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UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Biochemical Investigation of the Sea Urchin Clotting Response

A dissertation submitted in partial satisfaction
of the requirements for the degree

Doctor of Philosophy
in
Marine Biology

by

Brian J. Hillier

Committee in charge:

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The dissertation of Brian J. Hillier is approved, and it is
acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2005

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DEDICATION

For my grandmother, Mary Mulcahy.
In her wisdom, she valued education and offered generous support.

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ABSTRACT OF THE DISSERTATION

A Biochemical Investigation of the Sea Urchin Clotting Response

by

Brian J. Hillier

Doctor of Philosophy in Marine Biology
University of California, San Diego 2005

Victor D. Vacquier, Chair

The sea urchin, *Strongylocentrotus purpuratus*, has a fluid-filled body cavity, the coelom, containing four types of immunocytes called coelomocytes. Within minutes after coelomic fluid is removed from the body cavity, a massive cell-cell adhesion of coelomocytes occurs, referred to as clotting. One function of coelomocyte clotting could be to seal the body cavity following injury, as urchins are hard-bodied and unable to contract tissue around an injury that penetrates the test. Clotting is also thought to function in the cellular encapsulation of foreign material and microbes. Here I report that the intercellular clotting response is mediated by amassin, an extracellular coelomic plasma glycoprotein. Along its length, amassin contains these structural features: a signal peptide, a short predicted β-region, segments of dimerizing coiled-coils, and an olfactomedin (OLF) domain. Amassin forms disulfide-linked multimers required for its biological clotting activity. The functional activities of amassin's constituent protein regions were investigated using various truncated constructs expressed in the yeast, *Pichia pastoris*. This research revealed that the multimerization occurs by forming disulfide-linked dimers

through Cys[203] at the end of the coiled-coils region, then tetramers through the N-terminal β-region and/or the first segment of coiled-coils. The OLF domain itself exists in a monomeric state and contains two intradomain disulfide bonds. The OLF domain confers amassin's cell-binding activity and does so in a calcium-dependent manner. Utilizing data from the *S. purpuratus* Genome Project, I identified a family of OLF proteins, totaling five, that are all actively transcribed in coelomocytes. Phylogenetically, four of the five belong to a subgroup distinct from all other known OLF domains, and the fifth is similar to the most primitive subgroup known as the colmedins. The five OLF proteins of the sea urchin represent an intermediate diversification between those of the protostomes and the vertebrates. Due to the involvement of OLF domains in important developmental cellular events in other animals, I monitored the expression levels for the genes of all five OLF proteins throughout the larval life cycle. Transcripts were highly regulated, often coinciding with the formation of the rudiment, which eventually becomes the adult.

Brief Introduction

This dissertation began with an observation. When the coelomic contents of the sea urchin, *Strongylocentrotus purpuratus*, are removed, the cells rapidly and dramatically adhere together forming a large cellular clot (Fig. 1). The mechanism for this clotting response was unknown and wide open for investigation. I set out to study this phenomenon to increase the general knowledge concerning different molecular mechanisms of intercellular adhesion.

I was by no means the first to explore the sea urchin clotting event. Geddes (1880) made the first descriptions of the echinoid clotting response, and it was studied several times thereafter (reviewed in Endean, 1966). The remarkable cell adhesion event has continued to fascinate researchers since then. It has been determined to be a cell-cell adhesion, and not a passive entrapment of cells by a gelation of proteins in the plasma. There are at least four dominant cell types in the coelomic fluid of the sea urchin, and Johnson (1969) concluded that it was one particular cell type, the phagocytic coelomocytes, that first adhered. The other cell types were trapped later by some other means. Further experimentation revealed important features of the clotting response, namely a calcium requirement (Bookhout and Greenburg, 1940; Davidson, 1953; Donnellon, 1938) and dependence upon the formation of disulfides for clotting to occur (Bertheussen and Seijelid, 1978; Boolootian and Giese, 1959). My dissertation work is based upon their many careful observations obtained over more than a century, and with the help of modern molecular biology techniques I was able to extend the knowledge of this cell-adhesion event.

This dissertation is divided into four self-contained chapters that report my results, each containing its own introduction. To avoid excess repetition, I will introduce the investigations of each chapter at their most basic level.

Chapter I forms the basis for this dissertation and was published in *The Journal of Cell Biology* (2003) 160:597-604. Therein, I identify the protein component of the cell-free coelomic fluid that mediates the clotting response. This protein was named amassin, and its amino acid sequence was determined by cloning its transcript. Amassin contains a domain, known as olfactomedin (OLF), in its C-terminal half. Interest in the function of the OLF domain has intensified in recent years. This is primarily due to the identification of the OLF protein myocilin in the formation of some forms of inherited glaucoma, the most common cause of blindness in humans (Fautsch *et al.*, 2000; Hardy *et al.*, 2005; Nguyen *et al.*, 1998; Ray *et al.*, 2003; Stone *et al.*, 1997). Neurological effects of other OLF domain-containing proteins have increased the OLF domain's intrigue. For example, recent findings have demonstrated that similar proteins are involved in neural crest cell formation (Barembaum *et al.*, 2000; Moreno and Bronner-Fraser, 2001), organization of the neuromuscular junction (Loria *et al.*, 2004), and formation of the nodes of Ranvier (Eshed *et al.*, 2005). This chapter's results provided the first functional assay for an OLF protein, and my further work has benefited from it.

In Chapter II, I have expressed various portions of the amassin protein in the yeast, *Pichia pastoris*, in order to separate the structural features of amassin and ascertain their function in clotting. A calcium-dependent cell-binding activity was found for the OLF domain of amassin. Amassin forms disulfide-linked dimers through the coiled-coil region, but requires higher multimeric states in order to achieve its biological activity. A rough quaternary structural model for amassin is presented that summarizes the structural data.

To further study the OLF domain in the sea urchin, I have identified four additional OLF domain-containing proteins, bringing the total to five. This work utilized genomic assemblies from the Sea Urchin Genome Project (http://sugp.caltech.edu/) as the first step in cloning their transcripts. Their sequences are presented in Chapter III, along with a detailed analysis of their structural features, and their phylogenetic relationship to the OLF family as a whole. All five of the OLF family members are expressed in coelomocytes, with a potential involvement in the clotting response mediated by amassin. In addition to examining coelomocytes, I investigated the developmental regulation of OLF family members and report the results in Chapter III. Samples from the entire larval cycle of this indirect developing urchin, extending from the egg to metamorphosis at 35 days, were analyzed (Fig. 2). This analysis is fairly unusual, as most developmental studies in the sea urchin focus on the early embryonic stages, terminating at 1-3 days. Since *S. purpuratus* goes through an indirect development, the formation of the adult tissues occurs much later, and the functions of OLF family members might be overlooked if not examined at all stages of the larval cycle.

A three-dimensional structure has yet to be determined for the OLF domain. In light of its function in coelomocyte cell adhesion, and its emerging role in many important cellular events, I have performed the initial steps on the road to determining its crystal structure. These results are described in Chapter IV, the final chapter. The OLF domain of amassin, expressed in bacteria, was crystallized. A native data set was collected, extending to 2.7 Å, and molecular replacement was attempted. Unfortunately, this method was not successful. Experimental phases will most likely be required to solve the first structure of an OLF domain.

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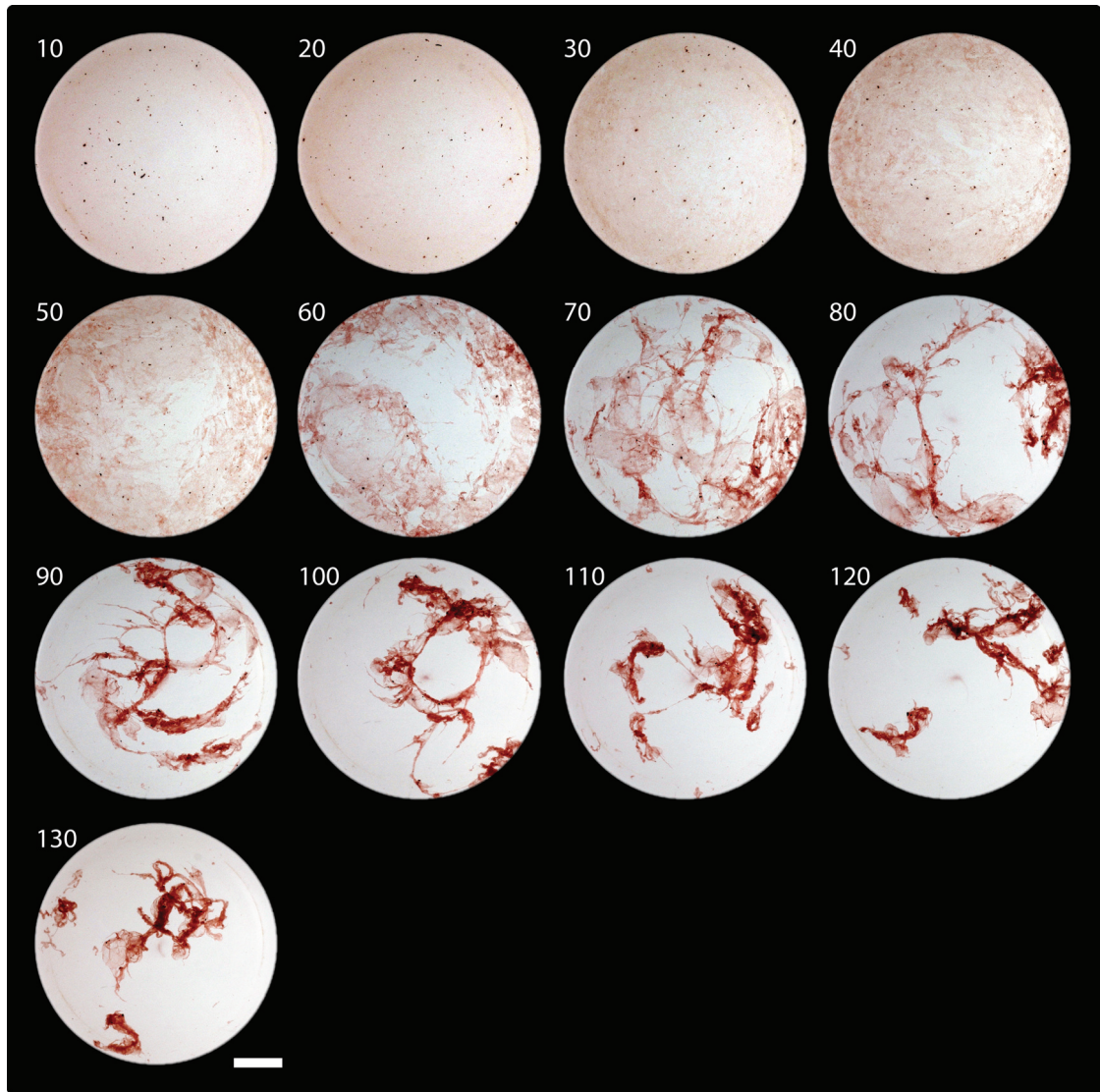
Figure 1
The clotting of sea urchin coelomocytes. Sea urchin coelomocytes form a massive cellular clot by rapid intercellular adhesion. The coelomic contents of one animal was poured into a beaker, constantly swirled, and top-view photographs were taken every 10 s. The coelomic fluid remains relatively uniform from 10-30 s, while at 50 s aggregates are clearly apparent and become progressively larger. After 70 s the clot begins to form with stringy cellular masses continuing to adhere to each other. By 130 s clot formation is complete. Scale bar = 1 cm.

Figure 2
Indirect development of the sea urchin, *S. purpuratus*. In its development, the larval sea urchin passes through many stages before metamorphosis to the adult. In this composite photograph, larvae are shown orientated with the oral surface towards the top, but orientation along other axes is not preserved. Top row: fertilized egg, 4-day-old late prism, 6-day-old early plutei, and 10-day-old pluteus. Second row: 15-day-old 4-arm stage, 20-day-old 6-arm stage, and 25-day-old 8-arm stage (early rudiment, which will become the adult, is visible to the left of its stomach). Third row: 30-day-old late larva (large rudiment on the right in this orientation), 35-day-old larva undergoing metamorphosis (a tube foot can be seen protruding from the right), and another larval metamorph (its five tube feet are extended and the larval body remains on its back). Bottom-left, a juvenile urchin (early spines and pedicellaria are present). Scale bar = 250 μm.

Chapter I

Amassin, an Olfactomedin Protein, Mediates the Massive Intercellular Adhesion of Sea Urchin Coelomocytes

ABSTRACT

*S*ea urchins have a fluid-filled body cavity, the coelom, containing four types of immunocytes called coelomocytes. Within minutes after coelomic fluid is removed from the body cavity, a massive cell-cell adhesion of coelomocytes occurs. This event is referred to as clotting. Clotting is thought to be a defense mechanism against loss of coelomic fluid if the body wall is punctured, and it may also function in the cellular encapsulation of foreign material and microbes. Here we show that this intercoelomocyte adhesion is mediated by amassin, a coelomic plasma protein with a relative molecular mass (M_r) of 75 kD. Amassin forms large disulfide-bonded aggregates that adhere coelomocytes to each other. One half of the amassin protein comprises an olfactomedin (OLF) domain. Structural predictions show that amassin and other OLF domain-containing vertebrate proteins, share a common architecture. This suggests that other proteins of the OLF family may function in intercellular adhesion. These findings are the first to demonstrate a function for a protein of the OLF family.

INTRODUCTION

Sea urchin coelomocytes are immune cells contained in the coelomic cavity at ~7.5 x 10^6 cells/ml (Coffaro and Hinegardner, 1977; Gross *et al.*, 2000; Pancer, 2000; Pancer *et al.*, 1999; Smith *et al.*, 1996). Four major types of coelomocytes are recognized: red amoebocytes (~15%, also called red spherule cells), colorless amoebocytes (~5%, also called colorless spherule cells), round vibratile cells with a single long flagellum (~14%), and phagocytes (~66%; Smith *et al.*, 1992). When sea urchin body fluid is removed from the animal, the coelomocytes rapidly adhere together, or clot, in a massive intercellular adhesion.

The literature from 1879 to 1959 on the clotting of coelomocytes of all echinoderm classes has been reviewed (Endean, 1966). Johnson (1969) summarized all previous work in this field and compared it to her observations on the behavior of sea urchin coelomocytes cultured in hanging drops. She concluded that clotting was a cell-cell adhesion of the phagocytic coelomocytes, the other three types of cells were passively trapped in, or adhered to, the surfaces of clots. Other results showed that the cell-free plasma (CFP) did not form a gel, and clot formation was irreversible. A calcium requirement for clotting had been demonstrated by others (Bookhout and Greenburg, 1940; Davidson, 1953; Donnellon, 1938). Clotting was also found to depend on the formation and presence of disulfide bonds (Bertheussen and Seijelid, 1978; Boolootian and Giese, 1959). More recent work showed that clotting was accompanied by a massive rearrangement of the actin cytoskeleton of the phagocytes that transformed from a petaloid to a filopodial morphology (Edds, 1977; Otto and Bryan, 1981).

The clotting of sea urchin coelomocytes is quite different from the mechanism of blood clotting in mammals (Doolittle, 1984), horseshoe crabs (Tai *et al.*, 1977), and lobsters (Fuller and Doolittle, 1971) because unlike these other animals, the protein concentration of sea urchin coelomic plasma is low (0.3-0.7 mg/ml) and varies among individuals and with the season (Holland *et al.*, 1967). Also, >50% of the soluble coelomic protein is a molecule of ~180 kD that is related to vertebrate transferrin (Brooks and Wessel, 2002) and has no clotting activity in our assay. We decided to study the biochemistry of clotting of sea urchin coelomocytes in an attempt to increase the general knowledge concerning different molecular mechanisms of intercellular adhesion.

RESULTS

The clotting of coelomocytes

The clotting of coelomocytes can be demonstrated by pouring coelomic fluid into a beaker, swirling it gently, and observing with the unaided eye. Clot formation is visible within 50 s and is complete by 150 s (Fig. 1). Clots settle onto the substratum; the cells spread out at the edges of the clots and appear viable for 1-3 d. To study the biochemistry of intercoelomocyte adhesion, we devised a reliable method to prevent clotting. This is accomplished by rapidly mixing whole coelomic fluid (WCF) with an approximately equal volume of an isosmotic, buffered calcium chelating solution. If calcium is then added to the clot-inhibited cells (to ~10 mM), then coelomocyte clotting proceeds normally as if just removed from the animal. However, if the coelomocytes are gently sedimented from the clot-inhibited WCF and washed free of the coelomic plasma, clotting does not occur following the addition of calcium. The supernatant coelomic plasma after removal of the cells, termed CFP, induces the clotting of the washed cells in a concentration-dependent manner (Fig. 2). Thus, addition of both CFP and calcium to the washed coelomocytes is required for clotting.

The plasma factor is a protein

A standard assay for coelomocyte clotting was developed (Fig. 2). Sufficient CFP to induce complete clotting was incubated for 10 min at temperatures 23-100°C before being assayed. A temperature of 70°C abolished clotting activity (unpublished data). CFP was also incubated for various times in 0.1 mg/ml pancreatic trypsin at 23°C. Soybean trypsin inhibitor was added (0.2 mg/ml) to stop the digestion, and the clotting assay performed. A 2-h digestion was needed to completely

abolish the clotting activity (unpublished data). The heat and protease sensitivity suggested that the clotting factor is a protein.

Identification of the plasma factor

CFP from clot-inhibited WCF was obtained and subjected to ultracentrifugation (150,000 g for 1 h). Clotting activity was present in both the supernatant and the pellet; however, there appeared to be more activity in the pellet. SDS-PAGE separation of the proteins in the supernatant and the pellet showed that the pellet had a less complex protein composition. The ultracentrifuge pellets were solubilized in a buffered salt solution and subjected to anion exchange chromatography on DEAE-Sepharose. After washing and eluting with a linear salt gradient, an aliquot of each fraction was taken and assayed for clotting activity. Silver-stained gels of the eluted fractions (Fig. 3) showed that the coelomocyte clotting activity was associated with a diffuse band of M_r ~75 kD (hereafter called amassin; fractions 10-17). The purity of the 75-kD protein in some of the fractions (fractions 12-16) suggested that it is the only plasma protein required for intercoelomocyte adhesion.

Antibody to amassin

Approximately 400 µg of amassin was purified by DEAE chromatography, and rabbit antiserum was raised to the protein. Specific IgG was affinity purified using a column of the full-length, bacterially expressed amassin protein (bacterially expressed amassin does not clot washed coelomocytes). Western blots of CFP showed that the purified antiserum recognized only the amassin band (Fig. 4 A). The whole antiserum inhibited amassin-induced clotting of coelomocytes, whereas control serum had no inhibitory activity (Fig. 4 B). Immunofluorescence localization shows

that amassin is present between adhering coelomocytes (Fig. 5). In larger clusters of coelomocytes, amassin is seen to bridge distant cells. Only minimal fluorescence is seen inside coelomocytes.

Coelomocyte clotting depends on amassin forming disulfide-bonded networks

Disulfide bond-reducing agents such as DTT inhibit coelomocyte clotting in the presence of calcium. If reducing agents such as DTT or β-mercaptoethanol are excluded from the SDS-PAGE sample buffer when the components of CFP are separated by SDS-PAGE, amassin does not enter the stacking gel (Fig. 6). Treatment of the CFP samples with different concentrations of reducing agent, followed by SDS-PAGE and Western blotting, shows the presence of monomers, dimers, and higher multimers of amassin (Fig. 6).

Clots of coelomocytes were washed with seawater to remove coelomic plasma proteins, treated with 20 mM DTT in seawater at pH 8, and gently agitated. In ~20 min, the clots dissociated into single cells. The cells were sedimented by low speed centrifugation, and the supernatant was centrifuged at 10,000 g for 30 min. SDS-PAGE analysis of the resulting supernatant shows that amassin is the major protein released as a clot is dissociated by DTT treatment into single cells (Fig. 7 A). The amassin recovered from clot dissociation was inactive as an inducer of coelomocyte clotting, showing that reduction of all disulfide bonds might denature the protein (unpublished data). Amassin is highly enriched in samples of coelomocytes allowed to clot (followed by washing) compared to washed clot-inhibited cells (Fig. 7 B). Only after long exposures is a slight detection of amassin possible in washed coelomocytes. Amassin seems to be most prominent in the plasma and in clotted coelomocytes.

The data obtained by the use of DTT suggest that amassin is present in CFP as disulfide-bonded aggregates. A sample of CFP was divided and one half treated with 10 mM DTT before ultracentrifugation (200,000 g for 2 h). A Western blot of the supernatant and the pellet showed that amassin was highly enriched in the supernatant of the DTT-treated sample but undetectable in the nontreated supernatant, indicating that the protein is present in CFP as disulfide-bonded aggregates (Fig. 7 C).

Cloning of the amassin cDNA

Using purified amassin, amino acid sequences were obtained from trypsin and cyanogen bromide-generated peptides. From this sequence, degenerate primers were designed (Table I) and used to obtain the complete 1,692 bp cDNA and derived amino acid sequences (sequence data available from GenBank/EMBL/DDBJ under accession no. AF533649). There is an 84-bp 5' untranslated region and a 123-bp 3' untranslated region containing a typical AATAAA polyadenylation signal 4 bp before the poly(A) tract begins. All seven sequenced peptides, totaling 112 aa, were found in the derived amino acid sequence of 495 residues, proving that the correct cDNA had been cloned. The antibodies made to native amassin specifically recognize a portion of the amassin sequence expressed recombinantly in bacteria (Fig. 8 A). Amassin has five potential N-linked glycosylation sites which probably carry oligosaccharide chains, since digestion with the enzyme peptide N-glycosidase-F (PNGase-F) decreases amassin's M_r from 75 to 60 kD (Fig. 8 B) which is close to the calculated molecular weight of 56.5 kD. The remaining molecular weight discrepancy could be due to other post-translational modifications such as O-linked glycosylation.

Structural analysis

The amassin sequence is similar in the COOH-terminal half (residues 230-477) to a family of proteins that contain an olfactomedin (OLF) domain. This domain is found in a variety of extracellular proteins. To investigate amassin's OLF domain in more detail, structural predictions were performed using alignments as input for a consensus prediction method (Fig. 9, A and B). This predicted what would be classified as an all β-strand structure for amassin's OLF domain (though one short α-helix is predicted), which most likely has a globular conformation. Additionally, by the same prediction criteria a two β-strand segment in the NH$_2$-terminal region (residues 39-59) is predicted (Fig. 9 B). The all β COOH-terminal half containing the OLF domain is in contrast to the NH$_2$-terminal half of amassin that is predicted to contain α-helical coiled-coils from residues 75-108 and 126-212 (Fig. 9 C). There is also a predicted signal peptide from residues 1-28.

