Echinonectin: a New Embryonic Substrate Adhesion Protein

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Abstract. An extracellular matrix molecule has been purified from sea urchin (*Lytechinus variegatus*) embryos. Based on its functional properties and on its origin, this glycoprotein has been given the name "echinonectin." Echinonectin is a 230-kD dimer with a unique bow tie shape when viewed by electron microscopy. The molecule is 12 nm long, 8 nm wide at the ends, and narrows to ~4 nm at the middle. It is composed of two 116-kD U-shaped subunits that are attached to each other by disulfide bonds at their respective apices. Polyclonal antibodies were used to localize echinonectin in paraffin-embedded, sectioned specimens by indirect immunofluorescence. The protein is stored in vesicles or granules in unfertilized eggs, is released after fertilization, and later becomes localized on the apical surface of ectoderm cells in the embryo. When used as a substrate in a quantitative in vitro assay, echinonectin is highly effective as an adhesive substrate for dissociated embryonic cells. Because of the quantity, pattern of appearance, distribution, and adhesive characteristics of this protein, we suggest that echinonectin serves as a substrate adhesion molecule during sea urchin development.

RECENT findings concerning the complexity of the embryonic extracellular matrix and adhesive events suggest that several molecules may participate in any single adhesive interaction (23). Analyses of those adhesive events have led to the discovery of a growing number of adhesive proteins such as uvomorulin, N-CAM, fibronectin (FN),[1] and laminin (11, 17, 23, 30, 31). There is little doubt these and other adhesive proteins are participants in the many processes that characterize morphogenesis. For developmental biologists the challenge is to understand how cells use these molecules for adhesion and as triggers for other morphogenetic phenomena.

In the sea urchin embryo the extraembryonic matrix is called the hyaline layer (HL). A major constituent of the HL is the high molecular weight glycoprotein, hyalin, which serves as a substrate adhesion molecule during early development (6, 24). Other proteins have been identified in the sea urchin embryonic HL as well (2, 15, 22, 25). These include antigens that cross-react with antibodies to vertebrate collagen, fibronectin, and laminin (33, 34). The spatial and temporal expression of these molecules is organized and complex, suggesting a potential for highly specific functions (25). However, information on the functions and interactions of these other HL components is sparse at best.

The assembly of the extraembryonic matrix is triggered by fertilization. Most, if not all HL components are stored in the unfertilized egg. Some of these proteins are secreted shortly after fertilization while others are reserved intracellularly until later in development (25). In still other cases, matrix proteins are synthesized by the embryo and become restricted in spatial distribution by mechanisms not yet clearly understood (25). Unique intracellular storage compartments and sequential release intimate an involved intracellular trafficking pattern before exocytosis of matrix proteins.

This study was initiated to isolate sea urchin fibronectin (FN) by gelatin agarose affinity chromatography for functional studies of embryonic cell adhesion. Instead, we have discovered a new molecule for which we propose the name echinonectin (EN). Aside from its collagen-binding activity, EN is distinct from FN as shown by immunological analysis, peptide mapping, and transmission electron microscopy of negatively stained specimens. EN is localized on the apical surface of the embryonic ectoderm. It is a highly effective substrate for dissociated cells when used in a quantitative in vitro adhesion assay. Given the quantity of EN present in embryos (0.05–0.1% of total protein), its apical extracellular location after fertilization, and its adhesive properties, it is likely that this protein is used for cell–substrate interactions during early embryogenesis. This paper describes the protein, its isolation, some of its physical properties, and provides evidence for its function.

Materials and Methods

Isolation of Echinonectin

Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were rinsed two times in artificial sea water then fertilized and cultured at room temperature (22–24°C). For isolation of EN, unfertilized eggs or late mes-

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1. *Abbreviations used in this paper*: EN, echinonectin; FN, fibronectin; HL, hyalin layer.

enchyme blastula/early gastrula stage embryos were homogenized and sonicated on ice in 3 vol of 50 mM Tris buffer, pH 7.5, containing 1% Triton X-100, 7.0 mM EDTA, and 1% DMSO (18, 31). In some experiments protease inhibitors were included to assess the possible effects of proteolysis. The sample was centrifuged for 45 min at 30,000 *g*. The supernatant was doubled in volume with homogenization buffer and applied to a 1.5 × 5.0-cm column of gelatin-agarose (Sigma Chemical Co., St. Louis, MO). The column was washed with 5 vol of 50 mM Trist/1% Triton buffer, 10 vol of 50 mM Tris/1.0 M NaCl, and eluted with 50 mM Tris/8.0 M urea (18, 31). All buffers were at pH 7.5. The column eluent was either dialyzed in 5 mM Tris and lyophilized, or stored frozen as collected. Absorbance at 280 nm, the dye binding protocol of Sedmack and Grossberg (32), or the heated biuret assay of Dorsey et al. (10) were used for protein estimation.

Electrophoresis

SDS-PAGE was performed using the buffer system of Laemmli (21). The apparent molecular weight of EN under native conditions was determined by retardation of mobility in 5.0, 6.5, 7.5, 10.0, and 12.5% gels according to Hedrick and Smith (16). Tube gel IEF was performed using the methods of O'Farrell (28) with minor modifications. Ampholytes (pH 3–10) were obtained from Sigma Chemical Co., as were isoelectric point standards. Proteolytic digests for one-dimensional peptide analysis were performed according to Cleveland et al. (7).

Immunological Analysis

Polyclonal antibodies were raised in rabbits by injection of EN excised from 7.5% polyacrylamide gels. The first injection included Freund's complete adjuvant (1:1). Subsequent injections included the EN/acrylamide emulsion only. Rabbits were injected every 28 d and bled on the seventh day after injection. For most experiments the serum was fractionated by ammonium sulfate precipitation at 50% saturation to isolate IgG, and dialyzed into PBS. The specificity of the anti-EN antibodies was tested by comparison with preimmune IgG, secondary antibody alone, antibody preabsorbed with purified EN, and anti-EN antibodies eluted from nitrocellulose blots of deglycosylated EN. Immunoblotting techniques were taken from Towbin et al. (35) and immunofluorescence methods from Wessel et al. (38).

