Intermediate Filaments in Bluegill Retinal Pigment Epithelial Cells

Thesis

Presented to the Graduate Council of

Southwest Texas State University

in Partial Fulfillment of the Requirements

for the degree of

Master of Science

by

Ernesto Pérez, Jr.

San Marcos, Texas

May 1999

TABLE OF CONTENTS

List of Figures.....iii

Acknowledgments.....v

Abstract.....vi

Introduction.....1

Methods.....11

Results.....17

Discussion.....40

Literature Cited.....45

LIST OF FIGURES

Figure 1. Illustration depicting the location of the retinal pigment epithelium in the vertebrate eye....2

Figure 2. Illustration depicting retinomotor movements involving the retinal pigment epithelium and the photoreceptors....3

Figure 3. Illustration depicting the secondary structures of the intermediate filament protein monomer....7

Figure 4. Photomicrographs showing the localization of circumferential microfilament bundles and filamentous actin in isolated bluegill retinal pigment epithelial cells....20

Figure 5. Photomicrographs showing the localization of intermediate filaments in isolated retinal pigment epithelial cells....22

Figure 6. Photomicrographs showing the localization of vimentin intermediate filaments in isolated retinal pigment epithelial cells....24

Figure 7. Image of a SDS-PAGE gel and corresponding Western blot illustrating the presence of vimentin protein in the retinal pigment epithelium....26

Figure 8. Transmitted light images of isolated retinal pigment epithelial cells with pigment granules dispersed and aggregated....28

Figure 9. Photomicrographs showing the distribution of vimentin intermediate filaments in isolated retinal pigment epithelial cells with pigment granules aggregated....30

Figure 10. Photomicrographs showing the distribution of vimentin intermediate filaments in isolated retinal pigment epithelial cells with pigment granules dispersed....32

Figure 11. Photomicrographs showing the absence and presence of cytokeratin intermediate filaments in isolated retinal pigment epithelial cells and in sections of bluegill liver respectively....34

Figure 12. Photomicrograph of control tissue for figure 11 (bluegill liver section)....36

Figure 13. Photomicrographs comparing the distribution of vimentin intermediate filaments in isolated RPE cells with pigment granules aggregated and dispersed.....38

ACKNOWLEDGMENTS

A once in a lifetime opportunity is rare. That rare opportunity became a reality the day Dr. Dana García offered to me a chance to conduct research in her laboratory. So, I am forever grateful to her for believing in and for having patience with me especially during my times of stress and uncertainty. She is a great mentor and a great friend.

I also thank Dr. Joseph Koke for his friendship, patience, and technical assistance throughout my graduate school experience at SWT. I am also grateful to Dr. Gary Aron for his understanding and patience during this thesis preparation experience. Additional thanks goes to Juan Herrera and David Zamora for recruiting me into Dr. García's laboratory. I can honestly say that their actions directed my career path toward science.

I also thank Annie LeMaster, Pat Stevenson, Sandra Bolanos, Gus Menger, Mark Hahn, Alfredo Gonzales, Christine Testa, Larry Contreras, and Dan Millikin. Equal thanks goes to my high school teacher and coach, Roddy Maddox, for believing in me. To my brothers, Luis, Carlos, and Johnny, and sister, Elizabeth, thank you for your friendship and support. Also, I can not forget about my nephew, Andrew, for making feeding the fish fun and my fiancée, Marisela, for bringing into my life a breath of fresh air. In addition, a thousand thanks goes to my extended family.

Finally, I am forever indebted to my parents, Ernesto and Catalina, for the many sacrifices they have experienced that has allowed for me to be at this time and place. This work was supported by a National Institutes of Health Initiative for Minority Student Development grant PAR-96-006 awarded to Dr. Damon C. Herbert at the University of Texas Health Science Center at San Antonio and Dr. Ronald B. Walter at Southwest Texas State University in San Marcos, Texas.

ABSTRACT

The retinal pigment epithelium (RPE) is a layer of cuboidal cells located between the neural retina and choroid layers of the eye. In teleosts, long and short apical processes are characteristic of these cells (Zinn and Marmor, 1979). Furthermore, unlike higher vertebrates, teleosts lack the ability to adjust their pupil diameter in accordance to different light intensities. However, they have evolved a process called retinomotor movements which involve changes in the relative positions of the rod and cone photoreceptors and melanin pigment granules within the RPE. In lit environments, the light sensitive rods elongate, burying their outer segments between the processes of the RPE, while cones contract away from the RPE. Pigment granules migrate into the apical processes where they function as a protective shield by absorbing light that would otherwise impinge on the rods. In dark environments, rods contract and cones elongate while pigment granules migrate into the cell body of the RPE (Burnside and Nagle, 1983).

In eukaryotes, cell shape, architecture, and motility depend on the cytoskeleton which is composed of microtubules (MTs; 25 nm diameter), microfilaments (MFs; 6-7 nm diameter) and intermediate filaments (IFs; 8-12 nm diameter) (Alberts *et al.*, 1994). Studies conducted on the microfilament and microtubule networks in teleost RPE cells have suggested a role for these cytoskeletal elements in the pigment granule translocation associated with light and dark adaptation (Klyne and Ali, 1981; Burnside *et al.*, 1983; Bruenner and Burnside, 1986; Troutt and Burnside 1989; Dearry *et al.*, 1990; King-Smith *et al.*, 1997). However, little is known of the role of intermediate filaments in teleost RPE cells or in eukaryotic cells in general.

The objective of this study was to determine the intermediate filament type or types expressed in bluegill RPE cells and to investigate whether the intermediate filament network is reorganized as pigment granules translocate. Previous immunolabeling experiments revealed the presence of intermediate filaments of unknown type extending from the level

of the circumferential microfilament bundles (CMBs) toward the apical processes in bluegill RPE cells. In this investigation, Western blot analysis has revealed the filamentous network to be composed of the protein vimentin with a relative molecular mass of 50 kDa. Corroborative immunolabeling studies have verified the intermediate filament type as vimentin and ruled out the presence of cytokeratin intermediate filaments in isolated RPE cells. Furthermore, when pigment granules were induced to aggregate into the cell body by the application of cAMP, the density of vimentin intermediate filaments increased in the apical processes of isolated RPE cells compared to the density of intermediate filaments in the processes of cells with pigment granules dispersed.

INTRODUCTION

The retinal pigment epithelium (RPE) is a monolayer of polarized columnar cells located between the neural retina and choroid layers of the eye (Fig. 1). These cells are differentiated from neighboring cell types by having long and short apical processes which interdigitate with the rod and cone photoreceptors (Fig. 2). In addition, as the name suggests, light-absorbing melanin pigment granules are found throughout the cytoplasm of these cells. RPE has numerous complex functions such as transport of metabolites between the photoreceptors and the choroid, phagocytosis of rod and cone outer segments, enhancement of image resolution by absorbing excess light, and maintenance of adhesion of the neural retina by the pumping of fluids from the vitreous to sclera (Zinn and Marmor, 1979). Furthermore, in teleosts the RPE plays a role in light adaptation.

Unlike higher vertebrates, teleosts and lower vertebrates have pupils of fixed diameter, thus they are unable to adjust their pupil diameter to changes in light intensity. Instead, changes in light intensity are compensated for by the adjustment of photoreceptor and pigment granule positions. The adjustments are referred to as retinomotor movements. In light, cone photoreceptors contract away from the RPE to maximize their exposure to the incoming light while rod photoreceptors elongate, burying themselves between the apical processes of the RPE in order to minimize their exposure to the light. During this process pigment granules of the RPE disperse into the apical processes, enabling them to function as a protective shield for the light sensitive rod photoreceptors. In dark, rod photoreceptors contract away from the RPE, thus maximizing their exposure to low levels of light. Furthermore, cone photoreceptors elongate toward the RPE while pigment granules migrate into the cell body (Fig. 2; Burnside and Nagle, 1983).

Pigment granule movement depends on the cytoskeleton of RPE cells, which is composed of microtubules, intermediate filaments, and microfilaments. In this

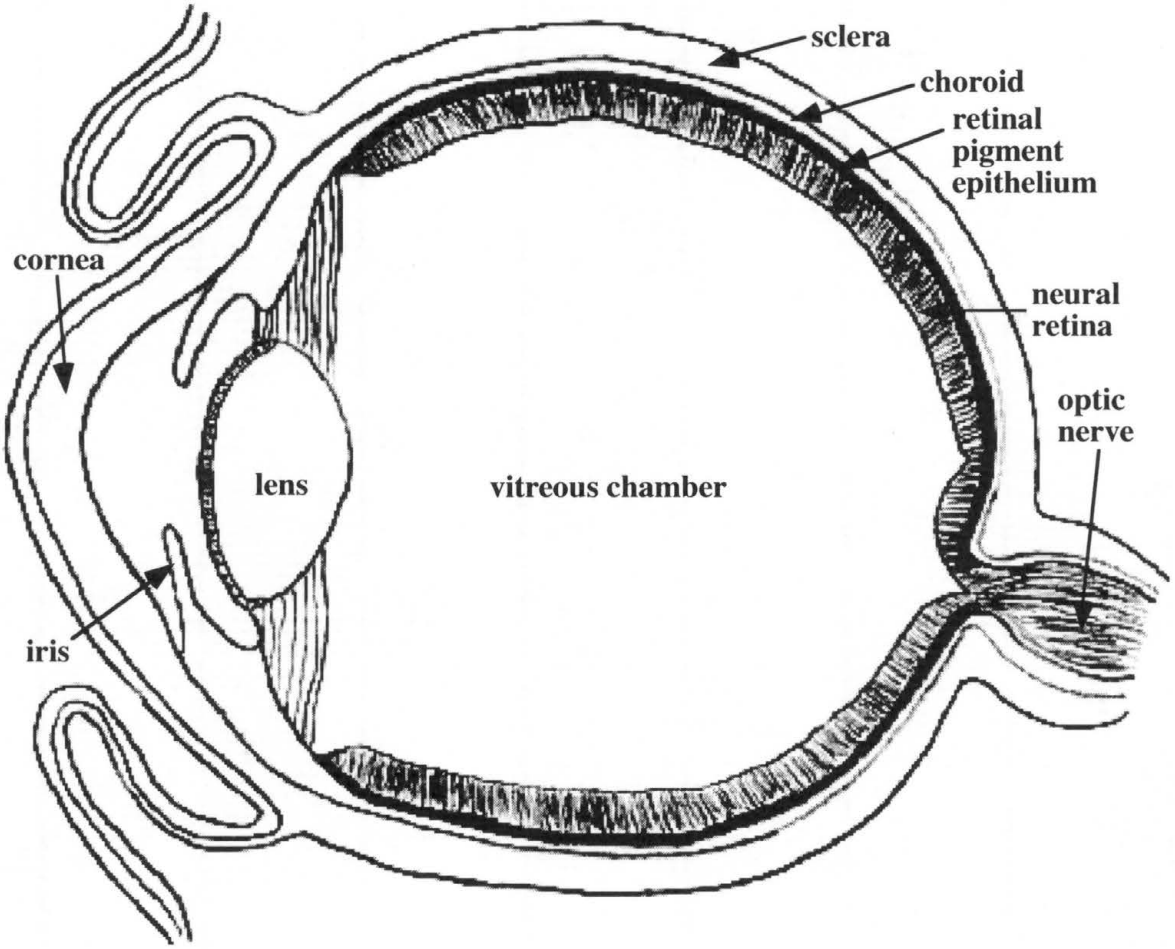


Figure 1. Illustration of the vertebrate eye showing the location of the retinal pigment epithelium (RPE) between the choroid and neural retina. Adapted from Adler and Farber, 1986 and Ayoub, 1996.

