Detection of vitellogenin in a subpopulation of sea urchin coelomocytes

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Sea urchin vitellogenin is a high molecular weight glycoprotein, which is the precursor of the major yolk protein present in the unfertilized egg. Vitellogenin processing into the major yolk protein and its further enzymatic cleavage during sea urchin embryonic devolopment, has been extensively described, and the adhesive properties of the processed molecule have been studied. The function of vitellogenin in the adult, where it has been found in the coelomic fluid of both male and female individuals, is still unknown, although its role on promoting the adhesion of embryonic cells has been shown.

In this report we describe the detection of vitellogenin in lysates of whole circulating coelomocytes of both male and female sea urchins of the species Paracentrotus lividus. By metrizoic acid gradients we purified total coelomocytes into six subpopulations that were tested for the occurrence of the molecule using vitellogenin-specific polyclonal antibodies. We detected vitellogenin only in the coelomocyte subpopulation called colorless spherule cells, packed in kidney-shaped granules located around the nucleus. We also showed that coelomocytes respond to stress conditions by discharging vitellogenin into the medium. This result together with previous observations on the adhesive properties of the molecule suggest a role for vitellogenin in the clotting phenomenon occurring after host invasion.

Introduction

Vitellogenin (Vtg) has been classically defined as the precursor of yolk proteins which are accumulated in the maturing egg. In the sea urchin, Vtg has an intriguing localization in the coelomic fluid of both male and female individuals [9, 23]. Furthermore the precise timing and pattern of cleavage occurring upon fertilization, operated by a cathepsin B-like enzyme [17, 19, 28], has been extensively described in different sea urchin species [2, 17, 19, 28]. In relation to these findings, its biological function as a mere reservoir of food for the growing embryo has been

questioned. We investigated the function of the molecule and found that it was able to promote the aggregation of cells dissociated from blastula-stage embryos [9]. We also showed that Vtg has a precursor-product relationship with a 22S glycoprotein complex isolated from blastula, whose function as cell adhesion molecule has been described [20]. It has also been shown that in the sea urchin species Strongylocentrotus purpuratus, Vtg is synthesized by the intestine in both males and females [23], whereas in the species Dendraster excentricus, a sand dollar also belonging to the Echinoids family, Vtg synthesis occurs in coelomocytes [15].

Coelomocytes are the circulating cells of the sea urchin and are part of the defence system that, in response to injury or host invasion, is capable of phagocytosis, chemotaxis or expression of cytotoxic agents. Coelomocytes have also been involved in graft rejection, and it has been shown that their response reactions produce the activation of a certain number of genes (for a review, see [25]).

In this report we investigate the occurrence of Vtg in circulating coelomocytes in adult male and female sea urchins of the species Paracentrotus lividus. Furthermore, taking advantage of the already described technique of coelomocyte purification into morphologically distinguishable cell types [13, 24], we investigate the cellular occurrence of Vtg. Finally, we present evidence that the molecule is released from cells, upon stress conditions, during experimental manipulation.

Materials and methods

Coelomocyte isolation and coelomic fluid preparations

Male or female sea urchins were bled individually through a cut in the peristomial membrane. The fluid, usually 10 ml, containing the total coelomocyte population was poured on 30 ml ice-cold anticoagulant solution consisting of 20 mM Tris-0.5 M NaCl-30 mM ethylenediaminetetraacetic acid pH 7.5 (ISO-EDTA). Cells were centrifuged at $50g$ for 10 min and the pellets resuspended in 1 ml ISO-EDTA. The supernatants were further centrifuged at $12\,000g$ for 10 min, dialyzed and concentrated by Amicon ultrafiltration,

exclusion 30 kDa, and referred to as coelomic fluid. Cells were separated by centrifugation on a discontinuous Na-metrizoate gradient in ISO-EDTA as described [13]. Bands were collected, diluted 1:10 in ISO and centrifuged as above. Pellets were resuspended in 1 ml ISO supplemented with a protease inhibitor cocktail: 2 µg/ml of leupeptin, aprotinin, pepstatin, antipain, 1 mM benzamidine, 0.3 mg/ml soybean trypsin inhibitor, sonicated (burst continuous, 3 × 20 sec cycles with 20 sec intervals), and used immediately or stored at −20 °C. Protein concentration was determined by readings at 280 nm, assuming that 1 OD corresponds to 1 mg of protein. All reagents used were from Sigma Chemical Company (St. Louis, MO/USA).

