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Carbohydrate-binding component of amphibian embryo cell surfaces: Restriction to surface regions capable of cell adhesion

(nonadhesiveness/cell recognition/endogenous lectins/concanavalin A/membrane domains)

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ABSTRACT **Superficial cells from early amphibian embryos display regional specializations of their cell surfaces. That portion of the plasma membrane facing the perivitelline space (apical surface) is nonadhesive, whereas, in the same cell, the lateral and basal portions of the plasma membrane will adhere to other cells. These adhesive differences are maintained on single cells that have been dissociated from the intact embryo. Extracts of cleavage-stage *Rana pipiens* embryos are capable of agglutinating formalinized sheep erythrocytes. The hemagglutination activity can be blocked by a yeast mannan and a family of glycoproteins containing high levels of mannose, indicating the presence of a lectin with oligomannosyl specificity. The cell surface location of this carbohydrate-binding component can be demonstrated by the ability of the formalinized sheep erythrocytes to form rosettes with living dissociated embryonic superficial cells. Rosette formation is blocked by the same inhibitors that are effective in blocking the activity of the crude extracts. The formalinized sheep erythrocytes form rosettes only to those cell surface regions of the superficial cells that are capable of adhering to other amphibian embryo cells. Receptors for concanavalin A, a lectin that binds D-mannose and D-glucose residues, have also been shown to be present exclusively over the adhesive regions of the superficial cells. The involvement of a carbohydrate-binding component with oligomannosyl specificity in the adhesive mechanisms of these cells is suggested by this restriction of both the embryonic amphibian lectin and its possible receptors (concanavalin A receptors) to adhesive regions of the cell surface.**

In his pioneering work on the adhesive characteristics of amphibian embryo cells, Holtfreter (1–3) described unusual properties for the embryonic amphibian superficial cell in regard to regional specialization of its cell surface. Those portions of the cell surface facing the outside of the embryo (apical surface) are nonadhesive, whereas lateral and basal portions of the plasma membrane are adhesive to other cells. These adhesive differences are maintained even on cultured dissociated cells (1–4). The original apical and lateral cell surfaces of animal hemisphere superficial cells can be distinguished on dissociated cells by the presence of abundant pigment granules, which underlie only the apical (nonadhesive) cell surface. We have previously demonstrated that superficial cells of the early cleavage stage embryo display a regional segregation of concanavalin A (Con A) receptors on their cell surfaces. Con A receptors were undetectable over the pigmented (nonadhesive) surfaces, whereas receptors were present on the unpigmented (adhesive) surface (5).

The ability of tissue cells to adhere to each other and to elements of the extracellular matrix is necessary for the construction of coherent tissues from populations of individual cells. Recently, it has been proposed that the interaction of cell surface lectins (multivalent carbohydrate-binding proteins) and the appropriate cell surface saccharide receptors contribute to cell-to-cell adhesion (6–10). In the present study we report the observation that extracts of cleavage stage embryos contain a Triton-solubilized lectin with oligomannosyl specificity. The presence of a carbohydrate-binding component at the cell surface was suggested by the ability of formalinized sheep erythrocytes to form rosettes with the superficial cells; rosette formation was inhibited by a yeast mannan, the same inhibitor that is effective in blocking the hemagglutination activity of the amphibian embryo extracts.

Few formalinized sheep erythrocytes bound to the apical (nonadhesive) portion of the cell surface, whereas the lateral and basal (adhesive) portions bound large numbers of erythrocytes. Thus both the cell surface oligomannosyl-specific carbohydrate-binding component (detected by erythrocyte rosetting) and cell surface mannose residues [detected by Con A binding (5, 11)] colocalize to those portions of the cell that are adhesive and are excluded from those portions that are nonadhesive. The correspondence of the distribution of the amphibian cell surface carbohydrate-binding component and the Con A receptors suggests that the Con A receptors could function as the endogenous receptors for the endogenous cell-surface lectin that has oligomannosyl specificity. The apparent restriction of the amphibian embryo lectin to only the adhesive surfaces of the superficial cell suggests that it could play a role in the adhesive interactions of these cells.

