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STRUCTURAL EVIDENCE OF RECEPTOR MEDIATED ENDOCYTOSIS AT POSTERIOR LENS SUTURES

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***Abstract*– *Purpose: To investigate whether lens sutures have structural parameters (clathrin coated vesicles) to support enriched transport of metabolites into the lens via receptor mediated endocytosis. Methods: Lenses from 20 normal 6 week old rats and 6 adult chickens were utilized for this study. A transmission electron microscopic (TEM) survey was conducted to establish the presence of coated vesicles/pits within suture planes and at apical and basal ends of elongating fibers. Coated vesicle density was quantified both within and outside sutural domains. Clathrin was localized by fluorescent immunolabeling and laser scanning confocal microscopic (LSCM) visualization in both rat and chicken lenses. Results: TEM examination demonstrated that abundant coated vesicles were present at the epithelial-fiber interface (EFI) in the apical domains of both epithelial cells and elongating fibers. Quantification revealed that although coated vesicle density was greater within than without anterior sutural domains, the difference was not significant. Abundant coated vesicles were also found at basal ends of elongating fibers, however their density was significantly greater within posterior sutural domains as compared to off-suture locations ($p<0.001$). Statistical comparisons of anterior vs. posterior locations showed that the density of coated vesicles was significantly different in both on- and off-suture locations. Immunolocalization demonstrated that clathrin was abundant at both anterior and posterior fiber ends in elongating fibers. Clathrin immunofluorescence was more pronounced at posterior sutures than at anterior sutures. This distribution was consistent in both rat and chicken lenses. Conclusions: Posterior lens sutures appear to facilitate transport of metabolites from the vitreous into lens fibers. Although suture planes are detrimental to lens optics, their anatomical arrangement along the visual axis may be important for optimal lens function. The lower amount of clathrin at anterior as opposed to posterior sutures is probably a result of the high transport capability of the anterior lens epithelium.*

Keywords – Crystalline Lens, Clathrin, Coated Vesicles, Receptor Mediated Endocytosis, Immunohistochemistry, Transmission Electron Microscopy, Laser Scanning Confocal Microscopy

INTRODUCTION

The ocular lens is a transparent, biconvex, asymmetrical spheroid comprised of the lens capsule, an anterior epithelial monolayer and the lens cells, called lens fibers due to their long, ribbon-like shape. The lens is often described as an inverted, stratified epithelium due to its mode of growth. Throughout life, new lens fibers are continuously added onto the exterior of the existing lens mass, resulting in a series of concentric growth shells with the oldest fibers at the center and the youngest fibers at the periphery. Within each growth shell, individual fibers extend from the anterior to the posterior aspect of the lens, while their ends abut with those of opposing fibers, thereby forming the lens sutures (see [1] for review).

Like many epithelial-derived tissues, the lens is avascular. This means that only the outermost lens cells, specifically the anterior epithelium and the basal ends of forming fibers, have direct access to the ions and metabolites which diffuse through the capsule. The underlying fibers must therefore rely on transport mechanisms, such as endocytosis, through the outer cell layers to obtain the required metabolites. Indeed, numerous investigations have shown that vesicular transport plays an important role in regulating the movement of various metabolites into lens fibers [2-10]. In particular, the identification of abundant coated pits and coated vesicles in both lens epithelial cells as well as superficial lens fiber cells indicates that receptor mediated endocytosis is a primary means of entry for macromolecules into the lens fibers [2,3]. In the most superficial lens cells, the majority of coated pits and vesicles are located at the basal cell surfaces [4], that is, they are adjacent to the capsule-epithelial interface anteriorly and the capsule-fiber interface posteriorly.

As stated above, sutures are formed when the elongating fibers of the lens meet and abut with opposing groups of fibers both anteriorly and posteriorly. Because new fibers are laid down atop previous layers, deep suture planes are formed which have a detrimental effect on lens optical quality [11-13]. It has long been know that the polar lens regions, which are coincident with lens sutures, contain morphological components able to support ion-linked fluid transport [14]. Thus, lens sutures also appear to form a natural conduit connecting the anterior and posterior portions of the lens. Such a conduit would be a prime location for transport of metabolites from the superficial layers into deeper region of the lens fiber mass. Therefore, the objective of this study was to evaluate whether lens sutures have structural parameters, specifically clathrin coated vesicles, to support enriched transport of metabolites into the lens interior via receptor mediated endocytosis. We performed quantitative transmission electron microscopy (TEM) of normal rat lenses and immunocytochemical localization of clathrin in both rat

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and chicken lenses. The data showed that posterior sutures, but not anterior sutures, appear to facilitate transport of metabolites into the cortical lens fibers via receptor mediated endocytosis.