Antibodies

Antisera were developed, as already described, by intraperitoneal injection of a 22S glycoprotein complex, purified from blastula cells, in mice [9] or in rabbits [14]. Sera were screened by enzyme-linked immunosorbent assay and only those having a titer higher than 1:12 500 were used in immunodotting assays, Western blots or immunoprecipitations. For immunohistochemistry, antisera aliquots (100 µl 1:250 dilution) were adsorbed to Vtg (10 µg), purified from coelomic fluid as previously described [9].

Immunoperoxidase staining of coelomocytes

Cells were prepared as described above, counted and adjusted to 0.5 × 10⁶ cells per ml, except that the anticoagulant solution consisted of Ca²⁺ and Mg²⁺-free sea water containing 0.01 M EDTA, pH 7.4 (CMFSW-EDTA), supplemented with 5% bovine serum albumin (CMFSW-EDTA-BSA) to prevent cell damage [1]. 200 µl of the cell suspension were layered onto glass slides precoated with 0.01% poly-L-lysine for 1 h. Cells were then fixed in 5% formic aldehyde in CMFSW-EDTA at room temperature for 15 min and washed once in distilled water for 15 min. Free sites were saturated by incubation with 5% BSA in CMFSW-EDTA at room temperature for 30 min. Slides were washed once with distilled water for 15 min and incubated overnight in humidified chamber with 1:250 diluted antisera at 4 °C. Slides were washed 3 times for 15 min with distilled water, incubated for 30 min with 1% H₂O₂ in methanol, washed once with distilled water for 15 min and incubated with the peroxidase-conjugated antibody (Bio-Rad Laboratories, Richmond, CA/USA) at room temperature for 30 min. After 3 × 15 min washes the staining was developed by dipping the slides into 0.06% 4-chloro-1-naphthol solution, and the reaction was terminated by the addition of distilled water.

SDS-PAGE and Western blot

Coelomic fluids and coelomocyte lysates were separated on 6% SDS-polyacrylamide minigels [18], and transferred to nitrocellulose paper as reported [27]. Mouse antiserum 014/8II or rabbit antiserum 202 were diluted 1:1000 or 1:2000, respectively. Second antibodies were peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit purchased from Sigma Chemical Company. 4-chloro-1-naphtol was used for detection of bands, and reactions were terminated by the addition of distilled water. Molecular weight markers from Bio-Rad Laboratories were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase-b (97 kDa), bovine serum albumin (66 kDa).

Immunoprecipitation

Total coelomocytes (26 × 10⁶ cells) were cultured in 3 ml CMFSW-EDTA for 1 h at 25 °C with occasional shaking. Cells were harvested by centrifugation at 300g for 5 min, washed once with ISO-EDTA and extracted for 20 min at 4 °C with 1 ml lysis buffer: 0.5% Triton X-100, 20 mM Tris-HCl pH 7.4, 150 mM NaCl,

1 mM CaCl₂ and 1 mM MgCl₂ (TBS) in the presence of a protease inhibitor cocktail (10 µg/ml leupeptin, 4 µg/ml pepstatin, 0.1 TIU/ml aprotinin). All reagents used were from Sigma Chemical Company. Lysed cells and culture medium were centrifuged at 12 000g for 10 min at 4 °C. The supernatants were preincubated with 200 µl of 50% protein A-Sepharose (Pharmacia, Uppsala/Sweden) in TBS, for 1 h at 4 °C. After centrifugation, supernatants were divided into two aliquots subsequently incubated overnight at 4 °C with 10 µl of preimmune serum or vitellogenin-specific serum. Immunocomplexes were bound to 100 µl 50% protein A-Sepharose (Pharmacia), washed three times, and bound material was eluted by boiling the beads in Laemmli buffer [18] for 5 min.

Results

Detection of vitellogenin in coelomic fluids and coelomocyte lysates of male and female sea urchins

