A Photoreceptor Calcium Binding Protein Is Recognized by Autoantibodies Obtained from Patients with Cancer-associated Retinopathy

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Abstract. Cancer-associated retinopathy (CAR), a paraneoplastic syndrome, is characterized by the degeneration of retinal photoreceptors under conditions where the tumor and its metastases have not invaded the eye. The retinopathy often is apparent before the diagnosis of cancer and may be associated with autoantibodies that react with specific sites in the retina. We have examined the sera from patients with CAR to further characterize the retinal antigen. Western blot analysis of human retinal proteins reveals a prominent band at 26 kD that is labeled by the CAR antisera. Antibodies to the 26-kD protein were affinity-purified from complex CAR antisera and used for EM-immunocytochemical localization of the protein to

the nuclei, inner and outer segments of both rod and cone cells. Other antibodies obtained from the CAR sera did not label photoreceptors. Using the affinity-purified antibodies for detection, the 26-kD protein, designated p^{26}, was purified to homogeneity from the outer segments of bovine rod photoreceptor cells by Phenyl-Sepharose and ion exchange chromatography. Partial amino acid sequence of p^{26} was determined by gas phase Edman degradation and revealed extensive homology with a cone-specific protein, visinin. Based upon structural relatedness, both the p^{26} rod protein and visinin are members of the calmodulin family and contain calcium binding domains of the E-F hand structure.

A variety of neurodegenerative diseases are known to be associated with different types of cancer, even though the tumor and its metastases have not invaded the nervous system (Brain and Norris, 1965; Brain and Wilkinson, 1965; Brain et al., 1951; Croft and Wilkinson, 1963; Denny-Brown, 1948; Hawley et al., 1980; Henson and Urich, 1982; Minna and Bunn, 1982; Norris, 1972). These "remote effects" of cancer, or paraneoplastic syndromes, are of undetermined etiology, although some studies have implicated viral, hormonal or toxic origins (Brain and Norris, 1965; Hawley et al., 1980; Sawyer et al., 1976; Schold et al., 1979). Alternatively, these degenerative diseases may stem from an autoimmune response that is directed towards antigens or epitopes found in both the tumor and specific neurons (Anderson et al., 1988; Keltner et al., 1983; Kornguth et al., 1982). Evidence supporting this connection has been presented in the case of Eaton–Lambert myasthenic syndrome, a disease of the peripheral nervous system, in which the binding of autoantibodies at the neuromuscular junction interferes with the calcium-induced release of neurotransmitter (DeAizpurua et al., 1988; Kim and Neher, 1988; Newsom-Davis, 1985). An autoimmune component also has been suggested in several diseases of the central nervous system, including paraneoplastic cerebellar degeneration (Anderson et al., 1988; Furneaux et al., 1990; Greenlee

and Lipton, 1986) and cancer-associated retinopathy (CAR)[1] (Buchanan et al., 1984; Grunwald et al., 1985; Keltner et al., 1983; Kornguth et al., 1982; Thirkill et al., 1987, 1989).

Like other retinal degenerations, CAR spares the inner retina while causing photoreceptors to degenerate (Buchanan et al., 1984; Keltner et al., 1983). However, during CAR there is no evidence of pigment migration or epiretinal membrane formation, as occurs in retinitis pigmentosa, nor are the vasculature or optic nerve head damaged (Buchanan et al., 1984). The inflammatory response characteristic of uveitis is not associated with CAR. In spite of these differences, autoantibodies may be involved in all of these degenerative diseases of the retina (see Tso, 1989 for review). Retinal antigens of 58–62, 145, and 205 kD have been observed to bind antisera obtained from patients with retinitis pigmentosa and age-related macular degeneration (Galbraith et al., 1986; Gurne et al., 1989). An earlier study of CAR also identified a retinal antigen of 65 kD, as well as a low molecular weight protein of ~20 kD (Grunwald et al., 1985). More recent and extensive studies of CAR have focused on a prominent retinal antigen of ~23 kD (Keltner et al., 1983; Thirkill et al., 1987, 1989). Several of these antisera cross-react with the

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1. *Abbreviations used in this paper*: BTP, bis [2-hydroxymethyl] (minotris-[hydroxymethyl]-methane; CAR, cancer-associated retinopathy; ROS, rod outer segment.

68-kD neurofilament protein, and this finding has led to the speculation that autoantibodies associated with retinal degenerations may be directed towards cytoskeletal elements found in the tumor and retinal cells (Grunwald et al., 1985; Gurne et al., 1989).

The results obtained from immunohistochemical studies using CAR antisera have been inconsistent; different populations of retinal cells have been labeled by different CAR antisera (Keltner et al., 1983; Kornguth et al., 1982; Thirkill et al., 1989). Antibodies from these complex human sera, however, have not been affinity-purified, therefore, it is difficult to associate a specific antibody with a particular labeling pattern. Nor have EM-immunocytochemical studies been performed. For these reasons, the identity of the retinal antigen that binds the CAR autoantibodies and its precise localization remain uncertain. The functional identification of the CAR antigen also has not been ascertained.

In this paper we describe the affinity purification of antibodies from CAR antisera, and the use of these antibodies in parallel Western blot analyses and EM-immunocytochemical studies. Using the sera from five individuals, we have identified a 26-kD CAR antigen that is localized with highest density to the cell bodies of the retinal rods and cones but also is found in the inner and outer segments of both types of photoreceptors. Further, we have purified the CAR antigen to homogeneity using a convenient, two-step chromatographic procedure. Protein fragments of the purified protein were subjected to amino acid sequence analysis, and the protein was identified as a member of a family of calcium binding proteins. With this identification in hand, it may now be possible to examine tumors from patients afflicted with CAR and determine whether a calcium binding protein or shared determinant is the antigenic source in their cancer cells. A further comparison is warranted for patients with similar cancers but who do not exhibit the retinopathy.

