

Amassin, an olfactomedin protein, mediates the massive intercellular adhesion of sea urchin coelomocytes

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Sea 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 $\sim 7.5 \times 10^6$ cells/ml (Coffaro and Hinegardner, 1977; Smith et al., 1996; Pancer et al., 1999; Gross et al., 2000; Pancer, 2000). Four major types of coelomocytes are recognized: red amoebocytes ($\sim 15\%$, also called red spherule cells), colorless amoebocytes ($\sim 5\%$, also called colorless spherule cells), round vibratile cells with a single long flagellum ($\sim 14\%$), and phagocytes ($\sim 66\%$) (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 (Donnellon, 1938; Bookhout and Greenburg, 1940; Davidson, 1953). Clotting was also found to depend on the formation and presence of disulfide bonds (Booolootian and Giese, 1959; Bertheussen and Seijelid, 1978). 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.

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*Abbreviations used in this paper: CFP, cell-free plasma; OLF, olfactomedin; PNGase-F, peptide *N*-glycosidase-F; WCF, whole coelomic fluid.

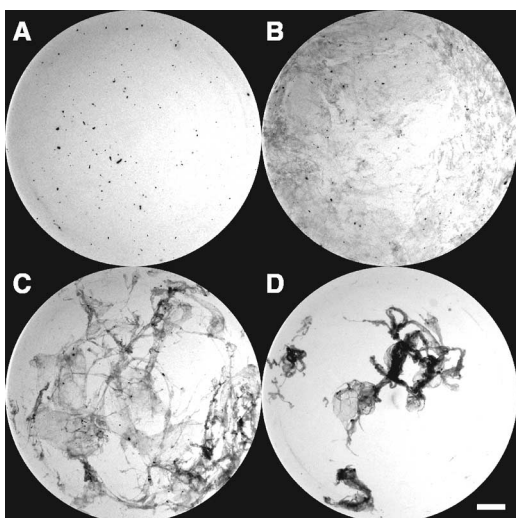


Figure 1. The clotting of coelomocytes. Sea urchin coelomocytes form a massive cellular clot by rapid intercellular adhesion. The contents of the coelomic cavity of one animal was poured into a beaker, constantly swirled, and top view photographs were taken. (A) After 10 s, aggregates of coelomocytes 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.

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 after 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 was performed. A 2-h digestion was needed to completely abolish the clotting activity (un-

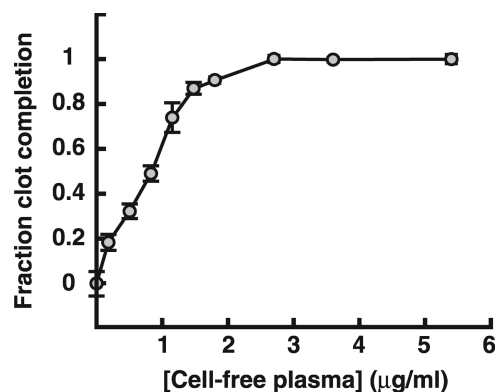


Figure 2. An assay for the level of coelomocyte clotting. The degree of coelomocyte clotting depends on the concentration of CFP. Complete clotting occurs at 3 µg CFP/ml. Data points are the mean of five experiments with the same batch of cells (standard error bars shown). Clot completion is defined as the level of clotting that 10 µg CFP would promote compared with no clotting with buffer alone.

published 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 $M_r \sim 75$ kD (hereafter called amassin; fractions 10–17). The purity of the 75-kD protein in

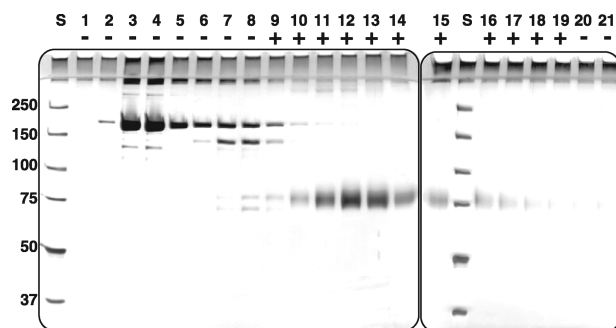


Figure 3. DEAE-Sepharose chromatography of cell-free coelomic plasma. A silver-stained 7.5% SDS-PAGE separation of proteins eluting from the DEAE column upon application of a linear salt gradient that increases left to right. Numbers on top of lanes represent individual fractions. A 10-µl aliquot of 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.

