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TEMPORAL-SPATIAL EXPRESSION OF TWO *PARACENTROTUS LIVIDUS* CELL SURFACE PROTEINS

DANIELE P. ROMANCINO, GIULIO GHERSI, ROBERTO GAMBINO, GIOVANNA MONTANA, CATERINA COSTA and MARTA DI CARLO*

Istituto di Biologia dello Sviluppo C.N.R. via Ugo La Malfa 153, 90146 Palermo, Italy

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The temporal expression of two cell surface proteins, called BEP1 and BEP4, during *Paracentrosus lividus* embryonic development was studied. These proteins are found in both monomeric and dimeric forms in egg and embryos and we have established that their specific form is related to their being in the cytoplasm or on the cell surface. The spatial distribution of BEP1 and BEP4 proteins in eggs and embryos was established by whole mount immunohistochemistry. These proteins are located in the animal part of unfertilized and fertilized eggs; thereafter they are much less represented in structures derived from the vegetal cells of the embryo such as the micromeres of the 16 cell stage, the primary mesenchyme of blastula and the gut of gastrula. At the prism stage BEP1 and BEP4 proteins are present to some ectodermal parts and thereafter, at the pluteus stage, to the oral region.

KEYWORDS: *Paracentrostus lividus*; cell surface proteins; spatial distribution; immunohistochemistry

INTRODUCTION

Cell contacts are fundamental in morphogenetic and histogenetic processes during development of multicellular organisms. The sea urchin embryo was one of the first organisms in which the role of cell interactions in controlling cell fate was studied. It is easy, indeed, to isolate and culture blastomeres and to dissociate the embryos into single cells that are able to reaggregate and differentiate when the physiological conditions are reconstituted (Horstadius, 1973; Giudice, 1962, 1973). Several cell surface proteins that have a role in cell adhesion during gastrulation, a period in which changes in specific adhesivity occur, have been identified in sea urchins (Hardin, 1994). Recently, two cadherin-like cell adhesion proteins, which are similar to vertebrate cadherins in their extracellular domains and in the last 26 amino acids of the carboxyterminal portion, have been identified (Ghersi and Vittorelli, 1990). One of the two molecules is expressed at all stages between the unfertilized egg and pluteus (Ghersi *et al.*, 1993). Moreover, Di Carlo *et al.* (1990) isolated from a λgt11 expression library, screened with antibodies against butanol-extracted proteins, two cDNAs called Bep1 and Bep4 (butanol-extracted proteins), and thereafter another one called Bep3, all belonging to the same multigenic family and transcribed by maternal mRNAs that are localized in the animal part of the eggs and embryos (Di Carlo *et al.*, 1994*a*, 1996; Montana *et al.*, 1996). Specific portions of both cDNAs were inserted in pEX31 expression vector, the fusion proteins produced were injected in rabbits, and the corresponding polyclonal antibodies generated (Romancino *et al.*, 1992). These antibodies were shown to recognize two specific 33 kDa antigens, as described by Romancino *et al.* (1992), and their localization on the cell surface was confirmed by immunofluorescence analysis. Treatment of dissociated embryos with Fabs against BEP1 and BEP4 antigens causes inhibition of cell reaggregation, showing that these antigens are involved in the process of cellular reaggregation. Moreover, Fabs anti-BEP1 and anti-BEP4 added at two cell stage embryos cause exogastrulation, indicating a possible role of these proteins in the establishment the animal-vegetal axis of the *Paracentrosus lividus*

*To whom correspondence should be addressed.

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egg (Di Carlo *et al.*, 1996). Recently a 32 kDa protein, transcribed by a maternal messenger, distributed throughout the cytoplasm of unfertilized egg and localized in the hyaline layer of the embryo, has been identified in *Strongylocentrotus purpuratus* (Brennan and Robinson, 1994). Comparative amino acid sequence analysis revealed a 47% homology with BEP1 and BEP4 proteins suggesting that all these sea urchin proteins are members of the same family.

