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Characterization of semenogelin proteins in the human retina

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Abstract

Semenogelin I and II are the major proteins present in semen coagulum. In the present study, semenogelin I and II were detected in human RPE lysates by proteomic analysis. We further analyzed the expression of these proteins in the retinal cells in vivo and in vitro. Western blots detected semenogelin I and II in both RPE and neural retina while the vitreous contained only SgII. Cryo and paraffin sections of human retina were processed for both immunofluorescence and DAB reaction with an antibody that recognizes both forms of semenogelin proteins. Retina and RPE total lysates were evaluated for the presence of these proteins and in a human RPE cell line (D407). Both proteins were detected by western blot in human RPE and in D407 cell lysates. Immunoreactivity was detected in the ganglion cell and photoreceptor layer of the retina. Our data support the expression of semenogelin I and II in the human retina in several different compartments. Further studies towards addressing the function of these proteins in the retina are in progress.

Keywords: semenogelins; immunohistochemistry; retina; RPE; proteomics

1. Introduction

Semen coagulum proteins are secreted at high concentration by the seminal vesicles. Humans produce semenogelin I (SgI) and SgII (SgII), whereas in rodents these proteins are known as SVS I–SVS VI. The genes of semenogelins (Sgs) and SVS II–SVS VI are homologous, but the proteins they give rise to have almost no structural similarity because of a rapid and unusual evolution (Lundwall, 1996; Lundwall and Lazure, 1995). The rapid evolution is also apparent within the primate linage. Closely related species like humans, chimpanzees and gorillas have Sgs of very different sizes (Jensen-Seaman and Li, 2003). There is also an intriguing interspecies size heterogeneity of Sg molecules.

SgI is a non-glycosylated protein with a molecular mass of 50 kDa (Lilja et al., 1989; Lilja and Lundwall, 1992). A few percent of the worlds population also carry an allele that gives rise to a truncated SgI molecule with a molecular mass of 43 kDa (Jensen-Seaman and Li, 2003; Lundwall et al., 2003; Miyano et al., 2003). Sg II, with a molecular mass of 63 kDa, has a potential site for N-linked glycosylation and approximately half of the molecules in seminal plasma are glycosylated, yielding two molecular species with an apparent mass difference of 5 kDa (Lilja and Laurell, 1985). Early studies suggested the Sgs to be exclusive for the male genital tract (Bjartell et al., 1996; Herr et al., 1986). More recent studies have demonstrated both SgI and SgII in a large number of tissues containing secretory epithelium, but also in non-glandular tissue such as skeletal muscle (Lundwall et al., 2002). Functional studies have indicated a role of Sgs in capacitation and motility of sperm, in vitro activation of hyaluronidase and the antibacterial activity of semen (Mandal and Bhattacharyya, 1995; Bourgeon et al., 2004).

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Studies have indicated a role of semenogelins related to capacitation and motility of sperm (de Lamirande et al., 2001; Robert and Gagnon, 1996). Other features of these molecules include the presence of a heparin-binding site (Malm et al., 1996). These findings have not been substantiated by independent studies and it is therefore still unclear whether the Sgs have any function beside their role as structural components of the seminal coagulum. However, the high Zn^{2+}-binding capacity of both SgI and SgII, as recently demonstrated (Jonsson et al., 2005), could suggest that Sg molecules might function as important regulators of extracellular Zn^{2+} homeostasis.

The purpose of this investigation was to identify novel components of the retinal pigment epithelium (RPE) starting with a proteomic approach. We found that extracts of RPE contained both SgI and SgII. In subsequent studies Western blotting and immunohistochemistry defined the expression and distribution of Sgs in the posterior part of the eye.

2. Materials and methods

2.1. Human eye tissue

Donor eyes were obtained from the Cleveland Eye Bank or through the Foundation for Fighting Blindness Eye Donor Program (Owings Mills, MD). Tissue from 14 different donors was analyzed. The donor ages varied between 50 and 82. The interval between death and tissue processing varied between 4 and 13.5 h. The immunocytochemistry and western analysis is exempt of IRB approval.