In Vitro Adhesion Assay

The adhesion assay was taken from methods developed by McClay et al. (26). Flexible microtiter assay plates (model 3912; Falcon Labware, Becton, Dickinson and Co., Oxnard, CA) were incubated for 2 h at room temperature with 50 μl of equimolar EN (5 μg/ml) or FN (10 μg/ml) in PBS, followed by several rinses in PBS. Wells were blanked for 2 h at room temperature with 5% Blotto (19) in artificial sea water. Control wells were treated with PBS alone and then with 5% Blotto. Mesenchyme blastula stage embryos were labeled for 4 h with [^{3}H]leucine in artificial sea water (100 μCi/30 ml concentrated embryos); rinsed two times in calcium-free sea water; dissociated on ice for 15 min in hyalin extraction medium (300 mM NaCl, 10 mM KCl, 20 mM $MgSO_4$, 300 mM glycine, 10 mM Tris, and 2 mM EGTA) (24); triturated; and passed through 28-μm Nitex mesh to remove clumps. Aliquots of the single cell suspension were added to substrate-coated wells. The microtiter plates were sealed and the cells centrifuged onto the substrates at 20 *g* for 8 min. More than 90% of the cells were centrifuged into the substrate under these conditions. The plates were immediately inverted and recentrifuged at 25 *g* to remove cells from the substrate. The plates were quick-frozen in dry ice/ethanol and the well bottoms containing bound cells were clipped and assayed in a liquid scintillation counter.

Sedimentation Analysis and Electron Microscopy of Echinonectin

Samples of affinity column eluent were dialyzed into 0.2 M ammonium bicarbonate (pH 9.5) and subjected to zone sedimentation analysis in a 15–40% gradient of glycerol in the same buffer. Gradients were centrifuged 18 h at 41,000 rpm (20°C) in a rotor (model SW41-Ti; Beckman Instruments, Inc., Palo Alto, CA) and the sedimentation coefficients were estimated by reference to standard proteins (13). 0.5-ml fractions were collected and analyzed for protein content on silver-stained SDS–polyacrylamide gels. Samples of protein-containing fractions were applied to carbon-coated, glow-discharged copper grids, negatively stained with 2% uranyl acetate, and viewed with an electron microscope, (model 300; Philips Electronic Instruments, Inc., Mahwah, NJ).

Cleveland and Amino Acid Analyses

To compare one-dimensional peptide maps of egg and embryo EN, an in-gel protocol was taken directly from Cleveland et al. (7). Briefly, 10-μg samples of EN were subjected to electrophoresis on a 7.5% polyacrylamide gel, stained, destained, excised from the gel, and equilibrated in Tris (7). The optimal enzyme/substrate ratio (yielding a well-resolved and reproducible ladder of bands) was determined empirically to be 0.05 μg papain/10 μg EN. The gel slices were placed in the wells of a second gel, overlaid with enzyme, and co-electrophoresed into the stacking gel. Power was interrupted for 30 min to facilitate proteolysis, and the resultant peptides were then separated in the 12.5% resolving gel.

This protocol did not permit adequate digestion of FN. Therefore, to compare peptide maps of EN to FN, separate conditions were established as follows. Equimolar EN and FN were incubated for 1, 2, and 4 h at room temperature with 2.0 μg/ml L-1-*P*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-treated porcine pancreatic trypsin (Sigma Chemical Co.) in 10 mM Tris, pH 7.8. Proteolysis was stopped by addition of 2.5 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.). After 1 min, samples were quick-frozen, concentrated by lyophilization, and resolved on a 12.5% polyacrylamide gel.

For amino acid analysis, EN was hydrolyzed in 6 N HCl for 24 h at 110°C in vacuo. Duplicate aliquots of the hydrolysate were analyzed on an automated amino acid analyzer (model D500; Durrum, Palo Alto, CA).

Other Methods

The protocol for alkylation with iodoacetimide was taken from Erickson and Carrell (13), and disruption of carbohydrates on blots and paraffin sections with sodium metaperiodate were performed according to Woodward et al. (39). The phenol-sulfuric acid assay of Ashwell (4) was used for estimation of carbohydrate.

Results

Purification and Initial Characterization

EN was isolated from embryo extracts by gelatin-agarose affinity chromatography. The protein was routinely eluted with 8.0 M urea as a single, sharp peak of absorbance at 280 nm (Fig. 1 *A*). In a typical preparation, 1.0 mg EN was recovered from 12 ml packed embryos. This represents ~0.1% of total embryo protein. It has been reported that FN can be completely eluted from gelatin agarose with 1.0 M guanidine-HCl (31). We used this approach for EN but found that only a minor proportion (10%) of bound protein was recovered from the column (Fig. 1 *B*). Elution with guanidine was subsequently accomplished using a gradient of 0.0–4.0 M. EN was recovered at 2.0 M guanidine, but this recovery still represented less than one half of bound protein. The remainder was eluted with 8.0 M urea.

When the peak fractions from the gelatin agarose column were analyzed by SDS-PAGE in the presence of dithiothreitol (DTT), one major protein band was observed with an apparent molecular mass of 116 kD (Fig. 2, lane *A*). The possible effects of proteolysis on the observed molecular mass of EN were considered. Preparations were maintained at 0–4°C to minimize degradation. The slightly basic pH of the isolation medium and EDTA suppress acid and metalloproteases, respectively. The presence or absence of serine and thiol protease inhibitors including phenylmethylsulfonyl fluoride (1.0 mM), aminocaproic acid (25 mM), Bowman-Birk inhibitor (50 μM) from soybean (inhibiting trypsin and chymotrypsin), and antipain (1.0 mM) had no effect upon the apparent molecular mass of EN.

