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THE BIOCHEMICAL IDENTIFICATION OF FIBRONECTIN IN THE SEA URCHIN EMBRYO

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Summary: We report the biochemical identification of fibronectin in the basal lamina of the sea urchin embryo. A. punctulata gastrula stage embryos were solubilized in Triton X-100 and the insoluble basal laminae extracted by incubation in buffer containing 8M urea, 2% 2-mercaptoethanol and 2% SDS. Extracted proteins were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose filters and probed with monospecific antibodies directed against human plasma fibronectin (pFN). Incubation in ^{125}I-labelled secondary antibody revealed a single band which co-migrates with human pFN at an apparent molecular weight of 220,000. This is the first direct biochemical demonstration of a fibronectin-like molecule in the sea urchin embryo which cross reacts with antibodies to vertebrate fibronectin.

The fibronectins are an interesting class of high molecular weight glycoproteins involved in a number of cellular processes including adhesion, differentiation, cytoskeletal organization, oncogenic transformation and cell migration. Structurally complex, these multifunctional proteins have been the object of intense investigation and a number of reviews are available which summarize recent advances [7,21].

Fibronectin is a commonly occuring component of vertebrate extracellular matrices (ECMs) and basal laminae. During normal embryonic development, fibronectin-rich ECM has been shown to line the migratory pathways of cells involved in a variety of morphogenetic events including gastrulation [3,4] and neural crest cell migration [2] in the chick, and gastrulation [1,12] and primordial germ cell migration [6] in amphibians.

Polyclonal antibodies directed against vertebrate fibronectins have been used by a number of investigators to localize fibronectin in the sea urchin embryo by indirect immunofluorescence. Spiegel et al. [15] first reported the presence of fibronectin at the cell surface and between cells of blastula and gastrula stage embryos. Subsequent studies localized fibronectin, as well as a number of other vertebrate

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ECM components, in the basal laminar region of early embryos and pluteus larvae [16,19]. Fibronectin and several other ECM components are localized in granules which are uniformly distributed throughout the cytoplasm of the unfertilized egg (19). Fibronectin has also been described on the cell surface of primary mesenchyme [10, 19].

Despite these immunocytochemical studies there has been little biochemical evidence reported to substantiate clearly the existence of the molecule in the sea urchin. Iwata and Nakano [8,9] have isolated a collagen-binding protein from adult sea urchin ovaries that co-migrates with human pFN as resolved by SDS-PAGE. Antibodies raised against this molecule did not cross-react with human pFN. The authors have also reported that antibodies prepared against human pFN did not cross-react with the isolated sea urchin fibronectin suggesting that the antigenic sites are not well conserved in evolution [8]. This suggests the possibility that antibodies used to localize the glycoprotein in the embryo may cross-react with antigenic determinants that are unrelated to vertebrate fibronectins. It is important therefore, to demonstrate biochemically the presence or absence of fibronectin in the sea urchin embryo. The present study reports the biochemical identification by Western blot analysis of a single band (~220,000 M_r) present in a urea extract of sea urchin embryo basal laminae that cross-reacts with antibodies directed against human plasma fibronectin.

Materials and Methods

Handling of Eggs and Embryos: Gametes were obtained from adult Arbacia punctulata and the egg fertilized as previously described [15].

Preparation of Basal Laminae: Late gastrula stage embryos were collected by hand centrifugation and washed three times in ASW followed by three times in Ca^{++}- and Mg^{++}-free seawater (CMFSW) containing 1mM EDTA. Approximately 5.0 X 10^5 embryos were resuspended in 5.0 ml of solubilization buffer [solubilization buffer: 0.1 M Tris-HCl (pH 8.1), 0.5% Triton X-100, 2mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO), 10 ug/ml leupeptin (Sigma), 10 ug/ml soybean trypsin inhibitor (STI, Worthington Diagnostic Systems Inc., Freehold, NJ), 10^{-7} M Pepstatin-A (Sigma) and 25 ug/ml DNase I (Sigma)]. All protease inhibitors were prepared fresh and added immediately before use. Insoluble material containing basal laminae was pelleted at 1,000 X g, resuspended in 5.0 ml of fresh solubilization buffer and left undisturbed for 30 min at room temperature. Basal laminae were then collected by centrifugation at 2,500 X g for 5 min.

