

Isolation of an Adhesion-mediating Protein from Chick Neural Retina Adherons

DAVID SCHUBERT and MONIQUE LACORBIERE

The Salk Institute, San Diego, California 92138

ABSTRACT Adherons are high molecular weight glycoprotein complexes which are released into the growth medium of cultured cells. They mediate the adhesive interactions of many cell types, including those of embryonic chick neural retina. The cell surface receptor for chick neural retina adherons has been purified, and shown to be a heparan sulfate proteoglycan (Schubert, D., and M. LaCorbiere, 1985, *J. Cell Biol.*, 100:56-63). This paper describes the isolation and characterization of a protein in neural retina adherons which interacts specifically with the cell surface receptor. The 20,000-mol-wt protein, called retinal purpurin (RP), stimulates neural retina cell-substratum adhesion and prolongs the survival of neural retina cells in culture. The RP protein interacts with heparin and heparan sulfate, but not with other glycosaminoglycans. Monovalent antibodies against RP inhibit RP-cell adhesion as well as adhesion-cell interactions. The RP protein is found in neural retina, but not in other tissues such as brain and muscle. These data suggest that RP plays a role in both the survival and adhesive interactions of neural retina cells.

A variety of proteins and a proteoglycan have been described which may be involved in the adhesive interactions of chick neural retina cells (1-7). At least two of these macromolecules, a 170,000-mol-wt protein (6) and a heparan sulfate proteoglycan (7), are involved in the interaction between cells and extracellular glycoprotein complexes, termed adherons, which have been isolated from the growth-conditioned medium of chick neural retina cells (6, 8, 9). Adherons are 15-20-nm particles composed of proteins and proteoglycans (8, 10, 11). They directly promote cell-cell and cell-substratum adhesion in many experimental systems and they may also serve as subunits for basement membrane assembly (10).

During the initial characterization of adherons from chick neural retina cells, it was shown that the glycosaminoglycans (GAGs)¹ heparin and heparan sulfate partially inhibit cell aggregation and completely block cell adhesion to adhesion-coated substrata; chondroitin sulfate and hyaluronic acid are ineffective (8). On the basis of this observation, a heparan sulfate proteoglycan was isolated from the surface of neural retina cells and was shown to be the cellular receptor for the adhesion-promoting particles (7). The next step in detailing the mechanisms involved in adhesion-mediated adhesion is the isolation of the material in the neural retina particles

which is responsible for the binding of the adherons to the cell surface heparan sulfate proteoglycan. The following paragraphs show that a 20,000-mol-wt protein component of neural retina adherons is able to selectively bind heparan sulfate proteoglycan, cause cell-substratum adhesion, and also prolong the survival of neural retinal cells in culture.

MATERIALS AND METHODS

Cells and Culture: Unless otherwise indicated, neural retina tissue was separated from the pigmented epithelium of 10-d leghorn chick embryos and incubated in HEPES-buffered Dulbecco's modified Eagle's medium (DME) with 0.5% (wt/vol) crude trypsin (Nutritional Biochemical Corp., Cleveland, OH) for 20 min at 37°C. The cells were then rinsed three times with DME containing Spinner salts and 1% newborn calf serum, dispersed by pipetting 15 times, and placed in Spinner culture flasks containing 20 µg/ml of deoxyribonuclease I (DNase, Worthington Biochemical Co., 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. Primary cultures from optic lobe, brain, heart, liver, and skeletal muscle were prepared in the same manner, except that they were plated directly into tissue culture dishes containing DME plus 10% newborn calf serum and incubated overnight.

Preparation of Conditioned Medium and Adherons: Conditioned medium was prepared 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. Adherons were prepared by centrifugation of the growth-conditioned medium at 100,000 g for 3 h (8). The washed pellet contained a relatively homogeneous population of 15-nm particles which was used as

¹ Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; RP, retinal purpurin.

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

Adhesion Assays: To assay cell-substratum adhesion, cells were labeled with [³H]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% bovine serum albumin (BSA) (Calbiochem-Behring Corp., San Diego, CA), and 0.2-ml aliquots were pipetted into 35-mm petri dishes to which material from growth-conditioned medium had been adsorbed. At the indicated times, the dishes were swirled 10 times, the medium was aspirated, and 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. Variation between duplicates was <5%.

Protein Assays and Blotting: Cells were labeled with [³⁵S]methionine and gel electrophoresis was done in gels containing 15% acrylamide and 0.1% SDS as described (12). Silver staining was done according to the protocol described by Wray et al. (13). In some cases after gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper using a blotting chamber and a buffer consisting of 25 mM Trizma Base, 192 mM glycine, and 20% methanol. After transfer, the cellulose nitrate was washed three times with 150 mM KCl, 10 mM imidazole, 5 mM MgCl₂, 0.3% Tween 20, and 0.1% CaCl₂, pH 7.3, containing 5% BSA. The nitrocellulose was incubated overnight with 1 × 10⁷ cpm of ³⁵S-SO₄ cell surface heparan sulfate proteoglycan or a 1:1,000 dilution of immune serum at room temperature. The strips containing the proteoglycan were washed three times for 2 min with the incubation buffer minus BSA and dried. The gels with antibody were washed for 2 h in incubation buffer minus BSA, incubated for 2 h with 1 × 10⁶ cpm of ¹²⁵I-protein A (New England Nuclear, Boston, MA), and extensively washed before drying and autoradiography.

