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Confocal immunolocalization of bovine serum albumin, serum retinol-binding protein, and interphotoreceptor retinoid-binding protein in bovine retina

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Purpose: Recently it has been shown that the transport as well as clearance of retinol from isolated rod photoreceptors requires an extracellular factor. Interphotoreceptor retinoid-binding protein (IRBP) is a component of the interphotoreceptor matrix (IPM) and is known to bind visual cycle retinoids. Serum albumin and serum retinol-binding protein (sRBP), proteins capable of binding retinoids, have also been reported to be components of the IPM. It is of interest to know the components present in the IPM that are capable of binding visual cycle retinoids and that also facilitate rhodopsin regeneration. The purpose of this study was to determine the localization of serum albumin, sRBP, and IRBP in bovine retina using immunofluorescence analysis.

Methods: Fresh bovine eyes, obtained from a local abattoir, were fixed immediately after enucleation. Tissue sections (100 μm) were incubated with primary antibodies to bovine serum albumin (BSA), sRBP, and IRBP. Sections were washed then incubated 4 h with 4'-6-Diamidino-2-phenylindole (DAPI), Alexa Fluor® 488 goat antimouse, and Alexa Fluor® 568 goat antirabbit secondary antibodies. Sections were analyzed using a laser scanning confocal microscope equipped with Nomarski optics. Western immunoblot analysis of bovine retinal tissues and protein standards was performed using the primary antibodies to BSA, sRBP, and IRBP to show specificity to their respective antigens.

Results: Immunoblot analysis showed that monoclonal anti-BSA was highly specific for BSA detecting only a single band at about 67 kDa. Antihuman sRBP and antibovine IRBP were also highly specific, recognizing a single band at about 25 and about 133 kDa, respectively. No immunopositive bands were observed in bovine neural retinal when probed with the anti-sRBP antibody; however, a single immunoreactive band at about 67 and about 133 kDa was detected in bovine neural retina by the anti-BSA and IRBP antibodies, respectively. Immunofluorescence analysis showed labeling for IRBP throughout the IPM. IRBP labeling was especially associated with the outer segments of photoreceptors and also with the apical surface of the retinal pigment epithelium. Immunofluorescence labeling for serum albumin was associated only with the lumen of retinal and choroidal blood vessels. Staining for both serum albumin and sRBP in the IPM was negative.

Conclusions: Immunofluorescence analysis of fresh bovine eyes using antibodies to BSA and sRBP clearly shows that serum albumin and sRBP are not components of bovine IPM. IRBP, on the other hand, is localized to the IPM where it is available for the binding and transport of visual cycle retinoids. From these data we conclude that serum albumin and sRBP are not factors that could participate in the binding as well as transport of visual cycle retinoids in the IPM of bovine retina.

The process of bleaching and regeneration of rhodopsin is indispensable to rod-mediated vision. This process, referred to as the visual cycle, is dependent on the exchange of retinoid, namely all-*trans* retinol and 11-*cis* retinal, between the rod photoreceptors and retinal pigment epithelium (RPE). Under bleaching conditions, all-*trans* retinol is generated within the rod photoreceptor and then is translocated from the outer segments to the interphotoreceptor space and taken up by the RPE. It is within the RPE that all-*trans* retinol is esterified for storage and isomerized to its 11-*cis* configuration, which is then oxidized to form 11-*cis* retinal. Transport of 11-*cis* retinal back to the rod outer segments, where it recombines with opsin to form rhodopsin, completes the visual cycle.

The movement of all-*trans* retinol and 11-*cis* retinal through the aqueous interphotoreceptor space is likely to involve a transport protein, for example, interphotoreceptor retinoid-binding protein (IRBP) [1,2], serum albumin [3], or other lipid binding proteins that may be present within the interphotorecepter space; although, transfer via the aqueous phase has also been proposed [4]. It has been demonstrated that IRBP plays a role in the uptake of retinol by the RPE [5] and specifically promotes the release of 11-*cis* retinal from the RPE apical surface [6-10], and recent studies suggest that in vivo, IRBP plays a direct role in the release of all-*trans* retinol from the rods during the visual cycle [11-13]. Nevertheless, questions have been raised concerning the physiological importance of IRBP in the visual cycle because of the finding in an IRBP null mouse that opsin is capable of regeneration to rhodopsin with fairly normal kinetics after bleaching [14,15]. It is, therefore, important to establish whether or not

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certain other retinoid-binding proteins such as serum albumin and serum retinol-binding protein (sRBP) are normal constituents of the interphotoreceptor matrix (IPM). Serum albumin has been demonstrated to bind the retinoids of the visual cycle [16,17] as does sRBP [17-19].