MATERIALS AND METHODS

Reagents and Media. Yeast mannan (baker's yeast), yeast invertase (grade X *Candida utilis*), porcine thyroglobulin, ovalbumin, fetuin, human transferrin, ovomucoid, bovine submaxillary gland mucin, dithiothreitol, trypsin (type XI), soybean trypsin inhibitor (type 1-S), deoxyribonuclease I (type III), and the simple sugars were obtained from Sigma. Endoglycosidase H was obtained from Miles. The unit A oligosaccharide from thyroglobulin was prepared by treatment of the intact thyroglobulin molecule with endoglycosidase H by the method of Tarentino *et al.* (12). Holtfreter's solution was prepared as follows: 3.5 g of NaCl, 0.05 g of KCl, 0.1 g of CaCl, and 0.2 g of $NaHCO_3$ were dissolved in 1 liter of glass-distilled H_2O and the pH was adjusted to 7.6 with NaOH. The periodate-oxidized invertase and thyroglobulin were prepared as follows: protein at 5 mg/ml in 0.05 M sodium acetate buffer, pH 5, was incubated at 4°C in the dark, with and without (control) 0.05 M sodium metaperiodate. The reaction was stopped by the addition of 0.1 M ethylene glycol and the samples were dialyzed extensively against 75 mM NaCl/75 mM Na_2HPO_4–KH_2PO_4, pH 7.2 (NaCl/P_i). Protein was determined by the method of

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Abbreviations; Con A, concanavalin A; NaCl/P_i, NaCl/sodium–potassium phosphate buffer.

Bradford (13). Carbohydrate concentration was measured by the phenol/sulfuric acid procedure (14).

Preparation of Cell Suspensions. *Rana pipiens* embryos were prepared by *in vitro* fertilization of eggs produced by hormonally induced ovulation (15). At the 32–64-cell stage, the jelly coats were removed manually with sharpened watchmaker's forceps. The pH of the medium was raised to 10.6 by the addition of KOH. When the embryo was dissociated into a mass of single cells (approximately 10 min), the vitelline envelope was removed with watchmaker's forceps. Any superficial cells with persisting adhesions were separated by the use of a hairloop. The superficial cells were sorted from cells that had lain deep from the surface with hairloops and were removed to another dish containing Holtfreter's solution at pH 7.6. All procedures were done in petri dishes coated with 2% agar to prevent adhesion of the cells to the surface of the dishes.

Preparation for Scanning Electron Microscopy. For observation with the scanning electron microscope, the superficial cells were fixed with 2% (wt/vol) paraformaldehyde/2% (vol/vol) glutaraldehyde/0.5% acrolein with 2 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer for 24 hr. The cells were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hr, dehydrated in ethanol, rinsed with amyl acetate, and dried from liquid CO_2 in a Bomar critical point drying apparatus. The dried specimens were coated with evaporated gold/platinum and observed with a Philips model 501 scanning electron microscope at 15 kV.

Preparation of Lectin Extract and Hemagglutination Assay. The jelly coats and vitelline envelopes were removed from 10 *R. pipiens* embryos at the 32–64-cell stage of development. The 10 embryos were homogenized with 25 strokes of a Teflon tissue grinder in 10 ml of NaCl/P_i and the homogenate was centrifuged at 100,000 × *g* for 1 hr. The supernatant was discarded and the pellet fraction was rehomogenized in 5 ml of NaCl/P_i with 2 mM EDTA/2 mM dithiothreitol/0.1% Triton-X 100. The suspension was centrifuged at 100,000 × *g* for 1 hr. The hemagglutination activity was present in the supernatant. Hemagglutination assays were performed in microtiter-V-plates (Dynatech, Alexandria, VA), using serial 1:2 dilutions of the extract. Each well contained 25 μl of a 4% suspension of sheep erythrocytes prepared by the method of Butler (16), 25 μl of the diluted extract in NaCl/P_i, and 25 μl of 0.01 M NaCl, with or without inhibitors added. The formalinized sheep erythrocytes were added last, after which the plates were shaken vigorously. Agglutination was scored after 90 min. Unagglutinated erythrocytes formed a clear dot on the bottom of the well, whereas agglutinated erythrocytes formed a diffuse mat on the bottom of the well. Agglutination activity was expressed as the reciprocal of the highest dilution giving agglutination. The minimal concentration of glycoprotein that reduced the number of serial dilutions that showed agglutination activity by half was taken to be the concentration that inhibits hemagglutination by 50%.