MATERIALS AND METHODS

Lenses:

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and all animal procedures were carried out in compliance with the Rush Institutional Animal Care and Use Committee approved protocol for this study. Lenses from normal 6 week old Sprague-Dawley rats were utilized for both TEM and laser scanning confocal microscopy (LSCM) of immunolabeled tissue in this investigation. A total of 20 animals were euthanized by intraperitoneal injection of sodium pentobarbital. Directly after animals were euthanized, eyes were enucleated, lenses were dissected from the orbit then fixed. Adult chicken lenses (n=6) were also utilized for LSCM and were obtained from a local poultry supplier/slaughterhouse immediately following decapitation. Lenses were dissected from the orbit and placed in fixative within 1 hour post-sacrifice.

TEM:

Lenses were fixed at room temperature for 2-3 days in 2.5% glutaraldehyde + 1% tannic acid in 0.07M sodium cacodylate buffer (pH 7.2) with fresh fixative changes daily. Lenses were washed in 0.2M sodium cacodylate buffer, post fixed overnight in 1% aqueous osmium tetroxide at 4 °C, washed in deionized water, then dehydrated through a graded ethanol series to propylene oxide. Specimens were infiltrated and flat embedded in epoxy resin then polymerized at 60 °C. Embedded lenses were bisected along the optic axis with a jeweler's saw and 1-2 µm thick sections were cut using a glass knife to locate suture planes. Sections were mounted on glass slides and stained with a dilute mixture of methylene blue and azure II. Mesas were raised which included either an anterior or posterior suture plane. Thin sections were cut at 60 nm, stained with uranyl acetate and lead citrate, then examined on a JEOL JEM 1200EX transmission electron microscope (JEOL USA, Peabody, MA) at 60 kV.

Quantification of Coated Vesicles/Pits:

A TEM survey of the density of coated vesicles/pits was conducted within sutural domains (both anterior and posterior) as well as outside the sutural domains. Coated vesicles within the lens epithelial cells were excluded from the survey. Due to the overlap and interdigitation of fiber ends, suture planes encompass an area approximately 25 µm wide. Consequently, a portion of the sutural domain 25 µm X 75 µm was defined at low magnification and eighteen to twenty-five micrographs at 20,000 X magnification were randomly taken within this area. Specifically, a raster-like pattern was followed during imaging to avoid overlap and/or duplication of areas and then the number of coated pits/vesicles was counted directly from the micrographs. Thus, coated vesicles/pits were quantified for approximately 15% of the defined sutural domain. The average number of coated vesicles was expressed as #/100 μm^2. In a similar fashion, areas > 100 µm away from the sutures (but less than 1000 µm away) were surveyed to determine the off-suture density of coated vesicles/pits at both anterior and posterior lens surfaces. The student's t-test was performed to compare the average number of coated vesicles in anterior versus posterior domains (both on and off suture). Statistical comparisons were also made to determine if on-suture densities differed significantly from off-suture densities for both the anterior and posterior surfaces.

Immunohistochemistry and LSCM:

