Identification of novel neural- and neural retina-specific antigens with a monoclonal antibody

(neural cell types/immunoblotting/immunocytochemistry/retina marker)

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ABSTRACT   A fluorescence-activated cell sorter screening method has been used to identify hybridomas that secrete monoclonal antibodies that can bind to viable subpopulations of embryonic chicken neural retina cells. One monoclonal antibody, C_1H_3, recognizes two nervous tissue-specific polypeptides that exhibit distinct developmental patterns. The monoclonal antibody reacts with a 140-kilodalton (kDa) polypeptide that is present at early stages of development (day 7) but is detected by immunoblotting in only negligible amounts at later times (day 17). In contrast, a 170-kDa polypeptide is first detectable by immunoblotting at day 10 and is the predominant C_1H_3 antigen at day 17. Analysis of proteolytic fragments of the two proteins indicates that the polypeptides are distinct molecules that share a common antigenetic determinant. Both polypeptides are neural-specific; the 140-kDa polypeptide appears to be retina-specific, while the 170-kDa polypeptide is also present in other areas of the nervous system. Metabolic labeling of retina cells in situ at early embryonic stages reveals only the synthesis of the 140-kDa polypeptide. When such cells are dissociated and labeled in vitro, they synthesize primarily the 170-kDa polypeptide. Thus, the differential rate of synthesis of these two polypeptides is controlled by environmental factors that possibly include cell–cell contacts or an unknown systemic factor. The 140-kDa polypeptide is a unique marker for early neural retina cells.

A goal of current research in neurobiology is the characterization of the molecular composition of defined cell types. The primary aim of these studies is to identify specific cell surface macromolecules that may serve as probes for the study of cell recognition phenomena or cell differentiation. Of particular interest is the search for molecules that may be specific to only a single cell type, thus providing the opportunity to follow the progress of the cells during development. The application of immunological techniques has facilitated the search for these unique neural cell markers, particularly after the advent of the hybridoma technology of Kohler and Milstein (for a general review, see ref. 1). Recent studies using monoclonal antibodies (MAbs) have described neural-specific markers in cerebellum (2–5), spinal cord (6, 7), and neural retina (8–10). MAbs also have been used to identify the neuron-specific A2B5 ganglioside (11), oligodendrocyte antigens (12), and central nervous system-specific (13) and peripheral nervous system-specific (14) antigens.

As part of a broad program to identify and characterize cell-specific surface antigens in the chicken neural retina, we identified two novel antigens with a MAb designated C_1H_3. Both of these proteins, which appear to be distinct, are localized exclusively in the embryonal nervous system. The first antigen, a polypeptide of 140 kilodaltons (kDa), is unique to early retinal cells and is present on the surface of 70% of dissociated retina cells. To the best of our knowledge, no antigen that is uniquely localized to the early retina has been previously described. The second polypeptide, of size 170 kDa, is common to all neuronal tissue examined. However, it only accumulates late in development in the neural retina, concomitant with the diminished expression of the 140-kDa protein. We report here the characterization of these two polypeptides as distinct proteins and describe their developmental regulation in the neural retina.

MATERIALS AND METHODS

Preparation and Screening of MAbs. MAbs were prepared by the method of Galfre et al. (15) as described (16). Briefly, day 7 retinas were mechanically dissociated, and the intact cells were injected intravenously into the tail vein of Sprague–Dawley rats. Hybridomas secreting antibodies against subpopulations of embryonic chicken retina cells were screened by using a fluorescence-activated cell sorter (FACS) method developed in this laboratory (16). Briefly, mechanically dissociated retina cells from day 9 retina were cultured in Costar microtiter plates for 2–3 hr, followed by a 1-hr incubation with culture supernatants containing MAbs. Cells were then labeled with fluorescein-conjugated goat anti-rat IgG (Cappel Laboratories, Cochranville, PA) and identified in a FACS IV (Becton Dickinson, Sunnyvale, CA), equipped with a logarithmic amplifier. Positive clones were recloned by using the FACS, and MAbs were isolated either by saturated ammonium sulfate precipitation of culture medium or from ascites fluid obtained after growing hybridomas in BALB/c nude mice.

