

## An Immunocytochemical Comparison of Müller Cells and Astrocytes in the Cat Retina

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Immunocytochemical localization, at the light and electron microscopic levels, of five different known glial proteins was used to compare Müller cells with astrocytes in the adult cat retina. Retina from two different areas of the eye was examined. A region of retina on the border of the optic nerve was used because of its large population of astrocytes, and a region away from the optic nerve was used to examine Müller cells (astrocytes are sparse in this region). Antibodies to cellular retinaldehyde binding protein and glutamine synthetase labeled the Müller cells but not the astrocytes, while labeling with anti-carbonic anhydrase C, anti-alpha crystallin and anti-glial fibrillary acidic protein was found in both Müller cells and astrocytes.

*Key words:* Müller cells; astrocytes; retina; glial fibrillary acidic protein; cellular retinaldehyde binding protein; glutamine synthetase; carbonic anhydrase C; alpha crystallin; immunocytochemistry.

### 1. Introduction

The vascularized retina of adult vertebrates contains two distinct macroglial cell types: Müller cells and astrocytes (Polyak, 1941). Although Müller cells are generally considered as highly specialized astrocytes, and both cell types differentiate from neuroectoderm, they assume different locations and have very different morphologies in the adult retina. Müller cell nuclei reside in the inner nuclear layer and their cellular processes are oriented radially between the outer retina, where they form junctions with each other and photoreceptor cells in an arrangement known as the outer limiting membrane, and the inner retina where their 'endfeet' border the vitreous cavity. The nuclei and cellular processes of the astrocytes are usually found closely associated with ganglion cell bodies or axons, and in the optic nerve region (Lessell and Kuwabara, 1963; Ramón y Cajal, 1972 translation; Ogden, 1978; Bussow, 1980; Schnitzer, 1985; Karschin, Wässle and Schnitzer, 1986a, b). An understanding of the differential expression of proteins by these cells will contribute to an understanding of their functions in the eye. In addition, because there is substantial interest in culturing these two cell types, it is necessary to find characteristics that allow them to be distinguished in conditions where they lose their highly differentiated morphologies.

Most immunocytochemical comparisons of Müller cells and retinal astrocytes have been restricted to glial fibrillary acidic protein (GFAP) (Björklund, Bignami and Dahl, 1985; Schnitzer, 1985; Karschin, Wässle and Schnitzer, 1986b), an intermediate filament (10-nm diameter) protein (Reuger, Huston, Dahl and Bignami, 1979; Eng, 1982). In this study we expand the comparison to include four other proteins thought to associate with one or the other of these cell types: glutamine synthetase (GS), an enzyme used for the conversion of glutamate to glutamine (Meister, 1974), carbonic anhydrase C (CAC), an enzyme that catalyzes the conversion of carbon dioxide to

carbonic acid (Carter, 1972), cellular retinaldehyde binding protein (CRALBP), a retinoid binding protein thought to be involved in the visual cycle (Futterman, Saari and Blair, 1977) and alpha crystallin (AC), a major lens protein (Piatigorsky, 1981) that has also been localized in Müller cells (Moscona, Fox, Smith and Degenstein, 1985). Using both light and electron microscopic immunocytochemistry we show here that all five antibodies are present in the Müller cells but only GFAP, CAC and AC are present in the retinal astrocytes.

## 2. Materials and Methods

### *Tissue preparation*

Adult cats ( $n = 3$ ) were killed by an overdose of sodium pentobarbital and the eyes were enucleated. After removal of the cornea, lens and vitreous the eyes were cut into quadrants and fixed for 1 hr with 1% paraformaldehyde and 1% glutaraldehyde in sodium phosphate buffer. The tissue was then dehydrated in increasing concentrations of methanol with the addition of uranyl acetate during the 70% incubation period and embedded in LR White resin (Polysciences; Erickson, Anderson and Fisher, 1987).

### *Antisera*

Rabbit anti-bovine-GFAP (DAKO Corp.) and rabbit anti-bovine-CRALBP (gift from Dr John Saari) were used at a dilution of 1:400. Rabbit anti-chicken-GS and CAC (gifts from Dr Paul Linser) and rabbit anti-mouse-AC (gift from Dr Joram Piatigorsky) were used at a dilution of 1:600. All antibodies were diluted with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Normal goat serum (NGS) (Vector Labs - diluted 1:75 with PBS) was used as a blocking agent on each section before applying the primary antibodies. Goat anti-rabbit IgG-40- or 20 nm gold (Janssen Pharmaceutica) was used at a 1:20 dilution for electron microscopic immunocytochemistry (EM ICC). Goat anti-rabbit IgG-5 nm gold (Janssen) was used at a 1:20 dilution for light microscopic immunocytochemistry (LM ICC) and the 5 nm gold was amplified with a silver-enhancing solution (Janssen).

### *Immunocytochemical procedures*

Sections (1  $\mu\text{m}$  thick) placed on Formvar-coated glass slides were used for the LM ICC procedure. The sections were covered with NGS for 30 min, blotted, then incubated overnight with the primary antibody. The next day, sections were rinsed with PBS-BSA and the secondary antibody was added for 1 hr. Sections were subsequently rinsed with PBS, post-fixed with 2% glutaraldehyde in PBS for 15 min, rinsed again with PBS and then with distilled water. At this point Janssen's silver enhancer kit was used; the enhancer was added to the sections for 5 min  $\times$  2 after which they were rinsed, fixed for 2 min in the supplied fixer, rinsed again and air-dried.