A structural comparison was made between amassin and four OLF family members: noelin, myocilin/TIGR, tiarin, and olfactomedin. Using the same prediction criteria, these five proteins share a striking similarity in the presence, size and order of motifs (Fig. 9 C). All five have a conserved cysteine (amassin residue 203) at or near the end of the coiled-coil domain in the proper location of a heptad repeat to form an interchain disulfide bond (Lupas *et al.*, 1991). Coiled-coils are common dimerizing motifs, and these are most likely parallel in orientation with a stabilizing disulfide bond. Other cysteine positions are also conserved; all five proteins contain 2-3 cysteines within, and just after, the NH$_2$-terminal β-region (Fig. 9, B and C). Four of the five proteins have a cysteine just before the first β-strand of the OLF domain, and all five have a cysteine at position 407 within the

domain (Fig. 9 A). With only 19-23% amino acid identity between amassin and these four other proteins, this structural comparison provides evidence that these five proteins may share a similar three-dimensional structure and thus might have a similar function.

Cell surface receptor for amassin

Washed coelomocytes were treated with 0.1 mg/ml pancreatic trypsin for 30 min followed by the addition of soybean trypsin inhibitor to a final concentration of 0.2 mg/ml. Cell free plasma (9 μg/ml) and calcium (10 mM) was then added, and the tubes gently mixed for 10 min. The cells exposed to trypsin did not clot, suggesting that amassin binds to a cell surface protein (unpublished data). A control sample in which trypsin and the inhibitor were added together for 30 min clotted normally.

DISCUSSION

Coelomocytes keep the sea urchin free of microorganisms by binding and phagocytosing foreign materials (Clow *et al.*, 2000; Pancer, 2000; Pancer *et al.*, 1999; Smith *et al.*, 1992; Smith *et al.*, 1996). Clotting of coelomocytes is thought to be a mechanism by which holes are plugged in the sea urchin's body wall and microbes and foreign particles are encapsulated (Endean, 1966; Johnson, 1969). Until now, the mechanism of coelomocyte clotting has not been studied biochemically.

The fact that calcium ions are required for clotting was known previously (Bookhout and Greenburg, 1940; Davidson, 1953; Donnellon, 1938), and chelation of calcium has been employed to block clotting by those studying these cells (Edds, 1977). This requirement was also found in our work and used as a means to prevent clotting from occurring as a first step in studying the process of clotting. Whether the calcium requirement is involved in exocytosis of a needed molecule or plays a role in the maintenance of protein structure or enzyme activation remains unknown.

We have shown here that plasma-free coelomocytes, calcium, and amassin are the only three components needed for clotting in our *in vitro* assay. We cannot rule out the involvement of other minor proteins in the plasma, which might have been present in our purified amassin, although by silver-staining amassin appears highly purified (Fig. 3).

The mechanism that triggers clotting remains unknown. An uninjured animal must have a way to keep clotting from occuring. The washed coelomocytes might have a mechanism to become adhesion competent within seconds after injury to the coelomic cavity. Since treatment of coelomocytes with protease prevents their

ability to clot, a cell-surface protein seems to be involved. This could be a receptor protein which is exposed or activated on the surfaces of coelomocytes in response to injury. Integrins, which are expressed on coelomocytes (Burke, 1999), or cell-surface lectins, would be excellent candidate amassin receptors which could also regulate downstream signaling events.

Essentially nothing is known about the production of coelomocytes by the adult sea urchin. Although the antibody to amassin does not stain coelomocytes brightly and Western blots of coelomocytes show a weak reaction, amassin cDNA can be obtained from total coelomocyte RNA, indicating that at least some amassin could be synthesized in coelomocytes. Amassin appears to be a minor component of CFP, since Coomassie-stained SDS gels of CFP show only minor bands in the 75-kD region. The 180-kD transferrin-like protein (Brooks and Wessel, 2002) is >50% of the total protein in CFP; it does not possess clotting activity in our assay.

The OLF domain present in amassin is found in many extracellular proteins including the following: noelin, which has a neurotropic effect in development with a role in neural crest formation (Barembaum *et al.*, 2000) and neurogenesis (Moreno and Bronner-Fraser, 2001); myocilin/TIGR, which is thought to have a role in the aqueous outflow of the vertebrate eye and is often mutated in some forms of glaucoma (Fautsch *et al.*, 2000; Nguyen *et al.*, 1998; Stone *et al.*, 1997; Tamm and Russell, 2001); tiarin, a protein mediating dorsalization of the amphibian neural tube (Tsuda *et al.*, 2002); and olfactomedin, a protein found in the mucus of bullfrog olfactory neuroepithelium (Snyder *et al.*, 1991; Yokoe and Anholt, 1993). Although their roles are being elucidated, a function for proteins with an OLF domain has remained unknown.

The previous finding that coelomocytes fail to clot in the presence of *n*-ethyl maleimide (Bertheussen and Seijelid, 1978) and reducing agents (Boolootian and Giese, 1959) suggested that disulfide bond formation is required for clotting. These observations can be explained by the sensitivity of amassin to reductants. Clotting, allowed to proceed to completion, can be reversed by incubation with DTT and in the process release amassin as the dominant protein (Fig. 7 A). Amassin appears to exist as homooligomers held together by disulfide bonds; monomers, dimers, and higher multimers resolve on SDS-PAGE depending on the concentration of disulfide-reducing agent (Fig. 6). The coiled-coil regions of amassin could be involved in dimer formation. In myocilin/TIGR, the coiled-coil region has been shown by yeast two-hybrid analysis to possess dimerizing ability (Fautsch and Johnson, 2001; Wentz-Hunter *et al.*, 2002). The oligomerization of the OLF family proteins TIGR (Nguyen *et al.*, 1998) and olfactomedin (Snyder *et al.*, 1991) has also been observed. Our structural analysis suggests that the other OLF family members (Fig. 9 C), known to reside in the extracellular matrix, might also be involved in formation of large polymer networks governing cell adhesion in a manner similar to amassin.

MATERIALS AND METHODS

Clot inhibition

Sea urchins (*Strongylocentrotus purpuratus*) were maintained in fresh flowing seawater. WCF was obtained by rapid removal of Aristotle's lantern (the jaws) and mixing of the coelomic fluid with an approximately equal volume of clot-inhibiting medium (CIM; 100 mM Hepes, 360 mM NaCl, 10.5 mM KCl, 29 mM Na_2SO_4, 2 mM $NaHCO_3$, 20 mM EGTA, pH 7.9). The isosmolarity with seawater of CIM was verified by vapor pressure osmometry. After mixing approximately equal portions of WCF and CIM the resultant pH was 7.5.

Preparation of washed coelomocytes and CFP

A three-step centrifugation method was used which sedimented enriched subpopulations of the four types of coelomocytes and were later combined. This was necessary to minimize damage to the fragile cells from excessive centrifugation, since the four types differ greatly in density. Clot-inhibited WCF was centrifuged (4^oC) for 2 min at 150 g, which sedimented the red and colorless amoebocytes (also called spherule cells). The supernatant was then centrifuged at 150 g for 5 min to sediment the vibratile cells. Finally, the resulting supernatant was centrifuged at 600 g for 10 min to sediment the remainder of cells, mostly phagocytes (to prepare CFP, the supernatant above the sedimented phagocytes was centrifuged at 10,000 g for 20 min). After removal of the coelomic plasma from each enriched subpopulation, the cells were washed by resuspension in buffered calcium-free artificial seawater (10 mM Hepes pH 7.5, 450 mM NaCl, 10.5 mM KCl, 26 mM $MgCl_2$, 30 mM $MgSO_4$, 2 mM $NaHCO_3$) and resedimented as before. All three enriched subpopu-

lations were resuspended in buffered calcium-free artificial seawater and combined at a concentration of 2×10^6 cells/ml.

Assay for clotting activity

Washed coelomocytes (1 ml) were combined with 20 µl of sample to be tested in a 1.5-ml microcentrifuge tube. One molar $CaCl_2$ was then added to a final concentration of 10 mM followed by end-over-end rotation for 10 min. Qualitative scoring could be accomplished at this stage by visual inspection and comparison to controls (buffer only for the negative control and 10 µg unfractionated CFP for the positive control). For a quantitative measure of degree of clotting, after the 10-min rotation the partially clotted cell suspension was passed through a nylon mesh of 70 µm (Spectra). The light-scattering ability of the filtrate was then measured at 350 nm and normalized to the same controls to determine fraction clot completion.

Heat and trypsin treatment of CFP

Samples of CFP (0.5 mg/ml) were incubated for 10 min at 23, 37, 42, 50, 70, and 100°C and then added at a final concentration of 5 µg/ml to 1 ml washed coelomocytes and scored for activity. CFP was also incubated for 0, 15, 30, 60, 120, and 240 min in 0.1 mg/ml pancreatic trypsin at 23°C. Soybean trypsin inhibitor was added (0.2 mg/ml) to stop the digestion, and the clotting assay was performed.

Purification of amassin

The protein from the CFP of 10 sea urchins (400 ml) was precipitated by addition of ammonium sulfate to 70% saturation. After centrifugation at 10,000 g (20 min), the pellet was redissolved in 8 ml CIM and centrifuged at 150,000 g for 45 min. The resulting pellet was redissolved in 10 mM Tris pH 8, 100 mM NaCl, 0.5

mM EDTA and loaded onto 10 ml DEAE-Sepharose (Amersham Biosciences) equilibrated in the same buffer. After washing with 10 column vol, 3-ml fractions were collected during elution with a linear gradient of NaCl (100-1,000 mM). Pure fractions of amassin were combined and concentrated by stirred cell ultrafiltration (Amicon) with a final yield of 0.4 mg. Estimating losses at all steps leads to an amassin protein concentration in the coelomic plasma at <1% of the total plasma protein. The concentration of total protein in CFP varies from 0.3 to 0.7 mg/ml.

DTT treatment to dissociate clots

Clots were dissociated into single cells by first washing them with excess filtered seawater, then incubating the washed clots in 0.45 μm filtered seawater containing 20 mM DTT and 20 mM Hepes, pH 8, with mild agitation for 20 min. Microscopic observation showed the coelomocytes were intact after dissociation. The cells were removed by sedimentation at 1,000 g for 10 min. The supernatant was then centrifuged at 10,000 g for 30 min to sediment debris. As a control, washed, clot-inhibited coelomocytes were treated in the same manner with DTT, and the supernatant isolated.

PNGase-F digestion

Purified amassin (3 μg in 10 mM Tris, 200 mM NaCl, and 0.5 mM EDTA) was brought to 0.5% SDS and 50 mM β-mercaptoethanol in a volume of 10 μl and then boiled for 5 min. Five μl of 7.5% NP-40 was added followed by 2 μl of PNGase-F and 13 μl water. The digestion was allowed to proceed overnight at 37ºC.

Antibody preparation

Purified amassin (400 μg) was used to raise rabbit antiserum (Strategic Biosolutions). Antiserum was affinity purified on a column of bacterially expressed full-length amassin coupled to Affigel-10 following the manufacturer's directions (Bio-Rad Laboratories).

Cloning amassin sequence

Purified amassin was separated and excised from an SDS-PAGE gel and sent to the PAN Facility at Stanford University, where tryptic fragments were generated and sequenced. Additional CNBr-generated fragments were transferred to PVDF membrane and sequenced at the University of California at San Diego Protein Microsequencing Laboratory. Total RNA was isolated from an entire 7 g sea urchin by homogenization in a Waring blender with 40 ml of Trizol (Invitrogen) followed by centrifugation at 150 g for 5 min to clear debris. The supernatant was then processed following the manufacturers instructions. PolyA+ RNA was purified (Ambion), and first-strand cDNA was synthesized with SuperScript II (Invitrogen) using random hexamers. Degenerate primers were designed from the peptide sequence and used to PCR amplify fragments of the amassin sequence (Table I). From the sequence of those PCR products, exact match primers were synthesized and used to complete the 1,692-bp amassin cDNA sequence by 5' and 3' rapid amplification of cDNA ends (Ambion). Sequence data for amassin is available from GenBank/EMBL/DDBJ under accession no. AF533649.

Western immunoblots

Proteins were resolved by SDS-PAGE then transferred to Immobilon-P membrane (Millipore) in a tank transfer apparatus (Bio-Rad Laboratories). The membrane was blocked in 5% dry milk in TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h, followed by incubation with a 1:25,000 dilution (0.5 mg IgG/ml stock) of purified antiamassin in blocking buffer for 1 h, and washed four times for 15 min each. The membrane was then incubated with goat anti-rabbit IgG HRP-conjugated secondary antibody (Jackson ImmunoResearch) at a 1:50,000 dilution in blocking buffer for 1 h, and washed four times for 15 min each. Amassin was detected with SuperSignal substrate (Pierce Chemical Co.).

Immunofluorescence

Coelomocytes in CIM were allowed to settle onto protamine-coated coverslips and were fixed for 45 min in seawater containing 20 mM Hepes, pH 7.5, 0.1% vol/vol glutaraldehyde and 3% wt/vol PFA. After washing, coverslips were blocked in TBST with 1% BSA and 5% normal goat serum. Affinity-purified antiamassin was used at various dilutions for 1 h. After washing, the secondary antibody, Alexa Fluor 546 goat anti-rabbit (Molecular Probes), was applied for 1 h at a 1:400 dilution. Negative controls of normal rabbit serum and no primary serum had absolutely no fluorescence after incubation with the Alexa Fluor secondary antibody.

Structural analysis

The five proteins (sequence data available from GenBank/EMBL/DDBJ) compared are: amassin from *Strongylocentrotus purpuratus* AF533649, noelin-1 from *Gallus gallus* Q9IAK4 (Barembaum *et al.*, 2000), myocilin/TIGR from *Bos taurus*

Q9XTA3 (Taniguchi *et al.*, 2000), tiarin from *Xenopus laevis* BAB85495 (Tsuda *et al.*, 2002), and olfactomedin from *Rana catesbeiana* Q07081 (Yokoe and Anholt, 1993). Assignment OLF domain positions were obtained with a Pfam search (Bateman *et al.*, 2002). Secondary structure predictions were performed by using a sequence alignment of the OLF domains or the NH_2-terminal β-regions from the five proteins as input for the consensus method utilized by Jpred (Cuff *et al.*, 1998). Signal peptides were predicted with the web server SignalP (Nielsen *et al.*, 1997). The program Coils (Lupas *et al.*, 1991) showed a high probability (>5 with a window of 28 residues) for the locations of the α-helical coiled-coils.

ABBREVIATIONS

CFP, cell-free plasma; OLF, olfactomedin; PNGase-F, peptide *N*-glycosidase-F; WCF, whole coelomic fluid.

ACKNOWLEDGMENTS

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Table 1
Amassin peptide sequences obtained, and corresponding degenerate oligonucleotide primers used to clone cDNA.

Peptide sequences (NH$_2$ to COOH termini)	Synthetic oligonucleotides (5' to 3')
Trypsin	
DII<u>HVSEPFT</u>VR (229-240)	CAYGTIWSIGARCCNTTYAC
	GTRAANGGYTCISWIACRTG
<u>I</u>GAW<u>FRDP</u>LQDYIK (246-259)	GGIGCNTGGTTYMGNGAYCC
	GGRTCNCKRAACCANGCICC
<u>LDPETLDV</u>IETWVAP (380-394)	GAYCCNGARACIYTNGAYGT
	ACRTCNARIGTYTCNGGRTC
LYG<u>WDNGHQV</u>VYDLTFDPPAR (460-480)	TGGGAYAAYGGNCAYCARGT
	ACYTGRTGNCCRTTRTCCCA
SQLLPT<u>DTNIPLNLQ</u> (481-495)	GAYACIAAYATHCCNYTIAA
	TTIARNGGDATRTTIGTRTC
Cyanogen Bromide	
RTTSGVV (40-46)	
MTLEGVLDGTVVVTVAELELVKEELKEM (117-144)	

NH$_2$-terminal peptide sequences obtained following trypsin or cyanogen bromide digestion. Peptide positions within the complete amassin protein sequence are noted in parentheses. Underlined amino acids represent the region to which the degenerate oligonucleotide primers were directed. Forward (top) and reverse (bottom) direction oligonucleotides were synthesized. Degenerate positions are denoted as follows: Y=C or T, N=A or C or G or T, R=A or G, I=inosine, M=A or C, K=G or T, W=A or T, S=C or G, H=A or C or T, D=A or G or T.
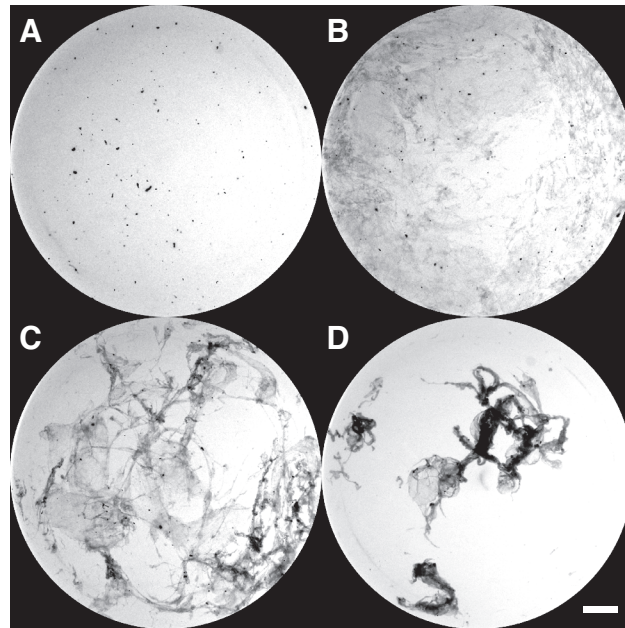
Figure 1
The clotting of coelomocytes. Sea urchin coelomocytes form a massive cellular clot by rapid intercellular adhesion. The contents of the coelomic cavity of one animal was poured into a beaker, constantly swirled, and top view photographs were taken. (A) After 10 s, aggregates of coelomocytes cannot be seen. (B) After 50 s, the cells have begun to form aggregations. (C) After 70 s, the clot begins to form with stringy cellular masses continuing to adhere to each other. (D) By 150 s, clot formation is complete. Bar, 1 cm.
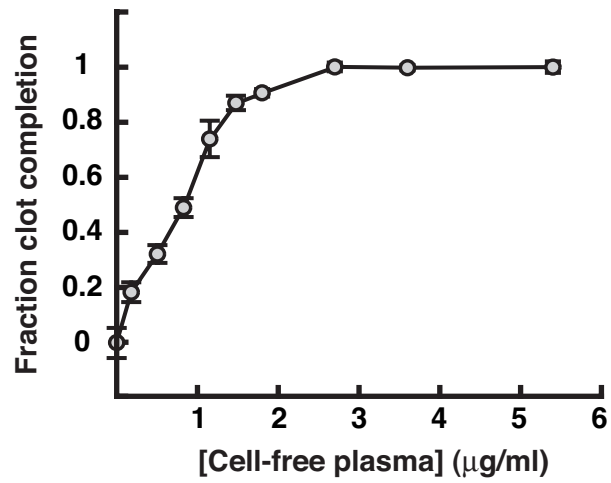
Figure 2
An assay for the level of coelomocyte clotting. The degree of coelomocyte clotting depends on the concentration of CFP. Complete clotting occurs at 3 µg CFP/ml. Data points are the mean of five experiments with the same batch of cells (standard error bars shown). Clot completion is defined as the level of clotting that 10 µg CFP would promote compared with no clotting with buffer alone.
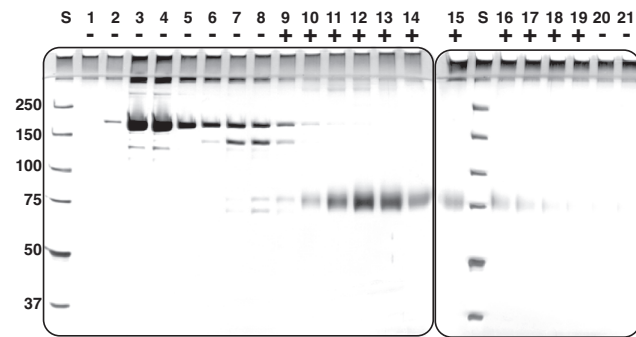
Figure 3

DEAE-Sepharose chromatography of cell-free coelomic plasma. A silver-stained 7.5% SDS-PAGE separation of proteins eluting from the DEAE column upon application of a linear salt gradient that increases left to right. Numbers on top of lanes represent individual fractions. A 10-µl aliquot of each fraction was subjected to the clotting assay; + denotes the ability to clot, and - fractions had no detectable clotting activity. The positive fractions are highly enriched for a 75-kD protein called amassin. S, molecular mass standards presented in kD.
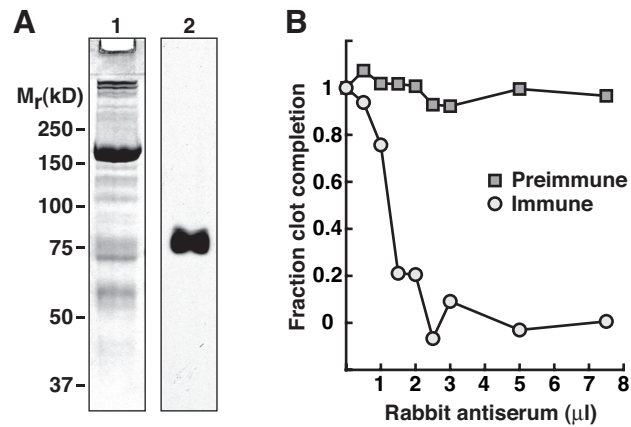
Figure 4