2.2. Preparation of human RPE lysates

RPE cells were isolated using the protocol initially described (Nakata et al., 2005) with mechanical removal of the retina and brushing of the RPE from the choroid. RPE cells were pelleted down, the PBS was removed and the fresh PBS containing protease inhibitors was added to the cells. The RPE cells were kept at −80 °C until used. When ready to use, RPE lysates were diluted 1:1 with 2× radioimmunoprecipitation buffer (RIPA) (0.2% SDS, 2% Triton X-100, 2% deoxycholate, 0.15 M NaCl, 4 mM EDTA, 50 mM Tris pH 7.4) containing a cocktail of protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Cells were lysed for 1 h at 4 °C in the rotator. Samples were centrifuged for 10 min at 14,000 rpm and the pellets were discarded.

2.3. LC MS/MS

For proteomics analyses, the RPE lysates were dissolved in 2× Laemmli buffer, boiled and proteins were resolved by SDS-PAGE on 4–15% gradient gels (Bio-Rad, Hercules, CA, USA). The gel lanes were cut from top to bottom into ~2 mm slices. Gel slices were washed, reduced, alkylated, digested with trypsin, extracted and resultant peptides subjected to LC MS/MS analysis using a QTOF2 mass spectrometer equipped with a CapLC System (Waters Corp, Milford, MA, USA). Protein identifications from MS/MS data utilized the Swiss-Prot, and the NCBI sequence databases and the search engines Protein Lynx TM Global server and Mascot (Matrix Science) as previously described (Bonilha et al., 2004; Crabb et al., 2002; West et al., 2003).

2.4. Immunohistology of tissue

To confirm the localization of some of the proteins identified by LC MS/MS analysis, immunohistochemical assays were performed using both paraffin and cryosections of human eyes in the peri-macular area. Eye pieces were cut and fixed by immersion in 4% paraformaldehyde made in PBS for 3 h at 4 °C. For paraffin processing fixed eye pieces were dehydrated and embedded in paraffin. Immunostaining was carried out on 5 μm sections. After deparaffinization and rehydration to PBS, sections were subjected to heat-mediated antigen retrieval by pressure cooking in 10 mM citric acid buffer, pH 6.0. Sections were probed with previously described rabbit antibodies to SgI and II in 5% BSA, PBS and 0.3% Triton X-100 overnight at 4 °C (Bjartell et al., 1996; Malm et al., 1996). Each antibody cross-reacts with the other semenogelin. The controls omitted the antibodies. Sections were washed, incubated with secondary antibody conjugated to biotin for 1 h at RT, washed, and incubated with avidin in PBS for 30 min, then developed with DAB for 2 min. The sections were examined with a Zeiss Axiophot light microscope and the images were digitized using a Hamamatsu CCD camera. Figure panels were composed using Adobe Photoshop 5.5.

For colocalization labeling, eyecups were fixed as described above, quenched with 50 mM NH_4Cl made in PBS for 1 h at 4 °C, infused successively with 15% and 30% sucrose made in the same buffer and with Tissue-Tek "4583" (Miles Inc., Elkhart, IN). Cryosections (12 μm) were cut on a cryostat HM 505E (Microm, Walldorf, Germany) equipped with a CryoJane Tape-Transfer system (Instrumedics, Inc., Hackensack, NJ, USA). For labeling, sections were blocked in PBS + 1% BSA (PBS/BSA) for 30 min, and incubated with the antibodies to SgI, monoclonal antibody B630N to rhodopsin (1:50, from Dr P. Hargrave, University of Florida, Gainesville, FL, USA), and the monoclonal antibody 7G6 to cone cytoplasm (1:100, from Dr P. MacLeish, Morehouse School of Medicine, Atlanta, GA). Cone-associated matrix was labeled with PNA-TRITC (1:100, Vector, Burlingame, CA), rod-associated matrix was labeled with WGA-TRITC (1:500), cell nuclei were labeled with TO-PRO®-3 iodide (Molecular Probes) and secondary antibodies were labeled with Alexa Fluor 488 (green) and Alexa Fluor 594 (red; Molecular Probes). Samples were observed in a laser scanning confocal microscope (TCS-SP2, Leica, Exton, PA). A series of 1 μm *xy* (en face) sections were collected. Each individual *xy* image of the retinas stained represents a three-dimensional projection of the entire cryosection (sum of all images in the stack). Microscopic panels were composed using Adobe Photoshop 5.5.