Immunocytochemical Localization of Antigens. Immunocytochemical localization of antibody binding was performed on 10-μm cryostat sections of retina fixed in periodate/lysine/paraformaldehyde (9) by the unlabeled enzyme method of Sternberger (17). Sections were incubated with culture supernatants and visualized by incubation with unlabeled goat anti-rat IgG and rat peroxidase-antiperoxidase (Pel-Freez) and by reaction with 3,3′-diaminobenzidine. Sections were photographed on a Zeiss Universal microscope with Kodak Tri-X Pan film.

Identification of Antigens Binding MAb. Retinas were removed from chickens of various embryonic ages and solubilized in boiling 2% NaDodSO$_4$/0.3 mM phenylmethylsulfonyl fluoride/1 mM benzamidine·HCl/1 mM EDTA. Proteins were electrophoresed on 5% polyacrylamide gels as described (9) and transferred to nitrocellulose following published methods (18). Nitrocellulose sheets were incubated with dilutions of ammonium sulfate-precipitated MAbs (equivalent to 10–15 ml of culture supernatant), followed by incubation with ^{125}I-labeled goat anti-rat IgG. Labeled antigens were visualized by exposing the nitrocellulose to Kodak X-Omat AR x-ray film.

Abbreviations: MAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; kDa, kilodalton.
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Characterization of Metabolically Labeled Antigen. Embryonic neural retinas of various ages (5×10^8 cells) were dissociated by either trituration or incubation with 0.05% trypsin. Cells were washed three times with methionine-free Dulbecco's minimal essential medium (DME, GIBCO) containing 10% dialyzed fetal calf serum (KC Biological, Lenexa, KS), glutamine (0.1 mg/ml) and 10 μM L-methionine. Cells were then incubated at 50 rotations per minute in 2 ml of medium containing either 100 μCi (1 Ci = 37 GBq) of [^{75}Se]selenomethionine (specific activity, 20–50 Ci/mmol, Amersham) or [^{35}S]methionine (specific activity, 1100–1200 Ci/mmol, New England Nuclear) for the indicated times. For pulse–chase experiments, cells were labeled with [^{35}S]methionine for 30 min, followed by transfer to media containing 2 mM L-methionine for the times indicated. After labeling, cells were washed three times in Hanks' balanced salt solution and homogenized in 0.4 ml of immunoprecipitation buffer (150 mM NaCl/20 mM Na$_2$HPO$_4$/1% Triton X-100/1% sodium deoxycholate/0.1% NaDodSO$_4$/1 mM benzamidine·HCl/0.3 mM phenylmethylsulfonyl fluoride/1.0 mM methionine, pH 7.5) as described (19). [^{35}S]Methionine incorporation was quantitated by precipitation of an aliquot of the homogenate in 10% trichloroacetic acid at 4°C for 30 min. Trichloroacetic acid-precipitable material then was collected by filtration on DA filters (Millipore) and washed twice with 5% trichloroacetic acid and once with absolute ethanol.

C$_1$H$_3$ antigen(s) was immunoprecipitated according to published methods (19). Briefly, homogenates were incubated at 4°C with appropriate dilutions of ammonium sulfate-precipitated MAb and were precipitated with goat anti-rat IgG-coated *Staphylococcus aureus* cells (Bethesda Research Laboratories). Then the isolated *S. aureus* cells were suspended in 50 μl of electrophoresis sample buffer and heated to 100°C for 5 min; immunoprecipitated polypeptides were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis.

For peptide mapping of C$_1$H$_3$ antigens, dissociated cells were labeled with [^{75}Se]selenomethionine as described (this radioactive precursor was used because of its high energy of emission and, thus, greater sensitivity in peptide mapping), followed by immunoprecipitation and polyacrylamide gel electrophoresis. Bands were identified by autoradiography, cut from the gel, and incubated overnight at 37°C with 300 μg of subtilisin BPN' protease (Sigma) in 0.05 M NH$_4$HCO$_3$ (pH 8.0) as described (20). The amount of protease used for digestion was in excess of substrate and therefore ensured complete digestion of the C$_1$H$_3$ antigens. Peptide fragments were then separated by HPLC on a Waters C$_{18}$ micro bonded column using a 0–60% gradient of acetonitrile programmed at 0.5%/min. Fractions (0.5 min) were collected and counted in a Beckman gamma counter.