Thin sections for EM ICC were cut from the same blocks of tissue that were used for LM ICC. The sections were first placed on nickel grids, incubated on drops of NGS for 30 min, blotted and incubated overnight on the primary antibody. The next day the grids were rinsed with PBS-BSA and transferred to drops of the secondary antibody for 1 hr. After rinsing with PBS-BSA and distilled water, the grids were stained with uranyl acetate for 15 min and lead citrate for 7 min in a LKB UltroStainer and exposed to osmium tetroxide vapor (2% osmium in PBS) for 1 hr and then carbon-coated in a vacuum evaporator.

Control sections for the ICC were processed at the same time as the experimental LM and EM sections. Normal rabbit serum or PBS-BSA was used in place of the primary antibody.

### *Protein gel electrophoresis and immunoblots*

Cat retinas ( $n = 3$ ) were homogenized in PBS containing 0.1 mM phenylmethylsulfonyl fluoride to inhibit protein degradation. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Fifty  $\mu\text{g}$  of protein was

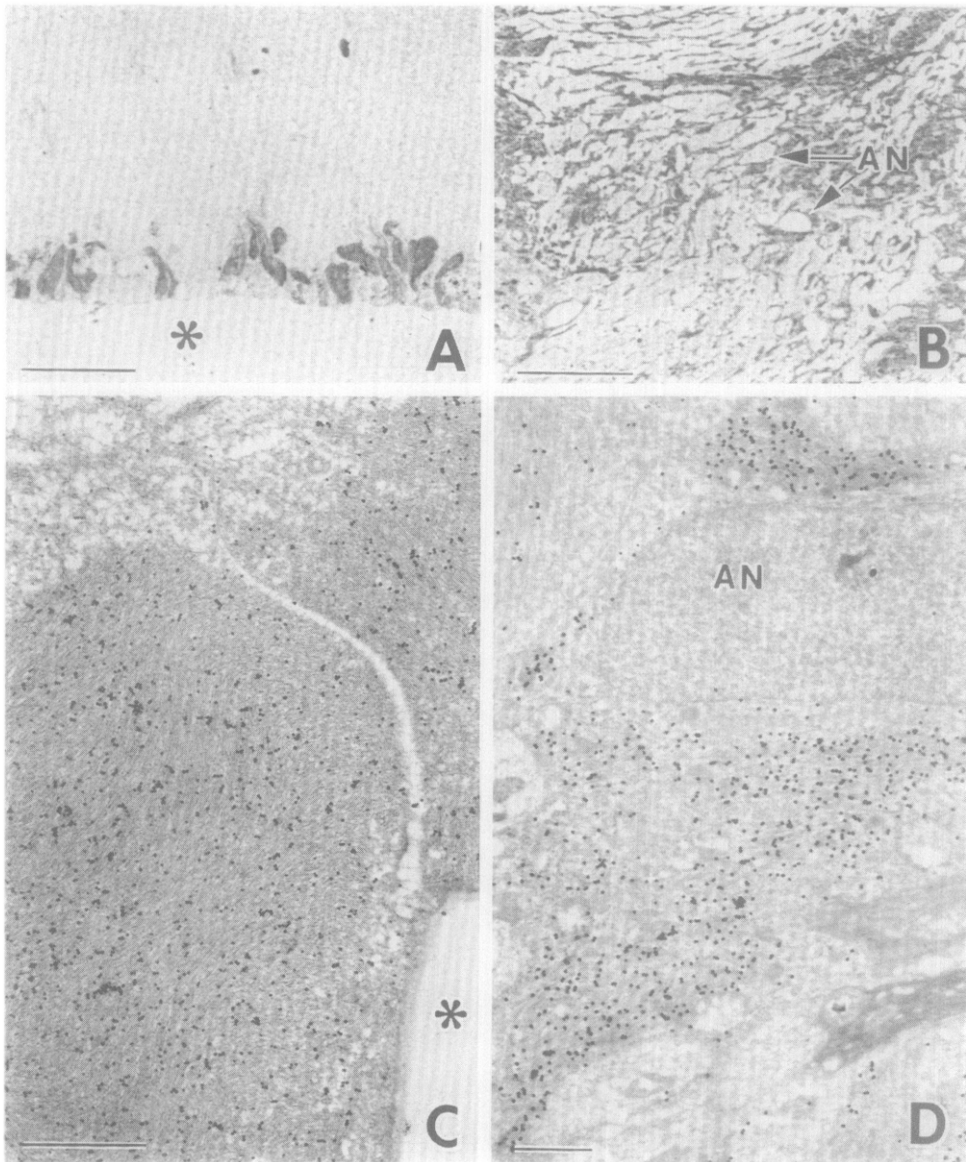


FIG. 1. Light (A,B) and electron (C,D) micrographs of anti-GFAP labeling. GFAP labeling is seen only over the Müller cell endfeet in the retina (A,C). In the optic nerve head, GFAP is present in the astrocyte processes (B,D), and can be seen encircling the numerous astrocyte nuclei (AN). In both cases, GFAP is located primarily over the 10-nm intermediate filaments. (Scale bars: A,B 25  $\mu$ m; C,D 1  $\mu$ m; \* vitreous cavity.)

analyzed by SDS-PAGE using a 10% gel. Proteins of known molecular weights (Bio-Rad) were used as standards.