2.5. Western blot analysis

Whole cell lysates from RPE, retina and vitreous collected from human eyecups, were solubilized in RIPA buffer supplemented with a cocktail of protease and phosphatase inhibitors (Sigma). Forty micrograms of protein per sample was resolved in a 10–20% SDS-PAGE (Novex, Invitrogen, Carlsbad, CA) and electro-transferred to Immobilon PVDF membranes (Millipore, Bedford, MA). Positive control was unliquefied seminal fluid in buffer containing 4 M urea. Membranes were incubated with antibodies to SgI (also react with SgII) in Blotto A buffer (20 mM Tris–HCl, 0.9% NaCl, 0.05% Tween 20 (TBST), 5% skimmed milk) for 1 h. Protein detection was performed with secondary antibodies conjugated to peroxidase and visualized using chemiluminescence Reagent Plus (NEN™ Life Science Products, Inc., Boston, MA) detection system. PVDF membranes were exposed to film, films were scanned and figures were composed using Adobe Photoshop 5.5.

3. Results

3.1. Identification of SgI and SgII peptides in RPE total lysates and characterization of the proteins in human eyes

In order to gain further understanding of the human RPE biology, total proteins were extracted and separated by SDS-PAGE, gel slices excised and proteins identified using well-established mass spectrometric and bioinformatic methods. SgI and SgII, proteins not previously identified in the eye, were frequently identified components in the human RPE lysates (Table 1). An overall protein coverage of ~23% and 10% of the proteins was observed for SgI and SgII, respectively. Subsequent Western analyses of different RPE from several human donors confirmed the presence of these proteins (Fig. 1A). To further characterize the presence of semenogelin proteins in the eye, neural retina and vitreous of different donors were also harvested, lysed and analyzed by Westerns (Fig. 1B and C). Both SgI and SgII are present in extracts of RPE and neural retina whereas the vitreous exhibits only SgII.

3.2. Presence of semenogelin proteins in choroid, RPE, photoreceptors, inner nuclear layer, and ganglion cell layer of the human retina

To dissect the molecular localization of semenogelin proteins in the neural retina, donor eyes were processed for paraffin embedding followed by immunohistochemistry. The distribution of semenogelin proteins was analyzed both in the retinal periphery of normal human donors (Fig. 2A–H). Sections were labeled with either antibody to SgI (Fig. 2B and E) and SgII (Fig. 2D and H), even though each antibody cross-reacts with the other semenogelin. Control sections (Fig. 2A, C, E and G) had the antibodies omitted. A striking distribution of SgI was observed throughout the choroid, RPE, photoreceptor cells, cells in the inner nuclear layer and the ganglion cell layer (Fig. 2B, inset and arrows) when compared to the control section. This unique distribution was also observed in sections labeled with the SgII antibody (Fig. 2D). However, the intensity of the staining was lower when labeling with the SgII antibody, leading us to use the antibody to SgI for the remaining histological studies. At higher magnification, semenogelin proteins could be observed in the choroid, RPE cone and rod inner and outer segments (Fig. 2F and H) and in the ganglion cells (Fig. 2B inset and D, arrows).

Table 1
Semenogelin peptides identified by LC MS/MS from RPE total lysates

Protein identified	Accession number[a]	Peptides matched	Protein coverage (%)[b]
Semenogelin I	P04279	8	22.5
Semenogelin II	Q02383	4	10.1

[a] Swiss Protein database accession numbers are shown; for links use the EXPASY server at http://us.expasy.org/sprot/.