Materials and Methods

Fluorescence Light Microscopy

Two initial observations were made regarding the binding of CAR antisera to sections of human retina. First, if eyecups or isolated retinas were fixed before rinsing with a Ringer's solution or other buffer, the affinity-purified anti-human IgG used as a secondary antibody labeled the subretinal space from the tips of the outer segments to the outer limiting membrane. The detection of IgG may result from a minor permeability of the blood-retinal barrier in vivo, or more than likely, a breakdown of the barriers upon death. In either case, isolated and rinsed retinas were used in the majority of these experiments to avoid background labeling. Secondly, the binding of CAR antisera to sections of human retina was suppressed if the tissue had been fixed with glutaraldehyde, therefore retinas were fixed for 3–12 h in 4% formaldehyde at room temperature. Retinas subsequently were embedded in acrylamide and frozen in cold isopentane before cryosectioning, as described by Johnson and Blanks (1984). The buffer used for fixation and embedding was 0.1 M sodium cacodylate, pH 7.2. Other reagents used for labeling cryosections were prepared with a Ringer's solution (130 mM NaCl, 2 mM KCl, 2 mM CaCl2, 2 mM MgSO4, 2 mM NaHCO3, 10 mM glucose, 50 mM sucrose, 10 mM Hepes, pH 7.5), and incubations were conducted at room temperature.

In parallel experiments, fixed retinas were embedded in a hydrophilic plastic, LR White or LR Gold, as described below, and thick sections were processed for fluorescence microscopy.

Sections were blocked for 30 min at room temperature using a Ringer's solution containing 1% wt/vol BSA and 1% vol/vol normal goat serum. Sections were washed and incubated with human CAR antisera or normal human sera at a dilution of 1:50 or 1:100 for 1 h. In some experiments, both cryosections and plastic sections were incubated overnight in a humidified

chamber with antibodies which had been affinity-purified from CAR antisera (see below). After several washes, sections were incubated for 1 h with a biotinyl-goat anti–human IgG at a concentration of 25 μg/ml. Sections finally were incubated for 1 h with a solution containing 50 μg/ml of a streptavidin-Texas red conjugate. After dehydrating with ethanol and clearing with xylene, sections were viewed with a Zeiss Universal fluorescence light microscope using X25 and X40 neofluor objectives.

Electron Microscopy

Isolated human retinas were rinsed with Ringer's solution, and 3-mm Trephine punches were fixed for 2 h or overnight with 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The fixed retinal pieces were rinsed three times for 10 min each in 0.1 M phosphate buffer and then dehydrated for 10 min each in 15, 30, and 50% ethanol. Some retinal pieces were postfixed for 1 h in 1% wt/vol uranyl acetate in 70% ethanol. The tissue was dehydrated further for 10 min each in 85 and 90% ethanol, and infiltrated for 30 min each in a mixture of LR-gold:ethanol (1:2, vol/vol; 2:1, vol/vol) at room temperature, followed by infiltration overnight in 100% LR-gold at 4°C. The samples then were embedded in fresh resin containing initiator and kept at 4°C for 2 h. Polymerization was accomplished by UV irradiation, overnight at −5°C. Embedding in LR White followed the manufacturer's recommendations, and polymerization was at 50°C.

Thin sections of human retina, collected on Formvar-coated nickel grids, were incubated in a blocking solution (PBS, pH 7.4, containing 1% wt/vol BSA, 1% vol/vol normal goat serum and 0.02% wt/vol azide) for 30 minutes at room temperature. The grids were blotted briefly with filter paper and then floated on 50-μl droplets containing either normal human serum or CAR antiserum at a dilution of 1:100, or affinity-purified antibodies obtained from CAR sera (see below). Incubation was conducted overnight in a humidified chamber at room temperature. After rinsing the grids in PBS containing 0.1% BSA and 0.1% normal goat serum, sections were incubated for 1 h at room temperature with AuroProbe EM goat anti-human IgG (Amersham Corp., Arlington Heights, IL) at a dilution of 1:25 in 0.1% BSA-PBS. After thorough washing in 20 mM Tris·HCl, 0.9% NaCl, pH 8.2 (Tris·NaCl), sections were fixed for 20 min in aqueous 2% glutaraldehyde and rinsed with water. Some sections were subsequently stained for 3 min in 2% uranyl acetate and 1 min in lead citrate. Sections were postfixed with 2% aqueous osmium tetroxide fumes for 1 h and viewed with Zeiss 10 C/A transmission electron microscope.

Preparation of Human ROS

Rod outer segments (ROS) were isolated from human retinas according to the procedures developed for bovine ROS, as described by Papermaster (1982). In some experiments, human retinas were disrupted using a Teflon pestle and solubilized with 0.15% wt/vol deoxycholate. After centrifugation at 100,000 g (SW28; Beckman Instruments, Fullerton, CA), the supernatant and previously isolated ROS were processed for SDS-PAGE. All isolations were done in the presence of 2 μg/ml leupeptin, 4 μg/ml PMSF, and 10 μg/ml aprotinin.

SDS-PAGE and Western Blot Analysis

Treatment of protein samples is described in detail elsewhere (Polans et al., 1979), as are the procedures for electrophoresis and transfer of protein to nitrocellulose (Polans and Burton, 1988). In some experiments, Immobilon (Millipore Corp., Bedford, MA) was used in place of nitrocellulose. Separation of peptide fragments was accomplished with a Tricine-SDS-gel electrophoresis system (Huang and Matthews, 1990; Schagger and von Jagow, 1987) using a 16% polyacrylamide gel. Protein determinations were made with a dye binding assay (Bradford, 1976).