Preparation of Rosettes. Approximately 100 superficial cells were cultured for 30 min in 5 ml of Holtfreter's solution at room temperature with or without inhibitors. After addition of 0.2 ml of a 10% suspension of formalinized sheep erythrocytes, the petri dish was gently shaken by hand. After 15 min the superficial cells were washed by gently pipetting them into a dish of fresh Holtfreter's solution. The amphibian embryo cells were fixed by treatment with 2% (wt/vol) formaldehyde in Holtfreter's solution and mounted on slides, and the first 50 cells encountered during examination of the preparation were scored for rosette formation. Only cells with three or more erythrocytes adhering to their surface were scored as rosettes.

RESULTS

Scanning Electron Microscopy of Superficial Cells. Scanning electron microscopy of the dissociated superficial cells revealed a sharp boundary between the adhesive and the nonadhesive regions. The nonadhesive (pigmented) portion of the cell surface appears as a contracted knob that is characterized by a convoluted appearance, with few microvilli. The adhesive surface has a much smoother appearance, with numerous microvilli (Fig. 1).

Hemagglutination Activity of Crude Embryonic Extracts. Extracts of the cleavage-stage *R. pipiens* embryos contain hemagglutinating activity against formalinized sheep erythrocytes. This hemagglutination activity is found exclusively in the particulate fraction; however, addition of 2 mM EDTA, 2 mM dithiothreitol, and 0.1% Triton-X-100 to the homogenization buffer results in the solubilization of the lectin activity. The soluble extracts had hemagglutination activities of 256 (320 μg of protein per ml) when activity was measured in the microtiter-V-plate assay.

The Triton-solubilized lectin activity was not blocked by the addition of the simple sugars D-glucose, D-mannose, D-galactose, L-fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, or sialic acid at final concentrations of 100 mM. However, the hemagglutination activity of the soluble extract could be blocked by yeast mannan and a family of glycoproteins containing high concentrations of mannose (Table 1). Mannan was capable of inhibiting 50% of the hemagglutination activity at concentrations as low as 18 μg/ml. Invertase and thyroglobulin required higher concentrations, 28 and 55 μg/ml, respectively, to inhibit 50% of the hemagglutination activity. Glycoproteins containing only complex oligosaccharide units, without terminal mannose, were not effective as inhibitors of hemagglutination. Included in this group of glycoproteins are transferrin, fetuin, ovomucoid, and bovine submaxillary gland mucin (Table 1). The reduced effectiveness of ovalbumin may be a result of the heterogeneity of the oligosaccharide unit. It has been reported that some of the oligosaccharide chains have *N*-acetylglucos-