[b] Peptides identified are shown in bold italic type on the protein sequence of: SEMENOGELIN I (P04279): 1MKPNIIFVLSLLLILEKQAAVMGQKGGSK GR***LPSEFSQFPHGQK***GQHYSGQKGKQQTESKGSFSIQYTYHVDANDH DQSRKSQQYDLNALHKTTKQHLGGSQQLLHNKQEGRDHDKSKGHFH RVVIHHKGGKAHRGTQNPSQDQGNSPSGK***GISSQYSNTEER***LWVHGLS KEQTSVSGAQKGRKQGGSQSSYVLQTEELVANKQQRETKNSHQNK***GH YQNVVEVR***EEHSSKVQTSLCPAHQDKLQHGSKDIFSTQDELLVYNKNQ HQTKNLNQDQQHGRKANK***ISYQSSSTEER***RLHYGENGVQK***DVSQSSIY SQTEEK***AQGKSQK***QITIPSQEQEHSQK***ANKISYQSSSTEERRLHYGENG VQKDVSQRSIYSQTEKLVAGK***SQIQAPNPK***QEPWHGENAKGESGQSTN R***EQDLLSHEQK***GRHQHGSHGGLDIVIIE QEDDSDRHLA QHLNNDRNP L FT **439**. SEMENOGELIN II (Q02383): 1MKSIILFVLSLLLILEKQAAVM GQKGGSKGQLPSGSSQFPHGQKGQHYFGQKDQQHTKSKGSFSIQHTY HVDINDHDWTRKSQQYDLNALHKATKSKQHLGGSQQLLNYKQEGRD HDKSKGHFHMIVIHHKGGQAHHGTQNPSQDQGNSPSGKGLSSQCSNT EKRLWVHGLSKEQASASGAQKGRTQGGSQSSYVLQTEELVVNKQQRE TKNSHQNK***GHYQNVVDVR***EEHSSKLQTSLHPAHQDRLQHGPK***DIFTTQ DELLVYNK***NQHQTKNLSQDQEHGRKAHKISYPSSRTEERQLHHGEKSV QKDVSKGSISIQTEEKIHGKSQNQVTIHSQDQEHGHKEN***ISYQSSSTEE R*** HLNCGEKGIQKGVSKGSISIQTEEQIHGKSQNQVRIPSQAQEYGHKEN KISYQSSSTEERRLNSGEKDVQKGVSKGSISIQTEEKIHGKSQNQVTIPS QDQEHGHKENKMSYQSSSTEERRLNYGGKSTQK***DVSQSSISFQIEK***LV EGKSQIQTPNPNQDQWSGQNAKGKSGQSADSKQDLLSHEQKGRYKQE SSESHNIVITEHEVAQDDHLTQQYNEDRNPI ST **559.**

During the ganglion cell layer (Fig. 2B, inset and arrows) when compared to the control section. This unique distribution was also observed in sections labeled with the SgII antibody (Fig. 2D). However, the intensity of the staining was lower when labeling with the SgII antibody, leading us to use the antibody to SgI for the remaining histological studies. At higher magnification, semenogelin proteins could be observed in the choroid, RPE cone and rod inner and outer segments (Fig. 2F and H) and in the ganglion cells (Fig. 2B inset and D, arrows).

3.3. Immunohistochemical distribution of SgI in the photoreceptor-associated matrix and cells

A detailed characterization of the distribution of Sgs in the photoreceptors cells was carried out in cryosections of eyes labeled with antibodies to both cone and rod protein markers. The rod outer segments were visualized through labeling with rhodopsin antibody (Fig. 3B). There was a great overlap between the rhodopsin labeling and the Sgs (Fig. 3A and D). Besides, cryosections were also labeled with an antibody specific to the cone cytoplasm (Fig. 3E). A low level of co-distribution of cone cytoplasmic marker and semenogelin proteins could be observed in these samples. This was due to the difference in signal intensity since Sgs can be observed both in the inner and outer segments of photoreceptor cells (Fig. 3A and D). Overlapping localization is shown as yellow in Fig. 3C and F. Cell nuclei were labeled with TO-PRO-3.

