In Vitro Biological Activities of Echinonectin†

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ABSTRACT: Echinonectin (EN) is a 230-kDa extracellular matrix glycoprotein found in the hyaline layer of sea urchin embryos. Dissociated embryonic cells attached strongly to EN-coated microtiter wells in a centrifugal-based in vitro adhesion assay, suggesting that EN is one of the hyaline layer proteins to which cells adhere in vivo (Alliegro et al., 1988). The present study examines the molecular properties of that adhesion using monoclonal antibodies as probes to block cell attachment, and also demonstrates that EN possesses lectin activity. EN binds tenaciously to agarose-based chromatography resins, such as Sepharose. The sugar-binding activity is associated with the polypeptide component of EN, and not with the carbohydrate moiety. Binding is inhibited with galactose and fucoidan, but not with glucose or locust bean gum. Although functional sites both for polysaccharide binding and for cell attachment are present on each subunit of the EN molecule, the sites appear to be functionally distinct because galactose and fucoidan are completely without effect on cell attachment in vitro. Proteolytic digestion of EN yields a highly limited set of immunoreactive peptides. Digestion with trypsin yields a 20-kDa fragment, chymotrypsin, a doublet at 20 kDa, and 20- and 23-kDa fragments with thermolysin. McAb's directed against these peptides block cell adhesion in vitro, suggesting that they possess the cell attachment domain of EN. This is supported by the observations that trypsin-digested EN is an effective substrate in adhesion assays and that adhesion to the tryptic fragments is also blocked by McAb's to the 20-kDa domain.

Early embryos of virtually all multicellular organisms are invested with one or more extracellular matrices (ECM's).[1] From functional studies on many different species, it is known that the ECM furnishes more than simple structural support for embryos; it provides an adhesive substrate and has a function in epigenetic regulatory pathways as well (Trelsted, 1984). ECM molecules are involved in feedback circuits and provide cues that help to guide morphogenesis. In a number of cases, these cues have been linked experimentally to the adhesive interactions between the cell and the ECM (McClay & Ettensohn, 1987).

The sea urchin embryo develops under the influence of three morphologically and spatially distinct ECM's. Similar structures are encountered in widely divergent species, including molluscs and mammals. The vitelline envelope is present on the egg surface prior to fertilization and regulates sperm adhesion and entry. The hyaline layer (HL) forms shortly after fertilization and closely surrounds the embryo throughout embryonic development. Lastly, the basal lamina is deposited on the basal surface of cells during blastocoel formation.

Located on the apical cell surface, the HL seems to play a role similar to that of a basal lamina. Experiments suggest that adhesion of cells to the HL is critical for several steps in morphogenesis. For example, disruption of cell–HL adhesion with monoclonal antibodies specific for hyalin (a major HL glycoprotein) results in altered gene expression and developmental arrest (Adelson & Humphreys, 1988). Also, removal of the HL with calcium-free seawater causes the embryo to dissociate (Herbst, 1900). Finally, certain cells lose affinity for HL proteins at specific stages in development coincident with their morphogenetic movement away from the HL (McClay & Fink, 1982; Fink & McClay, 1985).

A newly identified ECM molecule called echinonectin (EN) has recently been isolated from the HL of sea urchin embryos (Alliegro et al., 1988). EN is a relatively abundant glycoprotein (comprising 0.05–0.1% of total embryo protein) that is stored in the egg, released following fertilization, and later synthesized by cells of the ectodermal lineage. It supports cell attachment in in vitro adhesion assays (Alliegro et al., 1988), and the adhesive interaction is tissue specific (Burdsal et al., 1988, 1989). During ingression, primary mesenchyme cells selectively lose their affinity for EN as they invade the blastocoel. Until that time they, and their micromere progenitors, bind to EN with an affinity equal to that of other embryonic cells. Presumptive endoderm cells undergo a similar loss of adhesive affinity to EN later in development. Coincident with primitive gut formation in the embryo, endoderm joins mesenchyme cells in no longer binding to EN in vitro (nor are they in contact with the protein in vivo). These changes in affinity, timed to coincide with prominent developmental events, suggest that EN may participate in the cell rearrangements that characterize morphogenesis.

This study begins the functional characterization of the EN molecule in order to define its biological roles. We examine its binding properties and, using antibodies and proteolytic fragments, its functional domains.

Materials and Methods

Specimens and Reagents. Unless otherwise indicated, commercial reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Sea urchins (*Lytechinus variegatus*) were collected from the North Carolina Coast. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were rinsed twice in Millipore-filtered artificial seawater, and sperm were stored undiluted. Embryos were cultured at 20 °C. EN

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[1] Abbreviations: BSA, bovine serum albumin; CBP, carbohydrate-binding protein; ECM, extracellular matrix; EN, echinonectin; HL, hyaline layer; SBTI, soybean trypsin inhibitor.

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was isolated as previously described (Alliegro et al., 1988) and stored at −70 °C in 8 M urea/50 mM Tris, pH 7.5. Monomeric EN was produced by reduction with dithiothreitol in 4 M urea and alkylation with iodoacetamide (Erickson & Carrell, 1983).