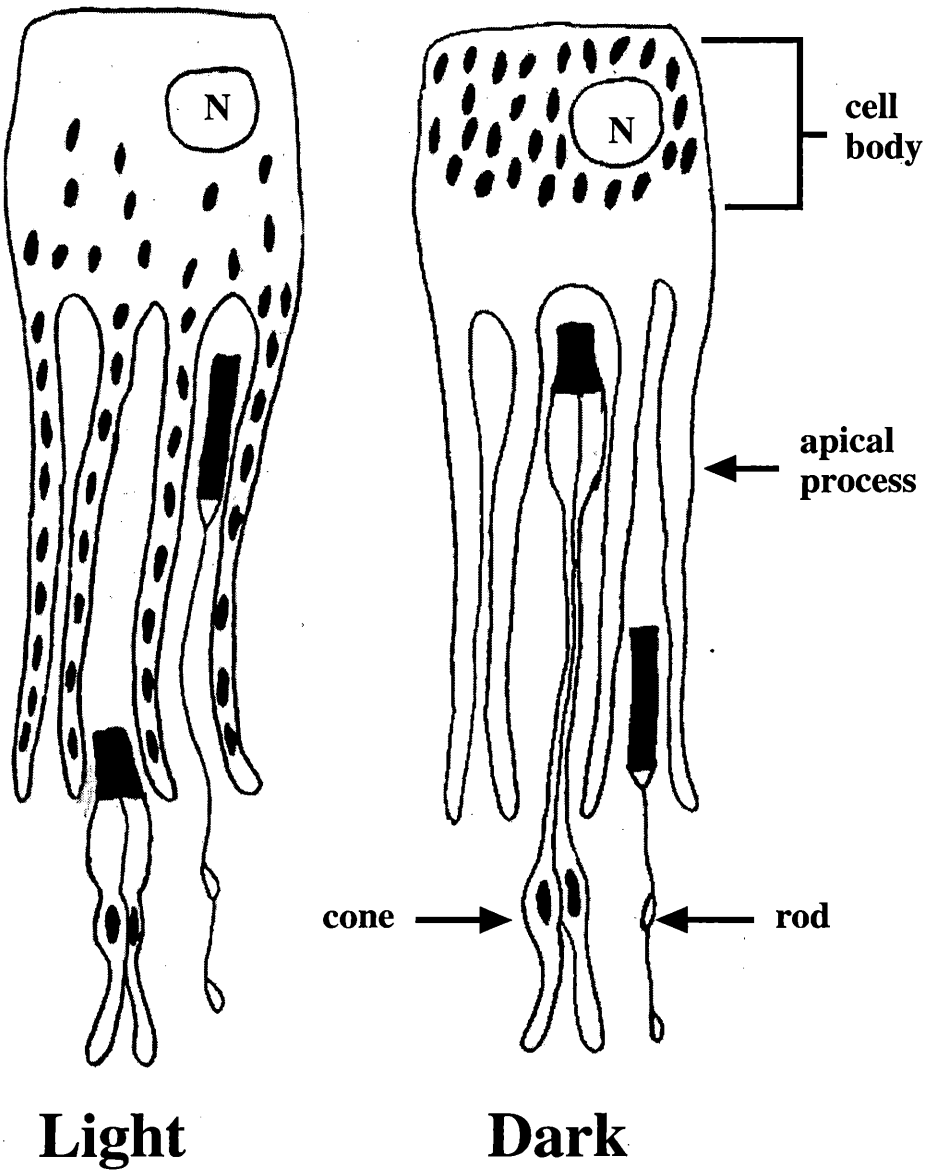


Figure 2. Illustration depicting retinomotor movements involving the retinal pigment epithelial cells and the rod and cone photoreceptors. In light, cones contract and rods elongate while pigment granules disperse into the apical processes. In dark, cones elongate and rods contract while pigment granules migrate into the cell body.

introduction, the assembly of the cytoskeleton and what is known about the cytoskeleton of RPE will be discussed. The role of microtubules and microfilaments in pigment granule translocation in teleost RPE will be examined. Finally, the present investigation to identify the intermediate filament type or types expressed in bluegill RPE and to determine if the intermediate filament network reorganizes in response to the aggregation and dispersion of pigment granules will be addressed.

Assembly of the Cytoskeleton

Microtubules consist of tubulin molecules which are heterodimers composed of globular polypeptides, _-tubulin and _-tubulin. The tubulin heterodimers associate in a row to form a protofilament which aligns linearly with 12 other protofilaments to form a hollow cylindrical structure (25 nm diameter) with a fast-growing, plus end and slow-growing, minus end giving the microtubule polarity. Furthermore, this cytoskeletal element is in a dynamic state of polymerization and depolymerization. It has been determined that the dynamic instability of microtubules depends on the hydrolysis of guanosine 5'-triphosphate (GTP). In this process GTP binds to the _-subunit of the tubulin molecule and upon addition to the end of a microtubule, GTP is hydrolyzed to guanosine 5'-diphosphate (GDP). However, in a growing microtubule, tubulin heterodimers are added to the polymer at a faster rate than the hydrolysis of the GTP bound to the _-subunit. As a consequence a stable GTP cap is formed which promotes polymerization because of its high affinity for heterodimers bound to GTP. Upon hydrolysis of GTP to GDP the bonds between tubulin molecules weaken, thus promoting disassembly of the protofilament into unpolymerized and free tubulin molecules (Mitchison, 1992; Alberts *et al.*, 1994).

Actin filaments are composed of globular actin (G-actin) monomers in a helical arrangement, forming a polymer of 6-7 nm diameter. As with microtubules, polymerization of G-actin monomers requires the presence of a nucleotide, in this case adenosine triphosphate (ATP), and the presence of K^+ and Mg^{2+}. Actin monomers carry a

bound ATP that is hydrolyzed to adenosine diphosphate (ADP) after the filament is assembled. Similar to GTP hydrolysis in microtubules, ATP hydrolysis promotes depolymerization by decreasing the affinity of the actin monomers for neighboring actin monomers in the polymer. In addition, these filaments maintain a state of polarity by having a slow-growing, minus end and a fast-growing, plus end (Mitchison, 1992; Alberts *et al.*, 1994).

The intermediate filament family is composed of three protein classes which include the cytokeratins, non-cytokeratins, and lamins. Classes are further subdivided into six types based on amino acid and cDNA sequence similarities. Type I are the acidic keratins, and type II are the neutral to basic keratins. Both Type I and II are specifically expressed in epithelial cells. Type III proteins are vimentin-related and include vimentin, expressed in mesenchymal cells; desmin, expressed in smooth, skeletal, and cardiac muscle cells; peripherin, found in neurons of dorsal root ganglia, sympathetic ganglia, cranial nerves, and ventral portion of the spinal cord; and glial fibrillary acidic protein (GFAP) specific to neuroglial cells. Type IV has four members, including the neurofilament-light chain (NF-L) , neurofilament-medium chain (NF-M), neurofilament-heavy chain (NF-H), which are expressed in the axons of motor and sensory neurons, and _-internexin which is specifically expressed in embryonic neurons. Type V are lamins A, B, and C, which are expressed in the nucleus, interconnecting nuclear pore complexes. Type VI is nestin, which is highly expressed in stem cells of the developing central nervous system and by somitic myoblasts (Fuchs, 1996; Kreis and Vale, 1993).

Intermediate filaments are composed of heterogeneous protein subunits which polymerize to form 8-12 nm filaments. The intermediate filament protein monomer is characterized by having three structural domains: (1) a highly conserved _-helical central rod domain, (2) a nonhelical head domain at the NH_2-terminus which varies in sequence and size in different intermediate filament proteins, and (3) a nonhelical tail domain at the COOH-terminus which also varies in sequence and size in different intermediate filament

proteins (Georgatos and Maison, 1996). Furthermore, the central rod domain is further subdivided into helices 1A, 1B, 2A, and 2B which are linked by nonhelical linker segments 1, 1-2, and 2 (Fuchs, 1996 and Fig. 3).

Intermediate filaments assemble in the absence of auxiliary proteins or factors (such as nucleotides), which suggests that assembly is directed by the primary structure of the intermediate filament polypeptide (Fuchs, 1996). In the assembly process, two _-helical rods intertwine in a parallel and left-handed fashion due to the presence of heptad repeats containing hydrophobic amino acids at the first and fourth positions. As a consequence, the two rods form a coiled-coil dimer. Dimers associate into half staggered tetramers in an antiparallel orientation. Tetramers then associate head to tail to form protofilaments. Two protofilaments intertwine to form a 4.5 nm protofibril, and finally, four protofibrils intertwine to form the 10 nm intermediate filament (Fuchs, 1996).

The Cytoskeleton of RPE

The organization of microtubules in RPE has been well described. These cytoskeletal elements extend from the perinuclear region toward the periphery of the cell (Owaribe, 1988). Ultrastructural studies conducted on light-adapted RPE of brook trout by Klyne and Ali (1981) revealed the presence of a microtubule network in association with mitochondria, endoplasmic reticulum, pigment granules, and 10-nm filaments in the basal area of the cells. Furthermore, microtubules were absent from the cell body of dark-adapted RPE. However, in ultrastructural studies conducted on green sunfish RPE comparing cells with pigment granules aggregated to cells with pigment granules dispersed, the number of microtubules in the apical processes increased when pigment granules were aggregated (Bruenner and Burnside, 1986). Investigations on the polarity of microtubules conducted by Troutt and Burnside (1988) suggested that in the apical processes of teleost

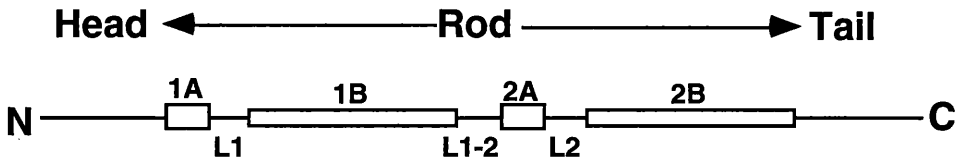


Figure 3. Illustration showing the secondary structures of the intermediate filament protein monomer. **N** and **C** represent the, NH_2-terminus and the COOH-terminus, respectively. Helices **1A**, **1B**, **2A**, and **2B** are linked by nonhelical linker segments **L1**, **L1-2**, and **L2** respectively. Adapted from Traub, 1995.

RPE, most microtubules are oriented with their fast-growing, plus end toward the cell body while the slow-growing, minus end is localized toward the tips of the processes. Similarly, studies conducted on chick RPE revealed that microtubules are oriented with their minus ends just under the apical process region while plus ends are toward the nucleus (Rizzolo and Joshi, 1993). This unusual microtubule orientation is opposite of most cell types, in which microtubules are oriented with their plus end toward the cell periphery (Euteneuer and McIntosh, 1981; McNiven *et al.*, 1984; Soltys and Borisy, 1985; Filliatreau and DiGiamberardino, 1981; Burton and Paige, 1981; Heidemann *et al.*, 1981).

In RPE, actin filaments exist as two distinct groups. Studies conducted on chick RPE revealed loosely packed actin filaments referred to as circumferential microfilament bundles in association with the zonula adherens region. These filaments display a nonrandom orientation with respect to polarity (Owaribe, 1988). Furthermore, it has been shown that isolated chick circumferential microfilament bundles are contractile structures which may possibly play a role in maintaining the polarity of the RPE and directly participate in changes of the cell shape and tissue shape (Owaribe and Masuda, 1982). In addition, actin filaments, referred to as the paracrystalline microfilament bundles, are present in the apical processes. Unlike the actin filaments of the circumferential microfilament bundles, these filaments are arranged in densely packed bundles and display the same polarity. It is thought that these filaments function in maintaining the structural integrity of the apical processes (Owaribe, 1988) and are likely to play a role in pigment granule movement (Burnside *et al.*, 1983; King-Smith *et al.*, 1997). Furthermore, studies using fluor-conjugated phalloidin as a marker for filamentous actin in bluegill RPE revealed the presence of the circumferential microfilament bundles and paracrystalline microfilament bundles *in situ* and *in vitro* (Zamora, 1997; Bolanos *et al.*, 1998; this thesis; Fig. 4).

The distribution of intermediate filaments in RPE has not been well characterized as microtubules and actin filaments. Ultrastructural studies conducted on the RPE of gold fish revealed the presence of 10 nm filaments confined to the basal and perinuclear regions but

not in the apical processes (Takeuchi and Takeuchi, 1979). These early investigations are corroborated by a later study in which 10 nm filaments were observed in the basal and perinuclear regions of brook trout RPE (Klyne and Ali, 1981). Later immunolabeling studies revealed that vimentin intermediate filaments are present in the cell body and absent in the apical processes of chick RPE (Philp and Nachmias, 1985). However, it has been shown that an intermediate filament network is present in the apical processes of chick RPE (Owaribe *et al.*, 1986). Furthermore, preliminary immunolabeling studies conducted on bluegill RPE utilizing anti-intermediate filament antigen (anti-IFA) IgG1 mouse monoclonal antibodies which recognize multiple types of intermediate filament proteins (Pruss *et al.*, 1981) revealed the presence of a cytoplasmic network of intermediate filaments extending from the level of the CMBs toward the apical processes (Zamora, 1997; Bolanos *et al.*, 1998; Fig. 5). Based on these observations, it seems more likely that intermediate filaments are present in the cell body and the apical processes in RPE.

The Role of the Cytoskeleton of Teleost RPE in Pigment Granule Translocation

Pigment granule movement may involve the microtubule network in teleost RPE. Studies in which colchicine was injected into the eyes of blue stripe grunt suggested that dispersion and aggregation of pigment granules within the RPE cell body is a microtubule dependent process; however, since microtubules within the apical processes remained intact, it could not be determined if microtubules are required for pigment granule movement within the apical processes (Burnside *et al.*, 1983). In subsequent microtubule disruption studies conducted on green sunfish RPE/retina, the application of nocodazole inhibited pigment granule aggregation, thus suggesting that microtubules somehow play a role in this process (Troutt and Burnside, 1989). However, microtubule disassembly did not affect pigment granule dispersion. Furthermore, based on the unusual microtubule polarity orientation and on the observation that microtubules may play a role in pigment

granule aggregation in teleost RPE, it was suggested that the microtubule-based motor protein, kinesin, may be involved directly in this process. Nevertheless, studies conducted by King-Smith *et al.* (1995) using function blocking antibodies strongly suggested that kinesin does not play a role in this process (although it is expressed in RPE).