RESULTS

In order to identify antigens present on the cell surface of retina cells that are viable in culture, our laboratory has developed a simple screening method using a FACS (16). One advantage of this method is that MAbs can be readily identified that bind to subpopulations of retina neurons. Because the antigen is restricted to the cell surface, it should be possible to isolate the subpopulations of cells and to maintain them in culture, thus permitting a more detailed biochemical analysis of the cells than can be obtained *in vivo*. In the present study, we concentrated on a single MAb identified by using the FACS screening protocol. The MAb C$_1$H$_3$ reacts with the majority of retina neurons (70% of total cells) as demonstrated by FACS analysis (Fig. 1). It can be seen in Fig. 1 that most retina cells possess a fluorescence intensity greater than background levels, indicating the presence of C$_1$H$_3$ antigen on the cell surface. The high fluorescence intensity of the positive cells also shows that a large number of
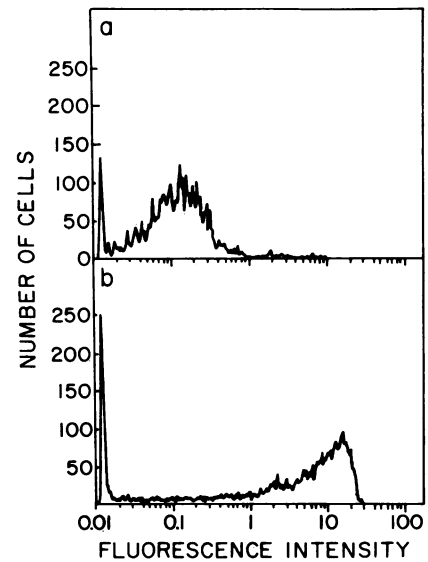

FIG. 1.   MAb binding to embryonic day 9 retina. Dissociated retinal cell monolayers were incubated with culture supernatants from parent cell line (*a*) or from C$_1$H$_3$ hybridoma (*b*). Binding was visualized with fluorescein-conjugated anti-rat IgG and analyzed on a FACS IV equipped with a logarithmic amplifier. These results show that the C$_1$H$_3$ MAb binds 70% of viable cells from cultured day 9 retina.

antigenic sites exist for C$_1$H$_3$ on the cell surface.

The localization of the C$_1$H$_3$ antigen to specific cell layers of the retina was determined by immunoperoxidase staining (Fig. 2). The distribution of the antigen in immature retina (day 7) is shown in Fig. 2*a*. The MAb appeared to stain most cell types at this age (in agreement with FACS analysis), particularly the ganglion cells. This conclusion is based on the pronounced staining of the most vitreal surface of the retina, which has been shown to contain the ganglion cells and the developing nerve fiber layer (21). In day 12 retina (Fig. 2*b*), the plexiform and nerve fiber layers were stained with the
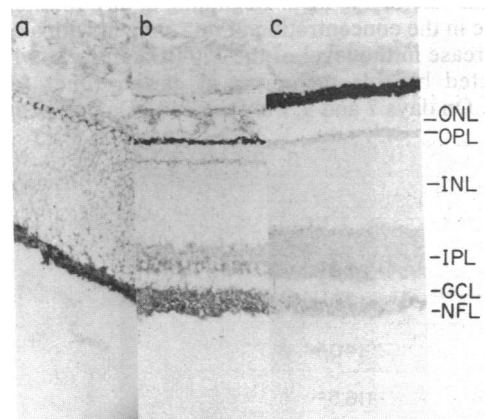

FIG. 2.   Immunocytochemical localization of C$_1$H$_3$ MAb binding. Cryostat sections (10-μm) from day 7 (*a*), day 12 (*b*), or day 19 (*c*) retina were incubated overnight at 4°C with culture supernatants. Sections were then incubated with goat anti-rat whole serum (1:10) and peroxidase-antiperoxidase (1:100). MAb binding was visualized by reaction with 0.05% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in 0.05 M Tris·HCl (pH 7.4). Note that most cells are labeled to varying extents at all ages; however, the most apparent developmental difference in staining is observed in the nerve fiber layer at days 7 and 12 relative to day 19. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. ($\times$140.)

