A Chick Neural Retina Adhesion and Survival Molecule Is a Retinol-binding Protein

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Abstract. A 20,000-D protein called purpurin has recently been isolated from the growth-conditioned medium of cultured embryonic chick neural retina cells (Schubert, D., and M. LaCorbiere, 1985, *J. Cell Biol.*, 101:1071–1077). Purpurin is a constituent of adherons and promotes cell-adheron adhesion by interacting with a cell surface heparan sulfate proteoglycan. It also prolongs the survival of cultured neural retina cells. This paper shows that purpurin is a secretory protein that has sequence homology with a human protein synthesized in the liver that transports retinol in the blood, the serum retinol-binding protein (RBP). Purpurin binds [^{3}H]retinol, and both purpurin and chick serum RBP stimulate the adhesion of neural retina cells, although the serum protein is less active than purpurin. Purpurin and the serum RBP are, however, different molecules, for the serum protein is ~3,000 D larger than purpurin and has different silver-staining characteristics. Finally, purpurin supports the survival of dissociated ciliary ganglion cells, indicating that RBPs can act as ciliary neurotrophic factors.

THE growth-conditioned medium of cultured cells contains molecules that promote the adhesive interactions of the homologous cell type (24). Since this initial observation, a variety of proteins and proteoglycans has been isolated from cultured cells that stimulate the rate of either cell–cell or cell–substratum adhesion. In the embryonic chick neural retina, a macromolecular complex containing several proteoglycans and proteins has been identified which, when adsorbed to an initially nonadhesive surface, greatly enhances the rate of cell–substratum adhesion (27). These particles, termed adherons, are 15 nm in diameter by electron microscopy and sediment at 12 S in sucrose gradients. Embryonic chick neural retina adherons mediate cell–substratum adhesion by interacting with a heparan sulfate proteoglycan on the cell surface (15, 29, 30). Two molecules have been isolated from neural retina adherons that bind to the heparan sulfate proteoglycan cell surface receptor. These include a 170,000-D protein isolated on the basis of a monoclonal antibody's ability to block cell–adheron adhesion (12–15), and a 20,000-D protein isolated from particles on the basis of its ability to directly stimulate cell–substratum adhesion (30). The latter protein also is a "trophic" or survival factor, for it prolongs the survival of a subset of neural retina cells in culture. Monovalent antibodies against the 20,000-D protein disrupt the histogenesis of embryonic chick neural retina in organ culture (31). The 170,000-D heparin-binding adheron protein has been identified as the neural cell adhesion molecule N-CAM (16), thus greatly extending the variety of adhesive interactions of a protein which was previously thought to interact only with itself (18). The following paragraphs show that the 20,000-D neural retina protein, called purpurin because of its silver-staining characteristics, is related to a group of known proteins—the retinol-binding proteins (RBPs)[1]. Purpurin has ~50% sequence homology with human serum RBP and it is able to bind [^{3}H]retinol. The neural retina protein is distinct from the chick serum RBP, although it is functionally related. These results indicate that the family of RBPs has a wider range of biological activities than simply the transport of vitamin A.

Materials and Methods

Cells and Culture

Unless otherwise indicated, neural retina tissue was separated from the pigmented epithelium (PE) 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 DNase I (Worthington Biochemical Corp., Freehold, NJ). The cells were incubated overnight at 100 rpm on a 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 the liver 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. Cultures of the retina glia (Müller cells) and PE were established by published procedures (2, 21, 23). The glia cultures were judged homogeneous by the observation that >95% of the cells stained positive for glial fibrillary acidic protein and by the absence of round, nerve-like cells. The nerve cell cultures, used 1 d after dissociation from 10-d embryonic neural retina, contained almost exclusively round, neurite-bearing cells (see also reference 2). The flat, glial fibrillary acidic protein-positive glial-like cells did not

1. *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; PE, pigmented epithelium; RBP, retinol-binding protein.

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appear until several days after dissociation. The relative homogeneity of the PE cultures was based on the dissection, the pigmented nature of the cells, and the lack of cells (neurons) containing neurites.

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 (27). The washed pellet contained a relatively homogeneous population of 15-nm particles which was used as 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 (BSA) was added.

Adhesion Assays

To assay cell-substratum adhesion, cells were labeled 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% BSA (Calbiochem-Behring Corp., La Jolla, 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 at the indicated time. Variation between duplicates was <5%.

