Isolation of a Cell-Surface Receptor for Chick Neural Retina Adherons

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ABSTRACT Embryonic chick neural retina cells release glycoprotein complexes, termed adherons, into their culture medium. When adsorbed onto the surface of petri dishes, neural retina adherons increase the initial rate of neural retina cell adhesion. In solution they increase the rate of cell-cell aggregation. Cell-cell and adheron-cell adhesions of cultured retina cells are selectively inhibited by heparan-sulfate glycosaminoglycan, but not by chondroitin sulfate or hyaluronic acid, suggesting that a heparan-sulfate proteoglycan may be involved in the adhesion process. We isolated a heparan-sulfate proteoglycan from the growth-conditioned medium of neural retina cells, and prepared an antiserum against it. Monovalent Fab' fragments of these antibodies completely inhibited cell-adheron adhesion, and partially blocked spontaneous cell-cell aggregation. An antigenically and structurally similar heparan-sulfate proteoglycan was isolated from the cell surface. This proteoglycan bound directly to adherons, and when absorbed to plastic, stimulated cell-substratum adhesion. These data suggest that a heparan-sulfate proteoglycan on the surface of chick neural retina cells acted as a receptor for adhesion-mediating glycoprotein complexes (adherons).

Several molecules have been isolated from the chick neural retina that may be involved in the adhesive interactions of this cell population. These include the following: a 50,000-mol-wt protein called cognin that was isolated on the basis of its ability to promote cell aggregation (1); a 10,000-mol-wt protein, ligatin, that inhibits cell-cell adhesions (2, 3); and 130,000- and 140,000-mol-wt proteins, characterized by their ability to neutralize the activity of antisera against the cell surface, that block spontaneous neural retina aggregation (4, 5). More recently, a glycoprotein complex, termed an adheron, has been isolated from the growth-conditioned medium of chick neural retina cells. Adherons directly promote cell-cell and cell-substratum adhesion (6). Finally, a monoclonal antibody against a 170,000-mol-wt protein component of adherons inhibits adheron-cell interactions (7).

Chick neural retina adherons are relatively homogeneous particles with respect to size on sucrose gradients and by electron microscopy. As a population, however, these adherons contain many proteins, perhaps reflecting the fact that the neural retina contains many distinguishable cell types (8). If the adheron particles play a role in determining the physical relationships of cells in the neural retina, then it is likely that individual particles interact with unique molecules on the cell surface to bind cells to extracellular matrix or to each other. In this paper, we describe the isolation and characterization of a cell-surface molecule that binds chick neural retina adherons. The description of a molecule in the adheron that interacts with the cell-surface receptor will be presented elsewhere.

MATERIALS AND METHODS

Cells and Culture: Unless indicated otherwise, neural retina tissue was separated from the pigmented epithelium of 10-d-old leghorn chick embryos and incubated in HEPES-buffered Dulbecco's modified Eagle's medium (DME)[1] with 0.5% (wt/vol) crude trypsin (Nutritional Biochemical Corp., Cleveland, OH) for 20 min at 37°C. For aggregation assays, the cells were then rinsed three times with DMEM containing Spinner salts and 1% newborn calf serum, dispersed by pipetting 15 times, and placed in Spinner culture flasks containing 20 μg/ml of DNase I (Worthington Biochemical Corp., Freehold, NJ). The cells were incubated overnight at 100 rpm on a Bellco Glass multistir apparatus (Bellco Glass, Inc., Vineland, NJ) at 37°C to allow for the recovery of surface molecules damaged by the trypsin.

Preparation of Conditioned Medium and Adherons: We prepared conditioned medium by washing the cells from Spinner cultures three times in serum-free DME and incubating them in serum-free DME for 20 h at 37°C. We prepared adherons by centrifugation of the growth-conditioned medium at 100,000 *g* for 3 h (6). The pellet contained a relatively homogeneous population of 15-nm particles that was used as substrates in the adhesion assays.

[1] *Abbreviations used in this paper:* anti-HSPG, anti-heparan-sulfate proteoglycan Fab'; DME, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; HSPG, heparan-sulfate proteoglycan.

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To prepare adheron-coated dishes, we placed suspensions of particles in 35-mm plastic petri dishes (Falcon Labware, Oxnard, CA) for 18 h at 37°C, and washed the dishes twice with HEPES medium. After the final wash, 2 ml HEPES medium containing 0.2% bovine serum albumin was added.

Adhesion Assays: To assay cell-substratum adhesion, we labeled cells with [^{3}H]leucine (5 μCi/ml) in DME minus calcium, plus 1% newborn calf serum, for 15 h. The cells were washed three times with HEPES medium containing 0.2% albumin, and 0.2-ml aliquots were pipetted into 35-mm petri dishes to which material from growth-conditioned medium had been adsorbed. At indicated times, the dishes were swirled 10 times, and the medium was aspirated; the remaining attached cells were dissolved in 3% Triton X-100, and their isotope content was determined. The data were plotted as the fraction of input cells that adhered as function of time. Variations between duplicates was <5%.

Cell aggregation was measured by the disappearance of single cells from an agitated suspension. Cells were washed twice in HEPES medium and added at 1×10^6 cells/ml to 0.5-ml aliquots of the test medium. The cells were agitated on a rotary shaker (100 rpm) at 37°C, and the disappearance of single cells was monitored with a Coulter counter (Coulter Electronics Inc., Hialeah, FL) (6).