Actin filaments may be required for pigment granule movement in teleost RPE. *In vivo* studies in which cytochalasin B was injected into the eyes of blue stripe grunt suggested that actin filaments are required for light-induced pigment granule dispersion and maintenance of the dispersed state (Burnside *et al.*, 1983). Later studies conducted on green sunfish RPE, isolated from the rest of the retina, suggested that actin filaments may play a role in pigment granule movements as indicated by the inhibition of pigment granule dispersion when teleost RPE was treated with cytochalasin D, an inhibitor of actin filament assembly (Dearry *et al.*, 1990). Recently, studies using green sunfish RPE cells cultured on glass coverslips suggested that both pigment granule dispersion and aggregation are actin-dependent processes and do not involve microtubules (King-Smith *et al.*, 1997).

Although much progress has been made in microtubule and actin filament studies, little is known about what role intermediate filaments play in pigment granule translocation in teleost RPE cells or in eukaryotic cells in general. In order to test whether intermediate filaments were involved in pigment granule movement, studies were first conducted to determine the type(s) of intermediate filament expressed by bluegill RPE. Western blot analysis was used to determine the intermediate filament type, and immunocytochemistry was then used to verify the identity of the intermediate filament type. To investigate whether the distribution of intermediate filaments in isolated RPE cells undergo a change when pigment granules are aggregated and dispersed, immunolabeling studies were conducted on RPE cells in the aggregated and dispersed states using confocal microscopy. Results suggest that vimentin intermediate filaments undergo a subtle reorganization concomitant with pigment granule translocation.

METHODS

Fish Culture

Use of animals was approved by the Southwest Texas State University Institutional Animal Care and Use Committee (protocol #1011). Bluegill fish (*Lepomis macrochirus*) were obtained from Johnson Lake Management Service located in San Marcos, Texas, and maintained in aerated, chlorinated tap water in 55 gallon laboratory aquaria. Fish were entrained to a 12 hr light/dark cycle for at least two weeks prior to experimentation.

Isolation and Preparation of Bluegill Tissues for Microscopy

RPE Prep #1

Dissociation of RPE cells was performed according to a procedure adapted from Owaribe *et al.* (1988). Bluegill fish were dark adapted for 30 min in aerated tanks in the afternoon. Fish were sacrificed in complete darkness by severing the spinal cord and double-pithing, and the eyes were enucleated and hemisected under dim white light. The cornea, vitreous humor, and lens were discarded. From the posterior eye cup, retina was carefully peeled free from the RPE and discarded. Eyecups were then incubated for 45 min to 1 hr at 37° C in phosphate buffered saline (PBS) (4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$, 1.4 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl; pH 7.3) containing 2 mM EDTA. Sheets of RPE were isolated by gently flushing the eyecup with a steady stream of PBS/EDTA solution. Afterwards, RPE sheets were transferred to a 15 ml plastic centrifuge tube containing PBS/EDTA solution and dissociated by gently triturating in a Pasteur pipet. Dissociated cells were seeded onto glass coverslips. Cells were allowed to settle and adhere onto the coverslips at room temperature for 1-1.5 hr or used immediately for immunolabeling.

Gel Electrophoresis and Immunoblotting

RPE sheets and neural retina were obtained from dark adapted fish and incubated separately in Ringer solution which contained (in mM) NaCl, 116.3; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 0.8; Na_2HPO_4, 1.0; glucose, 25.5; $NaHCO_3$, 4.0; HEPES, 21; pH 7.2. Bluegill heart, brain, and liver were also obtained and maintained in Ringer solution. Tissues were homogenized in 1.5 ml centrifuge tubes containing homogenization buffer (50 mM Tris-HCl, pH 7.4; 10 mM EDTA; 20 μm leupeptin; 1.5 μm aprotinin; 26 μm TAME; 0.5 mM PMSF). Retina, liver, heart, and brain homogenates were centrifuged at 800x*g* for 5 min at 4° C using a Spectrafuge Brushless Microcentrifuge with an 18x1.5 ml rotor (National Labnet, Woodbridge, NJ) (King-Smith *et al.,* 1995). RPE homogenate was centrifuged at 2000x*g* for 5 min at 4° C to bring down pigment granules. Supernatants were decanted into separate centrifuge tubes, and pellets were discarded. Supernatants were kept frozen at -80° C until protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA) with bovine plasma gamma globulin as a standard to ensure equal loading of protein samples during gel electrophoresis (Bradford, 1976).

Protein samples were diluted in reducing buffer containing 60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue. Samples were then boiled at 100° C in a water bath for 5 min to facilitate protein denaturation. Prestained broad range standards (Bio-Rad, Hercules, CA) and protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using gels composed of a 5% stacking gel and a 12% separating gel. Electrophoresis was done at a constant voltage of 200V with duplicate gels (Laemmli, 1970). Electrophoresis continued until the dye front migrated to about 1 cm from the bottom of the gels. One gel of the pair was stained in Coomassie gel stain solution (0.1% w/v Bio-Rad Coomassie brilliant blue-R-250, 45% methanol, 10% glacial acetic acid) for 30 min and de-stained in Coomassie gel destain solution (10% methanol, 10% glacial acetic acid).

The duplicate unstained gel and a nitrocellulose sheet were equilibrated in transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol; pH 8.1-8.4) for 30 min at room temperature. Proteins were transferred from the unstained gel onto nitrocellulose by electrotransfer using a Bio-Rad Mini Trans-blot transfer cell containing a frozen cooling unit at a constant voltage of 100V at room temperature for 1 hr. After transfer, nitrocellulose membrane was washed briefly with PBS and blocked for 2 hr in 20% non-fat powdered milk in PBS at room temperature. The membrane was washed 3X for 15 min with PBS and incubated for 1.5 hr at room temperature in anti-vimentin polyclonal antibodies developed in goat (Sigma, St. Louis, MO) diluted 1:700 in PBS containing 0.5% BSA. Nitrocellulose was washed 3X for 15 min with PBS and incubated for 1.5 hr at room temperature in rabbit anti-goat IgG alkaline phosphatase conjugated antibodies (Sigma, St. Louis, MO) diluted 1:30,000 in PBS containing 0.5% BSA. Membrane was washed 2 times with PBS and one time with alkaline phosphate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$; pH 9.5). Blots were developed by exposure to developing solution containing 6 mM *p*-nitro blue tetrazolium chloride and 11.5 mM 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphate buffer. Development was stopped by rinsing membrane with 20 mM EDTA in PBS.

Images of the stained gel and immuno-stained nitrocellulose membrane were recorded using a flat bed scanner and O-foto software. Images were prepared for presentation using Adobe Photoshop and Clarisworks software.

Isolation and Preparation of Bluegill Tissues for Microscopy

RPE Prep #2

Isolation and dissociation of RPE were performed according to a procedure adapted from King-Smith *et al.* (1997). Bluegill fish were dark adapted for 1 hr in aerated aquaria during their light period in the afternoon to facilitate separation of RPE from neural retina. In complete darkness fish were sacrificed by severing the spinal cord and double-pithing.

Under dim white light, eyes were enucleated and hemisected. Cornea, vitreous humor, and lens were removed and discarded. Neural retina was isolated from the posterior eye cup by carefully peeling it free from the RPE. Sheets of RPE were isolated by gently flushing the eye cup with a steady stream of Ringer solution using a plastic pipet. RPE sheets were enzymatically dissociated in digest solution containing 11.2 units/ml papain (Worthington, Freehold, NJ) activated 30 min before incubation with 3.5 mM cysteine and 0.14 mg/ml deoxyribonuclease I (DNase I; Sigma, St. Louis , MO) in calcium- and magnesium-free Ringer solution containing 1 mM EGTA. Incubation of RPE in digest medium lasted 30 min at room temperature. RPE was then transferred to a 15 ml plastic centrifuge tube and washed 3X with dissociation/wash solution containing Ringer solution, 0.5 % bovine serum albumin (BSA) (Sigma, St. Louis, MO) and 0.14 mg/ml DNase I. RPE was dissociated by trituration using a 10 ml glass serological pipet in dissociation/wash solution. Dissociated cells were seeded onto glass coverslips. Cells were allowed to settle and adhere onto the coverslips in a humidified chamber at room temperature for 4 hr. Two hours into the incubation period, incubation medium for half of the coverslips was replaced with Ringer solution containing 0.5 % BSA and 1 mM cAMP (free acid; Boehringer Mannheim, West Germany) to induce pigment granule aggregation.

Liver

A light adapted bluegill sunfish was sacrificed by severing the spinal cord and double-pithing. Liver was isolated and fixed by quick-freezing in -80° C methanol. Frozen tissue was then placed on a pre-cooled mounting stud and embedded in Tissue-Tek O. C. T. compound (Fisher Scientific, Houston, Texas, U. S. A.) at -23° C. Embedded liver was sectioned at 10 μm with a cryotome (Zeiss Microm HM 505 N, Thornwood, New York, USA). Sections were placed on 0.1 % poly-L-lysine (Sigma, St. Louis, MO) coated coverslips and allowed to dry overnight at 4° C prior to immunolabeling.

Immunofluorescence Microscopy

Labeling of RPE Cells (Prep # 1)

Dissociated RPE cells on coverslips were washed with PBS once and fixed by submerging them in -80° C hexane, incubating them in ice cold 5% acetic acid in ethanol for 10 min or incubating them in 4% paraformaldehyde for 10 min. Cells were then washed 3X for a total of 15 min with PBS containing 0.2% Tween-20 (PBST) and then blocked for 1 hr at room temperature in 20% non-fat powdered milk in PBST. Coverslips were washed 3X with PBST for a total of 15 min. RPE cells were incubated for 2 hr at room temperature in undiluted anti-intermediate filament antigen (anti-IFA) IgG1 mouse monoclonal antibodies or anti-vimentin IgM mouse monoclonal antibodies (clone 13.2; Sigma, St. Louis, MO) diluted 1:100 in PBST. Control cells were incubated for 2 hr at room temperature in normal mouse serum (NMS; Sigma, St. Louis, MO) diluted 1:200 or 1:100 in PBST. Cells were then washed 3X with PBST for a total of 15 min. RPE cells were incubated for 2 hr at room temperature in anti-mouse polyvalent FITC-conjugate antibodies (Sigma, St. Louis, MO) diluted 1:256 in PBST or goat anti-mouse IgG TRITC-conjugate antibodies (Sigma, St. Louis, MO) diluted 1:128 in PBST in a dark environment. Cells were then washed 3X for a total of 15 min with PBS. Afterwards, cells were incubated for 20 min at room temperature in Texas red-conjugated phalloidin or fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR) in a dark environment. Cells were then washed 3X for a total of 15 min with PBS. Coverslips were mounted in 90% glycerol in PBS containing 1% *p*-phenylenediamine and examined by a confocal microscopy system on an Olympus IX-70 inverted microscope coupled to a Bio-Rad MRC-1024 laser scanhead (Bio-Rad Laboratories, Hercules, California, U. S. A.). Images were acquired and processed using Bio-Rad Lasersharp software running on a Compaq PC.

Labeling of RPE Cells (Prep # 2) and of Liver Sections

Prior to the immunolabeling procedure differential interference contrast (DIC) images of representative RPE cells with pigment granules aggregated and dispersed were recorded using the Olympus IX-70 inverted microscope equipped for DIC. RPE cells or liver sections were permeabilized by 3 washes with 0.5% Tween-20 in PHEM buffer (50 mM PIPES, 25 mM HEPES, 8 mM EGTA, 2 mM $MgCl_2$, pH 7.0). Cells were then fixed for 5 min in methanol chilled to dry ice temperature. Fixed cells or sections were washed 3X for a total of 15 min with PHEM buffer and blocked for 1 hr at room temperature in 20% non-fat powdered milk in PHEM buffer. RPE cells were incubated for 2 hr at room temperature in anti-vimentin IgM mouse monoclonal antibodies (clone 13.2; Sigma, St. Louis, MO) diluted 1:100 in PHEM buffer or multi-keratin (recognizes keratins 4, 5, 6, 8, 10, 13, and 18) mouse monoclonal IgG_1 antibodies (Neomarkers, Clone C-11, Fremont, CA) diluted 1:300 in PHEM buffer . As a positive control for cytokeratin labeling, liver sections were incubated in multi-keratin mouse monoclonal IgG_1 antibodies. As a negative control for the staining procedure, RPE cells and liver sections were incubated in normal mouse serum (Sigma, St. Louis, MO) diluted 1:100 and 1:300 in PHEM buffer respectively. Afterwards, liver sections and cells were washed 3X for a total of 15 min with PHEM buffer. RPE cells or liver sections were incubated for 2 hr at room temperature in anti-mouse polyvalent FITC-conjugate (Sigma, St. Louis, MO) diluted 1:256 in PHEM buffer in a dark environment. Cells or liver sections were then washed 3X for a total of 15 min with PHEM buffer. Some of the cells were incubated for 20 min at room temperature in Texas red-conjugated phalloidin (Molecular Probes, Eugene, OR) in a dark environment. Cells were then washed 3X for a total of 15 min with PHEM buffer. Coverslips were mounted in 90% glycerol in PBS containing 1% *p*-phenylenediamine and examined by confocal microscopy.