Urea Extraction: Basal laminae were solubilized by a modification of the procedure used by Ohno et al. [14] to extract laminin from human placental basement membranes. The triton X-100 insoluble pellet was resuspended in 3 ml of 0.1 M phosphate buffer (pH 7.9) containing 8M urea, 2% 2-mercaptoethanol, 2% $NaDodSO_4$ (SDS), 5mM PMSF, 10 ug/ml STI,

10 ug/ml leupeptin and 10^{-7} M Pepstatin-A. Basal laminae were extracted for 2 hr at room temperature on an end-over-end mixer until completely solubilized. The extract was then dialyzed overnight in three changes of Tris buffered saline [TBS: 0.15M NaCl, 50mM Tris-HCl (pH 7.4), 1mM $CaCl_2$, 1mM $MgCl_2$] containing 1mM PMSF. Dialysed extract was centrifuged at 10,000 X g in an SS-34 rotor (Sorvall Instruments, Newtown, CT) for 10 min to remove any remaining insoluble material. Protein concentration was determined by absorbance at 280 nm (A_{280}). Aliquots were either stored at -80° C or prepared for electrophoresis by boiling for 5 min in 5X Laemmli sample buffer [11] containing protease inhibitors at the following final concentrations: 1mM PMSF, 1ug/ml leupeptin, 1 ug/ml STI and 10^{-7} M Pepstatin-A.

Preparation of Human Plasma Fibronectin: Human plasma fibronectin (pFN) was isolated by affinity column chromatography on gelatin-sepharose [5,20]. Aliquots of the pFN were stored in polypropylene tubes at -80° C or prepared in Laemmli sample buffer.

Preparation of Antibodies: Rabbit pre-immune and immune sera (raised against human pFN), were prepared as previously described [15], IgGs were prepared from ammonium sulfate precipitated fractions of whole sera on Whatman DE-52 ion exchange columns according to the methods of Cebra and Goldstein [2]. Monospecific and pre-absorbed IgG fractions were obtained by a modification of the procedure described by Talian et al. [17] for eluting specific antibodies from antigen immobilized on nitrocellulose. Monospecific antibody was eluted from the filters with 0.2 M glycine-HCl (pH 2.8) and the solution neutralized with 1 N NaOH as described by Talian et al. [17].

Gel Electrophoresis and Transfer to Nitrocellulose: Proteins were separated by SDS-PAGE [11] using a 5-15% polyacrylamide gradient. Prestained high molecular weight standards were obtained from BRL (Gaithersburg, MD). Separated proteins were either visualized by silver staining [13] or electrophoretically transferred to nitrocellulose using the Towbin et. al. [18] technique. Prior to transfer, gels were rinsed in tank buffer [192 mM glycine. 25 mM Tris], overlaid with a sheet of nitrocellulose (0.2 um pore size, BA-83, Schleicher and Schuell Inc., Keene, NH) and then sandwiched between two sheets of filter paper (3MM, Whatman Chemical Separation Inc., Clifton, NJ). Transfer was accomplished in tank buffer at 8 V/cm (1-2 A) for 1.5 hr in a Bio-Rad Trans-Blot Cell (Richmond, CA) with cooling coil in place. Following transfer, the nitrocellulose was incubated for 1 hr at 37° C in blocking buffer.

Incubation of Transfers in Antibodies: After incubation in blocking buffer as described, the nitrocellulose was cut into strips corresponding to sample lanes which were delineated by prestained molecular weight standards. Strips were incubated for 1 hr at room temperature with agitation in 3-5 ml of blocking buffer containing a 1:100 dilution of monospecific, pre-immune or pre-absorbed IgGs. Identical concentrations of IgG were used for each sample. The strips were washed in several changes of TBS and incubated for an additional hour in blocking buffer containing 1.0 X 10^6 cpm/ml donkey anti-rabbit ^{125}I-labelled $F(ab')_2$ (Amersham Corp., Arlington Heights, IL). The strips were again washed in several changes of TBS to remove non-specifically absorbed secondary antibody, air dried, and exposed to X-ray film (XAR-5, Kodak, Rochester, NY).

Results

Triton X-100 effectively removed all of the cells of the embryo as observed by phase and differential interference contrast microscopy. Only the basal lamina "bag" containing triradiate spicules was observed after the initial solubilization procedure. Histone proteins appeared to comprise the major cytoplasmic contaminants identifiable by SDS-PAGE after silver staining.