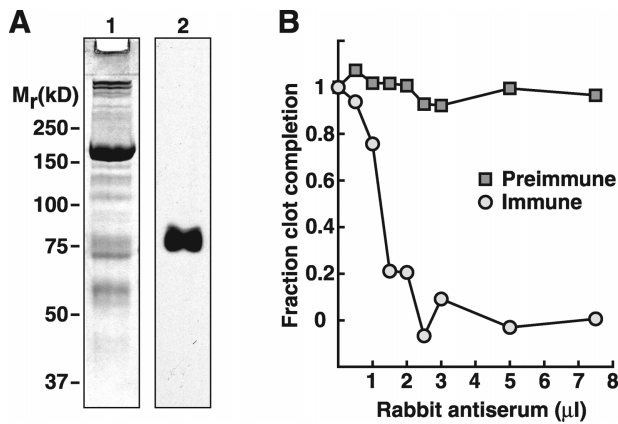


Figure 4. **Antibodies to amassin prevent clotting.** (A) Western blots of CFP show that the antibody reacts only with the 75 kD amassin. CFP (5 μg) was loaded onto lane 1 and stained with Coomassie. The sample for lane 2 was 300 ng CFP and detected by Western blot with anti-amassin. (B) Preincubation of CFP with anti-amassin 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.

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 pro-

tein. 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 and 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

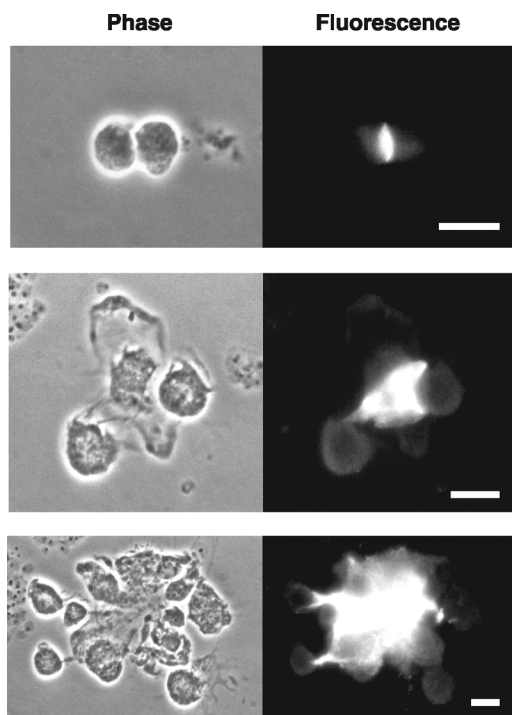


Figure 5. **Antibody localizes amassin between adhering coelomocytes.** The left panels are phase contrast, and the right panels are fluorescence with anti-amassin. In the top set, amassin binds two coelomocytes together. In the bottom two sets, larger groups of coelomocytes are shown. Bars, 15 μm.

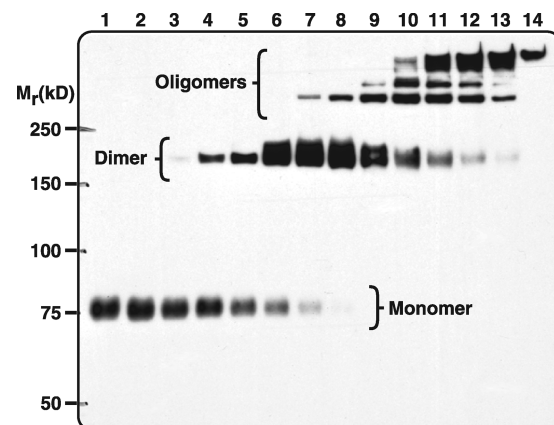


Figure 6. **Amassin is disulfide bonded into large aggregates.**

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 anti-amassin. 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.