In this paper we analyse the relationship between the temporal and spatial expression of BEP1 and BEP4 proteins and the corresponding transcripts.

MATERIALS AND METHODS

Gel electrophoresis and immunoblotting

In order to prepare total proteins, eggs and embryos were pelletted by a 5 min centrifugation at 1500 rpm and resuspended in 10 volumes of 1 × sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.7% β-mecaptoethanol, 0.1% bromophenol blue). Proteins extracted with butanol from eggs and from embryos dissociated into cells were prepared according to Romancino *et al.* (1992).

Samples by 30 µg were subjected to electrophoresis on 10% SDS polyacrylamide gels. When non-reducing conditions were utilized, pelletted eggs and embryos were resuspended in 1 × sample buffer without β-mercaptoethanol. Proteins were then either transferred onto nylon membranes using an LKB Multiphor apparatus (LKB, Uppsala, Sweden) under the conditions recommended in the manufacturer's instruction manual. Nitrocellulose filters were processed as described by Romancino *et al.* (1992). Before to use, anti-1C1 antiserum was preabsorbed on *Escherichia coli* extract containing 4A1 fusion protein and vice versa for 4A1 antiserum to eliminate the possibility of cross-reaction.

Purification of enriched cytosol and plasma membrane proteins

Sea urchin embryos at the blastula stage were resuspended in 3 volumes of 10 mM Tris–HCl, pH 7.6, homogenized and centrifuged for 10 min at 15,000 rpm. The supernatant, containing the enriched cytosol fraction was stored at − 80°C. The pellet was dissolved in 2.5 volumes of 10 mM Tris–HCl, pH 7.6 loaded onto a 45–60% sucrose gradient and centrifuged with a SW25 rotor for 16 h at 20,000 rpm at 4°C. The interface was recovered, resuspended in 5 volumes of water and centrifuged with a SW30 rotor for 2 h at 30,000 rpm. The supernatant was discarded and the pellet dissolved in 8 volumes of dextran-polyethylenglycol and centrifuged for 2 h at 5000 rpm. The interface was collected, resuspended in 10 volumes of water and centrifuged for 1 h with a SW75 rotor at 50,000 rpm. The pellet, containing a membrane enriched fraction, was stored at − 80°C.

Immunoprecipitation of sea urchin embryos labeled with ^{35}S-Methionine

An aliquot of fertilized sea urchin egg (50,000), was suspended in 2 ml of MFSW (Millipore-filtered sea water) containing 1 mCi/ml [^{35}S]methionine (Amersham) for 15 min, and another aliquot of fertilized sea urchin egg (50,000) was suspended in 2 ml of MFSW containing 1 mCi/ml [^{35}S]methionine and allowed to develop up to the blastula stage. The eggs and the embryos were washed twice with MFSW and then extracted with 1% TRITON X-100 in presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM benzamidine, 10 µm/ml soybean trypsin inhibitor and 10 µm/ml protease inhibitor. The suspensions were centrifuged twice for 2 min at 1500 rpm and once for 10 min at 10,000 rpm. Supernatants were collected and extensively dialysed against distilled water. The extracts were utilized for immunoprecipitation with anti-BEP antibodies. Immunoprecipitation of the same number of eggs and embryos was performed as described by standard procedures (Harlow and Lane, 1988). Precipitates were loaded loaded onto 10% SDS-PAGEs and the slab gels were exposed to X-ray films.

Whole mount immunohistochemistry

Unfertilized and fertilized eggs and embryos were fixed in 100% Bouin reactive (picric acid, 37% formaldehyde, acetic acid, respectively in ratio 15:5:1). Fertilization was carried out in 2 mM *p*-aminobenzoic acid (PABA) to prevent hardening of fertilization membrane. Fixed eggs and embryos were dehydrated with washes of ethanol from 50% to 70% and permeabilized in 2 mM EDTA, 100% methanol. After washing in 0.14 M NaCl, 0.2 M phosphate, pH 7.4 (PBS) the samples were incubated in PBST (PBS containing 0.1% Tween-20) with 3% BSA for 3 h at room temperature and then with primary antibodies diluted 1:500, overnight at 4°C. After three washings in PBST eggs and embryos were incubated with anti-rabbit HRP diluted 1:300 for 1 h at room temperature. After