Antibodies to amassin prevent clotting. (A) Western blots of CFP show that the antibody reacts only with the 75 kD amassin. CFP (5 µg) was loaded onto lane 1 and stained with Coomassie. The sample for lane 2 was 300 ng CFP and detected by Western blot with anti-amassin. (B) Preincubation of CFP with antiamassin inhibits coelomocyte clotting. Crude antiserum was incubated with sufficient CFP to induce complete clotting (3 µg/ml) for 15 min on ice in a volume of 20 µl before adding to washed coelomocytes in the clotting assay.
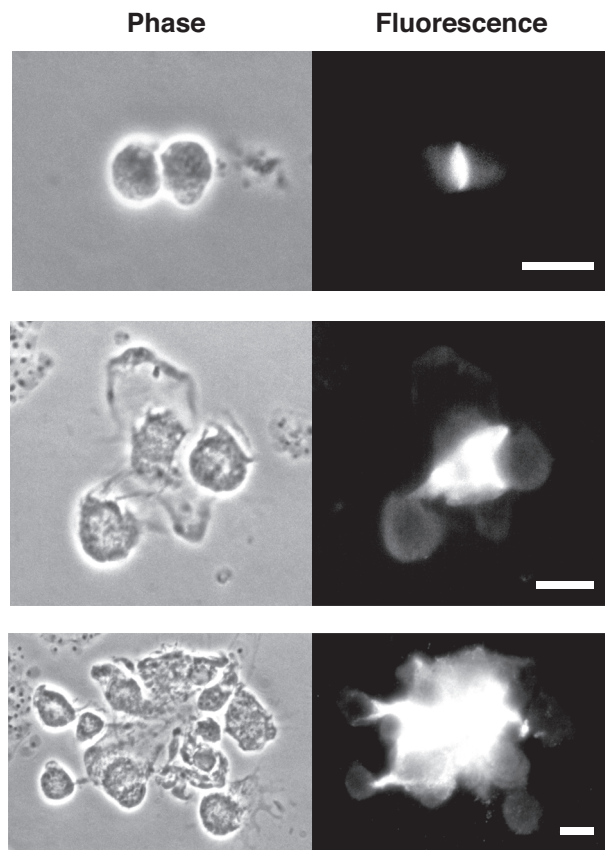
Figure 5
Antibody localizes amassin between adhering coelomocytes. The left panels are phase contrast, and right panels are fluorescence with antiamassin. In the top set, amassin binds two coelomocytes together. In the bottom two sets, larger groups of coelomocytes are shown. Bars,15 μm.
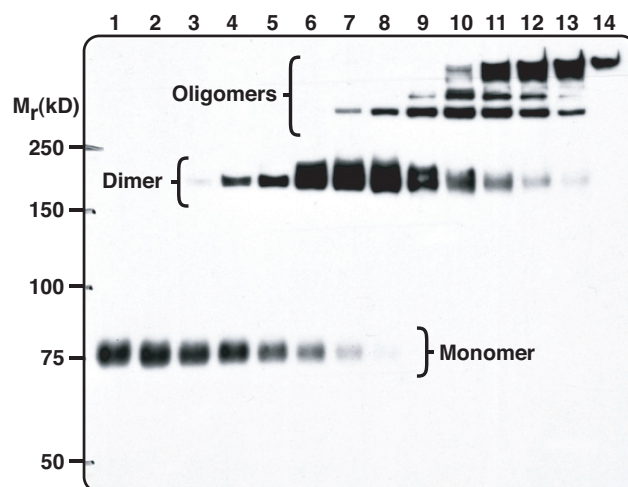
Figure 6

Amassin is disulfide bonded into large aggregates. Samples of CFP were incubated with decreasing concentrations of DTT (twofold dilutions starting from 100 mM in lane 1 to 24 µM in lane 13; lane 14 had no DTT) in SDS-PAGE gel loading buffer before separation on a 7.5% gel and immunoblotted with antiamassin. If disulfide bond-reducing agents are excluded from the sample buffer, amassin does not enter the 5% stacking gel (lane 14). Complete reduction of disulfides in lane 1 shows the 75-kD reacting band. The graded concentration series of DTT displays what can be classified as monomers, dimers, and oligomers of amassin.
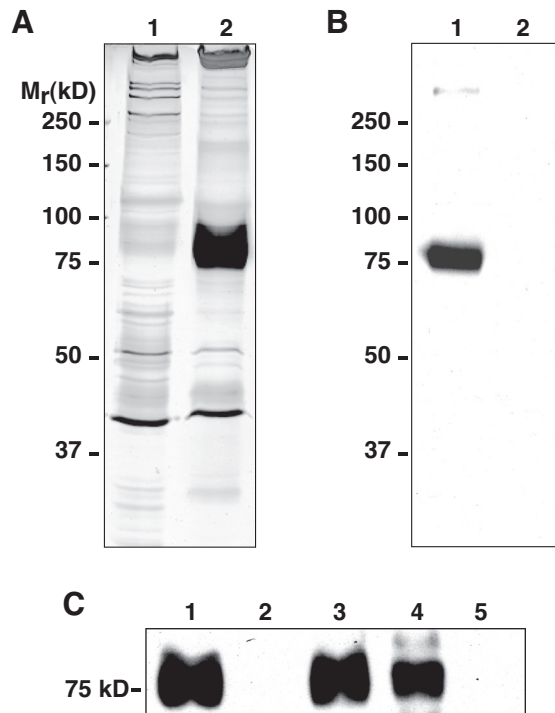
Figure 7

Amassin is released as coelomocyte clots are dissociated into single cells. (A) Washed clots were dissociated in DTT, and the 10,000-*g* supernatant recovered (lane 2). A control sample, lane 1 is the supernatant released from the same DTT treatment to washed, clot-inhibited coelomocytes run on a 10% silver-stained SDS-PAGE gel. DTT dissociates the clot into single cells and releases amassin. (B) An immunoblot probed with antiamassin. Amassin is present in clots of coelomocytes (lane 1), but almost undetectable levels (only after long exposures) are present in a sample of washed clot-inhibited coelomocytes (lane 2). (C) Samples of CFP (300 ng per lane, lane 1 no treatment) were subjected to ultracentrifugation either in the absence (lanes 2-3) or presence (lanes 4-5) of 10 mM DTT. The resulting supernatants (lanes 2 and 4) and pellets (lanes 3 and 5) detected by immunoblot with antiamassin show that amassin is present as a large, disulfide-bonded aggregate which can be dissociated with DTT.
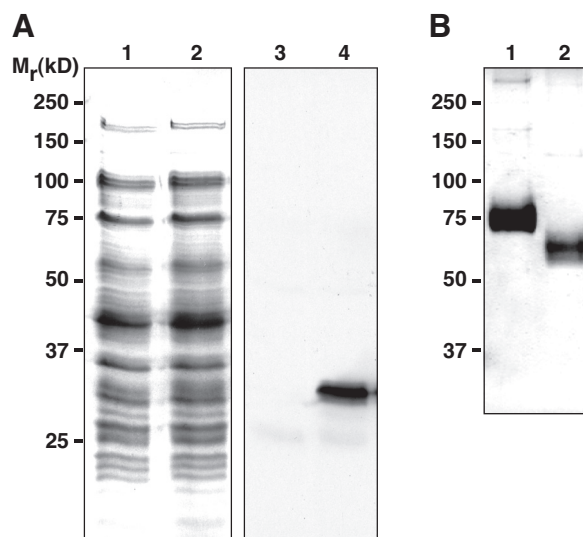
Figure 8

Confirmation of amassin cloning by recombinant protein reactivity and relative molecular mass agreement. (A) The bacterial expression of the derived amino acid sequence from the amassin OLF domain (aa 228-495) is recognized by the antibodies to native amassin. *Escherichia coli* lysates of preinduced (lane 1) and 30 min postinduction (lane 2) are shown stained with Coomassie; lanes 3 and 4 are the same samples but immunoblotted with antiamassin, which specifically detects the OLF domain of the expected size. (B) Amassin is heavily glycosylated. Purified amassin was digested with PNGase-F to cleave off the N-linked oligosaccharides. SDS-PAGE analysis of the digest (lane 2) showed a decrease in M_r relative to the minus enzyme control (lane 1), from 75 kD to ~60 kD.
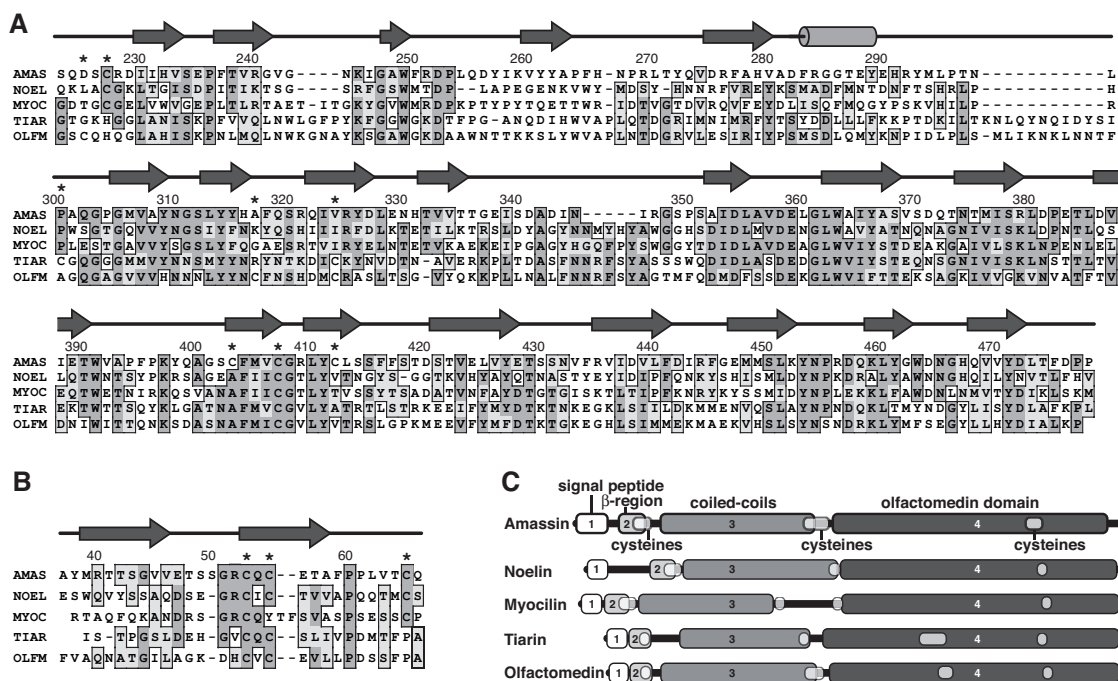
Figure 9

Analysis of the amassin sequence. Sequence alignment of five OLF family members in only the olfactomedin domains (A) or only the NH₂-terminal β-regions (B). AMAS, amassin; NOEL, noelin-1; MYOC, myocilin; TIAR, tiarin; and OLFM, olfactomedin. Amino acid identities are dark shaded, similarities are light shaded, and boxes enclose both. Positions which contain cysteines are noted by an asterisk above the alignment. Predicted secondary structural motifs are depicted with arrows for β-strands and a cylinder for an α-helix. (C) Comparison of key structural motifs in five full-length OLF family members. From NH₂ to COOH terminus there is a signal sequence (1), a short β-region (2), a long coiled-coil motif (3), and the OLF domain (4). All regions containing cysteines are shown. Sequence data for amassin is available from GenBank/EMBL/DDBJ under accession no. AF533649.

The text of Chapter I, in full, is a reprint of the material as it appears in: Hillier B. J. & Vacquier V. D. 2003. Amassin, an olfactomedin protein, mediates the massive intercellular adhesion of sea urchin coelomocytes. *The Journal of Cell Biology* 160(4):597-604 by copyright permission of The Rockefeller University Press. The dissertation author was the primary author, and co-author V. D. Vacquier directed and supervised the research, which forms the basis for this chapter.

Chapter II

Functional Identification of the Structural Features in Amassin, a Cell-binding Olfactomedin Protein

ABSTRACT

The protein amassin mediates a rapid cell adhesion event that tightly adheres sea urchin coelomocytes (body cavity immunocytes) together. Three major structural regions are found along its length: a short β-region, three coiled-coil segments, and an olfactomedin domain. Amassin contains eight bonded cysteines that upon reduction render the protein inactive. Various truncated forms of recombinant amassin were purified from *Pichia pastoris*, and their disulfide bonding patterns and activities investigated. Expressed alone, the olfactomedin domain contained two intramolecular disulfide bonds, existed in a monomeric state, and inhibited the amassin induced clotting of coelomocytes via a calcium-dependent cell-binding activity. The presence of the short N-terminal β-region, containing three cysteines, was not required for its clotting activity. The coiled-coils function to dimerize amassin in a parallel orientation through an essential homo-dimerizing disulfide bond. Neither amassin fragments disulfide-linked as dimers, nor engineered to exist as dimers, were sufficient to induce clotting. Instead, clotting ability required higher multimeric states of amassin, possibly tetramers, occurring through the N-terminal β-region and/or the first segment of coiled-coils. The quaternary structure of amassin may resemble that of other extracellular disulfide-linked multimers such as Acrp30/adiponectin or resistin.

INTRODUCTION

Sea urchins lack an adaptive immune response, but possess a strong innate response mediated mainly by phagocytic coelomocytes residing in the coelomic cavity and throughout their tissues (Coffaro and Hinegardner, 1977; Gross *et al.*, 2000; Johnson, 1969b; Nair *et al.*, 2005). The phagocytic coelomocytes engulf or encapsulate microbes and foreign particles (Johnson, 1969a; Pancer *et al.*, 1999; Smith *et al.*, 1992). When coelomic fluid is transferred from the animal to a beaker, the coelomocytes adhere together within three minutes in a dramatic cell-cell adhesion event referred to as clotting (Hillier and Vacquier, 2003). One function of coelomocyte clotting could be to seal the body cavity following injury, as urchins are hard-bodied and unable to contract tissue around an injury that penetrates the test (a shell-like skeleton) (Boolootian and Giese, 1959; Endean, 1966). This clotting event is unusual among cell adhesion phenomena in that it is rapid, and the cell-cell bonding is extremely tenacious. The evolutionary position of sea urchins as basal deuterostomes makes them more closely related to vertebrates than other non-chordate invertebrate model organisms. Thus, understanding the molecular mechanism of coelomocyte clotting could lead to novel insights into the evolution of cell-cell adhesion events in vertebrates.

Previous work has identified a single, 75 kDa coelomic fluid glycoprotein, named amassin, which mediates coelomocyte clotting (Hillier and Vacquier, 2003). The amino acid sequence of amassin[1] contains several structural domains. Most importantly, the C-terminal half of amassin is an olfactomedin (OLF)[2] domain (Snyder *et al.*, 1991; Yokoe and Anholt, 1993) comprising roughly 50% of the sequence. The function of this domain has been the subject of recent study in other

animals. For example, the human protein myocilin/TIGR has an OLF domain that when mutated is associated with inherited forms of glaucoma (Fautsch *et al.*, 2000; Hardy *et al.*, 2005; Nguyen *et al.*, 1998; Ray *et al.*, 2003; Stone *et al.*, 1997). The OLF domain of rat gliomedin participates in the formation of the peripheral nodes of Ranvier (Eshed *et al.*, 2005). Continuing with the neuronal associations of OLF family members is the protein UNC-122, which affects neuromuscular signaling in *Caenorhabditis elegans* and is thought to provide an organizational role at the neuromuscular junction (Loria *et al.*, 2004). Further examples include the neural crest cell stimulator, named noelin in chickens, and its mammalian homologs (Barembaum *et al.*, 2000; Danielson *et al.*, 1994; Moreno and Bronner-Fraser, 2001).

Other structural domains N-terminal to the OLF domain of sea urchin amassin include a signal peptide, a short β-region, and a coiled-coil region (Hillier and Vacquier, 2003). These same orderly features are found in an entire group of OLF proteins from diverse species including the above mentioned proteins myocilin, noelin, and pancortin, but also olfactomedin (Snyder *et al.*, 1991; Yokoe and Anholt, 1993), optimedin (Grinchuk *et al.*, 2005; Torrado *et al.*, 2002), tiarin (Tsuda *et al.*, 2002), and photomedin (Furutani *et al.*, 2005). Less similar in structural organization to the above "classical" OLF proteins are members of the recently described subgroup, the colmedins (Cao *et al.*, 2005; Eshed *et al.*, 2005; Franzke *et al.*, 2005; Graveel *et al.*, 2003; Loria *et al.*, 2004). Colmedins contain a short membrane-spanning region and collagen repeats in place of a signal peptide and coiled-coils (they include UNC-122 and gliomedin).

Research on OLF family members has consistently demonstrated the presence of intramolecular and intermolecular disulfide-bonds, and in some cases assigned

logical linkages between the cysteine residues (Ando *et al.*, 2005; Fautsch *et al.*, 2004; Furutani *et al.*, 2005; Nagy *et al.*, 2003; Russell *et al.*, 2001). The clotting of sea urchin coelomocytes also displays a strong dependence on disulfide bonds (Boolootian and Giese, 1959). Chemical reduction of amassin renders it inactive as a clot inducer, and also dissociates preformed clots (Bertheussen and Seijelid, 1978; Hillier and Vacquier, 2003). Unfortunately, lack of a functional assay for OLF proteins in vertebrates has precluded an analysis of the implications for these disulfides. The clotting of sea urchin coelomocytes by amassin provides such a functional assay. To gain insight into the function of structural features of this important class of proteins, we have constructed systematic truncations of the amassin sequence, thereby dissecting it into its constituent structural and functional domains.

RESULTS

The presence of free-sulfhydryls in amassin was investigated with the use of biotin probes attached to the reactive group maleimide under denaturing conditions. Purified natural amassin displays little, if any, detectable free-sulfhydryl compared to when the protein is fully reduced (Fig. 1). Full-length amassin (residues 29-495), expressed in the yeast (*Pichia pastoris*), gave a similar result, as did the OLF domain expressed by itself (residues 210-495). The amassin samples can be compared to BSA, known to contain only one free-sulfhydryl (Andersson, 1966; Gonias and Pizzo, 1983), which is strongly labeled even in the absence of reductant. With such a low level of labeling in the absence of reductant, the results indicate that most, if not all, cysteines in amassin are bonded. The slight labeling in unreduced samples could be attributed to a fraction of the molecules misbonded, or to the presence of a sensitive disulfide.

The mature amassin protein contains a total of eight cysteines (two additional cysteines are contained within the predicted signal peptide; Fig. 2). These cysteines can be grouped into three clusters. Group I contains three closely spaced cysteines (residues 53, 55, and 65) located mostly within the short N-terminal β-region. The spacing of these residues is conserved between many other OLF domain containing proteins, with the consensus sequence being C-X-C-X$_9$-C (Ando *et al.*, 2005). Group II contains one cysteine located near the end of the coiled-coils (residue 203). This cysteine occurs in the proper location within the heptad repeat of the coiled-coils to reside at the dimerizing face (Lupas *et al.*, 1991), promoting disulfide bond formation. A number of other OLF proteins—chicken noelin, human myocilin, *Xenopus* tiarin, and *Rana* olfactomedin—also contain a cysteine at a similar

position. Group III includes four cysteines that are mainly contained within the consensus OLF domain. The first, Cys^{227}, precedes the OLF domain boundary as defined in the Protein Family Database (Bateman *et al.*, 2002) by only three amino acids. With the exception of a few OLF proteins (rat gliomedin, human collomin, and *Xenopus* tiarin), all OLF family members contain a cysteine just preceding the OLF domain boundary, leading one to believe that the boundary designation could be extended. Further into the OLF domain of amassin, there is a closely spaced patch of three cysteines (residues 403, 407, 412) with the central cysteine highly conserved among OLF family members.

With the grouping of the cysteine residues, and other structural features of amassin (including the N-terminal β-region, coiled-coil segments, OLF domain, and C-terminal extension) in mind, nine different constructs of amassin were designed. Constructs were truncated from either or both termini, never was a portion deleted from within the protein. Truncated proteins were expressed by secretion from *P. pastoris* and purified on Ni-NTA resin. Full-length recombinant amassin (residues 29-495) was highly purified, and was glycosylated at N-linked sites in the yeast system yielding a relative molecular mass (M_r) of ~70 kDa (Fig. 3A), which is smaller than natural amassin by ~10 kDa when run on the same type of gel. Upon removal of N-linked glycosylation with PNGase-F, full-length recombinant amassin's M_r decreased to ~60 kDa (similar to natural amassin). The difference in M_r between natural and recombinant amassins indicates that the recombinant protein is lacking some post-translational modification. This difference becomes greater when one accounts for another ~2.5 kDa contained in the purification tag and pro-

tease site of the recombinant protein. Other truncated amassin constructs were also highly purified, as shown in Fig. 3B.

The presence of intermolecular disulfide bonds in amassin was revealed by stepwise reduction prior to resolution by SDS-PAGE (Fig. 4A). Three forms of amassin are discernible: the fully reduced form running at M_r ~80 kDa, the next larger form at ~300 kDa, and the third form is represented by a group of bands just entering the resolving gel or remaining at the stacking/resolving gel boundary. These forms have been assigned to be the monomer, dimer, and multimers of amassin (Hillier and Vacquier, 2003). Expressed full-length amassin (residues 29-495) shows the presence of the monomeric and dimeric forms, but no higher disulfide-linked multimers are visible (Fig. 4B). Also showing the same pattern of an intermolecular disulfide are the N-terminally truncated amassins (all lacking the Group I cysteines): 67-495, 131-495, and 185-495 (Fig. 4C-E). No higher-order multimers are detected (67-495 shows a very faint band running at high molecular weight). Further N-terminal deletion (to amino acid 210) removed the Group II Cys[203]; this construct displays no intermolecular disulfide bond (Fig. 4F), nor does the construct 223-476 (Fig. 4G), which is even further truncated at the N- and C-termini. There is, however, a slight increase in mobility coinciding with lack of reductant, a migration pattern indicative of intramolecular disulfide bonds. Any further deletion from the N-terminus to remove Cys[227] (construct 228-495) results in an aggregated and disulfide-scrambled protein not showing variation in response to reductant (data not shown). An amassin construct lacking the OLF domain but still retaining the Group I and II cysteines (residues 29-220), displays the presence of a intermolecular disulfide bond, as well as higher-order bonded species (Fig. 4H). Finally, the

construct containing only the coiled-coils and the Group II cysteine displays the pattern indicative of a disulfide-linked dimer (Fig. 4I).