Different experimental approaches have been utilized to examine the protein constituents of the IPM. When monkey eyes were obtained within 3 min after death and an in situ cannulation technique was then used to extract the components of the IPM while maintaining the topological integrity of the chorioretinal complex, IRBP was found to be the major protein present in monkey IPM and the only protein binding radiolabeled retinol in the IPM [20]. Immunohistochemical studies of mouse [21], rat [22], and human [23,24] eyes did not detect albumin in the interphotoreceptor space. In contrast, serum albumin was found in IPM samples from human donor eyes obtained by an IPM rinse technique and in the interphotoreceptor space by immunohistochemistry of a human retina obtained at 1 h postmortem [3,25]. An examination of IPM rinse samples from several different vertebrate species also showed the presence of variable amounts of albumin and IRBP [3].

In order to understand a dynamic process like the visual cycle, it is critical to evaluate the components present in the IPM that are capable of binding visual cycle retinoids and that also facilitate rhodopsin regeneration. The purpose of this study was to determine the localization of serum albumin, sRBP, and IRBP in the bovine retina using immunofluorescence analysis.

METHODS

Western blot analysis: Western immunoblot analysis of bovine retinal tissues and protein standards was performed using the primary antibodies to BSA, sRBP, and IRBP to show specificity to their respective antigens. Briefly, neural retinas (n=2) were carefully dissected from bovine eyes then homogenized in PBS containing Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The retinal homogenate was centrifuged at 100,000xg for 1 h at 4 °C, and protein concentration was determined on the supernatant by the Bradford method. Aliquots containing 1 μg total protein of the bovine neural retina supernatant and protein standards (positive controls) were subjected to SDS-PAGE using NuPage® 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were transferred to an Immobilon-FL polyvinylidene fluoride membrane (Millipore, Bedford, MA). After a brief wash in Tris-buffered saline containing 0.1% Tween-20 membranes were incubated overnight at 4 °C in Sea Block blocking buffer (Pierce Biotechnology, Rockford, IL). The rationale for using Sea Block blocking buffer, a fish serum-based blocking buffer, was that its nonmammalian nature would prevent cross-reactivity with antimammalian protein antibodies and therefore would also yield a low background. Membranes were then probed for BSA, sRBP, and IRBP by incubating 1 h at room temperature using the following primary antibodies diluted in blocking buffer: monoclonal antibovine serum albumin (Clone BSA-33, 1:4000; Sigma-Aldrich, Saint Louis, MO), monoclonal antihuman serum retinol-binding protein (Clone 42, 1:1000; BD Biosciences Pharmingen, San Jose,