three washings in PBST and one washing in 50 mM Tris–HCl, pH 6.8 the samples were stained with diamminobenzidine (DAB) buffer (10ml of 50 mM Tris–HCl, pH 6.8, 5 mg DAB, 10 ml 30% H_2O_2) for 10 min. Finally eggs and embryos were dehydrated with washes in 35%, 50%, 70%, 100% ethanol and resuspended in 80% glycerol in PBS.

RESULTS

Temporal expression of BEP1 and BEP4 antigens

Bep1 and Bep4 transcripts are maternal in origin, are accumulated in the egg and utilized till the gastrula stage. In order to investigate whether the same fate is shared by their translation products we first carried out Western blot analyses of these proteins extracted from various developmental stages. For this propose we analysed the presence of these two proteins in the butanol extracts of different developmental stages. Exposition of eggs and dissociated embryos to butanol removes the proteins of the cellular membrane with no cytolysis and without impairing the viability of the cells (Moscona, 1974; Noll *et al.*, 1979, 1985). Western blot of proteins obtained from butanol extraction of eggs and embryos were separately incubated with anti-BEP1 and anti-BEP4 as described in Materials and Methods. Figures 1B and 1D show the presence of both the antigens from the egg till the pluteus stage. The amount of these proteins per embryo decreases at the pluteus stage, and may be slightly at morula. Moreover, to confirm the specificity of each antibody we incubated two Western blot containing 1C1 and 4A1 fusion proteins (Romancino *et al.*, 1992) with anti-1C1 (Fig. 1A) and anti-4A1 (Fig. 1C) preabsorbed antibodies. These results demonstrate that, differently from what occurs for the bep transcripts, both the antigens are present in all the developmental stages.

BEP1 and BEP4 molecular structure during development

In order to establish whether these proteins are involved in higher order structures, such as dimers or heterogenic covalent complexes with other proteins in various *P. lividus* developmental stages, proteins extracted from eggs, 16 blastomere, morula, blastula, gastrula and prism embryos and a sample containing 1C1 or 4A1 fusion proteins, as control, were electrophoresed on two SDS-PAGEs under non-reducing conditions. After Western

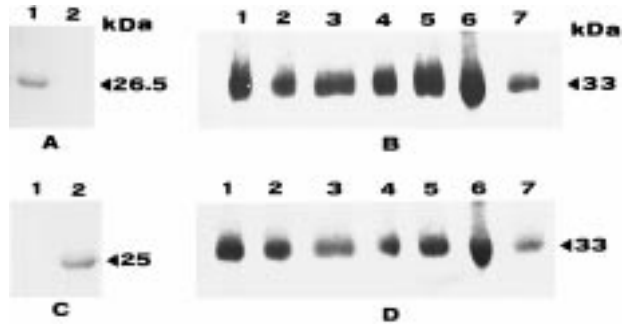

Fig. 1. Immunoblotting of 1C1 (1) and 4A1 (2) fusion proteins incubated against anti-1C1 preassorbed on 4A1 fusion protein (A). Immunoblotting of proteins obtained after butanol extraction of: (1) egg; (2) 16 cell stage; (3) morula; (4) blastula; (5) gastrula; (6) prism; (7) pluteus, and incubated with anti-BEP1 (B). Immunoblotting of 1C1 (1) and 4A1 (2) fusion proteins incubated against anti-4A1 preassorbed on 1C1 fusion protein (C). Immunoblotting of proteins obtained after butanol extraction of: (1) egg; (2) 16 cell stage; (3) morula; (4) blastula; (5) gastrula; (6) prism, egg; (2) 16 cell stage; (3) morula; (4) blastula; (5) gastrula; (6) prism; (7) pluteus, incubated with and anti-BEP4 (D). The molecular weight of the proteins is indicated by the arrowheads.

blots the filters were incubated with anti-BEP1 and anti-BEP4 antibodies. As shown in Figure 2A and 2B, in all the stages both the antibodies recognize a band of 66 kDa in addition to a band of 33 kDa. Moreover, two blots containing 1C1 and 4A1 fusion proteins incubated with the preassorbed anti-1C1 (Fig. 2A) and anti-4A1 (Fig. 2C) were carried out as controls. These data indicate that both antigens are disulfide bonded either to themselves or to other proteins. This is in agreement with the presence of four cysteine residues in the amino acid sequences of their cDNAs (Di Carlo *et al.*, 1990).