Protein and Glycosaminoglycan Assays: Cells were labeled with [^{35}S]methionine and gel electrophoresis was done in gels containing 15% acrylamide and 0.1% SDS as previously described (9). Glycosaminoglycan (GAG) analysis was carried out by labeling cells with [^{3}H]glucosamine or [^{35}S]sulfate, chromatographing the sample on a DEAE-cellulose column (10), and identifying the GAGs in the individual peaks by enzymatic methods (11). Streptomyces hyaluronidase (B grade) (Calbiochem-Behring Corp., San Diego, CA) was used at 50 U/ml for 24 h at 37°C. Chrondroitinase ABC and AC (Miles Laboratories Inc., Elkhart, IN) were used at 0.5 U/ml for 24 h at 37°C. GAG breakdown was monitored by column chromatography on Sepharose CL6B.

Preparation of Antiserum: Heparan-sulfate proteoglycans (HSPGs) were purified from conditioned medium as described in the text. For each injection, 100 μg of particulate material in saline was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into six spots on the backs of female white New Zealand rabbits. After four 100-μg injections equally spaced over 6 wk, the rabbits were bled and the serum was used in these experiments. IgG was purified by DEAE chromatography (12) and monovalent Fab' fragments were prepared according to Brackenbury et al. (13). Cells were stained for 30 min at room temperature, washed, exposed to the fluorochrome-labeled second antibody for 30 min, washed, and fixed with 95% ethanol (14).

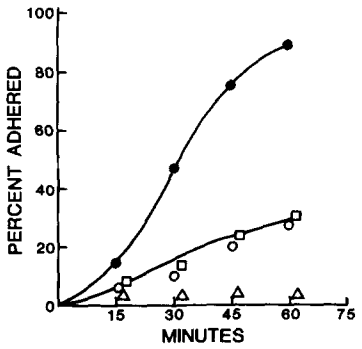

FIGURE 1 Cell-substratum adhesion-mediating material in growth-conditioned medium. Serum-free growth-conditioned medium was prepared from 11-d-old chick neural retina cultures. The conditioned medium was centrifuged for 5 min at 500 *g* to remove cells and at 20,000 *g* for 15 min to remove cell debris. These two initial centrifugations were done in all of the following experiments. Half of the medium was placed on ice, and the other half centrifuged at 100,000 *g* for 3 h, and separated into pellet and supernatant. The pellet was washed once with serum-free medium and resuspended to the original (same) volume as the medium and high speed supernatant. Equal amounts of each were incubated overnight in plastic petri dishes. In one case the pellet and high speed supernatant were recombined before the incubation. Using isotopically labeled material, it was shown that all of the macromolecular components in the culture medium adhered to the plastic. The rate of adhesion of isotopically labeled 10-d-old chick neural retina cells to surfaces coated with the above three fractions was determined. The percent of input cells (inferred from the fraction of isotope in the original cells) adhering to the dishes is plotted as a function of time. Less than 0.4% of the input cells adhered to petri dishes alone after 60 min (data not presented). The difference between duplicate samples was <5%. Adhesion of neural retinal cells to the following: (○) material adsorbed from total conditioned medium; (△) supernatant of growth-conditioned medium after centrifugation at 100,000 *g*. (●) Adhesion to 100,000 *g* pellet; (□) recombined pellet and 100,000-*g* supernatant.

RESULTS

Inhibition of Adhesion

When embryonic chick neural retina cells are incubated overnight in serum-free culture medium, an activity is released from the cells that promotes cell aggregation (15). If the growth-conditioned medium is centrifuged at 100,000 *g* for 3 h, all of the adhesion-promoting activity, defined by its ability to enhance the initial rates of cell aggregation and cell-substratum adhesion, is recovered in the pellet (6). Fig. 1 shows the ability of dissociated neural retina cells to adhere to petri dish surfaces to which material from complete growth-conditioned medium is adsorbed and to surfaces similarly coated with material from the high speed centrifugation pellet and high speed supernatant. These data indicate that there is more adhesion-promoting activity recovered in the pellet than was initially found in the total supernatant. To test the possibility that this is due to an inhibitory activity found in the 100,000 *g* supernatant, we mixed this supernatant with the pellet before adsorption to the petri dish. Fig. 1 shows that the total activity in the 100,000 *g* pellet was reduced to that of the complete growth-conditioned medium, suggesting that an inhibitory substance is present in the original conditioned medium that is not pelleted by high speed centrifugation. When an excess of the 100,000 *g* supernatant is used, adhesion to particle-coated substrata is completely blocked (data not presented).

In addition to the inhibitory activity in the supernatant, it was previously shown that the GAGs, heparin and heparan sulfate, completely block cell-substratum adhesion caused by neural retina adherons, whereas chondroitin sulfate and hyaluronic acid are ineffective (6). Since neural retina cells contain heparan sulfate (6, 16), these data suggest the possibility that a heparan-sulfate proteoglycan is involved in the adhesion process and may act as a cell surface receptor for the neural retina adheron. Since most cell surface molecules are shed into the culture medium (17, 18), it is possible that the inhibitory activity is a cell-surface receptor for the particle that is shed into the growth medium and acts as a monovalent hapten to inhibit adhesion. The following experiments describe the isolation of such a proteoglycan and show that antisera prepared against it alter the adhesion kinetics of neural retinal cells.