Immunoblots were performed by transferring the retinal proteins to nitrocellulose paper, blocking with 3% BSA in Tris-buffered saline (TBS) and incubating overnight in primary antibody (diluted 1:100 in 1% BSA in TBS). The next day the blots were washed in Tween-Tris-buffered saline (TTBS) (0.05% Tween 20) and incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Bio-Rad) for 1 hr. Blots were then washed

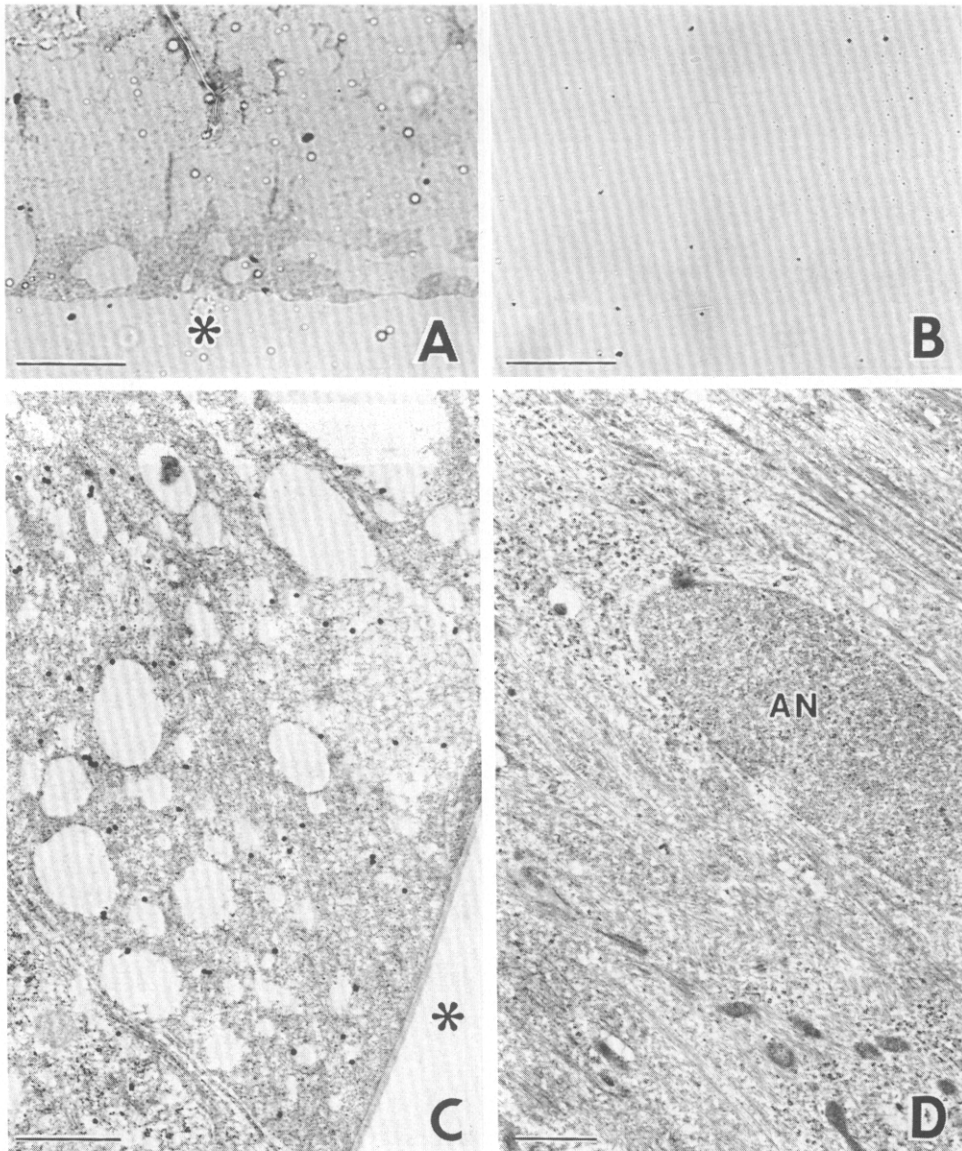


FIG. 2. Light (A,B) and electron (C,D) micrographs of anti-CRALBP labeling. CRALBP appears to be located only in Müller cells (A,C) with the heaviest labeling in the endfeet. No labeling is detectable in astrocytes (B,D) at either the light or electron microscopic level. (Scale bars: A,B 25  $\mu$ m, C,D 1  $\mu$ m; AN, astrocyte nucleus; \* vitreous cavity.)

in TTBS and then TBS and placed in HRP Color Development Reagent (Bio-Rad) for 15 min. After a distilled water rinse, the immunoblots were air-dried and photographed.

### 3. Results

The results of immunocytochemical labeling by the five antibodies at both the light and electron microscopic level are shown in Figs 1–6. Each figure compares sections