Affinity Methods. Purified EN for affinity chromatography was dialyzed into 50 mM Tris, pH 7.5, just prior to use. The source of fibronectin for positive controls was fetal bovine serum (Gibco Laboratories, Grand Island, NY). A gelatin–polyacrylamide matrix was constructed as described by Inman (1974) using the acyl azide reaction protocol. Methods for chromatography on BSA–agarose and uncoupled Sepharose 4B were identical with the gelatin–agarose purification already described (Alliegro et al., 1988). Column fractions were monitored by the absorbance at 280 nm or by the heated biuret protein assay (Dorsey et al., 1977). To obtain carbohydrate-free EN for Sepharose-binding experiments, the protein was translated in vitro from gastrula-stage poly(A)+ RNA. RNA was isolated according to Maniatis et al. (1982) and translated by using the protocol and reagents provided with a Promega (Madison, WI) rabbit reticulocyte in vitro protein translation kit.

In vitro translated EN was precipitated from solution with Sepharose 4B using a buffer system modified from Rohrschneider et al. (1979). One hundred microliter metabolically labeled samples were mixed with an equal volume of IP buffer [20 mM Tris (pH 7.2)/100 mM NaCl/2% sodium deoxycholate/2% Triton X-100/0.2% SDS/1 mM EDTA]. In vivo labeled samples were homogenized in IP buffer and centrifuged to remove nonsoluble cellular materials. Following addition of 25 μL of Sepharose 4B, samples were placed on an aliquot mixer at room temperature for 1–2 h. The Sepharose was then pelleted in a Beckman microfuge, washed 3 times by alternate resuspension in IP buffer and centrifugation, washed twice in IP buffer with 0.5 M NaCl, and boiled in electrophoresis sample buffer. Controls for these experiments included a "precipitation" without Sepharose. The results were negative.

Production and Characterization of Monoclonal Antibodies. EN antigen was excised from 7.5% polyacrylamide gels of affinity-purified protein. Prior to immunization of mice, the gel slices were treated with sodium metaperiodate to disrupt native carbohydrate structure (Bobbitt, 1956; Woodward et al., 1985). Experience with polyclonal sera had demonstrated that this treatment eliminated cross-reactivity with other sea urchin glycoproteins bearing shared carbohydrate epitopes. Hybridoma cell supernatants were screened for anti-EN activity by ELISA using purified EN as antigen. Positive cell lines were subcloned, and rescreened by indirect immunofluorescence, and on Western blots (Towbin et al., 1979) of purified EN and total gastrula protein.

Four monoclonal antibodies (McAb's) to EN were selected for use in various aspects of this study. These were examined to verify that they recognized peptide epitopes by treatment of Western blots with periodate or Pronase. The protocol for periodate treatment has been described previously (Woodward et al., 1985). Hydrolysis conditions for Pronase were optimized in preliminary experiments. Following electrophoretic transfer of EN onto nitrocellulose (Schleicher & Schuell, Keene, NH), strips were cut and incubated for 1 h at 25 °C in Tris buffer, pH 7.5, containing 0.1–5.0 μg/mL enzyme. The strips were then washed extensively with 50 mM Tris/0.9% NaCl, pH 7.5, and incubated with several changes of 1.0% BSA in the same buffer overnight. The extensive buffer rinse and overnight BSA incubation were required to remove all remaining proteolytic activity from the samples. Failure to do so resulted in decreased immunolabeling due to proteolysis of the *antibody* during subsequent incubations. Other evidence for the peptide nature of the epitopes was derived from proteolysis experiments described below.

Proteolytic Digestion of Echinonectin. Enzymes used for the digestion (Cleveland et al., 1977) of EN (with their peptide specificities) were bovine trypsin (lysine and arginine), bovine chymotrypsin (aromatic residues), and bacterial thermolysin (hydrophobic residues). Digestions were carried out at 25 °C. Incubation times and enzyme and substrate concentrations are provided with the results of each particular experiment. Proteolysis was terminated by quick-freezing in liquid nitrogen, lyophilization, and boiling in SDS electrophoresis sample buffer. Termination of proteolysis with the respective inhibitors of these enzymes yielded results identical with freezing followed by lyophilization.

Following proteolytic digestion of EN, peptides were separated on 15% polyacrylamide gels under reducing and denaturing conditions (Laemmli, 1970), blotted onto nitrocellulose (0.22-μm pore size), and probed with McAb's. McAb's were titrated in preliminary experiments to stain undigested EN with equal intensity. This was done because equalizing the McAb's on a simple microgram basis cannot account for differences in affinity for an antigen, which can vary by orders of magnitude. Under the present conditions, differences in staining could be attributed to proteolytic effects, and not differences in antibody titer.

To ensure that EN samples were identical in starting material and proteolytic products, protein was digested as a single batch (with one enzyme for each experiment), divided into equal aliquots for electrophoresis, blotted, and cut into separate strips for probing with the different McAb's. This approach enables a comparison of the relative reactivity of the different McAb's with individual proteolytic fragments.

These experiments were repeated with a slightly different format to directly compare the effects of trypsin, chymotrypsin, and thermolysin digestion on individual McAb epitopes. Separate (10 μg) aliquots of EN were digested with each enzyme separately, electrophoresed, and blotted onto nitrocellulose in parallel lanes, and probed with a single antibody. This process was repeated for each antibody. It also has the advantage of bypassing the requirement for pretitrating McAb's, since all three digests are incubated with the same antibody solution.