Isotopic Labeling, Gel Electrophoresis, In Vitro Translation, and Immune Precipitation

Cells were labeled with [^{35}S]methionine and gel electrophoresis was done in gels containing 15% acrylamide and 0.1% SDS as described (3). Silver staining was done according to the protocol described by Wray et al. (35). 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 nitrocellulose was washed three times with 150 mM KCl, 10 mM imidazole, 5 mM $MgCl_2$, 0.3% Tween 20, and 0.1% $CaCl_2$ (pH 7.3) containing 5% BSA. The nitrocellulose was incubated overnight with a 1:500 dilution of immune serum at room temperature. The nitrocellulose was then washed for 2 h in incubation buffer minus BSA, incubated for 2 h with 2×10^6 cpm of ^{125}I-protein A (New England Nuclear, Boston, MA), and extensively washed before drying and autoradiography.

To prepare poly(A)$^+$ mRNA, neuroretina cells were disrupted in 4 M guanidine thiocyanate with a motor-driven homogenizer on ice, and then passed several times through an 18-g needle. The RNA was precipitated with 95% ethanol on dry ice, extracted several times with water, guanidine HCl and the pellet resuspended in 0.1% lauryl sulfate-LiCl and incubated at 37°C for 10 min before poly(A)$^+$ selection. The sample was applied twice to an oligo dt column (Iso, Inc., Akron, OH), equilibrated in 0.1% lauryl sulfate-LiCl, and eluted with 5 mM Tris, 1 mM EDTA, and 0.1% SDS. The poly(A)$^+$ mRNA was translated in a rabbit reticulocyte lysate (Amersham Corp., Arlington Heights, IL) in the presence of [^{35}S]methionine (250 μCi/20 μl lysate). Incubation with 0.5 μg of lyophilized RNA was at 30°C for 60 min; the reaction was terminated by adding 1 μg RNase and further incubation for 10 min at 37°C. The sample was placed on ice and cold RIPA buffer (1% Na deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1% Trasylol, 10 mM sodium phosphate, 0.15 M NaCl, pH 7.2) was added. The relevant proteins were immunoprecipitated with 2 μl of antibody (30) overnight at 4°C, followed by removal of IgG with Pansorbin (Calbiochem Behring Corp.).

High-performance Liquid Chromatography Peptide Analysis and Amino Acid Sequencing

Embryonic chick neural retina purpurin was purified to homogeneity as described in the text. Initial attempts to determine the NH_2-terminal sequence showed that it was blocked, therefore chymotryptic peptides were generated. Dried purpurin was dissolved in 0.01 ml 0.5 M Tris-HCl, pH 7.7, 10 mM EDTA, and 6 M guanidine-HCl, and then diluted to 1 ml with 1% NH_4HCO_3. Chymotrypsin was added in a 1/50 (wt/wt) ratio, and the mixture incubated overnight at 37°C. The chymotryptic digestion fragments were purified on a reverse phase column (0.46 × 25 cm) (model RP-300, Brownlee Labs, Santa, Clara, CA) with a 0.1% trifluoroacetic acid/acetonitrile solvent system (19). The amino acid composition of each resolved peptide was determined before sequence determination. Amino acid analyses and gas phase microsequencing of purpurin digestion fragments were carried out by published methods (6, 20). Cysteine residues were oxidized with performic acid before amino acid analysis. Performic acid was generated by incubating 9 ml distilled formic acid with 1 ml 30% H_2O_2 at room temperature in a tightly capped tube for 1 h. 0.25 ml of this solution was employed to dissolve the dried peptides and the oxidation continued at 0°C for 2.5 h. The identification of cysteic acid in a given cycle was accomplished by comparison of the amino acid composition of the peptide and the remainder of its sequence as determined by Edman degradation.

Retinol-binding Assay

Vitamin binding was assayed by sucrose density centrifugation (10). Proteins were exposed to [^{3}H]retinol (New England Nuclear, 30 pmol/ml) for 2 h at 4°C in the dark in a buffer containing 50 mM Tris, 0.1 mM EDTA, and 10 mM KCl (pH 7.5). In some cases a 100-fold excess of unlabeled retinol was included. After incubation, the samples were loaded onto 5–20% sucrose gradients in the same buffer and centrifuged at 180,000 *g* for 20 h at 4°C in a rotor (model SW65; Beckman Instruments, Inc., Fullerton, CA). The tubes were punctured with a needle, and the fractions counted in a scintillation counter.

Ciliary Ganglion Cell Survival Assay

The survival assay was performed as described (4, 17) except that the protein was adsorbed to the substratum before the assay. Briefly, 35-mm tissue culture dishes (Falcon Labware) were precoated with 2 ml of a polyornithine solution (100 μg/ml in 0.15 M borate, pH 8.5) overnight at 4°C. The dishes were then washed twice and incubated in Hepes-buffered DME containing the test proteins overnight at 37°C. The dishes were washed twice with DME and seeded with 5,000 cells from 8-d embryonic chick ciliary ganglion per dish in DME plus 10% fetal calf serum. 24 h later cultures were assayed for viable cells either by direct visual count under phase contrast microscopy or with the use of 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide (25). The data are presented as the percent of the input cells surviving.