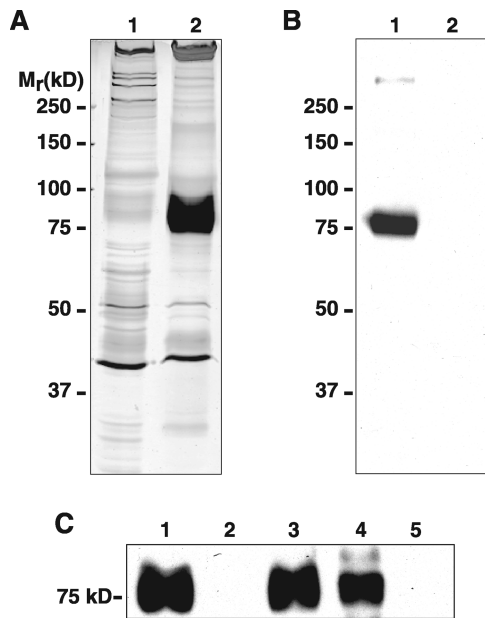


Figure 7. Amassin is released as coelomocyte clots are dissociated into single cells. (A) Washed clots were dissociated in DTT, and the 10,000-g supernatant was recovered (lane 2). A control sample, lane 1 is the supernatant released from the same DTT treatment to washed, clot-inhibited coelomocytes run on a 10% silver-stained SDS-PAGE gel. DTT dissociates the clot into single cells and releases amassin. (B) An immunoblot probed with anti-amassin. 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 anti-amassin show that amassin is present as a large, disulfide-bonded aggregate which can be dissociated with DTT.

in samples of coelomocytes allowed to clot (followed by washing) compared with washed clot-inhibited cells (Fig. 7 B). Only after long exposures is a slight detection of amassin

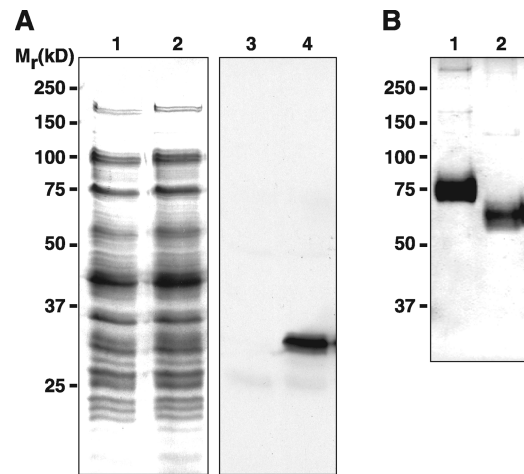


Figure 8. Confirmation of amassin cloning by recombinant protein reactivity and relative molecular mass agreement. (A) The bacterial expression of the derived amino acid sequence from the amassin OLF domain (aa 228–495) is recognized by the antibodies to native amassin. *Escherichia coli* lysates of 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 anti-amassin, 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.

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).

Table I. 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	
<u>D</u> I <u>I</u> H <u>V</u> S <u>E</u> P <u>F</u> T <u>V</u> R (229–240)	CAYGTIWSIGARCCNTTYAC GTRAANGGYTCISWIACRTG
<u>I</u> G <u>A</u> W <u>F</u> R <u>D</u> PLQDYIK (246–259)	GGIGCNTGGTTYMGNGAYCC GGRTCNCRAACCANGCICC
<u>L</u> D <u>P</u> E <u>T</u> L <u>D</u> V <u>I</u> E <u>T</u> W <u>V</u> A <u>P</u> (380–394)	GAYCCNGARACIYTNAYGT ACRTCNARIGTYTCNGRRC
<u>L</u> Y <u>G</u> W <u>D</u> N <u>G</u> H <u>Q</u> V <u>V</u> Y <u>D</u> L <u>T</u> F <u>D</u> P <u>P</u> A <u>R</u> (460–480)	TGGGAYAAAYGGNCAYCARGT ACYTGRTGNCCRTTRTCCCA
<u>S</u> Q <u>L</u> L <u>P</u> T <u>D</u> T <u>N</u> I <u>P</u> L <u>N</u> L <u>Q</u> (481–495)	GAYACIAAYATHCCNYTIAA TTIARNGGDATRTTIGTRTC
Cyanogen bromide	
<u>R</u> T <u>T</u> S <u>G</u> V <u>V</u> (40–46)	
<u>M</u> T <u>L</u> E <u>G</u> V <u>L</u> D <u>G</u> T <u>V</u> V <u>V</u> T <u>V</u> A <u>E</u> L <u>L</u> V <u>K</u> E <u>L</u> K <u>E</u> M (117–144)	

NH₂-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.

