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Protease-Insensitive Sea Urchin Embryo Cell Adhesions Become Protease Sensitive in the Presence of Azide or Cytochalasin B

Donald R. Bertolini, Michiko Watanabe, and Robert S. Turner, Jr.

Department of Biology, Wesleyan University, Middletown, Connecticut 06457

To understand the nature of the cell adhesions that must be modified during sea urchin embryo primary mesenchyme formation, we are studying the adhesive components of the hatched blastula stage embryo of Strongylocentrotus purpuratus. Pronase treatment conditions have been defined that leave the cells intact and able to recover from the effects of the protease upon its removal. Under these conditions, adhesion of the cells to tissue culture plates is totally eliminated, but cell-cell adhesion formation is only partially inhibited. Analysis of iodinated cell surface proteins indicates that most are affected by the pronase. Further studies of pronase effects found that sodium azide-treated cells are slightly adhesive and that pronase treatment of azide-treated cells totally eliminates cell-cell adhesions.

Key words: sea urchin, cell adhesions, cell-cell adhesions, protease effects, glycopeptides, cell surface protein, azide, cytochalasin B, protease release, endogenous inhibitor

Embryonic morphogenesis involves developmentally regulated modulation of cell associations. One example of this process is the release of single cells from embryonic epithelia. This event occurs during neural crest cell formation from vertebrate neural fold epithelium and during avian gastrulation to produce the mesoderm. We are analyzing a third example of this process, sea urchin embryo primary mesenchyme cell formation. These cells first express a differentiated function when they are released from the epithelium that comprises the hatched blastula-stage embryo and move into the blastocoel. This release event occurs shortly after hatching, at the mesenchyme-blastula stage.

We have demonstrated that the release event is accompanied by a decrease in cell-cell adhesiveness [1]. Therefore, a reasonable model for the molecular basis of primary mesenchyme formation is that cell release results from modification of the epithelial cell-cell adhesive components. This suggestion is supported by ultrastructural studies that detect changes in adhesive components as the primary mesenchyme forms [2,3]. These considerations indicate the importance of determining the biochemical nature of the hatched blastula adhesive components and of then defining the modifications that these components undergo during primary mesenchyme formation. This report describes some of the experiments we have conducted with these goals in mind.

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We have defined enzymatic treatment conditions of intact sea urchin embryo cells that detect developmental changes in their adhesions [4]. One finding was that pronase has a minor effect on hatched blastula cell adhesions. We now report that pronase has a major effect on adhesion formation by azide- or Cytochalasin B-treated cells.

MATERIALS AND METHODS

Obtaining and Culturing Embryos

Adult Strongylocentrotus purpuratus were purchased from Pacific Bio-Marine, Venice, CA. and maintained in Instant Ocean Aquaria (Aquarium Systems, Eastlake, OH) as previously described [4]. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Sperm was stored dry for up to one week at 4°C and diluted with artificial seawater (ASW) immediately before use. Eggs were washed with ASW and adjusted to 10,000/ml, which is a 1% (v/v) egg suspension. Fertilization was conducted by combining a 5% egg suspension with a 1:100,000 final dilution of dry sperm and the fertilized eggs washed with ASW. Embryos were cultured as a 1% suspension in plastic centrifuge bottles on a TC-7 New Brunswick Rotator at 16–18° C in a humidified Gem Blood Bank Refrigerator.

Embryo Dissociation and Cell Adhesion Assay

Hatched blastula embryos were dissociated by previously described modifications [4] of Giudice's procedure [5]. Briefly, the embryos are washed with cold calcium-free seawater (CFSW), gently sheared in a sucrose:EDTA solution, and the resultant cells washed with CFSW and then with ASW. The cell concentration is then adjusted to 1–3 $\times$ 10^6/ml in ASW.

The cell adhesion assay consists of determining the loss of single cells from the cell suspension as aggregation occurs. The assays were conducted in 18-ml Nalgene vials in a New Brunswick G-76 gyratory water bath shaker at 100 RPM at 16–18°C.