Results

Biosynthesis of Purpurin

Previous data suggested that purpurin is secreted by neural retina cells (30). If purpurin is a secretory protein, then it may be synthesized with an NH_2-terminal extension peptide or signal sequence (5). To test this possibility, cells were pulse-labeled for 5 min with [^{35}S]methionine, followed by a 1 h chase with unlabeled methionine-containing medium. The [^{35}S]methionine-labeled proteins were precipitated from cells and the culture medium at various times after the chase, and the immune precipitates run on SDS acrylamide gels along with immune precipitates using preimmune sera. Fig. 1 shows that purpurin after a 5-min pulse (lane *1*) is the same size as the secreted protein (lane *2*). The identity of the higher molecular mass extracellular protein is not known. Intracellular purpurin did not change in size throughout the 1-h chase (data not presented). However, when poly(A)$^+$-mRNA is isolated from embryonic neural retina cells, translated in a rabbit reticulocyte system, the proteins immune precipitated, and the precipitates run on SDS acrylamide gels, the sole immune precipitable translation product migrates with an apparent molecular mass of 23,000 D (lane *4*). These results indicate that neural retina cells synthesize purpurin as a primary translation product of 23,000 D, which is rapidly cleaved such that the larger protein is not detected after a 5-min pulse of [^{35}S]methionine.

Which cell types within the neural retina synthesize purpurin? There are three alternatives—Müller glial cells, PE cells, or members of the heterogeneous nerve cell population. To determine which cell type makes the protein, homogeneous populations of each were established in culture by pub-

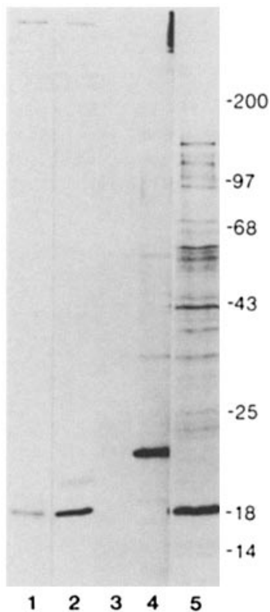

Figure 1. Pulse labeling of neural retina cells and translation of mRNA. 11-d embryonic neural retina cells were pulse labeled with [^{35}S]methionine for 5 min, and resuspended in isotope-free methionine-containing medium and incubated at 37°C. At various times the cells were pelleted, lysed, and purpurin was immune-precipitated as described in Materials and Methods. The immune precipitates were run on SDS acrylamide gels. In another experiment, poly(A)$^+$ mRNA was isolated from E11 neural retina cells, translated in a reticulocyte system, and the proteins immune-precipitated and run on SDS acrylamide gels. Lane *1*, 5-min pulse, anti-purpurin immune precipitate; lane *2*, anti-purpurin immune precipitate of 1-h secreted protein; lane *3*, translation product, preimmune serum; lane *4*, translation product, anti-purpurin; lane *5*, total extracellular protein. Purpurins immune-precipitated from pulse-labeled cells chased with cold methionine for 15, 30, and 60 min all co-migrated with the 5-min pulse and secreted forms.

lished procedures (2, 21, 23), and their growth-conditioned media were collected. The cultures release similar amounts of protein and 10 μg of each run on a SDS acrylamide gel, and the gel subjected to Western blot analysis using an antibody against purpurin. Fig. 2 shows that the antigen was present in the neural retina cultures, a trace was in the Müller cell cultures, and none in the PE. These results were confirmed with silver staining and the immune precipitation of isotopically labeled cellular and extracellular protein (data not shown). It is probable that the small amount of purpurin staining in the glial-culture-conditioned medium was due to contaminating nerve cells. No purpurin was detected in the culture medium of embryonic liver, although there was a weakly cross-reacting protein of ~24,000 D (Fig. 2, lane *5*).

Amino Acid Sequence Homology between Purpurin and Human Serum Retinol-binding Protein

Although purpurin has been purified to apparent homogeneity on SDS acrylamide gels (30), it is technically difficult to obtain good amino acid sequence data from proteins eluted from gels. Therefore 10-d embryonic chick neural retina cells were labeled with [^{35}S]methionine and the growth-conditioned medium was concentrated and run over a Sephadex G100 column in 4 M guanidine HCl. The fractions containing purpurin also contain four histone-like proteins (30). The histones were separated from purpurin by chromatography on CM 32 (Whatman Chemical Separation, Clifton, NJ) in 6 M urea and 0.01 M ammonium acetate using a gradient from 0 to 0.1 M NaCl. The resultant protein was free of histones and gave a single band on SDS acrylamide gels. The amino acid composition of this protein was determined, and several attempts were made to determine its NH_2-terminal amino acid sequence using a gas-phase sequencer. Repeated failure to detect any amino acid indicated that the NH_2-terminus was blocked. Approximately 1 μg of the protein was digested with chymotrypsin and the resultant peptides were purified