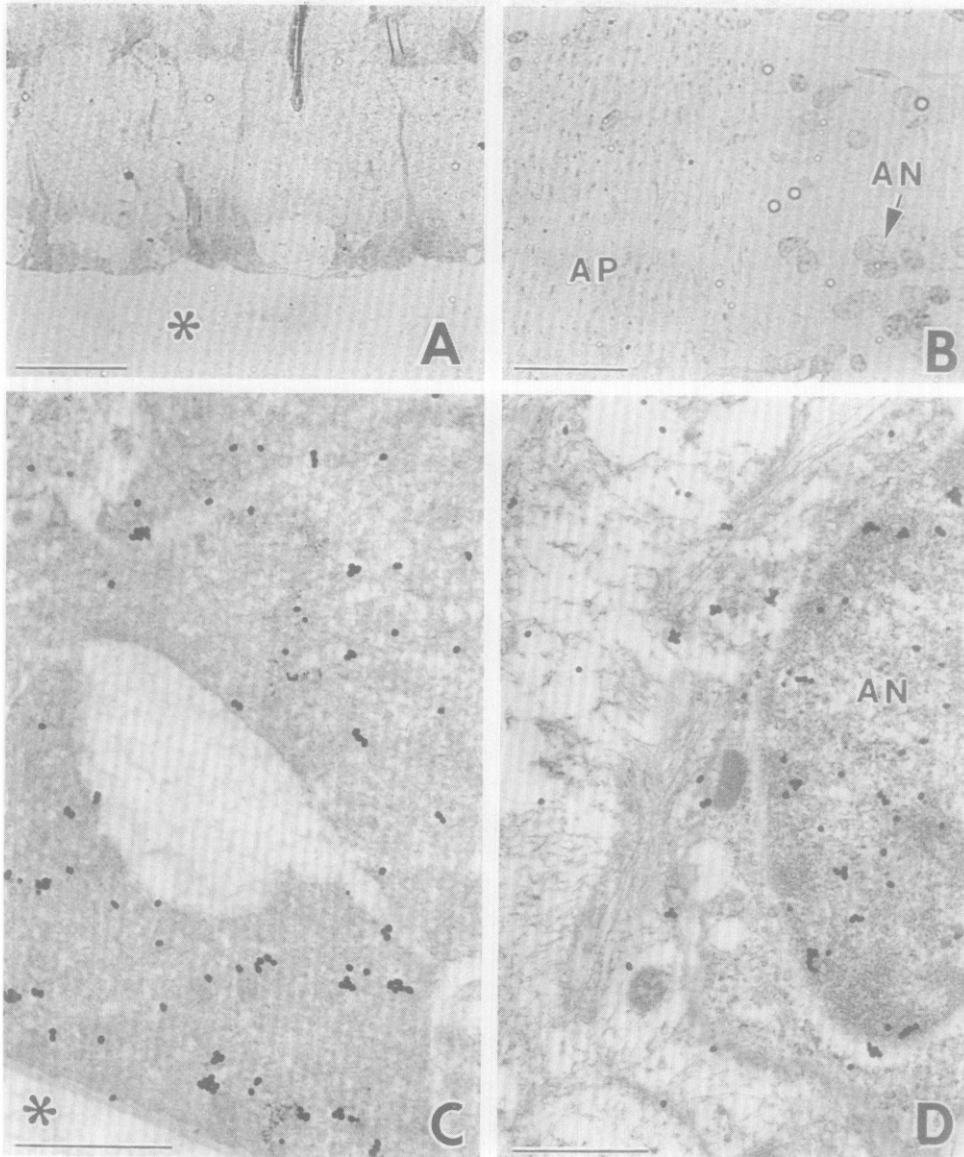


FIG. 3. Light (A,B) and electron (C,D) micrographs of anit-GS labeling. GS is located throughout Müller cells (A,C), with the heaviest labeling in the endfeet. No labeling is seen in astrocytes (B,D) at either the light or electron microscopic level. (Scale bars: A,B 25  $\mu$ m. C,D 1  $\mu$ m; AN, astrocyte nucleus; \* vitreous cavity.)

of optic nerve head, located directly adjacent to the retina where astrocytes are numerous (Bussow, 1980; Karschin, Wässle and Schnitzer, 1986b), with sections of retina away from the optic nerve head where Müller cells are the predominant glia. Astrocytes in the optic nerve head display the same labeling pattern as do the astrocytes within the retina. Only the endfeet of the Müller cells (adjacent to the vitreous border) are shown in the electron micrographs. It should be noted that the antibody labeling method used in this study is a post-embedding technique in which