Chemical cross-linking of amassin constructs followed by resolution on SDS-PAGE displays a similar result to that obtained by monitoring disulfide bonds. With an increase in concentration of cross-linker, all constructs that contain one segment of the coiled-coils are covalently linked as dimers (Fig. 5A-E and H-I). Natural amassin, and the 29-220 construct, show a diffuse band of higher molecular weight, potentially multimeric forms. A construct containing only the OLF domain (residues 210-495) remains as a monomer even at the highest concentration of cross-linker (Fig. 5F). The most truncated OLF construct (residues 223-476) is only partially cross-linked as a dimer (Fig. 5G). The transition to dimers never becomes complete, and is most likely the result of a misfolded fraction due to the extreme truncation at both termini. Cross-linking of the OLF domain constructs, 210-495 and 223-476, was also performed at a much higher protein concentration (1 mg/ml versus 10 μg/ml) with a similar result (data not shown), supporting the existence of monomeric recombinant OLFs.

The covalent capture of amassin as dimers by cross-linking depends on the presence of the disulfide bonds. In reactions with sufficient cross-linker to completely dimerize amassin, the presence of the reductant DTT prevents cross-linking (Fig. 5J). This indicates that one or more disulfides maintain the dimer; standard protein structure stability contributors, such as ionic and hydrophobic interactions, are insufficient.

The activity of all amassin constructs were assayed by monitoring their ability to clot washed coelomocytes (Fig. 6). Only two amassin constructs have clotting ac-

tivity: full-length (residues 29-495), and a construct lacking the N-terminal region (residues 67-495). This indicates that the N-terminal β-region is not necessary. Further N-terminal truncation to remove the first coiled-coil segment abolishes clotting activity. Amassin residues 67-495 represents the minimal requirement for maintaining coelomocyte-clotting activity.

Next, the ability of the recombinant amassins to inhibit the clotting induced by natural amassin was studied (Fig. 6). All constructs that were further N-terminally truncated—down to residue 223—had clot inhibitory activity. However, removal of Cys[227] in construct 228-495 renders it totally inactive as an inhibitor of clotting. Two constructs lacking the OLF domain while retaining the coiled-coils region (residues 29-220 and 67-220) also have no clotting or clot inhibiting activity. The C-terminal extension beyond the OLF domain boundary (residues 477-495) is not necessary for inhibitory activity, as construct 223-476 inhibited clotting by natural amassin. This construct represents the minimal content required for clot inhibition activity. An additional construct engineered to contain two linked OLF domains as part of one polypeptide chain (Dual 223-495) has inhibitory activity, but no clotting activity.

Expressed full-length amassin has sufficient affinity to bind coelomocytes and be retained following washing (Fig. 7). Binding of amassin to cells is calcium dependent. The level of binding in the absence of calcium is much lower, as evidenced by much fainter bands in those lanes. The truncated construct lacking the N-terminal β-region and the first coiled-coil segment (residue 131-495) also shows calcium-dependent affinity for coelomocytes. The OLF domain alone (residues 223-

495) binds to the cells. In contrast, the N-terminal half (residues 29-220) displays extremely low binding activity, at the limit of detection.

DISCUSSION

The OLF domain contains four cysteines assigned to Group III. While all four are disulfide bonded, they do not display characteristics of intermolecular bonds. Rather, in response to changing reducing conditions, constructs containing these four cysteines (residues 210-495 and 223-476; Fig. 4F-G) only gained a slight mobility by SDS-PAGE in the absence of reductant. This indicates that these constructs have intramolecular disulfide bonds. This was also found to be the situation for the OLF domain of human myocilin, which contains only two cysteines (Nagy *et al.*, 2003). The disulfide bonding pattern of the four cysteines of Group III can be predicted with this information. Amassin contains cysteines at the same highly conserved positions as human myocilin. Therefore, Cys^{227} is most likely bonded to Cys^{407}. This would leave Cys^{403} bonded to Cys^{412} since there are no intermolecular bonds and no free-sulfhydryls present (Figs. 1 and 8A). As mentioned in the "Results Section," the boundary of the OLF domain should probably be extended to include Cys^{227}, this position being highly conserved among OLF domain containing proteins. Proteins which do not contain this cysteine also do not contain its bonding partner (with the exception of *Xenopus* tiarin; Tsuda *et al.*, 2002), which is located at a position synonymous to amassin Cys^{407}, providing further support for the bonding assignment. Cys^{227} is also absolutely necessary for proper disulfide bond formation (data not shown), and the inhibitory activity of the domain towards clot induction by natural amassin (Fig. 6).

The monomeric state of the OLF domain was further supported by cross-linking experiments. Even when performed at high protein concentrations (1 mg/ml), which would favor the covalent capture of weak transient interactions, transi-

tion to dimer was not complete. The small amount of dimer captured by cross-linking in the construct 223-476 could be attributed to its lack of 19 amino acids C-terminal to the canonical OLF domain. This C-terminal extension may be involved in stabilizing the OLF domain fold, the lack of which could contribute to improper associations. Furthermore, a slightly extended construct (residues 210-495; Fig. 5F) remained completely uncrosslinked.

The OLF domain existed as monomers, but with the inclusion of amino acids N-terminal, the coiled-coils, disulfide-linked dimers formed. These must be joined by an intermolecular bond with the sole cysteine (Cys[203]) of Group II, since all constructs (including the coiled-coils expressed by itself, residues 67-220) displayed a large mobility shift following partial reduction and SDS-PAGE. The orientation of the coiled-coils, parallel or anti-parallel, could also be determined in these experiments. Because Cys[203] is located at one end of the coiled-coils, its intermolecular bonding would only allow a parallel orientation (Fig. 8B and C).

Two constructs that excluded the OLF domain (residues 29-220 and 67-220) displayed a mobility shift equaling roughly twice the molecular weight of the fully reduced monomer. Those including the OLF domain displayed a more dramatic shift, sometimes 3-fold that of the monomer. These were assigned to be dimers, not trimers or tetramers, based upon the aforementioned shift of non-OLF constructs and several other factors discussed below. The same mobility shifts were also observed in cross-linking experiments, which display their result by a completely different mechanism. Although the M_r on gels shifted (as the reductant was diluted, or cross-linker concentration was increased) from ~70-80 kDa to ~250-300 kDa in, for example, natural amassin and 29-495, no intermediate bands were ever seen. These

intermediates would be expected if the dimer band instead represented a higher multimeric state. The disulfide-linked dimers also ran at variable M_r, depending upon the type of gel used in the assay. Gels containing a high percentage of bis-acrylamide resulted in a lower M_r (~200 kDa; Hillier and Vacquier, 2003), while with gels cast with a more standard 2.67% (percentage of total acrylamide content) bis-acrylamide, as used in this study, the M_r was ~300 kDa. Thus, dimers formed by the disulfide between Cys^{203} residues, and containing the OLF domain, run anomalously at higher M_r on SDS-PAGE.

The bonding pattern of the three N-terminal, Group I cysteines could not be determined. They are most likely involved in forming higher order multimers, such as tetramers, which can be discerned as a high molecular weight band by SDS-PAGE with natural amassin. Unfortunately, yeast-expressed full-length amassin did not display these higher intermolecularly disulfide-linked forms, which might require further processing not possible in the yeast system. A discrepancy becomes apparent for this construct because little, if any, free-sulfhydryl was detectable (indicating that all cysteines are involved in disulfide bonds; Fig. 1), yet no higher-order multimers were detectable by SDS-PAGE. Two explanations may account for this, the first being that two of the three cysteines may be involved in an intramolecular bond, which would not contribute to a large mobility shift. Indeed, a slight downward shift is apparent in the samples exposed to the lowest reductant concentrations, indicative of an intramolecular bond. The second explanation resides in the possibility that the cysteines are not disulfide-bonded, but oxidized and thereby unreactive towards the biotin probe. Remaining undetected, higher-order multimers of recombinant amassin may have actually formed without the stabiliza-

tion of proper disulfides. The use of chemical cross-linking might reveal this occurrence, but it also did not capture these higher order structures (with the exception of slight cross-linking seen in natural amassin and 29-220). The first lysine (containing a primary amine capable of reacting with the cross-linker) is not until residue 138, which is well within the coiled-coils. Recombinant multimers may exist, but are not trapped by the amine reactive cross-linker used in our study.

The necessity of the N-terminal β-region and its three cysteines are questionable, because a construct lacking this region retained clotting activity. As the dimer-forming constructs (185-495 and 131-495) and the engineered dual OLF domain construct elicited no clotting, it may be that clotting activity requires tetrameric or higher multimer formation. The retention of activity in construct 67-495 opens the possibility that the first of the three coiled-coils segments forms a tetramer via an anti-parallel orientation (Fig. 8D), and thereby enables clotting activity. This structure might not be trapped by cross-linking due again to no available primary amines until residue 138 (within the second coiled-coils segment), and remain undetected by our methods.

All constructs containing the OLF domain have inhibitory activity towards clotting by natural amassin. The inhibition could be due to the OLF domain constructs binding natural amassin, or a receptor on the cell surfaces. Here, the cell-binding activity has been demonstrated (Fig. 7). The cell-binding activity is highly dependent upon the presence of calcium, and it remains unclear which component requires the calcium, the cell's receptor, or amassin. Motif searches do not identify any predictable calcium-binding regions in the amassin sequence. It is interesting to note that calcium removal by chelation is how we initially obtain clot-inhibited

coelomocytes, and disrupting the interaction between the OLF domain of amassin and its receptor may be the mechanism.

Further work should be directed at identifying the protease sensitive (Hillier and Vacquier, 2003) cell-surface receptor for the OLF domain of amassin. OLF domains can be involved in heterotypic OLF-OLF domain interactions, as those occurring between myocilin and optimedin (Torrado *et al.*, 2002). Hypothetically, there may be other OLF domain containing proteins anchored at the sea urchin coelomocyte surface, and amassin might bind these through its OLF domain in a calcium-dependent manner. But the receptor need not contain an OLF domain, as other binding partners for the domain have been demonstrated. For example, the OLF domain of the recently described protein gliomedin binds the cell adhesion molecules neurofascin and NrCAM (Eshed *et al.*, 2005), and myocilin's OLF interacts with the lipid raft protein flotillin (Joe *et al.*, 2005).

Taken together, amassin contains the structural units of the N-terminal β-region, the coiled-coils, and the OLF domain. The OLF domain contains two intramolecular disulfide bonds and exists as a monomer. The parallel coiled-coils act to dimerize amassin with a required disulfide bond. These features bear a rough resemblance to those of Acrp30/adiponectin (Tsao *et al.*, 2003). Electron microscopy revealed that its ball-and-stick-like structure contains trimeric coiled-coils, topped by a large globular sphere at one end, and a smaller sphere at the other. This also may be the case for amassin, with the dimeric coiled-coils forming a "stick," and two globular OLF domains existing as "balls" at one end. The N-terminal β-region (including the first segment of coiled-coils) of amassin may function by further multimerization, and form a smaller sphere at the N-terminal end of the

coiled-coils dimer. This general overall structure also exists in the serum protein resistin, where in its case hexamers are formed by disulfide-linkages between trimers at the N-terminal tips of the coiled-coils (Patel *et al.*, 2004). Continued research from a structural standpoint, using such tools as electron microscopy and crystallography, should yield insight into the exact nature of amassin's ability to form such disulfide-linked polymers and its effect upon the cell-binding activity of the OLF domain.

MATERIALS AND METHODS

Construction of Yeast Expression Vectors

A modified version of the *P. pastoris* expression vector pPICZαA (Invitrogen) was constructed. Briefly, the vector was altered following the α-factor signal sequence and Kex2 signal cleavage site to contain these features in order: a hexahistidine purification tag, a seven amino acid (DYDIPTT) spacer followed by a Tobacco Etch Viral (TEV) protease cleavage site, a *BamH* I restriction site (enabling insertion of the protein coding region of interest), and finally stop codons. This stretch of DNA was inserted by replacing the region between the *Xho* I and *Not* I sites of pPICZαA. The resultant vector, pBH91, is an intermediate vector coding for the expression of sea urchin REJ1 amino acids 67-328, and was constructed due to the many natural restriction sites within the coding region for amassin. Once the sequence was verified, pBH91 was used as the new parent vector by removal of the REJ1 coding region between the *BamH* I and *Not* I sites and replacement with various cassettes containing the desired coding regions of amassin. After midiprepping, all plasmids were verified by DNA sequencing of their entire coding regions.

These constructs encode proteins that contain α-factor signal sequences, directing their processing through the secretory pathway of the yeast. This provides an environment for eukaryotic protein modifications such as glycosylation and disulfide bond formation. During secretion, the signal sequence is removed by the *KEX2* gene product, rendering a new N-terminus containing the hexahistidine tag. The His-tags could be removed by the TEV protease, leaving the extra residues GS at the N-terminus. However, all proteins used in this study had their tags intact.

Protein Expression and Purification

The protocols, reagents, and media described in the EasySelect *Pichia* Expression Kit (Invitrogen) were used. Freshly prepared competent cells of *P. pastoris* strain KM71H were transformed with linearized vector by electroporation. Selection of genomic integrants was achieved by plating on YPDS agar containing 200 µg/ml of zeocin (Invitrogen).

Twelve colonies of each construct were screened for the highest expression levels by a small-scale expression screen as follows. Colonies were used to inoculate 50 ml BMGY and grown at 30 ℃ until OD_{600}=4-10. The yeast were then harvested by centrifugation and resuspended into 10 ml BMMY (1% methanol) for induction. Incubation with vigorous shaking in baffled flasks at 30 ℃ was continued for 5 days, with fresh methanol added to 1% every day. Samples were taken every day, and the yeast removed by centrifugation. The secreted protein samples were analyzed by western blotting with anti-amassin to determine the highest expressing colonies and the optimal time of induction.

Once the best colonies and expression conditions were determined for each construct, a larger scale induction was carried out for recombinant protein production. One liter of BMGY was inoculated with 2 ml of a starter culture grown to OD_{600}=6. The bulk growth phase was continued until OD_{600}=6-8 (16-24 h) when the yeast were harvested by centrifugation at 3,000 x g (10 min) and resuspended in 200 ml of BMMY (1% methanol) in 1 L baffled flasks. Induction was continued 2-5 days and the supernatant isolated by centrifugation at 3,000 x g (15 min) followed by 10,000 x g (15 min).

To the 200 ml supernatant was added EDTA to 10 mM, followed by ultrafiltration concentration. The concentrate was dialyzed overnight against 3 changes of binding buffer (BB; 50 mM NaH_2PO_4, 500 mM NaCl, pH 8) to remove low molecular weight components of the spent BMMY yeast media. The dialysate was centrifuged at 10,000 x g (15 min) before the addition of 1 ml of Ni-NTA (Qiagen) resin. Binding continued for 2 h with rotation. Then the resin was allowed to settle, and the unbound material removed. At this point the resin was transferred to a column and washed with 10 ml of BB, followed by 30 ml of wash buffer (BB + 15 mM imidazole), and finally eluted with 10 ml elution buffer (BB + 250 mM imidazole). The purified protein was then dialyzed against 10 mM Hepes pH 7.5, concentrated by ultrafiltration, and then frozen in a dry ice/ethanol slurry for storage at -80 $^{\circ}$C. Yields varied greatly between constructs and preparations: the highest expressing construct (residues 210-495) yielded 7 mg from 200 ml culture, while the lowest (residues 67-495) yielded only 75 µg.

Step-wise Reduction Mobility Shift Assays

Purified proteins (17 µg/ml in 50 mM Hepes, 450 mM NaCl, 20 mM EDTA, pH 7.5) were exposed to a step-wise dilution of the non-sulfhydryl reductant tris(2-carboxyethyl) phosphine (TCEP, Pierce Chemical Co.) by combining with an equal volume of 2x Laemmli sample buffer containing dilutions of TCEP (25 mM maximal final concentration). Samples were incubated 10 min at RT, boiled for 3 min, and then 50 ng/lane were resolved by SDS-PAGE. Precision Plus Protein Standards (Bio-Rad) were used on all gels as molecular weight markers. Western immunoblots were performed as described previously with the polyclonal anti-amassin antibody (Hillier and Vacquier, 2003).

Labeling Free-Sulfhydryls with Biotin

Purified proteins were diluted with water to 1.3 µg in a volume of 110 µl. The samples were divided, 55 µl each, and the following constituents were added: 12 µl MOPS pH 7, 1.2 µl 0.5 M EDTA, 2.4 µl of a 0.5 M solution of TCEP for the reduced control sample (water alone for the experimental), and water to a final volume of 83.5 µl. This solution was then added to a tube containing preweighed urea (55-60 mg), and dissolved by heating to 37 °C for 5 min. The volume was now ~120 µl. The final concentrations were: 7.6-8.3 M urea, 50 mM MOPS pH 7, 5 mM EDTA, and 10 mM TCEP for the reduced sample. Samples were then incubated at RT for 10 min. Biotin labeling proceeded with the addition of 1.2 µl of 5 mM maleimide PEO$_2$-Biotin (Pierce Chemical Co.) for 1 h. The reaction was quenched by adding 4 µl of 20 mM reduced glutathione for 30 min. Finally, 125 µl of 2x Laemmli sample buffer (containing 50 mM TCEP) was added, the samples boiled 3 min, and 5 µl/lane were resolved on by SDS-PAGE on a 4-15% gradient gel. The proteins were transferred to PVDF, and blocked for 1 h with 5% dry milk in Tris-buffered saline (TBS) containing 0.05% Tween 20. Following three rapid washes, the blot was probed with streptavidin-HRP at a 1:100,000 dilution (1 mg/ml stock, Sigma S-5512) for 1 h, and then washed four times for 15 min each. Biotinylated proteins were then detected with SuperSignal substrate (Pierce Chemical Co.).

Chemical Cross-linking

Dilutions of the water-soluble amine-reactive cross-linker bis(sulfosuccini-midyl) suberate (BS[3], Pierce Chemical Co.) were made in three-fold increments into which purified protein was added to 10 ng/µl. The maximum concentration of cross-linker was 1 mM. Buffer constituents for cross-linking were: 25 mM Hepes

pH 7.5, 225 mM NaCl, and 10 mM EDTA. The reaction continued at RT for 10 min followed by quenching with 50 mM Tris pH 7.5 (15 min). An equal volume of 2x Laemmli sample buffer (containing 50 mM TCEP) was added, the samples boiled 3 min, and 20 ng/lane were resolved by SDS-PAGE on 4-15% gradient gels.

Predictive Structural Analysis

Assignment of the amassin OLF domain position and N-terminal β-region was determined as previously described (Hillier and Vacquier, 2003). Coiled-coil positions were predicted with the program Coils (Lupas *et al.*, 1991), using the MTK matrix with no weighting of the a and d positions. A window size of 28 was used, with an assigned cutoff of 0.5 probability for the locations of the coiled-coil segments.

Coelomocyte Clotting/Inhibition Assays

Preparation of clot-inhibited coelomic fluid, washing of coelomocytes, and qualitative scoring of coelomocyte clotting activity was performed essentially as previously described (Hillier and Vacquier, 2003). Briefly, 0.5 ml of washed coelomocytes were combined with the purified protein of interest, followed by the addition of $CaCl_2$ to 10 mM. The tubes were then rotated end-over-end for 5 min before visual examination. Qualitative scoring of clotting was readily ascertained by comparison with a positive control (50 ng purified natural amassin), and with a negative control (buffer alone). A protein was scored as having no clotting ability if the addition of ≥20 μg did not induce complete clotting of washed coelomocytes. As a control, proteins were first boiled for 5 min, and then assayed for clotting ability. No boiled protein had clotting activity.

Clotting inhibition was assayed by adding recombinant amassin proteins to 0.5 ml washed coelomocytes with incubation at RT for 2 min. This was followed by the addition of 50 ng of natural amassin and 10 mM $CaCl_2$, and rotation end-over-end for 5 min. Qualitative scoring of clotting was performed as above. A protein was scored as having no inhibitory activity if the addition of ≥50 μg did not prevent clotting by natural amassin.

Cell-binding Assays

Purified amassin proteins (5 μg) were added to 0.5 ml washed coelomocytes, followed by either the addition of 10 mM $CaCl_2$, or buffered calcium-free artificial seawater (HCFSW; 10 mM Hepes pH 7.5, 450 mM NaCl, 10.5 mM KCl, 26 mM $MgCl_2$, 30 mM $MgSO_4$, 2 mM $NaHCO_3$). The tubes were rotated end-over-end for 5 min, then centrifuged at 500 x g (4 min). The supernatant was removed, and the cells were washed by the addition of either 1 ml buffered artificial seawater (HASW; 10 mM Hepes pH 7.5, 450 mM NaCl, 10 mM $CaCl_2$, 10.5 mM KCl, 26 mM $MgCl_2$, 30 mM $MgSO_4$, 2 mM $NaHCO_3$), or HCFSW. The tubes were rotated for 1 min, recentrifuged as before, and the supernatant discarded. Cell pellets were lysed in 100 μl 2x Laemmli sample buffer (containing 50 mM TCEP), boiled 5 min, and 5 μl/lane resolved by SDS-PAGE on an 11% gel.

FOOTNOTES

[1]The amino acid sequence of the amassin protein can be accessed through the NCBI Protein Database under NCBI Accession # AAO43562.

[2]The abbreviations used are: OLF, olfactomedin; TEV, Tobacco Etch virus; BB, binding buffer; TCEP, tris(2-carboxyethyl)phosphine; TBS, Tris-buffered saline; BS3, bis(sulfosuccinimidyl)suberate; HCFSW, Hepes-buffered calcium-free seawater; HASW, Hepes-buffered artificial seawater; BSA, bovine serum albumin; DTT, dithiothreitol.

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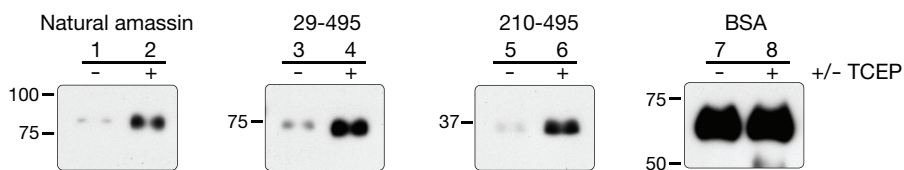
Figure 1

Amassin displays a lack of free-sulfhydryls. Urea denatured proteins were exposed to the sulfhydryl-reactive labeling reagent, maleimide PEO$_2$-biotin, in the absence of reductant (*lanes 1*, *3*, *5*, *7*), or in the presence of TCEP (*lanes 2*, *4*, *6*, *8*). A substantial increase in biotinylation occurred following reduction and was detected by probing a western blot with streptavidin-HRP. The expressed residues of each amassin protein are shown above; natural amassin was purified from the sea urchin. BSA (*lanes 7-8*), known to contain one free-sulfhydryl, was labeled in parallel as a control. Molecular weight standards, expressed in kDa, are shown on the left. Protein load was 13 ng/lane.