Rat lenses were fixed in 2% paraformaldehyde for 2 hours at room temperature, embedded in 3% agar, then sectioned parallel to the optic axis (Fig.1A) at 100 µm thickness, using a vibrating knife microtome (Lancer, St. Louis, MO). Chicken lenses were fixed in 4% paraformaldehyde for 2 hours at room temperature, embedded in 3% agar, then vibratome-sectioned parallel to the equatorial plane (Fig. 1B). Serial sections, 100 µm thick, were cut beginning at either the anterior or posterior pole to the equator. All sections were fixed for an additional 30 minutes, then washed in phosphate buffered saline (PBS). Immunolabeling was performed by immersing sections in drops of solution as follows: 0.1% Triton-X 100 in PBS, 30 minutes; 10% normal rabbit serum + 1% BSA in PBS (to block non-specific binding), 60 minutes; anti-clathrin antibody diluted to 1:100 in blocking solution (goat polyclonal; Sigma, C8034), overnight at 4° C; blocking solution, 3 x 10 minutes; rabbit anti-goat antibody conjugated to FITC at 1:200 dilution in blocking solution (Sigma, G4018), 2 hours in dark; PBS, 4 x 10 minutes. Labeled sections were mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlington, CA, U.S.A.) and visualized on a Zeiss 510 LSM confocal microscope (Carl Zeiss Inc., Thornwood, NY).

Negative controls for autofluorescence and immunolabeling were conducted on rat lenses. Unstained, fixed sections were mounted with Vectashield and viewed on the confocal to insure that the tissue did not auto-fluoresce. Negative immunolabeling controls were performed by replacing the anti-clathrin antiserum with normal goat serum (diluted to 1:100 in blocking solution) while executing the labeling protocol above.

RESULTS

TEM Survey and Quantification:

A survey showed the presence of clathrin coated vesicles and pits within both the anterior and posterior sutural domains of SD rat lenses. Anterior sutures were surveyed using TEM within a portion covering approximately 15% of the sutural domain, an area that measures 25 µm X 75 µm (Fig. 2). Anterior lens sections revealed that coated vesicles and pits were present at the epithelial-fiber interface (EFI) in the apical domains of both elongating fibers and epithelial cells. Coated vesicles were also located deeper within the sutural plane, although they were much less frequent than at the EFI (Fig. 3). Each sample survey, which consisted of 18-25 micrographs taken at 20,000 X, was counted and the vesicle density was expressed as #/100 µm². The quantitative results for all regions surveyed are summarized in Table 1. Quantification of the anterior sutural domain showed that the average number of vesicles per 100 µm² was 4.72. Coated vesicles were present in the posterior sutural domain (Figs. 4 and 5) as well as within the basal ends of the elongating fiber cells along the capsule-fiber interface (CFI; Fig. 5). As in

anterior sutural domains, coated vesicles were present at some distance from the CFI within posterior sutural domains. The average number of vesicles per 100 µm² was 7.19 (Table 1). Our analysis determined that the higher frequency of coated vesicles in posterior as compared to anterior sutural domains was statistically significant (p=0.018; Table 2).

Quantification of coated vesicles/pits in lens fibers was also conducted at off-suture locations both anteriorly and posteriorly (Table 1). Off-suture frequency of vesicles was significantly lower posteriorly than anteriorly (p=0.45; Table 2). Comparison of on-sutural vs. off-sutural locations showed that although coated vesicle density was greater within than without anterior sutural domains, the difference was not significant. However, posterior sutural domains had significantly more coated vesicles/pits than off-suture locations (p<0.001).

Immunolocalization of Clathrin:

Immunolocalization of clathrin was also performed on both rat and chicken lenses to determine the abundance of clathrin at both the anterior and posterior fiber ends in elongating fibers. Sections approximately 100µm thick were taken parallel to the optic axis of rat lenses and labeled with a primary anti-clathrin antibody as well as a secondary rabbit ant-goat antibody conjugated to FITC. In support of the TEM quantification results, it was discovered that clathrin immuno-fluorescence at the ends of fully elongated fibers was much more plentiful in the posterior fiber ends than the anterior fiber ends of rat lenses (Fig. 6). Negative controls (using normal goat serum in place of 1º antibody) were conducted on rat lens sections and confirmed that non-specific background staining was minimal (data not shown). A control to determine if auto fluorescence was a factor was also conducted and revealed no auto-fluorescence (data not shown).

To further confirm the localization results obtained in rat lenses, chicken lens sections were cut parallel to the equatorial plane and labeled for clathrin in the same manner as the rat lenses. Results showed that clathrin labeling was enhanced at both anterior and posterior sutures (Fig.7). Additionally, there was a much greater abundance of clathrin immunofluorescence in posterior polar sections as compared to anterior polar sections (compare Figs. 7A and 7C).