In order to look for the presence of Vtg in the coelomic fluids and in lysates of total coelomocytes of the sea urchin *Paracentrotus lividus*, we utilized immunological techniques for the detection of the antigen. To this purpose we used an antiserum, originally developed by immunizing mice with a 22S glycoprotein complex purified from blastula embryos and known to mediate cell adhesion [9]. As shown in Figure 1a, the 014/8II mouse antiserum strongly reacts with both male (m) and female (f) coelomic fluids. The antiserum can be considered to be monospecific, since
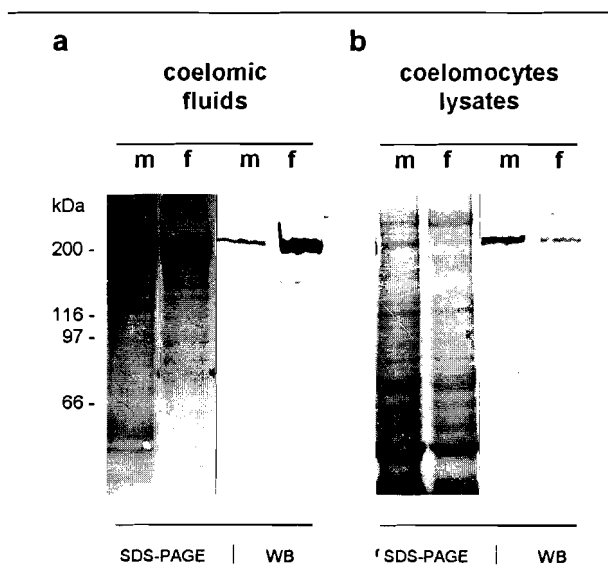

Fig. 1. Vitellogenin is found in coelomic fluids and coelomocyte lysates of male and female sea urchins. – **a.** Coomassie Blue-stained 6% SDS-PAGE (*left*) and Western blot (*right*) with the Vtg-specific mouse antiserum 014/8II of male (m) and female (f) coelomic fluids. About 20 µg of protein were loaded per each lane. – **b.** Coomassie Blue-stained 6% SDS-PAGE (*left*) and Western blot (*right*) with the Vtg-specific mouse antiserum 014/8II of male (m) and female (f) coelomocyte lysates. About 200 µg were loaded per each lane. Molecular weight markers are indicated.

it is able to recognize only a band with a molecular mass of 200 kDa corresponding to the Vtg molecule, whereas it failed to stain other components present in the coelomic fluids. We obtained identical results when using the 202 rabbit antiserum (not shown). As both sera could be used up to a dilution of 1:5000, they were utilized indifferently in the following experiments, and because of their specificity they will be indicated as Vtg-specific antisera.

It has been reported that, in the sea urchin, Vtg is secreted by the intestine into the perivisceral fluid and transported to oocytes [23]. Other authors claim that, in the sand dollar, the synthesis of Vtg occurs in coelomocytes [15]. Since we found Vtg in the coelomic fluid, we examined the possibility that Vtg could occur in the cells circulating in the coelomic fluid, namely coelomocytes. Consequently, lysates from male and female sea urchin coelomocytes were separated by SDS-polyacrylamide gels, transferred to nitrocellulose and probed with the Vtg-specific antiserum. As shown in Figure 1b, Vtg is present in coelomocyte lysates, regardless of the sex of the individual.

Detection of vitellogenin in a distinct coelomocyte subpopulation

It has been reported that in the circulating coelomic fluid of the sea urchin, there are at least five morphologically different cell types, namely amoebocytes, colored spherule cells, colorless spherule cells, vibratile cells and red cells [13, 24]. In order to assess if Vtg was present in all cell types or in a particular subpopulation, we separated total coelomocytes by density gradient and tested the lysates obtained from each cell type by immunodotting assay with Vtg-specific antiserum. Results obtained from purified

Fig. 2. Immunodotting assay of purified coelomocyte subpopulations. Cells were separated by Na-metrizoate step gradient and the corresponding bands (a–f) were collected. 200 µg of lysates from purified cells were dotted on nitrocellulose and probed with Vtg-specific mouse antiserum 014/8II.

cells of a single individual are shown in Figure 2. Only bands b, c and d that correspond to the so-called colorless spherule cells, show a strong positive reaction. The differences in the staining intensities among spherule cells may be due to amoebocyte contamination of the bands in the gradient. Furthermore, we have experienced that, depending on the preparation, not all the 6 bands in the metrizoic acid gradient are present, and the relative proportions of the bands vary among experiments. This is in agreement with many reports present in the literature (for reference, see [24]).