Preparation of Antiserum: The 20,000-mol-wt heparan binding protein was purified from conditioned medium as described in the text. For each injection, the protein from the neural retina culture supernatants of 36 10-d chick embryos (~2 μg) was mixed with complete Freund's adjuvant, and injected subcutaneously into six spots on the backs of a female white New Zealand rabbit. After four injections equally spaced over 6 wk, the rabbits were bled and the serum was used in these experiments. IgG was purified by DEAE chromatography (14) and monovalent Fab' fragments were prepared according to the procedures of Brackenbury et al. (15).

RESULTS

Isolation of Cell Binding Activity

Neural retina adherons are composed of at least two proteoglycans, hyaluronic acid, and numerous proteins (8). One or more of these components must be involved in the binding of the particles to the cell surface receptor proteoglycan. The isolation of these molecules is dependent upon devising conditions in which the particles can be dissociated and yet maintain the cell and heparan sulfate binding activity. Since it is unlikely that proteoglycans mediate the binding of adherons to heparan sulfate, conditions were used which separated the large proteoglycans from smaller proteins under dissociating conditions. Initial experiments showed that the adhesion-promoting activity in adherons is stable in 4 M guanidine HCl and that the proteoglycans can be separated from a low molecular weight heparan sulfate binding activity by chromatographing adherons on Sepharose CL4B in 4 M guanidine HCl. For better resolution, neural retina cells were labeled with [³H]leucine and [³⁵S]sulfate. Their adherons were isolated, dissolved in 4 M guanidine HCl, and chromatographed on a Sephadex G100 column in 4 M guanidine HCl. An equivalent amount of adherons was dissolved in 4 M guanidine HCl and refrigerated. Two peaks of radioactivity and optical density are resolved by the G100 column (Fig. 1). All of the sulfate-labeled proteoglycans elute with the void volume along with the majority of the protein. All of the material in the three indicated areas from the column were pooled, di-

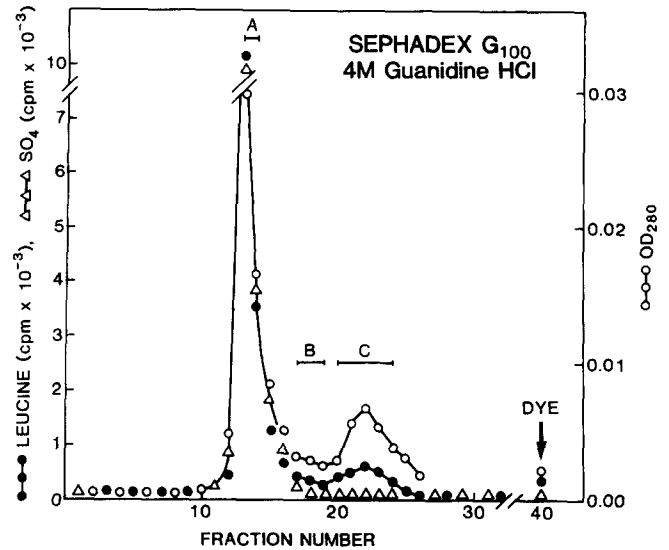


FIGURE 1 Separation of neural retina adheron components on Sephadex G100. Cultured 10-d embryonic neural retina cells were labeled in serum-free medium with [³H]leucine and [³⁵S]sulfate for 18 h. Adherons were prepared from the growth-conditioned medium and 200 μg of the isotopically labeled particles were dissolved in 1 ml of 4 M guanidine HCl, 0.5 M NaCl, and 5 × 10⁻⁴ M EGTA, pH 7. The samples were applied to a Sephadex G100 column at 4°C and fractions collected. The isotope in each sample was determined and the remainder used for adhesion assays (see Fig. 2). O, optical density at 280 nm; ●, [³H]leucine; Δ, [³⁵S]sulfate.