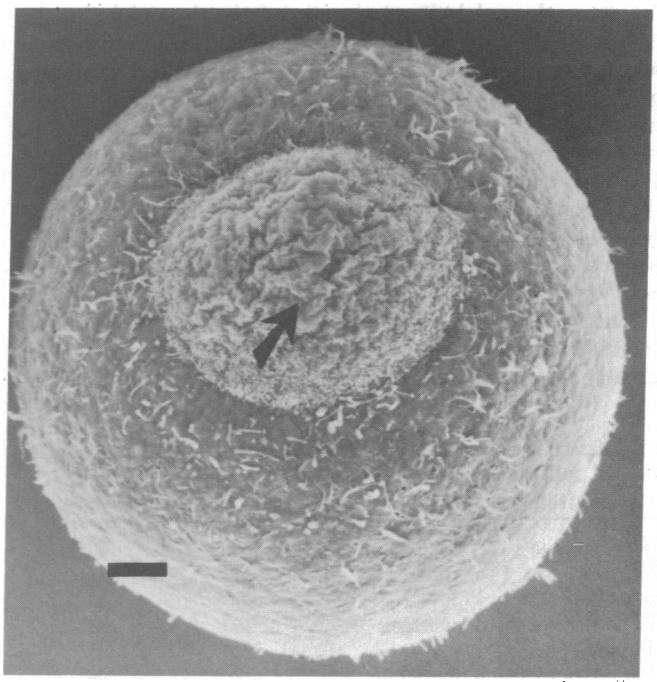

FIG. 1. Scanning electron micrograph of a dissociated superficial cell from an early cleavage stage *R. pipiens* embryo. The arrow points to the highly contracted portion of the cell, which is the pigmented (nonadhesive) region of the cell surface. Bar = 10 μm.

Table 1. Inhibition of hemagglutination activity of crude extracts

Inhibitor	Conc. required to reduce hemagglutination by 50%, μg/ml
Mannan	18
Invertase	28
Thyroglobulin	55
Ovalbumin	750
Fetuin	>3000
Transferrin	>3000
Ovomucoid	>3000
Submaxillary gland mucin	>3000
Periodate-oxidized invertase	1000
Periodate-oxidized thyroglobulin	1000
Unit A from thyroglobulin	1*

* Based on a phenol/sulfuric acid assay (13) with mannose as the standard.

amine at the nonreducing ends, whereas other chains are terminated by mannose (17).

Several lines of evidence indicate that the oligosaccharide rather than the protein component of the inhibitors is responsible for the inhibition of hemagglutination. Heating (100°C for 10 min) of the inhibitors does not significantly change the effectiveness of the inhibitors. Periodate oxidation of invertase and thyroglobulin does decrease their effectiveness. In addition, the oligosaccharide unit A from thyroglobulin, the simple high-mannose containing unit, is an effective inhibitor (Table 1).

The Triton-solubilized lectin could be inactivated by heating at 100°C for 10 min or by treatment with trypsin. The action of trypsin (100 μg/ml, 37°C, 3 hr, pH 7.2) was terminated after incubation with the Triton-solubilized lectin by the addition of excess soybean trypsin inhibitor (1 mg/ml). Addition of deoxyribonuclease I (200 μg/ml) to the assay system had no effect on the hemagglutination activity.

The formalinized sheep erythrocytes are able to form rosettes with 91% of the superficial cells (Table 2). Yeast mannan, yeast invertase, thyroglobulin, and unit A from thyroglobulin are effective inhibitors of the rosetting. Transferrin and bovine submaxillary gland mucin are not effective inhibitors. Periodate oxidation of invertase and thyroglobulin reduces their effectivness as inhibitors of the rosette-mediating component. Heating invertase, thyroglobulin, and unit A for 10 min at 100°C does not reduce the effectiveness of the inhibitors. Thus, the oligosaccharide specificity of the rosette-mediating component on the surfaces of the amphibian cells appears to be the same or similar to that of the Triton-soluble hemagglutination activity.

Preincubation of the superficial cells with mannan or other inhibitors, followed by washing to remove unbound inhibitors, greatly decreases rosette formation, indicating that the rosette-mediating component is associated with the amphibian embryo cell surface rather than the erythrocyte cell surface. Preincubation of the erythrocytes with the inhibitors had no effect on rosette formation.