greatest intensity. In contrast, the inner nuclear and ganglion cell layers showed only light staining. By embryonic day 19 (Fig. 2c), the pattern of staining was relatively unchanged, except that the intensity of staining of the nerve fiber layer had decreased when compared to day 12. This result may imply that there is an altered distribution of the C_1H_3 antigen in retina as the developing retina matures because with increasing age there would be an augmented concentration of neural membrane in the nerve fiber level and, hence, an anticipated increase in staining at day 19. These data also indicate that the C_1H_3 antigen is enriched in ganglion cell bodies and processes in early retina but is distributed more evenly in the fiber layers as the tissue matures. It was also apparent in day 19 retina that the inner nuclear and ganglion cell layers show low-level staining and that the outer nuclear and photoreceptor layers are not stained.

The antigen recognized by the C_1H_3 MAb was identified by immunoblotting of electrophoretically separated retina cell proteins. Fig. 3 shows the results of immunoblotting of protein extracts from embryonic day 9 retina and from other neural and nonneural tissues. In day 9 retina the C_1H_3 MAb reacted with a 140-kDa polypeptide. In contrast, in day 9 brain or tectum and in day 11 sympathetic ganglia, the antibody recognized a 170-kDa protein. The C_1H_3 MAb did not react with any polypeptide species in day 9 liver, heart, or fibroblasts (Fig. 3) or with any component in day 6 liver and heart or day 9 gut, esophagus, and thigh muscle (data not shown). These results indicate that the C_1H_3 antigen(s) is a neural-specific protein and illustrate the surprising observation that the MAb recognizes polypeptides of different apparent molecular size in neural retina and other regions of the nervous system.

As a first step in discerning the relationship between these two different polypeptides, we examined possible developmental changes in the antigen both in brain and neural retina. To assess developmental control of the antigen, proteins of different regions of the embryonic nervous system were separated on polyacrylamide gels and transferred to nitrocellulose. In the neural retina there was a striking change in this antigen during development; at early developmental times (before day 10), the only form of the antigen had an apparent molecular size of 140 kDa. Beginning at day 10, there was a decrease in the concentration of this antigen with a concomitant increase in the level of the 170-kDa antigen, which was undetected by this technique at earlier times (data not shown). On days 7 and 9, only the 140-kDa polypeptide was
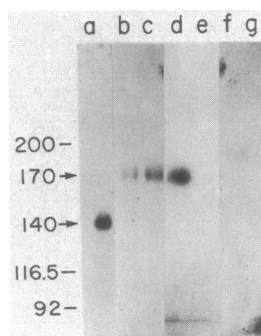
present in retina (Fig. 4a). By day 12 a significant amount of the 170-kDa polypeptide could be detected, in addition to the antigen of lower molecular size. By day 17 our results showed that the predominant C_1H_3 antigen in retina is the 170-kDa species.

We also carried out similar developmental studies using protein obtained from optic tectum and brain of the developing chicken embryo. Brain at all developmental stages contained only the 170-kDa antigen (Fig. 4b). The optic tectum also contained this antigen at all developmental stages; however, between days 12 and 17, the 140-kDa form of the antigen was present also in low amounts in the optic tectum. In light of evidence indicating that a small number of ganglion cell fibers begin to innervate the tectum at day 6, with the majority of fibers reaching the tectum at days 10–12 (22), it is likely that the 140-kDa component is only found in optic tectum as a result of innervation by ganglion cell axons.

Analysis of the C_1H_3 antigens by immunoblotting did not clarify the relationship between the 140- and 170-kDa polypeptides. Therefore, additional studies were necessary to gain insight into the relationship between these molecules. For example, the 140-kDa protein could arise from the 170-kDa polypeptide by proteolytic cleavage or the 170-kDa protein could arise from the 140-kDa protein by extensive glycosylation. In order to ascertain if the 140- and 170-kDa polypeptides are structurally related proteins (differing only as a result of processing) or if the polypeptides are distinct molecules sharing a common antigenic determinant, retina cells were metabolically labeled and the two antigens were isolated by immunoprecipitation and characterized as described below.