Subcellular localization of vitellogenin

In order to confirm the results obtained by the immunodotting assay and to obtain information on the subcellular localization of Vtg, we used Vtg-specific antisera for immunolocalization experiments on fixed total coelomocytes or enriched subpopulations. To assess the specificity of antibodies, a control experiment has been performed using Vtg-adsorbed mouse antiserum (Fig. 3a). The Vtg-specific 014/8II mouse antiserum stains about 48 % of total coelomocyte population (Figs. 3a, c), whereas using the Vtg-adsorbed antiserum (Fig. 3a) the number of cells stained is reduced at the level of the preimmune serum (Figs. 3a, b). Amoebocytes, purified from Na-metrizoate gradient, are not stained by Vtg-specific antiserum (Fig. 3d), whereas all of the colorless spherules were stained by the antiserum, as shown in Figure 3e. Clearly, the molecule is stored intracellularly and its subcellular localization is very intriguing. As seen at higher magnification in Figure 3f, Vtg is found in granules around the nucleus in a kidney-like shape. This image has a striking similarity to that of polymorphonucleated cells circulating in vertebrate blood.

Release of vitellogenin from cells upon stress conditions

During our studies we had often observed large differences in the amount of Vtg recovered from the coelomic fluid. Similarly, there were cases where cell lysates seemed devoid of the molecule, suggesting a release of Vtg into the medium occurring during cell manipulation. To test this hypothesis, coelomocytes were subjected to two washes and centrifugations immediately after collection, and lysates were used in Western blotting experiments. In parallel, coelomocytes were cultured for 1 h after their harvesting, cells and supernatant were then collected and used in imunoprecipitation experiments. Results shown in Figure 4 indicate a release of Vtg from cells after centrifugation (Fig. 4a). There is a large decrease in the amount of Vtg present in cell lysates for the individual # 2, whereas individual # 1 has a modest reduction in the content of Vtg, thus reflecting again a variability among individuals, in response to manipulation. On the other hand, no cell damage was observed by phase-contrast microscopic inspection of cell suspensions after each centrifugation. In the case of a 1 h incubation of cells (Fig. 4b), the culture medium is rich in the protein that, conversely, is practically absent in the cell lysate (not shown).
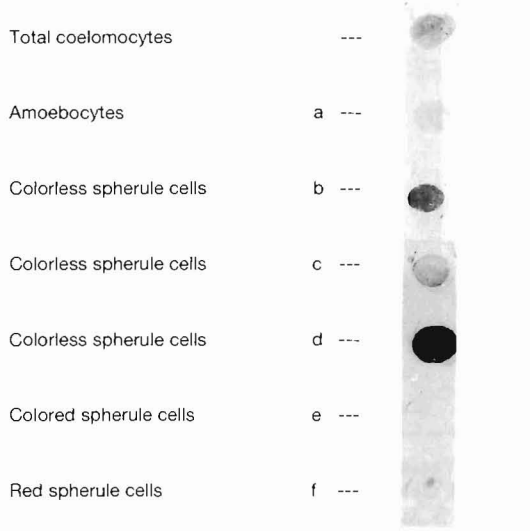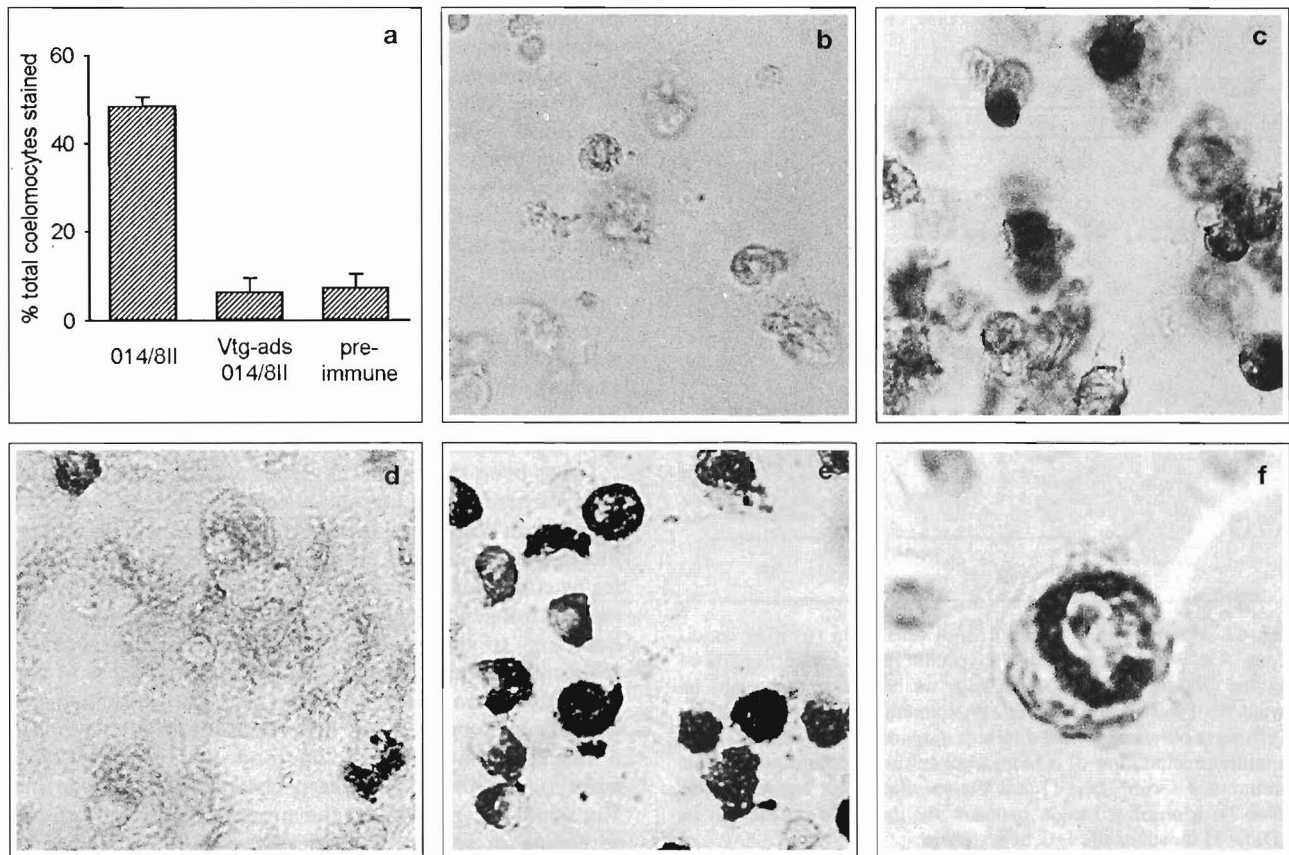
Fig. 3. Vitellogenin is located inside the colorless spherule cells. – **a.** Quantitative analysis of total coelomocytes stained using 014/8II Vtg-specific mouse antiserum, 014/8II Vtg-specific mouse antiserum preadsorbed with vitellogenin (purified from coelomic fluid as described in [9]) and preimmune serum. The values are the mean of the number of cells stained in 5 different fields where 100 cells were counted and for which the standard deviation has been calculated. – **b.** Preimmune serum. – **c.** Vtg-specific 014/8II mouse antiserum. – **d to f.** 202 rabbit antiserum on total coelomocytes (**b, c**), purified amoebocytes (**d**) and colorless spherule (**e, f**). – $200 \times$ (**b–d, f**), $800 \times$ (**e**).