Adheron Binding to Cells

The material in the high speed pellet to which the cells bind are adherons (6). These are relatively homogenous 15-nm particles as defined by electron microscopy and sucrose gradient analysis, and they consist of macromolecular complexes of glycoproteins and proteoglycans. To demonstrate that the adheron interacts directly with the cells, we prepared these particles from the growth-conditioned medium of [^{35}S]sulfate-labeled cells, and their interaction with neural retina cells was studied at 0°C. Fig. 2 shows that the particles bind to cells

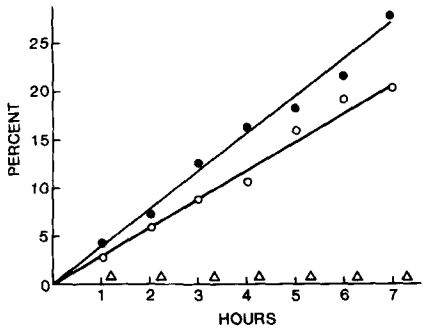

FIGURE 2 Direct binding of adherons to cells. Cells were labeled with $^{35}SO_4$ for 24 h, and the adherons were purified from the growth-conditioned medium by centrifugation at 100,00 *g* for 3 h. The particles (10^5 cpm per tube) were added to suspensions in HEPES medium of neural retina cells that had been dissociated with trypsin and allowed to recover their surface molecules in spinner culture for 16 h. The cells (1×10^6 per tube) were incubated with the particles at 0°C in the presence of the indicated GAGs at 200 μg/ml or growth-conditioned medium high speed supernatant. Samples of cells were periodically centrifuged at 2,000 rpm, washed once with cold medium, and the isotope in the cell pellet determined. Isotope adhered to blank tubes was subtracted from the data to eliminate nonspecific sticking. The data are presented as the percent of input bound. (●) Cells alone; (○) chondroitin-4-sulfate; (△) heparan sulfate. Heparin inhibited as well as heparan sulfate and the 100,000-*g* supernatant, while hyaluronic acid did not alter the adhesion kinetics.

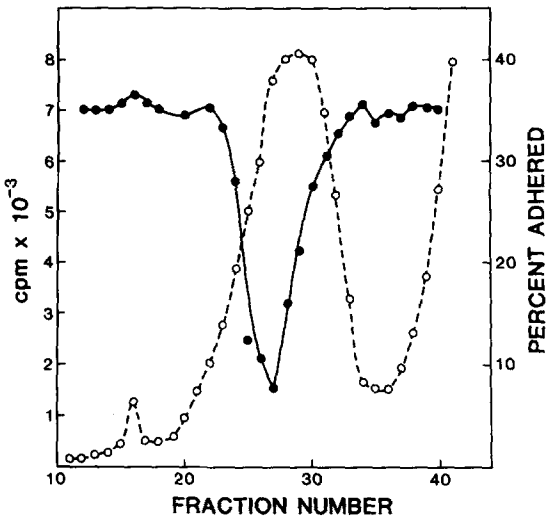

FIGURE 3 Sepharose CL4B chromatography of 100,000-*g* supernatant. Cells were labeled with $^{35}SO_4$ and the growth-conditioned medium prepared as in Fig. 1. The 100,000-*g* supernatant was then lyophilized, dissolved in 4 M guanidine, 0.5 M NaCl, 5×10^{-4} M EGTA (pH 7.2) and applied to a Sepharose CL4B column equilibrated with the same buffer. Each fraction was either counted for radioactivity (○) or dialysed against HEPES medium and placed in petri dishes that contained neural retina adherons adsorbed to their surface. These dishes were used to determine the ability of each fraction to inhibit the adhesion of neural retina cells. The fraction of input cells that adhered at 30 min is plotted (●).

and that this interaction is inhibited by heparin, heparan sulfate, and the 100,000 *g* supernatant of growth-conditioned medium; chondroitin sulfate and hyaluronic acid are less effective. The paragraphs below describe the purification of the inhibitory activity in the growth-conditioned medium and show that it is a HSPG.

Isolation of Inhibitory Activity

To test the possibility that the inhibitory activity in the culture supernatant is a HSPG, we labeled cells in serum-free medium for 20 h with $^{35}SO_4$, and we prepared and lyophilized the 100,000 *g* supernatant of the conditioned medium. The lyophylized material was dissolved in 4 M guanidine HCl, 0.5 M NaCl, 5×10^{-4} M EGTA (pH 7.2), and applied to a Sepharose 4B column equilibrated in the same buffer. The column fractions were assayed for radioactivity and for their ability to inhibit the adhesion of neural retina cells to adheron-coated substrata. Fig. 3 shows that the inhibitory activity migrated with the leading edge of an included peak of sulfate-labeled material. No inhibitory activity was associated with the excluded or the totally included volumes of the column. The fractions containing the inhibitory activity were pooled and further analysed by chromatography on a DEAE column. Fig. 4 shows that the material from the Sepharose 4B column separates into three peaks of radioactivity on the ion exchange column, and that the biological activity is associated with the second peak to elute from the column (II). An analysis of the two major sulfate-labeled peaks showed that this second peak is a HSPG, for when the material is digested with pronase and re-run on DEAE columns the GAGs co-migrate with heparan sulfate (Fig. 4*B*). These GAGs are degraded by nitrous acid but not by chondroitinase ABC, establishing that they are heparan sulfate. The third peak (Fig. 4*A*, III) is a chondroitin-sulfate proteoglycan, as its GAGs are degraded