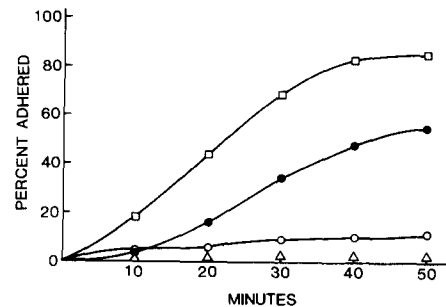


FIGURE 2 Cell-substratum adhesion. Column fractions containing all of the material in peaks A, B, and C (Fig. 1) were separately pooled. These fractions, and an amount of adheron (200 μg dissolved in 4 M guanidine) equivalent to that applied to the column, were dialyzed briefly against water, each diluted 1:10 into HEPES-buffered medium on 35-mm petri dishes, and incubated overnight at 37°C. Greater than 90% of the input protein from all three fractions and 83% of the proteoglycan (Fig. 1A) adsorbed to the substratum as determined by the recovery of the isotopically labeled material in the supernatant. The adhesion of 10-d embryonic neural retina cells to these surfaces was done as described in Materials and Methods. O, petri alone; Δ, Pools A and B from Fig. 1; ●, adheron; □, Pool C from Fig. 1.

alyzed, and 10% of each peak adsorbed to the surface of petri dishes. In addition, an amount of adheron equal to 10% of that loaded onto the guanidine HCl column was adsorbed to dishes, and the ability of each to stimulate cellular adhesion then assayed. Fig. 2 shows that the material in the void volume is unable to promote adhesion, whereas that from peak C is stimulatory. Since only one column fraction stimulated adhesion, it was possible to compare the activity in C with that of the complete particle. Slightly more than 100% of the adhesion-promoting activity is found in column fraction C than is

in the complete particle. There are several reasons why the biological activity of a fraction can be greater than that of the whole, but the most likely alternative is that the active elements in fraction C are less active when bound to (inhibitory) material in fraction A. This alternative was supported when the contents of each fraction were examined.

An analysis of the column fractions shown in Fig. 1 indicated that the void volume contains all of the GAGs, including hyaluronic acid; the protein distribution among the three pools is shown in Fig. 3. On the right hand side of each pair of gels is the autoradiograph of [³⁵S]methionine-labeled protein, and on the left hand side the silver-stained protein from the same sample. These gels show that while radioactive and stained proteins are qualitatively similar with respect to most bands, there are large quantitative differences throughout the gels and a striking difference in the low molecular weight material. The column peak with the adhesion-promoting activity contains a single protein which is labeled with [³⁵S]methionine (Fig. 3C [R, band 1]) and which stains bright purple with the silver method. The majority of the protein mass, as defined by silver staining, is, however, in proteins between the 12,000 and 15,000 molecular weight range (Fig. 3C [S, bands 2-4]). The following observations suggest that these proteins are histones which are released into the extracellular space. (a) The extracellular molecules co-migrate on SDS acrylamide gels with bona fide histones obtained by acid extraction (16) from the nuclear chromatin of chick neural retinal cells. (b) The histones of nondividing cells have a metabolic half life of several days and therefore would not be isotopically labeled as well as other proteins. The low molec-

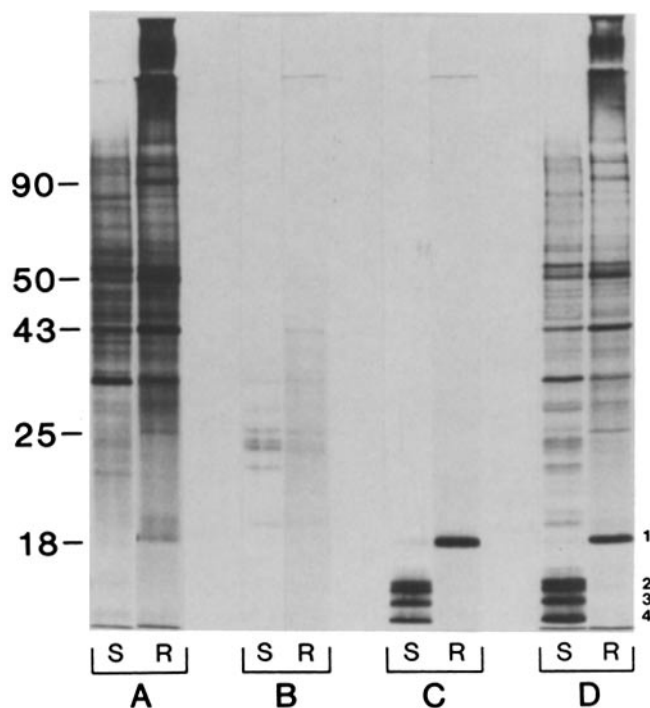


FIGURE 3 Gel electrophoresis of column fractions. Pooled fractions from the Sephadex G100 column depicted in Fig. 2 were pooled, dialyzed against water, lyophilized, and electrophoresed on 15% acrylamide gels containing SDS. One set of gels was exposed to film and the other stained by the silver technique. The silver-stained preparation is on the left (S) and the autoradiograph (R) on the right of each pair. A, pool A from Fig. 2; B, pool B from Fig. 2; C, pool C from Fig. 2; D, whole neural retina adheron.

ular weight proteins heavily stained with silver in Fig. 3 contain very little [³⁵S]methionine. (c) When nuclei prepared from Nonidet P-40-extracted cells, the four silver-stained bands which co-migrate with those found in adherons were detected, but the nuclei of chick neural retina did not contain a 20,000-mol-wt protein which was labeled with methionine (data not presented). It is therefore likely that during the preparation of these primary cultures cells lyse, the histones are released into the medium and adhere to negatively charged cell surface molecules, and that the histones are then released into the medium along with shed membrane components. Alternatively, the histones may bind directly to extracellular adherons.