Western blots were rinsed twice in Tris·NaCl, and nonspecific sites were saturated by incubation for 45 min at 37°C with a solution of Tris·NaCl containing 5% wt/vol BSA. The membrane then was incubated for 1–2 h either with CAR antisera at a dilution of 1:500 or affinity-purified antibody (see below). All incubations were performed in 0.1% BSA-Tris·NaCl. Blots were rinsed six times each for 5 min with 0.1% BSA-Tris·NaCl and then incubated for 2 h with a 1:50 dilution of goat anti-human IgG gold conjugate in 0.1% BSA-Tris·NaCl containing 0.4% wt/vol gelatin. After four rinses with 0.1% BSA-Tris·NaCl, blots were rinsed with water (twice for 30 s each) and silver-enhanced 10–15 min according to the manufacturer's procedures.

Affinity Purification of CAR Antibodies

Procedures for the affinity purification of complex antisera have been pub-

lished in detail elsewhere (Burton et al., 1989). Briefly, blots of human ROS protein were incubated with CAR antiserum. A single lane from the blot was stained using gold conjugates and silver enhancement as described above. The stained lane was realigned with the blot, and protein bands corresponding to the sites of antibody staining were excised separately. Antibody was eluted from excised bands using glycine·HCl, pH 2.2, followed by neutralization with Tris base. The antibody eluate was quantitated for human IgG using an immunogold procedure (Burton et al., 1989). After concentrating the eluate in a Centricon 30 tube (Amicon Corp., Danvers, MA), the sample was mixed to yield 0.1% BSA, 0.1% normal goat serum in either Tris·NaCl or PBS.

Purification of the 26-kD CAR Antigen

ROS from 150 bovine retinas were prepared as described previously (Wilden and Kühn, 1982) and finally resuspended in 20 ml of 50 mM Hepes buffer, pH 7.5, containing 1.0 mM EDTA and 100 mM NaCl. The suspension was homogenized with a glass-glass tissue homogenizer and centrifuged at 48,000 g (J 20.1; Beckman Instruments) for 10 min. The supernatant was collected and the extraction procedure was repeated three times. These steps were conducted at 4°C under dim red illumination. Calcium chloride then was added to the combined supernatants in order to yield a final concentration of 2 mM.

A Phenyl-Sepharose column (1.0 × 4.0 cm) was prepared and equilibrated with 50 mM Hepes buffer, pH 7.5, containing 2 mM CaCl$_2$ and 100 mM NaCl. The bovine ROS extract was applied to the column, and the column was washed with equilibrating buffer at a rate of 15 ml/h until the A$_{280}$ nm returned to baseline. Bound material was eluted with 50 mM Hepes buffer, pH 7.5, containing 10 mM EDTA and 100 mM NaCl at a rate of 5 ml/h. 1-ml fractions were collected, and aliquots were subjected to SDS-PAGE and Western blot analysis using CAR antiserum.

Fractions containing the 26-kD CAR antigen were combined and dialyzed against 1 liter of 10 mM bis[2-hydroxymethyl]iminotris[hydroxymethyl]-methane (BTP) buffer, pH 8.4. Aliquots containing 0.5–1.0 mg of protein were applied to a Mono Q column (HR 5 × 50 mm; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 10 mM BTP buffer, pH 8.4.

The column was developed with a linear gradient of NaCl (0–0.25 M) during 20 min at a rate of 0.5 ml/min. 0.5-ml fractions were collected, and aliquots were analyzed by SDS-PAGE and Western blot analysis using CAR antiserum. The 26-kD CAR antigen eluted at ~100 mM NaCl.

Amino Acid Sequence Analysis

The purified 26-kD protein was lyophilized and S-carboxymethylation was performed following the methods described by Crestfield et al. (1963). Cyanogen bromide (CNBr) cleavage of the S-carboxymethylated protein was performed over a 72-h period according to the methods published by Lai (1968). CNBr peptide fragments were separated by Tricine-SDS-gel electrophoresis (Huang and Matthews, 1990; Schagger and von Jagow, 1987) and electroblotted to Immobilon (Millipore Corp.) for sequence and amino acid analysis (Matsudaira, 1987). Additionally, the purified 26-kD protein was subjected to cleavage at lysyl residues with endoproteinase Lys-C (Boehringer-Mannheim Biochemicals, Indianapolis, IN), and peptides were isolated by narrowbone RP-HPLC (Vydac, Hesperia, CA) using the Applied Biosystems model 130 system (Applied Biosystems, Inc., Foster City, CA) (Crabb et al., 1986).

Phenylthiocarbamyl amino acid analysis was performed according to West and Crabb (1990) using an Applied Biosystems automatic system (models 420/130/920). HCl vapor phase hydrolysis was performed at 150°C for 1 h. Automatic Edman degradations were performed as previously described (Crabb et al., 1988) with an Applied Biosystems gas phase sequencer (model 470) and an on-line phenylthiohydantoin amino acid analyzer (model 120) using the 03RPTH sequencer program and the manufacturer's recommended program and solvents for the PTH analyzer.

