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[Short Communication]

Purification of EGIP-D-Binding Protein from the Embryos of the Sea Urchin *Anthocidaris crassispina*

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ABSTRACT—Exogastrula-inducing peptides (EGIPs) are intrinsic factors that are present in eggs and embryos of the sea urchin *Anthocidaris crassispina*. They induce exogastrulation when added exogenously to the embryos. In the present study, we isolated an EGIP-D-binding protein (EBP) from a homogenate of mesenchyme blastulae. EBP had an apparent molecular weight of 33,000. The N-terminal amino acid sequence of EBP had a sequence homology to HLC-32 and bep4 identified in other sea urchin embryos. In addition to its ability of binding to EGIP-D, EBP also inhibited exogastrulation induced by EGIP-D. These results suggest that EBP plays an essential role in EGIP-D-induced exogastrulation.

INTRODUCTION

Exogastrula-inducing peptides (EGIPs) are intrinsic factors that are present in eggs and embryos of the sea urchin *Anthocidaris crassispina*. They induce extrusion of the archenteron toward the outside of the embryo, leading to exogastrulation when added exogenously to embryos (Ishihara *et al.*, 1982; Suyemitsu *et al.*, 1989a). Four EGIPs, designated EGIP-A through EGIP-D, were purified from mesenchyme blastulae and the amino acid sequences of all the four peptides were determined (Suyemitsu *et al.*, 1989a, 1989b, 1990). In addition, the positions of the disulfide bonds in EGIP-D were determined (Suyemitsu, 1991). These sequence analyses together suggested that EGIPs are related to epidermal growth factor (EGF; Carpenter and Cohen, 1979), as indicated by the similarities in amino acid sequence and in the positions of disulfide bonds between EGIPs and EGF.

EGIPs are present in eggs and embryos throughout early development (Kinoshita *et al.*, 1992) and they are localized to the vesicular structures in the apical region of the cytoplasm of blastomeres (Mizuno *et al.*, 1993). Recently, Yamasu *et al.* (1995) isolated a cDNA clone for EGIPs which revealed the structure of the EGIP precursor. However, its function in the development of sea urchin embryos and the mechanism of the exogastrulation remain obscure.

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During our attempt to identify the receptors for EGIP-D on the surface of the cell membrane, we identified a protein that bound EGIP-D in the hyaline layer (HL) (Fujita *et al.*, 1994), which is the extracellular matrix that surrounds the embryo. In this report, we describe the purification of an EGIP-D-binding protein from a homogenate of mesenchyme blastulae.

MATERIALS AND METHODS

The fertilized eggs of *Anthocidaris crassispina* were cultured at 24°C in normal seawater. At the mesenchyme blastula stage, the swimming embryos were collected and stored frozen until use. The embryo pellet was homogenized and centrifuged as described previously (Kinoshita *et al.*, 1992). The supernatant was dialyzed against 0.1 M NaCl, 50 mM Tris- HCl buffer, pH 7.8, and an aliquot, equivalent to 60 mg of protein, was loaded onto a column (29 mm i.d. × 90 mm) of DEAE-cellulose (James River Corp., Berlin, NH, USA) that had pre-equilibrated with the dialysis buffer. The column was washed with the dialysis buffer, and the flow-through fraction was collected and concentrated by ultrafiltration on a YM-10 membrane (Amicon Corporation, Lexington, MA, USA). The flow-through fraction containing EGIP-D-binding activity was applied to a column (1.0 cm i.d. × 115 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was eluted with 10 mM Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl, and the elution was monitored at 280 nm. Each protein peak was concentrated, and the EGIP-D-binding activities of the peaks were assayed as described previously (Fujita *et al.*, 1994). The active fraction was dialyzed against 5 mM sodium acetate-acetic acid buffer (pH 5.0) overnight at 4°C and applied to a column (14 mm i.d. × 250 mm) of CM Toyopearl (Tosoh, Tokyo, Japan) pre-equilibrated with 5 mM sodium acetate-acetic acid buffer (pH 5.0). After washing with the same buffer, the column was eluted

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stepwise with 50 mM, 100 mM, and 500 mM sodium acetate-acetic acid buffer (pH 5.0).