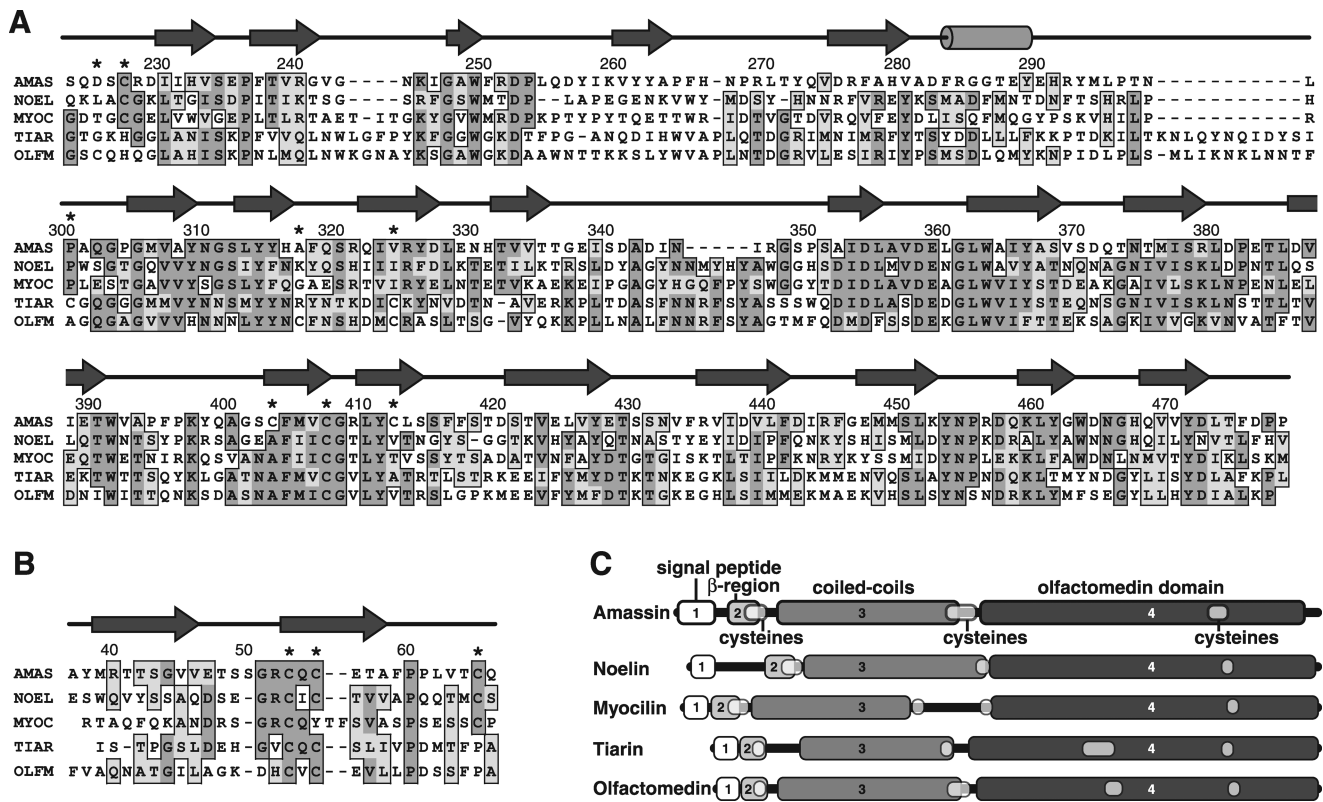


Figure 9. Analysis of the amassin sequence. Sequence alignment of five OLF family members in only the OLF domains (A) or only the NH₂-terminal β-regions (B). AMAS, amassin; NOEL, noelin-1; MYOC, myocilin; TIAR, tiarin; OLFM, olfactomedin. Amino acid identities are dark shaded, similarities are light 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/DBJ under accession no. AF533649.

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/DBJ 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 NH₂-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 NH₂-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 OLF. 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 NH₂-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 μ g/ml) and calcium (10 mM) was then added, and the tubes were 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 (Smith et al., 1992, 1996; Pancer et al., 1999; Clow et al., 2000; Pancer, 2000). 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 (Edean, 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 (Donnellon, 1938; Bookhout and Greenburg, 1940; Davidson, 1953), 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 occurring. 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 (Stone et al., 1997; Nguyen et al., 1998; Fautsch et al., 2000; 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 (Booolootian 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 Na₂SO₄, 2 mM NaHCO₃, 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°C) 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 MgCl₂, 30 mM MgSO₄, 2 mM NaHCO₃) and resedimented as before. All three enriched subpopulations were resuspended in buffered calcium-free artificial seawater and combined at a concentration of 2 × 10⁶ 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 CaCl₂ 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 and 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 was 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 37°C.