Materials

Human donor eyes were obtained from the Lions Eyebank of Oregon, Good Samaritan Hospital and Medical Center (Portland, OR). CAR antisera were the generous gift of Drs. C. Thirkill and J. Keltner (University of California at Davis). Sera also were obtained from Drs. R. Chenoweth and T. Shults (Good Samaritan Hospital and Medical Center) and Drs. R. Weleber and
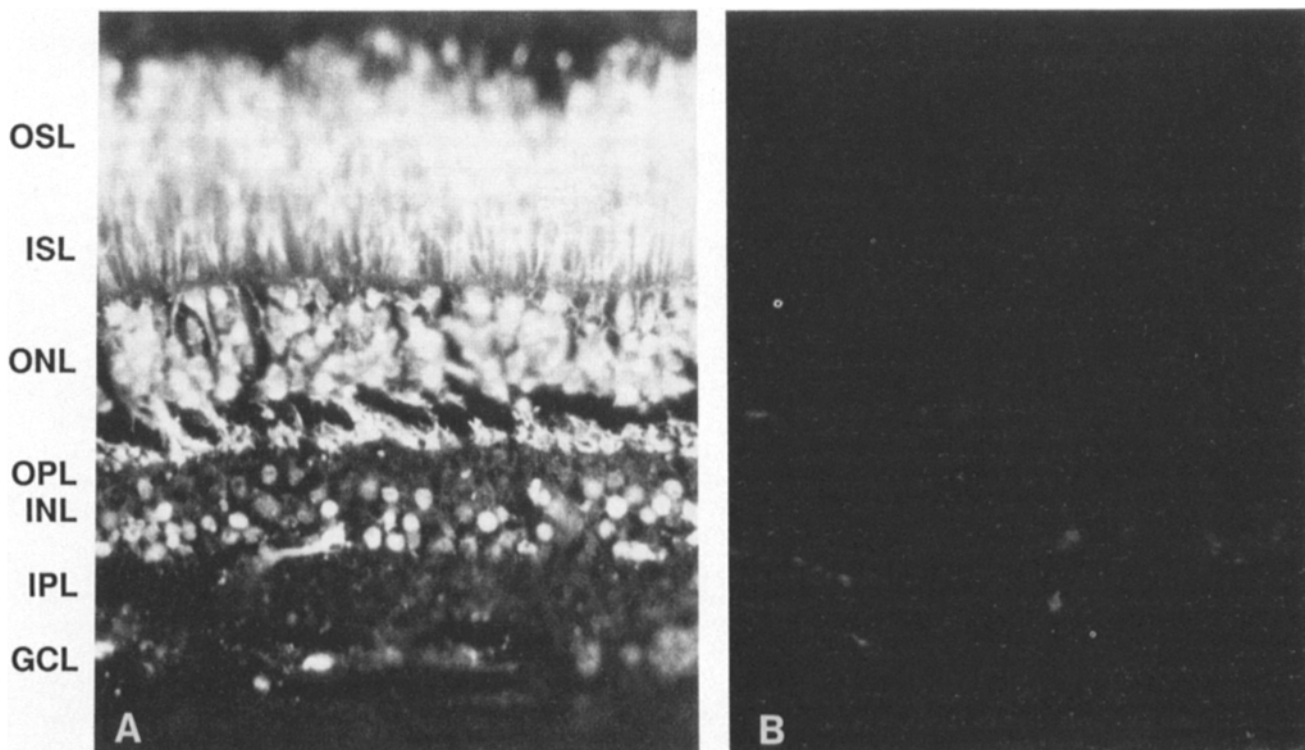
Figure 1. Fluorescent detection of CAR antiserum staining of human retina. Cryosections of rinsed and formaldehyde-fixed human retinas were incubated with a 1:100 dilution of CAR antiserum (*A*) or normal human serum (*B*) and subsequently incubated with a biotinyl-goat anti-human IgG and a streptavidin–Texas red conjugate. Abbreviations: *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *ISL*, inner segment layer; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *OSL*, outer segment layer.
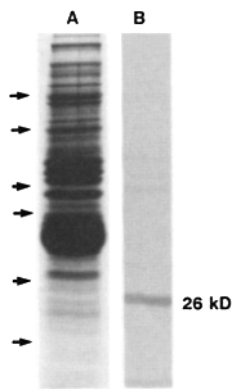
Figure 2. Binding of whole CAR antiserum to a nitrocellulose blot of human ROS proteins. After separation by SDS-PAGE, human ROS proteins were stained with Coomassie brilliant blue (*A*). Nitrocellulose blots of human ROS proteins were incubated sequentially with CAR antiserum (1:500 dilution), goat anti-human IgG gold complex and silver enhanced (*B*). A prominent band at 26 kD is labeled by the antiserum. Minor bands also could be detected in the 68–95 kD range. Molecular weight standards were: β-galactosidase (116 kD), phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), and soybean trypsin inhibitor (20.1 kD).

Results

Fig. 1 illustrates the binding of CAR antiserum to a cryosection of human retina, as detected by fluorescence light microscopy using a biotinyl-secondary antibody and streptavidin-Texas red conjugates. Fig. 1 *A* shows intense labeling by the CAR antiserum of the outer nuclear layer, as well as the inner and outer segment layers of the retina. A subpopulation of cell bodies in the inner nuclear layer also binds the antiserum. These results were obtained with CAR antisera from patients diagnosed either with oat-cell carcinoma of the lung or ovarian adenocarcinoma. In contrast, when normal human serum is substituted for the CAR antiserum, sections are not labeled (Fig. 1 *B*). These experiments confirm previous observations that CAR antisera contain autoantibodies that can bind to retinal sites (Grunwald et al., 1985; Keltner et al., 1983; Kornguth et al., 1982; Thirkill et al., 1987, 1989).

To identify the retinal antigen(s) that bind CAR autoantibodies, human ROS or retina were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose paper for staining with CAR antiserum. The protein staining pattern of human ROS obtained by SDS-PAGE is shown in Fig. 2 *A*, and Fig. 2 *B* shows the corresponding nitrocellulose blot reacted sequentially with CAR antiserum and goat anti-human IgG gold complex. A prominent band at 26 kD is labeled by the CAR antiserum, while several less intense bands in the 68–95-kD range also can be detected. We find that the 26-kD band is uniquely associated with the five CAR antisera tested. Human antisera which bind to different sites in the retina (Fig. 3 *A*), label, for instance, additional bands in the 68–95-kD range (Fig. 3 *B*). Labeling of the 26-kD band by antisera obtained from non-CAR patients was not observed in these experiments. Sera were tested from two pa-

tients diagnosed with retinitis pigmentosa, three individuals who were free of cancer but whose sera demonstrated retinal labeling, three cancer patients without any associated retinal labeling, and three individuals without cancer or detectable retinal labeling.