RESULTS

Immunolabeling of Bluegill RPE (Prep # 1)

In paraformaldehyde fixed bluegill RPE cells stained with Texas-Red conjugated phalloidin, circumferential microfilament bundles and filamentous actin were distinctly labeled (Fig. 4).

In hexane fixed bluegill RPE cells probed with anti-intermediate filament antigen antibodies and fluor-conjugated phalloidin, an intermediate filament network extending from the level of the circumferential microfilament bundles toward the apical processes was revealed; however, filamentous actin in the apical processes did not label (Fig. 5). No labeling was observed in cells incubated in normal mouse serum (Fig. 5).

In isolated bluegill RPE cells fixed in acetic acid in ethanol and probed with mouse anti-vimentin monoclonal antibodies and Texas-Red conjugated phalloidin, a filamentous network was revealed extending from the cell body toward and into the apical processes (Fig. 6). However, circumferential microfilament bundles and filamentous actin did not label (Fig. 6). No labeling was observed in cells incubated in normal mouse serum (Fig. 6).

Immunoblots of Bluegill Tissue Homogenates

In order to determine the intermediate filament type or types expressed in bluegill RPE, I subjected homogenates of RPE, neural retina (positive control), brain (positive control), liver (negative control), and heart (negative control) to SDS-PAGE and Western blot analysis. Blots were probed with anti-vimentin polyclonal antibodies developed in goat using purified vimentin protein from cultured human foreskin fibroblasts as the immunogen. Anti-vimentin polyclonal antibodies reacted with a polypeptide band with a relative molecular mass of 50 kDa in bluegill RPE, retina, and brain protein samples (Fig.

7). The anti-vimentin polyclonal antibodies did not label any polypeptide bands in bluegill heart and liver protein sample lanes (Fig. 7).

Immunolabeling of Bluegill Liver and RPE (Prep # 2)

In Prep # 1 bluegill RPE cells were obtained with a squid-like appearance which made it difficult to determine whether or not intermediate filaments are present in the apical processes. It is possible that the apical processes failed to adhere to the coverslips thus all or parts of the apical processes were washed off the coverglass during the immunolabeling procedure. In order to determine whether or not intermediate filaments are present in the apical processes, Prep # 2 was used to obtain dissociated RPE cells adhered onto glass coverslips with their apical processes spread out in a star-like formation and with pigment granules dispersed (Fig. 8). Through the use of Prep # 2, I was able to verify the expression of vimentin intermediate filaments by RPE cells and determine its distribution in isolated bluegill RPE cells using immunolabeling techniques.

Through immunolabeling techniques, anti-vimentin monoclonal antibodies, and anti-cytokeratin monoclonal antibodies were used to determine the intermediate type or types expressed in isolated bluegill RPE cells. Monoclonal anti-vimentin antibodies labeled a fine filamentous network throughout the cell body and the apical processes of isolated RPE cells (Fig. 9b and 10b). When normal mouse serum was used in place of primary antibodies, no labeling was observed in RPE cells (Fig. 9d and 10d). Furthermore, no labeling was observed in isolated RPE cells incubated in monoclonal anti-cytokeratin antibodies (Fig. 11b). However, the same monoclonal anti-cytokeratin antibodies labeled hepatocytes and endothelial cells lining blood vessels in sections of frozen bluegill liver (Fig. 11d). No labeling was observed in liver sections incubated in normal mouse serum (Fig. 12b). Additionally, bluegill RPE cells incubated in Texas-Red conjugated phalloidin labeled filamentous actin throughout the cell body and the apical processes (Fig. 4).

Spontaneous dispersion of pigment granules into the apical processes from dark-adapted fish has been observed to occur within 15 min after isolation of RPE sheets. (García and Burnside, 1994). Furthermore, studies have shown that addition of 1 mM cAMP to the incubation medium of isolated RPE cells, induces the aggregation of pigment granules (García and Burnside, 1994). In the present study, when isolated RPE cells with pigment granules dispersed were incubated in Ringer solution containing 1 mM cAMP, complete aggregation of the pigment granules was observed (Fig. 8a). Because pigment granules undergo spontaneous dispersion and cAMP triggers pigment granule aggregation, the distribution of intermediate filaments could be examined by confocal microscopy in the dispersed and aggregated state.

When isolated RPE cells were treated with cAMP, complete aggregation of the pigment granules into the cell body was observed (Fig. 8a). In RPE cells with pigment granules aggregated, vimentin intermediate filaments were observed throughout the cell body including the apical processes. Labeling of the vimentin intermediate filament network was greatest in the apical processes devoid of pigment granules and concentrated in a central region of the cell (Fig. 9b and 13a). However, in isolated RPE cells with pigment granules dispersed vimentin intermediate filament labeling was diffuse and less intense in the apical processes. Furthermore, vimentin intermediate filaments were present throughout the cell body but not concentrated in a central region as observed in a RPE cell with pigment granules aggregated (Fig. 10b and 13b).

Figure 4

Confocal micrographs of isolated bluegill retinal pigment epithelial cells stained with Texas-Red conjugated phalloidin labeling filamentous actin (F-actin) and the circumferential microfilament bundles (CMBs). **A**: Representative RPE cell fixed in 4% paraformaldehyde (PFA) illustrating a squid-like appearance. **B**: Representative cell fixed in -80° C methanol with apical processes spread out in a star-like shape. Arrows point to the CMBs, F-actin, and apical processes.

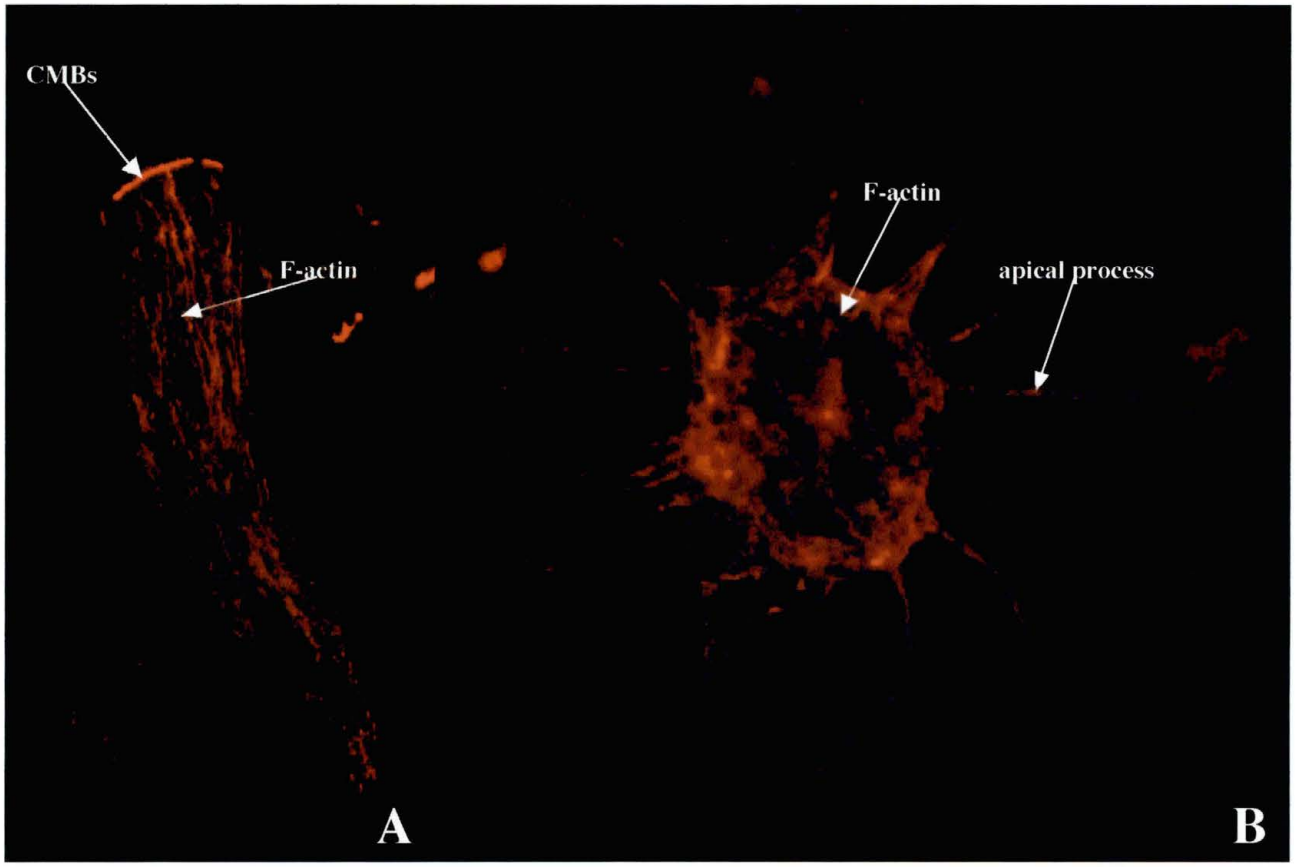

CMBs
F-actin
F-actin
apical process
A
B

Figure 5

Confocal micrographs of isolated retinal pigment epithelial cells fixed in -80° C hexane. RPE cells were incubated in anti-intermediate filament antigen IgG_1 monoclonal antibodies (Pruss *et al.*, 1981) and in anti-mouse polyvalent FITC-conjugate or anti-mouse IgG TRITC-conjugate secondary antibodies. Texas-Red conjugated phalloidin or fluorescein conjugated phalloidin were used to label the circumferential microfilament bundles (CMBs). **A** & **C**: Intermediate filaments (IFs) extend from the level of the CMBs toward the apical processes. Arrows point to the CMBs and the IFs. **B** & **D**: No immunolabeling of IFs was observed in cells incubated in normal mouse serum.