The data in Figs. 1 and 2 show that a cell binding activity can be found in the most included proteins (peak C) on a Sephadex G100 column. Fig. 3 indicates that this peak of activity contains at least five proteins, one of which is heavily labeled with [³⁵S]methionine. To determine which protein is responsible for adhesion, ~20 μg of pool C (Fig. 3) was electrophoresed on a preparative SDS acrylamide gel and stained by the silver method. The 20,000-mol-wt protein and the three best-resolved histone bands (Fig. 3C, proteins 2, 3, and 4) were excised from the gel and eluted with 0.1% SDS, 10 mM sodium acetate, pH 7.1, and 5% 2-mercaptoethanol. Greater than 95% of the isotopically labeled 20,000-mol-wt protein is extracted by this procedure. The majority of the SDS was then removed by chromatography on Sephadex G25 in 0.1 M acetic acid, the eluents were diluted into HEPES-buffered medium on 35-mm petri dishes, and the acid was neutralized. Over 90% of the input material adhered to the petri dish surface during the overnight incubation. The kinetics of adhesion of isotopically labeled neural retinal cells to the four fractions was then assayed. Fig. 4 shows that the cells adhered much better to the 20,000-mol-wt protein than to any of the three histone fractions, even though the apparent amount of silver-stained protein in the histones was much greater than the 20,000-mol-wt protein. The highest molecular weight histone was, however, able to stimulate the adhesion of neural retina cells to a limited extent. This is not an unreasonable fact since histones are highly positively charged molecules and could affect adhesion in much the same way

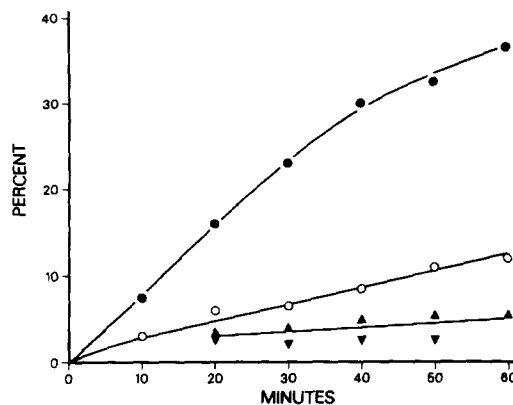


FIGURE 4 Adhesion of cells to acrylamide gel fractions. The proteins from gel fractions 1-4 in Fig. 3 were eluted and adsorbed to petri dishes in the same relative amounts as were resolved on the gels. The adhesion of isotopically labeled cells to each fraction was then determined as described in Fig. 2. ●, fraction 1 (20,000-mol-wt protein) from Fig. 3C; ○, fraction 2; ▼, fraction 3; ▲, fraction 4 and petri dish alone.

as polylysine.

To determine the relative potencies of each protein, the adhesion of neural retina cells to various amounts of adsorbed protein was determined. Fig. 5 shows that the 20,000-mol-wt protein is at least 300 times more effective in promoting the adhesion of neural retina cells than acid-extracted nuclear histones. The concentration at which the 20,000-mol-wt protein causes the adhesion of 50% of the input cells is ~100 ng per 35-mm petri dish as opposed to 30 μ g for the nuclear histones. It follows that although histones are able to increase the rate of cell-substratum adhesion of neural retina cells, the highest specific activity in the Sephadex G100 column fractions (Fig. 2C) is associated with the 20,000-mol-wt protein. This protein is the subject of the remainder of this manuscript and has been named retinal purpurin (RP) because of its silver-staining characteristics.

The Biological Activity of Retinal Purpurin

If the RP protein is involved in the cell binding activity of neural retina adherons, then at least four criteria must be met. (a) Exogenous heparan sulfate should block the binding of cells to RP; chondroitin sulfate and hyaluronic acid should be ineffective. (b) The RP protein should directly bind the heparan sulfate proteoglycan cell surface receptor for adherons. (c) Antibody against the RP protein should inhibit binding of adherons to cells. (d) There should be some tissue specificity with respect to the distribution of the RP protein.

To demonstrate the specificity of GAG inhibition, petri dishes were coated with the RP protein and the adhesion of neural retinal cells was determined in the presence of 200 μ g/ml of heparan sulfate, heparin, chondroitin sulfate, or hyaluronic acid. Fig. 6 shows that only the heparins significantly inhibit adhesion.