We have determined that single cell loss is not occurring due to cell lysis because the cells exclude Trypan Blue. Aggregate formation is visible only in those samples in which the single cell number is declining and the cells do not release significant amount of labeled RNA or protein during the reaggregation assay. Furthermore, the aggregates that form develop into embryo-like structures when left in culture overnight. Additional documentation for both the health of the cells and the functional nature of the adhesions that form is provided in Watanabe [6].

Single cell loss is monitored by withdrawing an aliquot from each sample at various times after initiating the assay and counting the single cell number in a hemacytometer. Both aliquot withdrawal and filling the hemacytometer were performed using capillary action to fill and empty the tip of a Pastuer pipette. The single cell number was then converted to *% reaggregation* by dividing the determination at each sampling time by the initial single cell number, subtracting from one, and multiplying by 100. These values were then plotted as a function of time after starting the assay.

Experimental Treatments of the Cells

Conditions have been established that allow continuous treatment of the cells with each of the agents used in this study by determining the concentrations of each agent that do not affect the ability of the cells to reconstitute embryo-like structures after removing them from the agent [4]. Furthermore, we have also restricted the treatment conditions by limiting the treatments to those that do not cause the cells to release significantly more labeled RNA than control cells.

Each of the reagent solutions was made fresh on the day of the experiment. All stock solutions were made by dissolving the reagent in ASW, except Cytochalasin B, which was dissolved in DMSO. The final DMSO concentration in the reaggregation vials was 0.03%, and DMSO by itself had no effect on the protease sensitivity of reaggregation.

Reaggregation vials contained the agent being tested, 67 units of penicillin, 67 μg of Streptomycin, and 100 μg of DNase in a volume of 0.25 ml. To each vial, 0.5 ml of cells were added and the cell adhesion assay was initiated.

Additional Procedures

The cell-plate binding assay and cell surface radioiodination were conducted as previously described [7,8].

Material

All chemicals were of reagent grade or better. Pronase was purchased from Calbiochem and trypsin was 2× crystalline from Sigma. All other chemicals were purchased from Sigma.

RESULTS

Addition of 300 μg/ml pronase to the reaggregation assays has a minimal effect on the adhesiveness of hatched blastula cells (Fig. 1A). This is in contrast to the marked effect of a lower concentration of pronase on the adhesion of these cells to tissue culture plates (Fig. 1B). Removal of the cells from the pronase by washing them with ASW at the end of the 2-hour reaggregation assay allows the cells to form embryo-like structures if they are returned to the vials on the gyratory shaker and cultured overnight. Alternatively, if the cells are incubated with pronase in stationary cultures, washed, and then returned to the tissue culture plates, they do not attach to the plates as control cells do, but the clusters that they form even in the presence of the pronase develop into embryo-like structures when cultured overnight.

Identical results are obtained with 900 μg/ml trypsin: Cell-cell adhesions are only slightly affected and cell-plate adhesions are substantially reduced. Inhibition of the trypsin by the addition of 1800 μg/ml soybean trypsin inhibitor after 2 hours allows reconstitution of embryo-like structures, but the cells do not attach to the tissue culture plates in stationary cultures.

Given the broad specificity of pronase, these results imply that cell-cell adhesions are not dependent upon cell surface proteins. This somewhat surprising result warranted further investigation, so we sought to determine if it