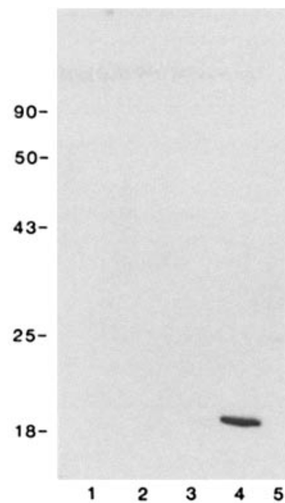

Figure 2. Cellular distribution of purpurin. Cultures of 11-d neural retina, PE, glial (Müller cells), and liver were prepared as described in the text. Serum-free, growth-conditioned medium was collected, concentrated, and 10 μg from each cell type run on 15% SDS acrylamide gels. The resolved proteins were then transferred to nitrocellulose, and reacted with anti-purpurin as described in Materials and Methods. Lane *1*, PE, unpassaged primary culture; lane *2*, PE, second passage in culture; lane *3*, Müller cells; lane *4*, neural retina; lane *5*, liver.

on a reverse phase high-performance liquid chromatography column. Fig. 3 shows that the [^{35}S]methionine isotopic label co-migrated with peptides detected by optical methods, indicating that the peptides that are being sequenced are those which are synthesized by the neural retina cells. Amino acid analysis of the [^{35}S]methionine-containing peptides confirmed that each had at least one methionine residue. The amino acid compositions of all of the well-resolved peptides were determined and the peptides completely sequenced. R. F. Doolittle (University of California, San Diego), using his protein sequence data base, matched these sequences with known sequence data. These data showed that within the 52 amino acid residues sequenced, purpurin is ~50% homologous to human serum RBP (Fig. 4). All of the uncontaminated (single sequence) peptides analyzed had sequence homology with human RBP.

Purpurin Binding of Retinol

The ability of a protein to bind retinol was assayed by mixing the protein with [^{3}H]retinol in the presence or absence of excess unlabeled retinol, centrifuging the protein into a sucrose gradient, and assaying the distribution of [^{3}H]retinol in the gradient (10). Fig. 5*b* shows that purpurin is able to specifically bind [^{3}H]retinol. Human serum RBP, which is structurally similar to purpurin, was used as a control (Fig. 5*C*). Both purpurin and human RBP migrate to about the same position within the gradient (2 S, 10). Since purpurin was initially found in neural retina adherons, cells were labeled with [^{35}S]sulfate to label glycosaminoglycans in adherons, the adherons prepared, mixed with [^{3}H]retinol, and centrifuged into a sucrose gradient. Fig. 5*A* shows that adheron particles are able to bind [^{3}H]retinol in a manner similar to the purified protein.

Relation of Purpurin to Chick Serum RBP

Since the serum RBP is synthesized in the liver and exported to the blood (10), the structural and functional relationships of purpurin to those of the chick serum protein were determined. Chicken serum RBP was purified by published procedures (1) and shown to bind [^{3}H]retinol by the centrifugation assay described in Fig. 5. When [^{35}S]methionine-labeled purpurin was run on SDS acrylamide gels with both human