Table 1: Average Number of Coated Vesicles/100µm²

Location	N	Mean ± SEM	Range
Anterior, On-Suture	7	4.72 ± 0.65	3.02 – 7.62
Posterior, On-Suture	7	7.19 ± 0.63	4.97 – 9.84
Anterior, Off-Suture	6	3.49 ± 0.41	2.54 – 5.40
Posterior, Off-Suture	6	2.49 ± 0.15	1.90 – 2.86

N = number of animals

Table 2: Statistical Analysis of Coated Vesicle Quantification

Comparison	P
Anterior vs. Posterior: On-Suture	0.018*
Anterior vs. Posterior: Off-Suture	0.045*
Anterior: On-Suture vs. Off-Suture	0.152
Posterior: On-Suture vs. Off-Suture	<0.001*

* Indicates a statistically significant difference

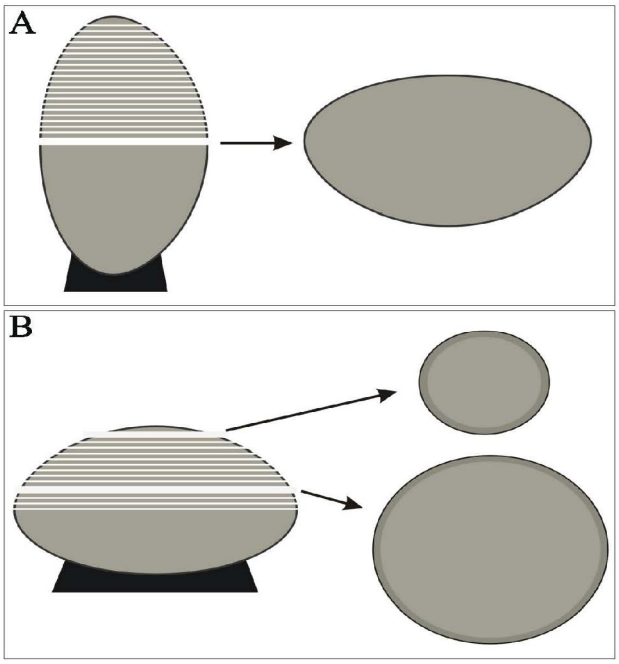

Figure 1. Diagrams of mounted lenses demonstrating the sectioning procedure with a vibrating knife microtome. (A) Rat lenses sectioned parallel to the optic axis for clathrin labeling. (B) Chicken lens sectioned parallel to the equatorial plane beginning at either the anterior or posterior pole.

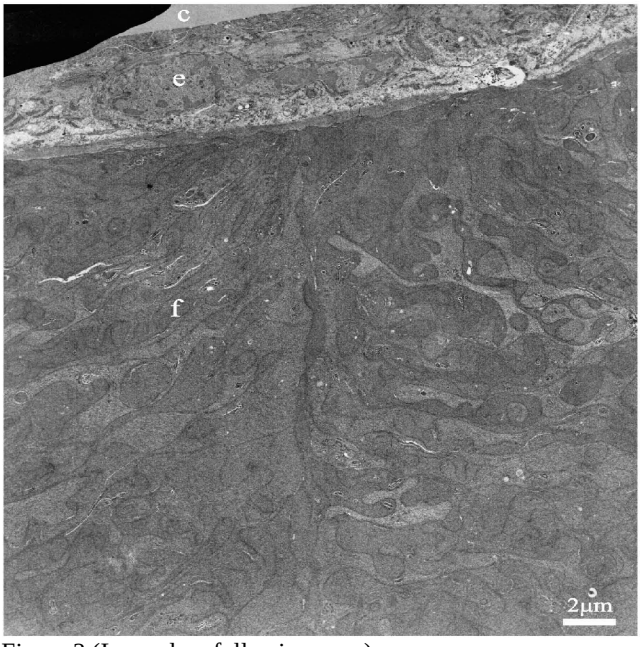

Figure 2 (Legend on following page)

Figure 2. (Prior page) Transmission electron micrograph of an anterior suture plane. Represented in the micrograph is the capsule (c), the epithelium (e), and fiber cells (f) which abut and interdigitate to form the suture.

