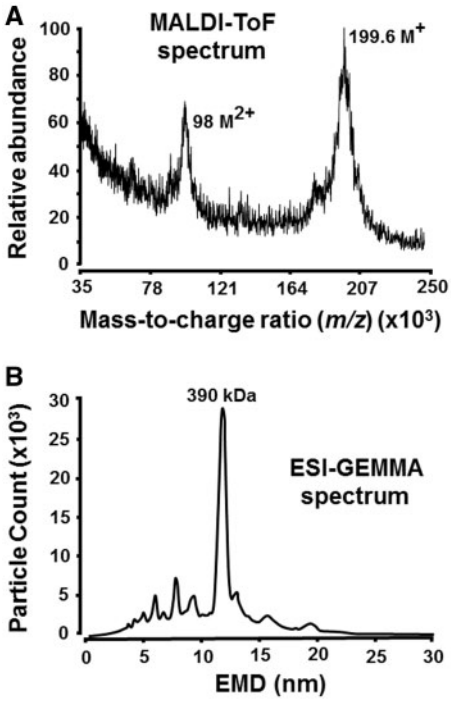

Fig. 2 Molecular weight determinations of the bioactive protein MULTIFUNCin and its native form. (A) A matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrum shows abundant peaks for the singly- and doubly-charged MULTIFUNCin ions corresponding to a molecular mass of 199.6 kDa in the intact molecule. (B) Electrospray-ionization gas-phase electrophoretic mobility molecular analysis (ESI-GEMMA) of the active LCA fraction (glycopeak) under native conditions (in 20 mmol L^{-1} ammonium acetate at pH 8.0). An abundant peak with an EMD of 12.9 nm indicates a molecular mass of ~ 390 kDa (± 5%), thus characterizing MULTIFUNCin as a homodimeric protein.

Table 1 Oligosaccharide analysis of MULTIFUNCin showing the concentration (μmol mg^{-1} protein) of each sugar moiety and molar percentage

Monosaccharide	Concentration (μmol mg^{-1})	Molar percentage
Mannose	121.8	58.5
Glucose	33.9	16.3
Glucosamine	27.5	13.2
Galactose	9.6	4.6
Fucose	9.0	4.3
Galactosamine	6.4	3.1
Total	208.2	100.0

Note: Analytical determinations were made using HPAEC-PAD chromatography.

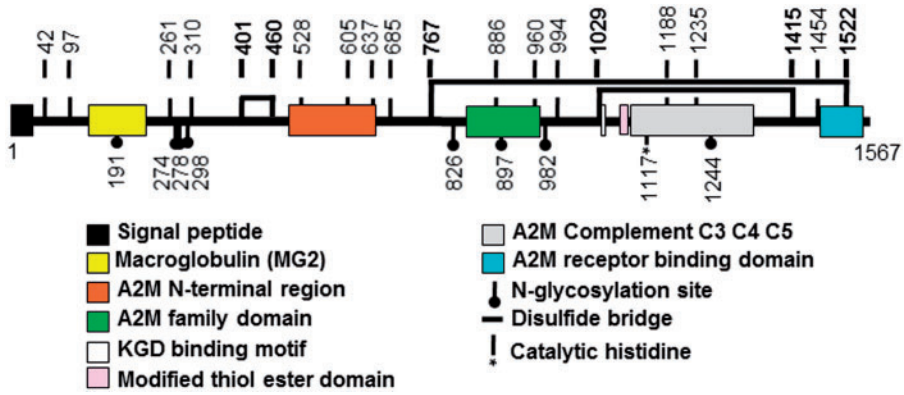

Fig. 3 (A) Structural architecture and domain organization of barnacle (*Balanus glandula*) MULTIFUNCin, a 199.6-kDa glycoprotein cue with 1567 amino acids (1 = amino terminus; 1567 = carboxy terminus). A signal peptide, modified thiol ester motif, and conserved domains and regions are denoted by color-specific rectangles. Amino acid positions of N-glycosylation sites are provided (closed circles), as well as the site of a putative catalytic histidine (*). Vertical lines with **bold** numbers denote cysteine positions. The estimated locations of disulfide bridges are marked with horizontal lines connecting **bold** cysteine residues. Like C5 complement factor proteins, MULTIFUNCin lacks a classical sequence (GCGEQ at positions 1124–1128) for the thiol ester domain, substituting glycine for cysteine and proline for glutamine. A2M: α2- macroglobulin; KGD: lysine-glycine-aspartic acid; C3, C4, and C5: complement factor proteins 3, 4, and 5.