Adhesion Assay. The in vitro adhesion assay has been described previously (McClay et al., 1981; Alliegro et al., 1988; Lotz et al., 1989). Reagents used to block cell attachment were added to the EN-coated microtiter wells and washed out prior to addition of radioactive probe cells. Antibodies used in adhesion assays were purified from tissue culture supernatants by affinity chromatography on protein A–Sepharose (RepliGen, Cambridge, MA) as described by Ey et al. (1978). Specific assay conditions are provided with the results of each adhesion experiment.

Results

Polysaccharide-Binding Activity of Echinonectin. Our initial approach to purifying EN was to use gelatin–agarose affinity chromatography (Alliegro et al., 1988). This method was effective for obtaining purified preparations of the protein, but it did not prove that EN binds to collagen. Indeed, it was observed that in small batch preparations (such as for immunoprecipitations) EN could also bind to Sepharose alone. We therefore wished to determine if carbohydrate binding, gelatin binding, or both were the basis for EN isolation. We used two approaches in examining this question. First, embryo

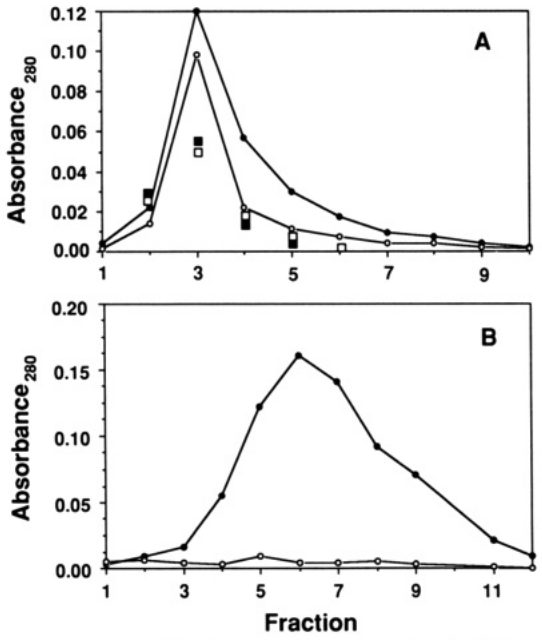

FIGURE 1: Retention of echinonectin by agarose-based affinity resins. (A) Five milliliters of unfertilized eggs was homogenized in the Tris/Triton buffer described under Materials and Methods, divided into equal aliquots, and applied to gelatin–agarose, BSA–agarose, or uncoupled Sepharose 4B. The columns were washed extensively and eluted with 8 M urea or 0.5 M galactose in 50 mM Tris, pH 7.5. (●) Gelatin–agarose eluted with urea; (○) BSA–agarose eluted with urea (the complementary urea elution of Sepharose 4B for this experiment is not shown); (□) galactose elution of EN from Sepharose 4B; (■) control urea elution of Sepharose. For these latter two, the ordinate represents protein concentration in milligram per milliliter. SDS–polyacrylamide gel electrophoresis of the respective peaks yielded a single 116-kDa band in each case. (B) Fibronectin (●) binds to a gelatin–polyacrylamide affinity matrix, but echinonectin (○) does not. Starting materials were 20 mL of fetal calf serum for fibronectin and 3.0 mL of unfertilized eggs for echinonectin. Identity of the fibronectin peak was confirmed by electrophoresis and immunoblotting.

lysates were applied to minicolumns (0.75-cm diameter × 1.0 cm) of gelatin–agarose, BSA–agarose, or uncoupled agarose (Sepharose 4B; Sigma), and quantitative retention of EN by these three resins was compared. We found that, regardless of the affinity matrix, equal quantities of EN were recovered from equally divided starting materials (Figure 1A).

The possibility remained that the EN carbohydrate-binding activity might be a collagen carbohydrate recognition property. To test this possibility, we immobilized gelatin on a matrix of polyacrylamide for use as an affinity column. Fibronectin was used as a positive control and was found to bind to and elute from the column under the standard conditions for fibronectin purification (Figure 1B). EN failed to bind to the gelatin–polyacrylamide column under the same buffer conditions in which it bound to the gelatin–agarose column (Figure 1B). Thus, EN binding to gelatin–agarose appears to have been due to an interaction with agarose alone.

Strumski (1985) and Strumski and Kinsey (1983) reported on the isolation of a 110-kDa protein from sea urchin eggs and embryos with carbohydrate-binding activity (designated carbohydrate-binding protein; CBP). The immunofluorescent distribution, amino acid composition, and other characteristics of the CBP suggested that it may be similar to or the same as EN. We found that polyclonal antibodies directed against the CBP reacted strongly with purified EN and also specifically labeled 116-kDa EN on immunoblots of whole gastrula protein (data not shown). Moreover, EN could be eluted from agarose columns with galactose (Figure 1A), as previously described (Strumski, 1985). On the basis of these observations,