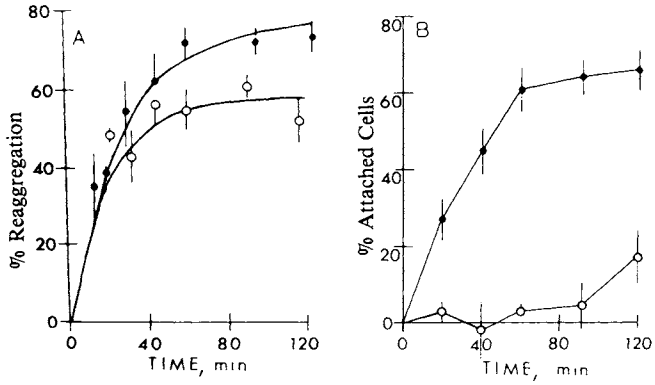

Fig. 1. Kinetics of cell-cell and cell-plate adhesion formation in the presence and absence of pronase. Hatched blastula embryos were dissociated into single cell suspensions. A) Kinetics with which the cells reaggregate in the rotation-mediated assay. The points are the means, with their standard errors, for the data from at least six experiments with duplicate samples counted at each time in each experiment. Closed symbols indicate the control cell kinetics; open symbols are the kinetics of adhesion formation in the presence of 300 μg/ml pronase. B) Similar analysis of the kinetics of attachment of the cells to tissue culture plates. The pronase concentration was 100 μg/ml (open circles) and not 300 μg/ml as in Figure 1A. Control cells are indicated by the closed circles.

were possible that the surface proteins of these cells were refractory to pronase treatment.

To test this point, cell surface proteins were labeled using lacto-peroxidase-mediated radioiodination. The surface-labeled cells were then incubated in a reaggregation assay for 40 minutes, collected, lysed, and subjected to analysis by SDS gel electrophoresis under reducing conditions. This study indicates that the majority of the cell surface proteins are sensitive to pronase (Fig. 2), but that incubation of the cells in the reaggregation assay conditions without pronase has no effect.

In a second series of studies, we analyzed pronase effects on cell surface lectin receptors. To inhibit reaggregation so that lectin agglutination could be observed, we treated the cells with sodium azide. One of the controls that we conducted consisted of treating azide-treated cells with pronase. Surprisingly, we found that these two agents had a synergistic effect on cell-cell adhesiveness (Fig. 3). Even the small cell clusters present in our initial cell suspension were dissociated under these conditions.

To evaluate this result, we determined the effects of pronase or trypsin treatment of cells treated with inhibitors of microfilament organization and of microtubule assembly.

The results indicate that inhibiting microtubule assembly did not cause the cell adhesions to become protease sensitive, but that inhibition of either microfilament organization or of energy production made them totally protease sensitive (Table I). Furthermore, the adhesions of the appropriately inhibited cells were sensitive not only to pronase, but also to trypsin.

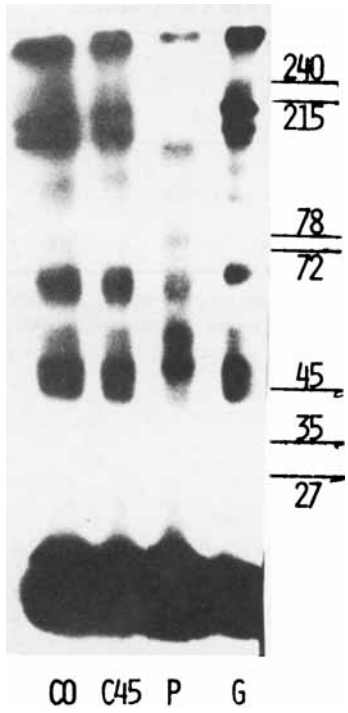

Fig. 2. Pronase effects on cell surface proteins. Dissociated hatched blastula cells were radioiodinated using glucose oxidase and lactoperoxidase. Reaggregation assays were conducted in the presence of pronase or of a mixed exoglycosidase. The cells were washed, lysed, and the proteins separated by SDS gel electrophoresis. The gels were used to produce the autoradiogram shown in the figure. The numbers indicate the apparent molecular weights of red blood cell ghost proteins. Lane CO) Control cells kept on ice while the remaining samples were used for the reaggregation assay; C45) control cells used in the reaggregation assay; P) reaggregation in 300 μg/ml pronase; G) reaggregation in 3 mg/ml mixed exglycosidase from Turbo cornutus (Miles).