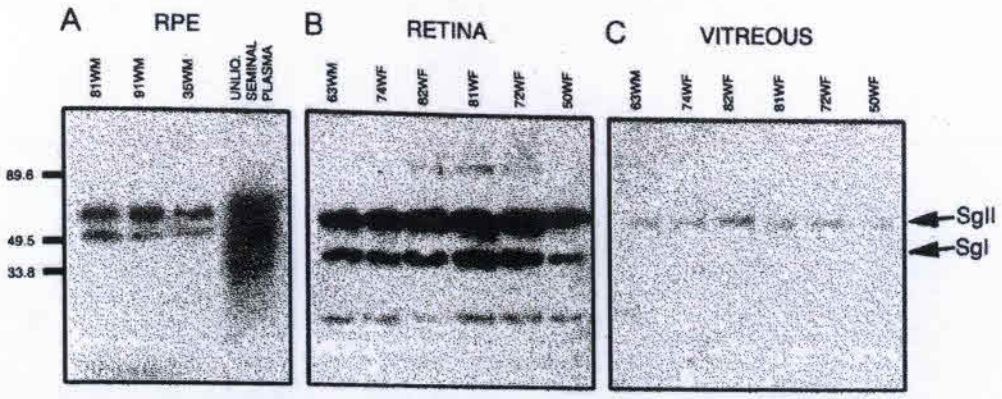

Fig. 1. Western analysis of RPE and retina exhibits both SgI and SgII while vitreous just exhibit SgII. RPE (A), neural retina (B) and vitreous (C) from six different human donors were harvested, lysed, 40 μg of total protein of each sample was separated on a 10–20% SDS gel, transferred to PVDF membranes and probed with an antibody specific to SgI, which also reacts with SgII, followed by ECF detection of immunoreactivity. The age, ethnical background and gender of the donors is indicated on the top of each lane. Positive control is unliquefied human seminal plasma.

The distribution of Sgs was also compared to the distribution of the rod and cone- associated matrix through the labeling of sections with WGA (rod-associated) and PNA (cone-associated) lectins (Fig. 4). High levels of colocalization could be observed between Sgs (Fig. 4A) and WGA (Fig. 4B) then between Sgs (Fig. 4D) and PNA (Fig, 4E). However, Sgs are observed both at the photoreceptor inner and outer segments. The low degree of colocalization between Sgs and the cone-associated matrix is probably due to the difference in the intensity of the labelings.

The distribution of Sgs was also investigated in the D407 human RPE cell line, which preserves native RPE characteristics including the ability to perform phagocytosis (Davis et al., 1995). Analysis of these cell lysates revealed the presence of both SgI and II in D407 cells (data not shown). The localization of Sgs within these cells revealed a cytoplasmic, mostly perinuclear, distribution; co-labeling studies confirmed the co-distribution of Sgs with calnexin, indicating the presence of Sgs in the endoplasmic reticulum of these cells (data not shown).

4. Discussion

Semenogelin proteins were originally identified as the major protein components present in the seminal vesicle secretion (Lilja and Laurell, 1985). Subsequently, both semenogelin proteins were identified in several other tissues throughout the human body as well as in several malignant tissues and cell lines (Hienonen et al., 2005; Lundwall et al., 2002; Rodrigues et al., 2001; Zhang et al., 2003). Furthermore, both SgI and SgII expressed sequence tags (EST) have been identified in skeletal muscle, breast tissue, kidney, mammary gland, and colon RNA in the UniGene database (http://www.ncbi.nlm.nih.gov/UniGene). No EST sequences for either semenogelin proteins have been detected in the eye. The SgI and SgII sequences in the EST database suggests that both molecules are always synthesized with a signal peptide for secretion. Most likely, this is also the case in the eyes and RPE cell culture as is suggested by the presence of Sgs staining in D407 cells in the ER. Our previous proteomic analysis of human RPE (West et al., 2003) failed to detect semenogelins and to our knowledge this is the first demonstration of the presence of these proteins in the human eye. The initial observation was confirmed through several experimental methodologies.

Our data suggests that RPE and neural retina contain both SgI and SgII, while vitreous contains only SgII. The functional significance of Sgs in the vitreous is not known but it could be important for the establishment of the vitreous gel. In vitreous samples SgII is a major component of it through observation of the total protein profile of SDS-PAGE gels (data not shown). In the seminal vesicles SgI and SgII are the major components of the seminal vesicles secretion. Semenogelin proteins together with fibronectin from the seminal vesicles form the gel-like coagulum of newly ejaculated semen (Lilja and Laurell, 1985; Malm et al., 1996). Similarly, the vitreous is known to be enriched in fibronectin (Menasche et al., 2001; Menasche et al., 1997; Probst et al., 2004). The presence of Sgs and vitronectin in the vitreous may be important for its gel property.