Organization of BEP proteins in the cell

In order to investigate the dimerization state of BEP proteins in different cell compartments, we extracted proteins from purified membranes and from cytoplasmatic components of blastula stage embryos. These proteins were loaded onto SDS-PAGE under non reducing conditions. Figure 3 shows the results obtained by a Western blot incubated with anti-BEP4 antibodies (3A) and with a preimmune serum obtained from the same rabbit then utilized to produce BEP4 antibodies (3B). A 66-kDa band was detected in the lane in which the proteins extracted from purified membrane were loaded, whereas only a 33-kDa band was found in the lane in which the cytoplasmatic proteins are loaded. The band of 55 kDa present in lane 2 (Fig. 3A) is due to an unspecific reaction of a serum

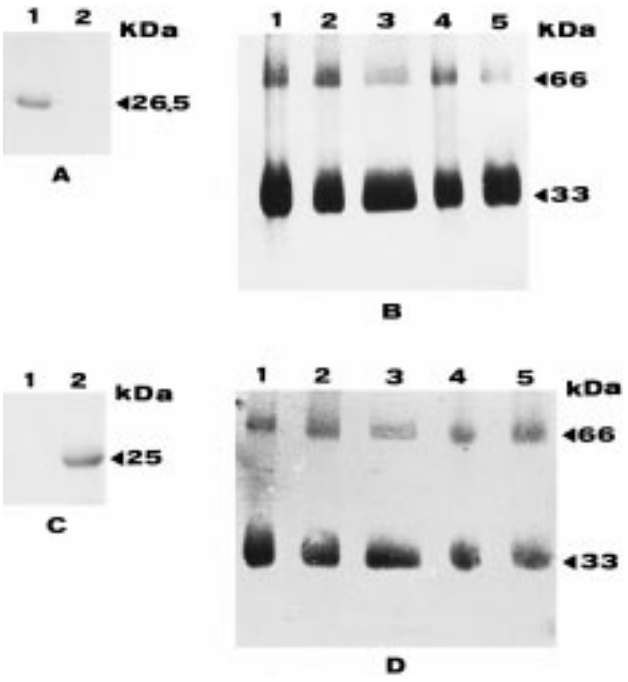

Fig. 2. Immunoblotting of 1C1 (1) and 4A1 (2) fusion proteins incubated against anti-1C1 preassorbed on 4A1 fusion protein (A). Western blot of SDS-PAGE under non reducing conditions of proteins extracted from: (1) egg; (2) 16 cell stage; (3) blastula; (4) gastrula; (5) prism, and probed with anti-BEP1 (B). Immunoblotting of 1C1 (1) and 4A1 (2) fusion proteins incubated against anti-4A1 preassorbed on 1C1 fusion protein (C). Western blot of SDS-PAGE under non reducing conditions of proteins extracted from: (1) egg; (2) 16 cell stage; (3) blastula; (4) gastrula; (5) prism, and probed with anti-BEP4 polyclonal antibodies (D). The molecular weights of the bands are indicated by the arrowheads.

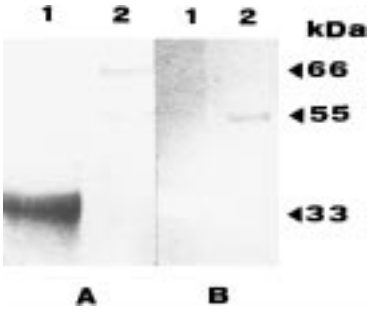

Fig. 3. Western blot of SDS-PAGE in non-reducing conditions of: (1) blastula stage cytosol proteins; and (2) blastula stage plasma membrane proteins, incubated with anti-BEP4 (A) and with preimmune serum (B). The arrowheads indicate the molecular weight of the bands obtained.