Under nonreducing conditions EN migrates as a dimer at 230 kD (Fig. 2, lane *B*). EN was also analyzed by native gel electrophoresis by measuring the retardation of mobility in gels of varying acrylamide concentration (16). The molecu-

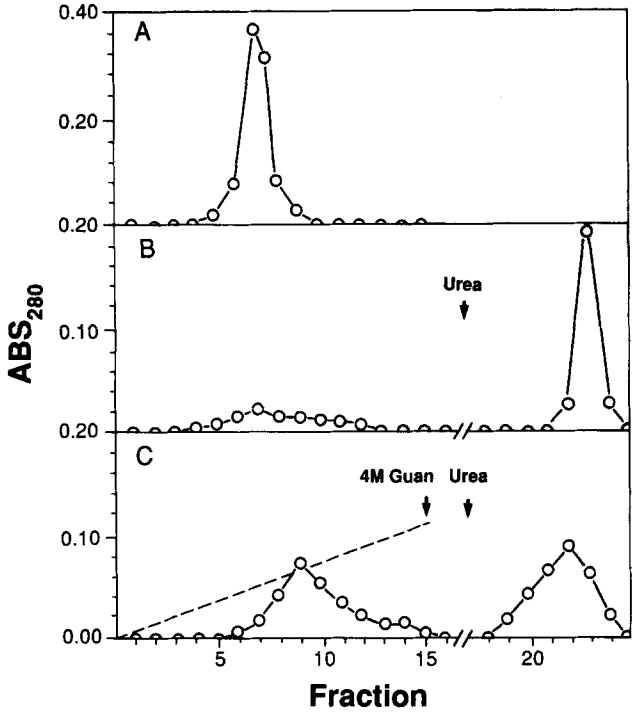

Figure 1. Elution of EN from gelatin agarose. Fraction volume = 2.0 ml. (*A*) Elution with 8.0 M urea in 50 mM Tris, pH 7.5. (*B*) Elution with 1.0 M guanidine-HCl in 50 mM Tris, followed by elution with 8.0 M urea/50 mM Tris. (*C*) Elution with 0–4 M gradient of guanidine followed by urea. The columns were reequilibrated with Tris between guanidine and urea addition (indicated by discontinuous abscissa in *B* and *C*).

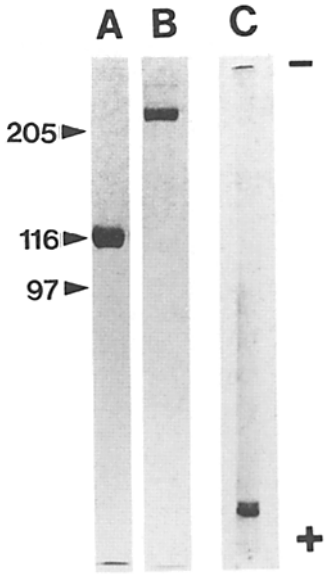

Figure 2. Electrophoretic analysis of gelatin agarose peak. (*A*) Coomassie Blue-stained 7.5% acrylamide reducing (0.5 M DTT) gel. Protein load, 15 μg. (*B*) Identical sample subjected to electrophoresis under nonreducing conditions. EN is a 230-kD disulfide-bonded dimer. Numbers at left indicate positions of molecular mass markers in kD. (*C*) IEF of EN in the presence of 8 M urea in a pH 3–10 gradient of ampholytes.

lar mass estimate derived from this method was 205 kD, or 25 kD less than expected for the dimer. This result suggested that EN carries a net negative charge, which was borne out by IEF experiments. In the presence of 8.0 M urea, EN was resolved into two acidic charge isomers with isoelectric points of approximately 5.0 and 5.2 (Fig. 2, lane *C*). In the absence of urea the protein did not focus well.

EN was isolated from unfertilized eggs in quantities comparable to those observed in embryos. Egg and embryo EN are identical in molecular mass and in peptide maps of papain-digested material (Fig. 3, lanes *A* and *B*). Moreover, EN from the two sources was equivalent in adhesion assays (see below). For efficiency of time and yield (especially avoiding embryo loss during culture), our source of protein for most analyses was unfertilized eggs.

We next compared the one-dimensional peptide map of EN to that of FN. Under the conditions established for papain digestion of egg and embryo EN (see Materials and Methods for details), FN was poorly digested (Fig. 3, lane *C*). Increasing the enzyme/substrate ratio 10-fold resulted in a complete loss of the 116-kD EN band with most material running at or near the dye front; however, FN remained undegraded under these circumstances as well. This result indicates that the primary structures of EN and FN are indeed different from each other. Conditions were subsequently established to digest FN using porcine pancreatic trypsin. The results are shown in Fig. 3, lanes *D–M*. Trypsin proteolysis of FN resulted in a number of products, the major ones approximating 180, 100, 50, and 25 kD. There was a progressive loss of the highest molecular mass bands (>200 kD) with increased proteolysis time (Fig. 3, lanes *D–G*). On the other hand, digestion of EN resulted in the formation of a single product at 40 kD. Proteolysis appeared to be completed by the earliest time point—1 h (Fig. 3, lanes *I–L*). Chymotryptic digests of EN and FN were also carried out (2.0 μg/ml chymotrypsin pretreated with tosyl-L-lysine chloromethyl ketone, 20 h at 25°C). The digest resulted in the formation of several minor EN bands, including ones at 52, 21, and 15 kD (not shown). One major peptide migrating at 39 kD was formed. The FN digest resulted in the formation of several minor and intermediate bands, including only one that comigrated with EN products at 39 kD, and with one major band migrating at 32 kD. These observations support the conclusion reached using papain—that EN and FN exhibit considerable structural differences.

EN contains 6% carbohydrate by weight. Notable features of the amino acid composition (Table I) are the high ratio of polar/nonpolar residues (2:1) and the paucity of positively charged amino acids (9%). The isoelectric point(s) observed by focusing suggest(s) that a preponderance of the asx and glx represent aspartic and glutamic acids, respectively.