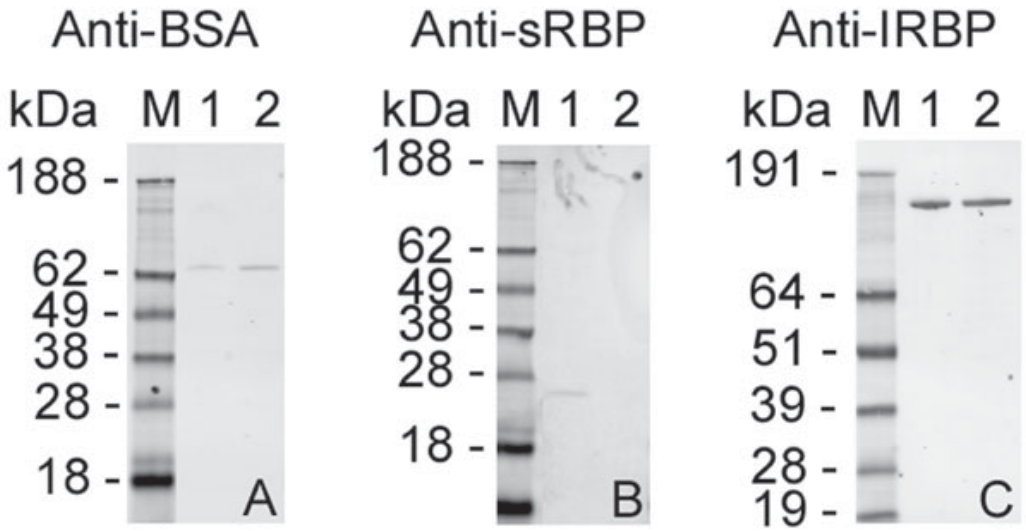

Figure 1. Specificity of the primary antibodies to their respective antigens. Positive controls (1 ng total protein) loaded into lane 1 were purified bovine serum albumin (BSA; **A**), fetal bovine serum (**B**), and purified bovine interphotoreceptor retinoid-binding protein (IRBP; **C**). Bovine neural retina supernatant (1 μg total protein) was loaded into Lane 2 (all panels). The supernatant was prepared by homogenizing bovine neural retinas (n=2) in PBS containing Complete protease inhibitor cocktail (Roche Applied Science) followed by centrifugation at 100,000xg for 1 h at 4 °C. Protein concentration was determined on the supernatant by the Bradford method. Western immunoblot analysis was performed using (**A**) monoclonal anti-BSA (Clone BSA-33, 1:4000; Sigma-Aldrich), (**B**) monoclonal antihuman serum retinol-binding protein (Clone 42, 1:1000; BD Biosciences Pharmingen), and (**C**) rabbit antibovine IRBP (1:5000). The secondary antibodies used were Qdot® 655 conjugated goat F(ab')2 antimouse (1:1000; **A**, **B**), and Qdot® 655 conjugated goat F(ab')2 antirabbit (1:1000; **C**; Quantum Dot Corporation). SeeBlue® (Invitrogen) molecular weight markers are shown in Lane M (all panels). The differences seen in migration of the SeeBlue® molecular weight markers for panels **A** and **B** compared to **C** is due to using either MES- (**A**, **B**) or MOPS- (**C**) SDS running buffer.

CA), or rabbit antibovine interphotoreceptor retinoid-binding protein (1:5000). Purified bovine IRPB protein [1,26] was used for immunization of New Zealand rabbits (Biocon, Rockville, MD) and the antiserum was used as the primary antibody. After washing, membranes were incubated for 1 h at room temperature with either Qdot® 655 conjugated goat F(ab')2 antimouse (1:1000), or Qdot® 655 conjugated goat F(ab')2 antirabbit (1:1000; Quantum Dot Corporation, Howard, CA) secondary antibodies. Detection of labeled proteins was achieved by direct fluorescence analysis using a Typhoon® 9400 imaging system (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry: Bovine eyes were obtained from a local abattoir (J.W. Treuth and Sons Inc., Baltimore, MD). To minimize potential postmortem changes in the distribution of serum proteins, all eyes used for immunolocalization studies were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.3) within 15 min of the animal's death. To facilitate rapid fixation, a 3 cm incision was made through the sclera, posterior to the limbus. Eyes were immersed in an excess (250 ml) of fixative and held on ice for 3 h, then transferred to PBS.

Pieces of retina with attached RPE-choroid were dissected, washed in PBS, and embedded in 7% low gelling temperature agarose. Tissue sections (100 μm thick) were cut with a vibrating microtome (Leica Microsystems, Bannockburn, IL). For confocal immunolocalization studies, sections were incubated for 24 h with the following primary antibodies: rabbit antibovine IRPB (polyclonal, 1:200), mouse antibovine serum albumin (Clone BSA-33, 1:100; Sigma-Aldrich, Saint Louis, MO) or mouse antihuman sRBP (Clone 42, 1:100; BD