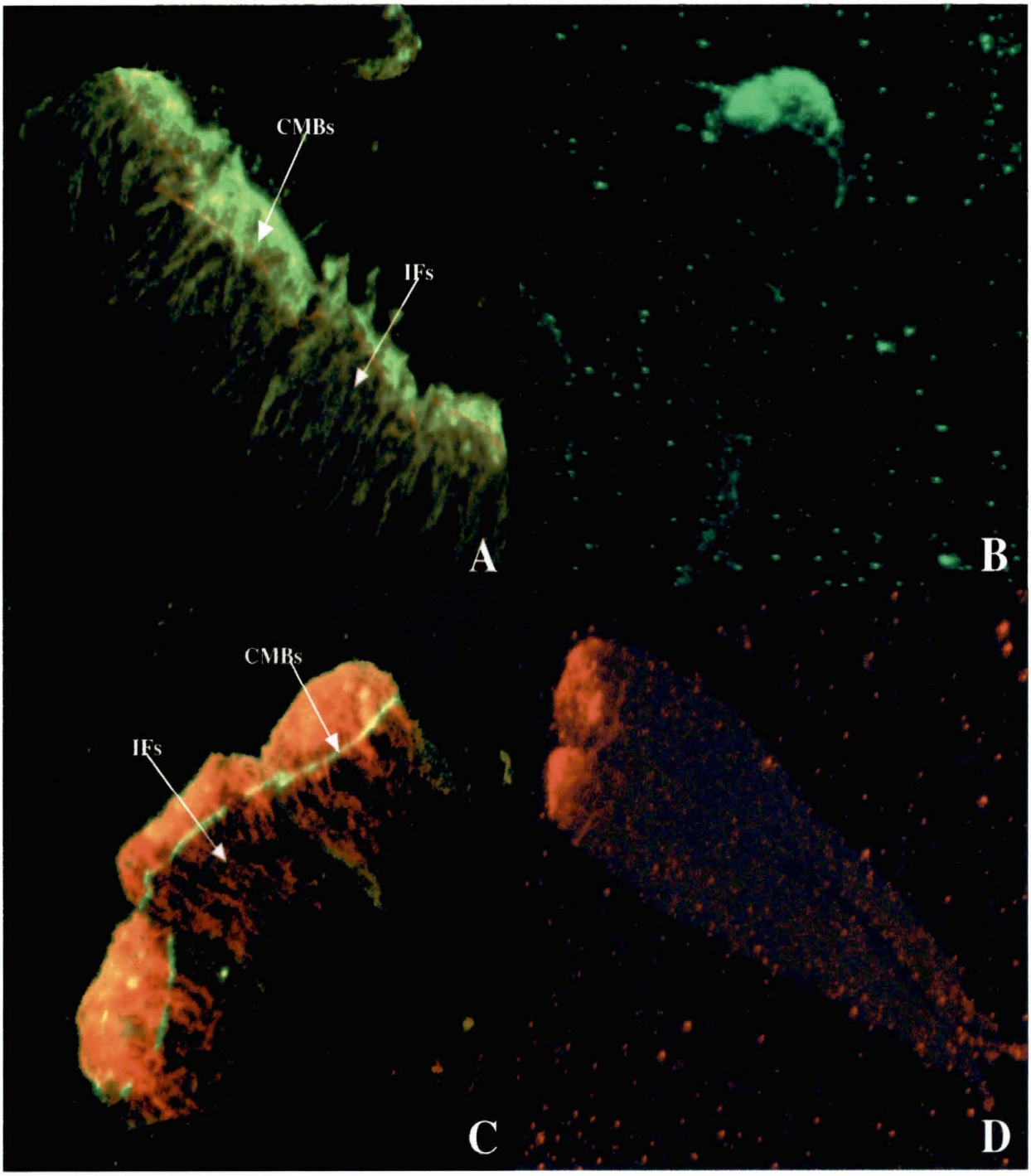

CMBs
IFs
A
B
CMBs
IFs
C
D

Figure 6

Confocal micrographs of isolated retinal pigment epithelial cells fixed in ice cold 5% acetic acid in ethanol. RPE cells were incubated in mouse anti-vimentin IgM monoclonal antibodies and in anti-mouse polyvalent FITC-conjugated secondary antibodies. Cells were also incubated in Texas-Red conjugated phalloidin. **A**: Vimentin intermediate filaments extend from the level of the cell body toward and into the apical processes. Circumferential microfilament bundles and filamentous actin did not label. Arrow points to the intermediate filaments. **B**: No immunolabeling of intermediate filaments was observed in cells incubated in normal mouse serum.

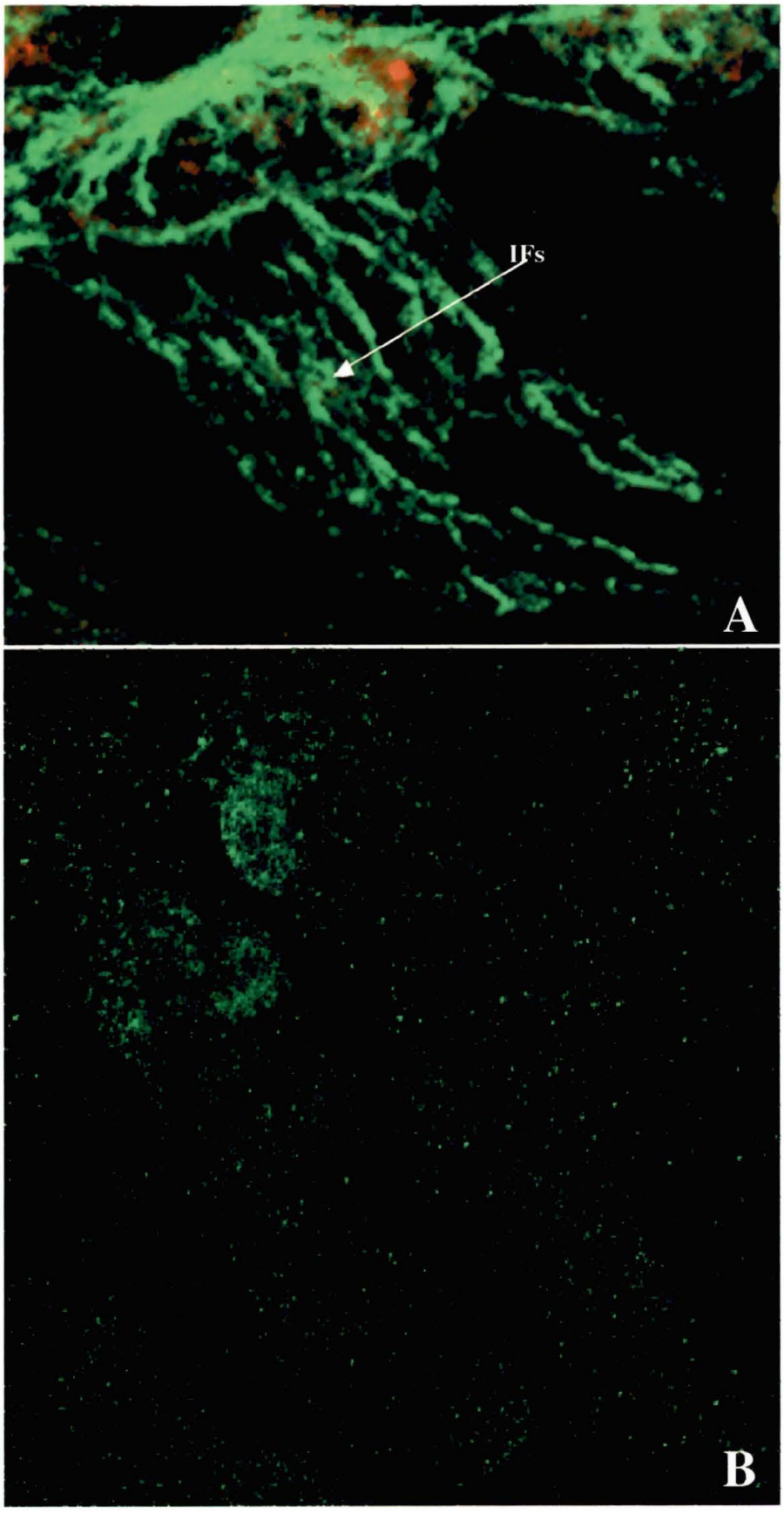

IFs
A
B

Figure 7

Western blot analysis of bluegill RPE . **A**: SDS-PAGE gel stained with Coomassie blue showing separation of proteins in bluegill RPE, liver, neural retina, heart, and brain homogenates respectively. **B**: Corresponding immunoblot probed with goat anti-vimentin polyclonal antibodies, showing a prominent band at 50 kDa in RPE, neural retina, and brain.

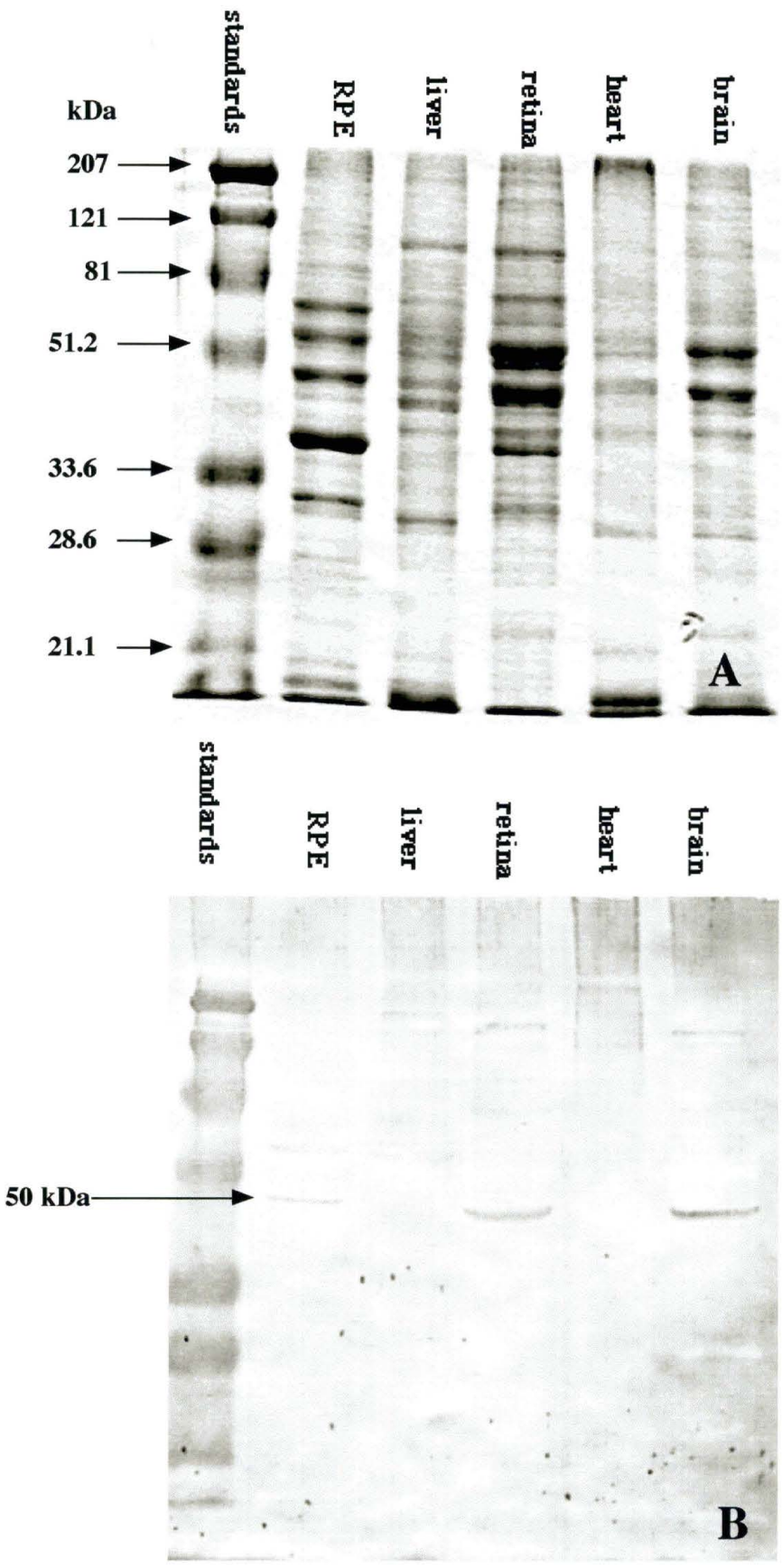

standards
RPE
liver
retina
heart
brain
kDa
207
121
81
51.2
33.6
28.6
21.1
A
standards
RPE
liver
retina
heart
brain
50 kDa
B

Figure 8

Transmitted light images of isolated bluegill RPE cells illustrating pigment granule aggregation and dispersion prior to the immunolabeling procedure. **A**: RPE cell treated with cAMP to induce pigment granule aggregation into the cell body. **B**: RPE cell without cAMP treatment showing spontaneous pigment granule dispersion into the apical processes. Arrows point to pigment granules and apical processes. Calibration bars represent 50 μm (A) and 10 μm (B).

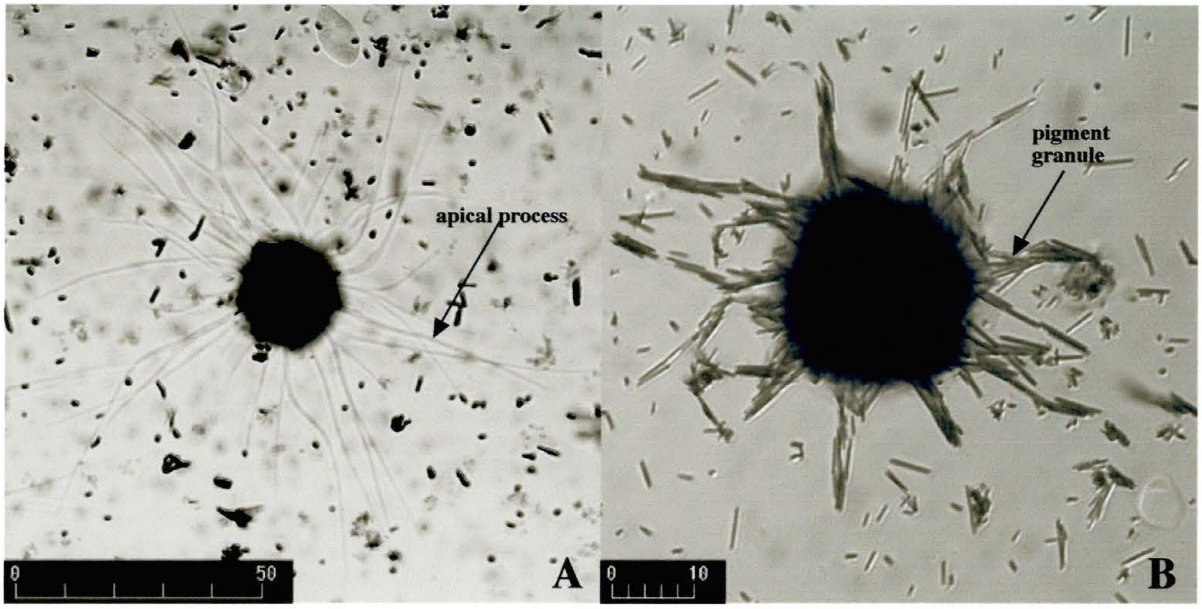

apical process
0
50
A
pigment
granule
0
10
B

Figure 9

Distribution of vimentin intermediate filaments in RPE cells with pigment granules aggregated. Sequential Z-plane optical sections were taken through the RPE cells to compose a stacked image projection of 13.3 μm depth. **A** & **C**: DIC images of isolated RPE cells treated with cAMP to induce pigment granule aggregation. Apical processes are devoid of pigment granules. **B**: Confocal micrograph of RPE cell in **A** after incubation in mouse anti-vimentin monoclonal antibodies and in anti-mouse polyvalent FITC-conjugated secondary antibodies. Vimentin intermediate filaments are localized throughout the cell body including the apical processes. Intense labeling of the vimentin intermediate filament network was in the apical processes devoid of pigment granules and concentrated in a central region of the cell body. **D**: Confocal micrograph of RPE cell in **C** after incubation in normal mouse serum in place of the primary antibody which resulted in no labeling. Arrows point to apical processes. Calibration bars represent 10 μm.