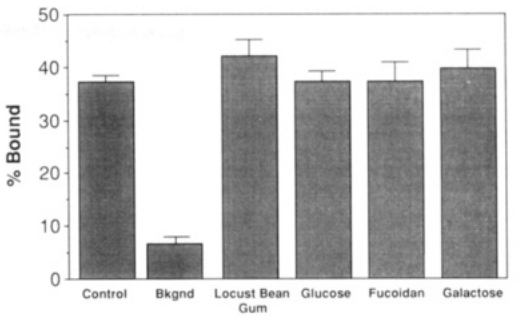

FIGURE 2: Competitors for the echinonectin polysaccharide-binding activity (galactose and fucoidan) do not interfere with cell attachment in vitro. Microtiter wells were coated with 50 μL of 10 m>/mL EN in 50 mM Tris, pH 7.5, washed 2 times with 100 μL of Tris, incubated with the saccharides (50 μL) for 1 h, and washed an additional 3 times with Tris. Concentrations used: galactose and glucose, 0.5 M; fucoidan and locust bean gum,[2] 5.0 mM. "Control" is cell attachment to EN treated with buffer only. "Bkgnd" shows background level of binding to wells without EN, blocked with Blotto (Johnson et al., 1984). Data are expressed as percentage of total input counts.

we concluded that EN and the egg CBP described by Strumski (1985) were identical.

We used the available information on the carbohydrate specificity of CBP (Strumski, 1985) to test the hypothesis that cell attachment to EN observed in vitro is mediated by the polysaccharide-binding activity. The approach taken was to perform adhesion assays using as potential inhibitors saccharides that specifically compete for the binding activity of EN to carbohydrate. In addition to galactose (200 mM–1.0 M), fucoidan dissociated EN from agarose (Strumski, 1985; personal observation). Carbohydrate binding was reduced by more than 75% with 4 mM fucoidan (Strumski, 1985). Glucose and certain other polysaccharides, such as locust bean gum, were without effect.

In cell adhesion assays, neither galactose (500 mM) nor fucoidan (5 mM) reduced cell attachment to EN (Figure 2).[2] Levels of cell binding were, in each case, identical with controls in a quantitative assay where even partial interference should be detectable (McClay et al., 1981; Lotz et al., 1989). We examined the possibility that these results were due to an inability of plastic-adsorbed EN to bind sugars. Microtiter plates were prepared as for an adhesion assay, except the galactose and glucose solutions were spiked with 10 μCi of their respective tritiated forms. The control for this experiment measured the binding of tritiated sugar to microtiter wells without EN. The wells were then washed with buffer in the usual manner and prepared for scintillation counting. The result was that galactose (20 490 ± 2100 cpm) but not glucose (627 ± 81 cpm) bound to EN-coated microtiter wells. Control binding was 488 (±33) cpm for galactose and 516 (±70) cpm for glucose. It appears that carbohydrate binding and cell attachment are two distinct activities of the EN molecule.

We found that the polysaccharide-binding domain of EN lies in the polypeptide, and not the carbohydrate moiety of the protein. Initially, this was suggested by the observation that EN, treated with 20 mM sodium periodate, bound to agarose columns at levels equal to controls (unpublished observation). To better examine the notion that the polypeptide domain is responsible for the polysaccharide-binding activity, carbohydrate-free EN was prepared by translation in vitro. From either in vivo or in vitro translations, only one polypeptide was subsequently found to be precipitable with Sepharose (Figure

[2] The locust bean gum concentration reported was at the upper limit of its solubility in Tris buffer. The actual concentration in solution may be slightly lower.

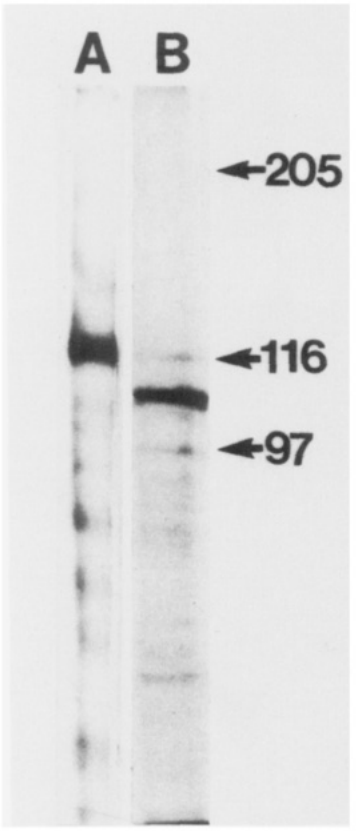

FIGURE 3: Carbohydrate-free EN exhibits polysaccharide-binding activity. EN was translated in vitro from 0.5 μg of gastrula-stage poly(A)+ RNA and precipitated with Sepharose 4B. (A) Control (in vivo) ^{35}S-labeled EN; (B) in vitro translated EN. Numbers at left indicate molecular weight standards (×10^{-3}).