The ability of heparan sulfate proteoglycan to bind to RP was assayed by transferring the adheron proteins separated on SDS acrylamide gels to nitrocellulose (blotting), and then binding radioactive heparan sulfate proteoglycan to the transferred proteins. Fig. 7 shows that heparan sulfate proteoglycan bound only to RP in neural retina adherons (lane 6). There was no binding to proteins in adherons from liver, heart, and

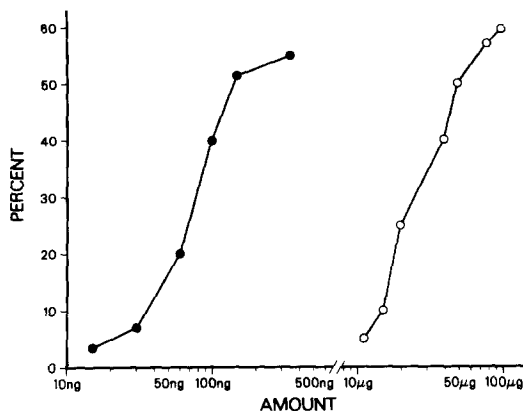


FIGURE 5 Concentration dependence of adhesion to the 20,000-mol-wt protein and histones. The 20,000-mol-wt protein was prepared by elution from acrylamide gels and total nuclear histones were isolated from chick neural retina cells by acid extraction. The adhesion of neural retina cells to various amounts of adsorbed protein was determined and the percent input cells adhered at 60 min plotted against concentration. ●, the 20,000-mol-wt protein; ○, histones.

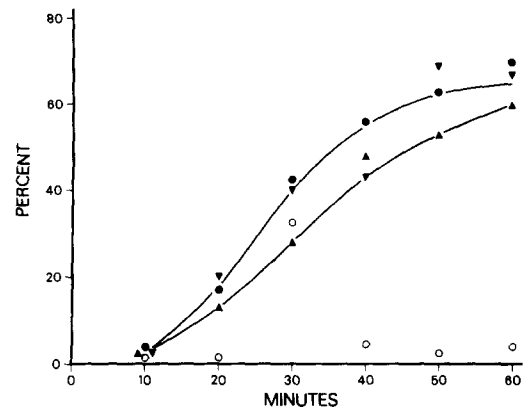


FIGURE 6 GAG inhibition of cell binding to RP. 35-mm petri dishes were coated with 100 ng of the RP protein and the adhesion of 10-d embryonic neural retina cells determined in the presence of 200 μ g/ml of heparan sulfate, heparin, chondroitin sulfate, or hyaluronic acid as described in Fig. 2. ●, control; ○, heparan sulfate (or heparin); ▲, chondroitin sulfate; ▼, hyaluronic acid.

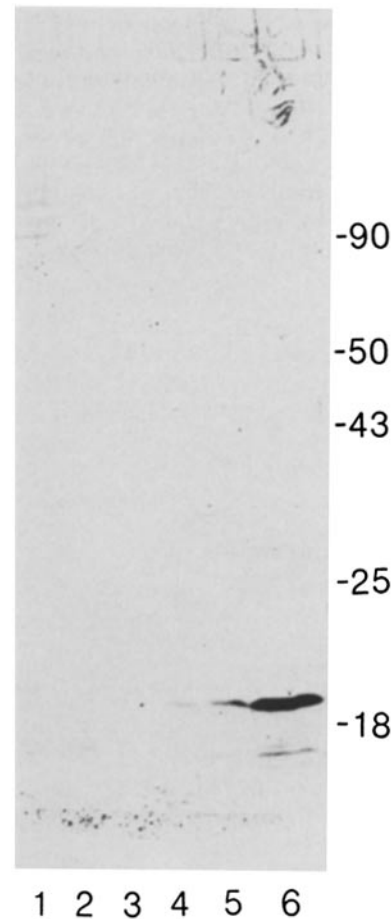


FIGURE 7 Binding of heparan sulfate proteoglycan to RP. Adherons were prepared from the culture medium of several cell types from 10-d embryonic chicks and 20 μ g of protein was electrophoresed on 15% SDS acrylamide gels. The proteins were transferred to nitrocellulose and reacted with [35 S]heparan sulfate proteoglycan prepared from chick neural retinal cells as described in Materials and Methods. Lane 1, skeletal muscle; lane 2, liver; lane 3, heart myoblasts; lane 4, brain; lane 5, optic lobe; lane 6, neural retina.

skeletal muscle. Very weak binding to optic lobe (lane 5) and brain (lane 4) was detectable. The binding of the heparan sulfate proteoglycan was inhibited by heparin and heparan

sulfate but not by chondroitin sulfate or hyaluronic acid. Chondroitin sulfate proteoglycan isolated from the cell surface or conditioned-medium bound to no protein in the blot. It follows that RP is able to bind to a cell surface heparan sulfate proteoglycan.