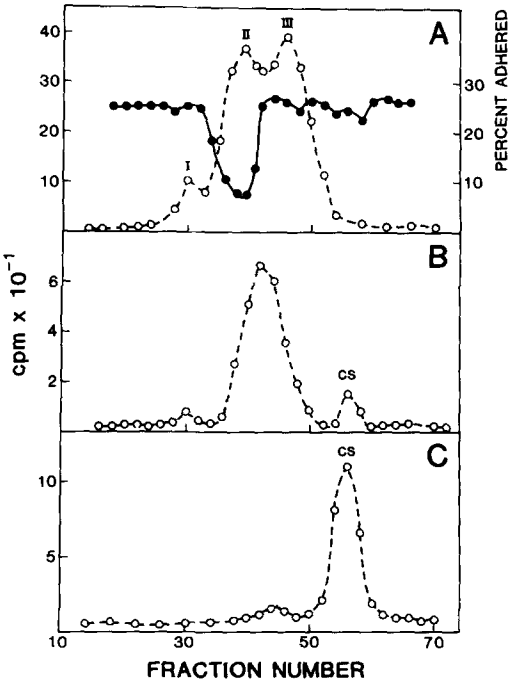

FIGURE 4 Ion exchange chromatography of activity from Sepharose CL4B. The fractions that contained the inhibitory activity on the Sepharose 4B column (Fig. 3, fractions 24–30) were dialysed against starting buffer and rechromatographed on a DE52 column. Each column fraction was assayed for radioactivity and biological activity as described in Fig. 3. (*A*) DE52 chromatography of proteoglycans (●) Biological activity. (○) ^{35}S-sulfate. (*B*) Peak II of proteoglycan column (see *A*) digested with pronase and rechromatographed on DE52. (*C*) Peak III of proteoglycan column (see *A*) digested with pronase and chromatographed on DE52.

by chrondroitinase ABC but not by nitrous acid. Material in this peak co-migrated with authentic chondroitin sulfate on a DEAE column (Fig. 4*C*[CS]). These data, in conjunction with those showing that heparan sulfate is a potent inhibitor of cell-substratum adhesion, suggest that a HSPG is involved in adheron-mediated adhesion. To examine this further, we purified the cell-surface HSPG, and observed that this molecule can directly stimulate adhesion.

Isolation and Characterization of Cell-surface Proteoglycan

The cell-surface HSPG was purified from $^{35}SO_4$-labeled cells by initially chromatographing cell lysates on Sepharose 4B in guanidine HCl, followed by resolving the proteoglycans on a DEAE column. As in Fig. 4*A*, two proteoglycans were resolved on the ion exchange column. One was a HSPG as determined by rechromatography of the pronase-digested proteoglycan on DEAE and subsequent chemical and enzymatic analysis as described for the secreted proteoglycan. The second was a chondroitin-sulfate proteoglycan as determined by the same criteria (data not presented).

Since it is likely that reciprocal adhesive interactions take place between cells or between cells and cell-surface-bound adherons, the ability of the purified cell-surface molecule to stimulate adhesion was determined. Two assays were employed, the stimulation of cell-substratum adhesion and a bead assay. When the purified proteoglycan is adsorbed to the surface of petri dishes, it stimulates cell-substratum adhesion; the chondroitin-sulfate proteoglycan is ineffective (Fig. 5). Like cell-adheron adhesion, cell-proteoglycan adhesion was inhibited by heparin and heparan sulfate, but not by chondroitin sulfate or hyaluronic acid. Through the use of isotopically labeled proteoglycan, it was shown that all of the input material was adsorbed to the petri dish surface and that it was not released by heparan sulfate. The HSPG isolated from growth-conditioned medium also stimulates cell-substratum adhesion when adsorbed to the surface of petri dishes. It was, however, not completely adsorbed after an 18-h incubation, and it gave variable results with respect to adhesion kinetics.

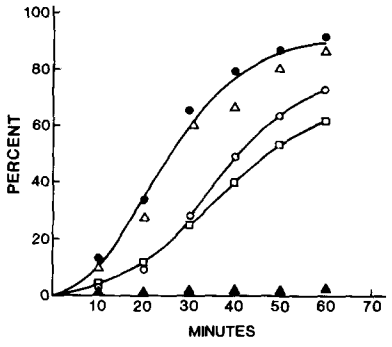

FIGURE 5 Proteoglycan-stimulated adhesion of neural retina cells. Retina cells were labeled for 20 h with $^{35}SO_4$ and the cellular HSPG was prepared as described in Fig. 4. The proteoglycan was adsorbed to the surface of petri dish by overnight incubation at 37°C in HEPES medium. Greater than 90% of the 5 μg of protein per 35-mm dish adhered. Adhesion of dissociated neural retina cells was carried out with or without the indicated GAGs at 200 μg per dish as described in Fig. 1. (●) Control; (△) hyaluronic acid; (○) chondroitin-4-sulfate; (□) chrondroitin-6-sulfate; (▲) heparin or heparan sulfate.