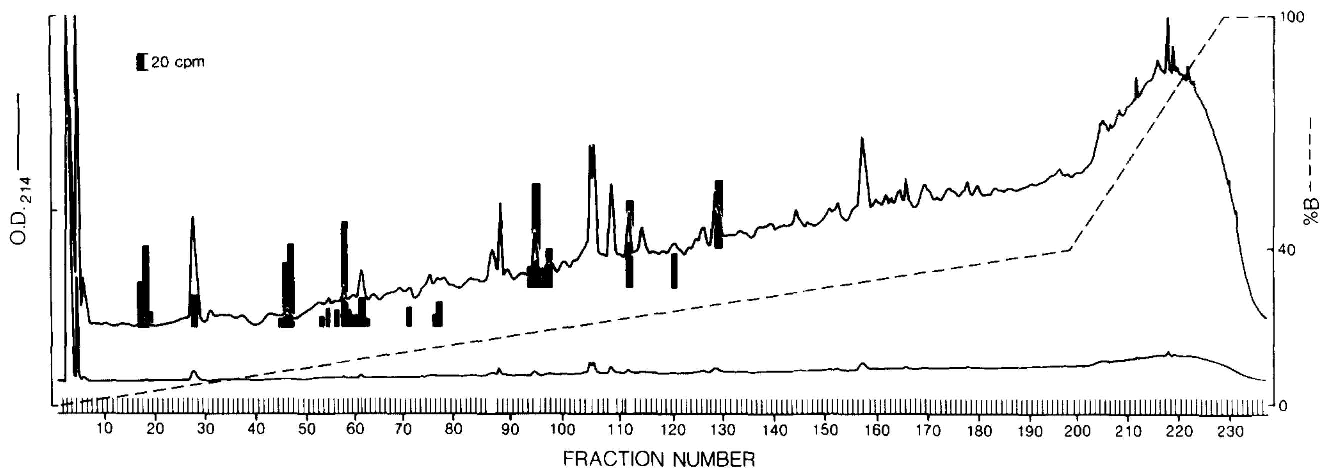

Figure 3. Reverse phase liquid chromatography of chymotrypic digest. Approximately 1 μg of purpurin was digested with chymotrypsin and pumped onto a 0.46 × 25 cm reverse phase column (model RP-300; Brownlee Labs) using a 0.1% trifluoroacetic acid/acetonitrile solvent system and eluted at 0.6 ml/min with a 90-min gradient of 8–40% acetonitrile (---). Chromatography was at room temperature and the isotope in 5% of each fraction was determined in a liquid scintillation counter (black bars).

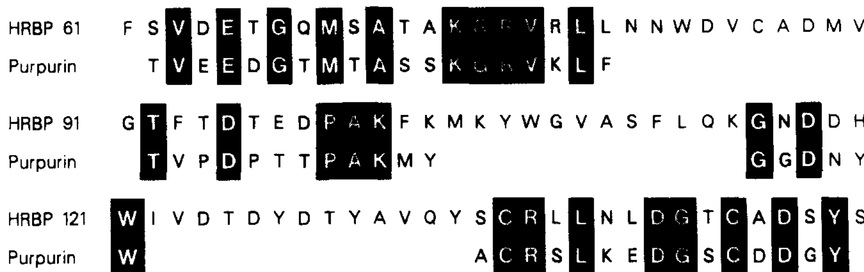

Figure 4. Sequence homology between purpurin and human serum RBP. Data from the sequence analysis of purpurin chymotryptic peptides were compared with those of human RBP (32). Identical residues are outlined in black. The residue number from the NH_2-terminus of human serum RBP is indicated at the left.

and chick RBP, purpurin migrated at a faster rate than either serum protein (Fig. 6). The human protein has been sequenced and has a molecular mass of 21,200 D (32). The chicken RBP is slightly larger than the human, and both are larger than purpurin. Neither the chick nor human RBPs stained purple with the silver stain as is characteristic of purpurin. The antigenic relationship between the three proteins was assayed by Western blotting with an antiserum against purpurin. Fig. 6 shows that rabbit antipurpurin reacted with all three proteins. The antipurpurin sera reacted much more weakly with the serum RBPs than with purpurin, since it would not blot the RBPs if 50 ng of each were run on gels; this concentration of purpurin gave a strong signal. Approximately 100-fold more serum RBPs than purpurin were loaded on the gels shown in Fig. 6 (lanes *4* and *6*).

Neural retina purpurin stimulates the rate of cell–substratum adhesion when adsorbed to a nonadhesive surface, an interaction which is blocked by exogenous heparin and heparan sulfate (30). Since the serum RBP must interact with the cell surface in order to transfer bound retinol to the cell (10), it may also cause cellular adhesion. Fig. 7 shows that purpurin is very efficient in stimulating adhesion and that chick serum RBP enhances the adhesion of chick neural retina cells with a half maximum stimulation at a concentration slightly greater than fibronectin. It should be noted, however, that the maximum effective concentration of fibronectin only causes the adhesion of 11% of the input cells over 1 h, while purpurin causes 60% of the cells to adhere, and with serum RBP 95% of the input cells adhere. Since the neural retina is an extremely heterogeneous cell population, the cells adherent to each molecule probably reflect adhesive preferences of subsets of cells within the population. Serum RBP is, however, almost 20 times less efficient than purpurin in stimulating adhesion with respect to the molar amount of protein required for half maximum adhesion. Similarly, heparan sulfate and heparin were less efficient in blocking the adhesion of neural retina cells to serum RBP than to purpurin (Fig. 8). It follows that the serum RBP and purpurin are distinct molecules, but that they are antigenically related and have similar activities with respect to retinol binding and cellular adhesion.