Table 2 Peptide fragment sequences obtained from LC-MS/MS fingerprinting of the 199-kDa MULTIFUNCin and its 98-kDa cleavage product, including the origin of each fragment (from SDS-PAGE isolated protein bands) and sequence location

Sequence	Origin	Fragment location
ACLSLFNLAGPNR	199, 98 kDa	N-terminal
ETFLTLVQTDK[a]	199, 98 kDa	N-terminal
ESGEVISDSR	199, 98 kDa	Internal
AAPNSCVGISAVDK	199, 98 kDa	Internal
TSITTFKPFFTEVSPYSLK	199, 98 kDa	Internal
GGEAALTAFVM	199 kDa	Internal
FSSTLEVPDPAFSLGVAAK	199, 98 kDa	C-terminal
VHDFYRPEER	199 kDa	C-terminal

[a]Denotes sequence used for degenerate primer design.

(Tables 2 and 3). Third, ESI-GEMMA determinations were made of the bioactive LCA fraction under native solution conditions. This analysis yielded a lone compound having an EMD of 12.9 nm and a molecular mass of 390 kDa (± 5%) (Figure 2b). Taken together, the findings reveal a single bioactive glycoprotein of 199 kDa, and following secretion, a 390-kDa homodimer in its native state. This active compound has been named MULTIFUNCin in recognition of the multiple roles played as sensory cues to whelks and barnacle larvae, and as a likely component of adult barnacle structural and/or immunological defense (cuticle) (see "Discussion: MULTIFUNCin structure-activity relations" section).

Localization and distribution of protein cue in adult barnacle tissues

The expression of MULTIFUNCin occurs in association with a surface cuticle of epidermal origin. Combining SDS-PAGE-isolated materials from 30 live barnacles, expression was limited to the exposed surfaces of thoraces (body cuticle), feeding appendages (cirri), and shells (Figure 4a). No MULTIFUNCin, however, was detected when analyses were repeated on substances from 30 "dead" (uninhabited) shells (Figure 4a). An IHC survey, using a monoclonal antibody raised against the single, bioactive protein, both confirmed and extended results. Microscopic observations of the same tissues (thoraces, cirri, and shells) of live barnacles revealed localization of MULTIFUNCin to epicuticle (outer/old) and procuticle (inner/new) layers that surround the epidermis (Figure 4b, e, f). There was no evidence for the secretion and subsequent accumulation of MULTIFUNCin on the outer body surface. Additional IHC efforts probed the penis and hindgut, both of which are covered with cuticle in barnacles (Rainbow and Walker 1977). Here, MULTIFUNCin was embedded in cuticle, but not in striated muscle and other tissues of non-epidermal origin (Figure 4d). The monoclonal labeling of developing barnacle larvae (nauplius stage) localized MULTIFUNCin to embryonic cuticle (Figure 5). Expression patterns are thus consistent across ontogenetic stages. Because no labeling was associated with dead (uninhabited) shells of adults (Figure 4g), MULTIFUNCin specifies immediately upon contact the presence of a live barnacle.

Table 3 Complete amino acid sequence of *Balanus glandula* MULTIFUNCin and its alignment with *Amphibalanus amphitrite* SIP (from Dreanno et al. 2006a)

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B. glandula:   MARSLLVLLAALAATHAVKVPERGYLLTAPKVLQAGTSERACLSLFNLAGPNRLLKLAFF  60
A. amphitrite:MGGPVVLLVALATASAVKVPESGYLFTAPKVLQAGTDERACLSLFNLPGPNRALKLKFY  60

B. glandula:   ETNVPQKLSDTLDSDDFLLFRTQTYVPDSVDPNGEYCFDLSIPSKITVNFATLKLELSTD 120
A. amphitrite:ERDVPSSLSTTLDKSDFLLEETNTAVPDSVAENGEYCFDITIPSKVVARSADMHMELTAG 120