If RP is involved in adheron-cell adhesion, then antisera against this protein should block adheron-cell adhesions. An antiserum was prepared in a rabbit by the injection of protein extracted from acrylamide gels following silver staining (Fig. 3). The ability of Fab' fragments to inhibit the adhesion of neural retina cells to adheron-coated dishes was assayed along with their effect on the adhesion of isotopically labeled adherons to neural retina cells. Fig. 8 shows that anti-RP inhibited both the binding of adherons to cells and the adhesion of cells to adheron-coated substrata by ~50%. This inhibition appears to be the maximal obtainable by the antiserum, for the use of five times the Fab' concentration had no additional inhibitory effect. It is likely that only half of the adherons from the heterogeneous neural retina cell population contain the RP protein, for anti-RP antibody is only able to precipitate half of the [³⁵S]methionine-labeled adherons from neural retina growth-conditioned medium. In contrast to anti-RP, an antiserum prepared against intact adherons (8) completely blocks the adheron-cell interaction (Fig. 8) and precipitates all of the adherons from growth-conditioned medium. Finally, Fab' fragments of anti-adheron, anti-heparan sulfate proteoglycan, and anti-RP completely inhibit the binding of isotopically labeled RP protein to cells, and the binding of cells to RP protein-coated substrata (data not presented).

Tissue Distribution

The tissue distribution of retina purpurin was assayed using

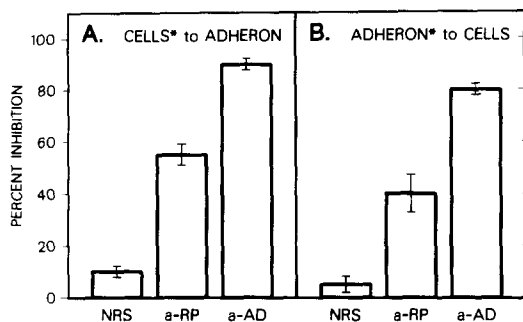


FIGURE 8 Antibody inhibition of RP protein-cell adhesion. (A) Petri dishes were coated with neural retina adherons (30 μ g) as described in Materials and Methods. The coated dishes were then exposed to Fab' fragments at 200 μ g per dish of IgG prepared from anti-purpurin or pre-bleed serum for 20 min at room temperature. The dishes were then washed three times and the adhesion of isotopically labeled 10-d embryonic neural retina cells was assayed. The data are presented as the percent inhibition of control input cells adhered at 40 min, plus or minus the error of the mean of triplicate determinations. (B) 5×10^6 neural retina cells were incubated at 4°C to prevent internalization of material bound to the cell surface in 2 ml of HEPES medium plus the Fab' fragments (200 μ g/ml) of the various antisera and 2×10^6 cpm of [³⁵S]methionine-labeled neural retina adherons. At various times the cells were centrifuged, washed twice, and counted. Kinetic analysis of this preparation indicated that adheron binding reached a maximum level between 4 and 6 h (see also reference 11). 65% of the input isotope adhered to the cells at 6 h and the data are presented as the percent inhibition of the control binding at 6 h by the various antibodies plus or minus the error of the mean of triplicate determinations.