Initial attempts to label C_1H_3 antigens involved labeling of trypsin-dissociated retinal cells from 12-day-old embryos with either [^{35}S]methionine or [^{75}Se]selenomethionine. The 170-kDa component was the major protein detected after immunoprecipitation (Fig. 5a). Labeling of trypsin–dissociated or mechanically dissociated day 7 retina cells surprisingly also showed that the 170-kDa polypeptide was the major C_1H_3 reactive antigen detected, although small amounts of the 140-kDa protein also were synthesized (Fig. 5b). This is in sharp contrast to immunoblotting analysis, which indicated that the 170-kDa protein was not detectable in day 7 cells.

It appeared possible that the discrepancy between data obtained by immunoblotting, which measures the steady-state level of the antigen, and the metabolic labeling experiments,
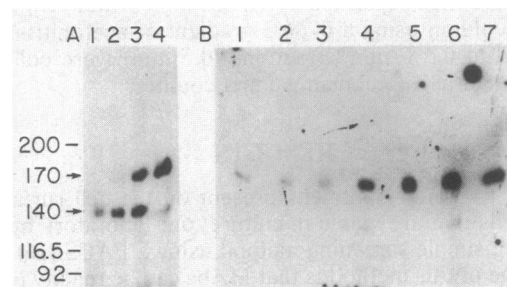

FIG. 3. Identification of C_1H_3 antigen and its tissue distribution by immunoblotting. Extracts from various embryonic tissues (75 µg of protein per lane) were separated on 5% polyacrylamide gels and transferred to nitrocellulose. The C_1H_3 antigen was visualized by incubation with MAb, followed by ^{125}I-labeled goat anti-rat IgG. Antigens were then visualized by autoradiography. Lanes: a, embryonic day 9 retina; b, day 9 brain; c, day 9 optic tectum; d, day 11 sympathetic ganglia; e, day 9 liver; f, day 9 skin fibroblasts; g, day 9 heart. Sizes are shown in kDa. Protein standards included myosin heavy chain (200 kDA), β-galactosidase (116.5 kDa), and phosphorylase b (92 kDa).

FIG. 4. Developmental regulation of the C_1H_3 antigen in retina (A) and brain (B). NaDodSO$_4$ extracts from embryonic retina, brain, or optic tectum (50 µg of protein each) were separated on 5% polyacrylamide gels and transferred to nitrocellulose as described in Fig. 3. (A) Developmental regulation of C_1H_3 antigen in neural retina. Lanes: 1, day 7; 2, day 9; 3, day 12; 4, day 17. (B) Developmental regulation of C_1H_3 antigen in embryonic brain and optic tectum. Lanes: 1, day 7 brain and optic tectum (at this stage of development, the developing optic tectum cannot be separated from the embryonic brain); 2, day 9 optic tectum; 3, day 9 brain; 4, day 12 optic tectum; 5, day 12 brain; 6, day 17 optic tectum; 7, day 17 brain. Note the presence of small amounts of the 140-kDa antigen in only day 12 and 17 optic tectum. Sizes are shown in kDa.
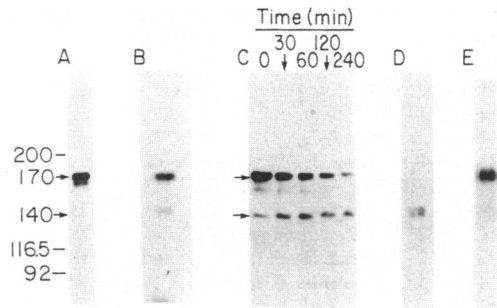
Neurobiology: Cole and Glaser