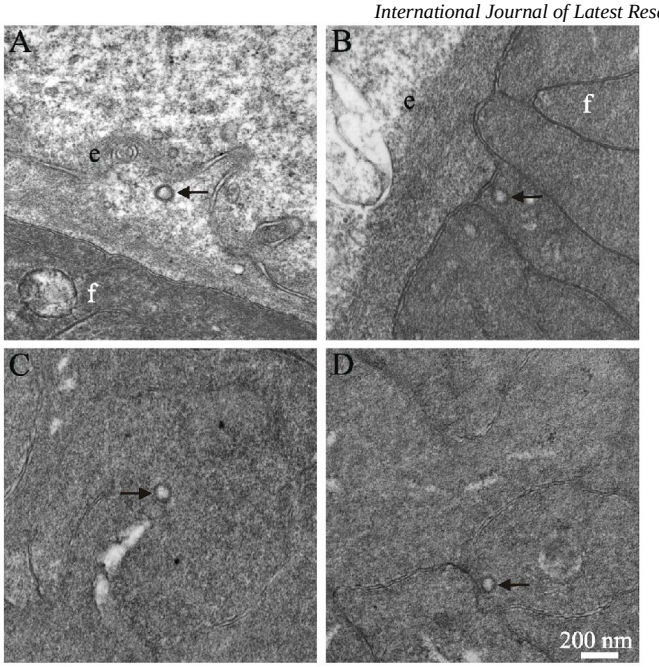

Figure 3. A gallery of transmission electron micrographs displaying coated vesicles within the epithelium (e) and anterior sutural domains of fibers (f) in normal rat lenses. (A) A coated vesicle in the epithelium (arrow). (B) A coated vesicle (arrow) within a fiber cell adjacent to the EFI. (C and D) Coated vesicles (arrows) within the sutural domain and deep to the EFI were noted within the first 4-5 fiber layers. Panels A-D are at identical magnification.

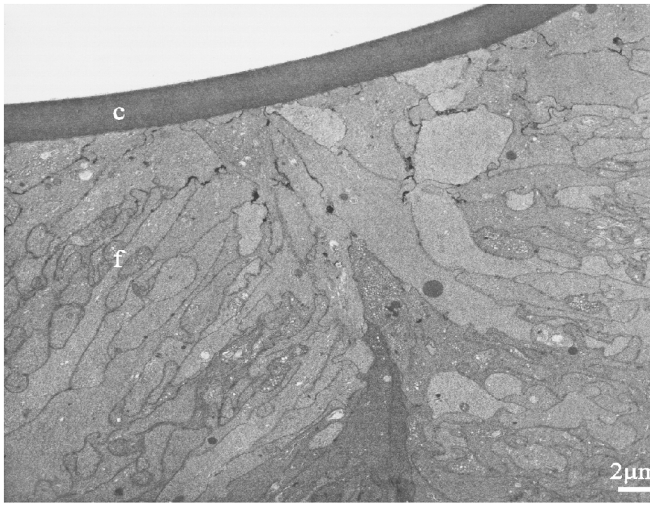

Figure 4. Transmission electron micrograph of a posterior suture plane. The capsule (c) and fiber cells (f) interdigitating at the suture are evident.