Table 2. Inhibition of rosette formation

Additions to Holtfreter's solution	Conc., μg/ml	% cells that form rosettes
None	—	91
Mannan	250	15
Invertase	250	21
Periodate-oxidized invertase	250	83
Thyroglobulin	250	16
Periodate-oxidized thyroglobulin	250	71
Unit A from thyroglobulin	50	31
Transferrin	250	86
Submaxillary gland mucin	250	89

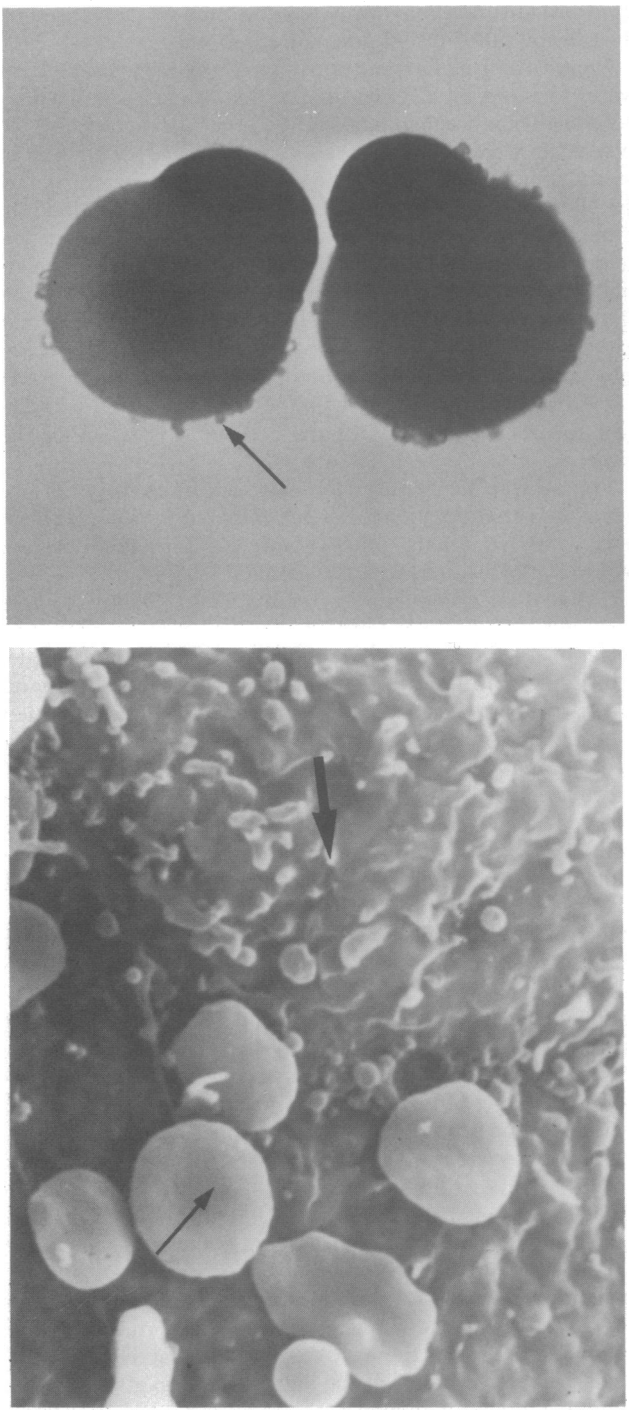

FIG. 2. Rosettes. (*Upper*) Photomicrograph. The arrow points to one of the sheep erythrocytes attached to the surfaces of the dissociated superficial cells. Note that the erythrocytes are almost entirely absent from the pigmented (nonadhesive) portion (one erythrocyte may be associated with the pigmented surface of the right embryonic cell). (×500.) (*Lower*) Scanning electron micrograph. The thin arrow points to an erythrocyte on the lateral surface and the thick arrow points to the pigmented surface, which has no erythrocytes attached to it. (×1125.)