HSPG-adheron adhesion could be reconstituted by coupling the proteoglycan to agarose beads that are ~100 μm in diameter, and adsorbing the adherons to negatively charged latex beads that are ~3 μm in diameter. The beads were mixed in various combinations for 6 h at 0°C, and the number of small beads associated with the circumference of the agarose beads was counted under the phase-contrast microscope. Table I shows that beads containing adheron or the cell-surface proteoglycan interacted with each other in a manner similar to the cell-substratum and particle-cell interactions. Again, heparin and heparan sulfate inhibited the adhesive interactions, whereas hyaluronic acid and chondroitin sulfate were less effective.

Effect of Anti-HSPG on Adhesion

To further test the role of the HSPG in the adhesive interactions, we made antisera in rabbits against the proteoglycan purified from the growth-conditioned medium of chick neural retina cells. This antiserum precipitated HSPG, as determined by column chromatography on DE52, from both growth-conditioned medium and the cell surface; it did not precipitate additional metabolically labeled proteins as assayed on SDS acrylamide gels. Monovalent Fab' fragments were prepared from this anti-serum and the pre-immune serum of the same rabbit. The ability of these monovalent antibodies to affect cell-substratum adhesion and spontaneous cell aggregation were then assayed. Fig. 6 shows that the rabbit antiserum completely blocked the adhesion of neural retina cells to neural retina adheron-coated substrata. As positive and negative controls, chondroitin sulfate did not alter the adhesion kinetics, whereas heparan sulfate was inhibitory. Neither the antibody nor heparan sulfate detached adsorbed adherons as determined by the use of isotopically labeled particles. In contrast to cell-substratum adhesion, the Fab' only inhibited spontaneous aggregation by ~40%, even at concentrations up to 4 mg/ml (Fig. 7). When we tested the ability of heparin and heparan sulfate to inhibit aggregation, both GAGs inhibited the spontaneous aggregation of the cells to a similar extent as the antibody. Hyaluronic acid and chondroitin sulfate were again ineffective.

TABLE I
Reconstitution of Adhesion System

Sepharose beads	Latex beads	Medium	Percent
Nothing	Nothing	—	0
HSPG	Nothing	—	0
Nothing	Adheron	—	0
HSPG	Adheron	—	100
HSPG	Adheron	Heparan sulfate	6
HSPG	Adheron	Chondroitin sulfate	91
HSPG	Adheron	Hyaluronic acid	97
HSPG	Adheron	*NRS Fab'	87
HSPG	Adheron	anti-HSPG Fab'	7

Cell-surface HSPG was covalently coupled to CNBr-activated Sepharose 4B and the adherons adsorbed to carboxylated latex beads. The various combinations were mixed and gently agitated in HEPES medium for 6 h at 0°C. The beads were then diluted and the number of latex beads on the circumference of the Sepharose beads determined on at least 100 beads by examination with phase-contrast optics. The HSPG-adheron pair were taken as maximum rosetting (27+/−8 latex beads per large bead), and the data for other conditions are presented as a percent of this number. The GAGs were used at 10^{-3}M with respect to uronic acid and the Fab' at 2 mg/ml.
* NRS, normal rabbit serum Fab'.

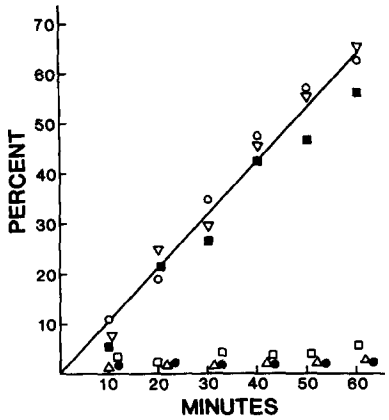

FIGURE 6 Anti-HSPG inhibition of adheron substratum-cell adhesion. Antisera were prepared against the extracellular proteoglycan and monovalent Fab' fragments prepared as described in Materials and Methods. The cells were incubated in HEPES buffer containing 0.2% BSA and 2 mg/ml of antibody at 0°C for 1 h and then diluted directly into the neural retina adheron-coated culture dishes, and adhesion was determined as described in Fig. 1. In some cases GAGs (200 μg/ml) were added to the incubation mixture. (○) Control (no added antibody); (▽) normal rabbit serum Fab'; (●) petri dish alone (no adsorbed adheron); (△) anti-HSPG Fab'; (□) heparan sulfate; (■) chondroitin sulfate.

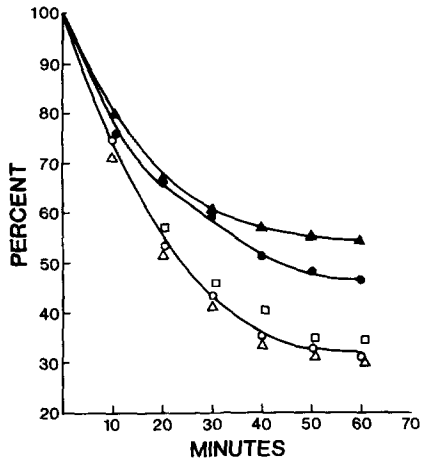

FIGURE 7 Anti-HSPG- and GAG-inhibition of spontaneous neural retina aggregation. Antisera and GAGs were used as described in Fig. 6. Cells were incubated for 1 h at 0°C with 2 mg/ml of Fab', washed once at 0°C, and then diluted to a final concentration of 2 × 10^6 cells/ml and placed on a rotary shaker at 37°C. Aggregation was assayed by measuring the disappearance of single cells with a Coulter counter. The data are plotted as the percent of single cells remaining as a function of time. In some cases cells were not exposed to antiserum but diluted directly into media containing GAGs. (○) Control; (▲) anti-HSPG; (●) heparan sulfate; (△) hyaluronic acid; (□) chrondroitin-4-sulfate.