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 manufacturer's 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/DBJ under accession no. AF533649.

Western immunoblots

Proteins were resolved by SDS-PAGE and 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 anti-amassin 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 Laboratories) 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 anti-amassin 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/DBJ) 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 NH₂-terminal β-regions from the five proteins as input for the consensus method used 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.

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References

- Barembaum, M., T.A. Moreno, C. LaBonne, J. Sechrist, and M. Bronner-Fraser. 2000. Noelin-1 is a secreted glycoprotein involved in generation of the neural crest. *Nat. Cell Biol.* 2:219–225.
- Bateman, A., E. Birney, L. Cerruti, R. Durbin, L. Etwiller, S.R. Eddy, S. Griffiths-Jones, K.L. Howe, M. Marshall, and E.L. Sonnhammer. 2002. The Pfam protein families database. *Nucleic Acids Res.* 30:276–280.
- Bertheussen, K., and R. Sejelid. 1978. Echinoid phagocytes in vitro. *Exp. Cell Res.*

- 111:401–412.
- Bookhout, C.G., and N.D. Greenburg. 1940. Cell types and clotting reactions in the echinoid, *Mellita quinquiesperforata*. *Biol. Bull.* 79:309–320.
- Boooloatian, R.A., and A.C. Giese. 1959. Clotting of echinoderm coelomic fluid. *J. Exp. Zool.* 140:207–229.
- Brooks, J.M., and G.M. Wessel. 2002. The major yolk protein in sea urchins is a transferrin-like, iron binding protein. *Dev. Biol.* 245:1–12.
- Burke, R.D. 1999. Invertebrate integrins: structure, function, and evolution. *Int. Rev. Cytol.* 191:257–284.
- Clow, L.A., P.S. Gross, C.S. Shih, and L.C. Smith. 2000. Expression of SpC3, the sea urchin complement component, in response to lipopolysaccharide. *Immunogenetics.* 51:1021–1033.
- Coffaro, K.A., and R.T. Hinegardner. 1977. Immune response in the sea urchin *Lytechinus pictus*. *Science.* 197:1389–1390.
- Cuff, J.A., M.E. Clamp, A.S. Siddiqui, M. Finlay, and G.J. Barton. 1998. JPred: a consensus secondary structure prediction server. *Bioinformatics.* 14:892–893.
- Davidson, E. 1953. Clotting of the perivisceral fluid of the sand dollar, *Echinarachnius parma*. *Biol. Bull.* 105:372.
- Donnellon, J.A. 1938. An experimental study of clot formation in the perivisceral fluid of *Arbacia*. *Physiol. Zool.* 11:389–398.
- Doolittle, R.F. 1984. Fibrinogen and fibrin. *Annu. Rev. Biochem.* 53:195–229.
- Edds, K.T. 1977. Dynamic aspects of filopodial formation by reorganization of microfilaments. *J. Cell Biol.* 73:479–491.
- Edean, R. 1966. The coelomocytes and coelomic fluids. In *Physiology of Echinodermata*. R.A. Boooloatian, editor. Interscience Publisher, New York. 301–328.
- Fautsch, M.P., and D.H. Johnson. 2001. Characterization of myocilin-myocilin interactions. *Invest. Ophthalmol. Vis. Sci.* 42:2324–2331.
- Fautsch, M.P., C.K. Bahler, D.J. Jewison, and D.H. Johnson. 2000. Recombinant TIGR/MYOC increases outflow resistance in the human anterior segment. *Invest. Ophthalmol. Vis. Sci.* 41:4163–4168.
- Fuller, G.M., and R.F. Doolittle. 1971. Studies of invertebrate fibrinogen. II. Transformation of lobster fibrinogen into fibrin. *Biochemistry.* 10:1311–1315.
- Gross, P.S., L.A. Clow, and L.C. Smith. 2000. SpC3, the complement homologue from the purple sea urchin, *Strongylocentrotus purpuratus*, is expressed in two subpopulations of the phagocytic coelomocytes. *Immunogenetics.* 51:1034–1044.