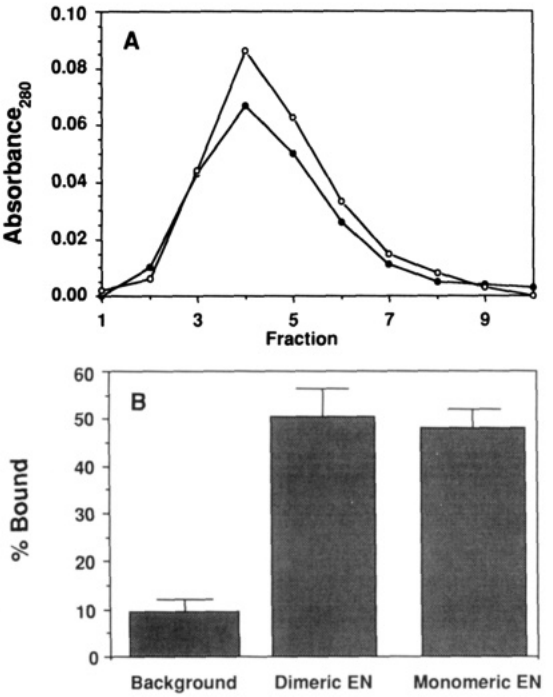

FIGURE 4: Monomeric echinonectin retains polysaccharide and cell attachment activities. For Sepharose binding, a 500-μg sample of alkylated or control (dimeric) EN was applied to a 2-mL column of Sepharose 4B in a 5-mL graduated pipet and washed and eluted by using the conditions described for Figure 1. Parallel samples of alkylated and control EN were applied in adhesion assays using the standard protocol as described under Materials and Methods. (A) Elution of monomeric (●) and dimeric (○) echinonectin from Sepharose 4B with 8.0 M urea. Results with galactose elution were identical. (B) Results of in vitro adhesion assay using monomeric or dimeric echinonectin. Controls for the effects of alkylation included untreated EN, urea alone (no DTT or iodoacetamide added), urea + DTT (no iodoacetamide), and urea + iodoacetamide (no DTT). These latter three yields results equivalent to untreated EN, which was simply allowed to incubate at room temperature for the same time period as the others. All samples were dialyzed into 50 mM Tris and adjusted to equal concentrations of protein (20 μg/mL for adhesion assay) prior to use.

3). The estimated M_r for the protein translated in vitro was 110K, vs 116K in vivo. M_r 110K is in precise agreement with our previous estimate for the EN polypeptide chain based on carbohydrate analysis (Alliegro et al., 1988). Carbohydrate-free EN thus appears to exhibit polysaccharide-binding activity.

Monomeric Echinonectin Retains Binding Function. Echinonectin is a dimer composed of two 116-kDa subunits. The subunits are U- or V-shaped and are held together in back-to-back fashion by disulfide bonds. We wanted to know if higher order structure (disulfide bonds and dimer assembly) was necessary for the two activities discussed above, or if reduced/monomeric EN alone possessed functional polysaccharide-binding sites and could support cell attachment as well. Monomeric EN was produced and stabilized by alkylation with iodoacetamide as previously described (Alliegro et al., 1988). The monomers were assayed for function by chromatography on agarose and as substrate in adhesion assays. Prior to use, samples of the alkylated EN were subjected to electrophoresis under nonreducing conditions to determine whether the treatment was effective. We found that at least 90% of the sample was reduced to monomeric form. This monomeric EN was subsequently found to bind to agarose and support cell attachment at levels equal to dimeric EN (Figure 4). Thus, neither disulfide bonds nor the dimeric structure of EN is required for cell attachment or polysaccharide binding.

Monoclonal Antibodies to EN Block Cell Attachment in Vitro. The four McAb's used in this study are designated 1b2, 3c6, 5e1, and 2/1b7. Each selectively labeled the 116-kDa band on Western blots of total gastrula homogenate (Figure 5A). Three of the antibodies localized to the apical ECM (hyaline layer) of embryos using indirect immunofluorescence (Figure 5B). This is the distribution previously observed for EN with polyclonal sera (Alliegro et al., 1988). McAb 2/1b7 was negative by immunofluorescence, suggesting that its epitope was destroyed by fixation. The antibodies apparently recognized polypeptide epitopes on the molecule in that immunoreactivity was retained after periodate treatment, but was abolished by Pronase treatment of blots (Figure 5C). Non-periodate-treated EN and other proteins with carbohydrate epitopes (Alliegro & McClay, 1988) or peptide epitopes (Adelson & Humphreys, 1988) were blotted and probed with their respective antibodies to test the validity of the periodate and Pronase treatments.

The McAb's described above were tested for their ability to inhibit cell adhesion in the in vitro assay. While nonspecific antibodies had no measurable effect on adhesion of cells to EN, all four of the McAb's directed against EN were effective to varying degrees (Figure 6). The antibody effect was concentration dependent: McAb 1b2 inhibition was reduced by 62% with a 50% decrease in antibody, and 5e1 inhibition was reduced by 21% with a 33% decrease in antibody.

Very few immunoreactive peptides were generated by proteolytic digestion of EN with thermolysin, chymotrypsin, or trypsin. There was considerable overlap in the recognition of these EN fragments with the four McAb's. This suggested that the epitopes were in close proximity to each other on the