The observation that the antiserum against the HSPG only partially inhibits spontaneous cell aggregation suggests that it distinguishes at least two populations of cells within the dissociated neural retina. Therefore, dissociated cells were allowed to adhere to polylysine-coated substrata and stained with the anti-HSPG antiserum. Fig. 8 shows that ~40% of the cells became stained with the antiproteoglycan; the preblead serum stained none of the cells. These data indicate that about one-third of the dissociated cell population has a detectably higher surface density of HSPG than the remaining cells.

The Specificity of Adhesions

To assay the specificity of the adhesive interactions that are mediated by the cell-surface HSPG, we assayed adhesion to plastic (sulfonated) tissue culture dishes in the presence and absence of the anti-HSPG and several exogenous glycosaminoglycans. Fig. 9 shows that, unlike adhesion to neural retina adherons, hyaluronic acid, heparan sulfate, and chondroitin-4-sulfate all inhibit cell-tissue culture plastic adhesion to about the same extent. The monovalent antibody against the proteoglycan is ineffective. These data show that cells use alternative adhesion mechanisms when presented with different substrata. The heparan-sulfate moiety is not rate-limiting in the adhesion of cells to tissue culture plastic.

DISCUSSION

The above data suggest that a cell-surface HSPG is able to interact with neural retina adherons to promote cell-substratum and cell-cell adhesion. This conclusion is based upon the following observations. (*a*) Exogenous heparan sulfate acts as a hapten to inhibit cell aggregation and the adhesion of cells to substrata coated with adherons. Heparan sulfate also blocks the direct binding of adherons to neural retina cells. (*b*) A HSPG isolated from growth-conditioned medium inhibits cell-substratum adhesion when present in the assay medium. (*c*) A similar proteoglycan, purified from neural retina cells, promotes cell-substratum adhesion when adsorbed to the surface of a petri dish. (*d*) When bound to Sepharose beads, the cellular proteoglycan causes the adhesion of neural retina adherons. (*e*) Monovalent Fab' fragments of an antiserum against the extracellular HSPG inhibit cell-substratum adhesion and partially block spontaneous aggregation.

HSPG may exist as an integral membrane protein, as a component of the extracellular matrix, or in a soluble form (19, 20). In the case of liver cells, the extracellular form appears to be derived from the membrane via the cleavage of the hydrophobic portion of the core protein that anchors the proteoglycan in the membrane (19). Most extracellular glycoproteins are derived in a similar manner from the plasma membrane (17, 18). All these forms may play a role in cellular adhesion, for HSPGs have been implicated in the cell-substratum adhesion of fibroblastlike cells (21) and the adhesion of sympathetic neurons to smooth muscle cells (22). More recently, it was demonstrated that exogenous heparinlike GAGs inhibit the motility of smooth muscle cells, while the other GAGs are ineffective (23). These data suggest that HSPGs play a role in the adhesive interactions of many cell types.

Since cells bind to both adheron-coated and cell-derived HSPG-coated surfaces, and since the reconstitution experiments described in Table I show that the proteoglycan can interact directly with adherons, it follows that both the cell surface and the adherons must have receptors capable of recognizing the proteoglycan. If there are multiple binding sites within the particle, the particles could cross-link cells and lead to their aggregation. Alternatively, cell-surface-bound particles may interact directly with each other to effect cell-cell adhesion. Although the latter mechanism is clearly involved in myoblast adhesion (24), neural retina adhesion is more complex, for purified neural retina adherons do not spontaneously aggregate (6). Therefore, although reciprocal interactions between adherons bound to the surface of two