- Holland, L.Z., A.C. Giese, and J.H. Phillips. 1967. Studies on the perivisceral coelomic fluid protein concentration during seasonal and nutritional changes in the purple sea urchin. *Comp. Biochem. Physiol.* 21:361–371.
- Johnson, P.T. 1969. The coelomic elements of sea urchins (*Strongylocentrotus*). I. The normal coelomocytes; their morphology and dynamics in hanging drops. *J. Invertebr. Pathol.* 13:25–41.
- Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science.* 252:1162–1164.
- Moreno, T.A., and M. Bronner-Fraser. 2001. The secreted glycoprotein noelin-1 promotes neurogenesis in *Xenopus*. *Dev. Biol.* 240:340–360.
- Nguyen, T.D., P. Chen, W.D. Huang, H. Chen, D. Johnson, and J.R. Polansky. 1998. Gene structure and properties of TIGR, an olfactomedin-related glycoprotein cloned from glucocorticoid-induced trabecular meshwork cells. *J. Biol. Chem.* 273:6341–6350.
- Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10:1–6.
- Otto, J.J., and J. Bryan. 1981. The incorporation of actin and fascin into the cytoskeleton of filopodial sea urchin coelomocytes. *Cell Motil.* 1:179–192.
- Pancer, Z. 2000. Dynamic expression of multiple scavenger receptor cysteine-rich genes in coelomocytes of the purple sea urchin. *Proc. Natl. Acad. Sci. USA.* 97:13156–13161.
- Pancer, Z., J.P. Rast, and E.H. Davidson. 1999. Origins of immunity: transcription factors and homologues of effector genes of the vertebrate immune system expressed in sea urchin coelomocytes. *Immunogenetics.* 49:773–786.
- Smith, L.C., R.J. Britten, and E.H. Davidson. 1992. SpCoel1: a sea urchin profilin gene expressed specifically in coelomocytes in response to injury. *Mol. Biol. Cell.* 3:403–414.
- Smith, L.C., L. Chang, R.J. Britten, and E.H. Davidson. 1996. Sea urchin genes expressed in activated coelomocytes are identified by expressed sequence tags. Complement homologues and other putative immune response genes suggest immune system homology within the deuterostomes. *J. Immunol.* 156:593–602.
- Snyder, D.A., A.M. Rivers, H. Yokoe, B.P. Menco, and R.R. Anholt. 1991. Olfactomedin: purification, characterization, and localization of a novel olfactory glycoprotein. *Biochemistry.* 30:9143–9153.
- Stone, E.M., J.H. Fingert, W.L. Alward, T.D. Nguyen, J.R. Polansky, S.L. Sunden, D. Nishimura, A.F. Clark, A. Nystuen, B.E. Nichols, et al. 1997. Identification of a gene that causes primary open angle glaucoma. *Science.* 275:668–670.
- Tai, J.Y., R.C. Seid, Jr., R.D. Huhn, and T.Y. Liu. 1977. Studies on *Limulus* amoebocyte lysate II. Purification of the coagulogen and the mechanism of clotting. *J. Biol. Chem.* 252:4773–4776.
- Tamm, E.R., and P. Russell. 2001. The role of myocilin/TIGR in glaucoma: results of the Glaucoma Research Foundation catalyst meeting in Berkeley, California, March 2000. *J. Glaucoma.* 10:329–339.
- Taniguchi, F., Y. Suzuki, H. Kurihara, Y. Kurihara, H. Kasai, S. Shirato, and M. Araie. 2000. Molecular cloning of the bovine MYOC and induction of its expression in trabecular meshwork cells. *Invest. Ophthalmol. Vis. Sci.* 41:2070–2075.
- Tsuda, H., N. Sasai, M. Matsuo-Takasaki, M. Sakuragi, Y. Murakami, and Y. Sasai. 2002. Dorsalization of the neural tube by *Xenopus* tiarin, a novel patterning factor secreted by the flanking nonneural head ectoderm. *Neuron.* 33:515–528.
- Wentz-Hunter, K., J. Ueda, and B.Y. Yue. 2002. Protein interactions with myocilin. *Invest. Ophthalmol. Vis. Sci.* 43:176–182.
- Yokoe, H., and R.R. Anholt. 1993. Molecular cloning of olfactomedin, an extracellular matrix protein specific to olfactory neuroepithelium. *Proc. Natl. Acad. Sci. USA.* 90:4655–4659.