Discussion

In this paper we describe the detection of Vtg in whole circulating coelomocytes of both male and female adults of the sea urchin Paracentrotus lividus. The analysis has been extended to the six cell populations into which total coelomocytes can be separated by metrizoic acid gradients. We found that only the so-called colorless spherule cells contain measurable amounts of Vtg, packed in kidney-shaped reservoirs, located around the nucleus. Upon stressing conditions, coelomocytes appear devoid of the molecule which is found in the culture medium.

In a series of studies, coelomocytes have been classified into cell types, on the basis of their morphology (for a review, see [24]). The proportion among the subclasses is very variable from species to species and even from individual to individual, depending on their physiological conditions or geographical locations. The different amounts of Vtg that we found in the bands b, c and d of the colorless spherule cells may reflect a different stage of maturity in that cell type. In fact, it has been questioned if these categories correspond to functionally different cell types, or if they represent different developmental stages of the same cell line [24].

Despite the large amount of morphological studies, little information on the possible function of spherule cells is available. There are some studies in which these cells have been shown to retain antibacterial activity [13]. In other studies an arylsulfatase activity has been found in total coelomocyte population, and histochemically the enzyme was detected in granules of spherule cells [5, 6]. It seems therefore that spherule cells function as immune effectors operating after host invasions.