A

Group I

YMRTTSGVVETSSGR(C)Q(C)ETAFPPLVT(C)QDYVETGYPERL

53 55 65

Group II

TRPTEVRVLQRKIDDLKSKLAE(C)ERYTRTEDESLFQPWSD

203

heptad repeat: f g a b c d e f g a b c d e f g a b c d e f g a b c d e f g a

Group III

DFPSQDS(C)RDIIHVSE •••• KYQAGS(C)FMV(C)GRLY(C)LSSFF

227 403 407 412

B

Group I   Group II   Group III

β-region
signal peptide   coiled-coils   olfactomedin domain

C
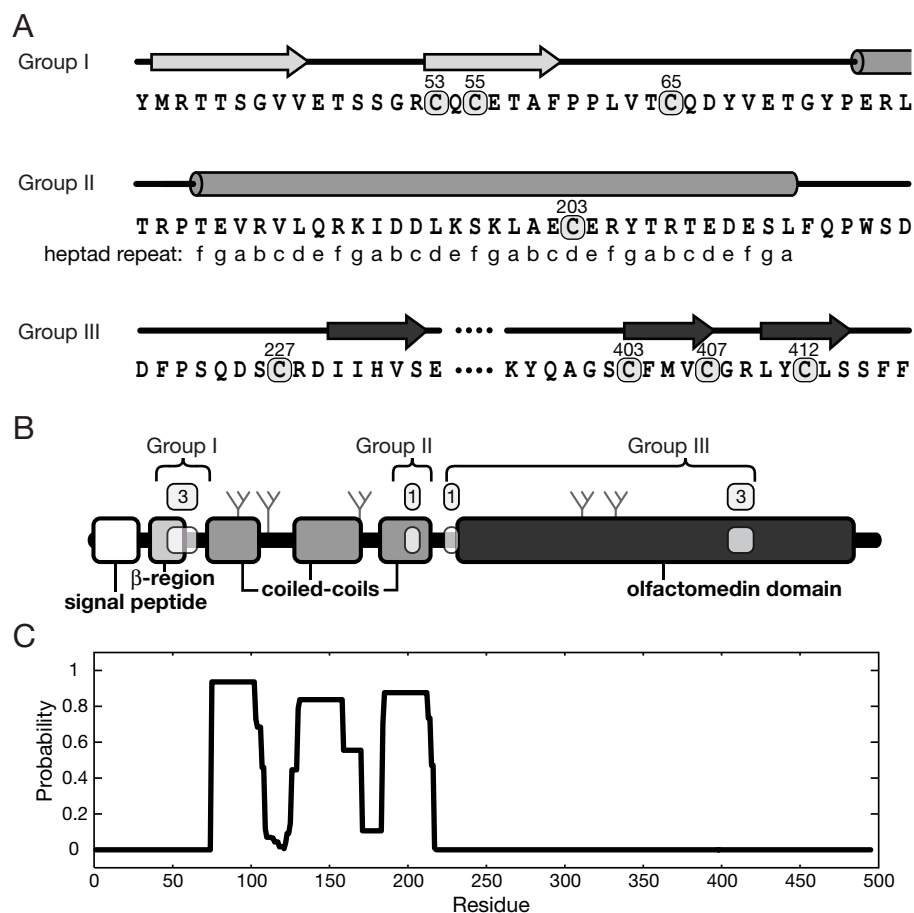
Figure 2

Grouping cysteine positions and structural organization of amassin. *A*, the eight cysteines of amassin occur in three groups. Shown are the amino acid sequences in the proximity of each group, a box surrounds each cysteine. Predicted secondary structural elements are depicted as arrows for β-strands, and cylinders for α-helices. Labeled below the Group II sequence is the position within the heptad repeat of the third coiled-coils segment, the a and d positions reside along the dimerizing interface. *B*, a schematic of the amassin sequence is shown with structural regions outlined by rounded rectangles. From the N-terminus there is a signal sequence, a short predicted β-region, three segments of coiled-coils, and the OLF domain (labeled below the schematic). Cysteines occurring within five residues of each other are enclosed by small rectangles. A duplicate is shown above the schematic labeled with the number of cysteines bounded by the rectangle. The three cysteine clusters are enclosed by brackets labeled Groups I, II, and III. Branch-like symbols denote the position of predicted N-linked glycosylation sites. *C*, coiled-coils are predicted in three segments (residues 75-106, 130-170, and 184-214) by the program Coils as scored with the MTK matrix in a 28-residue window (graphical output shown). Probabilities greater than 0.5 are considered likely.
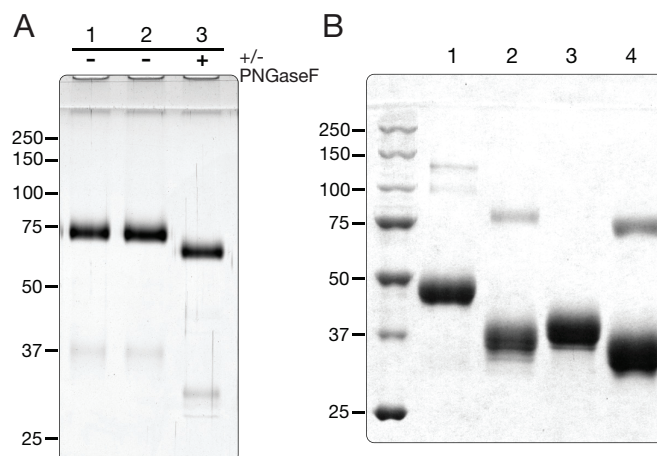
Figure 3

Yeast expressed amassin proteins are glycosylated, and purified to a high degree. *A*, full-length expressed amassin is N-linked glycosylated. Incubation with PNGase-F increased its mobility to ~60 kDa (lane 3) as compared to that before treatment (*lane 1*) running at M_r ~70 kDa (*lane 2* is a minus enzyme control treated identically to *lane 3*). Digestion was performed as previously described (Hillier and Vacquier, 2003) and 0.1 μg was resolved by SDS-PAGE on a 10% gel followed by silver staining. *B*, hexahistidine-tagged amassin constructs were highly purified from yeast media supernatant. Five μg/lane were resolved by SDS-PAGE on a 9% gel run under reducing conditions and stained with Coomassie. Amassin constructs 131-495 (*lane 1*), 185-495 (*lane 2*), and 223-495 (*lane 4*) show the presence of some disulfide-linked dimers most likely due to reoxidation upon resolution through the gel, whereas construct 210-495 (*lane 3*) ran solely in its reduced form. Molecular weight standards, expressed in kDa, are shown on the left.
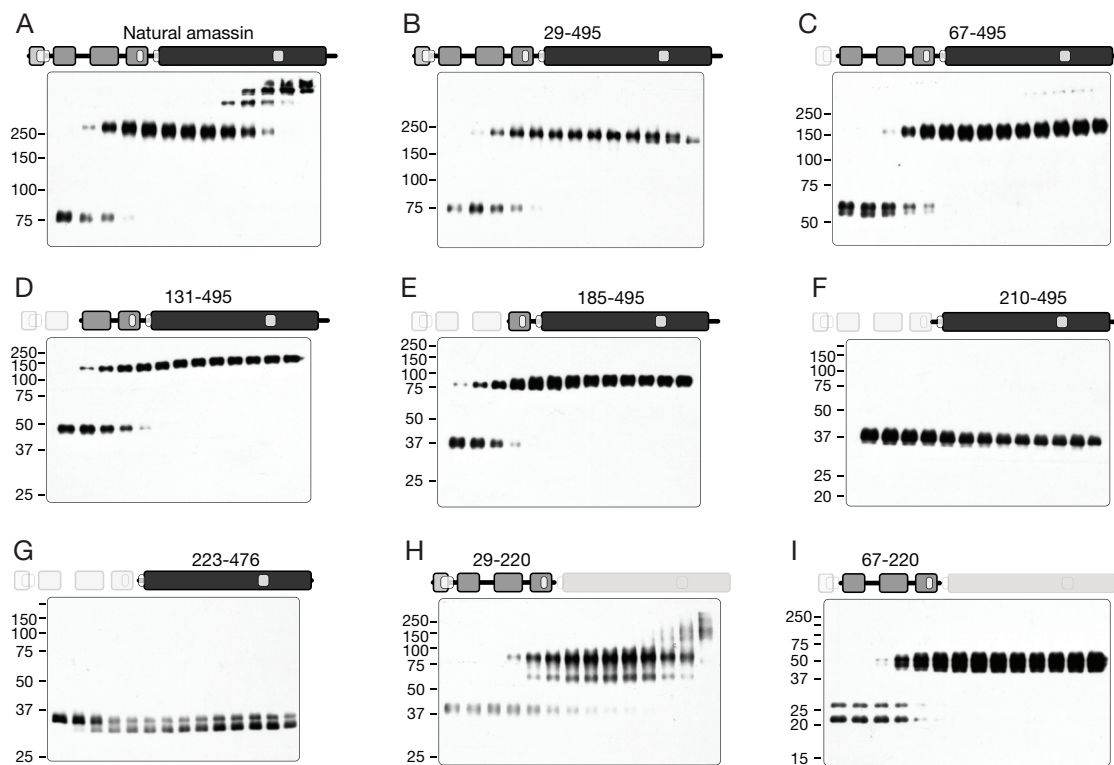
Figure 4

Intermolecular disulfide-bonds become apparent by a step-wise TCEP reduction mobility shift assay. Samples of protein were exposed to a step-wise gradient of TCEP prior to resolution by SDS-PAGE. The maximum concentration was 25 mM TCEP (far-left lanes), and was diluted 2-fold in each lane to the right. The far-right lanes had no reductant. Proteins (50 ng/lane) were detected by immunoblot with antiamassin. *A-E* and *H-I*, amassin constructs containing the Group II Cys[203] at the end of the coiled-coils experienced a mobility shift, indicative of a intermolecular disulfide bond, which increased their M_r to a value ~2-3 times greater. Those constructs containing only the OLF domain (*F* and *G*) displayed a minor mobility shift due to their intramolecular disulfide bonds. Schematic diagrams above each western blot denote the structural regions contained within the constructs (as in Fig. 2*B*), light-shaded regions were truncated and are shown only for reference. Molecular weight standards, expressed in kDa, are shown on the left.
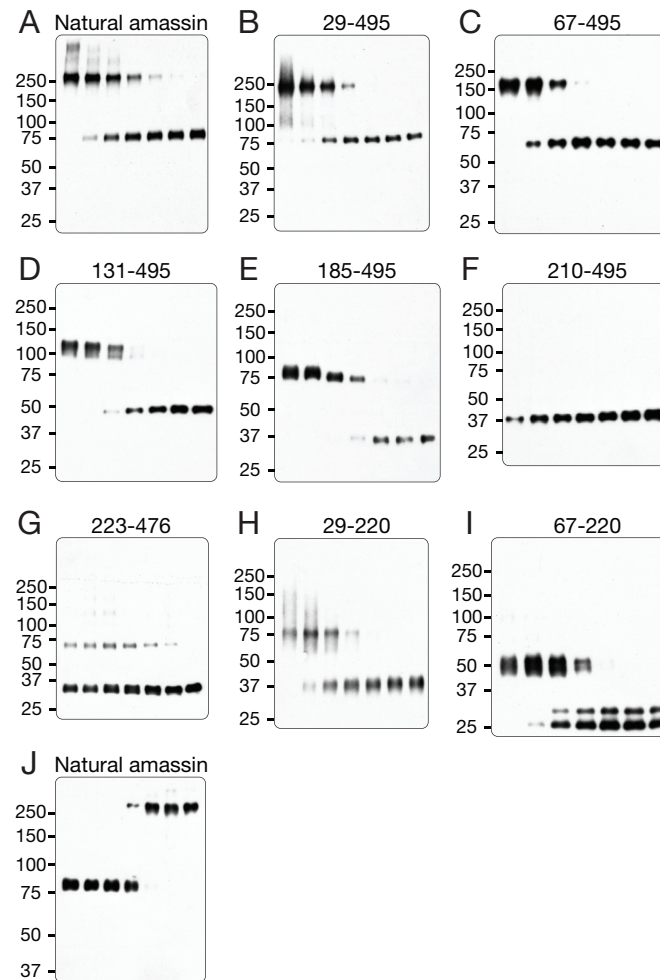
Figure 5

Chemical cross-linking of amassin constructs reveals multimeric states, and is disulfide-bond dependent. *A-I*, natural and truncated amassin proteins were cross-linked with various concentrations of BS^3 ranging from a maximum of 1 mM (far-left lanes), and decreasing in three-fold steps to the right. The far-right lane of each blot had no cross-linker. Constructs containing at least one of the coiled-coils segments (*A-E* and *H-I*) displayed the complete conversion from monomers to dimers upon cross-linking. Those constructs containing only the OLF domain were either totally non-cross linked (*F*), or only partially cross-linked (*G*). *J*, disulfide-bonds are essential for the capture of multimeric forms of amassin by cross-linking. Natural amassin was exposed to 1 mM BS^3 in the presence of varying concentrations of DTT ranging from a maximum of 100 mM on the far-left, and diluted in three-fold steps to the right. The far-right lane was not exposed to reductant. All blots were detected with the anti-amassin antibody. Molecular weight standards, expressed in kDa, are shown on the left. Protein load was 20 ng/lane.
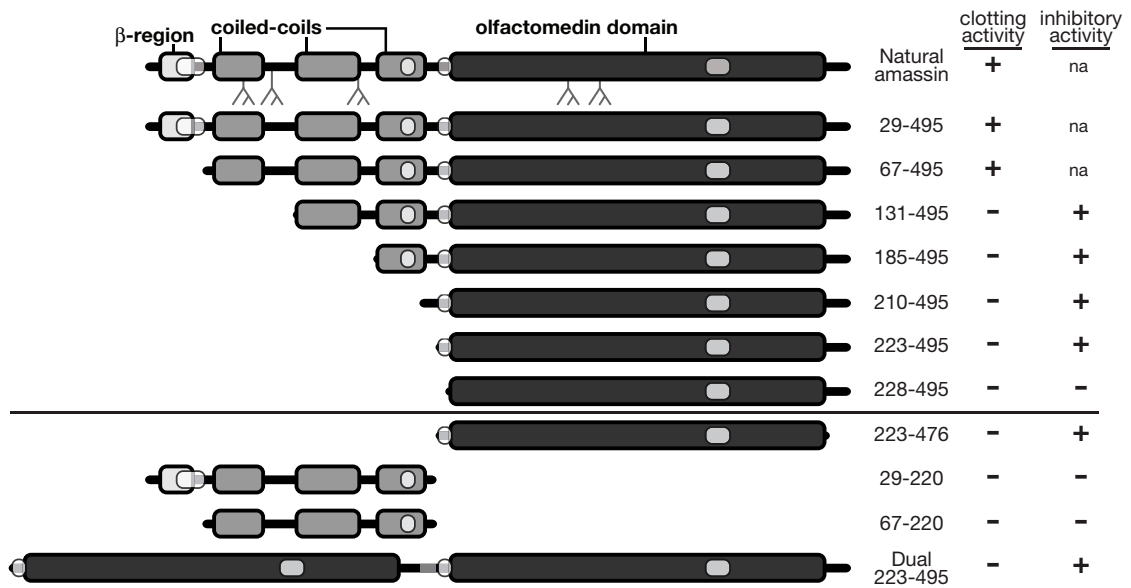
Figure 6

Functional portions of amassin are determined by monitoring the clotting or inhibitory activity of truncation constructs. Schematic representations of the structural elements are shown (as in Fig. 2*B*) for each construct. Those above the horizontal line belong to a sequential N-terminal truncation series of amassin constructs. Those constructs below the line are truncated from either the C-, or both termini. An additional recombinant construct was utilized (labeled Dual 223-495) that encoded two adjacent OLF domains joined by a flexible (Gly-Ser)$_5$ linker. The clotting and/or inhibitory activity of each protein was determined (as described under "Materials and Methods") and denoted in the columns as present (+), or absent (-).
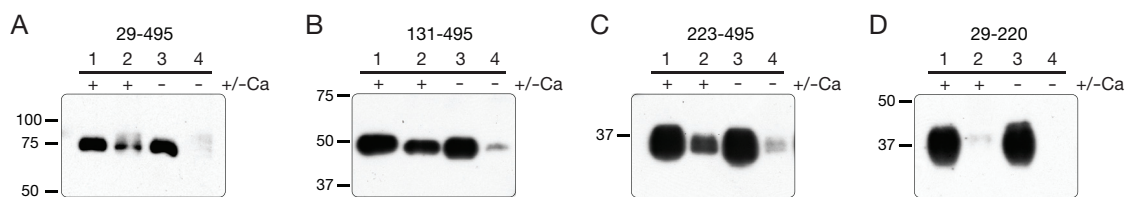
Figure 7

The OLF domain in isolation has calcium-dependent cell-binding activity. Proteins were incubated with washed coelomocytes in the presence (*lanes 1-2*), or absence of calcium (*lanes 3-4*). Unbound material (*lanes 1* and *3*) was removed, followed by washing, before resolving the cell pellets (*lanes 2* and *4*) by SDS-PAGE and immunoblotting with anti-amassin. Full-length recombinant amassin (*A*), 131-495 (*B*), and the OLF domain 223-495 (*C*) retain proteins on the coelomocytes, while the N-terminal half of amassin (residues 29-220; *D*) displays almost no detectable binding activity. Molecular weight standards, expressed in kDa, are shown on the left.
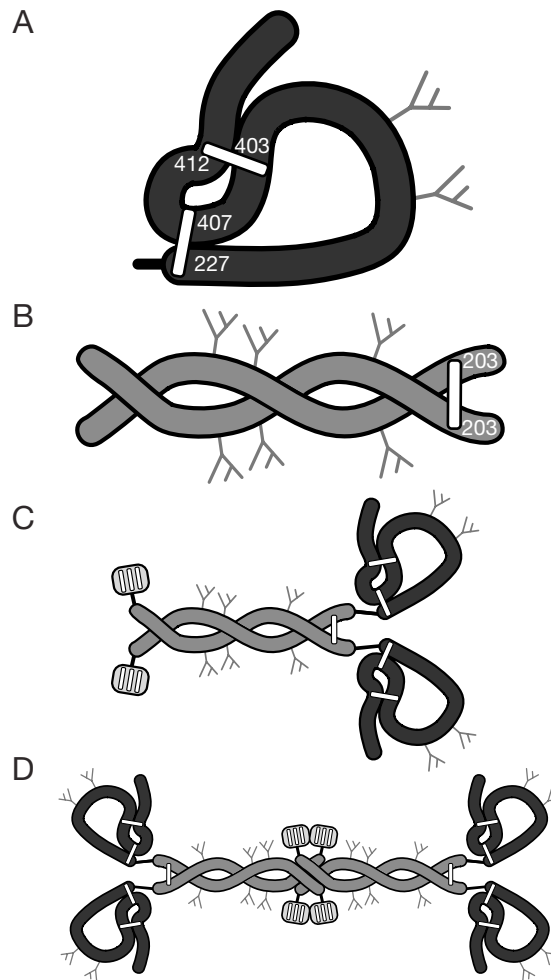
Figure 8
Hypothetical model of structural features. Shown are highly schematic representations of the structural features of amassin in cartoon form. The experimental evidence supports disulfides within the monomeric OLF domain (*A*), and between coiled-coils (*B*), as labeled by their residue number. *C*, the predominant form of amassin is found as a disulfide-linked dimer. *D*, the active form (most likely tetramers) of amassin may form in a manner roughly similar to that depicted. White bars represent cysteine residues, and branched symbols are placed at the approximate locations of the N-linked glycosylation sites.

The text of Chapter II, in full, is being prepared for publication. The dissertation author was the primary author, and co-author V. D. Vacquier directed and supervised the research, which forms the basis for this chapter.

Chapter III

The Diversity of Olfactomedin Proteins in the Sea Urchin

ABSTRACT

Increasing numbers of proteins containing an olfactomedin (OLF) domain are being identified that maintain novel extracellular protein-protein interactions in diverse animal phyla. To date, only one OLF family member, amassin, has been described from the sea urchin, *Strongylocentrotus purpuratus*, a basal invertebrate deuterostome. Amassin mediates the rapid intercellular adhesion of sea urchin coelomocytes (immunocytes). Here we describe the identification and protein structural features of four additional OLF family members, bringing the total number of OLF proteins in the sea urchin to five. Phylogenetically, four of the five proteins—here named the amassins—form a unique subgroup among all previously identified OLF proteins. The fifth shares similarity to the OLF family subgroup known as the colmedins, containing a type II transmembrane domain, collagen repeats, and an OLF domain. The OLF proteins of the sea urchin represent an intermediate diversification between those of the protostomes and the vertebrates. Transcripts of all five OLF family members were found in the coelomocytes as well as adult radial nerve tissue. Transcript levels of the OLF proteins were highly regulated on the time scale of the complete larval life cycle. In one case, levels were dramatically upregulated one-million-fold, coinciding with formation of the adult urchin rudiment within the larval body.

INTRODUCTION

Previous work has identified the extracellular protein, amassin, as an essential factor in the rapid and dramatic cell adhesion phenomenon (clotting) that occurs between coelomocytes in the sea urchin, *Strongylocentrotus purpuratus* (Hillier and Vacquier, 2003). Amassin contains an olfactomedin domain (OLF) in the C-terminal half of the protein, and it is through this domain that amassin is capable of binding the coelomocyte surface (Chapter II). Amassin belongs to a family of proteins that contain an OLF domain. OLF family members occur in diverse animal phyla including nematodes, arthropods, echinoderms, and chordates. The number of OLF proteins continues to grow. Recently described members with neurological roles include UNC-122, which functions by organizing the neuromuscular junction (Loria *et al.*, 2004), and gliomedin, which promotes the formation of the nodes of Ranvier (Eshed *et al.*, 2005). These OLF family proteins bind their protein targets through the OLF domain. Mutations in the OLF domain of human myocilin are associated with inherited forms of glaucoma (Fautsch *et al.*, 2000; Hardy *et al.*, 2005; Nguyen *et al.*, 1998; Ray *et al.*, 2003; Stone *et al.*, 1997). Myocilin binds its target, another OLF protein named optimedin, through the OLF domain (Torrado *et al.*, 2002). These data from disease states to developmental neurogenic events underscore the importance of understanding the functions of OLF domain-containing proteins.