Antibodies to EN

The 116-kD band was excised from polyacrylamide gels and used as an immunogen for raising polyclonal antibodies in rabbits. The antibodies obtained stained 1.0 μg of purified EN on Western blots at serum dilutions as great as 1:2,000 (Fig. 4, lanes *A* and *D*). When tested against whole embryo homogenates, however, they recognized several other bands as well. Reactivity against all of the bands was eliminated by preabsorption of sera with purified EN, and results with preimmune sera were negative. To test the possibility that the cross-reactivity was due to shared carbohydrate epitopes, blotted proteins were treated with periodate before they were reacted with antiserum. Under these circumstances, the anti-EN antibodies recognized only the 116-kD polypeptide in blots of whole embryo homogenate (Fig. 4, lanes *B* and *E*). The results were the same with serum from each of the three

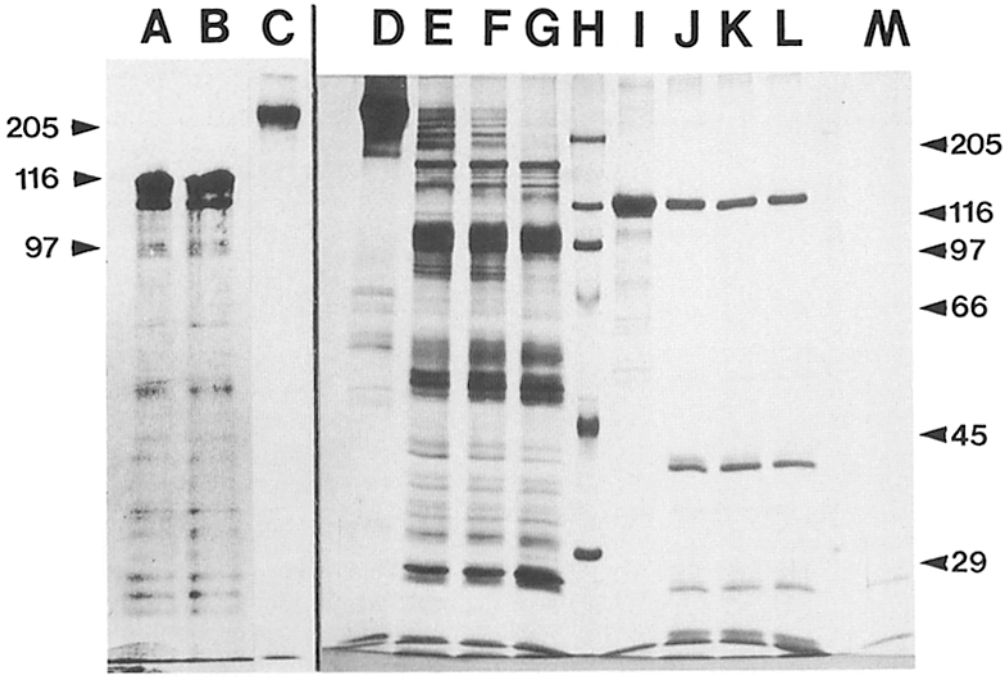

Figure 3. Partial proteolytic digests of egg and embryo EN, and gelatin agarose-purified fetal calf serum FN. In *A–C*, proteins were isolated from 7.5% SDS gels and digested with 0.05 μg papain within the stack of the second gel (12.5% acrylamide, silver stain) according to Cleveland et al. (7). (Lane *A*) embryo EN; (lane *B*) unfertilized egg EN; (lane *C*) fibronectin. In *D–L*, proteins were digested in solution with trypsin (2.0 μg/ml). (Lane *D*) FN control (incubated 4 h without enzyme); (lanes *E–G*) FN incubated with trypsin for 1, 2, and 4 h; (lane *H*) molecular mass standards; (lane *I*) EN control; (lanes *J–L*) EN incubated with trypsin for 1, 2, and 4 h; (lane *M*) 2.0 μg/ml trypsin plus 2.5 μg/ml soybean trypsin inhibitor (no substrate) to show positions of these two added proteins. Numbers indicate positions of molecular mass markers in kilodaltons.

rabbits immunized. Subsequent immunoblots and immunofluorescence were carried out on periodate-treated material, or with antibody affinity purified (29) by adsorption to periodate-treated EN polypeptide. Both approaches resulted in selective staining of the 116-kD band on Western blots. When the anti-EN antibodies were reacted with blots of fetal calf serum FN, labeling was rarely observed, and then only faintly (Fig. 4, lanes *C* and *F*). In the reverse reaction, polyclonal antimammalian FN antibodies (made in goat or rabbit) do not react with EN (Fig. 4, lanes *A* and *G*). Considering the protein loads on these heterologous immunoblots, we conclude that EN and FN are, at best, weakly cross-reactive.

Table I. Amino Acid Composition of Echinonectin

Amino acid	Mole
	%
asx	11.86
thr	6.91
ser	7.26
glx	9.54
pro	6.16
gly	14.92
ala	7.46
cys	0.46
val	5.62
met	1.50
leu	6.75
ile	4.38
tyr	2.67
phe	3.28
his	1.98
lys	2.36
arg	6.87
trp	ND

When visualized by indirect immunofluorescence, EN is found distributed throughout the unfertilized egg cytoplasm with a granular, or punctate, appearance. After fertilization it is transported to the cell surface. EN later becomes concentrated on the apical surface of ectoderm cells during germ layer differentiation (Fig. 5). Mesoderm and endoderm cell lineages do not express the antigen at levels detected by immunofluorescence. Some EN is carried inward with the archenteron during primary invagination but is soon lost from the primitive gut structure.