Electrophoresis was carried out on 12.5% polyacrylamide gels in the presence of SDS (SDS-PAGE) as described by Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250 (Laemmli, 1970). A molecular-marker kit containing phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and β-lactoalbumin (Pharmacia Fine Chemicals) was used for calibration of the gels for estimation of the molecular weight of the EGIP-D-binding protein.

The peptides were cross-linked by disuccinimidyl suberate (DSS; Pierce Chemical Co., Rockford, IL, USA) for 20 min on ice and the reaction was quenched by the addition of 100 mM Tris-HCl buffer (pH 7.8). The reaction mixture was dissolved in sample buffer (Laemmli, 1970) for electrophoresis on a 12.5% polyacrylamide gel, and then proteins were transferred to a nitrocellulose sheet. The sheet was probed with an antiserum against EGIP-D for detection of the EGIP-D-binding protein as described previously (Fujita *et al.*, 1994).

Exogastrula-inducing activities were examined by monitoring the morphological changes of sea urchin embryos after the addition of EGIP-D to the culture medium, as described previously (Ishihara *et al.*, 1982). In co-incubation experiments, the purified binding protein and EGIP-D were incubated together at 24°C for 1 hr prior to the addition to the embryo suspension. The rate of exogastrulation was expressed as an average of duplicate experiments.

Twenty micrograms of purified protein (approximately 600 pmol) was subjected to SDS-PAGE on a 12.5% polyacrylamide gel. After electrophoresis, protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Trans-Blot; BioRad, Richmond, CA, USA) by the metod of Matsudaira (1987). The membrane was stained with 0.1% Amido black in 20% methanol for 1 min, and then it was washed in distilled water for 10 min. The stained protein bands were cut from the membrane. The N-terminal amino acid sequence of the protein was analyzed with an automated protein sequencer (PSQ-2; Shimadzu Corporation, Kyoto, Japan).

RESULTS AND DISCUSSION

Purification

The mesenchyme blastulae were homogenized and centrifuged as described in Materials and Methods. An aliquot of the supernatant after dialysis equivalent to 60 mg of protein was applied to a column of DEAE-cellulose. The EGIP-D-binding activity was detected only in the flow-through fraction which cotained 31 mg of protein. The flow through fraction was further fractionated on a Sephadex G-100 column. As shown in Fig. 1, five fractions were obtained by the gel filtration chromatography. Among them, the EGIP-D-binding activity was restricted to fraction III which contained 7.5 mg of protein. Fraction IV which contained no EGIP-D-binding activity was used as a source for purification of EGIP-D, as described previously (Kinoshita *et al.*, 1992). Fraction III was applied to a column of CM-Toyopearl for further purification. The binding activity was exclusively found in fraction C which had been eluted with 100 mM sodium acetate-acetic acid buffer (pH 5.0) and fraction D which was eluted with 500 mM sodium acetate-acetic acid buffer (pH 5.0).

Gel electrophoresis

Figure 2 shows the results of SDS-PAGE under non-reducing and reducing conditions of fractions D from the column of CM-Toyopearl. Under non-reducing conditions, frac-