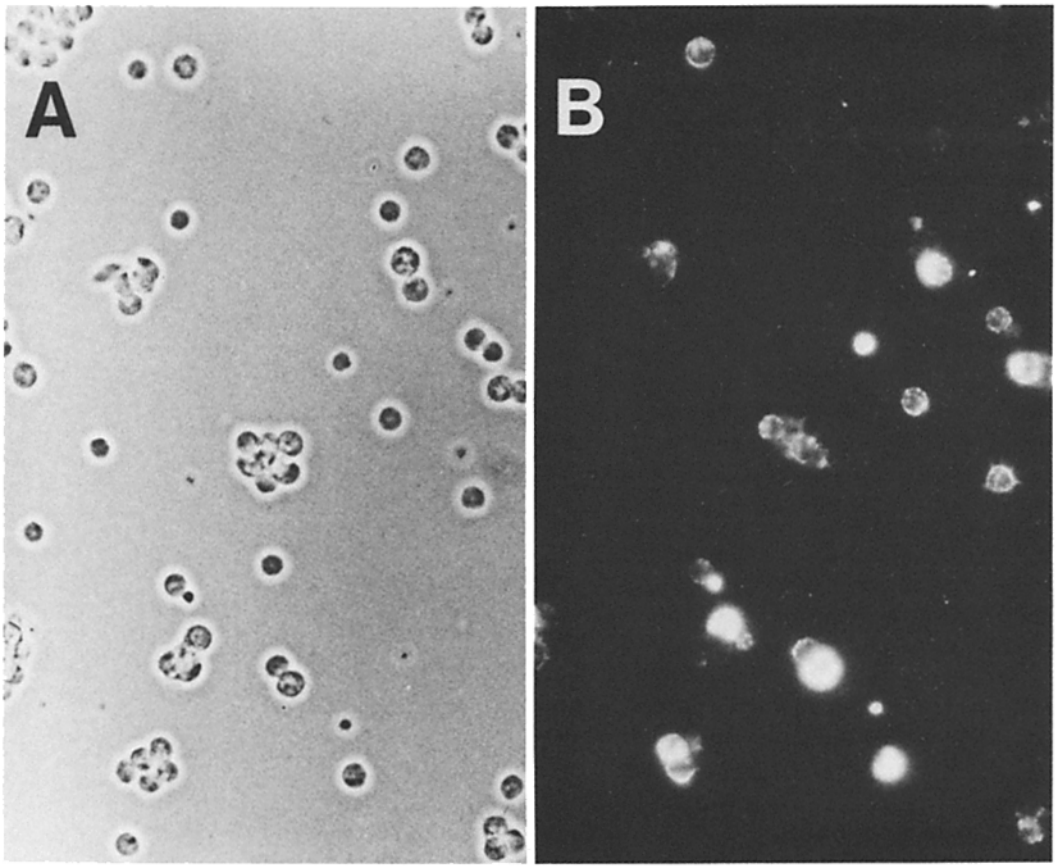

FIGURE 8 Immunofluorescence detection of proteoglycan antigen on dissociated cells. Cells were dissociated with trypsin from 11-d-old embryonic neural retina, placed overnight in spinner culture, and cultured on polylysine-coated substrata for 5 h. They were then treated either with rabbit anti-HSPG or pre-injection serum, followed by fluorescein goat anti-rabbit antibody. The prebleed antibody did not stain. (*A*) Phase-contrast photomicrograph. (*B*) Fluorescent stain. Average cell diameter, 15 μm.

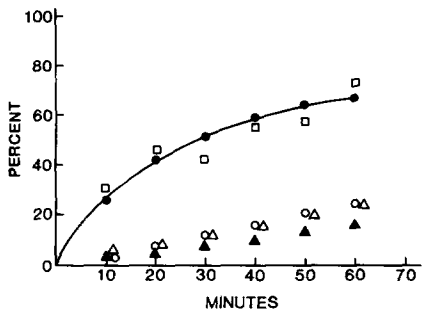

FIGURE 9 Specificity of substratum adhesion. Dissociated neural retina cells were isotopically labeled and their adhesion to plastic tissue culture dishes assayed as described in Fig. 1. In some cases the cells were treated with 4 mg/ml rabbit anti-heparan sulfate Fab' fragments as described in Fig. 7. The GAGs were used at 200 μg/ml. (●) control (no additive); (□) anti-proteoglycan (normal rabbit serum Fab' gave similar results); (○) hyaluronic acid; (△) chondroitin-4-sulfate; (▲) HSPG.

cells are possible, it is more likely that individual adherons are multivalent with respect to proteoglycan binding. Since the proteoglycan-cell adhesion is inhibited by exogenous heparan sulfate but not by chondroitin sulfate, it follows that proteoglycan-cell binding is not simply a function of high charge density, for chondroitin sulfate is more highly charged than heparan sulfate.

Exogenous heparan sulfate and monovalent Fab' fragments against the proteoglycan block particle-cell adhesion, but only partially block spontaneous cell aggregation (Figs. 6 and 7). Staining of dissociated cell cultures with the anti-HSPG shows that only ~40% of the cells have a sufficiently high density of receptor to detect (Fig. 8). These data suggest that lower surface densities of the HSPG can mediate cell-substratum adhesion than can initiate cell-cell interactions in short term aggregation assays. Since both heparan sulfate and monovalent antisera inhibit aggregation to similar extents, it is unlikely that these activities reflect nonspecific steric hindrances. The inhibition may be a reflection of either the density, distribution, or mobility of the molecules in the membrane.

Neural retina cells do not adhere to adherons from skeletal muscle, indicating that there is some inherent specificity in adheron-mediated adhesion (6). Neural retina cells also appear to use different or overlapping mechanisms when interacting with different substrata. For example, the adhesion of neural retina cells to plastic tissue culture dishes, which are more highly sulfonated than the nonadhesive petri dishes used in adheron-induced binding assays, is blocked by heparan sulfate in a manner analogous to the adhesion of cells to adheron-coated substrata (Fig. 9). However, the tissue culture dish-cell interaction is also inhibited by hyaluronic acid and chondroitin sulfate at concentrations that do not alter the adhesion to adherons. In addition, a monovalent antiserum against the HSPG does not inhibit adhesion to tissue culture plastic. These data suggest that a less specific set of electrostatic interactions may mediate the adhesion to charged plastic

surfaces. The HSPG may be involved, but other proteoglycans play a role as well. This multiplicity of interactions may explain the large numbers of molecules that have been extracted from neural retina tissue and are thought to be involved in the adhesion of these cells.