B. glandula:   GYEPLTETAKVSLKKETFLTLVQTDKSKYQPGQKVLFRVVSLSHDLTALNNVLEEVWITT 180
A. amphitrite:-EGVWKEESVVTLKSETFLTLVQTDKSKYQPGQKVLFRVVTLSHDLTALNNDLNEVWITT 179

B. glandula:   PDGIRVSQWKNVSTNTGMVQLDMQLTEEPPLGSWVIHVKTASQTITKRFSVEEYELPTFE 240
A. amphitrite:PDNIRVAQWKNVKTNTGMVQLELQLTEEPPLGSWTIHVLTTQDTYTKRFTVEEYVLPTFE 239

B. glandula:   VEIEAPSSLERSERTITTKICAKYTFGKPLIGANVTINVTASGIGDWEYRNNKELVKNIS 300
A. amphitrite:LEIEAPESLESNEKTVTVKVCAKYTFGKPLIAANVSINATARGIGSWQYNNNPDLLRNIS 299

B. glandula:   DYQLSDEKGCAIFDLVVSKIGIGHPGISSGNTAVMTVVEEEGTGLKQVEVKRVTQSYSF 360
A. amphitrite:DYQPSDEQGCAIFDLVVSKIGIGHRNIGGGNTVIITIDVEEQGTGLRQVEVKEVSQAYSF 359

B. glandula:   IRLSQSDNAQAFLKPKLPFYGEYKLKIKDGKEAKNEIVKVCYTAKYKERAISSEDKQAIP 420
A. amphitrite:INLRQSDNAQKFLKPKLPFYGEYTLSMRDGKAAKNEIVKVCYTAKYKERVISDE-KKPTP 418

B. glandula:   DDPIHSTTKKYEEHVETVFGYTPFFWETEGPNQRTTSGECREYRTDAEGRVVYYIPPQAE 480
A. amphitrite:DDPVYSTHKKYESHVKTEFGYTPFFWETSEPNRRTTGGECREYKTDENGRIVYYIPPQAE 478

B. glandula:   DIDSISISTSTKVGSDSDSRQNTLKAFFSPSHSYLSIDAHELPEQLPCTGDVTVKLLTTE 540
A. amphitrite:DIDSIDISTSTSVGGDSDSSHSTLTAFFSPSHSYLSIDAHELPEQLPCSGDVTVKLLSTE 538

B. glandula:   EGPPPAMVYKIMSRGRIIKAGNMNTDSLTFPVLAKMGPEFKLLVYYIKESGEVISDSRVF 600
A. amphitrite:EGPVPAMVYKILSRGKIIKAGNMNTNILTFPVLPKMGPEFKLLVYYIKESGEVVSDSRVF 598

B. glandula:   KVNKCFPNTVGVSWDQETVKPGDSASFTIRAAPNSVCGISAVDKSTELLGTSNQITLDTV 660
A. amphitrite:KVDKCFPNTVQVSWDQKTVKPGDSASFTVRASPNSVCGISAVDKSTELLGTSNQITLDTV 658

B. glandula:   FNKLDQFIINRYARPQQVDGDSVYCKELQISLVETLRSGGATEAELT-RAPENTASDGDL 719
A. amphitrite:FSKLQQFIINSFESPNQVRSPGDYCRELQLSLVDTLRSGGATVAELTGQSTPEGTPESET 718

B. glandula:   SGSSSPSFGRFPPPRSERYRTDRDDAITPFDQSGFLVISNLALETRPCYKRVNAKEIPGA 779
A. amphitrite:SGAAHSSLFIPPPTRSQRFRTDREDAIKPFDEAGFLVLSNLALETRPCYKRVEAKELP-E 777

B. glandula:   STTESIQPMRAHAESVDYEDSDSAPVFEAALAFSKSDAASSSFGDPNQSRITAGTGGSTG 839
A. amphitrite:LTEDKIQASRDGEE-ELLDDILDSP---VPALAFSKESADASREAAEGG----VSGGGGAA 829

B. glandula:   PPQEDQVRDDFPEAFLFSIETLDAYGVKTLTSEMPDTITSWVGSAICTNSESGFGVSNKT 899
A. amphitrite:PPQEDQVRDEFPEAFLFSIETLDAEGVKTVTSEMPDTITSWVGSAICTNSKDGFGISNKT 889