Our finding that Vtg is specifically located in a particular cell type, together with its occurrence in male sea urchins, argues for a role of the molecule in the adult. We have already shown that vitellogenin is the precursor of an independently discovered 22S cell adhesion molecule, called toposome, which is able to promote the aggregation of cells dissociated from sea urchin blastula embryos [9, 10,
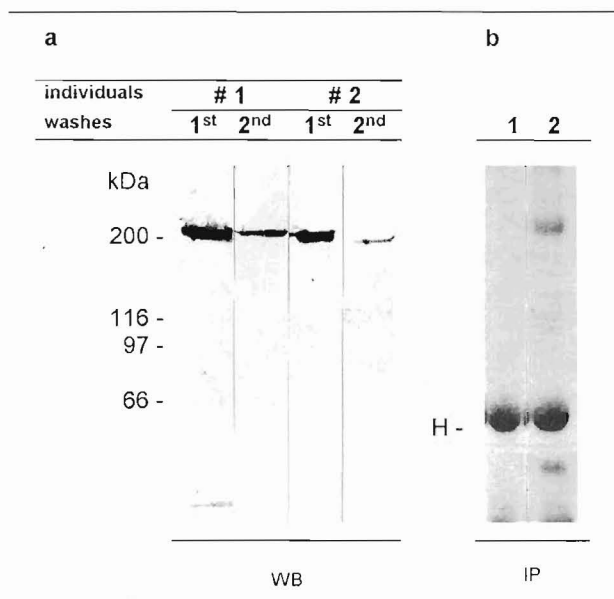
a   b

individuals	# 1		# 2				
washes	1st	2nd	1st	2nd		1	2

kDa

200 -

116 -
97 -

66 -

H -

WB   IP

Fig. 4. Vitellogenin is released from cells upon stressing conditions. – **a.** Western blot with Vtg-specific rabbit antiserum 202 of lysates (200 µg per lane) from cells washed once (1st wash) or twice (2nd wash) with ISO-EDTA immediately after collection. Cells were obtained from 2 different individuals: # 1 and # 2. – **b.** Immunoprecipitation of coelomocyte culture medium with rabbit preimmune serum (*lane 1*) and Vtg-specific rabbit antiserum 202 (*lane 2*). Molecular weight markers are indicated on the left in kDa. – H indicates the IgG heavy chain.

22]. More recently we have also demonstrated a multifunctionality of the toposome, namely its ability in mediating the adhesion of cells to the substrate [21].

On the other hand, the presence in the coelomic fluid of an adhesive activity has been already shown [8]. The component, purified from the coelomic fluid of the sea urchin Paracentrotus lividus, has an approximate molecular mass of 200 kDa and is able to promote cell-cell adhesion of autologous coelomocytes and their spreading onto the substrate.

It is generally accepted that the invertebrate host defence functions, at first, by immobilizing and then encapsulating the invasive microorganism. For this process to occur, there must be the active adhesion between immune effector cells that are circulating in the body fluid, namely hemocytes or coelomocytes. This hypothesis has been well documented for the crustacean Pacifastacus leniusculus, where for the first time a cell adhesion factor has been shown to occur in hemocytes, circulating in the perivisceral fluid. The factor is actively secreted after host invasion and is able to mediate the adhesion of autologous cells to the substrate [16]. Similarly, the occurrence of a lectin-type molecule in hemocytes of the tunicate Phallusia mamillata has been described. The release of the lectin takes place independently of active protein synthesis, and therefore indicates that the lectin is stored inside the cells and released upon in vitro stimulation [1]. Microcultures

of Phallusia mamillata hemocytes fractionated by a Percoll density gradient, showed that multivacuolated cells, named "compartment cells", release lectin [4]. Moreover, it has been shown that coelomocytes from the echinoid Holoturia polii release an aggregating factor, having a molecular mass of 220 kDa, that is able to agglutinate cells and is possibly responsible for cell clotting [7].

The release of vitellogenin into the medium that we found, can be the result of stressing conditions due to manipulation, and therefore it can reflect the mechanism operating in the living animal when it is attacked by invasive organisms. In fact it has been shown in the case of the sea urchin Strongylocentrotus purpuratus, that the mere puncture of the peristomial membrane can activate the expression of a single copy gene called Spcol1, a sea urchin analog of mammalian profilin [25, 26].

It has been shown that invertebrate vitellogenin shares some sequences in its amino-terminal region with the human von Willebrand factor, whose function in vertebrate blood clotting is well known [3]. Furthermore, other studies by computer alignment of the peptide sequence of lobster fibrinogen, have shown that this molecule shares homology to vitellogenins [12]. These data suggest that a relationship may exist between certain precursors of egg yolk proteins and plasma proteins [11]. These findings give a molecular support to our hypothesis that vitellogenin has a function other than being food for the developing embryo. On the whole, these observations suggest that Vtg could be involved in the processes of adhesion and spreading of sea urchin coelomocytes, being utilized in vivo after host invasion. Functional assays using purified vitellogenin, as well as gene cloning analysis, are needed in order to better elucidate this matter.

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