Since more than one retinal antigen appears to be recognized by the CAR antiserum, the identity of the antibody-antigen interaction that underlies the observed labeling of the retina is uncertain. To address this issue, complex CAR antisera were fractionated to provide affinity-purified antibodies (see Materials and Methods). Fig. 4 *A* shows the results of applying affinity-purified antibody against the 26-kD protein to cryosections of human retina. As shown previously with whole antiserum, antibodies to the 26-kD protein intensely labeled the outer nuclear layer and inner and outer segment layers of the retina. Antibodies eluted from proteins in the 68–95-kD range only slightly labeled the plexiform layers of the retina (Fig. 4 *B*). We can conclude from these experiments that the principal, if not sole, retinal antigen recognized by CAR autoantibodies appear to be the 26-kD protein. Interestingly, application of CAR antisera to sections of human retina which had been embedded in plastic resin, either LR-gold or LR-white, showed a diminution of label (Fig. 4 *C*). Whereas binding of anti-26-kD antibodies to the outer nuclear layer was still visible, binding to the outer segments fell below detection by fluorescence light microscopy. The difference in labeling intensity between frozen tissue and tissue embedded in plastic may reconcile our results with a previous report that demonstrated CAR labeling of the outer nuclear layer but not the inner and outer segments of retinal tissue which had been embedded in LR-white (Thirkill et al., 1989).

The binding of CAR antisera to plastic-embedded tissue could be detected by an EM-immunocytochemical approach. In preliminary experiments, we observed that the binding of CAR antisera was suppressed in tissue that had been treated with glutaraldehyde. Although the ultrastructural preservation of donor tissue normally obtained with glutaraldehyde was difficult to maintain with formaldehyde, antibodies to the 26-kD protein were found to label nuclei and the surrounding cytoplasm of photoreceptor cell bodies in the outer nuclear layer (Fig. 5 *A*). Successive sections, incubated either with normal human IgG or antibodies to the 68–95-kD proteins, were not labeled upon further incubation with the immunogold complex (Fig. 5 *B*). In contrast to controls, the inner segments (Fig. 5 *C*) and outer segments (Fig. 5 *D*) of both rods and cones also were observed to bind affinity-purified antibodies to the 26-kD protein. The EM-immunocytochemical results, therefore, support our fluorescence studies which demonstrated the presence of the 26-kD antigen in the inner and outer segment layers of the retina, as well as the outer nuclear layer. These immunocytochemical studies are further corroborated by our Western blot analyses, in which preparations of outer segments (Fig. 2 *B*) and of the remnant retina both contained the 26-kD protein (data not shown).

To characterize the 26-kD CAR antigen further, the protein was purified by a two-step chromatographic procedure. Bovine ROS were homogenized in the presence of 1 mM EDTA and no added calcium. Under these conditions the 26-kD protein was extracted into a soluble fraction, whereas, in the presence of excess calcium, the protein mostly remained bound to ROS membranes. An aliquot of the soluble extract was subjected to SDS-PAGE, and the protein staining
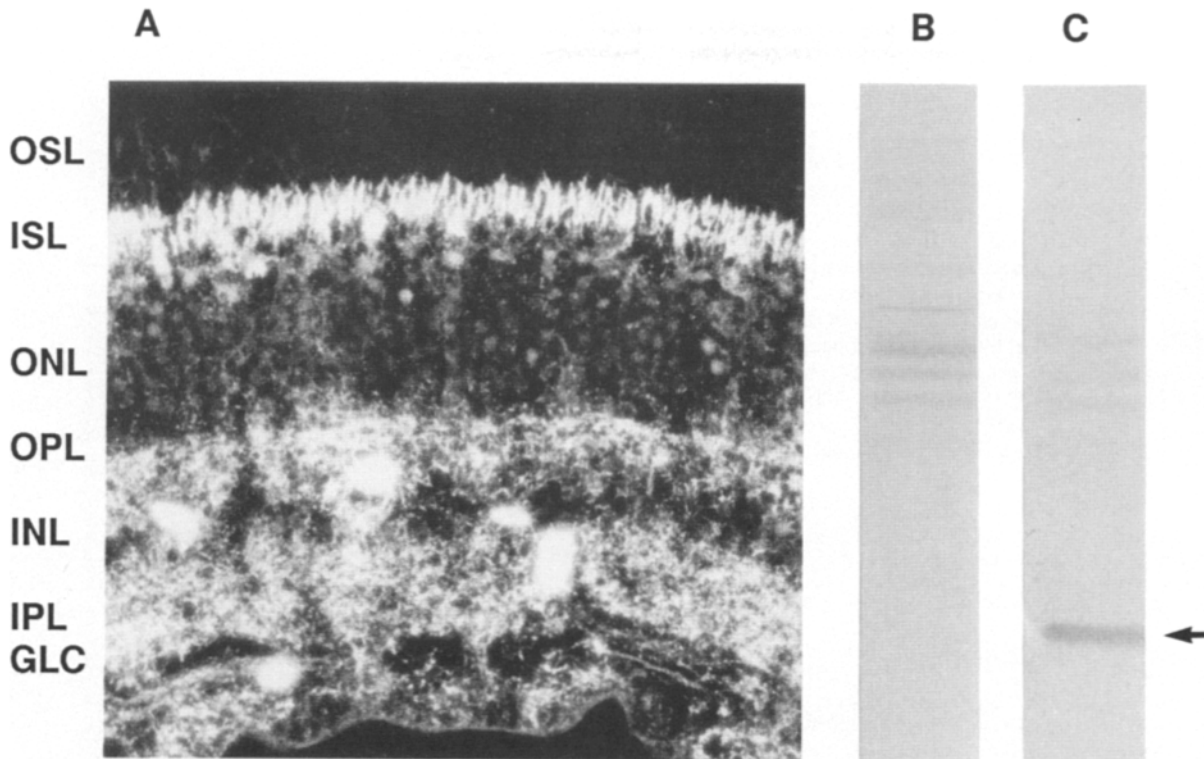
Figure 3. Binding of a non-CAR antiserum to human retina. Cryosections of retina were incubated with a human antiserum obtained from a patient without cancer but whose serum did bind to retina (*A*). Note the intense staining of the inner segment layer and both plexiform layers, whereas the outer segments and outer nuclear layer were unlabeled. Nitrocellulose blots of human ROS protein stained with the same antiserum (*B*) did not label the 26-kD band (*C*). Additional bands in the 68–95-kD range were labeled by this antiserum. All incubations were conducted as described in the legend of Fig. 1 and Materials and Methods.