The Sgs distribution in the human eye tissues was associated with the choroid, RPE, photoreceptor cells, cells in the inner nuclear layer, and ganglion cell layer. In the photoreceptor cells, Sgs were observed in the inner and outer segments and IPM. The IPM is located between the outer limiting membrane of the retina and the apical border of the retinal pigment epithelium (RPE). Structure-function activities of fundamental importance to vision occur within this matrix, including the trafficking of retinoids and other metabolites between photoreceptors and the RPE; retinal attachment; maintenance of photoreceptor specific microenvironments; photoreceptor alignment; and cell–cell interactions involved in outer segment shedding and RPE phagocytosis. The molecular interactions responsible for these activities are not known. Semenogelin molecules are proteins that may modulate and interact with proteoglycans in the IPM. It has been shown that hyaluronan (HA) and several HA-binding proteins are key participants in the organization of the IPM and in retinal attachment (Becerra et al., 1999; Berman, 1969; Chaitin et al., 1999; Hollyfield, 1999; Hollyfield et al., 1999).

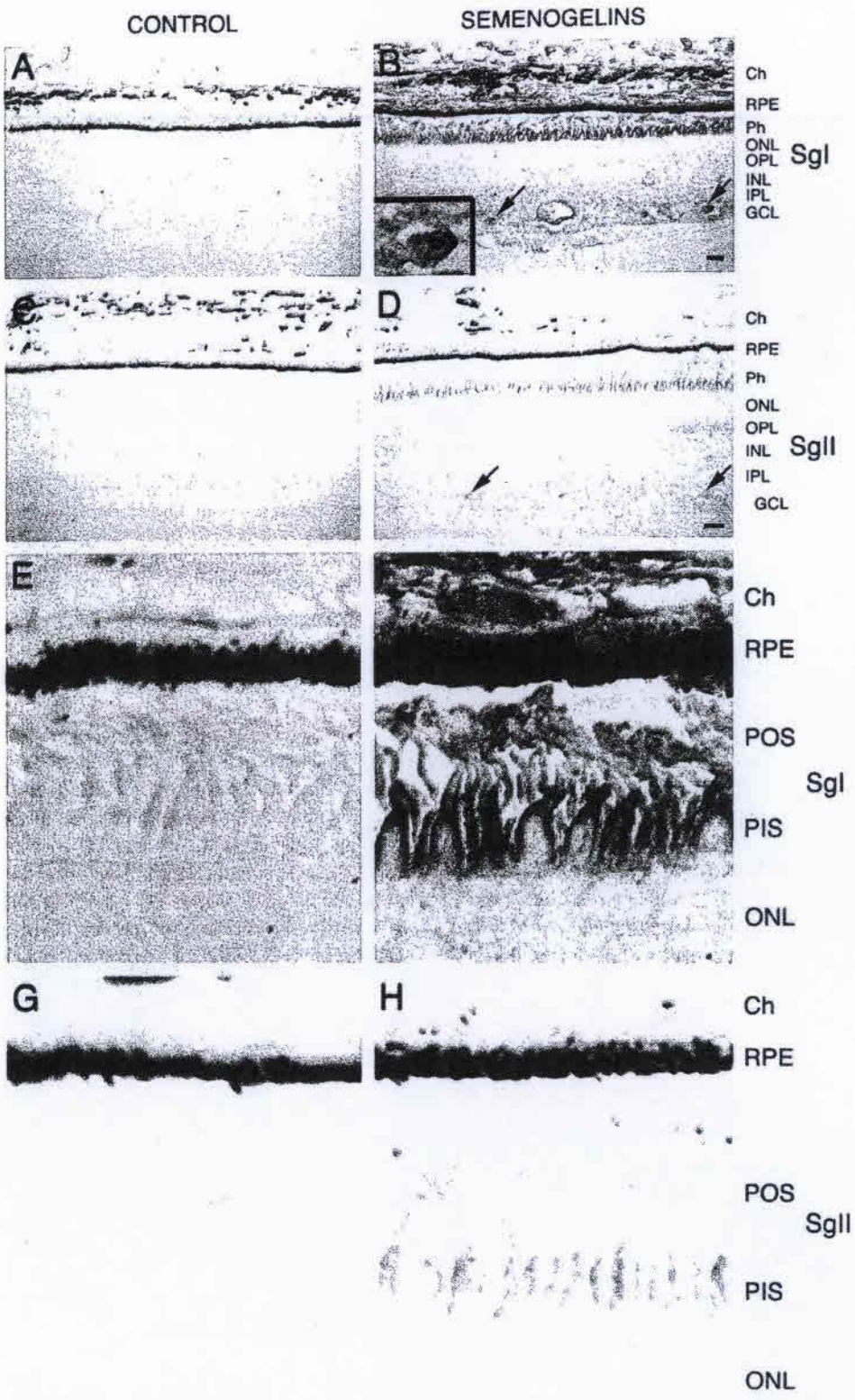

Fig. 2. Immunohistological demonstration of Sgs in photoreceptors, RPE, choroid, inner nuclear layer, and ganglion cell layer of the human retina. Paraffin sections (5 μm) of human donors were probed with SgI (B and F) and SgII (D and H) antibodies. Each antibody cross-reacts with the other Sg. In the controls (A, C, G and E) the antibodies