EN Is an Adhesion Protein

To examine the possible functions of EN, its ability to act as an adhesive substrate for embryonic cells was tested. Mesenchyme blastula-stage embryos were dissociated into single cells in calcium-free media, and their binding to EN was determined using FN as a standard for comparative purposes. It has been shown previously that sea urchin cells will bind to FN (14). At equimolar concentrations of EN and FN, EN was found to support cell binding at a higher level (68%) than FN (53%) in the centrifugal adhesion assay, with background levels of binding at 12% (Fig. 6). In both cases, prior control experiments measured concentration optima for the substrates. In experimental comparisons between the substrates, both were used within that optimal concentration range. Periodate treatment of EN had no effect on cell adhesion, suggesting that the cell binding domain lies in the polypep-

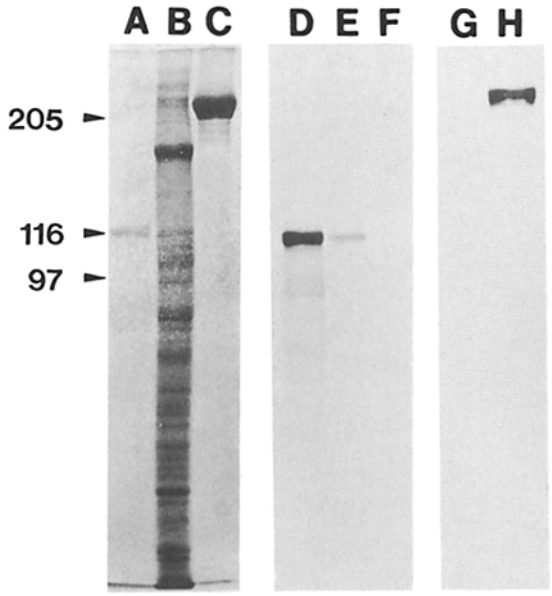

Figure 4. Immunoblot analysis of polyclonal anti-EN. (Lanes *A–C*) Coomassie Blue-stained 7.5% acrylamide gel of purified EN (2.5 μg), whole gastrula homogenate (50 μg), and purified fetal calf serum FN (10 μg); (lanes *D–F*) immunoblot (35) of *A–C* with anti-EN antiserum diluted 1:500 in PBS; (lanes *G* and *H*) immunoblot of EN (10 μg) and FN (5 μg) with goat anti-human plasma FN antibody (Organon Technika) diluted 1:500 in PBS. Positions of molecular mass markers are indicated at left.

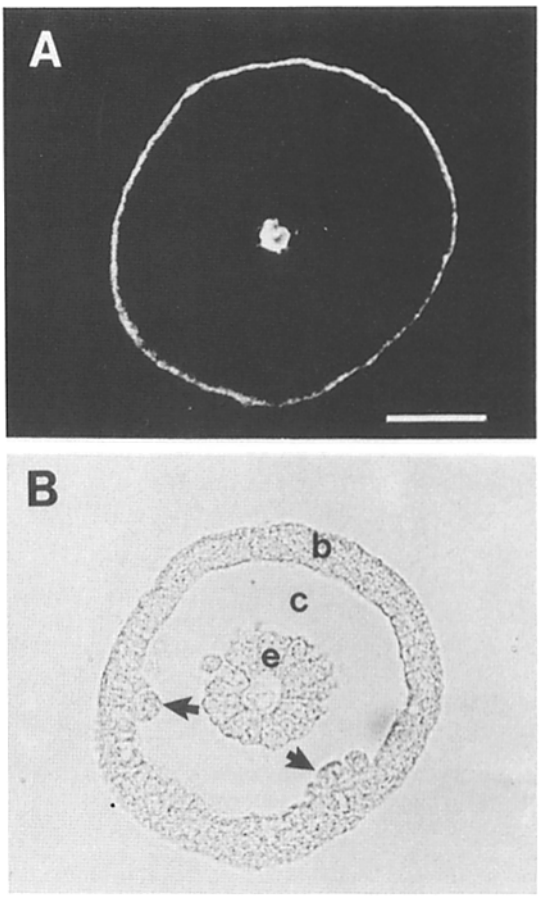

Figure 5. Immunofluorescence localization of EN in paraffin-sectioned gastrula-stage embryo of *Lytechinus variegatus* (*A*) and corresponding brightfield micrograph (*B*). Cross section showing the epithelium, or blastoderm (*b*), blastocoel (*c*) with primary mesenchyme cells (*arrows*), and the endodermal lining of the archenteron, or primitive gut (*e*). Immunofluorescent staining was abolished by preabsorbing the antiserum with purified EN, and no staining was observed with preimmune serum. Bar, 20 μm.

tide and not in the carbohydrate moiety. EN isolated from eggs or embryos supported binding to an equal extent. Storage in 8.0 M urea, Tris, or phosphate buffers did not appear to diminish the adhesive properties of EN; however, lyophilization reduced cell binding to slightly above control levels.

Ultrastructure

Purified EN dimer was subjected to zone sedimentation analysis in 15–40% glycerol gradients. A major peak was recovered at 10S, with a minor trailing edge (Fig. 7). Samples across the glycerol gradient were prepared for ultrastructural observation by negative staining with 2% aqueous uranyl acetate. The structure of the protein recovered in the 10S peak resembled a bow tie with dimensions of 12 nm in length, 8 nm in width at the widest points, and 4–5 nm in the constricted central region (Fig. 8, *A* and *C*). The ends of the molecule usually contain a cleft, but in some cases they appear to form a loop. Probably these differences are due to different orientations on the grid. In three dimensions the arms of the molecule may curve so that some orientations show the ends separate and others show them overlapped (in projection). The molecules all seem to lie with their long axis parallel to the carbon film, but there is probably no preference for rotation about this axis. Although at first the images appear to be symmetric about the long axis, close inspection shows that the two arms are different. One is shorter and slightly more flared; the other is longer, thicker, and runs more parallel to the long axis.