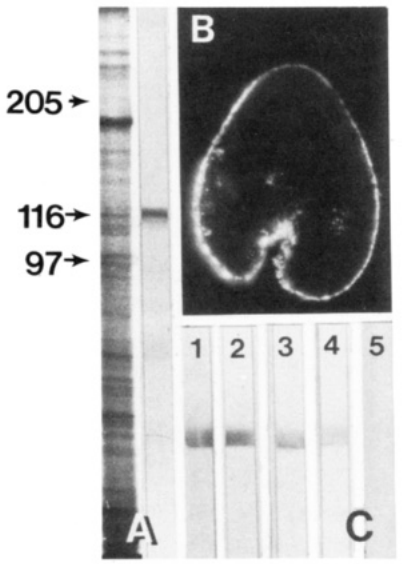

FIGURE 5: Characterization of anti-echinonectin monoclonal antibodies. (A) Coomassie blue stained gel (left) and immunoblot (right) of gastrula homogenate. Staining with McAb 5e1 is illustrated. (B) Immunofluorescence pattern observed with McAb 3c6. The distributions observed with 1b2 and 5e1 were identical. McAb 2/1b7 was negative by immunofluorescence. (C) Epitope characterization. Lane 1 is 0.5 μg of echinonectin, blotted onto nitrocellulose, and probed with McAb 3c6. In lane 2, the nitrocellulose strip with echinonectin was treated with 20 mM periodate. No reduction in staining was observed. Lanes 3, 4, and 5 were treated with 0.1, 1.0, and 5.0 μg/mL Pronase, respectively.

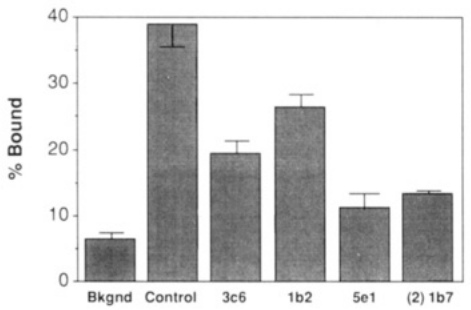

FIGURE 6: Inhibition of in vitro cell adhesion with monoclonal antibodies to echinonectin. Antibodies were purified from tissue culture supernatants by protein A affinity chromatography. Five micrograms (50 μL of a 100 μg/mL solution) was applied to EN-coated microtiter wells for 1 h at room temperature, and washed out with 3 volumes of Tris. The cell attachment assay was then performed in the standard manner. Controls for antibody adhesion assays included P3× (parent hybridoma), McAb's to other sea urchin proteins, and buffer without antibodies.

EN molecule. However, several differences were found, indicating that the four epitopes were not identical. Digestion of EN with thermolysin resulted in the formation of one principal immunoreactive fragment at 20 kDa, and another at 23 kDa (Figure 7A). Three of the McAb's, 1b2, 3c6, and 5e1, recognized the 20-kDa fragment on immunoblots. McAb 2/1b7 immunoreactivity was destroyed by thermolysin digestion. On the other hand, chymotrypsin digestion of EN destroyed 3c6 immunoreactivity, leaving the epitopes for 1b2, 5e1, and 2/1b7 intact. Trypsin digestion of EN resulted in the formation of two polypeptide products at 40 and 20 kDa.[3] The 40-kDa band was not recognized by any of the McAb's

[3] The 40-kDa fragment is the major product and has been reported previously. The 20-kDa band is relatively minor and was not previously observed because it is masked on protein-stained gels by comigration with SBTI.

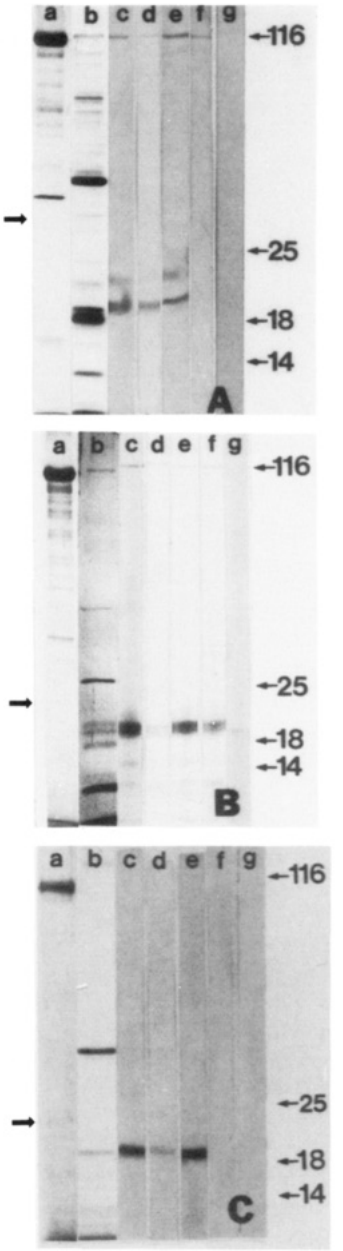

FIGURE 7: Immunological analysis of echinonectin digested with the proteases thermolysin (panel A), chymotrypsin (panel B), and trypsin (panel C). Reaction conditions: 5 μg of EN (50 μL of a 100 μg/mL solution in 50 mM Tris, pH 7.5); 50 ng of enzyme (0.5 μL of a 100 μg/mL solution in Tris; 18–20 h, 25 °C. In each panel: lane a, silver-stained 15% polyacrylamide gel of undigested EN; b, protease digest, silver stain; c–f, immunoblots with McAb's 1b2 (c), 3c6 (d), 5e1 (e), and 2/1b7 (f); g, immunoblot of each respective enzyme alone with McAb 1b2. This control was performed with each McAb. Positions of molecular weight standards ($\times 10^{-3}$) are indicated on right side for each panel. Arrows on left indicate positions of enzymes on gels.

(Figure 7C). The 20-kDa band stained strongly on Western blots with McAb's 1b2 and 5e1, weakly with 3c6, and not at all with 2/1b7.