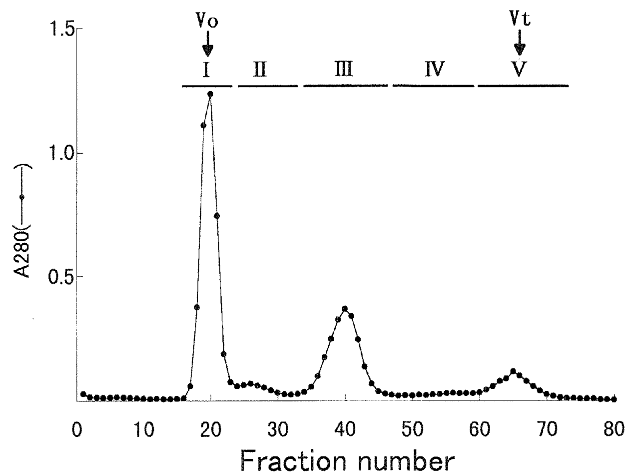

Fig. 1. Elution profile of the active fraction from a Sephadex G-100 column. The active fraction from the DEAE-cellulose column was applied to a Sephadex G-100 column and proteins were eluted with 0.1 M NaCl in 10 mM Tris-HCl buffer (pH 7.8). Two milliliters of eluate was collected in each tube and the absorbance was measured at 280 nm (●). Vo and Vt show the positions of void volume and column volume, respectively.

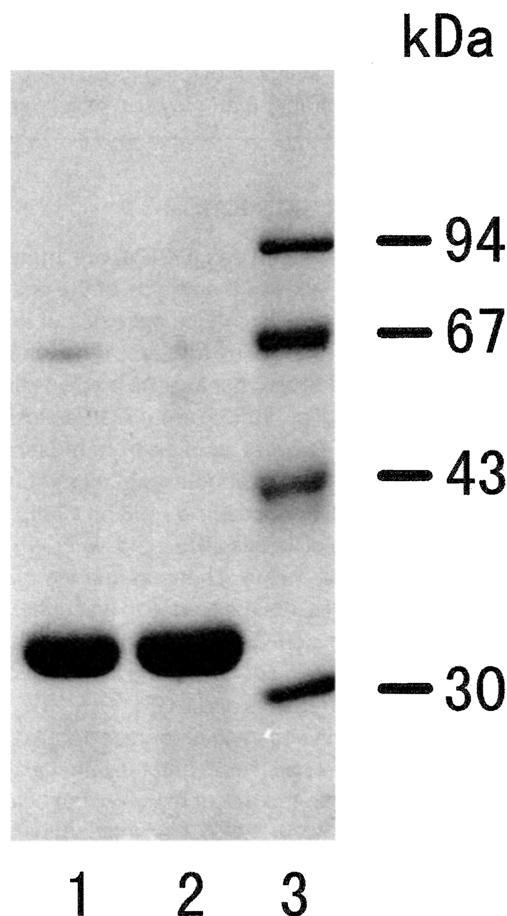

Fig. 2. SDS-polyacrylamide gel electrophoresis of EBP (fraction D). Lane 1, non-reduced EBP; lane 2, reduced EBP; lane 3, standard proteins: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

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tion D was separated into major 33-kDa and minor 67-kDa proteins. Under reducing conditions, however, fraction D migrated on SDS-PAGE as a single protein with a molecular weight of 33 kDa. These results suggest that the 67-kDa protein was formed as a dimer of the 33-kDa protein. In fraction C, however, there were some contaminations as well as 33-kDa and 67-kDa proteins in so far as analyzed by SDS-PAGE (data not shown). Finally, we purified 3.3 mg of the EGIP-D-binding protein (fraction D) from a supernatant of mesenchyme-blastula homogenate, equivalent to 60 mg of protein. The purified EGIP-D-binding protein was named EBP.

When EBP was cross-linked with EGIP-D by DSS and separated by SDS-PAGE under non-reducing conditions followed by Western blotting probed with an anti-EGIP-D antibody, only one immunoreactive band was specifically detected with the molecular mass of 39 kDa (Fig. 3). This value is in good accordance with the sum total of the sizes of EBP and EGIP-D, suggesting equimolar association of the two proteins. It is uncertain whether EGIP-D binds to the 67-kDa protein.