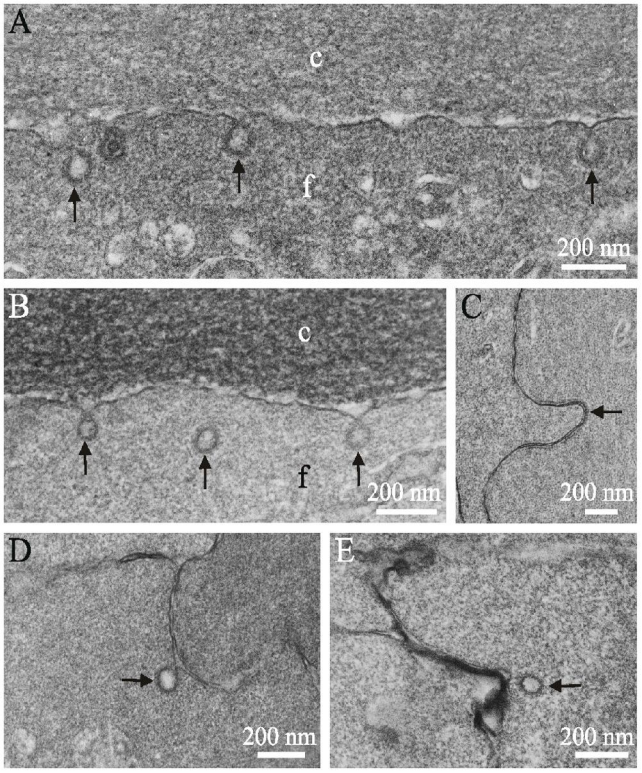

Figure 5. A gallery of transmission electron micrographs showing coated vesicles both within and outside of posterior sutural domains in normal rat lenses. (A) A series of three coated vesicles, marked by black arrows, within a sutural domain and adjacent to the CFI; c = capsule, f = fibers. (B) A series of three coated vesicles (black arrows) in an area lateral to a posterior suture plane; c = capsule, f = fibers. (C) A coated pit within a posterior sutural domain (arrow). (D and E) Two coated vesicles (black arrows) located deep to the CFI and within a suture plane.

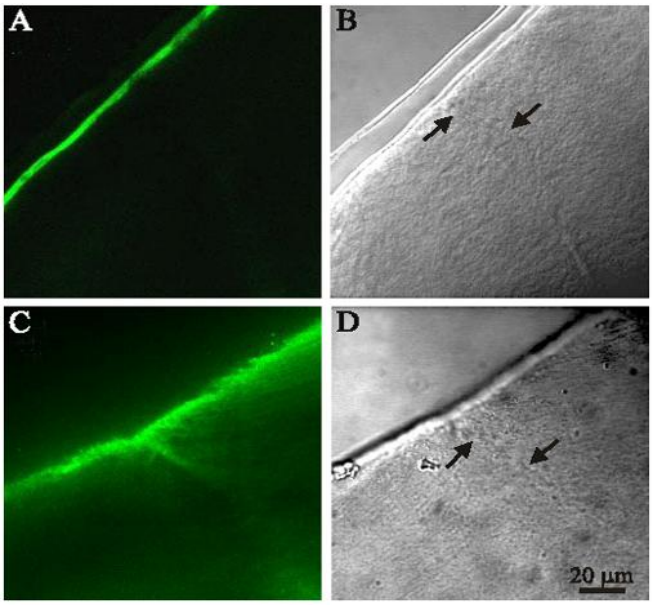

Figure 6. (Legend on following page)

Figure 6. (Prior page) LSCM images of clathrin localization in rat lenses sectioned parallel to the optic axis. (A and B) Paired fluorescent (A) and differential interference contrast (B) micrographs of an anterior suture plane. In the anterior suture plane (indicated with arrows) clathrin immunofluoresence is localized to the epithelial cell layer/EFI with little or no detectable clathrin at the interdigitated fiber ends. (C and D) Paired fluorescent (C) and differential interference contrast (D) micrographs of a posterior suture plane. In contrast to the anterior sutures, heavy labeling can be observed at the posterior ends of elongating fibers and in the superficial cortical fibers which have detached from the capsule to interdigitate at the posterior suture (arrows indicate the suture plane). Panels A-D are at identical magnification.