pattern is given in Fig. 6 *A*. Excess calcium was added to the remaining soluble extract, and this material was applied to a Phenyl-Sepharose column. The majority of extractable protein ran unretarded through the column (Fig. 6 *B*), whereas bound material, consisting primarily of the 26-kD

protein, could be eluted from the Phenyl-Sepharose column by the addition of EDTA (Fig. 6 *C*). This calcium-dependent binding to Phenyl-Sepharose strongly suggested that the 26-kD protein might be a calcium-binding protein. Final purification of the protein was accomplished by a second chro-
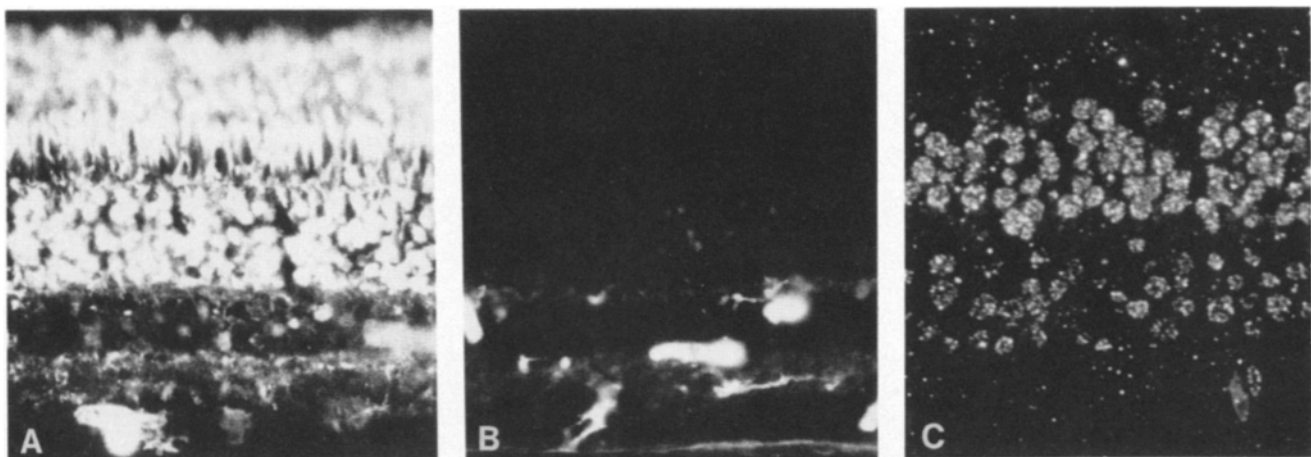
Figure 4. Affinity-purified CAR antibody staining of cryosections and plastic sections of human retina. Affinity-purified antibodies to the 26-kD protein (see Materials and Methods) were used to stain cryosections of retina (*A*) according to the procedures given in the legend of Fig. 1 and Materials and Methods. Like the CAR antiserum, antibodies to the 26-kD protein intensely labeled the ONL, ISL and OSL. Antibodies to the 68–95-kD proteins of human ROS which are depicted in Fig. 2 only slightly stained the plexiform layers of cryosectioned retina (*B*). CAR antiserum showed reduced labeling of the outer nuclear layer and no labeling of the inner and outer segment layers when the retina was embedded in a plastic resin, LR-white (*C*).
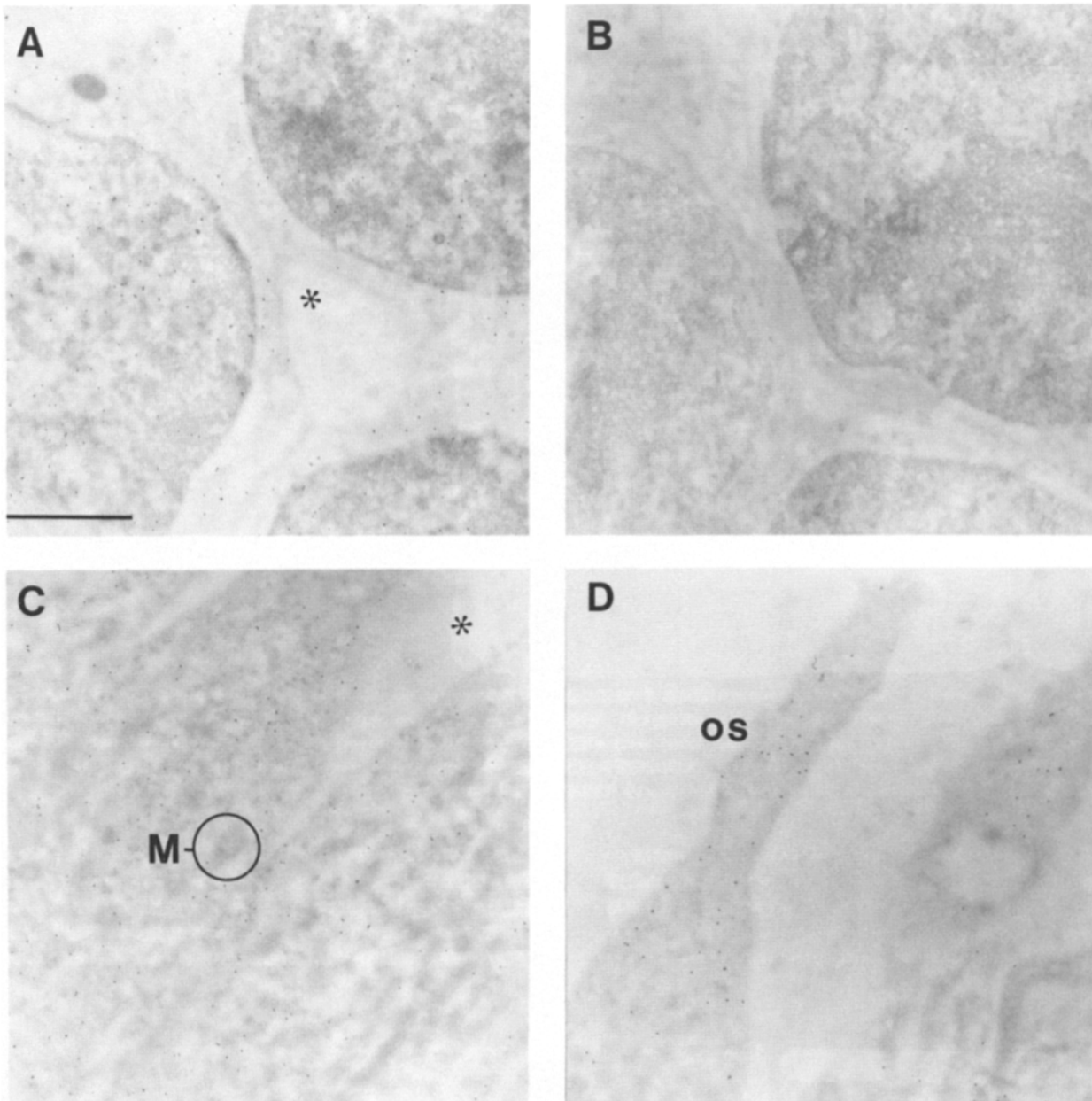
Figure 5. Retinal binding of affinity-purified antibodies to the 26-kD protein, as detected by EM. Human retinas were fixed in formaldehyde and stained with affinity-purified antibodies to the 26-kD protein. Antibody binding was detected with a goat anti–human IgG gold complex (10 nm). Human donor retina showed intense labeling of the nuclei and surrounding cytoplasm of photoreceptor cell bodies in the outer nuclear layer (*A*). The asterisk indicates the absence of background label in a region between three cell bodies. Sections incubated with normal human IgG were not labeled upon subsequent incubation with the anti-human IgG gold complex (*B*). The inner segments of three photoreceptors are labeled with antibodies to the 26-kD protein (*C*), while the surrounding plastic support is devoid of label (*asterisk*). The darker profiles (*M*) are mitochondria which appear almost negatively stained when embedding a nonosmicated tissue in a hydrophilic plastic like LR-gold. Photoreceptor outer segments (*OS*) also are labeled (*D*).