Relatively few OLF family members have been identified from non-chordate invertebrates: one from *Drosophila melanogaster*, two from *Caenorhabditis elegans*, and one, amassin from *Strongylocentrotus purpuratus* (Hillier and Vacquier, 2003; Loria *et al.*, 2004). The situation is quite different in vertebrates where OLF domain

proteins are much more common. For example, in humans there are at least 13 OLF domain proteins. This disparity between animal groups in the number of OLF family members makes it seem that vertebrates were the primary OLF innovators. Using the publicly available assemblies of the sea urchin genome at the Baylor College of Medicine, Human Genome Sequencing Center (http://www.hgsc. bcm.tmc.edu/projects/seaurchin/), we have identified four additional OLF family members, bringing the total to five for this sea urchin species. The results of this paper illuminate another line of OLF domain evolution specific to the sea urchin.

As stated above, the original sea urchin OLF family member is amassin (Hillier and Vacquier, 2003). In the present work, we have renamed it amassin-1 to avoid confusion with the three newly identified members that share high similarity and which are named amassin-2, amassin-3, and amassin-4. The fifth and last newly identified OLF family member is named colmedin, due to its similarity to proteins of the OLF colmedin subfamily (Cao *et al.*, 2005; Eshed *et al.*, 2005; Franzke *et al.*, 2005; Graveel *et al.*, 2003; Loria *et al.*, 2004). Here we describe the protein features of these new OLF family members. Additionally, we have quantified transcripts for each of the five OLF family members through the complete course of development, from the egg to embryo to larva to metamorphosis. The results are unusual and display a dramatic regulation of OLF transcription, implying important, as yet unknown roles for these proteins in development.

RESULTS

Structural features of the sea urchin OLF family

An assembly of the sea urchin genome provided large genomic contigs to search for OLF domain-containing proteins. Starting from predicted coding regions of similarity to OLF domains and amassin's OLF domain, we isolated the entire coding regions of four additional OLF proteins (Fig. 1). This brings the total to five OLF proteins in this sea urchin species. The newly identified proteins are named amassin-2, amassin-3, amassin-4, and colmedin. Their NCBI accession numbers are, respectively: DQ250734, DQ250735, DQ250736, and DQ250737.

Members of the OLF family in the sea urchin share several features (Fig. 1 and Table 1), most notably an OLF domain located at the C-terminal end. Differentiating the amassins (amassin-1, -2, -3, and -4) from colmedin is the N-terminal half. This is exemplified by their overall sequence identity to amassin-1: 29% amassin-2, 28% amassin-3, 27% amassin-4, and 12% colmedin.

All amassins contain coiled-coils as the next structural motif N-terminal to the OLF domain. This region functions to homo-dimerize amassin-1, and is stabilized by a disulfide bond near the C-terminal end of the coiled-coils (Chapter II). Every amassin also contains a cysteine at a similar position, and in the proper position within the heptad repeat of the coiled-coils to reside at the dimer interface (Lupas *et al.*, 1991). Colmedin, on the other hand, contains a longer region encoding collagen repeats (Gxx) in place of the coiled-coils of the amassins (Fig. 1 and Table 1). Colmedin's three regions of collagen repeats encode a total of 249 amino acids, or 83 repeats of Gxx. Surrounding the first repeat region, and preceding the second, are sets of two closely spaced cysteine residues. Since collagen is typically a triple

helix, each of these dual cysteine patches could form a stabilizing ring around a colmedin trimer. Although the coiled-coils of the amassins and the collagen repeats of colmedin are very different in sequence, both types of domains may function to multimerize OLF protein monomers in a parallel orientation with stabilization by disulfide bonds (Chapter II).

Preceding the coiled-coil segments of all four amassins is a short predicted β-region (Fig. 1 and Table 1; Hillier and Vacquier, 2003). This region contains three cysteines arranged in the consensus $CxCx_9C$ pattern, as found in other OLF family members (Furutani *et al.*, 2005). This region may act to multimerize amassin-1 to higher states than dimers, but is not necessary for its biological activity of clotting coelomocytes (Chapter II). Dissimilarly, the region N-terminal to the collagen repeats of colmedin (residues 36-180) has no significant similarity to any protein in the NCBI database. This region is predicted by the DisEMBL server (Linding *et al.*, 2003) to be a region of intrinsic disorder. However, one significant feature of colmedin, a furin cleavage site, RARR, is present in this region at positions 139-142 (Fig. 1 and Table 1).

At the N-terminus of all amassins is a predicted signal peptide of 23-28 residues (Fig. 1 and Table 1). Within two amino acids of the predicted cleavage site, all amassins contain a conserved exon-exon junction. A signal peptide can loosely be predicted for colmedin, but its confidence is low because it lacks a signal peptidase site. Instead, it is predicted to be an anchor signal, containing a type II transmembrane domain from residues 13-35 (Fig. 1 and Table 1). This would leave a short intracellular N-terminus of only 12 amino acids. The N-terminal region of colmedin is fairly rich in cysteine residues, containing a total of five. There are two cysteines

in the short intracellular segment (Cys^9 and Cys^{11}), one within the transmembrane (Cys^{26}), and two located just extracellularly (Cys^{38} and Cys^{49}).

OLF domain characteristics

All sea urchin OLF family members contain one OLF domain, invariably at the C-terminal end. A conserved exon junction precedes the OLF domain boundary by the equivalent of 1-5 amino acids, and is followed by another conserved junction ~45 amino acids downstream (Figs. 1 and 2). Identity in the OLF domain is higher than in the protein as a whole. Identity to amassin-1 is 35% for amassin-2, 34% to amassin-3, 33% to amassin-4, and 25% to colmedin. Only 40 residues in the OLF domain of ~250 amino acids are absolutely conserved between all members (Fig. 2). The two longest stretches of high conservation are from amassin-1 residues 354-366 (DxAVDExGLWxIY) where 10 of 13 are identical, and from residues 451-466 (LxYNPRDxxLY) where 8 of 11 are identical.

The position of a cysteine residue is absolutely conserved (amassin-1 Cys^{227}; Fig. 2), just preceding, or coincident with the defined OLF boundary. This cysteine most likely forms an intramolecular disulfide bond with the central cysteine of a patch of three in the amassins (amassin-1 Cys^{407}), or the only other cysteine in colmedin, located at the same position. The amassins likely form an additional intramolecular disulfide between the peripheral cysteines of this patch, as is the case for amassin-1 (Cys^{403} bonded to Cys^{412}; Chapter II). Any additional cysteines beyond those already mentioned typically occur in pairs in OLF domains, and so are most likely disulfide bonded. Amassin-2 and -4 contain two additional cysteines—predicted to disulfide bond—whereas amassin-3 contains only one. Amassin-3 is very similar to amassin-4, sharing 87% identical amino acids. Even so, instead of the second

matching cysteine found in amassin-4, amassin-3 contains an arginine, which is also present in amassin-1 and -2. Amassin-3 may therefore contain an unbonded, free cysteine.

Diversity of the OLF family

A phylogenetic tree was constructed for OLF family proteins from a dozen animal species by aligning their OLF domains (Fig. 3). Major groupings correspond to those obtained in recent work by Zeng *et al.* (2005). The subgroups, labeled I–VII, represent these dominant members: (I) noelins and optimedin, (II) latrophilins, (III) myocilins, (IV) photomedins, (V) olfactomedin and tiarin, (VI) colmedins, and (VII) olfactomedin-like. However, previous phylogenetic work was inconsistent in assigning amassin-1 to any one group. In one instance it was assigned to subgroup VII, but in another tree, it was placed in subgroup V (Zeng *et al.*, 2005). Here, we show that the four amassins identified from the sea urchin are more similar to themselves than to any other group. Thus, the sea urchin amassins form an eighth distinctive OLF subgroup.

By far, the most diverse subgroup is the colmedins (named by conjoining <u>col</u>lagen and olfacto<u>medin</u>; Loria *et al.*, 2004), subgroup VI (Fig. 3). To date, colmedins are represented by members from four animal phyla: *Nematoda*, *Arthropoda*, *Chordata*, and now *Echinodermata*. Sea urchin colmedin shares the characteristic presence of a short intracellular N-terminal portion, followed by a transmembrane domain and collagen repeats. This is the only subgroup representing both protostomes and deuterostomes; all other subgroups have only deuterostome members. As such, it seems that this is the most primitive subgroup. Further diversification of OLF members has only occurred within the deuterostomes. With the ex-

ception of the colmedins, the only non-chordate deuterostome known to contain an OLF domain protein is the sea urchin. The sea urchin is intermediate to the diversity of the vertebrates, being represented in two of the eight phylogenetic subgroups. Human sequences are present in seven of the eight OLF subgroups.

OLF presence in coelomocytes

A highly conserved region among all sea urchin OLF family members (amassin-1 residues 452-468) was used as a peptide epitope to raise a polyclonal antibody (anti-P452). Amassin-2 contains 14 of 17 identical residues in this region, amassin-3 12 of 17, amassin-4 12 of 14, and colmedin 11 of 17 (Fig. 2). Because of the high identity, the antibody would be expected to react with all members of the sea urchin OLF family.

Indeed, in coelomocyte lysates analyzed by western blotting, there are multiple reactive bands (Fig. 4). In contrast, when reacted with anti-P452, cell-free coelomic fluid displays only a single reactive band running at ~80 kDa. This band is determined to be amassin-1 due to reactivity with its polyclonal antibody at an identical relative mobility (M_r). Washed coelomocytes are lacking in amassin-1. This is evidenced by faint reactivity at 80 kDa when probed with highly specific antiamassin-1, and the lack of a prominent band when probed with anti-P452. However, amassin-1 is abundant in lysates produced from clotted coelomocytes, as seen with both antibodies.

By inference, the other reacting bands present in washed coelomocytes can be ascribed to the remaining OLF family members. Colmedin has a predicted molecular weight of 85.6 kDa (Table 1). This is in close agreement with the reacting band migrating at ~90 kDa. Collagen repeat-containing proteins are typically pre-

sent in a triple helix, and can be cross-linked through lysine residues by a lysyl-oxidase type enzyme (Jourdan-Le Saux *et al.*, 1999; Maki *et al.*, 2001; Molnar *et al.*, 2003). A covalently cross-linked colmedin trimer would have a calculated molecular weight of 257 kDa, similar to the reactive band migrating at ~240 kDa in washed coelomocytes. Indeed, a protein similar to human lysyl-oxidase-like2 was cloned from coelomocytes during this study (NCBI accession number DQ250738), leaving the occurrence of a covalently linked trimer possible. Interestingly, when coelomocytes are induced to clot, the presumed colmedin bands are no longer present. Instead, there is a band at ~190 kDa. This may be due to cleavage at the furin site (RARR)—removing the first 142 residues of each subunit in the covalently linked trimer—which would reduce its calculated molecular mass to 208 kDa. Monomeric colmedin would decrease to a calculated molecular mass of 69.4 kDa and be obscured by the other intense bands in that region.

There remain two discernible bands, one at ~60 kDa, and the other at ~70 kDa (Fig. 4). For certain they are not amassin-1, as they are unreactive with its specific antibody, antiamassin-1. The protein sequences of amassin-3 and -4 are very similar, and may migrate together as one band on gels. For these remaining amassins, there is not sufficient experimental evidence at this time to assign them to a reactive band on a western blot.

Regulation of OLF family members throughout larval stages

The regulation of OLF family transcript levels at eleven developmental stages were investigated by real-time quantitative PCR (RTQ-PCR). Samples were taken at these stages: unfertilized egg, 2-day-old late gastrula, 4-day-old late prism, 6-day-old early pluteus, 10-day-old pluteus, 15-day-old 4-arm stage, 20-day-old 6-arm

stage, 25-day-old 8-arm stage, 30-day-old late larvae, 35-day-old metamorphs, and from an entire small 7 g adult. Previous work on sea urchins has shown that the ubiquitin transcript (*UBIQ*) level remains constant through early development (Nemer *et al.*, 1991; Ransick *et al.*, 2002). We found that the copy number of *UBIQ* transcripts remains fairly constant throughout development at ~10^4-10^5 copies per 4.2 ng total RNA, until reaching the adult which contains 1.9 x 10^3 copies (Fig. 5). The copy number of *UBIQ* transcripts was used for normalization of OLF family transcript levels.

The amassin transcripts are developmentally regulated. A dramatic regulation is observed for *AMAS1* expression (Fig. 5). No transcripts are detectable until the 25-day-old larvae—a stage where the developing rudiment is visible. By the adult, its levels increase one-million-fold to 1.1 x 10^6 copies. *AMAS2* is also highly enriched in the adult, rising to 3.3 x 10^5 copies. But *AMAS2* is also found at very low levels in earlier stages of development (from 10-62 copies), with undetectable levels until six days, and cannot be detected at 15 days. *AMAS3* is primarily expressed in late development and the adult, with a slight detectable expression in the egg (6.9 copies). Although *AMAS4* is very similar to *AMAS3* in sequence, regulation of its gene is quite different. Unique among all OLF members, *AMAS4* is detectable in all stages of development. In general, its levels increase through development until 15 days, with a gradual loss to metamorphosis, and again a high level in the adult.

The colmedin transcript (*CLMDN*) is also regulated throughout development (Fig. 5). Although absent in the unfertilized egg, it is detectable at all other stages. Its levels peak at 10 days, followed by a reduction at 20 days, and then an increase through metamorphosis.

Proteins were also analyzed from the same developmental samples by western analysis. Interestingly, the amassin-1 protein remained undetectable throughout development, rendering no reactivity with the specific antiamassin-1 IgG (data not shown). With the anti-P452 antibody, which should recognize all OLF family members, several bands are visible (Fig. 6). The ~240 kDa band is present in all stages of development, with lower intensity in the egg and 35-day metamorphosis samples. This band is hypothesized to react with a cross-linked trimer of colmedin, described previously. A dramatic increase in the presence of the ~60 kDa band occurs beginning at 25 days, and continues through metamorphosis. Upon overexposure, faint, possibly doublet bands are also visible at ~70 kDa in most developmental stages. It is undetectable in the egg and 10-day samples.

DISCUSSION

Common structure of sea urchin OLF proteins

There are five OLF domain-containing proteins in this sea urchin species, four amassins and one colmedin. Among the four amassins, the overall structural elements are remarkably conserved. Beginning from the N-terminus, all amassins have a signal peptide that would direct their secretion to the extracellular space, as is the case for amassin-1, which is found in the coelomic fluid. Having no doubt arisen by gene duplications, the four amassins have a common exon junction close to the C-terminal end of the signal peptide and another further downstream following the next shared protein feature (Fig. 1). This next feature is a short predicted β-region that partially encloses three cysteines. This region is involved in forming higher multimeric states in amassin-1 (Chapter II). Further C-terminal, there are segments of coiled-coils that end with a cysteine in the proper location to form a dimerizing disulfide. The OLF domains comprise the C-terminal half of all four amassins (Fig. 1).

With the exception of the latrophilins, a similar domain arrangement can be found in all OLF proteins. These similarities include the presence of signal peptides that can be found in every subgroup aside from the colmedins, subgroup VI. The conservatively spaced cysteines, assigned to be part of the N-terminal β-region, are found in a smaller subset, which excludes the colmedins (subgroup VI) and the olfactomedin-like proteins (subgroup VII). But common to every OLF group is a helical multimerizing motif located N-terminal to the OLF domain. In most OLF proteins these are dimeric coiled-coil regions, while in the colmedins they are tri-meric collagen repeat regions. This would result in a common structure of multiple

OLF domains being displayed together as part of one larger molecule. Because it is the OLF domain that is the functional portion of these proteins that bind their target proteins via protein-protein interaction, the result of closely spaced multiple OLF domains would be an increase in avidity. This could be the reason for the marked conservation of structure of OLF proteins.

The OLF domain itself displays further similarities among all five sea urchin OLF family members. Exon junctions are well conserved, supporting their evolution by gene duplication followed by functional specialization (Fig. 1). Cysteine positions, probably involved in intradomain disulfides, are also conserved.

OLF presence and function in coelomocyte clotting

Overall identity between the OLF domains was fairly low, ranging from 25-35% identity to amassin-1, with only two stretches of high percentages of identity among all proteins. One of these stretches was utilized to produce an antibody that recognizes all OLF family members. Multiple reactive bands resulted from sea urchin protein preparations probed with this antibody and logical inferences were made in assigning probable OLF proteins to specific bands. Of note is the assignment of the bands at 90 and 240 kDa in coelomocyte preparations. The calculated molecular masses of 85.6 kDa for monomeric colmedin, and 257 kDa for cross-linked trimeric colmedin led to the assignment of these two bands as the colmedins. Attempts to identify the antibody-reacting bands by mass spectrometry were unsuccessful.

There are three facts supporting the hypothesis that colmedin is a likely candidate to be the binding partner of the coelomocyte clot mediator, amassin-1. First, heterotypic OLF-OLF interactions have previously been demonstrated for other

proteins, for example, myocilin and optimedin (Torrado *et al.*, 2002). Second, like myocilin and optimedin, the isoelectric points of amassin-1 and colmedin are separated by at least four units (Table 1), which could be an indicator of complementarily charged interaction surfaces. Third, colmedin contains a transmembrane domain, providing a membrane anchor and extending its OLF domains away from the cell surface, where it could interact with OLF domains of amassin-1 and bind coelomocytes together in clots. Additionally, the colmedin reactive band could be truncated, possibly at the furin cleavage site, following clot formation (Fig. 4). Coelomocytes remain tightly bound during this stage, and secondary cell-adhesion events may be occurring. Interestingly, all the OLF family proteins identified in this work are expressed in the coelomocytes.

OLF family gene regulation during development

Because many OLF family proteins are developmentally regulated, especially in neuronal tissue, the appearance of their transcripts during development was investigated. The sea urchin under investigation, *S. purpuratus*, goes through indirect larval development. The free-living larva bears no resemblance to the developing adult urchin body.

Amassin-1 gene expression displays a rather unusual developmental profile. It is not detectable until well into larval development, first appearing at 25 days when the adult forming rudiment becomes visible. To our knowledge, this is the latest developmental gene activation known for this species. Beginning at 25 days, amassin-1 expression increases dramatically, climbing to a one-million-fold increase in the young adult. The mechanism of its transcriptional regulation would be important to study. Perhaps its expression is regulated in introns or 3' to the gene,

such as in the OLF family member UNC-122 (Loria *et al.*, 2004). The amassin-1 protein is undetectable at all stages until the adult, where it is found in the coelomic fluid and expressed by coelomocytes. These observations indicate that amassin-1 is an adult functioning protein, and most likely plays no developmental role until metamorphosis to the adult.

The transcriptions of the other amassin genes are also highly regulated. The lowest expressing amassin, amassin-2, is present in small amounts in the latter half of development, and then is plentiful in the adult. Amassin-3, displays a marked increase beginning at 20 days, again coinciding with rudiment formation. An OLF family protein is detectable which matches this profile; its presence begins five days later at 25-days and it migrates at 60 kDa on gels. This band may correspond to amassin-3. The final amassin, amassin-4, displays a more constant level of expression and is found throughout development. The OLF family member running at 70 kDa may correspond to this protein, as it is faintly detectable in most stages and its sequence corresponds to that molecular mass. Regulation of colmedin follows a cyclic profile, with an early peak at 10-days then a later peak in the adult. The 240 kDa band has been assigned to this protein, and is found throughout development. Functionally, colmedin may follow that of other related proteins, UNC-122 and gliomedin, with neurogenic roles important during development (Eshed *et al.*, 2005; Loria *et al.*, 2004). It is important to note that the same pools of larval cDNA were used in the analysis of all genes in this work.

In summary, these data show that the genes coding for OLF family members in sea urchin are highly transcriptional regulated during larval development, which most probably is a reflection of their as yet unknown functional importance. Neu-

rological roles for the OLF family members in the sea urchin should be seriously considered, as transcripts for all members can be easily amplified from cDNA prepared from adult radial nerve tissue (data not shown), although no other tissues were analyzed. Further research should reveal interesting functions for sea urchin OLF proteins.

MATERIALS AND METHODS

Immunoblots and antibodies

Immunoblots were performed as described previously with the antiamassin-1 specific antibody (Hillier and Vacquier, 2003). Antiamassin-1 was used at a 1:25,000 dilution. The monoclonal anti-α-tubulin (Sigma T5168) was used in immunoblots at a dilution of 1:20,000.

An 18-mer peptide was synthesized (Bio-Synthesis Inc.) corresponding to amassin residues 452-468 with the addition of one cysteine residue at the N-terminus (amine-CKYNPRDQKLYGWDNGHQ-amide). The peptide was coupled to maleimide-activated mariculture keyhole limpet hemocyanin (Pierce Chemical Co.) and used to raise rabbit antiserum (Strategic Biosolutions). The peptide was also linked to a solid support (SulfoLink Coupling Gel, Pierce Chemical Co.) following the manufacturer's instructions. This peptide-linked gel was used to affinity purify the antiserum. The antibodies (anti-P452) were concentrated to 0.5 mg/ml with the addition of 10 mg/ml BSA. Anti-P452 was used at a dilution of 1:250 for western blots.

Cloning full-length cDNAs

The publicly available assemblies of the *S. purpuratus* genome at the Baylor College of Medicine, Human Genome Sequencing Center (http://www.hgsc.bcm.tmc.edu/blast/) contigs dated November 23, 2004 were searched using tBLASTn. Queries were submitted in an exhaustive search using most well characterized OLF domains currently recognized in the Pfam database as search models. The genomic search results were manually scored for confidence by the presence of appropriately spaced conserved sequence features typically present in an OLF domain. Few ab-

solutely conserved stretches of residues actually exist between all OLF domains, but the tripeptide GLW present midway in the domain at amassin residues 361-363, and the highly conserved cysteine (Cys[407]) located ~44 residues later were most useful. Four unique OLF domain-containing genomic sequences (besides amassin) were identified after the positive contigs were tabulated and redundancies removed.

PCR primers were designed and synthesized which would amplify hypothetical exon regions. Total RNA was prepared from a whole adult animal (Hillier and Vacquier, 2003), and from total coelomocytes with the Trizol reagent (Invitrogen). First-strand cDNA was synthesized by priming with either random decamers or oligo(dT)$_{12\text{-}18}$ (Invitrogen), and extension with SuperScript III (Invitrogen) following the manufacturer's protocol. PCR products were cloned by TOPO-TA (Invitrogen) and plasmids sequenced.