Ciliary Ganglion Nerve Cell Survival Activity

The 20,000-D chick retina purpurin molecule promotes the survival of a subset of dissociated embryonic neural retina cells in culture (30). A protein of similar size increases the survival of dissociated chick ciliary ganglion cells (4, 17, 26). Fig. 9 shows that purpurin is able to increase the survival rate of cells in dissociated ciliary ganglion cultures; serum RBP does not reproducibly promote survival at concentrations up to 10^{-9} mol/dish. Fibronectin is also inactive. Due to the relative insolubility of purified purpurin in physiological salt solutions it was necessary to adsorb the protein to the substrata of the assay dishes from dilute solutions of urea before the test cells were added. It has been demonstrated that substratum-bound ciliary neurotrophic factors can promote cell survival (9), and the two experiments shown in Fig. 9 indicate that purpurin has this biological activity. Histones, which were separated from purpurin during the last phase of purification on a CM-32 ion exchange column, did not support the

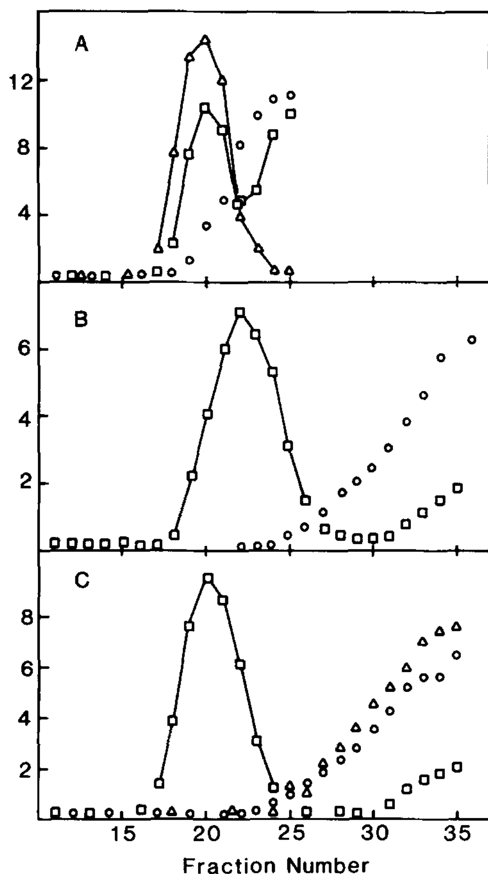

Figure 5. Purpurin binding of [^{3}H]retinol. [^{35}S]Sulfate-labeled adherons and unlabeled purpurin were prepared from 11-d embryonic chick neural retina as described in text, and purified human serum RBP was obtained from Dr. Johan Sundelin (University of Uppsala, Sweden). 2 μg of each was mixed with 30 pmol/ml of [^{3}H]retinol (New England Nuclear) with or without a 100-fold molar excess of retinol, and, in the case of purpurin and human RBP, centrifuged into a 4-ml, 5–20% sucrose gradient for 24 h at 180,000 *g*. The adherons were centrifuged into a 12-ml, 5–20% sucrose gradient (in 0.01 M Tris, pH 7.2) for 16 h at 100,000 *g*. The fractions were counted and, in the case of the [^{35}S]sulfate-labeled adherons, corrected for isotopic overlap. (*A*) Adherons: △, $^{35}SO_4$; □, [^{3}H]retinol; ○, [^{3}H] retinol plus cold retinol. (*B*) Purpurin: □, [^{3}H]retinol; ○, [^{3}H]retinol plus cold retinol. (*C*) Human RBP: □, [^{3}H]retinol; ○, [^{3}H]retinol plus cold retinol; △, [^{3}H]retinol with no protein. cpm (× 10^{-4}) are indicated on left.

survival of the ciliary ganglion cells under identical assay conditions (data not shown).

Discussion

The following conclusions can be made from the above data. (*a*) Embryonic retina purpurin, a secreted protein with a molecular mass of ~20,000 D, is translated as a 23,000-D protein (Fig. 1), suggesting that it has an NH_2-terminal signal sequence. (*b*) Purpurin has amino acid sequence homology with human serum RBP (Fig. 4), and is able to bind retinol (Fig. 5). (*c*) Although antigenically related, purpurin is distinct in structure and function from chicken serum RBP, for purpurin has a lower apparent molecular mass (Fig. 6) and is more active than serum RBP in stimulating nerve cell adhesion (Fig. 7). (*d*) Purpurin promotes the survival of embryonic chick ciliary ganglion cells (Fig. 9).