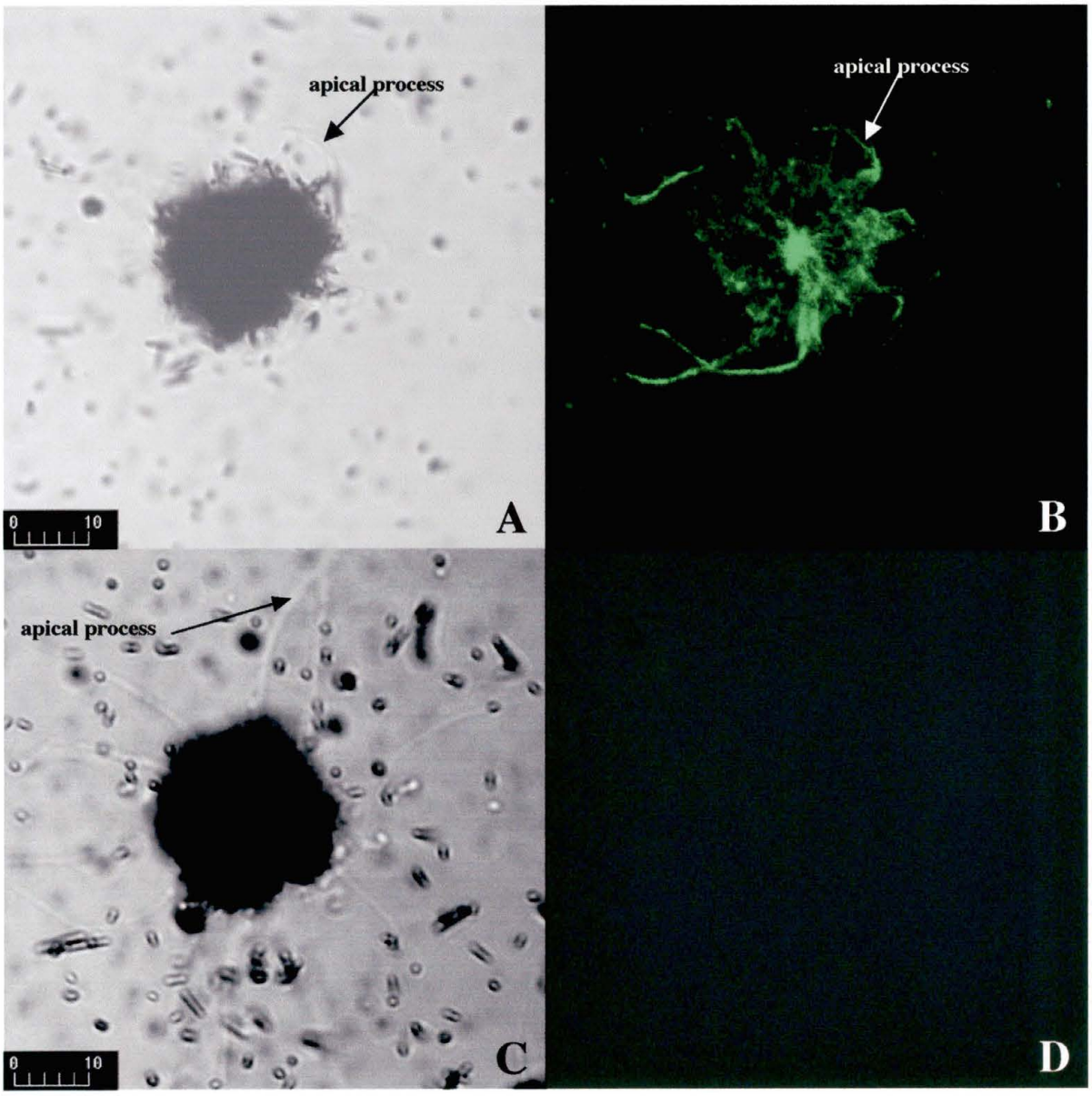

apical process
A
apical process
B
apical process
C
D

Figure 10

Distribution of vimentin intermediate filaments in RPE cells with pigment granules dispersed. Sequential Z-plane optical sections were taken through the RPE cells to compose a stacked image projection of 4.4 μm depth. **A** & **C**: DIC images of isolated RPE cells without cAMP treatment displaying pigment granule dispersion into the apical processes. **B**: Confocal micrograph of RPE cell in **A** after incubation in mouse anti-vimentin monoclonal antibodies and in anti-mouse polyvalent FITC-conjugated secondary antibodies. Vimentin intermediate filaments are distributed throughout the cell body including the apical processes. Labeling of vimentin intermediate filaments appears diffuse and less intense in the apical processes. Vimentin intermediate filaments are labeled throughout the cell body but are not concentrated in one central area. **D**: Confocal micrograph of RPE cell in **C** after incubation in normal mouse serum in place of the primary antibody which resulted in no immunolabeling of intermediate filaments. Arrows point to apical processes. Calibration bars represent 10 μm.

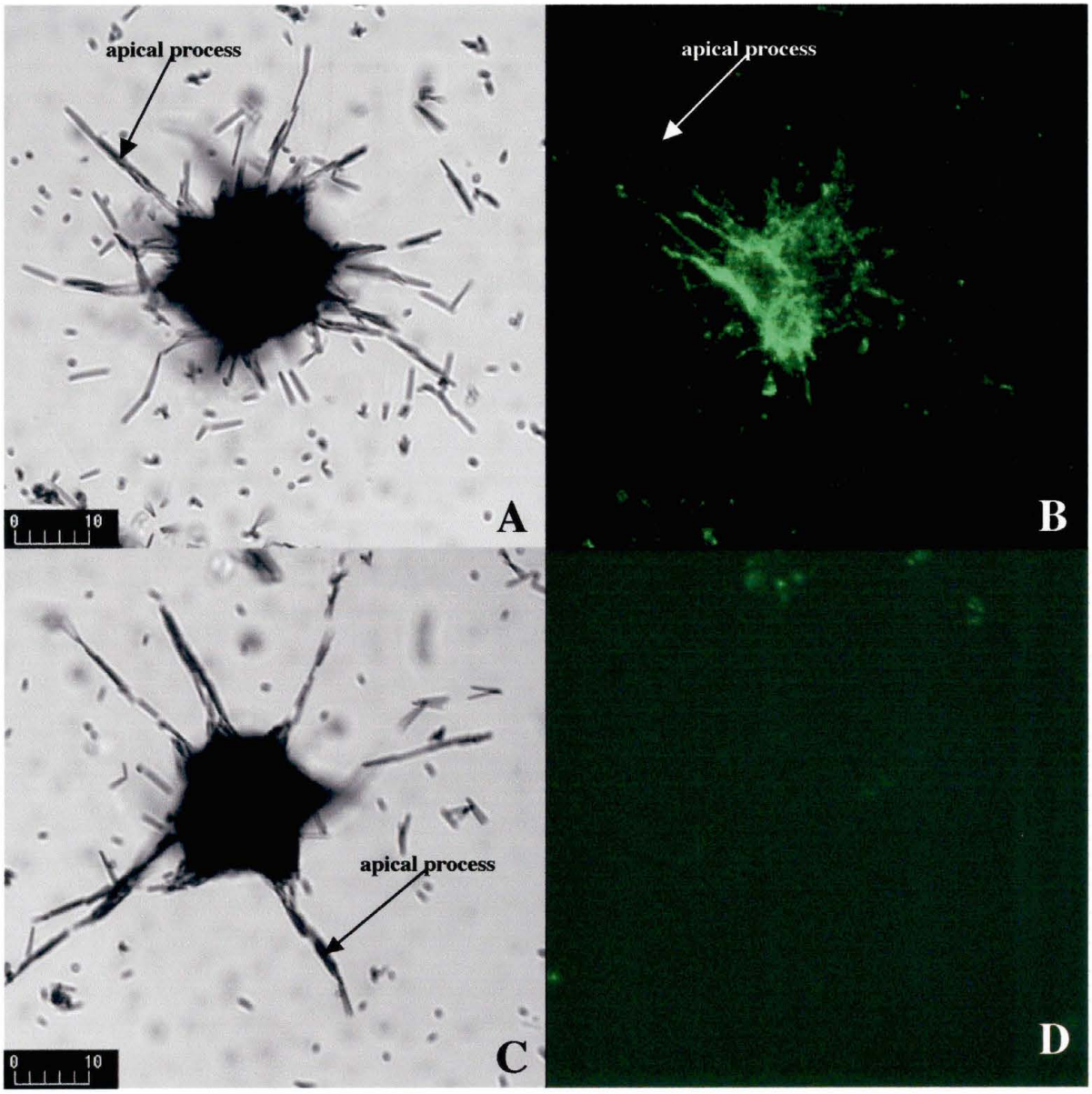

apical process
A
apical process
B
apical process
C
D

Figure 11

Immunolabeling of a bluegill RPE cell and a liver section for cytokeratin. **A & C**: DIC image of an isolated RPE cell displaying dispersed pigment granules and of a liver section through a blood vessel. **B**: Confocal micrograph of RPE cell in **A** after incubation in mouse anti-cytokeratin monoclonal antibodies and in anti-mouse polyvalent FITC-conjugated secondary antibodies. No immunolabeling of intermediate filaments was observed. **D**: Sequential Z-plane optical sections were taken through the liver section to compose a stacked image projection of 18 μm depth. Confocal micrograph of liver section in **C** after incubation in mouse anti-cytokeratin monoclonal antibodies and in anti-mouse polyvalent FITC-conjugated secondary antibodies displaying positive labeling of cytokeratin intermediate filaments in the endothelial cells of a blood vessel and in hepatocytes. Arrows point to apical process, blood vessel, and hepatocytes. Calibration bar represents 10 μm (A) and 50 μm (C).