To summarize, digestion of EN with three enzymes yielded a restricted number of immunoreactive products, all approximating 20K in M_r. Epitopes for the four McAb's seemed to be in proximity to one another on the molecule because of overlap in recognition of these fragments. The epitopes were not all identical based on differential sensitivity to the proteases. McAb's 1b2 and 5e1 seemed to be most consistent in their recognition of fragments generated by any of the three enzymes.

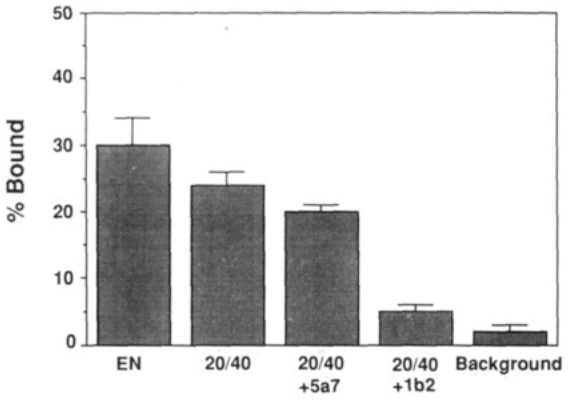

FIGURE 8: Cell attachment to tryptic fragments of EN. EN (20 μg/mL) was digested for 2 h at 25 °C with 5.0 μg/mL bovine pancreatic trypsin. The reaction was terminated by addition of 12 μg/mL soybean trypsin inhibitor (SBTI), and the digest was adsorbed to microtiter wells for substrate in adhesion assays as usual. Controls included trypsin/SBTI-coated microtiter wells (no EN). Levels of cell attachment to trypsin/SBTI were slightly below background. EN, undigested EN; 20/40, trypsin-digested EN, indicating the presence of 20- and 40-kDa polypeptides; 20/40+5a7, digested EN plus McAb 5a7, which is directed against another sea urchin cell surface protein and does not recognize EN; 20/40+1b2, digested EN plus McAb 1b2 directed against the 20-kDa fragment of EN; background, cell attachment to blocking solution, 10% Blotto (Johnson et al., 1984). McAb 5e1 was used to inhibit cell attachment to trypsin-digested EN in separate experiments. Adhesion was reduced from 34% in controls (20/40) to 22% ($p < 0.05$).

Localization of the McAb epitopes to a ~20-kDa fragment(s), coupled with the ability of these McAb's to block adhesion, suggested that the cell attachment activity of EN might be associated with the 20-kDa domain. To examine this question further, tryptic digests containing a mixture of the 20- and 40-kDa fragments were used as a substrate in adhesion assays. Cells attached to the trypsin-digested EN at levels slightly below controls (Figure 8), indicating that at least one of the two fragments possessed a functional cell attachment domain. McAb's 1b2 and 5e1 were used to distinguish between the two, since these antibodies did not recognize the 40-kDa peptide but exhibited strong recognition of the 20-kDa peptide on Western blots (Figure 7). Both McAb's significantly reduced adhesion of cells to the EN matrix (McAb 5e1 by 35% and 1b2 by 79%). These results provide strong evidence that a domain critical for cell–substrate adhesion lies within the 20-kDa proteolytic fragment of EN.

Discussion

To understand the role of EN in development, we have begun by dissecting the known functional activities of the molecule, and by identifying domains containing those activities. How EN interacts with other cell surface components to impart structural support and/or physiological cues remains to be examined. The observations presented in this report provide evidence, for, and some characteristics of, two functions of EN: lectin activity and cell–substrate attachment activity.

Alkylation of EN with iodoacetamide was used to analyze the potential role of disulfide-dependent structure in polysaccharide binding and cell attachment. EN is a disulfide-bonded dimer, and our previous observations suggested the presence of intrachain disulfides as well (Alliegro et al., 1988). However, it appears that neither lectin nor cell attachment activities require disulfides, since reduced EN was equal to control EN in both agarose-binding and cell adhesion assays. These results also indicate that active sites for the two activities are not formed by dimer assembly but are present in at least one copy on each subunit of the protein. This is the most common situation for lectins (Lis & Sharon, 1986). Bivalency is also a feature shared by a variety of other substrate adhesion molecules such as fibronectin (Rouslahti et al., 1982) and laminin (Martin & Timpl, 1987).

Previous observations on the periodate insensitivity of cell attachment (Alliegro et al., 1988) and the present data on epitope mapping with adhesion-inhibiting McAb's suggest that the cell attachment domain of EN is a peptide and not a carbohydrate portion of the molecule. The same seems to be true for the lectin activity since periodate-treated EN retains its agarose-binding affinity, as does EN translated in vitro. It is possible, in the latter experiment, that the 110-kDa Sepharose-binding protein translated in vitro was not EN. However, since only one Sepharose-binding protein is known in sea urchin embryos, and since the M_r of carbohydrate-free EN is 110K, this is considered unlikely. In several respects, EN is functionally similar to disocoidin I, another developmentally regulated substrate adhesion molecule isolated from *Dictyostelium* (Barondes & Springer, 1987). Discoidin also possesses a galactose-specific lectin activity. Both the polysaccharide-binding site and the cell attachment site reside in polypeptide domains of the molecule, and are independent of each other (Barondes & Springer, 1987).