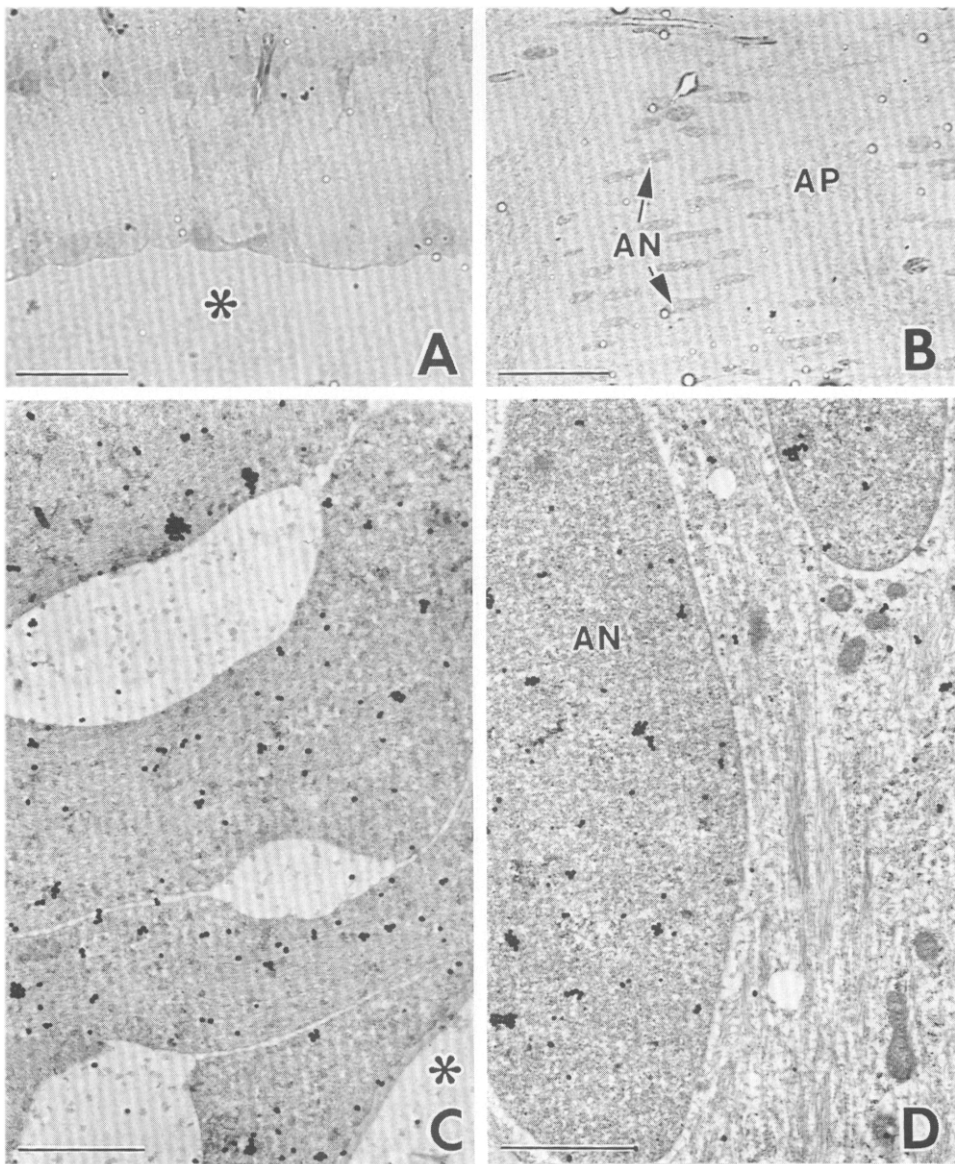


FIG. 4. Light (A,B) and electron (C,D) micrographs of anti-'CAC' labeling. 'CAC' appears to be present in Müller cells (A,C) and in the cytoplasmic processes (AP) and nuclei (AN) of astrocytes (B,D). (Scale bars: A,B 25  $\mu$ m, C,D 1  $\mu$ m; \* vitreous cavity.)

only surface antigens on the sections are labeled. This can result in a labeling intensity that appears less than in studies utilizing frozen or paraffin sections or whole cells where antigen throughout the thickness of the preparation may be labeled.

Anti-GFAP labeling of Müller cell endfeet occurred mostly over intermediate (10-nm diameter) filaments, with some diffuse cytoplasmic labeling also present (Fig. 1A,B). Anti-GFAP also labeled intermediate filaments and a diffuse cytoplasmic component of the astrocytes in the retina and optic nerve region (Fig. 1C,D).

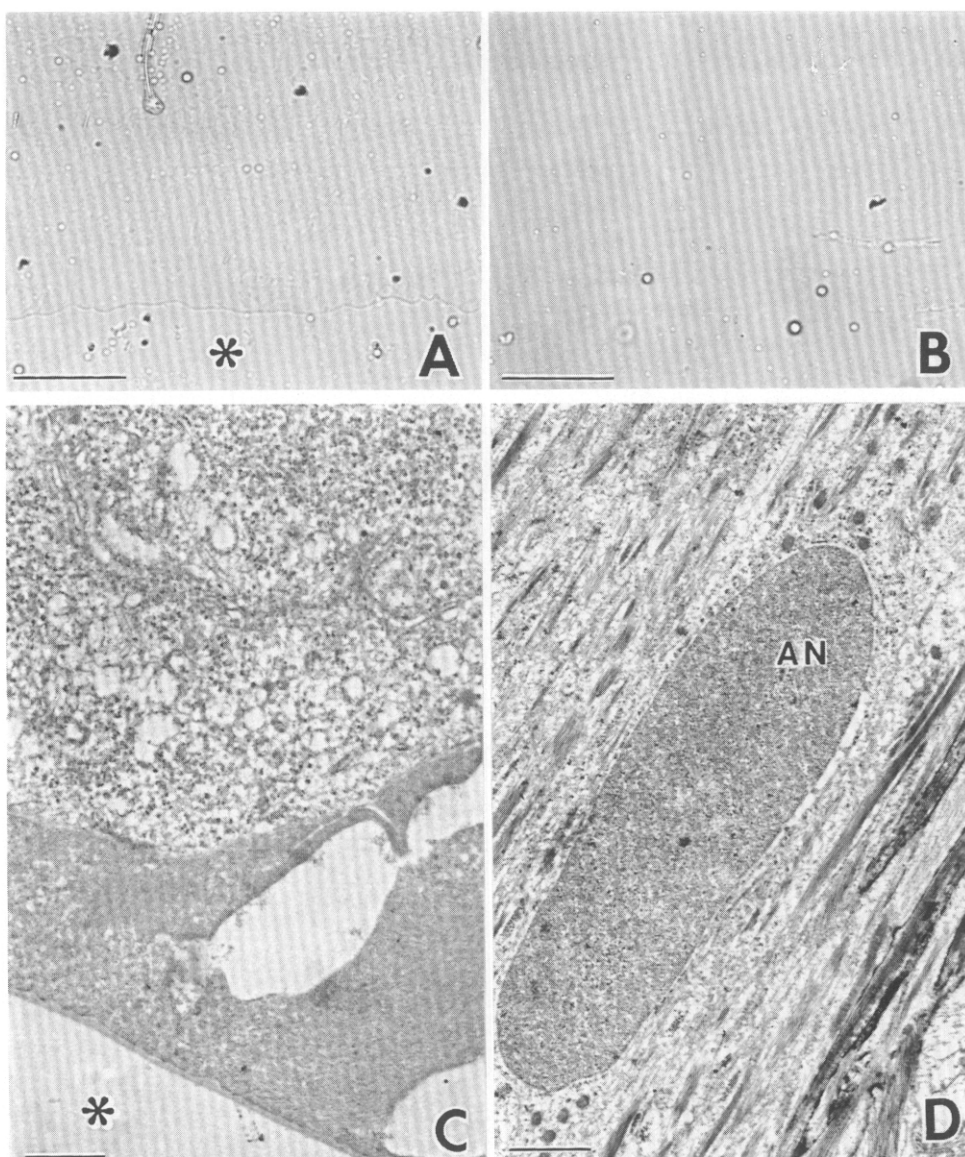


FIG. 5. Light (A,B) and electron (C,D) micrographs of anti-AC labeling. AC is located in the Müller cells (A,C) and in the processes (AP) and nuclei (AN) of astrocytes (B,D). (Scale bars: A,B 25  $\mu$ m, C,D 1  $\mu$ m; \* vitreous cavity.)