The images and sedimentation data are consistent with a single molecule of 230 kD. A cylinder 8 nm in diameter and

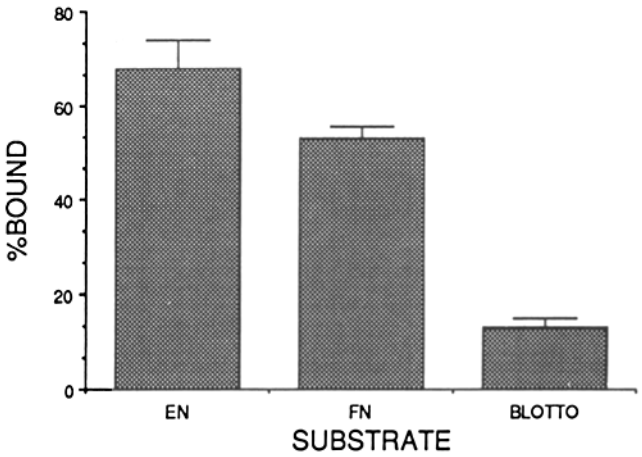

Figure 6. Adhesion of embryonic cells to EN-coated substrates. The data is expressed as a percentage of the total input counts remaining bound to the substrate (± SEM) after centrifugation at 25 *g* to dislodge unbound cells. 22 nanomolar solutions of each protein were used to coat their respective microtiter wells. When protein levels were made equal on a microgram basis, adhesion of cells to EN more than doubled adhesion to FN.

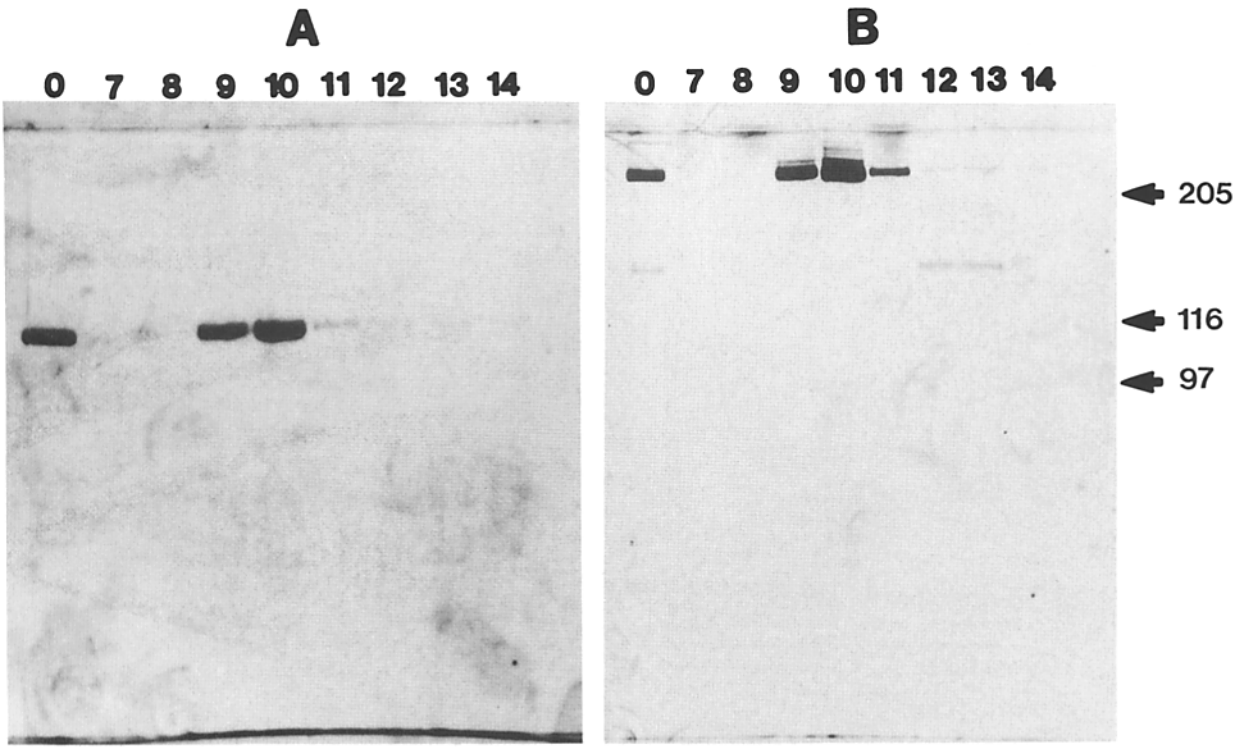

Figure 7. Zone sedimentation analysis of EN in a 15–40% gradient of glycerol in ammonium bicarbonate, pH 9.5. Gradient fractions (indicated by numbers at top) are shown after electrophoresis on a 7.5% acrylamide gel. (*A*) Reduced. (*B*) Nonreduced. Lanes *O* are samples of starting material diluted 1:10. EN dimers sediment at 10S. Numbers at right indicate positions of molecular mass markers.

12 nm long could hold 500 kD of densely packed protein (at 1.33 g/cm^3). Thus the 230 kD estimated for the dimer would fill about half the volume of this enclosing cylinder. This is consistent with the images, considering the clefts at the ends and at the waist of the molecule. The 10S sedimentation coefficient indicates a frictional ratio $f/f_o = 1.4$ (where f_o is the frictional coefficient of an unhydrated sphere of protein of 230 kD). This is similar to values for slightly elongated proteins such as tubulin and phosphofructokinase.

When trailing fractions from the glycerol gradient were viewed by negative staining, the possible subunit structure was discerned. A majority of the molecules in the 7.5S fractions appeared as U-shapes (Fig. 8 *B*) that could be arranged in back-to-back fashion to form the 230 kD (10S) bow tie–shaped protein. The dimensions of these structures were identical to each half of the bow tie. However, the putative half-molecules from the glycerol gradients migrated slightly above 116 kD on nonreducing SDS gels (150 kD in 7.5% acrylamide and 130 kD in 5% acrylamide). They could be induced to migrate at 116 kD by the addition of DTT (compare corresponding fractions in Fig. 7, *A* and *B*).