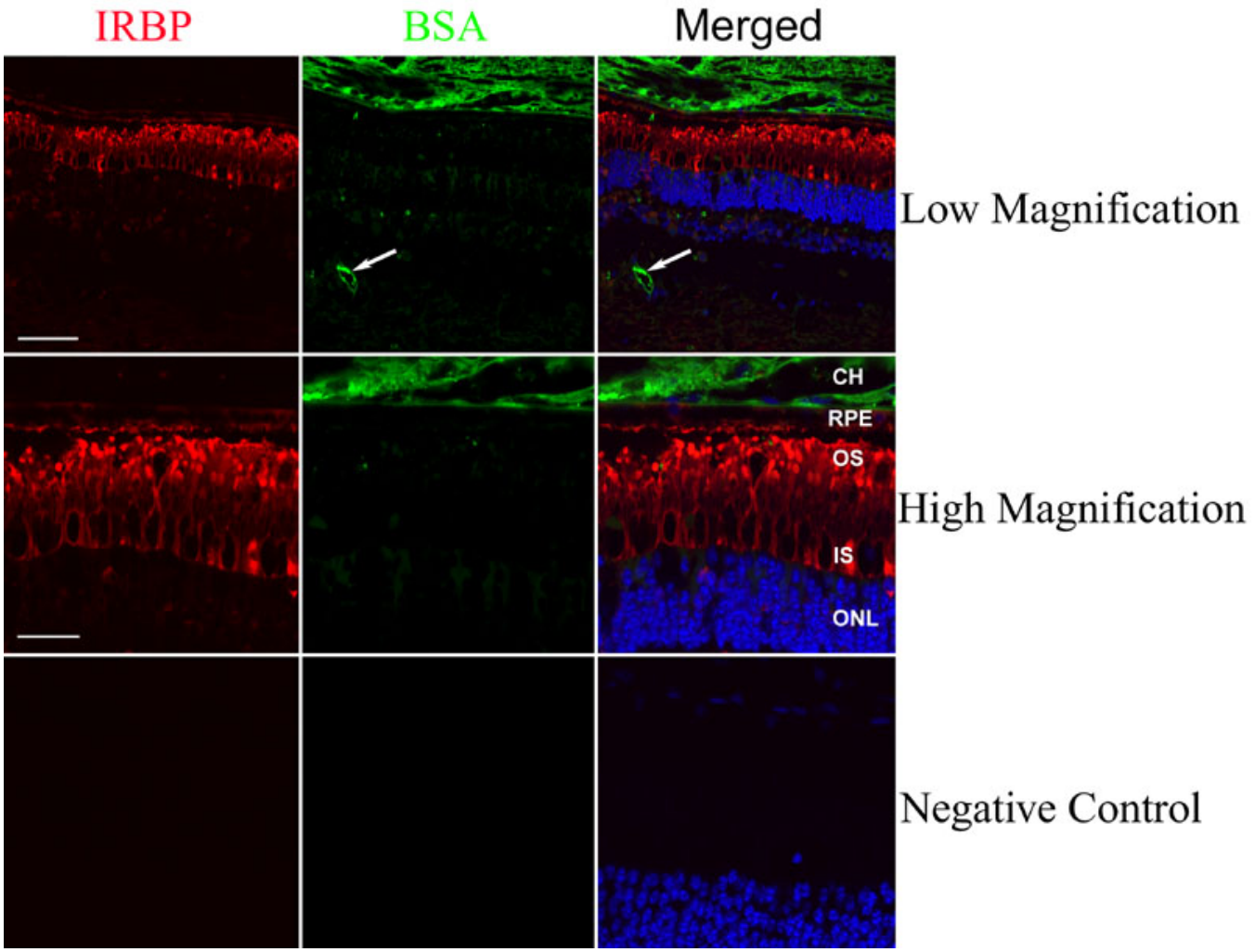

Figure 2. Confocal immunofluorescence analysis of interphotoreceptor retinoid-binding protein and bovine serum albumin in bovine retina. Immunoreactivity for interphotoreceptor retinoid-binding protein (IRBP; red fluorescence) is visible throughout the interphotoreceptor matrix (IPM). Labeling for bovie serum albumin (BSA; green fluorescence) is associated only with the lumen of retinal (arrows) and choroidal blood vessels. There is no significant labeling for BSA in the IPM, as defined by IRBP labeling (red). The dark ovals within the IPM and just above the outer nuclear layer are cone photoreceptor inner segments. Cell nuclei appear blue after DAPI staining. The following abbreviations are used: choroid (CH), retinal pigment epithelium (RPE), outer segment (OS), inner segment (IS), outer nuclear layer (ONL). Top row: Scale bar represents 75 μm; middle and bottom rows: Scale bar represents 30 μm.