Anti-CRALBP diffusely labeled the entire Müller cell in the retina (Fig. 2A,B). Astrocytes were not labeled with this antibody by either the LM or EM procedure (Fig. 2C,D) even though on the same tissue section the Müller cells were clearly labeled.

Anti-GS labeled Müller cells in the retina (Fig. 3A,B) but it did not label the astrocytes (Fig. 3C,D). The GS localized in Müller cells appeared throughout the cell cytoplasm including some nuclei, with the highest concentration in the endfeet.

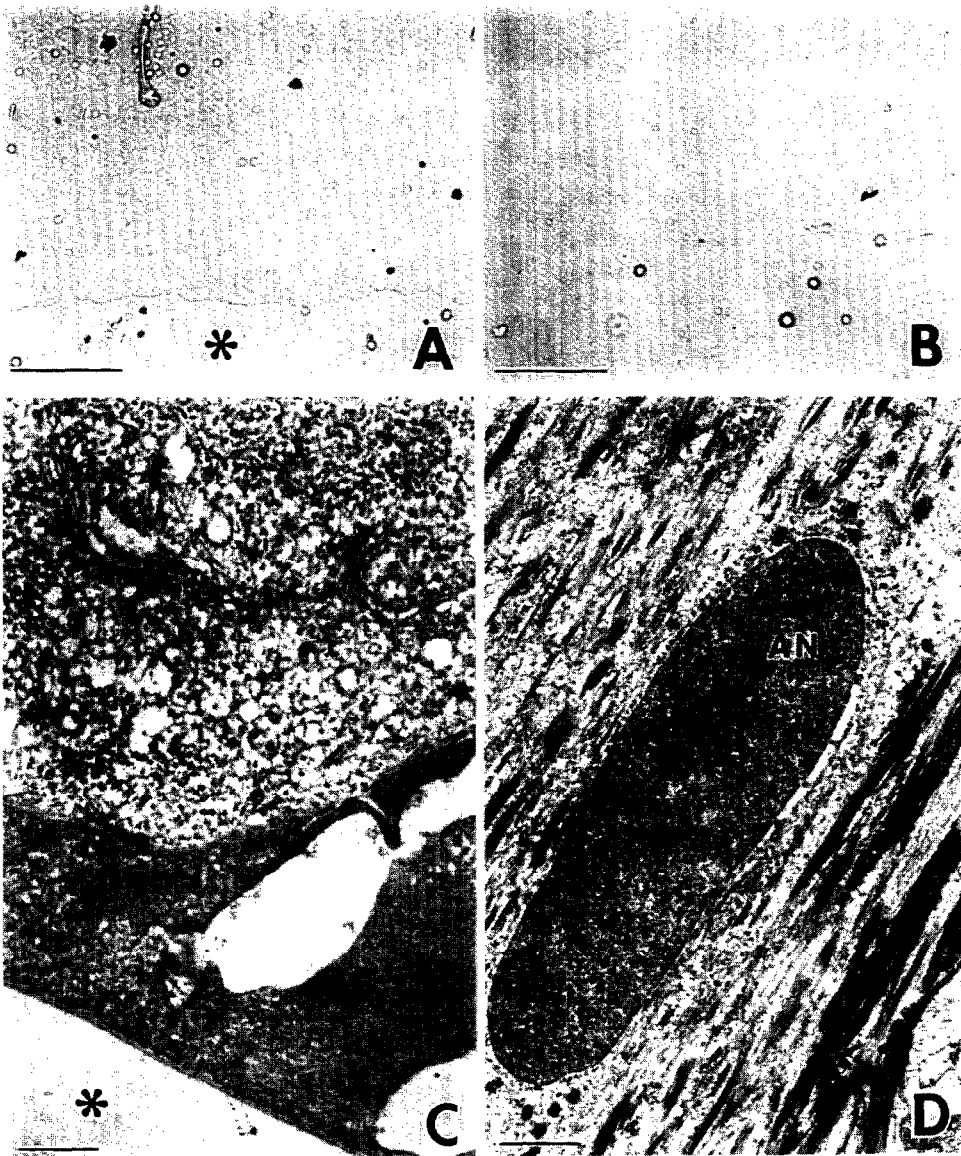


FIG. 6. Immunocytochemistry control sections of retina (A,C) and optic nerve head (B,D) at both the light (A,B) and electron (C,D) microscopic level are unlabeled. (Scale bars: A,B 25  $\mu$ m. C,D 1  $\mu$ m; \* vitreous cavity; AN, astrocyte nucleus.)

Anti-CAC (Fig. 4) and anti-AC (Fig. 5) bound to both Müller cells and astrocytes throughout the cell including some nuclei. The ICC control sections show essentially no gold particles in either the retina or optic nerve head region (Fig. 6).

Immunoblots (Fig. 7) of whole retina homogenates show the presence of single bands at appropriate molecular weight for each antibody used with the exception of AC (Fig. 7F). With AC, two bands are seen, one at 20000 and one at 22000 KDa. These have previously been identified as the alpha and beta subunits of AC (Bloemendal, 1981).