were omitted. The presence of Sgs was visualized through incubation with secondary antibody conjugated to biotin followed by reaction with DAB. Comparison of the samples showed that SgI antibody displays stronger reaction with the photoreceptor inner segments (PIS), photoreceptor outer segments (POS), choroid (Ch), retinal pigment epithelium (RPE), cells in the inner plexiform layer (IPL), and the ganglion cell layer (GCL). The SgII antibody displayed similar localization with the exception of the reaction with the Ch and lower intensity. PIS, photoreceptor inner segments; POS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar = 200 μm.

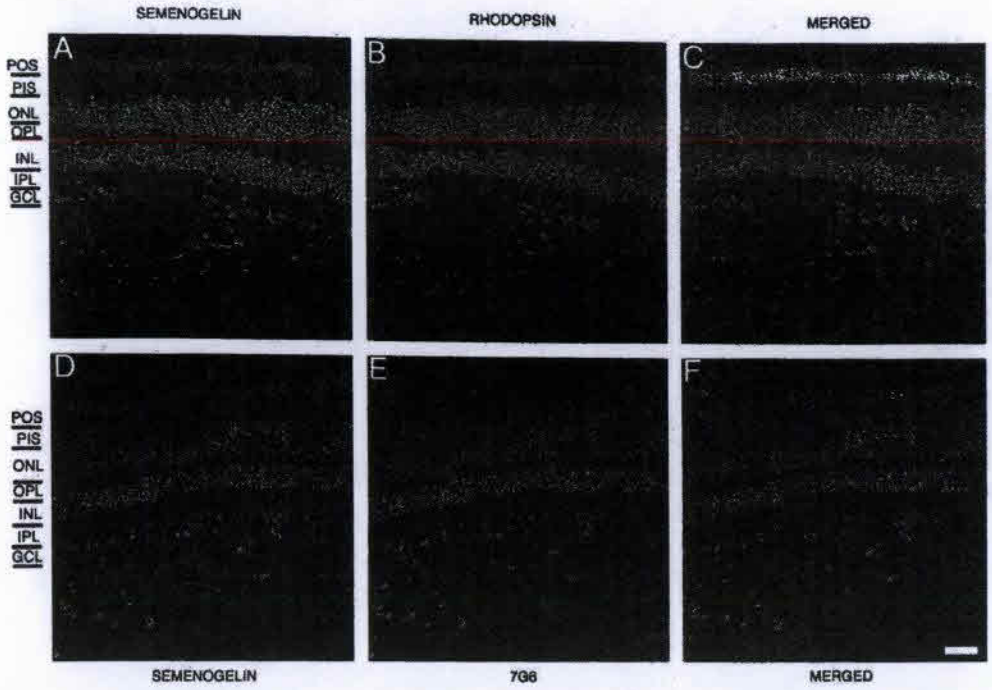

Fig. 3. Higher levels of colocalization of Sgs with rhodopsin then with a cone cytoplasmic marker. Cryosections (12 μm) of human donors were labeled with antibodies specific to SgI (A and D) and with the anti-rhodopsin (B) and the cytoplasmic cone marker 7G6 (E). Cell nuclei were labeled with TO-PRO-3 (blue). Overlaid images are shown in C and F. Sections were analyzed using a Leica laser scanning confocal microscope (TCS-SP2, Leica, Exton, PA). A series of 1 μm *xy* (en face) sections were collected. Each individual *xy* image of the retinas stained represents a three-dimensional projection of the entire cryosection (sum of all images in the stack). Comparison of the samples showed that SgI colocalizes with rhodopsin in the outer segments of the rods. PIS, photoreceptor inner segments; POS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar = 40 μm.