Biosciences Pharmingen, San Jose, CA). Normal serum and serum proteins such as BSA were omitted from the immunolabeling process to prevent potential contamination of bovine sections with exogenous serum proteins. Sections were washed in modified immunolabeling buffer (PBS containing 0.1% Tween 20 and 0.05% sodium azide) then incubated for 4 h in the following fluorochrome conjugated secondary antibodies (goat antimouse Alexa Fluor® 488, goat antirabbit Alexa Fluor® 568 and DAPI; Molecular Probes). Primary antibodies were omitted from sections used as negative controls. Sections of labeled bovine retina were washed, mounted in Gel-Mount (Biomeda, Foster City, CA), and cover slipped. A Leica SP2 confocal microscope was used to image samples. Gain and off-set (black level) values were kept constant for each set of experimental and negative control samples.

To identify regions of colocalization, cytofluorogram scatterplots were generated using images collected in sequential scan mode. In a scatterplot correlating red and green channels, the pure red and green pixels cluster near the axes of the plot. Fluorophore colocalization, if present, is represented by pixels falling near the center (i.e., x=y) and upper right-hand corner of the scatterplot. Pixels from areas of signal colocalization were identified in scatterplots and mapped back to the original image.

RESULTS

Western immunoblot analysis: The specificity of the primary antibodies to BSA, sRBP, and IRBP was validated by western immunoblot analysis (Figure 1A-C, respectively). Monoclonal antibovine serum albumin (Clone BSA-33) was highly specific for bovine serum albumin (Figure 1A, Lane 1) and also a single immunoreactive band at about 67 kDa in bovine neural