Retinol is circulated in the blood via a serum RBP synthesized in the liver (10). The retinol-RBP complex is in turn associated with prealbumin. This trimolecular complex interacts with the cell surface of target cells and the retinol is transported into the cell where it affects many developmental events by undefined mechanisms (10). There is also a class of proteins that are thought to be exclusively intracellular, which may be involved in the transport of retinol from the cell surface to the nucleus (see, for example, reference 34). Sequences of both classes of molecules have been reported, and there appears to be no homology between the cellular and serum RBPs (33). The neural retina protein purpurin shares ~50% of its known sequence with the human serum RBP (Fig. 4). This observation, plus the fact that purpurin is a retinol-binding secretory protein, suggests that it is a member of a class of molecules in the neural retina which is thought to be involved in the transport of retinol between cells (see, for example, reference 7). Since retinol is removed from serum by the PE, where it is esterified and stored until it is transported to the photoreceptor outer segments for use in phototransduction (10), it is possible purpurin is involved in this process. However, RBPs have been purified that are thought to be involved in this process, and all are considerably larger than chick purpurin (7, 22). In addition, when relatively pure primary culture populations of PE, nerve, and Müller cells were assayed for their ability to synthesize and secrete purpurin, only the nerve cell cultures synthesized large amounts of this protein. Since these primary nerve cultures are thought to contain both photoreceptors and nerve (2), it is possible that a subset of this population is releasing an RBP which interacts with another target cell. In situ hybridization using probes generated from cloned cDNA sequences will be required to exactly define the site of purpurin synthesis within the retina.

Although an antiserum prepared against embryonic chick neural retina purpurin cross-reacts with the chick serum RBP, the two proteins differ in both molecular masses and biological activities. The human serum RBP cDNA has been cloned and sequenced, and the protein determined to have a molec-

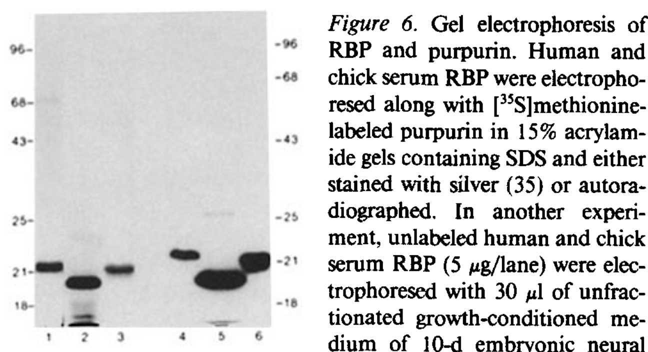

Figure 6. Gel electrophoresis of RBP and purpurin. Human and chick serum RBP were electrophoresed along with [^{35}S]methionine-labeled purpurin in 15% acrylamide gels containing SDS and either stained with silver (35) or autoradiographed. In another experiment, unlabeled human and chick serum RBP (5 μg/lane) were electrophoresed with 30 μl of unfractionated growth-conditioned medium of 10-d embryonic neural retina, which contained <20 ng of purpurin. The proteins were transferred to nitrocellulose, reacted with a 1:500 dilution of antipurpurin or preimmune serum, followed by ^{125}I-protein A. Preimmune serum alone labeled none of the proteins. Lane *1*, chick RBP, silver stain; lane *2*, [^{35}S]methionine purpurin radioautograph; lane *3*, human RBP, silver stain; lane *4*, chicken RBP blot; lane *5*, neural retinal-conditioned medium blot; lane *6*, human RBP blot. Molecular mass standards (× 10^{-3}) are indicated on right and left.

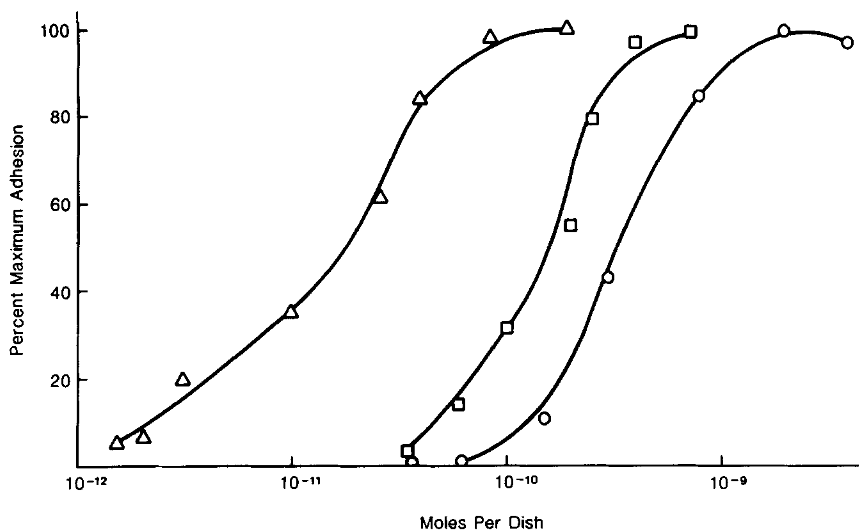

Figure 7. Effect of purpurin, serum RBP, and fibronectin on neural retinal cell adhesion. 35-mm plastic petri dishes were coated with the indicated amounts of each protein and the adhesion of isotopically labeled 11-d neural retina cells determined as described in Materials and Methods. Adhesion was done for 1 h and the data presented as percent of input cells that adhered minus adhesion to petri dishes alone (<2% of input). Each point is the average of triplicate determinations. O, chick serum RBP; Δ, purpurin; □, human fibronectin (Collaborative Research). 60% of the input cells adhered to purpurin, 11% to fibronectin, and 95% to chick serum RBP.