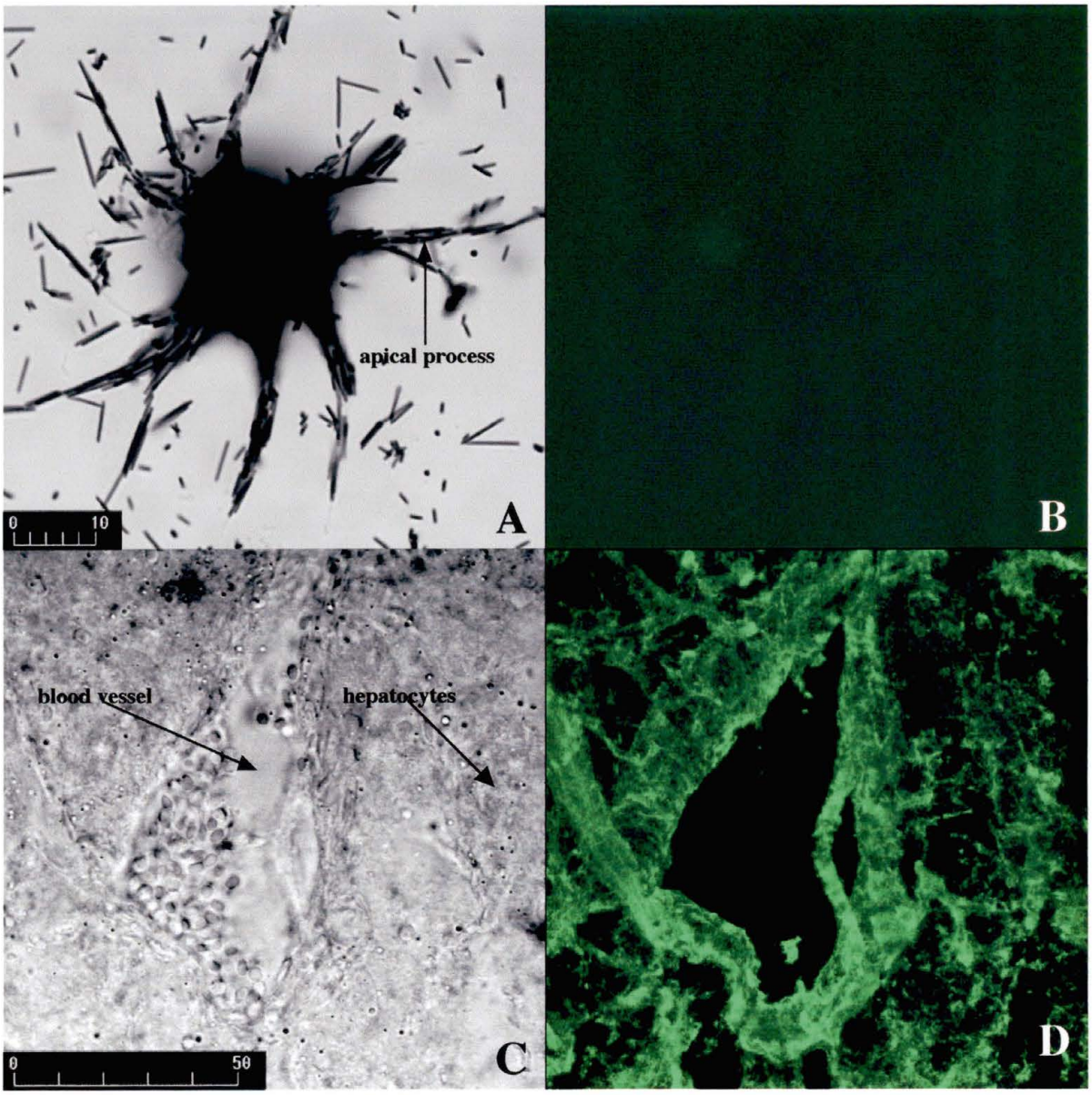

apical process
0
10
A
B
blood vessel
hepatocytes
0
50
C
D

Figure 12

Photomicrograph of a control tissue for figure 11 (bluegill liver section). **A**: DIC image of liver section through a blood vessel. **B**: Sequential Z-plane optical sections were taken through the liver section to compose a stacked image projection of 18 μm depth. Confocal micrograph of bluegill liver section in **A** after incubation in normal mouse serum and in anti-mouse polyvalent FITC-conjugated secondary antibodies; no labeling was observed. Arrow points to blood vessel. Calibration bar represents 50 μm.

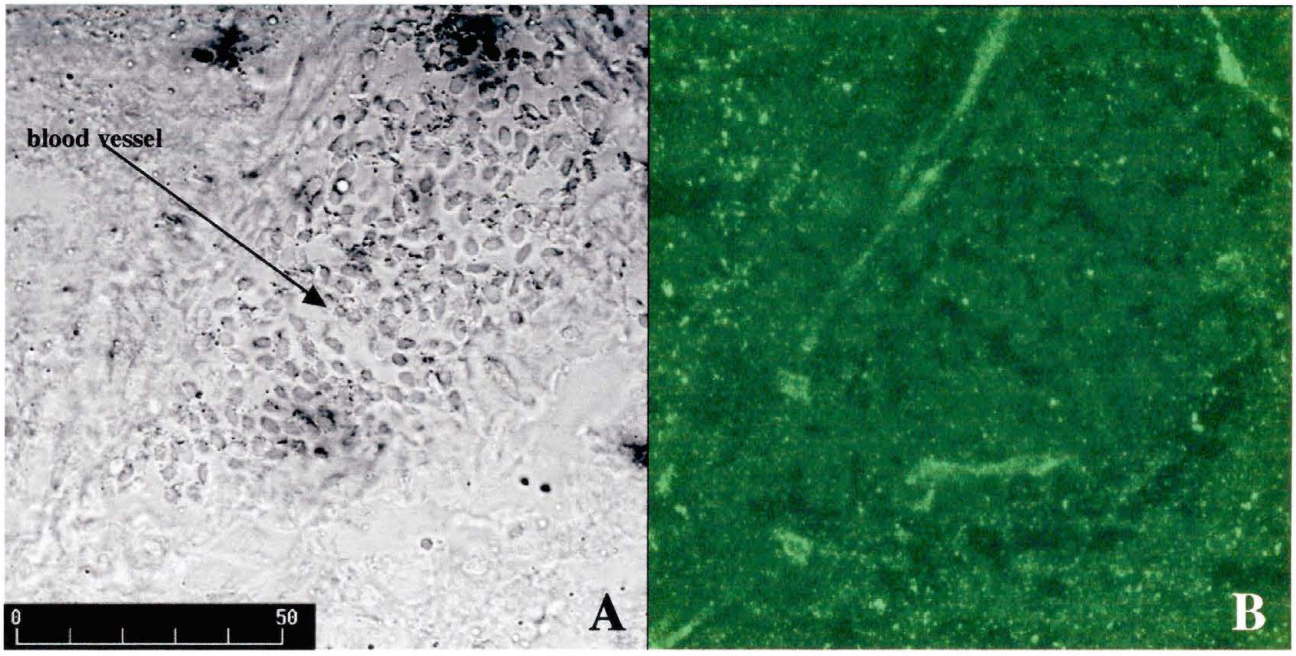
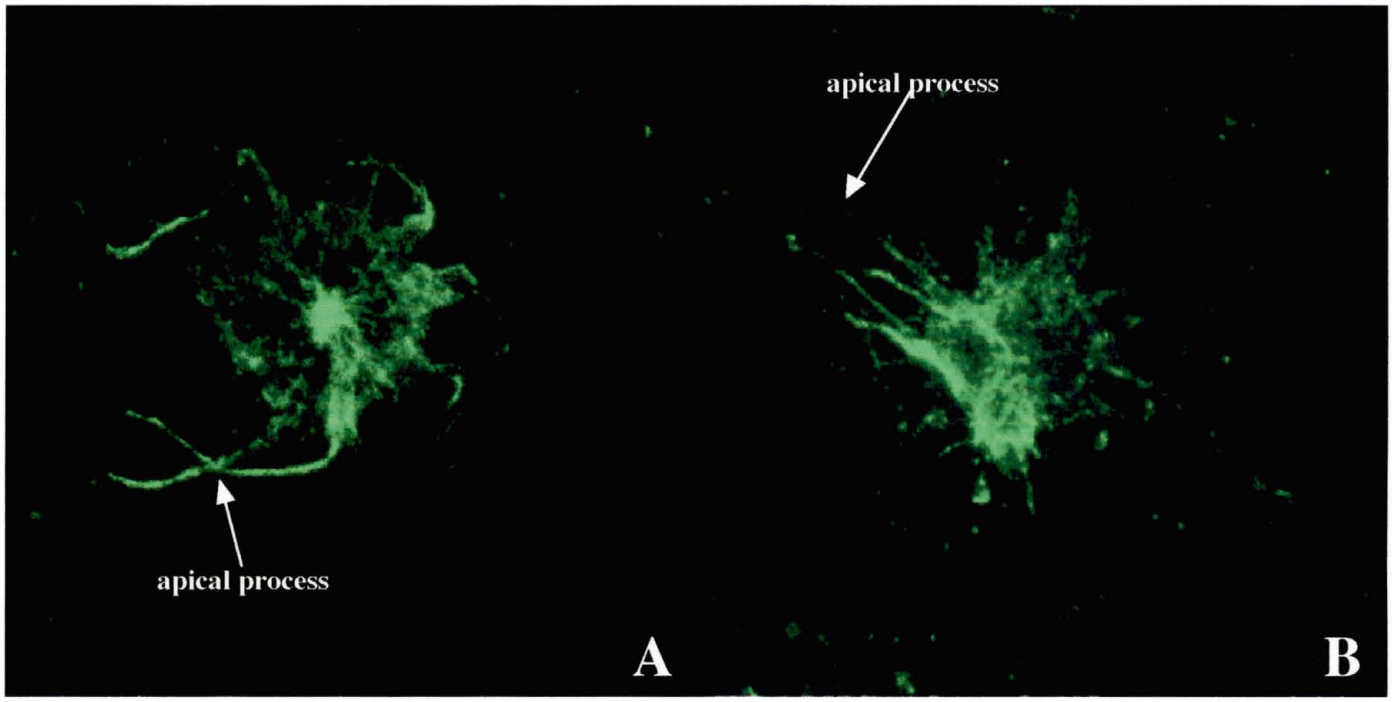

blood vessel
0
50
A
B

Figure 13

Vimentin intermediate filaments undergo a subtle reorganization in isolated RPE cells with pigment granules aggregated and dispersed. A comparison of figures 9 and 10. **A**: Confocal micrograph showing the distribution of vimentin intermediate filaments in a bluegill RPE cell with pigment granules aggregated. The intensity of vimentin intermediate filament labeling is greater in the apical processes of the RPE cell with pigment granules aggregated. Furthermore, vimentin labeling is concentrated in a central region of the cell body. **B**: Confocal micrograph showing the distribution of vimentin intermediate filaments in a bluegill RPE cell with pigment granules dispersed. Compared to **A** vimentin intermediate filament labeling is less intense in the apical processes. Furthermore, vimentin intermediate filaments labeled throughout the cell body, but are not concentrated within a specific area of the cell body. Arrows point to apical processes.


apical process
apical process
A
B

DISCUSSION

In this investigation, results suggest that vimentin intermediate filaments are expressed in isolated bluegill RPE cells. In addition, bluegill vimentin appears to have a relative molecular mass of 50 kDa. Results have also indicated that RPE cells do not express cytokeratin intermediate filaments. Furthermore, results have revealed that the vimentin intermediate filament network of bluegill RPE cells undergoes a subtle reorganization in accordance with pigment granule aggregation and dispersion.

Preliminary studies have suggested that the intermediate filament protein expressed in bluegill RPE is vimentin as indicated by immunoblotting using polyclonal anti-vimentin antibodies (García and Koke, 1996). Also, immunolabeling studies using anti-intermediate filament antigen monoclonal antibodies (Pruss *et al.*, 1981) revealed the presence of intermediate filaments extending from the level of the circumferential microfilament bundles toward and possibly into the apical processes of isolated bluegill RPE cells (Fig. 5). In contrast, *in situ* immunolabeling studies by Zamora (1997) using the same antibodies suggested that bluegill RPE cells express a network of intermediate filaments extending from the base of the RPE cells toward but not into the apical processes. Furthermore, immunolabeling studies using a monoclonal antibody specific for vimentin intermediate filaments revealed the presence of a filamentous network extending from the cell body into the apical processes in isolated bluegill RPE cells (Fig. 6). Based on these observations, the possibility arose that bluegill RPE expresses a vimentin intermediate filament network in the cell body and in the apical processes.

Through the use of Western blot analysis, a polypeptide band with a relative molecular mass of 50 kDa was detected in bluegill RPE (Fig. 7). In molecular biology studies of trout RPE, a gene coding for vimentin was isolated and determined to code for a polypeptide with a predicted molecular weight of 53, 325 Da (Herrmann, *et. al.*, 1996). In the same report, *in situ* studies using monoclonal antibodies raised against vimentin from

Xeponus laevis revealed the presence of vimentin intermediate filaments in trout RPE, neural retina, and brain. Recently, the gene coding for vimentin was isolated from zebrafish and was determined to code for a polypeptide with a predicted molecular weight of 52, 597 Da (Cerda, *et al.*, 1998). Furthermore, *in situ* studies, using monoclonal antibodies raised against human vimentin (clone V-9), revealed the presence of vimentin intermediate filaments in zebrafish RPE and neural retina (Cerda, *et al.*, 1998). Interestingly, efforts to label bluegill RPE using clone V-9 antibodies failed. Nevertheless, evidence that bluegill RPE expresses vimentin with a relative molecular mass of 50 kDa is consistent with studies reported in trout and zebrafish (Fig. 7).

To corroborate immunoblot evidence that bluegill RPE expresses vimentin intermediate filaments, immunolabeling studies were conducted using isolated RPE cells. Since it has been shown that RPE from other vertebrate species express both vimentin and cytokeratin intermediate filaments (Owaribe *et al.*, 1988; Hunt, 1994) and various teleost tissues express cytokeratin intermediate filaments (Markl and Franke, 1988; Markl *et al.*, 1989), studies were also conducted to determine whether bluegill RPE expresses cytokeratin intermediate filaments. Through the use of confocal microscopy, immunolabeling studies on isolated RPE cells using a monoclonal antibody specific for vimentin revealed the presence of a network of vimentin intermediate filaments extending from the cell body into the apical processes in isolated bluegill RPE cells (Fig. 9b and 10b). In contrast, when isolated RPE cells were probed with monoclonal antibodies specific for cytokeratin intermediate filaments, no labeling was observed (Fig. 11b). In order to demonstrate that the monoclonal antibodies recognize bluegill cytokeratin intermediate filaments, sections of bluegill liver were also probed. Cytokeratin monoclonal antibodies labeled a filamentous network in endothelial cells of blood vessels and hepatocytes in bluegill liver (Fig. 11d). Taken together, these observations indicate that bluegill RPE cells express vimentin intermediate filaments and not cytokeratin. Again, these findings are

consistent with *in situ* studies revealing the presence of vimentin intermediate filaments in trout and zebrafish RPE (Herrmann, *et. al.*, 1996; Cerda, *et al.*, 1998).