Extension of the clones further terminal (5' and 3') was accomplished by a combination of two techniques. First, contigs were translated *in silico* in all possible reading frames and examined for the presence of structural features typically found in OLF family members, namely coiled-coils or collagen repeats. Once these regions were found, additional primers were designed to amplify a larger region of cDNA. Second, complete coding regions were identified using 5' and 3' rapid amplification of cDNA ends (Ambion). Additional primers that would anneal in the newly identified untranslated regions were used in combination with a proofreading polymerase (Platinum *Pfx* Polymerase, Invitrogen) to verify complete sequences. Using the complete cDNA sequences as queries for a BLAST search against the genomic database, other contigs were identified which individually

spanned one or more exons. Exon boundaries were identified by manual comparison of the cDNA and genomic contig sequences with conformation to the typical AG-exon-GT rule (Mount, 1982; Shapiro and Senapathy, 1987), the results of which are tabulated in the Supplemental Materials (Table S1).

Primary sequence analysis

The location of the OLF domain boundaries were obtained from the Pfam database (Bateman *et al.*, 2002). Coiled-coil predictions were made with the Coils server (Lupas *et al.*, 1991) using the MTIDK matrix, with no weighting, and a window size of 21. Continuous regions with a probability score >0.5 were considered likely. The presence and location of signal peptides were predicted with SignalP (Nielsen *et al.*, 1997), and a transmembrane domain identified with the TMHMM Server v2 (Sonnhammer *et al.*, 1998). The short N-terminal predicted β-region was identified by alignment with the amassin sequence. The furin cleavage site and location of predicted N-linked glycosylation sites were found with assistance from the MacVector (Accelrys) computer program.

Phylogenetic analysis

A multiple sequence alignment was constructed from the OLF domains of 34 proteins with the default settings of the ClustalW function of MacVector. NCBI accession numbers of the proteins used can be found in the Supplementary Materials (Table S2). The highly conserved cysteine located just N-terminal to the presently defined OLF domain boundary (positions synonymous to amassin Cys[227]) and five additional residues beyond were included. An unrooted distance-based

neighbor-joining tree was created from this alignment with PAUP* (Swofford, 1998).

Coelomocyte preparations

Washed clot-inhibited coelomocytes were prepared as previously described (Hillier and Vacquier, 2003). Washed cells (0.5 ml at 2×10^6 cells/ml) were pelleted by centrifugation at 500 x g for 4 min. Cells were lysed by resuspension into 100 μl 2x Laemmli sample buffer, boiled 5 min, and 10 μl/lane resolved by SDS-PAGE on an 8% gel. Clotted cell samples from the same batch of coelomocytes were prepared similarly. To the washed cells were added 250 ng purified amassin and 10 mM $CaCl_2$ with rotation end-over-end for 5 min to induce complete clotting. Further processing of the clotted samples was identical to that of the washed-alone samples.

Culture of larvae and preparation of their biomolecules

Sea urchin larvae were reared essentially as described (Wray *et al.*, 2004). Larvae were incubated at 14°C, with no added agitation. The media, 0.45 μm filtered natural seawater, was replaced every 2 days by gentle capture of larvae on a 20 μm mesh of nytex, and resuspension in fresh seawater. Separate cultures of the unicellular algae, *Rhodomonas*, were maintained and added to the media after every seawater change.

Biomolecules were prepared at various stages of their developmental growth by taking samples of the larvae after a seawater change, but before diluting the culture and adding algae. Sufficient larvae were pelleted and washed with filtered seawater to give a pellet of ~100 μl. Protein lysates from each larval stage were prepared by resuspension in 200 μl 2x Laemmli buffer with the aid of a micro-pestle and boiling

for 5 min. The protein lysates were clarified by centrifugation for 5 min at 14,000 x g and stored at -20°C until western analysis. From identical samples, total RNA from each stage was prepared. RNA was solubilized by resuspension in 1 ml of Trizol reagent (Invitrogen), and total RNA purified according to the standard procedure. Synthesis of cDNA was performed with 1 μg of total RNA using the Super-Script III polymerase and oligo(dT)$_{12\text{-}18}$ primers by standard procedures (Invitrogen) resulting in a final volume of 24 μl.

Real-Time Quantitative PCR (RTQ-PCR)

Primers were designed to amplify the specific genes as follows (5' to 3'): *AMAS1* CAACTGCGTGAAGAACTG and AGACGGAGGCATAGATTGC (622 bp product), *AMAS2* GAGGACCTGGCACAATGCGA and CATTCTCATCCACAG-CAAAG (544 bp product), *AMAS3* CGATCAATATATCCTTGACC and GTAAGATAGACGAAGTATA (356 bp product), *AMAS4* TGATCAATTTAT-CATCGTCA and ATAGGATAGACGAAGCTGT (356 bp product), *CLMDN* GTTTGGACCGAAAGGAATC and GGATGATAGTTGGAACAAGTCG (655 bp product), *UBIQ* CGAGTATTTGCCAGATGAACCC and ATTGGATTTTTTG-CCCCTGC (233 bp product; NCBI accession number M61772). These primer pairs were used to PCR amplify cDNA prepared from whole adult animal. The products were gel purified (Qiagen), and concentrations determined with the PicoGreen reagent (Molecular Probes). Standard template quantities, expressed as number of copies, were prepared from these products by serial dilution. RTQ-PCR was performed on a MX3000P instrument with the Brilliant SYBR Green reagent (Stratagene) in a 96-well format. A passive reference dye (monitored on the ROX channel)

was included in all samples. Template cDNA from each unknown (staged larvae) was added at 0.1 μl/reaction. A standard curve, run on the same plate as unknowns, was generated by determining the threshold cycle of each dilution of standard. Copy number of unknowns, obtained in triplicate and analyzed individually, were then determined from this curve. Variation between stages was corrected by normalization to the determined copy number of ubiquitin. The product specificity of each RTQ-PCR was assayed by a dissociation (melting) curve analysis, and by agarose gel electrophoresis.

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Table 1
Summary of protein features

protein	amino acids	m.w.[a] (kDa)	signal peptide[b]	t.m.[c]	N-term. β-region	coiled-coils	OLF domain	N-linked glycosylation[d]	pI[e]
amassin-1	495	56.5	SKC^QE 28^29	-	39-59	75-101 130-209	230-477	5 (95, 113, 170, 310, 330)	4.4
amassin-2	480	53.9	VSA^QC 23^24	-	29-47	78-112 123-160 167-197	221-480	2 (74, 308)	4.2
amassin-3	474	53.6	ANA^QC 28^29	-	34-52	65-111 152-202	223-472	2 (37, 164)	4.1
amassin-4	469	52.7	ANA^QC 23^24	-	29-47	60-80 150-198	218-467	2 (32, 159)	4.0
colmedin	794	85.6	RARR^N[b] 142^143[b]	13-35	-	262-282 181-250[f] 295-331[f] 343-484[f]	535-790	6 (125, 255, 262, 276, 539, 614)	8.5

[a]Calculated molecular weight.

[b]Shown are the amino acids in the vicinity to the cleavage site, denoted by a carat. For colmedin, the furin cleavage site is shown.

[c]Location of transmembrane segment.

[d]Number of N-linked glycosylation sites, the positions are in parentheses.

[e]Calculated isoelectric point.

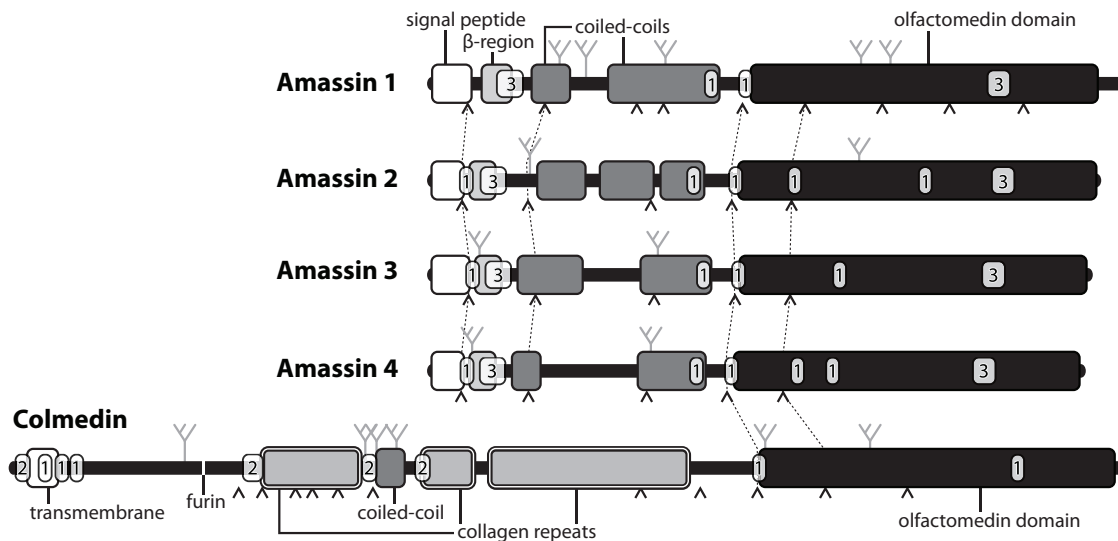[f]For colmedin, denotes the position of the collagen repeat regions.

Figure 1
All five OLF family members share a similar architecture. A schematic outlining the structural features within the OLF proteins is shown. Rounded rectangles filled with a different shades of gray denote the location of each feature, and are also labeled above and below. In the amassins, from the N-terminus there is a signal peptide, a β-region, segments of coiled-coils, and the OLF domain. Colmedin also contains an OLF domain at the C-terminus, but further N-terminally it contains three segments of collagen repeats, a small coiled-coil segment, a type II transmembrane helix, and a short intracellular segment. Smaller rectangles represent the location of cysteine residues (within a proximity of 10 residues to each other), and the numeral inside indicates the quantity of cysteines contained. Exon junction locations are denoted by carat symbols (^). Several of these junctions are well conserved within the OLF family members, and are shown linked by dashed-lines. Branched symbols symbolize the sites of predicted N-linked glycosylation. The furin cleavage site is labeled on the schematic for colmedin.
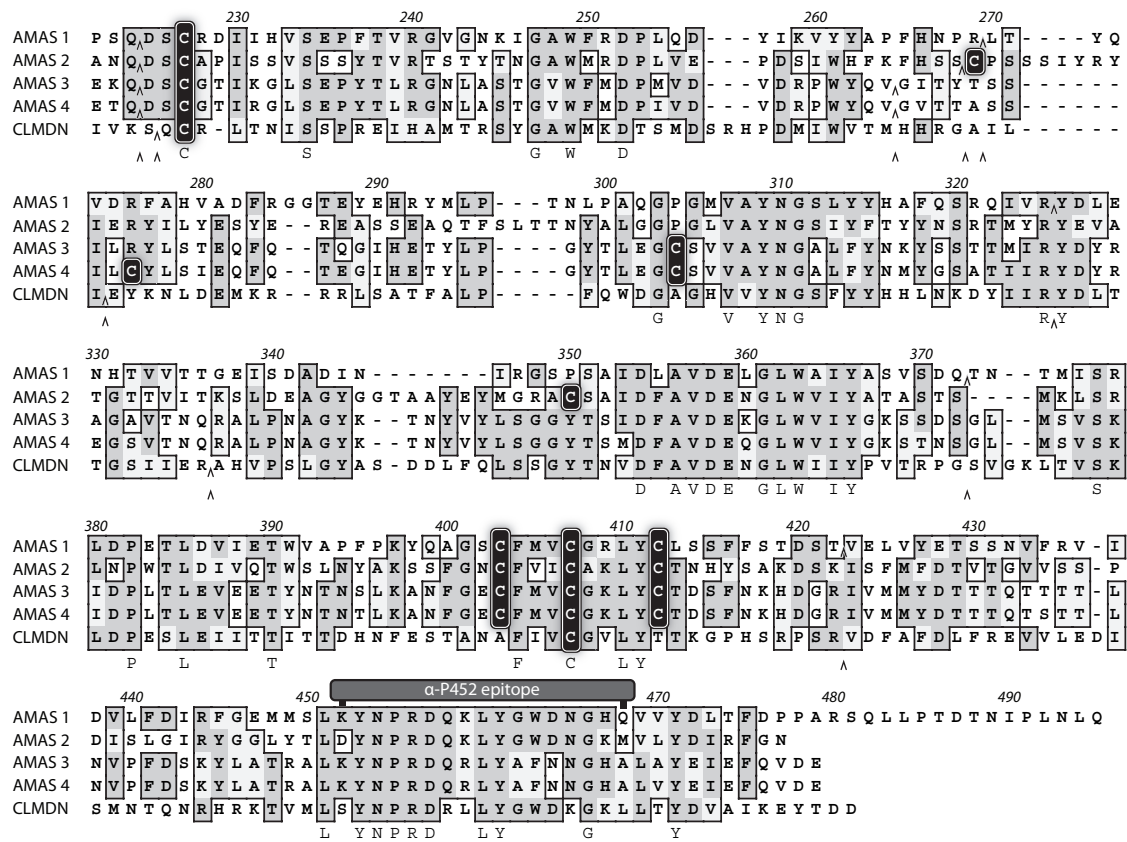
Figure 2

Conservation in the OLF domain. Multiple sequence alignment of the OLF domains from all five family members. AMAS1, amassin-1; AMAS2, amassin-2; AMAS3 amassin-3; AMAS4, amassin-4; CLMDN, colmedin. Locations of cysteines are highlighted by black rectangles. Amino acid identities are dark shaded, similarities are light shaded, and boxes enclose both. The 40 amino acids that are absolutely conserved are shown below the colmedin sequence. The numbering is for the amassin-1 protein sequence. Carat symbols (^) mark the location of exon junctions. The anti-P452 peptide epitope (amassin-1 residues 452-468) is indicated.
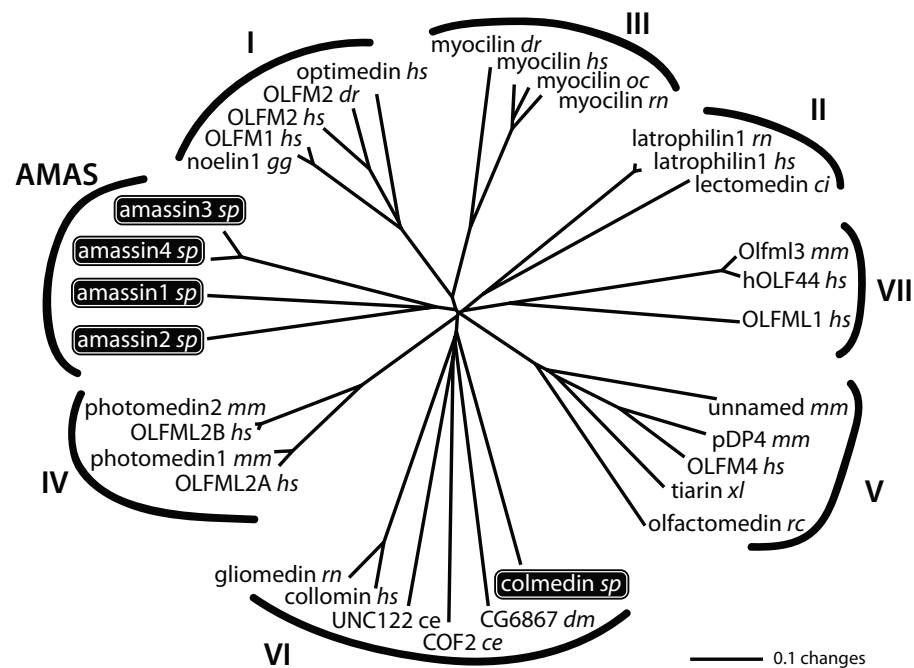
Figure 3
Amassins-1, -2, -3, and -4 form their own subgroup of OLF proteins. An unrooted phylogenetic tree made from an alignment of the OLF domains is shown. Seven previously described (Zeng *et al.*, 2005) subgroups are apparent (labeled I-VII), with one additional subgroup containing the four amassins from sea urchin. Sea urchin colmedin is found in subgroup VI. Following the protein name, species names are abbreviated as follows: sp *Strongylocentrotus purpuratus*, hs *Homo sapiens*, ce *Caenorhabditis elegans*, dm *Drosophila melanogaster*, rn *Rattus norvegicus*, ci *Ciona intestinalis*, mm *Mus musculus*, gg *Gallus gallus*, dr *Danio rerio*, oc *Oryctolagus cuniculus*, xl *Xenopus laevis*, rc *Rana catesbeiana*. NCBI accession numbers of all proteins used in this tree can be found in the Supplemental Materials (Table S2).
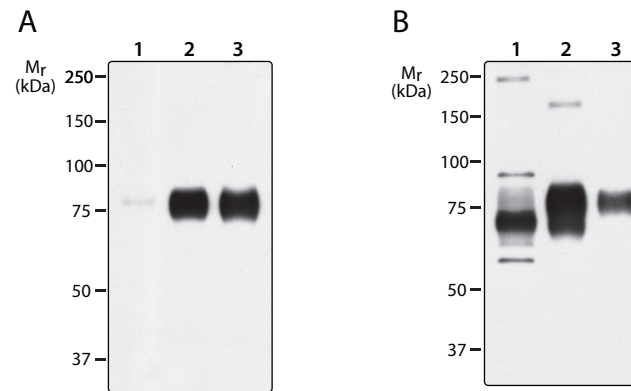
Figure 4
OLF family proteins are in coelomocytes. Lanes were loaded identically in both A and B. Washed coelomocytes (lane 1), clotted coelomocytes (lane 2), and cell-free coelomic fluid (lane 3) were analyzed by immunoblots probed with antiamassin-1 (A), or anti-P452 (B). Molecular mass standards, shown on the left, are in kDa.
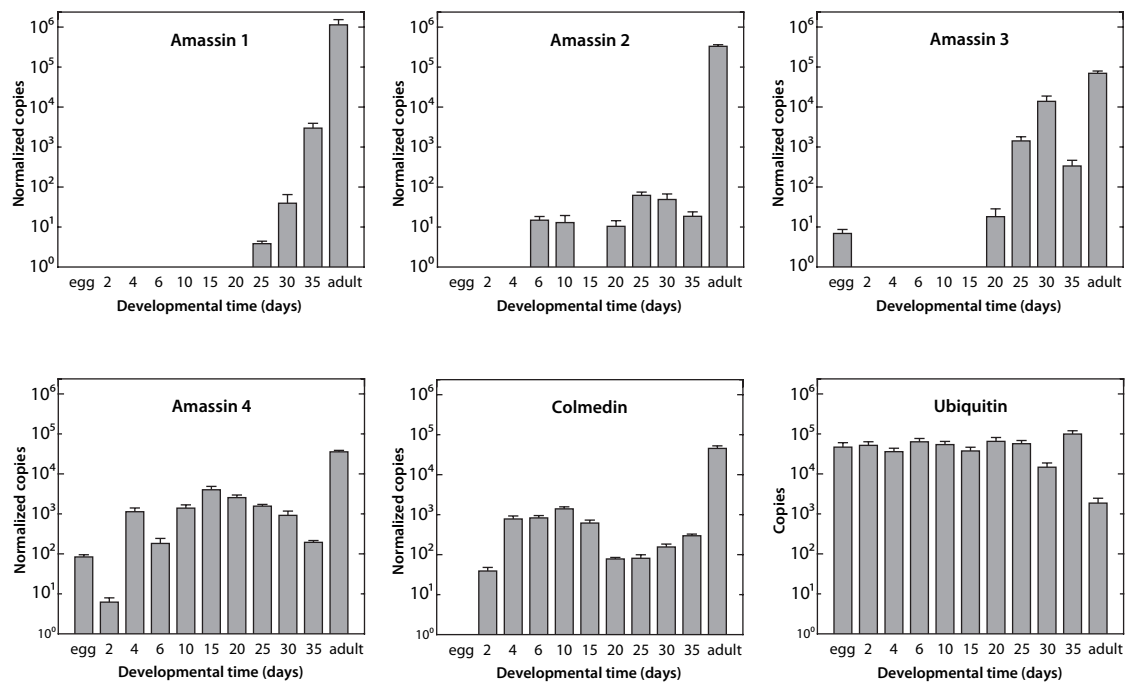
Figure 5

Regulation of OLF transcript levels throughout all stages of development. Transcript copy numbers (per 4.2 ng total RNA) at each of 11 developmental stages for all five OLF family members are shown. Obtained by RTQ-PCR, results are the averages of three replicates, standard error is shown. Copy numbers were calculated from a standard curve generated in the same run, and were normalized to the level of ubiquitin transcript. The transcripts of all six genes were analyzed from the same pools of cDNA.
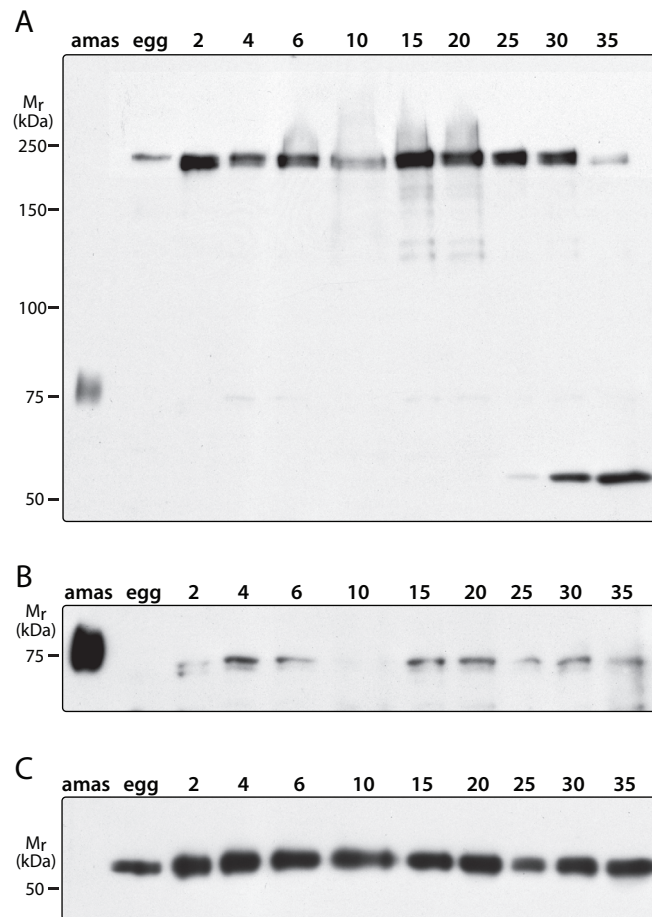
Figure 6

OLF family proteins are present in development. Protein lysates from each developmental stage were resolved by SDS-PAGE on a 6% gel and immunoblotted with anti-P452 which should react with all OLF proteins. (A) The 240 kDa band is present throughout development, while the 60 kDa band is only present beginning at day 25. (B) In an overexposure, a reactive band becomes visible at 70 kDa (possibly a doublet) in the majority of stages except for egg and 10 day. (C) Immunoblotting with anti-α-tubulin controls for equal protein load. Onto the lane labeled amas was loaded 10 ng of purified amassin-1.