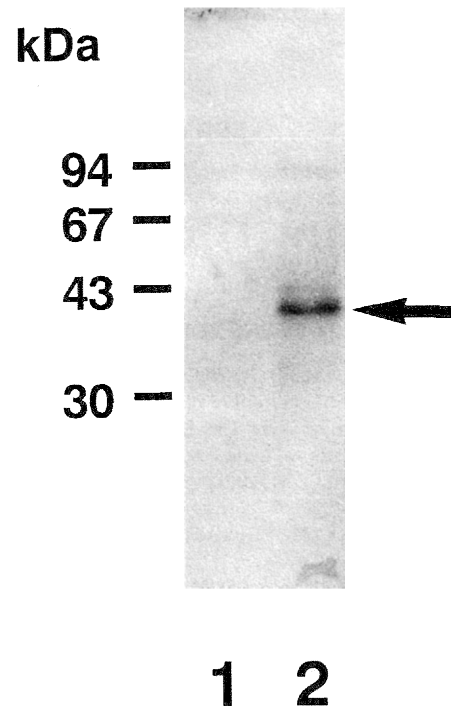

Fig. 3. Affinity labeling and Western blotting. Fraction D from the CM-Toyopearl column was incubated with EGIP-D, chemically cross-linked with each other by DSS and analyzed by SDS-PAGE under non-reducing conditions. Proteins were transferred to a nitrocellulose sheet and probed with EGIP-D-specific antibody. Lane 1, untreated EBP; Lane 2, EBP cross-linked with EGIP-D. The arrow indicates a band with the molecular mass of 39 kDa.

Analysis of N-terminal amino acid sequence

The N-terminal amino acid sequence of EBP was analyzed with an automated protein sequencer and determined to be SQDDVIERLQALKLREAAN. This N-terminal amino acid sequence showed a close similarity to those of two proteins in standard databases, HLC-32 (Brennan and Robinson, 1994) and bep4 (Di Carlo *et al.*, 1990, 1994), as shown in Fig. 4.

HLC-32 was reported to be a 32-kDa protein component of HL found in the embryo of the sea urchin *Strongylocentrotus purpuratus*, while bep4 is a butanol-extractable 33-kDa protein that was isolated from the embryos of the sea urchin *Paracentrotus lividus*. Fab fragments of antibodies against bep4 inhibited reaggregation of dissociated cells from blastula-stage embryos, indicating that bep4 plays a role in cell interaction during embryonic development (Romancino *et al.*, 1992).

EBP inhibits the exogastrulation induced by EGIP-D

To examine whether EBP could inhibit the EGIP-D-induced exogastrulation, mesenchyme blastulae were cultured with 5 μg/ml EGIP-D in the presence or absence of 50 μg/ml EBP. Exogastrulation was observed in 91% of embryos treated with EGIP-D alone. In contrast, when the embryos were treated simultaneously with EGIP-D and EBP, the rate of exogastrulation induced by EGIP-D declined to 63% and the remaining embryos developed normally. All the embryos treated only with EBP also developed normally. When embryos were treated with EGIP-D in the presence of bovine serum albumin (BSA; 500 μg/ml) as a control, induction of exogastrulation by EGIP-D was not affected.

In the present study, we purified the EGIP-D-binding protein (EBP) from a homogenate of mesenchyme blastulae. However, it is still unclear why exogenous EGIPs induce exogastrulation. Burke *et al.* (1991) reported that an antibody against fibropellins, the apical lamina proteins in HL, caused exogastrulation of sea urchin embryos, as do EGIPs. EGIP-D might affect EBP in HL, for example, by inducing a conformational change of the protein, resulting in perturbation of the morphogenesis of sea urchin embryos.

The functions of EGIP-D and its binding protein in normal

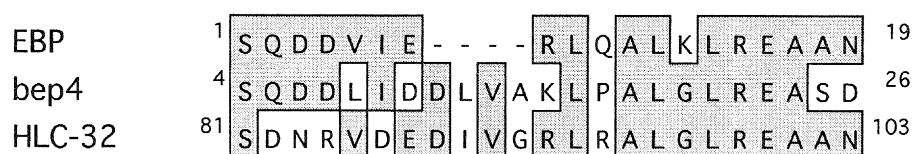

Fig. 4. Multiple alignment of the partial amino acid sequences of EBP and other proteins from sea urchins. Identical amino acid residues are boxed and shaded.

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development are still obscure. Further studies of EBP should lead to a clearer understanding of the biological significance of EGIP-D.

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