B. glandula:   SITTFKPFFTEVSLPYSLKRGEVLSMSASVFNFMDSSLSVYLELGASDQYESQGEVGYDL 959
A. amphitrite:SITTFKPFFTEVSLPYSMKRGEILSMSVSVFNFLDSSLSVYLEVGASDQYEISGEVAMGL 949

B. glandula:   CIAAGRTAVKNFFLTFSALGEVNITVSAKVKDGNCEQPNTVAPGSDTVIRPIVLKPEGFP 1019
A. amphitrite:CIAAGRTEVRSFEVNFLGLGEVNITVTARAQDGYCDEGNTIAPGSDTVIRPIVVKPEGFP 1009

B. glandula:   QEVTRSRFICLDKGDENEVETVEMPVPEGLVADSERAYFSVIGDLLGPTYQGLEGGLIAS 1079
A. amphitrite:QEVTHSRFICLDKDDDHETETVNLPVPEGLVPDSQRAYFSVIGDLLGQTFQGLEGGLIKS 1069

B. glandula:   PTGGGEPNMITLVPNIYVRDYLERTGVLTEEQRRKTDHNMKSGYQRQLRFRRSDDSFSSY 1139
A. amphitrite:PTGAGEPNMITLVPNIYIRRYLETTGQLNERQRRQLEHNMKSGYQRQLRFRRYDGSFSSY 1129

B. glandula:   GNDDSQGSLWLTAFVVKSFQEAAKYIDIDDKVISKARDWILKKQQDNGCFPRIGELIHKE 1199
A. amphitrite:GNEDPQGSMWLTAFVVKAFREASEYIEIDETIINKAKDWILKKQNTTGCFPRFGELIHKE 1189

B. glandula:   LKGGTERGGEAALTAFVMLALKDIATTNQLASSFACLEDGILLTNKTLYSEILIAHTYLK 1259
A. amphitrite:LKGGTERGGEAALTAFVMLALKDIATTNELANGFACLEDGLLLPNKTLYSEILLAYTYLN 1249

B. glandula:   MGQIVKGERLVAKLMEKAKREGDDVLYWEGDRISLFGGSKAVDVEMTSYMVLSLLHISGK 1319
A. amphitrite:MGQDVKGERLVNKLMSKAKREGDDILYWEGDRDSLFGGSRAVDVEMTAYMALSLMHISGK 1309

B. glandula:   GYLEEAARAVRWINTQRNSHGGFITTMDTMTAVKALTEFAVRTYASELTTTVSVTAGGAP 1379
A. amphitrite:GNMEEAARAIRWINTQRNSNGGFKSTQDTIVAVEALSEFASRTFASDLATSVSVTAGGET 1369

B. glandula:   AELTVDANNRLLLQQSKVPDLTLPGTVTFEVSPPGCVVVQNIFRFSSTLEVPDPAFSLGV 1439
A. amphitrite:VQRMVDGDNRLLYQESKVPDLTLPGTMNFDVSPPGCVVYQSIFRFSSTLEVPDPAFSLGV 1429

B. glandula:   AAKRRGRNGYELEVCTSYLRTGPGSVDRVILEVEMPSGYIPVDKTLRDLRRQGDNSKPAV 1499
A. amphitrite:AAKKRGRTGYELEVCTSFLRN-SGAVDRAILETELPSGYVAVDSTLRDLRRG-----SAV 1483

B. glandula:   RQYKAKEGEVTFTLQGVSEDKTCLKFRIIQQNEVEQLKPSVAKVHDFYRPEERNIQEYEL 1559
A. amphitrite:RSYEIKEGKVIFTLQGVAEDKTCLPFRIIQENEVEQLKPSIVKVHDFYRPEERNIQEYEL 1543

B. glandula:   VPTAAASA 1567
A. amphitrite:TPAA---- 1547
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Notes: Identical and fully conserved amino acids are denoted by black and grey highlighting, respectively. Peptide fingerprints are bolded and underlined as identified for MULTIFUNCin and its 98-kDa cleavage product. GenBank accession #s: *B. glandula* MULTIFUNCin, KC152471; *A. amphitrite* SIPC, AY423545.