Supplemental Table 1
Exon positions in the OLF family cDNAs

gene	cDNA feature		NCBI accession number
AMAS1	5'-UTR	1-84	AF533649
	exon 1	1-162	
	exon 2	163-333	
	exon 3	334-533	
	exon 4	534-585	
	exon 5	586-747	
	exon 6	748-890	
	exon 7	891-1058	
	exon 8	1059-1201	
	exon 9	1202-1350	
	exon 10	1351-1700	
	3'-UTR	1573-1700	
AMAS2	5'-UTR	1-90	DQ250734
	exon 1	1-162	
	exon 2	163-303	
	exon 3	304-558	
	exon 4	558-742	
	exon 5	743-872	
	exon 6	873-1613	
	3'-UTR	1534-1613	
AMAS3	5'-UTR	1-90	DQ250735
	exon 1	1-177	
	exon 2	178-321	
	exon 3	322-573	
	exon 4	574-748	
	exon 5	749-866	
	exon 6	867-1578	
	3'-UTR	1516->1578[a]	
AMAS4	5'-UTR	<1-39[a]	DQ250736
	exon 1	1-111	
	exon 2	112-255	
	exon 3	256-507	
	exon 4	508-682	
	exon 5	683-800	
	exon 6	801-1938	
	3'-UTR	1450-1938	
CLMDN	5'-UTR	1-111	DQ250737
	exon 1	1-600	
	exon 2	601-652	
	exon 3	653-724	
	exon 4	725-760	
	exon 5	761-814	
	exon 6	815-889	
	exon 7	890-1462	
	exon 8	1463-1588	
	exon 9	1589-1711	
	exon 10	1712-1858	
	exon 11	1859-2031	
	exon 12	2032-2538	
	3'-UTR	2497-2538	

[a]Incomplete untranslated region.

Supplemental Table 2
Proteins used to generate phylogenetic tree

protein name	species	NCBI accession number
AMAS		
amassin-1	*Strongylocentrotus purpuratus*	AF533649
amassin-2	*Strongylocentrotus purpuratus*	DQ250734
amassin-3	*Strongylocentrotus purpuratus*	DQ250735
amassin-4	*Strongylocentrotus purpuratus*	DQ250736
I		
noelin-1	*Gallus gallus*	AF182815
OLFM1	*Homo sapiens*	BC008763
OLFM2	*Homo sapiens*	AF131839
OLFM2	*Danio rerio*	BC044164
optimedin	*Homo sapiens*	Q96PB7
II		
latrophilin1	*Rattus norvegicus*	O88917
latrophilin1	*Homo sapiens*	AF307079
lectomedin	*Ciona intestinalis*	AY261876
III		
myocilin	*Danio rerio*	NM_001015062
myocilin	*Homo sapiens*	NM_000261
myocilin	*Oryctolagus cuniculus*	AY191317
myocilin	*Rattus norvegicus*	NM_030865
IV		
photomedin2	*Mus musculus*	BC025654
OLFML2B	*Homo sapiens*	NM_015441
photomedin1	*Mus musculus*	AK035313
OLFML2A	*Homo sapiens*	AB119055
V		
olfactomedin	*Rana catesbeiana*	L13595
tiarin	*Xenopus laevis*	AB075925
OLFM4	*Homo sapiens*	NM_006418
pDP4	*Mus musculus*	XM_139161
unnamed	*Mus musculus*	BC058791
VI		
colmedin	*Strongylocentrotus purpuratus*	DQ250737
CG6867	*Drosophila melanogaster*	NM_133034
COF2	*Caenorhabditis elegans*	AY494975
UNC-122	*Caenorhabditis elegans*	AY494976
collomin	*Homo sapiens*	AK172756
gliomedin	*Rattus norvegicus*	AY266116
VII		
Olfml3	*Mus musculus*	NM_133859
hOLF44	*Homo sapiens*	AY464015
OLFML1	*Homo sapiens*	AY358591

The text of Chapter III, in full, is being prepared for publication. The dissertation author was the primary author, and co-author V. D. Vacquier directed and supervised the research, which forms the basis for this chapter.

Chapter IV

Expression, Purification, Crystallization and Preliminary X-ray Analysis of the Olfactomedin Domain from the Sea Urchin Cell-Adhesion Protein Amassin

ABSTRACT

A family of animal proteins is emerging which contain a conserved protein motif known as an olfactomedin (OLF) domain. Novel extracellular protein-protein interactions occur through this domain. The OLF family member amassin, from the sea urchin *Strongylocentrotus purpuratus*, has previously been identified to mediate a rapid cell-adhesion event resulting in a large aggregation of coelomocytes, the circulating immune cells. In this work, heterologous expression and purification of the OLF domain from amassin was carried out and initial crystallization trials were performed. A native data set has been collected, extending to 2.7 Å under preliminary cryoconditions, using an in-house generator. This work leads the way to the determination of the first structure of an OLF domain.

INTRODUCTION

Originally identified in its namesake protein isolated from the olfactory neu-roepithelial mucous layer in the bullfrog, *Rana catesbeiana* (Snyder *et al.*, 1991; Yo-koe and Anholt, 1993), the number of proteins identified as containing an olfac-tomedin (OLF) domain continues to grow. Currently, members are found in diverse animal groups including nematodes, arthropods, echinoderms, and vertebrates (Bateman *et al.*, 2002). An interest in the OLF domain has arisen in part as a conse-quence of the identification of the ability of proteins containing this domain to stimulate neurogenic events (Moreno and Bronner-Fraser, 2001; Tsuda *et al.*, 2002) such as neural crest cell formation (Barembaum *et al.*, 2000) and the formation of nodes of Ranvier (Eshed *et al.*, 2005). A majority of research also centers around the finding that mutations in the human protein myocilin, which has been implicated in at least one form of glaucoma, are concentrated within the OLF domain (Adam *et al.*, 1997; Ray *et al.*, 2003; Stone *et al.*, 1997; Tamm and Russell, 2001). The func-tions of the OLF-family members are often not understood, but recently described activities of these proteins point towards a general structural/organizational role on the extracellular surface. For example, the OLF protein UNC-122 in *Caenorhabditis elegans* appears to organize components at the neuromuscular junction (Loria *et al.*, 2004). Additionally, a unique cell-adhesion activity has been identified for the OLF-family member amassin (Hillier and Vacquier, 2003), the subject of this re-search. Purified amassin mediates a rapid intercellular adhesion of sea urchin (*Strongylocentrotus purpuratus*) coelomocytes.

Amassin is a 495-amino-acid extracellular and glycosylated protein found in the coelomic fluid. Along its length are several structural features: a signal peptide di-

recting its secretion, a predicted short β-region, a region of dimerizing coiled-coils and finally the OLF domain at its C-terminus. Previous research utilized various truncated amassin proteins heterologously expressed in the yeast *Pichia pastoris* to find that the OLF domain alone was the portion responsible for coelomocyte-binding activity (Chapter II). While the receptor for amassin's OLF domain on the cell surface has not been identified, other OLF-family members have also been found to bind their targets through this domain. Examples include the protein gliomedin that binds its targets neurofascin and NrCAM through the OLF domain (Eshed *et al.*, 2005) and a heterotypic OLF-OLF interaction occurring between myocilin and optimedin (Torrado *et al.*, 2002).

OLF domains have been found by secondary-structure prediction (Hillier and Vacquier, 2003) and by circular dichroism (Nagy *et al.*, 2003) to adopt an all-β structure. The domain typically spans ~250 amino acids. No further structural information is known for these domains. To increase our knowledge of this domain, we have set out to obtain the three-dimensional structure of the OLF domain from amassin. To that end, proteins heterologously expressed in *P. pastoris* and *Escherichia coli* have been screened for crystallization ability. Owing to the identification and requirement for proper disulfide bonds in the entire amassin protein, as well as within its OLF domain (Chapter II), expression by secretion in the eukaryotic yeast host was initially utilized. This was followed by prokaryotic expression in a bacterial strain that favors disulfide formation.

PROTEIN EXPRESSION AND PURIFICATION

The region encoding amassin residues 210-495 (NCBI accession number AAO43562) was inserted into the bacterial expression vector pBH8 (Hillier *et al.*, 1999). This vector codes for the following features: start codon, hexahistidine tag, linker region of amino acids DYDIPTT, tobacco etch viral (TEV) protease cleavage site, the amino acids of interest (preceded by two amino acids GS) and finally stop codons. Upon ligation and subsequent transformation into *E. coli* strain TOP10 (Invitrogen), the entire coding region was verified by DNA sequencing.

For expression, the plasmid was transformed into *E. coli* strain Rosetta-gami B(DE3)pLysS (Novagen) and plated onto LB agar containing carbenicillin, chloramphenicol, tetracycline, and kanamycin (100, 34, 12.5, and 15 mg ml^{-1}, respectively). This feature-rich expression strain was chosen mainly owing to the mutations in its thioredoxin reductase and glutathione reductase genes, which greatly enhance the formation of disulfide bonds in the bacterial cytoplasm (Bessette *et al.*, 1999; Prinz *et al.*, 1997). A single fresh transformant colony was used to inoculate a starter culture in LB with the same antibiotics and grown to an OD$_{600}$ of 0.6; it was then stored at 277 K overnight. The starter bacteria were harvested by centrifugation, resuspended in fresh media and used to inoculate five 1 l flasks. On reaching OD of 0.6, these cultures were induced with 1 mM IPTG and incubated at 310 K for 2 h. The bacteria were harvested by centrifugation at 4000g for 15 min, resuspended in 160 ml lysis buffer (50 mM sodium phosphate pH 8, 500 mM NaCl, 10 mM imidazole) and frozen at 203 K.

Cell suspensions were thawed, lysed by sonication and Triton X-100 added to 0.5%. The lysate was cleared by centrifugation at 10 000g. 1 ml of Ni-NTA Fast Flow

(Qiagen) was added to the supernatant and allowed to bind in batch with tumbling for 2 h. The resin was settled and the unbound material removed, then transferred to an empty column. The column was washed with three cycles of 30 ml lysis buffer, then 30 ml of lysis buffer with 20 mM imidazole, and finally the protein was eluted with 10 ml of lysis buffer with 250 mM imidazole. The eluate was then dialyzed against cleavage buffer (25 mM Tris pH 7.5, 50 mM NaCl). The hexahistidine purification tags were removed by incubation with sufficient TEV protease to completely digest within 2 h at 298 K. The protein was then loaded onto a 2 ml HiTrap Q FF anion-exchange column attached to an ÄKTA FPLC system (Amersham Biosciences). An extended elution gradient of 25 mM to 1 M NaCl over 100 column volumes was necessary to resolve the OLF domain from most contaminants (Fig. 1). Monitoring the elution-profile chromatogram, the core of the protein peak was pooled and dialyzed with three changes against 10 mM HEPES pH 7.5. The protein was concentrated by ultrafiltration (Amicon) to 10 mg ml^{-1}, filtered using a 0.22 μm syringe and then frozen in a dry ice-ethanol slurry and stored in aliquots at 203 K. Final yields were typically ~1-3 mg from a 5 l culture.

The amassin OLF domain was expressed to a sufficient level to allow crystallization trials to be performed. However, its purification was not as simple as would be expected for a histidine tagged protein. Major contaminants were retained and coeluted from the Ni-NTA column even under more stringent conditions, including the presence of reductants, hydrophobic washes and limiting quantities of chromatography resin (data not shown). The anion-exchange chromatography step was able to remove the majority of the contaminants by careful fractionation dur-

ing an extended elution. Enhanced purity came at a significant loss, as ~30% of the applied protein was lost in the final purification step.

CRYSTALLIZATION EXPERIMENTS

Sparse-matrix screening kits Crystal Screens I and II (Hampton Research) were used for all initial crystallization trials using the sitting-drop vapour-diffusion method. 2 μl protein solution was mixed with 2 μl well solution and placed into Cryschem plates (Hampton Research) sealed with tape. The trays were incubated in a laboratory cabinet at ~298 K.

Initial trials used the OLF domain (residues 223-476 and 223-495) of amassin expressed in the eukaryotic host *P. pastoris* (Chapter II). However, no crystals were obtained using these proteins. The proteins were *N*-linked glycosylated in the yeast system and it may be that the glycosylation was heterogeneous, inhibiting the formation of crystals. To remedy this, the two N-linked sites were removed by mutating the asparagine residue of the site N-*X*-T/S to aspartate or alanine. These proteins were secreted from *P. pastoris* and the purified proteins indeed appeared to lack glycosylation. This was evidenced by sharp-running bands on SDS-PAGE and no mobility shift upon incubation with PNGaseF (data not shown). Unfortunately, yields from these mutants were too low to warrant crystallization trials. Additionally, expression in the presence of the glycosylation inhibitor tunicamycin (Sigma) with the wild-type sequence yielded extremely low protein levels. Attempts were also made to deglycosylate the yeast-expressed proteins. However, this was not feasible on the scale required for crystallization because the proteins were fairly resistant, requiring large amounts of enzyme (data not shown).

To avoid glycosylation events entirely, an extended residue range (210-495) containing the OLF domain was expressed in the prokaryotic host *E. coli*. The calculated molecular weight after removal of purification tags is 32.8 kDa. Sparse-

matrix screens revealed several conditions in which crystals formed within a month. Hampton Screen I condition No. 9 (0.1 *M* sodium citrate pH 5.6, 30% PEG 4000, 0.2 *M* ammonium acetate) was most promising, although its crystals were malformed and walnut-shaped (Fig. 2*a*). A more directed screen surrounding this condition ensued that maintained the buffer composition and substituted PEG 4000 with PEG 3350. We found a marked improvement in crystal appearance coinciding with lower concentrations of ammonium acetate. Additionally, lower PEG concentrations promoted fewer crystal initiating events, leading to larger crystals. The optimized condition was 0.1 *M* sodium citrate pH 5.6 with 20% PEG 3350. After approximately 1 week, crystals formed from within a light precipitate. At least two gross crystal morphology differences could be observed from within the same drops, one with a tapered appearance and the other more squared (Fig. 2*b*). As growth continued, much less precipitant was visible and the size of the crystals gradually increased for 2-3 weeks (Fig. 2*c*).

DATA COLLECTION AND X-RAY CRYSTALLOGRAPHIC ANALYSIS

A cryoprotecting procedure was used which involved first transferring the crystal to a drop containing a slightly higher concentration of precipitant than that used for crystallization, with the addition of glycerol (0.1 M sodium citrate pH 5.6, 23% PEG 3350, 15% glycerol). The crystal remained in the cryoprotectant for ~10 s before removal with a nylon loop and flash-cooling by direct placement into a liquid-nitrogen stream at 110 K. A data set was collected on an in-house R-AXIS IV imaging-plate detector using Cu $K\alpha$ radiation from a Rigaku RU200 rotating-anode generator equipped with Osmic mirrors. Data were collected, indexed and scaled with the *CrystalClear* (Rigaku) software package. The crystal diffracted to 2.7 Å under this cryoprotectant procedure with 0.3° mosaicity. Systematic absences indicated the crystal belongs to space group $P2_12_12_1$, with unit-cell dimensions of $a = 66.71$, $b = 72.28$, $c = 113.35$ Å. Crystal parameters and diffraction statistics are shown in Table 1. Assuming the presence of two OLF domains in the asymmetric unit, the Matthews coefficient is 2.1 Å^3 Da^{-1} and the solvent content is 40.1% (Matthews, 1968).

While no structure has yet been determined for an OLF domain, an effort was made to use the molecular-replacement method using theoretically predicted models. The structure-prediction tool *3D-PSSM* (Kelley *et al.*, 2000; MacCallum *et al.*, 2000), using only the primary sequence as input, predicted by threading onto a library of structures several high-confidence scoring solutions. The top-scoring PDB entries and their confidence scores (E values) were as follows: 1lpx (Springer, 1998) 0.064, 1ndx (Springer, 1998) 0.194 and 1rwi (Good *et al.*, 2004) 0.239. These in turn were used as models, with and without side chains, with the *CCP4* package compo-

nent *Phaser* in an attempt to phase the data (Collaborative Computational Project, Number 4, 1994; Read, 2001; Storoni *et al.*, 2004). While a reasonable solution was obtained by this maximum-likelihood technique, the resultant density maps were not sufficiently robust to rebuild or even properly orient the molecule. This may have been expected, as the highest sequence identity for a search model was merely 16%.

Structure solution of the OLF domain will most likely require the acquisition of experimental phases. The OLF domain expressed and crystallized here contains six methionine residues out of a total of 288. Since it can be expressed in bacteria, the substitution of those residues for selenomethionine is possible, leading the way to structure determination by multiwavelength anomalous diffraction. With the first OLF domain structure in hand, we hope to gain valuable insight into how this domain of emerging importance is able to function and bind its extracellular targets.

ACKNOWLEDGMENTS

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Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell

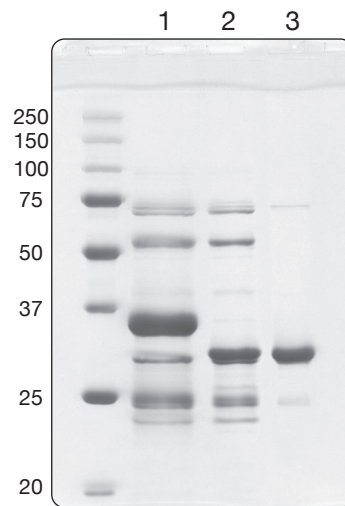|	
Wavelength (Å)	1.5418
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 66.71, b = 72.28, c = 113.35$
Resolution range (Å)	29.9-2.7 (2.80-2.70)
No. of reflections	80424 (7440)
Unique reflections	27497 (2704)
Completeness (%)	94.5 (92.2)
Redundancy	2.92 (2.75)
R_{merge} (%)	10.8 (34.3)
$<I/\sigma(I)>$	7.3 (2.9)

Figure 1
Purification of the bacterially expressed amassin OLF domain. The preparation was fairly impure following elution from the Ni-NTA column (lane 1). The tagged protein ran at a relative mobility of ~35 kDa, and upon tag removal at ~30 kDa (lane 2). Subsequent anion-exchange chromatography improved the purity substantially (lane 3). Proteins were resolved by SDS-PAGE on a 10% gel, followed by staining with Coomassie Blue. Shown on the left are molecular weight standards labeled in kDa.
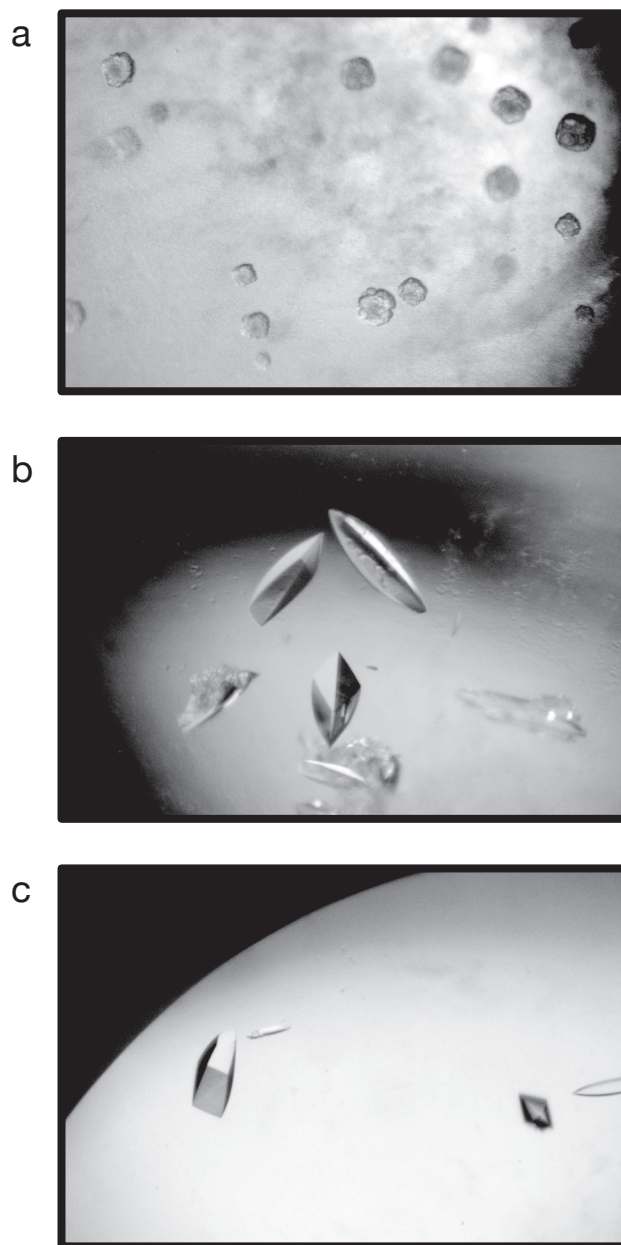
Figure 2
Photographs of amassin OLF domain crystals. Walnut-shaped crystals formed in the initial screen with Hampton Crystal Screen condition No. 9 (*a*). Following optimization of conditions, a single drop may yield several crystal morphotypes; the common form was fusiform-like, while thicker, more rectangular forms also were found (*b*). The crystal used for data collection (leftmost crystal) was of the rectangular type with approximate dimensions of 0.3 x 0.2 x 0.15 mm (*c*).

The text of Chapter IV, in full, is being prepared for publication. The dissertation author was the primary author, and co-author V. D. Vacquier directed and supervised the research, which forms the basis for this chapter. Crystallographic data collection and analysis was performed at The Scripps Research Institute by the dissertation author and V. Sundaresan, under the direction of C. D. Stout.