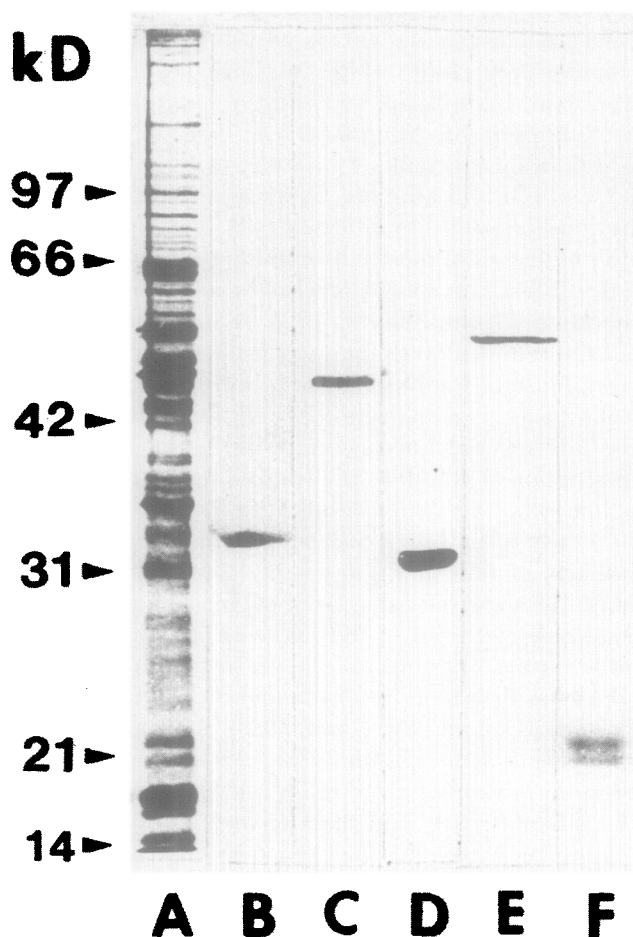


FIG. 7. Coomassie Blue-stained SDS-PAGE gel of proteins of cat retinal homogenates (Lane A) and Western blots (Lanes B-F). Bands at the appropriate molecular weights are shown for all antibodies used. Anti-CRALBP labels a band at 33 kDa (Lane B), anti-GS at 45 kDa (Lane C), anti-CAC at 31 kDa (Lane D), anti-GFAP at 51 kDa (Lane E) and anti-AC at 20- and 22 kDa (Lane F). The doublet shown in Lane F probably represents the alpha and beta subunits of AC.

#### 4. Discussion

The purpose of this study was to make a detailed comparison of antigens present in Müller cells and astrocytes by using antibodies to five different known glial proteins. Such a comparison and the subcellular localization of each protein may help us to understand the specialized functions of these two cell types. The results reported here show that while some proteins are common to both glial cell types, others are not.

The intermediate filament protein, GFAP, has previously been shown to occur in normal Müller cells (Björklund, Bignami and Dahl, 1985; Bignami and Dahl, 1979; O'Dowd and Eng, 1979; Ohira, Oshima and Kikuchi, 1984; Erickson, Fisher, Guerin, Anderson and Kaska, 1987a) and in normal retinal astrocytes (Schnitzer, 1985; Karschin, Wässle and Schnitzer, 1986a, b; Björklund, Bignami and Dahl, 1985) using

immunocytochemical labeling as the criterion. It was, however, commonly reported that GFAP occurred in most species only in Müller cells after retinal injury or during degeneration (Bignami and Dahl, 1979; Miller and Oberdorfer, 1981; Shaw and Weber, 1983; Eisenfeld, Bunt-Milam and Sarthy, 1984). This may have been due to differences in procedures and to the difficulty in detecting the protein by using LM ICC; the low level of labeling is difficult to detect in normal eyes. By using LM silver enhancement ICC and EM ICC, however, the presence of GFAP is clearly seen in both cell types. As previously reported by Erickson et al. (1987a), labeling was restricted to the endfeet of the Müller cells, mostly over the intermediate filaments. It has been suggested that the diffuse component labeled by anti-GFAP may represent GFAP monomers (Schachner, Hedley-Whyte, Hsu, Schoonmaker and Bignami, 1977). Because GFAP monomers will assemble into filaments *in vitro* (Lucas, Bensch and Eng, 1977; Rueger, Huston, Dahl and Bignami, 1979), the suggestion that the diffuse cytoplasmic labeling may be monomers of GFAP is consistent with the observation that this diffuse labeling only occurs in cells that contain GFAP intermediate filaments. The restriction of GFAP to the endfoot region of normal Müller cells may represent some functional compartmentalization within these cells. Other studies have shown that the Müller cell is indeed regionally specialized; the membrane bordering the vitreous containing a high density of potassium channels (Brew, Gray, Mobbs and Attwell, 1986) and increased levels of glucose 6-phosphatase (Cameron and Cole, 1965), and the outer border of the cell, with its microvilli extending into the interphotoreceptor space, having a concentration of certain glycoproteins (Reichenbach, Dettmer, Bruckner, Neumann and Birkenmeyer, 1985), and a sodium/potassium ATPase (McGrail and Sweadner, 1986). Although the specific function of the intermediate filaments is not known, it has been suggested that in some cells they may be involved in the anchoring of the cell nucleus to components of the membrane skeleton and the differentiation and maintenance of cell shape (Lazarides, 1980, 1987). Müller cells are known to alter their shape dramatically and to undergo nuclear migration after injury to the retina (Erickson, Fisher, Anderson, Stern and Borgula, 1983) and this may relate to the increased production of intermediate filaments in the injured retina (Bignami and Dahl, 1979; Eisenfeld, Bunt, Milam and Sarthy, 1984; Erickson et al., 1987a).