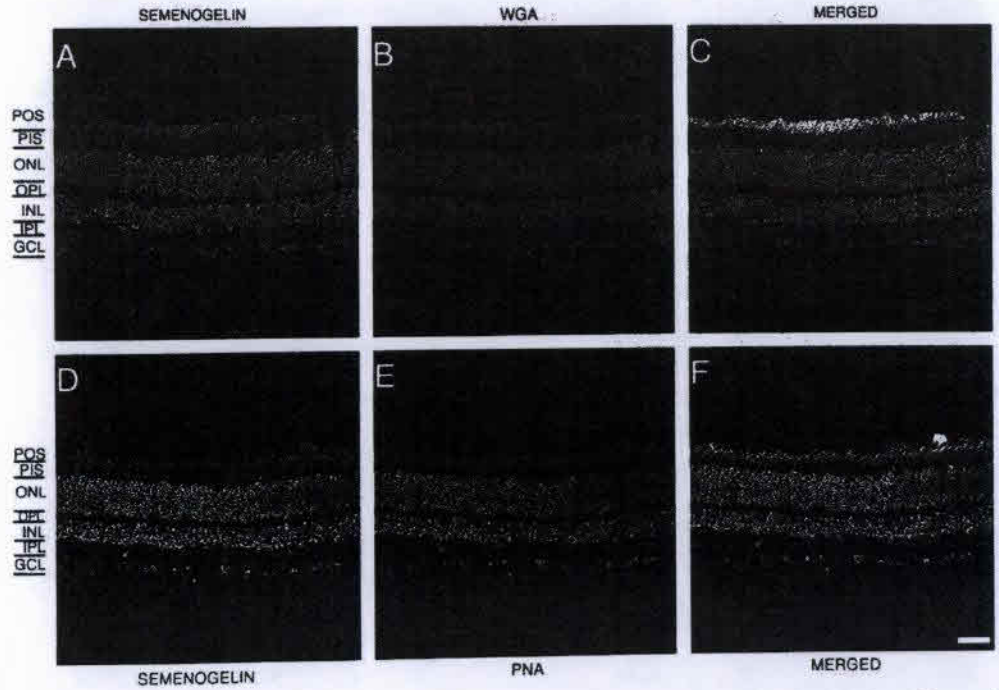

Fig. 4. Higher levels of colocalization of Sgs with rods than with cones in the interphotoreceptor-matrix (IPM). Cryosections (12 μm) of human donors were labeled with antibodies specific to SgI (A and D) and with the lectins WGA (B) and PNA (E). Cell nuclei were labeled with TO-PRO-3 (blue). Overlaid images are shown in C and F. Sections were analyzed using a Leica laser scanning confocal microscope (TCS-SP2, Leica, Exton, PA). A series of 1 μm *xy* (en face) sections was collected. Each individual *xy* image of the retinas stained represents a three-dimensional projection of the entire cryosection (sum of all images in the stack). Comparison of the samples showed that semenogelin colocalizes with WGA but not with PNA. Bar = 40 μm.

HA-binding sequences have been well characterized in proteins such as RHAMM, CD44 and SPACRCAN and were determined to be specifically spaced basic amino acids (Chen et al., 2004; Wang et al., 1996; Yang et al., 1994). Analysis of the SgI and SgII sequence does not indicate the presence of any of the traditional HA-binding sequences. However, a heparin-binding site has been identified in Sgs (Malm et al., 1996). Heparin binding domains in molecules such as RHAMM have been located in amino acid segments containing HA binding motifs (Yang et al., 1994). This poses semenogelin proteins as potentially capable of binding HA present in the IPM.

A recent report indicates that both SgI and II bind zinc (Jonsson et al., 2005). Earlier clinical trial data found a significant decrease in the progression of age-related macular degeneration (AMD) in individuals supplemented with antioxidants and zinc (Clemons et al., 2005; Schmidt-Erfurth, 2005). The cellular targets in AMD are the RPE and macular photoreceptors (Penfold et al., 2001). Our observations that Sgs are localized to photoreceptors and the RPE may point to a function related to the ability of these cells to sequester zinc for protection against AMD. In this regard, it would also be interesting to define the distribution of Sgs in the eyes from donors with diagnosed AMD.

In conclusion, we report here the identification and distribution of the semenogelin proteins in the vitreous associated with several cells of the human neural retina.

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