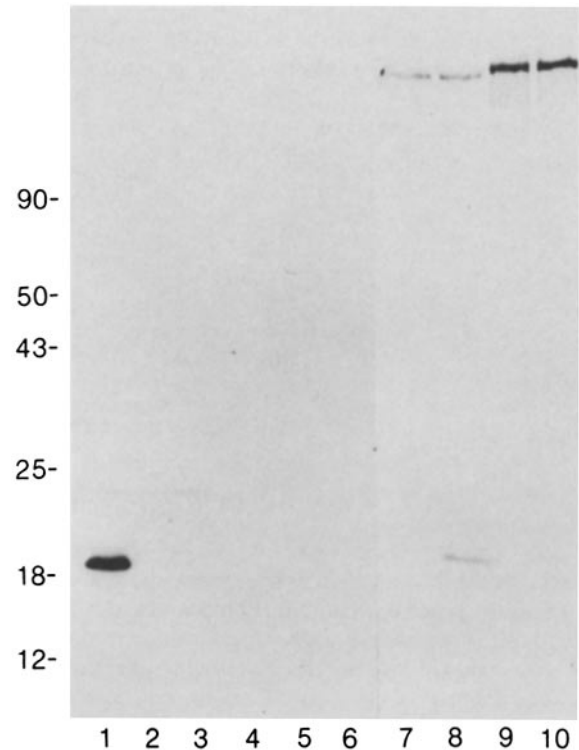


FIGURE 9 Tissue distribution of RP protein. Primary cultures were prepared from 10-d embryonic chick neural retina, optic lobe, brain, liver, heart, and skeletal muscle as described in Materials and Methods. After 2 d in culture, the cultures were washed twice with serum-free medium and incubated in serum-free medium for 18 h, in some cases in the presence of 50 μ Ci/ml of [³⁵S]methionine. The unlabeled medium was passed through a Sephadex G25 column in water to remove salt, lyophilized, and the proteins were separated on 15% acrylamide gels. The proteins were then transferred to nitrocellulose and reacted with anti-RP protein and ¹²⁵I-protein A as described in Materials and Methods. The whole cells were lysed with 1% Nonidet P-40 and either subjected to acrylamide gel electrophoresis (unlabeled) or immunoprecipitation (³⁵S-labeled). Isotopically labeled protein was also immunoprecipitated directly from the growth-conditioned medium. Lanes 1-6, Western blotting of extracellular proteins with anti-RP serum; lanes 7-10, immunoprecipitated proteins. Lane 1, neural retina; lane 2, optic lobe; lane 3, brain; lane 4, heart myoblasts; lane 5, liver; lane 6, skeletal muscle myoblasts. Lanes 7-10, immunoprecipitation from growth-conditioned medium. Lane 7, neural retina, anti-RP pre-bleed; lane 8, neural retina, anti-RP; lane 9, skeletal muscle, anti-RP pre-bleed; lane 10, skeletal muscle, anti-RP. With the exception of neural retina whole cell blots and immunoprecipitates, all other cells were RP negative.

the rabbit antiserum. Two assays were used—immune precipitation and Western blotting. Both total cellular protein and proteins released into the culture medium were assayed. Fig. 9 shows the results of Western blotting and immunoprecipitation of extracellular proteins with anti-RP serum. The antibody reacted only with extracellular RP protein from neural retina. The extracellular proteins of optic lobe, brain, liver, heart, and skeletal muscle did not contain detectable antigenicity, nor did these cells secrete proteins with the mobility and silver staining characteristics of the neural retina RP protein. Immunoprecipitation and Western blotting of total cellular protein gave similar results. Although the rabbit antiserum against RP only reacts with an antigen found in the neural

retina, this observation does not prove that proteins with related primary structures are not synthesized by other tissues. Secondary modification of the extracellular protein used as immunogen may create epitopes unique to this tissue.

Cell Survival Activity

Since many growth factor activities have been associated with heparan binding proteins with molecular weights of ~20,000 (see Discussion), the ability of RP to support the survival of embryonic neural retina was determined. Cells were placed on polylysine-coated tissue culture dishes to which RP was previously adsorbed. Since >95% of these cells are neurons (17), there is little or no cell division in the cultures. Fig. 10 shows two independent experiments in which the survival of cells on purpurin substrata were compared to polylysine substrata alone. In both cases a subpopulation of the cells survived longer in the RP-coated dishes than on polylysine alone. The RP protein had no reproducible effect on neurite outgrowth.

DISCUSSION

The above data provide the following information. (a) A 20,000-mol-wt protein, named retinal purpurin (RP), was isolated from embryonic chick neural retina adherons on the basis of its ability to stimulate neural retina cell adhesion (Figs. 1–5). (b) Like the adhesive interaction between adherons and cells (8), RP–cell adhesion is inhibited by heparin and heparan sulfate, but not by chondroitin sulfate or hyaluronic acid (Fig. 6). (c) The RP protein binds to the heparan sulfate proteoglycan cell surface receptor for neural retina adherons (Fig. 7). (d) Fab' fragments of rabbit antiserum against the RP protein inhibit RP–neural retina cell and partially inhibit adheron–neural retina cell interactions (Fig. 8). (e) The RP protein is apparently localized to the neural retina, for it is not detected by immunoblotting or immunoprecipitation in brain, optic lobe, liver, heart, or skeletal muscle (Fig. 9). (f)

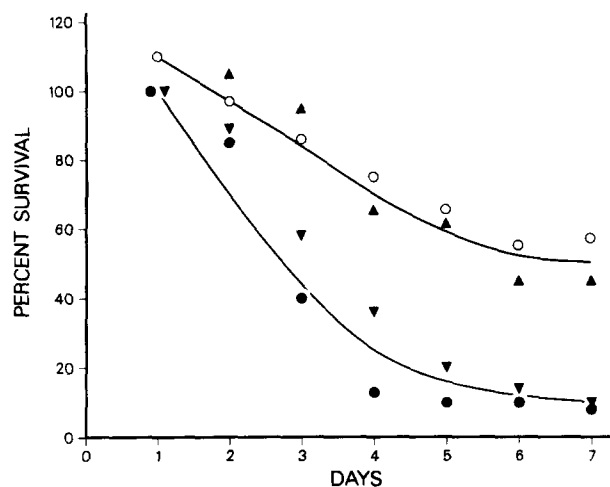


FIGURE 10 Survival activity of the RP protein. 10-d embryonic-neural retina cells were dissociated with trypsin and plated at 2×10^4 cells per 35-mm tissue culture dishes coated with polylysine and, where indicated, the RP protein isolated from acrylamide gels (80 ng per dish). As a control, an area on the gel between RP and the highest molecular weight histone (see Fig. 3) was excised from the gel and treated the same way as the RP protein. The number of adhered viable cells was counted daily (20 fields \times 2 dishes). There was variability between experiments with respect to the kinetics of cell death; two separate experiments are shown. (Experiment 1) O, RP; ●, control. (Experiment 2) ▲, RP; ▼, control.