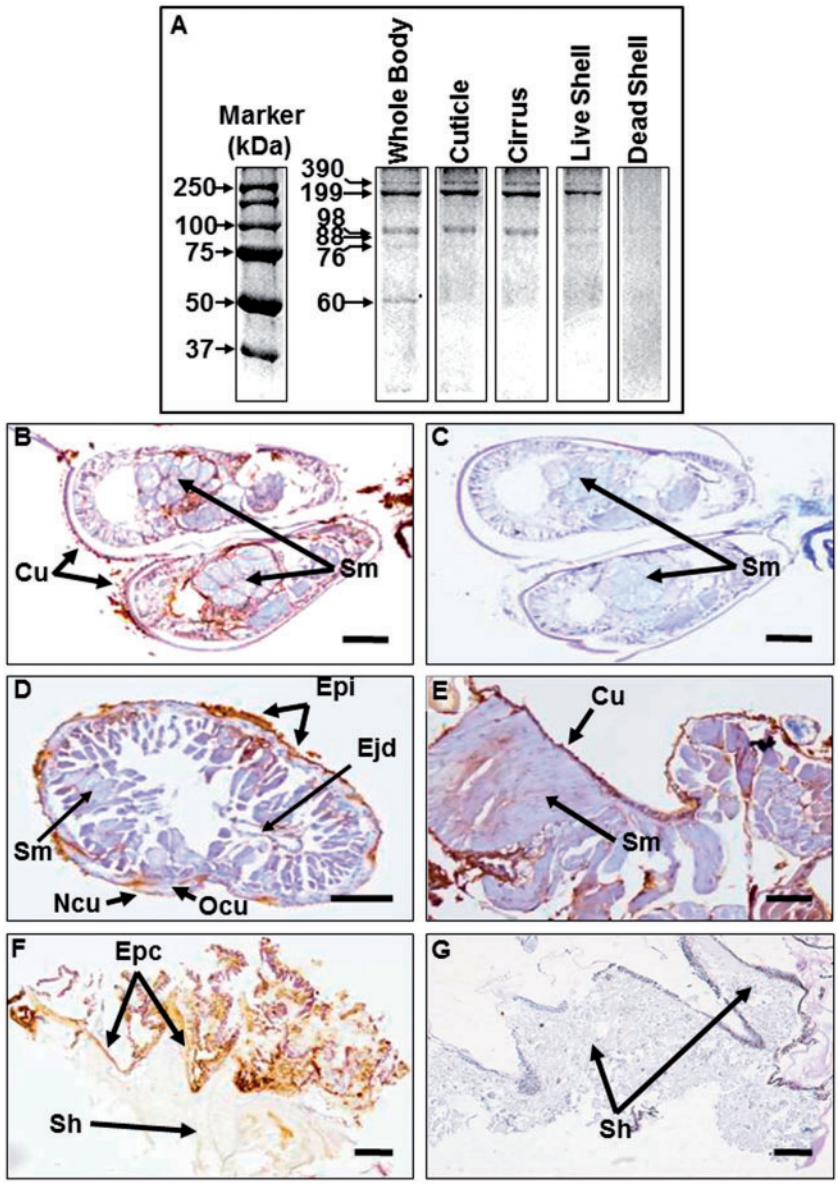

Fig. 4 SDS-PAGE and IHC surveys of MULTIFUNCin expression in select adult barnacle (*Balanus glandula*) tissues. (A) SDS-PAGE profile of extractions from intact live barnacles, thoracic (body) cuticles, cirri (external feeding appendages), scrapings from "live" shells, and scrapings from "dead" shells (empty tests without live animals). Protein bands, matching the molecular weight of MULTIFUNCin (199 kDa), were identified in all lanes containing live tissues and shell. No protein was identified in dead shell. (B–G) Immunolabeling of MULTIFUNCin in tissue sections of adult *B. glandula*. Red/brown color indicates the presence of a monoclonal antibody raised against a MULTIFUNCin epitope, whereas blue represents hematoxylin counter-stain of cell nuclei. All images were from transverse sections and thin slices (7 μm thick). (B) Two barnacle cirri showing localized staining of the cuticle in association with expressed MULTIFUNCin. (C) The two barnacle cirri in (B), as stained alternatively with non-immune mouse serum; no cross-reactivity was observed between the serum (control) and any barnacle proteins. (D) Barnacle penis in which MULTIFUNCin was localized to both the old/outer epicuticular and new/inner procuticular layers. (E) Barnacle thoracic wall (organs excised) displaying MULTIFUNCin immunolabeling limited to the cuticle. Residual tissues attached to the integument (e.g., striated muscles) failed to positively stain for the bioactive product. (F) A live barnacle shell with a MULTIFUNCin-positive epicuticular layer on the outer, exposed, surface. (G) A dead barnacle shell did not stain positively for the bioactive protein. All IHC surveys were performed at a monoclonal antibody dilution of 1:500 in blocking buffer. Results are representative of five replicate surveys per tissue. Cu: cuticle; Sm: striated muscle; Epi: epidermal cells; Ncu: new procuticule; Ocu: old epicuticle; Ejd: ejaculatory duct; Epc: shell epicuticle; Sh: shell. Scale bars = 100 μm.