To address more directly the question of subunit structure, EN monomer was generated by reduction with DTT followed by alkylation with iodoacetimide. The alkylated monomers were next analyzed by zone sedimentation and negative staining. Under these conditions EN was recovered exclusively in the 7.5S peak of glycerol gradients and migrated at 116 kD in nonreducing SDS gels (Fig. 9 *A*). In the electron microscope the alkylated molecules seemed to be somewhat swollen, and the concavity was largely obliterated. However, the overall shape and size of the 7.5S/116-kD molecule were similar to the 6 nm U-shapes observed previously (Fig. 9 *B*). These data support the idea that the 230-kD EN dimer is composed of two U-shaped, 116-kD monomers attached to each other at their apices by disulfide bonds. In addition, the data suggest that EN contains intrachain disulfide bonds as well as the interchain disulfides that hold the bow tie together. This is supported by our ability to alter the mobility of "spontaneous" monomers in SDS gels with thiol reagents. The change in appearance of the 7.5S monomers in the electron microscope following alkylation is not in disagreement with this hypothesis. However, an alternative explanation is that a small polypeptide is attached to EN that is obscured by the dye front on 7.5% acrylamide reducing gels. This is considered less likely in view of the results just described, and as a result of analysis on heavily loaded, silver-stained 15% acrylamide gels.[2]

Discussion

Based on results with osmotic shock, Dan (8) proposed that the HL serves as the first adhesive substrate for embryonic cells. Maintenance of cell–substrate interactions, he proposed, was critical for the formation of the blastocoel (8). It is now known that the HL is composed of a number of polypeptides (2, 15, 22) organized into discrete layers (5),

2. Using as an estimate the minimum molecular mass of 14 kD and a minimum number of one polypeptide per EN dimer, an 18-μg protein load on a gel should include slightly over 1 μg of the putative peptide. This is within the detection limits of Coomassie Blue, and should yield a strong band with silver stain.

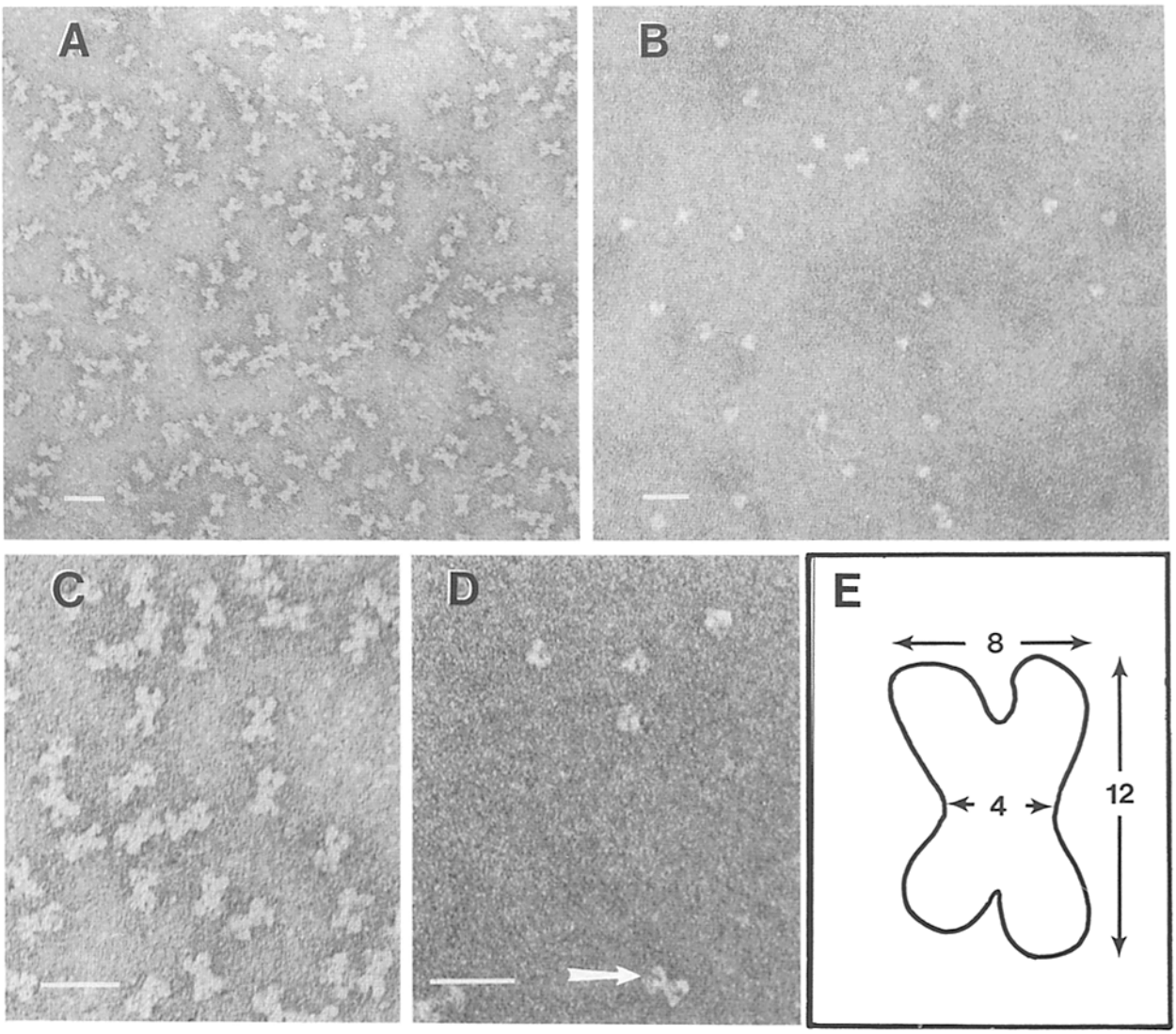

Figure 8. Ultrastructural analysis of glycerol gradient fractions 10 and 12, negatively stained with 2% uranyl acetate. (*A*) Lower magnification field view of fraction 10 showing whole EN dimers with characteristic bow tie shape. (*B*) Field view of fraction 12. The molecules are U-shaped and smaller, appearing to be half-EN. (*C* and *D*) Higher magnification views of fractions 10 and 12, respectively. Arrow in *D* highlights a whole molecule, providing a comparison within the sample. Bars, 20 nm. (*E*) Diagrammatic representation of a typical EN molecule showing dimensions. Traced from a projected negatively stained image.

and that its composition changes during early development. Adhesive interactions between cells and the HL are dynamic and germ layer specific (14). These features suggest a complexity of structure and function making the HL an important component of the embryo. At the molecular level, until now, the only protein analyzed for function has been hyalin.