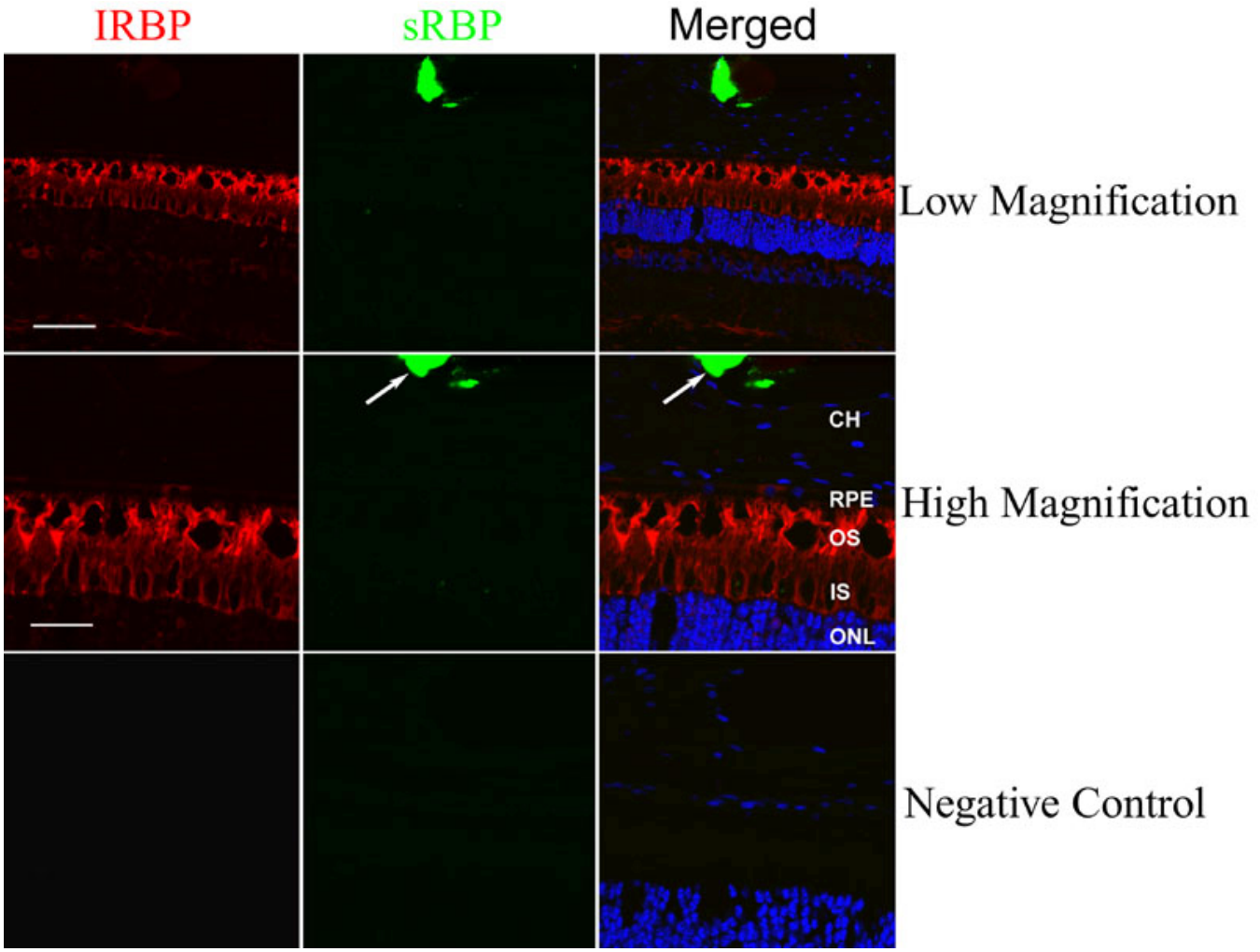

Figure 3. Confocal immunofluorescence analysis of interphotoreceptor retinoid-binding protein and serum retinol-binding protein in bovine retina. Immunoreactivity for interphotoreceptor retinoid-binding protein (IRBP; red fluorescence) is throughout the interphotoreceptor matrix (IPM). Labeling for serum retinol-binding protein (sRBP; green fluorescence) is associated with the lumen of a choroidal blood vessel (arrows). There is no significant labeling for sRBP in the IPM, as defined by IRBP labeling (red). The dark ovals within the IPM and just above the outer nuclear layer are cone photoreceptor inner segments. Cell nuclei appear blue after DAPI staining. The following abbreviations are used: choroid (CH), retinal pigment epithelium (RPE), outer segment (OS), inner segment (IS), outer nuclear layer (ONL). Top row: Scale bar represents 75 μm; middle and bottom row: Scale bars represents 36 μm.

retina (Figure 1A, Lane 2). Monoclonal antihuman serum retinol-binding protein (Clone 42) and polyclonal rabbit antibovine interphotoreceptor retinoid-binding protein were also highly specific for their respective antigens recognizing a single immunoreactive band at about 25 (Figure 1B, Lane 1) and about 133 kDa (Figure 1C, Lane 1), respectively. No immunoreactive bands were detected in bovine neural retina by the anti-sRBP antibody (Figure 1B, Lane 2); however, a single immunoreactive band at about 133 kDa was detected in bovine neural retina by the anti-IRBP antibody (Figure 1C, Lane 2).

Confocal immunofluorescence analysis: The localization of BSA, sRBP, and IRBP in bovine retina was examined by confocal immunofluorescence analysis (Figure 2, Figure 3). The immunolocalization of IRBP (red fluorescence, Figure 2, Figure 3) was restricted to the area between the external limiting membrane and the apical surface of the RPE with more intense labeling around the outer segments of photoreceptors. This region defines the limits of the interphotoreceptor matrix. Labeling for BSA (green fluorescence, Figure 2) was associated only with the choroid and the lumen of retinal blood vessels. Labeling for sRBP, (green fluorescence, Figure 3) was associated with material in the lumen of a choroidal blood vessel. No immunolabeling for either BSA or sRBP was observed in the IPM of bovine retina. Cytofluorogram analysis provided additional evidence that neither BSA nor sRBP is present in the IRBP-rich IPM (Figure 4A,B, respectively). Note that the area denoted by the white box within the scatterplots (inset) of Figure 4A,B is essentially devoid of any signal arising from colocalized fluorophores.