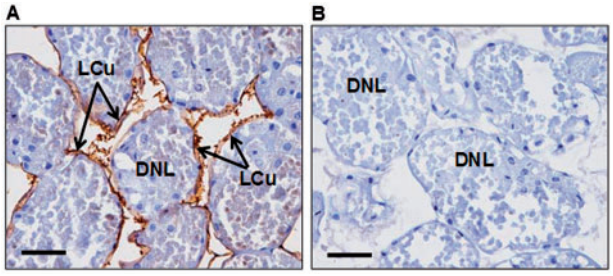

Fig. 5 IHC survey of MULTIFUNCin expression in developing *Balanus glandula* nauplius larvae obtained from excised egg cases. Red/brown color indicates the presence of a monoclonal antibody raised against a MULTIFUNCin epitope, whereas blue represents hematoxylin counter-stain of cell nuclei. All images were from transverse sections and thin slices (7 μm thick). (A) Nine developing nauplius larvae showing positive immunolabeling of the larval cuticle in association with expressed MULTIFUNCin. (B) Eight developing nauplius larvae as stained alternatively with non-immune mouse serum; no cross-reactivity was observed between the serum (control) and any barnacle protein. All IHC surveys were performed at a monoclonal antibody dilution of 1:500 in blocking buffer. Results are representative of five replicate surveys per tissue. LCu: larval cuticle; DNL: developing nauplius larva. Scale bars = 100 μm.

Discussion

MULTIFUNCin structure-activity relations

Shared, conserved domains place MULTIFUNCin (of *Balanus glandula* origin), as well as SIP (of *Amphibalanus amphitrite* origin; also known as settlement inducting protein complex, SIPC; Matsumura et al. 1998; Dreanno et al. 2006a), in the α_2-macroglobulin (A2M) subgroup of the thioester-containing protein (TEP) family (Figure 6). The α_2-macroglobulins are abundant plasma proteins of many metazoan species, including crustaceans, and are vital to structural defense (Armstrong and Quigley 1999). They also function naturally as broad-spectrum protease inhibitors (Blandin and Levashina 2004; Armstrong 2010). Akin to the A2Ms of vertebrates and invertebrates, MULTIFUNCin has one domain (positions 846–981) similar to the variable A2M bait region (Saravanan et al. 2003; Ma et al. 2010), and a second domain indicating a receptor binding site (positions 1455–1547) near the carboxy terminus