matographic step using a Mono Q column. The column was developed with a linear gradient of NaCl, and the purified protein, which we designate as p[26], was eluted at ∼100 mM NaCl. Fig. 6 *D* shows a sample from the purified protein fraction; no other protein bands could be detected. Molecular weight standards were run in lane *E*. Approximately 275 μg of purified protein was obtained from 150 retinas. An aliquot of the purified protein was subjected to SDS-PAGE and immunoblotting with a CAR antiserum, and the positive staining of the 26-kD band is shown in Fig. 6 *F*.

Our initial attempt to sequence purified p[26] revealed that the NH₂ terminus was blocked. Therefore, the protein was cleaved with CNBr. The CNBr fragments were resolved by modified SDS-PAGE and electroblotted to Immobilon for direct sequencing. Edman analysis of the immobilized CNBr peptides yielded the partial sequence GFFGKKDDDKLT-EKEFIEGXLAN. The purified protein (∼7 μg) also was subjected to enzymatic digestion at lysyl residues with endoproteinase Lys-C, and fragments were purified by narrowbore RP-HPLC before sequencing. Table I shows the partial
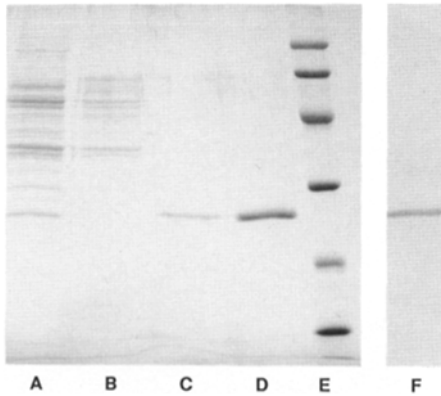
Figure 6. Purification of the CAR antigen. Bovine rod outer segments were extracted with EDTA, and the soluble fraction (after adjusting the calcium concentration) was applied to a Phenyl-Sepharose column. An aliquot of the soluble extract was separated by SDS-PAGE, and the protein staining pattern is shown in lane *A*. Protein which did not bind to the column is shown in lane *B*, whereas bound material which could be eluted with EDTA is stained in lane *C*. The 26-kD protein represented the majority of protein which was bound to the column. The eluted material depicted in lane *C* was further purified by Mono Q chromatography, and an aliquot of the final sample is shown in lane *D*. Molecular weight standards in lane *E* were: phosphorylase b (94 kD); bovine serum albumin (67 kD); ovalbumin (43 kD); carbonic anhydrase (30 kD); soybean trypsin inhibitor (20.1 kD); and α-lactalbumin (14.4 kD). An immunoblot of the purified fraction shown in lane *D* was prepared according to the procedures given in the legend of Fig. 2 and in Materials and Methods. CAR staining of the immunoblot is shown in lane *F.*

amino acid sequence of p^{26} and the homology it shares with a cone photoreceptor-specific protein, visinin (Yamagata et al., 1990). The two proteins share ∼60% identical amino acids in the 157 residues that have been sequenced in p^{26} and almost 90% homology if considering conservative replacements (Dayhoff et al., 1969). Three regions within the visinin sequence are clearly homologous with calmodulin and troponin C (Yamagata et al., 1990) and represent calcium binding domains of the E-F hand structure. Corresponding regions within the p^{26} sequence revealed ∼90% homology to visinin. Recently, a more complete amino acid sequence of p^{26} has been determined by others characterizing a protein they term recoverin (Hurley, J., K. Walsh, and A. Dizhoor, personal communication). In concurrence with our results, these investigators also have recognized the homology with visinin, as well as with other calcium binding proteins of the calmodulin family.