DISCUSSION

For this study we evaluated the presence of serum albumin, serum retinol-binding protein, and interphotoreceptor retinoid-binding protein, in the IPM of bovine eyes using confocal immunofluorescence analysis. Our primary interest in doing this type of study was to establish whether or not these proteins are normal constituents of the IPM. One of our initial concerns for this study was postmortem changes in retinal tissues. It has been reported [27] that degenerative changes in postmortem retinal tissues occur within 15 min of death when tissues were maintained at room temperature before being fixed. Our goal was to maintain the integrity of retinal tissues and to minimize the potential for postmortem changes that could result in diffusion of serum as well as intracellular proteins into the IPM. Therefore, we obtained fresh bovine eyes from a local abattoir where eyes were enucleated, placed on ice, and fixed by immersion in ice cold 4% paraformaldehyde within 15 min of the animal's death.

The IPM is an extracellular matrix that fills the interphotoreceptor space separating the neural sensory retina from the RPE. The IPM of the mammalian retina surrounds

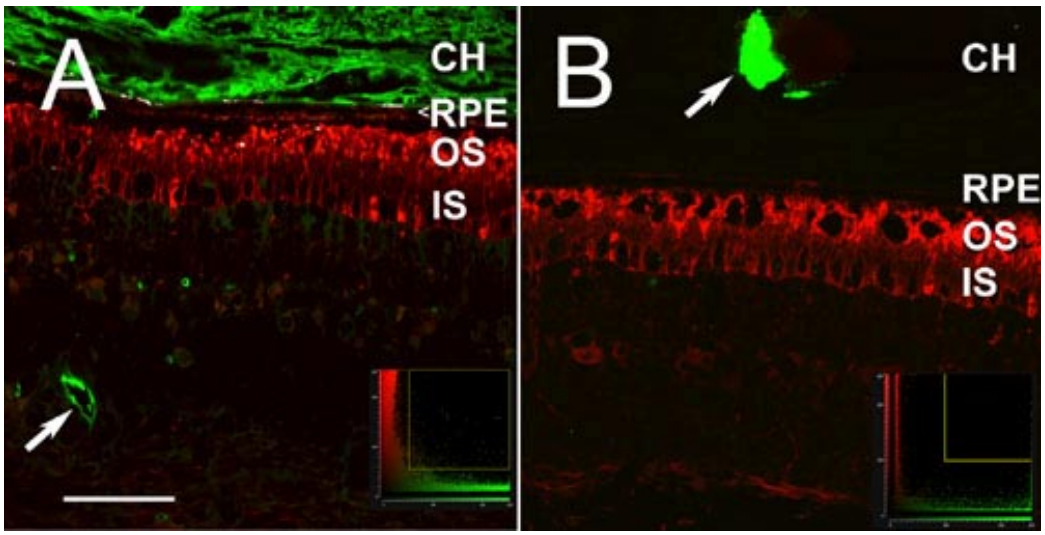

Figure 4. Colocalization analysis of bovine serum albumin, serum retinol-binding protein, and interphotoreceptor retinoid-binding protein in bovine retina. To determine colocalization of fluorophores, we performed scatterplot analysis using composite images which were generated from two independent channel single-wavelength acquisitions. The composite image showing bovine serum albumin (BSA; green channel) and interphotoreceptor retinoid-binding protein (IRBP; red channel) is displayed in **A** and serum retinol-binding protein (sRBP; green channel) and IRBP (red channel) in **B**. Scatterplots (panel insets) that correlate the red and green channels, show the pure red and green pixels clustering near the axes of the plot, while colocalized pixels, if present, fall near the center (i.e., x=y) and upper right-hand corner of the scatterplot. To perform the colocalization analysis, we employed an area of interest (AOI), denoted by the white box within the scatterplot, to identify pixels from regions of colocalization. These pixels (shown in white) were mapped to the image. The AOI's for both scatterplots are essentially devoid of any signal arising from colocalized fluorophores with only scant colocalization, consistent with background labeling, visible in **A** and no areas of colocalization visible in **B**. Arrows point to the lumen of a retinal blood showing labeled BSA (**A**) and a choroidal blood vessel showing labeled sRBP (**B**). The following abbreviations are used: choroid (CH), retinal pigment epithelium (RPE), outer segment (OS), inner segment (IS). (**A**, **B**) Scale bar represents 75 μm.

apices of Müller, photoreceptor cells, and RPE and is bounded by two distinct diffusional barriers. One is formed by junctional complexes composed of tight junctions, or zonula occludens, between the cells of the RPE and constitutes the outer blood retinal barrier [28]. The other diffusional barrier is formed by the zonulae adherentes of the external limiting membrane. The cells surrounding the IPM in conjunction with the diffusional barriers allow for a unique environment by either restricting movement of material (e.g. protein) into or out of the IPM. In cases where the blood retinal barrier was compromised by disease or injury, serum proteins were found to accumulate within the IPM [23,24,29].