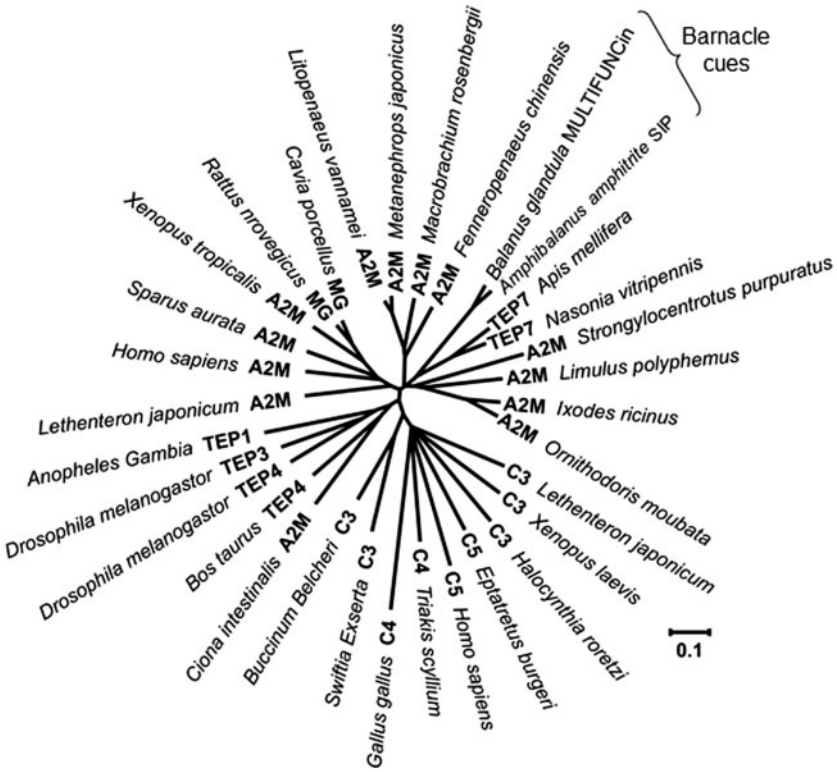

Fig. 6 Unrooted phylogenetic tree illustrating the evolutionary relationships of barnacle MULTIFUNCin (*Balanus glandula*) to SIP (*Amphibalanus amphitrite*), and to members of the α2-macroglobulin (A2M), murinoglobin (MG), thioester-containing (TEP), and complement factor (C3-5) protein families. Evolutionary history was inferred using the neighbor-joining method, with the optimal tree as shown (sum of branch length = 7.532). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances employed to determine the phylogenetic relationships. Distances were computed using the Poisson correction method, and are in units of the number of amino acid substitutions per site.

(Figure 3) (Sottrup-Jensen et al. 1990; Solomon et al. 2004). Three additional motifs in MULTIFUNCin are characteristic of most invertebrate A2Ms and serve critical structural and functional roles. These motifs include: (1) a RxxFPExxL region (positions 861–869), (2) a QTD dynein light chain (DYNLL) binding site (positions 141–145), and (3) an FxVxxYxxPFE promoter (positions 229–240) (Figure 3) (Solomon et al. 2004; King 2008; Wu et al. 2012). From combined results, we infer that MULTIFUNCin and SIP share a common α_2-macroglobulin-like protein ancestor. Orthologues to MULTIFUNCIn and SIP also have been found in other barnacles (*Semibalanus cariosus*, *Chthamalus fissus*, and *Magabalanus coccopoma*; Yorisue et al. 2012; Ferrier et al. unpublished data), and may well exist in additional barnacle and/or other crustacean species.

The C5 complement components of vertebrates are A2Ms and are highly immunoreactive. They form nuclei in membrane attack complexes that disrupt the phospholipid bilayers of bacterial pathogens (Nauta 2002). Whereas MULTIFUNCin shares a large, conserved domain (positions 1121–1360) with the C5 components, it lacks a critical netrin motif (NTR-C345C) that could act in recognition of non-self cell surfaces (Rajasekharan and Kennedy 2009). Still, other mechanisms might act in recognition and binding of MULTIFUNCin to the membranes of pathogens and drive anti-microbial function. The presence of a KGD binding motif (positions 1032–1034), as well as a catalytic histidine (position 1097), would allow MULTIFUNCin to recognize the hydroxyl groups on microbe surfaces and, therein, immobilize and disrupt the membrane integrity of pathogenic attackers (Drickamer 1992; Gadjeva et al. 1998).

Among arthropods (including barnacles), a cuticle is the first line of defense against biological vectors and physical environmental challenges. Gene expression of A2M-like proteins is upregulated immediately before and after an animal moults (sheds its hard exoskeleton) (Lin et al. 2008; Ma et al. 2010). Such over-expression aids in building new cuticle and in protecting organisms against physical disturbance and external attack by microbes during the moulting process (Wu et al. 2012). Although MULTIFUNCin is a previously undescribed glycoprotein, it shares 78% nucleotide sequence homology with the settlement-inducing pheromone (SIP) of the barnacle, *Amphibalanus amphitrite* (Table 3 and Figure 6) (Dreanno et al. 2006a; Clare 2011). All 20 cysteine residue positions are common to both molecules, indicating similar, or identical, locations of disulfide bridges. In addition, six out of eight N-linked glycosylation sites are conserved between MULTIFUNCin and SIP. Based on combined evidence, we propose that these two proteins are orthologues and that they evolved ancestrally in structural and immunological roles. More recently, they became exploited as contact-chemical cues with key ecological consequences for con- and heterospecific organisms, alike.