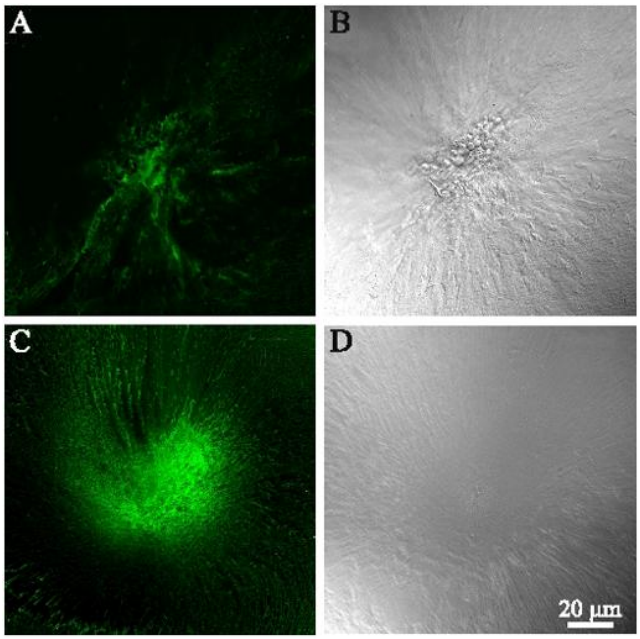

Figure 7. LSCM images of clathrin localization in normal chicken lenses sectioned parallel to the equatorial plane. (A and B) Paired fluorescent (A) and differential interference contrast (B) micrographs of an anterior point suture. (C and D) Paired fluorescent (C) and differential interference contrast (D) micrographs of a posterior point suture. The micrographs show clathrin labeling was much less prominent in the anterior polar section (A) than the posterior polar section (C) where heavy clathrin labeling was noted as the fibers come to confluence at the suture. Panels A-D are at identical magnification.

DISCUSSION

It is generally accepted that the lens epithelium plays a vital role in transport of metabolites from the aqueous humor into underlying lens fiber cells. Early investigations focused on the abundance of gap junctions in the lens as the most likely transport mechanism [15,16], in spite of parallel evidence that receptor mediated endocytosis occurred in the lens [17]. Indeed, subsequent investigations revealed that abundant clathrin coated vesicles were present in lens epithelial cells and were also present in the underlying anterior fibers [2,3,5]. Lo and coworkers [4] documented the presence of clathrin coated vesicles in posterior segments of lens fibers, revealing that receptor mediated endocytosis was responsible for entrance of metabolites from the vitreous humor into the lens at its posterior aspect. In that study, various membrane domains were examined, revealing that more than 80% of coated vesicles were present adjacent to the lens capsule, i.e. the basal membrane domains of both lens epithelial cells and elongating fibers. The results of the present study confirm earlier results showing coated vesicles in both the lens epithelium and superficial fiber segments (anterior and posterior) and importantly, extend those findings by quantifying the distribution of coated vesicles at lens sutures.

The results of the present study revealed that posterior sutural domains had significantly more coated vesicles than anterior sutural domains. However, in off suture locations, anterior fiber segments had higher numbers of coated vesicles than posterior fiber segments (Table 2). The quantification data was entirely consistent with our localization studies which showed more prominent clathrin labeling at posterior as compared to anterior sutures in both rat and chicken lenses (Figs. 6 and 7). This data is only partially in agreement with earlier findings that showed a gradual decrease in the frequency of coated vesicles from the anterior to the posterior of the lens [4]. However, it should be noted that the aforementioned prior study [4] did not assess the sutural regions at either anterior or posterior lens surfaces. Further, our results quantified and compared the coated vesicles within fibers only, whereas the prior study [4] included comparisons of the epithelium to the fibers for several parameters. These differences in study design and analysis are likely responsible for some of the perceived differences in results.

Comparisons of on suture versus off suture locations also provided valuable insights into coated vesicle transport in the lens. Specifically, there was no statistically significant difference in the frequency of coated vesicles between on suture and off suture locations anteriorly. Further, this quantification data was in concert with our clathrin immunolocalization data in rat lens sections, which showed a homogeneous layer of fluorescence in the epithelium and at the EFI (Fig. 6A). This data agrees with prior studies showing the prevalence of receptor mediated endocytosis within the entire lens epithelial layer as well as at the EFI [3-5,7,18]. In contrast to anterior domains, there was a significant difference between posterior on sutural versus posterior off suture regions (Table 2), with sutural regions having more than a three-fold greater frequency of coated vesicles. These results indicate that posterior sutures are highly important areas for transport of metabolites from the vitreous humor into the lens. In fact, recent evidence has underlined the importance of vesicular transport of growth factors, lipoproteins and cholesterol from the vitreous humor into posterior segments of lens via receptor mediated endocytosis [19].

In conclusion, our data indicates that the posterior, but not anterior sutures are regions of enriched receptor mediated endocytosis. It also confirms that vesicular transport into the lens via the vitreous humor is an important conduit for macromolecules. Although suture planes exert a negative effect on lens optics, their anatomical arrangement along the visual axis may be important for optimal lens function.

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