Proc. Natl. Acad. Sci. USA 81 (1984) 2263

FIG. 5. NaDodSO₄/polyacrylamide gel electrophoresis of metabolically labeled and immunoprecipitated C_1H_3 antigens. Cultures of embryonic retinal cells were incubated at 50 rotations per min and 37°C in the presence of either [^{35}S]methionine or [^{75}Se]selenomethionine as described. For immunoprecipitation, cell extracts were incubated with MAb and *S. aureus* cells coated with goat anti-rat whole serum. Immunoprecipitated polypeptides were then separated on 5% polyacrylamide gels and visualized by fluorography or autoradiography. Arrows denote the location of the C_1H_3 antigens; sizes are shown in kDa. (*A*) Immunoprecipitate of C_1H_3 antigens from day 12 retinas labeled 4 hr with [^{75}Se]selenomethionine. (*B*) Immunoprecipitate of C_1H_3 antigens from day 7 retinas labeled 4 hr with [^{75}Se]selenomethionine. (*C*) Pulse–chase labeling of day 7 retinal cell proteins. Cells were labeled for 30 min with [^{35}S]methionine and chased for the times indicated in cold methionine. C_1H_3 antigens were immunoprecipitated as described. The band at 160-kDa most likely represents a proteolytic fragment of the 170-kDa species because the 160-kDa fragment is degraded with similar kinetics and is not always detected after immunoprecipitation (as seen in *B*). (*D*) Immunoprecipitate of C_1H_3 antigens after *in vivo* labeling of day 7 chicken embryos. [^{35}S]Methionine (50 μCi) was injected into the vitreous of one eye, and retinas were isolated 3 hr after injection. C_1H_3 antigens were immunoprecipitated as described. Note only the synthesis of the 140-kDa C_1H_3 antigen by intact day 7 retinas. (*E*) Immunoprecipitate of C_1H_3 antigens from incubation medium of retinal cells pulse-labeled 6 hr with [^{75}Se]selenomethionine. Only the 170-kDa C_1H_3 antigen is released from the cells into the medium.

which measure the rate of antigen synthesis, could be due to changes in the biosynthetic program induced by cell dissociation. To test this possibility, retinas were labeled *in vivo*. Under these conditions only the 140-kDa polypeptide was detected by immunoprecipitation of the radioactive cells (Fig. 5*d*). This observation indicates that day 7 retina has the potential of synthesizing the 170-kDa protein but it does not accumulate in the cells in detectable amounts. One possible explanation for these results is that the 170-kDa protein is a precursor of the 140-kDa protein and that post-translational modification of the polypeptide is not completed in dissoci-

ated cells. When retina cells in culture were pulse-labeled 30 min with [^{35}S]methionine and then transferred to unlabeled medium, the 170-kDa polypeptide showed a rapid apparent turnover while the 140-kDa polypeptide was relatively stable (Fig. 5*c*). These data appear to rule out a precursor–product relationship between the two antigens. Treatment of the 170-kDa polypeptide with exo- and endoglycosidases also did not give rise to a protein that comigrates with the 140-kDa polypeptide, and metabolic labeling of the antigens with [^{3}H]glucosamine showed that the 140-kDa component was labeled to a greater extent and, thus, more glycosylated than the 170-kDa antigen (data not shown). In addition, the apparent turnover of the 170-kDa polypeptide was accounted for, at least in part, by secretion into the surrounding medium. After incubation of neural retina cells with [^{35}S]methionine, the 170-kDa polypeptide, but not the 140-kDa polypeptide, could be detected in the medium (Fig. 5*e*).

A precursor–product relationship between these two antigenically related proteins would be precluded if the proteins were found to be largely distinct by peptide mapping. Fig. 6 shows peptide maps obtained by HPLC of [^{75}Se]selenomethionine-labeled C_1H_3 antigens isolated by immunoprecipitation. The absence of pronounced similarities in the elution pattern of the peptide fragments in Fig. 6 demonstrates conclusively that the 140- and 170-kDa polypeptides are distinct proteins that share a common antigenic determinant.

DISCUSSION

To the best of our knowledge an antigen that uniquely identifies cells at early stages of development in the chicken neural retina (or retinas of other species) has not been described. The results described in this study strongly suggest that the 140-kDa protein identified in early neural retina represents such a cell surface marker. While the C_1H_3 MAb recognizes a second polypeptide present in nervous tissue, the two polypeptides appear to be distinct molecules that are not biosynthetically related but share an antigenic determinant. The recognition of several distinct proteins by one MAb has been documented (23, 24). The apparent change in histological staining of the antigens recognized in neural retina by C_1H_3 also suggests that the 140- and 170-kDa polypeptides have different cellular distributions or, at least, are present in the same cell types but in different concentrations. For example, the apparent reduction of immunoperoxidase staining in the nerve fiber layer (Fig. 2 *b* and *c*) is also temporally correlated with the shift in appearance of the two antigens.