MULTIFUNCin and sensory exploitation

Chemical recognition via surface glycoproteins is by no means limited to barnacles and whelks. These molecules mediate many different types of biological interactions. They serve, for example, as signals in pheromone communication between sperm and egg and act as stringent pre-zygotic barriers that block hybridization among gametes of different species (Vacquier 1998; Swanson and Vacquier 2002). Their role in reproduction is not restricted to germ cells. Species-specific mate recognition might be directed by such compounds, when bound in the integument of adult rotifers and crustaceans (Snell and Carmona 1994; Snell et al. 2009; Caskey et al. 2009a, 2009b). Cell recognition and adhesion processes also are dependent on glycoprotein ''lock and key'' systems. Here, glycoprotein signals direct cell proliferation, migration and differentiation, and ultimately determine the developmental fates of specific cell lines within eukaryotic organisms (Sharon and Lis 1989; Chen et al. 2005).

Requisite for internal (cell level) and external (organism level) communication, glycoproteins are well suited for sensory exploitation. A prime example is the targeting of host-expressed mucin and collagen by pathogenic bacteria for attachment and colony establishment (Roos and Jonsson 2002). Moreover, a single glycoprotein—for example, MULTIFUNCin—can carry with it information that conveys different messages to different species. Promoting habitat selection via settlement on the one hand, and death via predation on the other, MULTIFUNCin simultaneously mediates opposing demographic processes toward structuring both predator and prey populations. For whelks and barnacles, sensory recognition mechanisms have converged across phylogenetically diverse species and different life history stages to promote the exploitation of a valuable, shared, resource. Combined, our results are the first to establish a significant effect of a fully purified (and identified) natural product on larval settlement within field habitats (Ferrier 2010; Zimmer et al. in press; Ferrier et al. present study). Our findings also provide the first complete structural elucidation of

any natural product exploited by a marine predator tracking a live, intact prey.

In the future, it will be interesting to determine if the MULTIFUNCin protein has other functions than those documented in this report. For example, is it a protease-binding protein (functioning like a classical α_2-macroglobulin), or is it a participant in target opsinization or cytolytic attack on colonizing algae, fungi, or microbes (functioning like a C3/C4/C5 membrane attack complex)? In this vein, the α_2-macroglobulins have been shown to execute a number of functions such as cytokine binding that are quite independent of their best-characterized role in clearing from the internal chemical milieu proteases of differing enzymatic classes. Barnacles, similar to all marine metazoans, are subject to colonization by epibionts and it is possible that MULTIFUNCin operates as a defense against epibionts other than conspecific larvae. Thus, the compound may have additional adaptive value besides acting as a conspecific settlement cue to promote gregariousness, group living, and sexual reproduction.

Supplementary information

Supplementary Data available at *ICB* online.

Acknowledgments

The authors thank Dr Rachel Ogorzalek Loo for assisting with the *de novo* peptide sequencing; Dr Deborah Leon for contributing to protein mass spectrometry analysis; and, Dr Paul Barber for providing lab space, instrumentation, and insights on gene identification and isolation procedures. Dr Jeffrey Riffell and Dr Ashley Rowe graciously invited this contribution to the symposium on neuroecology, and generously provided funds for travel and publication through the Society of Integrative and Comparative Biology, the National Science Foundation, and the Company of Biologists, Limited. We are grateful to an anonymous reviewer, who wrote the final paragraph of the Discussion (subsequently edited by the authors). These words and ideas skillfully captured the spirit of our work, and outlined an excellent plan for future investigation.

Funding

This research was supported by awards from the National Science Foundation (OCE 08-52361 and IOS 11-21692 to R.K.Z.), the National Institutes of Health (R01GM104610 and R01GM103479 to J.A.L.), and the UCLA Council on Research.

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