Two enzymes characteristic of glial cells in general are CAC and GS. Their appearance in specific types of glial cells, however, is variable. In brain CAC has been reported in both oligodendrocytes and astrocytes (Ghandour, Langley, Vincendon and Gombos, 1979; Kumpulainen, Dahl, Korhonen and Nystrom, 1983; Linser, 1985; Cammer and Tansey, 1988) while GS has been found only in astrocytes (Tholey, Ghandour, Bloch, Ledig and Mandel, 1987). In the retina, both GS (Riepe and Norenberg, 1978; Sarthy and Lam, 1978; Linser and Moscona, 1979; Moscona and Degenstein, 1981b; Moscona, 1983; Trachtenberg and Packey, 1983; Linser, Smith and Angelides, 1985) and CAC (Bhattacharjee, 1971, 1976; Musser and Rosen, 1973; Moscona and Degenstein, 1981b; Kumpulainen, 1980; Linser and Moscona, 1981; Lutjen-Drecoll and Lonnerholm, 1981; Parthe, 1981; Kumpulainen, Dahl, Korhonen and Nystrom, 1983; Lutjen-Drecoll, Lonnerholm and Eichhorn, 1983; Linser, Smith and Angelides, 1985) have been shown to occur in the Müller cells of many species. While the results of this study confirm that, as in other species, cat retinal Müller cells express both GS and CAC, they also demonstrate that retinal astrocytes, unlike astrocytes in the brain, express only CAC. This differential distribution of these two enzymes between the two types of retinal glia allows GS to be used as a specific

marker for cat Müller cells and highlights the differential functions of these two glial cell types. Both enzymes have been shown to be involved in the regulation of the neuronal environment, with GS aiding in the recycling of amino acid neurotransmitters released from nerve terminals (Kuffler and Nichols, 1976; Stewart and Rosenberg, 1979) and CAC controlling sodium and potassium ion movement and fluid balance (Carter, 1972). Müller cells, therefore, are probably involved in both functions in the retina. Indeed, Müller cells have been shown actively to take up GABA in a number of species (Graham, 1972; Neal and Iverson, 1972; Marshall and Voaden, 1974; Lam and Holyfield, 1980; Memo, Riccardi, Trabucchi and Spano, 1981; Sarthy, 1982). The lack of GS and presence of CAC activity in retinal astrocytes, however, probably indicates a different role for them than astrocytes elsewhere in the brain. Whereas the brain astrocytes appear to be involved in both biochemical functions and the oligodendrocytes have just one, in the case of the retina, Müller cells have both functions and the astrocytes have just one.

Müller cells and astrocytes also differ in their expression of CRALBP, a retinoid binding protein involved in the transport of vitamin A. CRALBP has previously been localized immunocytochemically to Müller cells and retinal pigment epithelial cells (Bunt-Milam and Saari, 1983; Bok, 1985, Anderson et al., 1986). Our results simply confirm immunocytochemical labeling of Müller cells in another species but in addition they demonstrate that the antibody does not label astrocytes, even on the same tissue section, making it likely that the protein is absent in these cells. Since Müller cells are intimately associated with the photoreceptors and contain this retinoid binding protein, it has recently been suggested that they may participate in some way in the visual cycle (Saari, Bunt-Milam, Bredberg and Garwin, 1984; Bok, 1985). Whatever the function, the presence of anti-CRALBP labeling in Müller cells clearly distinguishes them from astrocytes.

It was previously shown that Müller cells *in vitro* can acquire certain lens-like characteristics including the expression of specific lens cytoplasmic and membrane antigens (Moscona and Degenstein, 1981a; Moscona, Brown, Degenstein, Fox and Soh, 1983; Moscona and Linser, 1983). Subsequently, antibodies to the specific lens fraction, alpha crystallin, were shown to bind molecules expressed in normal, intact retinal Müller cells. The exact identity of these molecules, however, has not been found (Moscona, Fox, Smith and Degenstein, 1985). It has been suggested by the same group that the labeled Müller cell molecules may be only partly co-antigenic with AC since AC has an amino acid sequence showing some homology with heat shock proteins (Ingolia and Craig, 1982). Nevertheless, in this study we show that antibodies to AC bind molecules in Müller cells and retinal astrocytes, revealing another similarity between these two cell types.

As pointed out in the Results section, CAC and AC appear to label a nuclear component of these cells in addition to the cytoplasmic labeling. CAC has previously been found in the nuclear fraction of some cells (Karler and Woodbury, 1959); however, histochemical techniques have produced mixed results. Because nuclear labeling was found only in tissue that was fixed and not in unfixed tissue (Hansson, 1967, 1968; Lutjen-Drecoll and Lonnerholm, 1981; Lutjen-Drecoll, Lonnerholm and Eichhorn, 1983), it has been suggested that this nuclear localization may be due to non-specific diffusion into the nucleus. Although the immunocytochemistry and the labeling of a specific band on the immunoblots suggests that this does not represent the binding to some other molecular species, it is conceivable that these soluble proteins diffuse into the nucleus either before or after a relatively mild fixation.

This comparison of Müller cells and astrocytes demonstrates that while there are some similarities in the expression of various proteins by these two retinal glial cells, there are also some important differences. Localization of the proteins is the first step to understanding their specific functions within each cell type. In a subsequent article (Lewis, Kaska, Vaughan and Fisher, 1988), we report that cultured Müller cells continue to express all five of the proteins discussed here. The availability of well-characterized cell cultures, in which these proteins continue to be expressed, may help in understanding their functions in the retina.

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