Analysis of the developmental expression of the two antigens in brain and tectum suggests that the 140-kDa polypep-
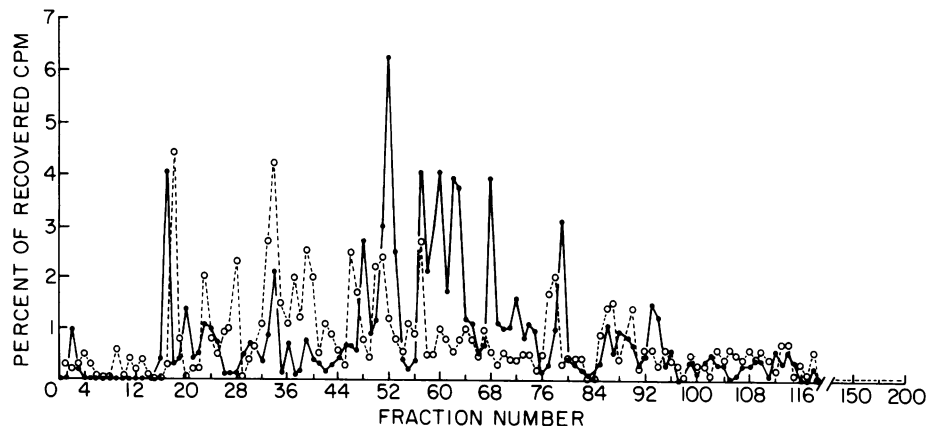

FIG. 6. Analysis of peptide fragments of C_1H_3 antigens by HPLC. Immunoprecipitated C_1H_3 antigens were separated on 5% polyacrylamide gels and visualized by autoradiography, and then the bands were cut from the gel. The polypeptides were then treated with 300 μg of subtilisin BPN protease as described. Peptide fragments were separated on a Waters C_{18} micro bonded column with a 0.5%/min gradient of acetonitrile. Individual fractions (0.5 min) were graphed with respect to radioactivity: ●, 170-kDa polypeptide; ○, 140-kDa polypeptide.

tide is retina-specific. Small quantities of the 140-kDa polypeptide are detected in the optic tectum; however, expression of this antigen in the tectum strongly suggests that it only appears in the tectum after innervation by retina ganglion cell axons. To unequivocally demonstrate that the 140-kDa protein is retina-specific, it will be necessary to show that the expression of the molecule in the tectum is dependent on innervation by ganglion cell axons. Disruption of innervation by removal of the optic cup should indicate if such a dependence exists.

Although the function of the two C_1H_3-reactive polypeptides is unknown, their expression during development implies that their functions are also distinct. At the present time, we can only speculate on the possible function of these molecules. The presence of a cell surface marker that uniquely identifies early retina cells directly leads to the postulate that this molecule participates in cell recognition processes and/or in the identification of retinal cells as a distinct subpopulation of differentiating cells. It should be feasible in the future to select monoclonal antibodies that only recognize the 140-kDa polypeptide and to use these antibodies to assess the function of this protein by impairing normal development. The highly restricted cellular localization of the 140-kDa antigen also should permit studies aimed at the delineation of the appearance and migration of presumptive neural retina cells from the primitive ectoderm of the developing chicken embryo.

The broad distribution of the 170-kDa polypeptide does not provide any obvious clues regarding the role of this nervous tissue-specific antigen in neural retina development. Immunocytochemical localization of the 170-kDa polypeptide during late stages of development has shown that it is enriched in the nerve fiber and plexiform layers, which suggests that the molecule could be localized to synapses. In the light of the demonstration that *in vitro* the 170-kDa polypeptide is secreted by retinal cells, one may speculate that this molecule functions in cell adhesion systems. This proposal is based on evidence that other cell adhesion molecules, such as N-CAM (25) and D2 (26), have been shown to be released from the cell surface. In addition, a polypeptide of similar molecular size is present in embryonic chicken retinal cell adherons (27).

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