The results of the Western blot analyses of human pFN and sea urchin basal laminae extracted in 8M urea are summarized in fig. 1. The pFN standard was prepared as described from human plasma after several lots of a commercially available source of pFN showed severe proteolytic degradation. Fig. 1A (open arrow) indicates the pFN standard incubated in monospecific anti-pFN IgG. A silver stain of a gel sample identical to that used to generate the blots in fig. 1(A-C) is shown in fig. 1G. The pFN, resolvable by coomassie-blue or silver staining, migrates as a doublet (~220,000 M_r) on a 5-15% polyacrylamide gel. The pFN appears as a single diffuse band on autoradiographs (fig. 1A) due to the high specific activity of the ^{125}I-labelled secondary donkey anti-rabbit antibody. The urea extract of sea urchin basal laminae contains a single band (as resolved on autoradiographs) which

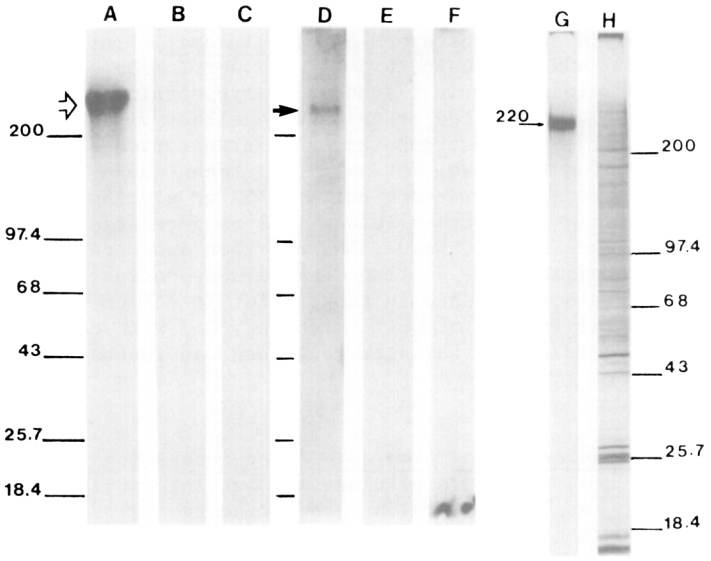

Fig 1. Western blot analyses of human plasma fibronectin and urea extracts of sea urchin basal laminae. (A-C,G) Human pFN standard. (D-F,H) Urea extract of sea urchin basal laminae. Nitrocellulose blots probed with, (A,D) anti-pFN monospecific I_gG, (B,E) pre-immune I_gG, (C,F) pre-absorbed I_gG. (G) Silver stained gel lane of human pFN standard ~2.5ug). (H) Silver stain of sea urchin urea extract (~20ug). Arrows indicate the co-migrating fibronectin bands in each panel (molecular weight markers X 1,000). (G,H) Molecular weight markers on right.

cross reacts with the monospecific anti-pFN IgG and co-migrates with the pFN doublet (fig. 1D, closed arrow). A silver stained gel lane of the urea extract is shown in fig. 1H. Antibody controls included pre-immune (fig. 1 B,E) and pre-absorbed (fig. 1 C,F) IgGs as well as omission of the first antibody (data not shown). In all cases, only background binding of the iodinated secondary antibody was noted in the control lanes.

Triton X-100 insoluble basal laminae can be solubilized prior to urea extraction by boiling in Laemmli sample buffer for ~15-30 min. Samples prepared in this manner did not cross react with the antibody following transfer of the separated proteins onto nitrocellulose. If the solubilization and extraction procedures used to generate the sea urchin samples were carried out using a single protease inhibitor (PMSF) instead of several then proteolysis and loss of antigenicity were noted. The combination of protease inhibitors included in the extraction procedure, at the concentrations listed, greatly reduced proteolytic degradation of the sea urchin fibronectin.

Discussion

In the present study, we present biochemical evidence of a ~220,000 M_r protein from sea urchin embryo basal laminae which cross reacts with anti-human plasma FN antibodies. This result confirms the existence of a fibronectin-like molecule in the sea urchin embryo. It further indicates, in contrast to the report of Iwata and Nakano [8], that fibronectin is conserved across phylogenetic lines. The difficulties encountered in such a biochemical characterization are primarily due to both the presence of proteases which are released upon disruption of the embryo and the relative insolubility of the fibronectin. Extraction of basal laminae in 8M urea, 2% 2-mercaptoethanol and 2% SDS in the presence of several protease inhibitors appears to have helped maintain the antigenicity of the molecule by circumventing the problems normally associated with isolation. Because our basal laminae were prepared by solubilizing whole embryos in Triton X-100, we cannot be absolutely certain that the fibronectin detected by immunoblotting was derived exclusively from the basal lamina. It is possible that the Triton X-100 insoluble contents of the blastocoel matrix, mesenchymal and epithelial cells (which normally appose the basal lamina) still remain after solubilization and were present in the urea extract. It is clear, however, from direct observation by optical microscopy, that no intact cells or

morphologically distinct cellular components (i.e., nuclei) are present following solubilization. In addition, initial washes in CMFSW reduce the number of cells and the amount of outer ECM present prior to Triton X-100 extraction. These results confirm that fibronectin is a major component of the basal lamina at the gastrula stage [16,19].

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