Although the lectin and cell attachment sites of EN share several features in common, they appear to be functionally separate. Galactose at 0.5 M blocks the lectin activity of EN by greater than 95% (Strumski, 1985; this report) and 5 mM fucoidan by greater than 75% (Strumski, 1985). Yet, at these same concentrations, both were completely without effect in an in vitro adhesion assay which can resolve differences in cell binding of less than 10% (McClay et al., 1981; Lotz et al., 1989). Thus, cell attachment appears to not be mediated by the polysaccharide-binding site. The independence of the two functions in vitro does not, however, preclude their cooperativity in vivo. Until the orientation of EN in the ECM is known and EN-binding proteins are identified, any relationship between the two biological activities will be open to question. One possibility, for example, is that the molecule attaches to the cell surface via the antibody-inhibitable cell attachment site, and is anchored to another ECM molecule by the lectin activity. However, at this juncture, several equally viable models can be proposed.

Antibody studies, in conjunction with protease digestion, point to a ~20-kDa proteolytic domain as having the cell attachment activity of EN. Digestion with trypsin, thermolysin, or chymotrypsin results in the formation of a limited number of immunoreactive products, each approximating 20K in M_r. All four McAb's recognize similar domains on Western blots and significantly inhibit cell adhesion in vitro. For further evidence, a mixture of 40- and 20-kDa proteolytic fragments of EN was prepared by trypsin digestion and used as a substrate in adhesion assays. If the binding activity resided in the 20-kDa tryptic fragment, we then expected to inhibit cell attachment with McAb's that selectively recognized this fragment. This was the result, providing substantial support for the hypothesis that the adhesive activity is in the 20-kDa domain.

Having established the cell attachment and lectin activities of EN, the next goals are to further localize the cell attachment domain, to identify a putative membrane receptor, and an endogenous ligand for the lectin activity. Preliminary data have also indicated that EN binds to other ECM proteins. This suggests the presence of yet additional functional domains. Further structural analysis should reveal the regions of the EN molecule important for these interactions.

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REFERENCES

Adelson, D. L., & Humphreys, T. (1988) *Development 104*, 391–402.

Alliegro, M. C., & McClay, D. R. (1988) *Dev. Biol. 125*, 208–216.

Alliegro, M. C., Ettensohn, C. A., Burdsal, C. A., Erickson, H. P., & McClay, D. R. (1988) *J. Cell Biol. 107*, 2319–2327.

Barondes, S. H., & Springer, W. R. (1987) in *Genetic Regulation of Development* (Loomis, W. F., Ed.) pp 129–140, Alan R. Liss, Inc., New York.

Burdsal, C. A., Alliegro, M. C., Ettensohn, C. A., & McClay, D. R. (1988) *J. Cell Biol. 107*, 382a.

Burdsal, C. A., Alliegro, M. C., Ettensohn, C. A., & McClay, D. R. (1989) *Dev. Biol.* (submitted for publication).

Cleveland, D. W., Fischer, M. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Dorsey, T. E., McDonald, P. W., & Roels, O. A. (1977) *Anal. Biochem. 78*, 156–164.

Erickson, H. P., & Carrell, N. A. (1983) *J. Biol. Chem. 258*, 14539–14544.

Ey, R. L., Prowse, S. J., & Jenkins, C. R. (1978) *Immunochemistry 15*, 429–436.

Fink, R. D., & McClay, D. R. (1985) *Dev. Biol. 107*, 66–74.

Herbst, C. (1900) *Wilhelm Roux' Arch. Entwicklungsech. Org. 9*, 424–463.

Inman, J. K. (1974) *Methods Enzymol. 34*, 31–58.

Johnson, D. A., Gautch, J. W., Sportsman, G. R., & Elder, G. H. (1984) *Gene Anal. Tech. 1*, 3–11.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lis, H., & Sharon, N. (1986) *Annu. Rev. Biochem. 55*, 35–67.

Lotz, M. M., Burdsal, C. A., Erickson, H. P., & McClay, D. R. (1989) *J. Cell Biol. 109*, 1795–1805.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Press, Cold Spring Harbor, NY.

Martin, G. M., & Timpl, R. (1987) *Annu. Rev. Cell Biol. 3*, 57–86.

McClay, D. R., & Fink, R. D. (1982) *Dev. Biol. 92*, 285–293.

McClay, D. R., & Ettensohn, C. A. (1987) *Annu. Rev. Cell Biol. 3*, 319–345.

McClay, D. R., Wessel, G. M., & Marchase, R. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4975–4979.

Rohrschneider, L. R., Eisenman, R. N., & Leitch, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4479–4483.

Rouslahti, E., Hayman, E. G., Pierschbacher, M., & Engvall, E. (1982) *Methods Enzymol. 82*, 803–831.

Strumski, M. A. (1985) Ph.D. Dissertation, University of Miami.

Strumski, M. A., & Kinsey, W. H. (1983) *J. Cell Biol. 97*, 29a.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Trelsted, R. L., Ed. (1984) *Role of the Extracellular Matrix in Development*, 643 pp, Alan R. Liss, Inc., New York.

Woodward, M. P., Young, W. W., & Bloodgood, R. A. (1985) *J. Immunol. Methods 78*, 143–153.