It is widely accepted that IRBP is the most abundant glycoprotein of the IPM [20,30-32], accounting for >70% of the soluble protein. IRBP mRNA expression was demonstrated by in situ hybridization [33,34] and localized to the inner segments of both rod and cone photoreceptors. This mRNA is translated and IRBP is secreted into the IPM [35-37]. Due to its large size, (R_s=55 Å; M_r equal to 140 kDa), IRBP is limited to the confines of the IPM [38]. IRBP is known to bind endogenous retinol [30,39,40] and is hypothesized to be the transport vehicle for visual cycle retinoids between the neural retina and RPE. Recent reports provide evidence suggesting that, in vivo, IRBP plays a direct role in the release of all-*trans* retinol from rod photoreceptors following rhodopsin bleaching [11,12]. In our study, we clearly show the immunolocalization of IRBP is indeed within the limits of the IPM using confocal immunofluorescence analysis. However, we did not see any immunolabeling of cone inner segments. This pattern of immunolabeling is consistent with earlier studies using immunocytochemical techniques [31,41,42] which demonstrate the immunolabeling for IRBP to be extracellular and restricted to the IPM. Furthermore, labeling of inner segments of either rod or cones was not apparent in these studies [31,41,42].

The presence of other proteins capable of binding visual cycle retinoids, namely serum albumin and sRBP, have been demonstrated in IPM samples obtained by gently washing excised retinas and the apical surface of intact RPE of several vertebrate species including human [3,25,43]. Furthermore, Adler et al. [3] were able to show the presence of albumin in the IPM of a human retina fixed 1 h post mortem by using immunohistochemical analysis. Other studies [23,24], however, found albumin present in human IPM that resulted from pathological abnormalities, particularly in cases when the outer blood retinal barrier was compromised. In these studies it was shown that an intact blood retinal barrier was effective in excluding albumin from the inner and outer retina. That is, positive staining for albumin was observed only in the choroid and within retinal blood vessels. In our study we could not detect albumin within the bovine IPM. There was no evidence of albumin diffusing across the outer blood retinal barrier or the intercellular junctions (i.e., zonulae adherents) of the external limiting membrane. We could only observe immunofluorescence for albumin in the choroid and within the lumen of a retinal blood vessel.

Previous work by others has demonstrated the presence of sRBP in the neurosensory retina by using immunocytochemical techniques [44,45]; however, neither study was able to detect sRBP within IPM [44,45]. It is interesting to note that within ocular tissues, the mRNA for sRBP uniquely localizes to the RPE [45]. Furthermore, Ong et al. [46] were able to show that synthesis of sRBP in cultured RPE cells does indeed occur and that this sRBP was secreted into both the apical and basal culture media, although recovery of the secreted sRBP by these cultured RPE cells was much higher in the apical medium [46]. If the apical secretion of sRBP by the RPE does occur in vivo, the RPE could serve as a likely source of sRBP to the neurosensory retina. In our study, immunofluorescence for sRBP was limited to material within the lumen of a choroidal blood vessel. We found no labeling within the IPM or other cells of the neurosensory retina for sRBP. It is possible that the level of sRBP, if present in the bovine IPM, was below the limit of detection for the methods used in our study.

In conclusion, we have shown that serum albumin and sRBP do not immunolocalize to the bovine IPM. Therefore, we believe that serum albumin and sRBP are not physiologically normal constituents of the bovine IPM, and it is unlikely that either of these two proteins are involved in the bovine visual cycle. On the other hand, our data clearly shows that IRBP, a protein known to bind visual cycle retinoids, localizes exclusively to the IPM of the bovine retina. These data, in conjunction with several recent studies [6-12], provide additional evidence supporting the role of IRBP in the binding and transport of visual cycle retinoids.

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