In this investigation, evidence suggests that a subtle reorganization of vimentin intermediate filaments accompanies dispersion and aggregation of pigment granules in bluegill RPE (Fig. 13). When pigment granules were induced to aggregate by cAMP treatment, the density of vimentin intermediate filaments appeared greater in the apical processes and concentrated in a central area of the cell body, raising the possibility that the number of vimentin intermediate filaments increases during pigment granule aggregation. In contrast, when pigment granules were dispersed into the apical processes, the level of fluorescence was less in the apical processes suggesting that the number of vimentin intermediate filaments decreases in this region during pigment granule dispersion (Fig. 13). However, vimentin intermediate filaments were present throughout the cell body but not distinctly concentrated in a central area of the cell body (Fig. 13). Nevertheless, the observed change in the distribution of vimentin intermediate filaments between RPE cells with pigment granules dispersed and aggregated is not conclusive evidence to support a role in pigment granule translocation.

Reorganization of the cytoskeleton has been implicated to be concomitant with changes in pigment granule distribution in pigmented cells (Murphy and Grasser, 1984; Bruenner and Burnside, 1986; Palazzo *et al.*, 1989a; Walker *et al.*, 1989; Rodionov *et al.*, 1996). In ultrastructural studies conducted on black tetra chromatophores by Murphy and Grasser (1984), observations were made of pigment granules entrapped within and making direct contact with an intermediate filament network. Based on these observations, the authors proposed that intermediate filaments may increase the efficiency of pigment granule migration by interconnecting all pigment granules into a single physical mass. In immunolabeling studies conducted on goldfish xanthophores by Walker *et al.* (1989), intermediate filaments were observed to be distributed throughout the cytoplasm of cells with carotenoid droplets dispersed; however, when the carotenoid droplets were aggregated

intermediate filaments were largely excluded from the aggregated pigmented mass but instead formed a band that circumscribed the aggregated mass. Based on these observations, it was proposed that intermediate filaments may play a role in carotenoid droplet aggregation or the stabilization of the aggregated pigmented mass.

More recently, ultrastructural investigations by Leonova (1992) revealed that in black tetra melanophores with pigment granules dispersed, a dense network of vimentin intermediate filaments was observed surrounding pigment granules while microtubules displayed a wavy appearance; however, when pigment granules were in the aggregated state, the intermediate filaments and microtubules reorganized into radially oriented structures. Based on these observations, the author suggested that aggregation is a microtubule dependent process which may also involve intermediate filaments. Other studies conducted on black tetra melanophores have indicated that intermediate filaments cross-link pigment granules prior to the onset of pigment granule aggregation, raising the possibility that pigment granule motion toward the center of the cell may depend on this interaction (Rodionov *et al.*, 1996). Based on such studies conducted on teleost dermal pigmented cells and on recent findings implicating that pigment granule translocation in isolated teleost RPE cells is an actin dependent process (King-Smith *et al.*, 1997), the possibility arises that vimentin intermediate filaments in association with microfilaments or microtubules may play a supporting role in the coordination of pigment granule movement and stabilization of the dispersed and aggregated states in bluegill RPE.

To investigate the subtle reorganization of vimentin intermediate filaments in RPE cells with pigment granules aggregated and dispersed further, quantitative studies would be appropriate to determine if such a change in vimentin intermediate filament distribution is significant. Specifically, immunofluorescence labeling of vimentin intermediate filaments would be analyzed and compared quantitatively between groups of bluegill RPE cells with pigment granules aggregated and dispersed. Furthermore, it would be of interest investigating whether vimentin intermediate filaments play a possible role in pigment

granule movement. Protocols would have to be developed to disrupt the vimentin intermediate filament network without altering the microtubule and microfilament networks, and isolated bluegill RPE cells could then be exposed to drugs such cAMP to induce aggregation or dopamine to induce dispersion and the role of intermediate filaments in pigment granule movement could be inferred.

LITERATURE CITED

Adler, R., and Farber, D. (1986). *The Retina.* (p. 3) New York: Academic Press.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994). *Molecular Biology of the Cell.* (pp. 803-810, 821-825) New York: Garland Publishing.

Ayoub, G. (1996). On the design of the vertebrate retina. *Origins & Design.* 17(1): 19-22.

Bolanos, S. H., Zamora, D. O., García, D. M., and Koke, J. R. (1998). An _-actinin isoform which may cross-link intermediate filaments and microfilaments. *Cytobios.* 94: 39-61.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

Bruenner, U., and Burnside, B. (1986). Pigment granule migration in isolated cells of the teleost retinal pigment epithelium. *Invest. Opthamol. Vis. Sci.* 27: 1634-1643.

Burnside, B., and Nagle, B. (1983). Retinomotor movements of photoreceptors and retinal pigment epithelium: mechanisms and regulation. In: Osborne, N., Chader, G., eds. *Progress in Retinal Research, Vol. 2.* (pp. 67-109). New York: Pergamon Press.

Burnside, B., Adler, R., and O'Connor, P. (1983). Retinomotor pigment migration in the teleost retinal pigment epithelium. *Invest. Opthamol. Vis. Sci.* 24: 1-15.

Burton, P. R., and Paige, J. L. (1981). Polarity of axoplasmic microtubules in the olfactory nerve of the frog. *Proc. Natl. Acad. Sci. USA.* 78: 3269-3273.

Cerdá, J., Conrad, M., Markl, J., Brand, M., and Herrmann, H. (1998). Zebrafish vimentin: molecular characterization, assembly properties and developmental expression. *Eur. J. Cell Biol.* 77: 175-187.

Dearry, A., Edelman, J. L., Miller, S., and Burnside, B. (1990). Dopamine induces light-adaptive retinomotor movements in bullfrog cones via D2 receptors and in retinal pigment epithelium via D1 receptors. *J. Neurochem.* 54: 1367-1377.

Euteneuer, U., and McIntosh, J. R. (1981a). Polarity of some motility-related microtubules . *Proc. Natl. Acad. Sci. USA.* 78: 372-376.

Filliatreau, G., and DiGiamberardino, L. (1981). Microtubule polarity in myelinated axons as studied after decoration with tubulin. *Biol. Cell.* 42: 69-72.

Fuchs, E. (1996). The cytoskeleton and disease: genetic disorders of intermediate filaments. *Annu. Rev. Genet.* 30: 197-231.

García, D. M., and Burnside, B. (1994). Suppression of cAMP-induced pigment granule aggregation in RPE by organic anion transport inhibitors. *Invest. Ophthalmol. Vis. Sci.* 35: 178-188.

García, D. M., and Koke, J. R. (1996). The cytoskeleton of the retinal pigment epithelium. *Advances in Structural Biology*. 4: 151-174.

Georgatos, S. D., and Maison, C. (1996). Integration of intermediate filaments into cellular organelles. *Inter. Rev. Cyto.* 164: 91-138.

Heidemann, S. R., Landers, J. M., and Hamborg, M. A. (1981). Polarity orientation of axonal microtubules. *J. Cell Biol.* 91: 661-665.

Herrmann, H., Münick, M. D., Brettel, M., Fouquet, B., and Markl, J. (1996). Vimentin in a cold-water fish, the rainbow trout: highly conserved primary structure but unique assembly properties. *J. Cell Sci.* 109: 569-578.

Hunt, R. C. (1994). Intermediate filaments and other cytoskeletal structures in retinal pigment epithelial cells. *Progress in Retinal and Eye Research.* 13: 125-145.

King-Smith, C., Bost-Usinger, L., and Burnside, B. (1995). Expression of kinesin heavy chain isoforms in retinal pigment epithelial cells. *Cell Motil. Cytoskel.* 31: 66-81.

King-Smith, C., Paz, P., Lee, C. W., Lam, W., and Burnside, B. (1997). Bidirectional pigment granule migration in isolated retinal pigment epithelial cells requires actin but not microtubules. *Cell Motil. Cytoskel.* 38: 229-249.

Klyne, M. A. and Ali, M. A. (1981). Microtubules and 10 nm-filaments in the retinal pigment epithelium during the diurnal light-dark cycle. *Cell and Tissue Res.* 214: 397-405.

Kreis, T. and Vale, R. (eds.) (1993). *Guidebook to the Cytoskeletal and Motor Proteins.* New York, NY: Oxford University Press.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.

Lenova, E. V. (1992). Aggregation of melanosomes in melanophores is accompanied by the reorganization of microtubules and intermediate filaments. *Cell Biol. Inter. Rep.* 16: 955-965.

Markl, J. and Franke, W. W. (1988). Localization of cytokeratins in tissues of the rainbow trout : fundamental differences in expression pattern between fish and higher vertebrates. *Differentiation.* 39: 97-122.

Markl, J., Winter, S. and Franke, W. W. (1989). The catalog and the expression complexity of cytokeratins in a lower vertebrate: biochemical identification of cytokeratins in a teleost fish, the rainbow trout. *Eur. J. Cell Bio.* 50: 1-16.

McNiven, M. A., Wang, M., and Porter, K. R. (1984). Microtubule polarity and the direction of pigment transport reverse simultaneously in surgically severed melanophore arms. *Cell.* 37: 753-765.

Mitchison, T. J. (1992). Compare and contrast actin filaments and microtubules. *Mol. Biol. Cell.* 3: 1309-1315.

Murphy, D. B. and Grasser, W. A. (1984). Intermediate filaments in the cytoskeletons of fish chromatophores. *J. Cell Sci.* 66: 353-366.

Owaribe, K., and Masuda, H. (1982). Isolation and characterization of circumferential microfilament bundles from retinal pigmented epithelial cells. *J. Cell Bio.* 95: 310-315.

Owaribe, K., Sugino, H., and Masuda, H. (1986). Characterization of intermediate filaments and their structural organization during epithelium formation in pigmented epithelial cells of the retina in vitro. *Cell Tissue Res.* 244: 87-93.

Owaribe, K. (1988). The cytoskeleton of retinal pigment epithelial cells. *Progress in Retinal Research.* 8: 23-49.

Owaribe, K., Kartenbeck J, Rungger-Brändle E, and Franke, W. W. (1988). Cytoskeletons of retinal pigment epithelial cells: interspecies differences of expression patterns indicate independence of cell function from the specific complement of cytoskeletal proteins. *Cell Tissue Res.* 254: 301-315.

Palazzo, R. E., Lynch, T. J., Taylor, J. D. and Tchen, T. T. (1989). Rearrangements of pterinosomes and cytoskeleton accompanying pigment dispersion in goldfish xanthophores. *Cell Motil.* 13: 9-20.

Philp, N. J. and Nachmias, V. T. (1985). Components of the cytoskeleton in the retinal pigmented epithelium of the chick. *J. of Cell Bio.* 101: 358-363.

Pruss, R. M., Mirsky, R., and Raff, M. C. (1981). All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell* 27: 419-428.

Rodionov, V. I., Hope, A. J., Urven, L. E., Svitkina, T. M., and Borisy, G. G. (1996). Possible role of intermediate filaments in pigment transport in fish melanophores. *Mol. Biol. Cell* 7: 1314.

Soltys, B. J., and Borisy, G. G. (1985). Polymerization of tubulin *in vivo*: direct evidence for assembly onto microtubule ends and from centrosomes. *J. Cell Biol.* 100: 1682-1689.

Takeuchi, I. K., and Takeuchi, Y. K. (1979). Intermediate filaments in the retinal pigment epithelial cells of the goldfish. *J. Electron Microsc.* 28: 134-137.

Traub, P. (1995). Intermediate filaments and gene regulation. *Physiol. Chem. Phys. & Med. NMR.* 27: 377-400.

Trout, L. L., and Burnside, B. (1988). The unusual microtubule polarity in teleost retinal pigment epithelial cells. *J. Cell Bio.* 107: 1461-1464.

Trout, L. L., and Burnside, B. (1989). Role of microtubules in pigment granule migration in teleost retinal pigment epithelial cells. *Exp. Eye Res.* 48: 433-443.

Walker, G. R., Taylor, J. D. and Tchen, T. T. (1989). Immunofluorescence evidence for cytoskeletal rearrangement accompanying pigment redistribution in goldfish xanthophores. *Cell Motil. Cytoskel.* 14: 458-468.

Zamora, D. O. (1997). Localization of cytoskeletal elements in teleost retinal pigment epithelium. M. S. Thesis, Southwest Texas State University, San Marcos, TX.

Zinn, K. M., and Marmor, M. F. (eds.) (1979). *The Retinal Pigment Epithelium.* Cambridge, MA: Harvard University Press.