Discussion

We have confirmed previous reports that the sera from CAR patients contain autoantibodies that bind to the retina (Keltner et al., 1983; Kornguth et al., 1982; Thirkill et al., 1987, 1989). We have affinity-purified the autoantibodies from complex CAR sera to identify the principal, if not sole, retinal antigen as a 26-kD protein. In EM immunocytochemical experiments, we localized the 26-kD protein to the cell bodies, inner and outer segments of both rods and cones. We purified the rod protein by a simple chromatographic procedure and

demonstrated, using partial amino acid sequence analysis, that the protein shares extensive homology with a cone-specific protein, visinin. Both visinin and the rod protein, p^{26}, contain within their sequences the proper motifs for the binding of calcium. Concurrent investigations in several laboratories are examining the role which these calcium binding proteins might play in phototransduction, perhaps by regulating guanylate cyclase activity (Hurley, 1990; Koch, 1990; Yamagata et al., 1990).

Two laboratories recently have presented preliminary data on the isolation of a protein similar to visinin but isolated from rods (Hurley, 1990; Koch, 1990). A comparison of amino acid sequences demonstrates that p^{26} is the same protein (Hurley, J., K. Walsh, and A. Dizhoor, personal communication). Thus, CAR autoantibodies recognize a retinal protein that is involved, at least, in the calcium-dependent regulation of guanylate cyclase. Since CAR antisera label both rods and cones, it appears likely that p^{26} and visinin are the respective antigens, although p^{26} may be present in both types of photoreceptors and is the sole antigen. Although two other calcium-binding proteins, calretinin and calbindin, are present in cones but not rods and may bind CAR autoantibodies, they share little homology with visinin or p^{26} (Rogers, 1987; Yamagata et al., 1990). Nonetheless, the definitive identification of the cone antigen awaits the purification of these calcium binding proteins in order to determine which ones, in addition to p^{26}, bind CAR autoantibodies. While discussing other retinal antigens, we also should consider that CAR, like retinitis pigmentosa, may consist of a group of retinal degenerations. As studies continue, other retinal antigens may be associated with the autoimmune component of the disease. The identification and localization of these antigens may help establish a correlation between the affected retinal antigen and, for instance, the severity and progression of the disease.

Owing to the characterization of p^{26}, it now should be possible to examine biopsy tissue obtained from CAR patients and look for the expression of an immunoreactive calcium-binding protein or shared determinant. The examination of tissue from a patient with a similar tumor but no associated retinopathy would determine the extent to which the expression of the protein correlates with the retinopathy, and whether additional mechanisms dictate which cancer patients will develop the retinal disorder. Finally, it is interesting to note that autoantibodies generated in Eaton-Lambert myasthenic syndrome bind to calcium channels (DeAizpurua et al., 1988; Kim and Neher, 1988; Newsom-Davis, 1985); thus, recognition sites for calcium may be a common element among different paraneoplastic syndromes.

Table I. Homology of p[26] with Visinin

Visinin	1	M G N S R S S A L S R E V L Q E L R A S T R Y T E E E L S R W Y E G F Q R Q C S	40

```
                    .  . . .    .   . .         .      . . . . . . .          .
                    . . . . . . . .                   . . . . . . . .    . . . .
p26                 S G A L S K E I L E E L Q L N T K F T E E E L S S W Y Q S F L K E S P
                    |---------|------|--------------|-------------------------|
                        Endo 1        Endo 2              Endo 3
```

	41	D G R I R C D E F E R I Y G N F F P N S E P Q G Y A R H V F R S F D T N D D G T	80

```
                    . . .              . .          . .                   . .
                    . . . . .      . . . .      . . . . . . . .      .    . . . . . . . .
                    S G R I T R Q E F Q T I Y S   F F P E A D P K   Y A Q H V F R S F D A N
                    |--------------------------|  |-------------|  |--------------------|
                            Endo 4                    Endo 5              Endo 6
```

	81	L D F R E Y I I A L H L T S S G K T H L K L E W A F S L F D V D R N G E V S K S	120

```
                    . .    . . . .    . .    . . .       . . . . . . . . . . .    . . .   . .
                    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
                    E Y V I A L H M T S A G K T N Q K L E W A F S L Y D V D G N G
                    |------------------------|---------------------------------|
                              Endo 7                    Endo 8
```

	121	E V L E I I T A I F K M I P E E E R L Q L P E D E N S P Q K R A D K L W A Y F N	160

```
                        . .        .    . . . . . . . . .    . .        . . . . .    .
                        . . . . .      . . . . . . . . . . . .
                        M I S P E D T K H L P E D E N T P E K       I W G F F G
                        |-----------|-------------------------|    |-----------
                           Endo 9           Endo 10                 |------
```

	161	K G E N D K I A E G E F I D G V M K N D A I M R L I Q Y E P K K

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                    .    . .       .   . . .      .        .    . . . . .    . .
                    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
                    K K D D D K L T E K E F I E G T L A N K E I L R L I Q F E P Q K
                    --------------------|                 |---------------------------|
                          Endo 11                                 Endo 12
                    ----------------------------------|
                              CNBr
```

The amino acid sequence of p[26] and visinin (Yamagata et al., 1990) are given in single letter code (*Eur. J. Biochem.* 1968, 5:151–153). The double and single dots indicate identical amino acids and conserved replacements, respectively (Dayhoff et al., 1969). The peptide fragments from p[26] are underlined according to their isolation either by Endoproteinase Lys-C cleavage (*Endo*) or CNBr cleavage.

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