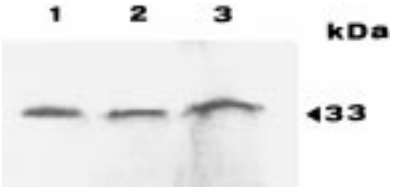

Fig. 4. Immunoprecipitation with BEP4 antibodies of: (1) egg; (2) 16 cell stage; (3) blastula, ^{35}S labelled, newly synthesized proteins. The molecular weights of the immunoprecipitated protein are indicated by the arrowhead.

protein, as demonstrated by its presence also in lane 2 of Figure 3B. The same results were obtained when anti-BEP1 was utilized (data not shown). This result suggests that these proteins are assembled on the cell surface as dimers. The band of 33 kDa was much stronger, which indicates that only a minor portion of BEP4 lays in the membranes.

Immunoprecipitation of BEP1 and BEP4 antigens

In order to investigate whether the BEP1 and BEP4 antigens are synthesized in the stages in which the transcripts are present, we incubated fertilized eggs and embryos with [^{35}S]met. The proteins were extracted from fertilized eggs, 16 blastomeres and blastula stages and immunoprecipitated with anti-BEP1 and anti-BEP4 polyclonal antibodies. Figure 4 shows the electrophoretic pattern of the *in vivo* labeled proteins after immunoprecipitation with anti-BEP4. A 33-kDa band corresponding to the analysed antigen is shown, which demonstrates that BEP4 is synthesized at all the investigated stages. The same result was obtained when anti-BEP1 was immunoprecipitated (data not shown).

Spatial distribution of BEP1 and BEP4 proteins

We have recently demonstrated that Bep1 and Bep4 transcripts are localized in the animal part of the egg and are not present in micromeres of the 16 cell stage, in primary mesenchyme cells of blastula and in archenteron of gastrula (Di Carlo *et al.*, 1994*a*, 1996). In order to investigate whether the same fate is shared by their proteic products and to establish in which territory they are present after the gastrula stage, when their transcripts are no longer present, we prepared eggs and embryo for whole mount immunostaining reaction. Figure 5 shows the results obtained after incubation with polyclonal antibodies against BEP4 and with preimmune serum obtained from the same rabbit then utilized to produce anti-BEP4. A gradient of staining is visible in about three quarters of the unfertilized egg (5A′) whereas following fertilization only one side is stained suggesting a movement toward the cortical region (5B′). When immunoreaction was carried out on whole mount embryos at the 16

cell stage (5C′) little or no staining was detected in the micromeres, suggesting that BEP4 protein is localized in the animal part of the egg. Subsequently, in the later stage, the signal present in the primary mesenchyme cells of blastula or in the upper part of the archenteron of gastrula was less intense than elsewhere (5D′–E′). At the prism stage (5F′) the staining pattern was more intense than in elsewhere in the ectodermal cells of the top and bottom of the archenteron and thereafter, at the pluteus stage (5G′), essentially a cluster of oral cells was stained. From this result we can deduce that BEP4 protein is localized in one side of the egg, which is the animal part, as demonstrated by Di Carlo *et al.* (1996), and mostly in the part of the embryo derived from this half till the gastrula stage. Later on, when differentiation proceeds, and when the transcript is not present, this protein is present only in a few regions, and eventually to part of the oral structures. No staining reaction was observed when preimmune serum was utilized (Fig. 5A–G). The same localization was found for BEP1 protein and when the same experiment was carried out utilizing immunohistochemistry on sections (data not shown).

DISCUSSION

In this paper we have examined temporal expression and spatial distribution, during *P. lividus* development, of two cell surface antigens belonging to a multigene family.