There are at least 50 distinguishable cell types within the neural retina, including several morphologically distinguishable nerve and glial cell types, and many subsets of neurons defined on the basis of neurotransmitter content (25). Since the cytoarchitecture of the neural retina is both ordered and complex, it is highly unlikely that a single molecule is responsible for its assembly. In addition to the proteoglycan described here, at least five molecules thought to be involved in this process have been purified (1–5, 7). A number of independent adhesion mechanisms for neural retina cells have also been described based on ion and proteolytic sensitivities (26). Assuming that all of the various in vitro assays employed in the isolation of these molecules are valid, is the multiplicity of adhesion-mediating molecules within one tissue predictable?

According to the elegant work of Steinberg (27), an individual molecule could be uniquely responsible for the proper sorting of cells in a complex tissue such as the neural retina. Since cells sort out according to their relative affinities for each other, it is possible to define a hierarchy of adhesive affinities by varying either the surface density or surface distribution of one molecule (27). This model makes three predictions with respect to the neural retina: (*a*) All cells have the antigen; (*b*) its surface density on the various cell types must be different; and (*c*) monovalent antibodies against the antigen should inhibit adhesion completely in an aggregation assay. In contrast, if different molecules expressed on the surfaces of different cell types were rate-limiting in the developmental adhesive interactions, it is likely that (*a*) only a subset of cells in the retina would have the antigen, and (*b*) monovalent antibodies should only partially inhibit aggregation of the whole population. On the basis of published data, it is quite likely that more than one molecule is involved in the assembly of the neural retina.

The adhesion-mediating activities in the neural retina are described on the basis of several assays, but the most common assay is the inhibition of spontaneous aggregation by a monovalent antibody. Antisera against cognin, N-CAM, and a 130,000-mol-wt cell-surface component inhibit most spontaneous neural retina aggregation over a period of 1 h (4, 5, 28). In contrast, anti-HSPG is only partially effective in inhibiting spontaneous aggregation, and the antigen is most prevalent on only a fraction of the neural retinal cells. There are, however, no published data showing that any of these proteins are differentially expressed within the developing neural retina population, although the amount or activity of the antigens varies with embryonic age (6, 28, 29). These observations suggest that there is a set of molecules involved in the overall, relatively nonspecific cell-cell interactions of most of the cell types within this tissue, and a subset that is rate-limiting in the adhesion of smaller groups of cells. In addition, the molecules involved in cell-cell or cell-substratum (matrix) adhesion may be the same or distinct.

There are a number of models that can be used to describe adhesion. The two simplest are the interaction between two "receptors," R_1-R_2 (a situation analogous to antigen-antibody binding), and the bridging of similar receptors by another ligand, R_1-L-R_1 (two antibodies and a multivalent antigen). In the neural retina and probably most other systems, neither model appears to be sufficient, for specific antibodies against several different antigens block adhesion. This could be explained by three basic mechanisms. (*a*) Only one determinant is directly involved in adhesion, but a large number of others are clustered around it such that antibodies against these neighboring determinants are sufficient to sterically inhibit ligand binding to the receptor and thus inhibit cellular adhesion. The inhibition of ligand binding by antibodies bound to different abundant surface antigens has been well-documented in the immune system (30). Even extended monovalent Fab′ fragments are large (6 × 4 nm) relative to neural retina adherons (15-nm in diameter) (31). This alternative is compatible with the observations that relatively few homogenous molecules (cognin, a 170,000-mol-wt adheron protein, and a proteoglycan [7, 28]) have been found that directly stimulate cell adhesion, whereas antibodies against at least five defined antigens can block it. (*b*) Adhesion is a multistep process involving the sequential intervention of multiple surface components in addition to the ligand (L) binding to the receptor (R), (L-R→A→B→C). In this case, the L-R interaction is sufficient to initiate adhesion, but antibodies against A, B, or C may block adhesion even though these molecules are not involved in the initial binding event. Inhibition of adhesion of a subpopulation of neural retina cells could be explained if the inhibiting antibody were against a unique receptor or against a component of one of the subsequent steps found only within that subpopulation. This model is compatible with the observation that adhesion is a relatively slow, energy-dependent process. The mechanism may be either an allosteric change within one complex or a series of sequential changes analogous to a biosynthetic pathway. For example, fibronectin requires "activation" through ligation with collagen and/or proteoglycans before it is maximally effective (32). Adherons may be such an activated complex of macromolecules (33). (*c*) Adhesion is a multisite process involving the sequential or simultaneous intervention of multiple surface receptors with different ligands (L_1-R_1→L_2-R_2→L_3-R_3). If all of the reactions are required for effective adhesion, the antisera against any component of the system may block adhesion. For example, fibronectin contains multiple sites for binding to the cell, collagen, and proteoglycans, all of which may be involved in adhesion (34). An example of a sequential process is the possible requirement of cell surface receptors to migrate in the membrane to the sites of adhesive contact (35). It follows that the observation that an antibody blocks adhesion does not prove that its antigen is the unique "adhesion molecule." Necessary and sufficient criteria should be that the molecule either stimulates (if multivalent or on a neutral substratum) or inhibits (if in excess in solution) adhesion and that antibodies against it block adhesion.

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