At the stage of embryonic development used, the blastomeres are large, permitting determination of the regional localization of erythrocyte binding. The formalinized sheep erythrocytes

adhere almost solely to the nonpigmented (adhesive) regions of dissociated superficial cells, indicating that the rosette-mediating component is confined to the adhesive regions of the cell surfaces (Fig. 2). Rosettes will also form between sheep erythrocytes and dissociated blastomeres of the 32–64-cell stage amphibian embryo that were deep from the surface of the embryo. These cells lack the nonadhesive (pigmented) portion of the cell surface and bind erythrocytes over the entire cell surface.

DISCUSSION

The interaction of lectins and their endogenous receptors appears to mediate a variety of processes, such as the binding of nitrogen-fixing bacteria to host root cells (6), binding of bacteria to host cells (7), clearance of serum glycoproteins by the liver (18), binding of glycoproteins by macrophages (19), and intercellular adhesion (8–10). Hemagglutinating activity has also been demonstrated to be associated with thrombin-activated platelets (20), chicken embryo tissues (21), and the cell surface and intercellular matrix protein fibronectin (22).

Our results indicate that a lectin is present in crude Triton extracts of early cleavage stage amphibian embryos. The presence of the lectin is demonstrated by the ability of the extracts to agglutinate formalinized sheep erythrocytes. The hemagglutination activity of the extracts can be blocked by the addition of mannan or one of a family of glycoproteins that have oligomannosyl units with mannose in the terminal position. Hemagglutination is not blocked by the addition of glycoproteins that have complex oligosaccharide units without terminal mannose.

The cell surface location of an amphibian embryo carbohydrate-binding component is indicated by the ability of the amphibian embryo cells to form rosettes with the formalinized sheep erythrocytes. Rosette formation can be greatly reduced by the addition of the same inhibitors that were effective in blocking the hemagglutination activity of the soluble extracts, suggesting that the two components might be identical. Only those cell surface regions of the superficial cells that are capable of adhering to other amphibian embryo cells are capable of binding the formalinized sheep erythrocytes. The formalinized sheep erythrocytes fail to bind to the pigmented (nonadhesive) surface of the cells, suggesting that the carbohydrate-binding component is excluded from parts of the cell surface that are incapable of adhesion. We have previously demonstrated that Con A, a lectin specific for D-mannose and D-glucose residues (11), binds only to the adhesive (unpigmented) regions of the surface of the superficial cells (5). The colocalization of the oligomannosyl-recognizing carbohydrate-binding component and its potential receptor oligosaccharide chains to those portions of the cell surface that are adhesive suggests that their interaction may be involved in adhesion of amphibian embryo cells, but our results do not rule out the possibility that the carbohydrate-binding component functions in other recognition capacities. A cell surface carbohydrate-binding component with a similar specificity (i.e., recognition of oligomannosyl residues) has recently been reported to be involved in the cell–cell adhesion of teratocarcinoma stem cells (10). Preliminary experiments that attempted to inhibit cell adhesion with yeast mannan and thyroglobulin were unsuccessful. Because the embryonic blastomeres are large, they are easily broken, making it impossible to use standard rotation-mediated quantitative assays for cell aggregation (23, 24). Instead, a stationary aggregation assay capable only of distinguishing adhesion from nonadhesion was used; subtle effects on adhesiveness could not be detected. In addition, the lack of demonstrable effect of the inhibitors on adhesion might result from the affinity of the cell surface lectin for its natural endogenous receptor being higher than that for the oligosaccharide chains of the inhibitors.

It is now necessary to purify and characterize the lectin. The existence of the purified lectin will facilitate further studies on the precise nature of the relationship between the Con A receptors and the amphibian embryo endogenous lectin and will allow further studies on its role in embryonic development.

The observation that the formalinized sheep erythrocytes form rosettes with only the unpigmented surfaces of dissociated cells further demonstrates that the cells maintain distinct membrane domains even after dissociation of the embryo into single cells. The mechanism that maintains these distinct membrane domains is not known; however, the ability of single cells to maintain them indicates that it is not dependent on cell contact or intact membrane junctions.

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