BEP1 and BEP4 proteins are always present from unfertilized egg to pluteus. During the embryogenesis period both proteins are present as dimers and as monomers at the same time, indicating a possible role in specific homophilic or heterophilic cell–cell binding interactions. Moreover, Western blot analysis of proteins extracted from cytoplasm and purified membranes indicates that when these proteins are on the membranes they are in the form of dimers, whereas when they are in the cytoplasm they are in the form of monomers. These results suggest that probably when these proteins are employed in cell interaction function they are in the form of dimers, whereas when they are stored in some cytoplasmatic organelles they are in the form of monomers. On the other hand their involvement in cell interactions has been extensively demonstrated by experiments in which Fabs against BEP1 and BEP4 antigens were shown to inhibit reaggregation of dissociated cells (Romancino *et al.*, 1992). Presently we do not know if these proteins are directly involved in cell adhesion or have an effect on other phenomena relevant to cell–cell interactions. Nevertheless, the presence of potential Ca^{2+} binding domains in their sequences and the dissociation effect caused by Fabs suggests that they might be mainly involved in cell-to-cell contact (Takeichi, 1991; Di Carlo *et al.*, 1990, 1994*b*).

Furthermore, new synthesis of BEP1 and BEP4 proteins, in the stages in which the transcripts are present, was confirmed by experiments of *in vivo* [^{35}S]methionine labelling followed by immunoprecipitation. Therefore BEP1 and BEP4 proteins are both utilized by the cell in the stages in which the transcripts are available and probably stored in some cellular organelles such as yolk granules in egg, before being employed on the cell surface, after differentiation when the mRNAs are absent. Storage in yolk granules of a precursors of some cell surface protein complexes, also extractable with butanol, called toposomes. was found by Cervello and Matranga (1989). Also Gratwhol *et al.*, (1991) detected by electron microscopy the toposome in cortical granules and yolk of sea urchin eggs.

We have also established that the BEP proteins have the same spatial distribution as the corresponding RNAs till the gastrula stage, whereas specific localization is detectable in later stages, such as prism and pluteus when the transcripts are no longer present (Di Carlo *et al.*, 1994*a*). Immunohistochemistry, indeed, of whole mount eggs and embryos at several developmental stages shows spatial distribution both for BEP1 and BEP4 proteins. These proteins are localized as a gradient in a part of the unfertilized egg, identified as the animal side (Di Carlo *et al.*, 1996), and are contained in lower amount in the structures deriving from the vegetal side of the egg such as the micromeres of the 16 cell stage, the primary mesenchyme cells of blastula and the gut of gastrula. The spatial distribution in the egg recalls the location of other proteins and mRNAs studied in other embryonic systems such as *Drosophila* and *Xenopus* (St. Johnson and Nusslein-Volhard, 1992; Melton, 1987). It has been hypothesized that the compartmentalization of mRNAs might be used to concentrate translation and sorting of proteins which are necessary for cell specification (Ding and Lipshitz, 1993). The presence of *bep1* and *bep4* mRNAs and of their corresponding translation products in the same position in the egg and in the embryos may support this hypothesis. These mRNAs and proteins, however, are the first molecular examples of localized macromolecules in the unfertilized sea urchin egg. The localization of BEP1 and BEP4,