In this report we have purified and described EN, another extracellular matrix protein from the sea urchin embryo. With the identification of a cell–substrate adhesive function for EN, there are now at least two demonstrated adhesion proteins in the HL (the other being hyalin). Hyalin and EN may play very different roles in development, or they may have similar functions and act in an additive or cooperative fashion with each other. Primary mesenchyme cells and presumptive endoderm detach from both of these proteins during their respective morphogenetic movements. Experimentally, it has been shown that primary mesenchyme cells selectively lose an affinity for hyalin (14). The cellular release from hyalin and perhaps EN may mediate or trigger aspects of differentiation. For example, hyalin binding to the blastoderm can be disrupted with small amounts of a monoclonal antibody to hyalin. The loss of binding is accompanied by altered expression of several gene products, including actin (1). It is therefore possible that an association exists between gene expression and correct cell–substrate contact. Such an association has been demonstrated in other systems (36). It is also possible that the function of either one of these proteins is mediated through its interaction with the other. Preliminary results indicate that hyalin and EN may in fact bind to each other, but that EN is more tightly associated with the cell surface than hyalin.

The potential binding of EN to collagen warrants a more detailed examination, particularly with regard to sea urchin collagen. The latter has been localized both to the basal lam-

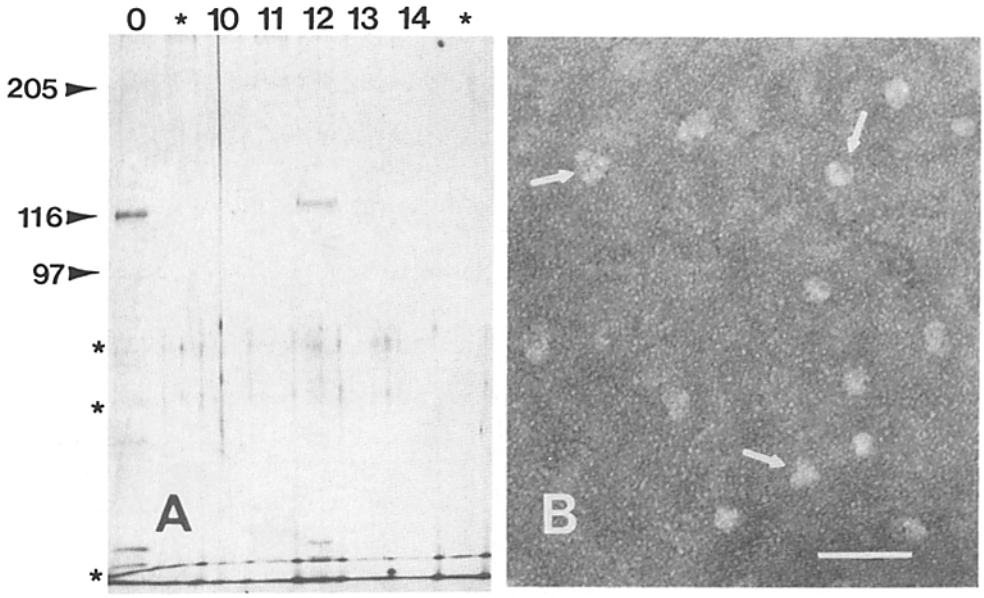

Figure 9. Sedimentation analysis and electron microscopy of alkylated EN. (*A*) silver-stained 7.5% nonreducing acrylamide gel of glycerol gradient fractions (indicated by numbers at top). Lane *O* is starting material, diluted 1:5. Arrowheads at left indicate positions of 205-, 116-, and 97-kD molecular mass markers. Alkylated EN sediments in the same position as the "spontaneous" half-molecules observed earlier (7.5S). Asterisks at left indicate contaminating bands in the gel. These are present in unloaded lanes as well (indicated by asterisks at the top). (*B*) Negative stain electron microscopy of fraction 12 shows the distorted appearance of the 7.5S/116-kD monomers. The concavity, or cleft in the "U" is largely obliterated. Arrows are included to indicate the probable orientation of several molecules, pointing into the concavity. Bar, 20 nm.

ina and to the HL in sea urchin embryos (33, 37, 38). Of possible interest is the observation that EN could be eluted from gelatin-agarose in two fractions. One fraction is recoverable with guanidine-HCl; the other fraction requires subsequent elution with urea. On SDS gels, both peaks yielded the 116-kD protein. Whether these two elution peaks represent functional (physiological) variations is not known at present. The relationship, if any, to the charge isomers demonstrated by IEF may also be of interest. A more extensive study of EN under native conditions may be helpful in answering these questions.

Although EN was isolated from eggs and embryos by gelatin agarose affinity chromatography using a protocol developed for FN, the two proteins differ according to a number of important criteria, including molecular mass, amino acid composition, peptide mapping, immunological analysis, and ultrastructure. Furthermore, EN is associated with ectodermal structures in the embryo whereas FN is generally considered to be of mesenchymal origin. It is still possible that EN is simply a sea urchin equivalent of FN, but the great differences in structure and distribution between the two do not readily support this hypothesis. In addition, there is some evidence that a more typical FN-like molecule is present in urchins (9, 14, 18, 20, 34, 38). Another possibility is that EN is a functional analog of FN in ectoderm (e.g., serving some functions generally attributed to the mesenchymal FN), perhaps through a few conserved, though limited domains. Under these circumstances, one might expect to observe low or no immunological cross-reactivity. Sequence data are needed before this question can be resolved.

The identification of adhesion molecules in the HL plus the presence of antigens that cross-react with antibodies to vertebrate collagen (33, 37), FN (34), laminin (34), and heparin sulfate proteoglycan (38) suggest that the HL serves functions similar to that of the basal lamina before its formation in the blastula. The discovery of EN and its apparent role in adhesion adds to the complexity of the HL. An ability to manipulate the cells and matrix components of this embryonic system offers advantages for the detailed study of extracellular matrix function in early development.

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