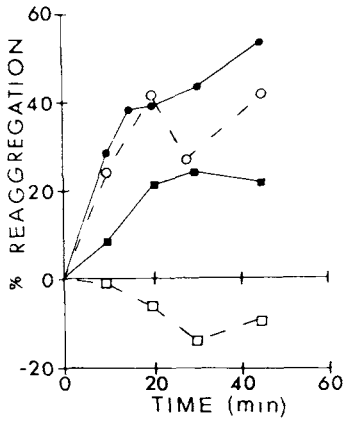

Fig. 3. Reaggregation of cells treated with Azide and pronase. Reaggregation kinetics of hatched blastula cells in the presence of 0.4% sodium azide and 300 μg/ml pronase. Control (●), pronase only (○), azide only (■), azide and pronase (□).

TABLE I. Effectors of Hatched Blastula Cell-Cell Adhesions

Inhibitor	No protease	Protease addition	
		pronase	trypsin
None	100	65	94
Azide (0.1–0.4%)	35	–24	–24
Cytochalasin B (3 μg/ml)	65	–18	ND
Colchicine (0.1 mM)	100	62	ND

Reaggregation assays were conducted in the presence of the indicated concentrations of inhibitors. Protease final concentrations were 300 μg/ml of pronase and 900 μg/ml of trypsin. The numbers are the maximum amount of reaggregation relative to the no inhibitor, no protease control. ND, not determined.

DISCUSSION

The results presented above offer some insight into the relatively minor effect of proteases as inhibitors of sea urchin embryo cell-cell adhesions.

Previously presented control experiments indicate that the protease conditions we have employed confine the action of the protease to the cell surface [4]. Under these conditions, cell-plate adhesions are markedly affected, most cell surface proteins are altered by the protease, but cell-cell adhesions are only slightly reduced. The most obvious conclusion from these observations is that the cell-cell adhesions do not involve cell surface proteins.

This conclusion is contraindicated by the ability of azide and Cytochalasin B to render the cell-cell adhesions totally protease sensitive. These agents may be acting to increase the protease sensitivity of the cell-cell adhesions by inhibiting secretion or affecting rearrangement of the adhesive components on the cell surface.

A block of secretion by azide or Cytochalasin B could account for our results if the cells contain an intracellular reserve of the protease-sensitive, cell-cell adhesive components. In the presence of the inhibitors, the cells would be unable to replace any protease-inactivated adhesive components to their surfaces from the intracellular reserve. This would produce cells that are totally nonadhesive (in the presence of both the protease and the inhibitor) and have increased levels of the adhesive components in their intracellular reserve. This intracellular adhesive component reserve should then be protected from the action of the proteases in the presence of the inhibitors.

Definitive examination of this possibility requires isolation of the component and production of immunological probes directed specifically against it. Such probes could then be used to determine the ratio of protease-insensitive to protease-sensitive adhesive component in cells treated with pronase in the presence and in the absence of either azide or Cytochalasin B.

Such probes are also necessary to investigate the second major mechanism by which the metabolic inhibitors might increase the protease sensitivity of cell-cell adhesion formation: By affecting rearrangement of the adhesive components on the cell surface.

It has been demonstrated that organization of cell surface components is affected by cytoskeletal elements, particularly microfilaments, and this organization is frequently found to be energy dependent [9]. Recently it has been shown that inhibition of energy metabolism disorganizes microfilaments, even though the cell shape changes induced by Cytochalasin B are not affected by inhibiting energy production [10]. It therefore seems possible that our experiments using either azide or Cytochalasin B are disrupting microfilaments.

Such microfilament disruption could alter the organization of the adhesive components on the cell surface if the components interact either directly or indirectly with the microfilaments. This altered organization could render the adhesive components accessible to proteolytic attack. For example, a microfilament-dependent process may cluster the adhesive components on the cell surface and the clustered components may be sterically inaccessible to the proteases. In the presence of the inhibitors, the microfilaments would be prevented from clustering the adhesive components, which would then become exposed to the proteases.

The considerations discussed so far indicate the desirability of obtaining adhesive component-specific probes. These probes can only be generated after isolation of the adhesive components, which is the aim of our current research.

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