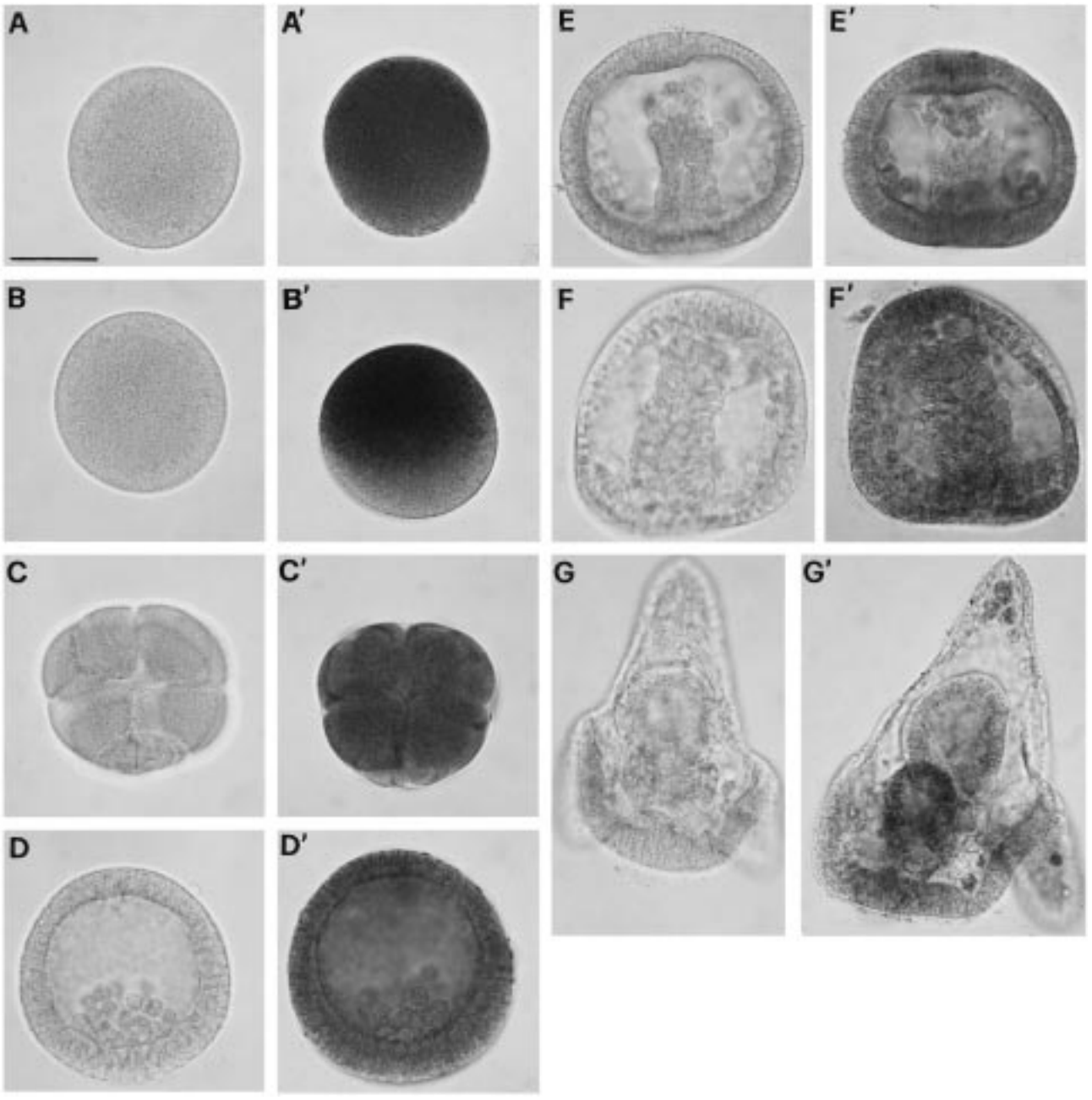

Fig. 5. Localization of BEP4 protein during sea urchin development. Three dimensional images of whole mount immunohistochemistry of *P. lividus*: (A,A′) unfertilized egg; (B,B′) fertilized egg; (C,C′) 16 cell stage; (D,D′) blastula; (E,E′) gastrula; (F,F′) prism; (G,G′) pluteus, stained with preimmune serum (A–G) and anti-BEP4 (A′–G′). Bar=25 m.

cell surface proteins, suggests that they might be important for establishing and maintaining intracellular connections of the animal part of the embryo and participating in cell lineage determination. Recent results indicate that BEP1 and BEP4 are relevant to morphogenesis (Di Carlo *et al.*, 1996). Actually, Fab fragments against these antigens are able to alter cell morphology, producing a vegetalizing effect. These proteins might therefore have a function in the control of gene expression, by down regulating genes which enhance vegetal potentialities.

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