In addition to its adhesion-stimulating function, RP promotes the survival of a subpopulation of dissociated neural retina cells (Fig. 10). These data suggest that RP is directly involved in the adhesive interaction between neural retina cells and neural retina adherons.

At least six molecules have been purified which are thought to be involved in neural retina adhesion (1–7, 18), and undoubtedly more will be isolated. Some of these molecules may be involved directly in cell–cell adhesion, while others may participate predominately in cell–matrix interactions. In addition to RP, another protein has been described which is in neural retina adherons, and which interacts with the heparan sulfate proteoglycan cell surface receptor for neural retina adherons. Among a collection of monoclonal antibodies directed against neural retina cells, one antibody bound specifically to a 170,000-mol-wt adheron protein and also inhibited adheron–cell adhesion (6, 18, 19). The interaction between cells and the 170,000-mol-wt protein is inhibited by heparan sulfate but not other GAGs. This protein also binds directly to heparan sulfate, for an antiserum against the heparan sulfate proteoglycan cell surface receptor blocks the adhesion of cells to substrata coated with the 170,000-mol-wt protein (9). The RP and the 170,000-mol-wt proteins do not immunologically cross-react as assayed by immunoprecipitation and Western blotting (Cole, G. J., and D. Schubert, unpublished observations). However, the adhesion-promoting activity of the 170,000-mol-wt protein was not detected in our assays because it co-migrates with the heparan sulfate proteoglycans on the Sephadex column (Fig. 1), and once the guanidine HCl is removed, the proteoglycan and the protein may interact, neutralizing the biological activity of the 170,000-mol-wt protein. Alternatively, guanidine may directly inactivate the protein. These data show, however, that adherons contain at least two proteins which can interact with the same cell surface receptor. Since anti-RP only inhibits adheron–cell adhesion by 50% (Fig. 8) and only precipitates half of the particles in the growth-conditioned medium, it is possible that the two proteins are in different adherons. Heterogeneity of the particles is not surprising since the neural retina is extremely heterogenous with respect to cell type. The functional relationship between both proteins in mediating adheron–cell interactions is under investigation.

Adherons can be selectively incorporated into extracellular matrix (10), and they are ultrastructurally similar to particles which may be subunits of extracellular matrix (20–22). Since purpurin binds to cells and is isolated from adherons, RP may be a component of the extracellular matrix. Because RP is able to cause cellular adhesion via its interaction with a proteoglycan cell surface receptor, part of the molecule must be free to interact with cells, while another maintains its interaction with the adheron particle. Whether or not there are multiple heparan sulfate binding sites in RP remains to be established.

In addition to the stimulation of cellular adhesion, RP promotes cell survival (Fig. 10). A large number of growth factor activities have recently been associated with 16,000- to 20,000-mol-wt proteins which are able to bind heparin (23–26). Although retinal purpurin does not cause the division of the postmitotic neural retina cells used here, it does support the survival of at least a subpopulation of these cells cultured from 10-d embryonic chicks. There are no dividing flat glial or fibroblast-like cells in the population. An activity has also been isolated from retinal cells which supports the survival of cultured chick ciliary ganglion cells (27, 28). This activity

apparently resides in a 20,000-mol-wt protein. Although there is no published material showing that it is a heparan sulfate binding molecule, it is possible that the ciliary ganglion "trophic factor" and RP are the same. In addition, the heparin binding domain of laminin promotes the survival of cultured chick sympathetic neurons (29). The shared features between the various endothelial growth factors and RP (similar molecular weights and heparan sulfate binding activities) suggest that they may be a related group of molecules with similar biological functions. Sequence data will be required to solve this problem. It is, however, necessary to establish the biological specificity of these molecules, for many appear to stimulate the growth of a variety of cell types, and they are found in many tissues.

The mechanism by which RP and the other heparin-binding growth factors promote cell survival is not clear. Since many cell types require an adhesive surface on which to attach in order to survive in culture (see, for example, reference 30), and since many nonbiological surfaces such as tissue culture plastic and polylysine can fulfill these requirements, it follows that simply an increase in cell-substratum adhesion can be of survival value for cultured cells. In addition to survival per se, cell-substratum adhesion can have profound effects on cellular metabolism (see, for example, reference 31). In contrast to survival "factors," molecules which stimulate cell division may function by the perturbation of cell-substratum adhesion. For example, mild trypsinization of cultured fibroblasts induces mitosis (32) and some growth factors, such as glial growth factor, have proteolytic activity (33). Perhaps any weakening of cell-substratum adhesion can induce mitosis. If cells are tightly associated with a layer of matrix via a heparan sulfate proteoglycan, heparin binding proteins may act as haptens and block the matrix-bound heparin-cell surface interaction (7, 8). This interaction could lead to a weakening of cell attachment and induce mitosis. If adhesion, survival, and growth-promoting activities are all contained within the same heparin binding molecule, then its biological effects must be dependent upon its extracellular concentration and solubility. If the protein is tightly associated with matrix, then it would promote cell-matrix adhesion and survival; the soluble form may be mitogenic.

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