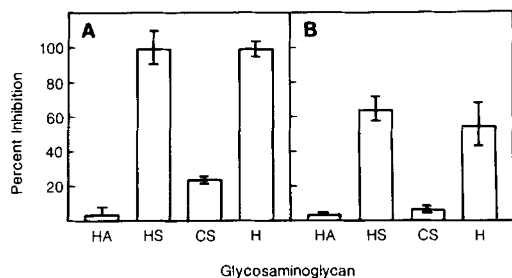

Figure 8. Glycosaminoglycan inhibition of cell adhesion to purpurin and serum RBP. Neural retina cells were adhered to chick serum RBP (5×10^{-10} mol/dish) or purpurin (10^{-11} mol/dish) for 1 h as described in Fig. 7 in the presence or absence of 200 μg/ml of the different glycosaminoglycans. The data are presented as the mean inhibition ± SEM for quadruplicate determinations. (*A*) Cells to purpurin substratum. (*B*) Cells to chick serum RBP substratum.

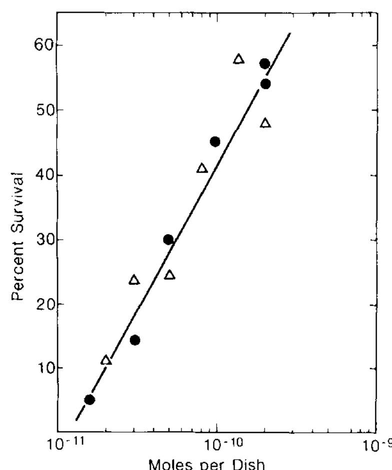

Figure 9. Effect of purpurin on ciliary ganglion cell survival. Various concentrations of purpurin were adsorbed to the surface of polyornithine-coated 35-mm tissue culture dishes and the survival of dissociated ciliary ganglion cells from 8-d embryonic chicks determined as described in Materials and Methods. The data are plotted as the percent of input cells (determined by cell counts 4 h after plating) which survived for 18 h. Δ and O, purpurin, two different experiments.

ular mass of 21,200 D (11, 32). The chick serum protein is ~22,000 D, and purpurin is ~20,000 D. Purpurin has 50% of its maximum adhesion-promoting activity at 2×10^{-11} mol/35-mm dish while that of chick serum RBP is 4×10^{-10} mol and human fibronectin 2×10^{-10} mol. These data show that purpurin is ~10-fold more effective than fibronectin in stimulating cellular adhesion. Heparan sulfate blocks the adhesion of cells to both purpurin and chick serum RBP, suggesting that the RBP protein interacts with neural retina cell surface heparan sulfate in a manner analogous to purpurin (30).

The observation that purpurin promotes the survival of both chick neural retina cells (30) and ciliary ganglion cells (Fig. 9) shows that RBP may play a significant role as trophic factors in the development of the nervous system. This is not surprising within the eye, for the phototransduction mechanism is dependent upon exogenous retinol. The photoreceptor cells comprise about half of the cells in 10-d embryonic neural retina cultures (2); purpurin allows the survival of ~50% of the cells in cultured embryonic 10-d neural retina (30).

The data showing that purpurin promotes ciliary ganglion cell survival is also consistent with what is known about this biological activity. The ciliary neurotrophic factor activity is found in high concentrations in the eye (4, 17, 26). The RBP purpurin is located in the eye along with several other RBPs (see, for example, references 7 and 10). In addition, purpurin has a molecular mass of 20,000 D, similar to the eye-derived ciliary neurotrophic factor (4, 17, 26). These data suggest that purpurin and the eye-derived ciliary survival factor described by others may be the same molecule.

Purpurin is associated with adhesion-mediating glycoprotein complexes called adherons in the growth-conditioned medium of cultured chick neural retina (30). Chick neural retina adherons are 15 nm in diameter and contain several proteoglycans and proteins. They mediate both cell–cell and cell–substratum adhesion (27), they can be incorporated into extracellular matrix, and may be a subunit of this matrix (see reference 28, for discussion). Purpurin and another heparan-binding protein, recently shown to be the neural cell adhesion molecule N-CAM (16), are both found in neural retina adherons. Since both molecules promote cell–matrix interactions via binding to cell surface heparan sulfate proteoglycan (15, 16, 30), and antibodies against each disrupt normal

histogenesis (8, 31), it is probable that both purpurin and the neural cell adhesion molecule N-CAM are involved in maintaining neural retina cytoarchitecture.

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