MFG-E8 in the retina and retinal pigment epithelium of rat and mouse

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Purpose: To study the distribution of milk fat globule epidermal growth factor E8 (MFG-E8) in the rodent eye and to investigate a potential role for this molecule in the phagocytosis of photoreceptor outer segments (POS) by the retinal pigment epithelium (RPE).

Methods: We have used immunohistochemistry, in situ hybridization, Northern and Western blotting to demonstrate the presence and distribution of MFG-E8 in the rat and mouse retina. siRNA technology was used to knock down MFG-E8 mRNA and to study the effect of such knockdown on the phagocytosis of POS by the RPE.

Results: We identified a novel long form of this protein (MFG-E8L) in rat tissues, which contains a 56 amino acid insert that is rich in proline and threonine. This is the first demonstration that MFG-E8L is present in a species other than the mouse. Immunohistochemistry and in situ hybridization demonstrate that MFG-E8 is present in the retina and RPE. Northern blotting and PCR show that the short form of MFG-E8 (MFG-E8S) is present in both the retina and RPE, but MFG-E8L is found only in the RPE. Our results do not demonstrate a role for MFG-E8 in POS phagocytosis by cultured RPE cells.

Conclusions: In all tissues in which MFG-E8 has been localized, it has been shown to perform an important role in cell-cell binding, or in promoting phagocytosis. The localization of this glycoprotein in the retina and RPE, and particularly the specific localization of MFG-E8L in the RPE, suggests that this molecule may play an important, but as yet unknown role in retinal function.

The circadian shedding of the tips of photoreceptor outer segments (POS), and their engulfment by the adjacent retinal pigment epithelium (RPE) is vital to the survival of the retina. When this process is aberrant, as in the Royal College of Surgeons (RCS) strain of rat, degeneration of the underlying photoreceptor cells occurs [1-4]. The phagocytic defect in the RCS rat has been shown to be due to a mutation in the Merkt gene [5-8], a cell surface receptor tyrosine kinase that plays a key role in initiating the events that result in the ingestion of the shed POS. While Merkt, after activation by its ligands Gas6 and PS [9-12] is responsible for the signaling step(s) leading to POS ingestion, it is unlikely that this receptor is responsible for binding of shed POS to the RPE surface, since the RCS rat RPE in tissue culture binds POS normally, but, as is found in vivo, does not ingest them [13]. A number of candidate molecules have been proposed to fulfill the role of the receptor responsible for binding shed POS, viz. the mannose receptor [14], CD36 [15] and a αβ5 integrin [16-19].

Milk fat globule epidermal growth factor E8, (MFG-E8, PAS 6/7; lactadherin; SED1; BA46; p47; rags) [20] was first isolated from milk fat globule membranes as a 66 kDa glycoprotein. MFG-E8 and its orthologs are heavily glycosylated proteins consisting of an N-terminal signal peptide, two EGF-like repeat domains (EGF1 and EGF2), the second of which contains an RGD-integrin binding motif, followed by two discoidin domains (C1 and C2) found in blood clotting factors V and VIII [21]. The presence of an RGD motif and a discoidin domain in this molecule suggested that it may play a role in adhesion of cell surface phosphatidylserine (PS)-rich apoptotic cells through one or both of the discoidin repeats to integrin molecules on the surface of an acceptor cell [21]. Indeed, studies have shown that MFG-E8 binds to PS coated plates [22,23] and to a αβ3 or a αβ5 integrin expressing cells [24,25]. Akakura et al., [25] have suggested that “MFG-E8, a αβ5 integrin and DOCK180 function as a module to generate Rac1 mediated phagocytosis of apoptotic cells”, thus elevating MFG-E8 to the status of a functional signaling ligand, rather than merely a bridging/binding molecule. Wu, Tibrewal and Birge [26] have recently provided a comprehensive review of the state of the field.

The presence of a long form of MFG-E8 (MFG-E8L), containing a 37 amino acid proline/threonine rich insert between the EGF2 and C1 domains, was first shown in lactating mouse mammary gland [27]. Subsequently MFG-E8L has been found in activated mouse macrophages [23,28] as well as in immature dendritic cells, Langerhans cells of the skin [29] and in epidermal keratinocytes [30]. However, this long form of MFG-E8 has so far been found only in mouse tissue, while the short form (MFG-E8S) is widely distributed.

Hanayama et al. [23,28] and Miyasaka et al. [29] have showed that MFG-E8 facilitated the engulfment of apoptotic thymocytes by macrophages by binding to PS on the apoptotic cell plasma membrane and a αβ3 integrin on the phagocyte.
While both the long and short forms of MFG-E8 could facilitate engulfment, the long form was about 8 times more effective than the short form, on a molar basis [23]. Interestingly, MFG-E8 is secreted by the macrophage itself. Dasgupta and Thiagarajan [31] have shown that the phagocytosis of sickle cell erythrocytes by peripheral macrophages is stimulated in the presence of bovine lactadherin and that phagocytosis is partly inhibited by an antibody to αvβ3 integrin, but not by an antibody to the Fcγ receptor. Presumably it is the short form of MFG-E8 which was active in this study, since MFG-E8L has not been found in bovine mammary gland. Nakatani et al. [32] recently showed that MFG-E8 is upregulated in involuting mouse mammary glands, at the time when the glands undergo a substantial increase in the rate of epithelial cell apoptosis. This MFG-E8 was present in membrane vesicle fractions, rather than in milk fat globule fractions. Ensslin et al. [29] have shown that SED1 is secreted by cells in the initial segment of the epididymis and binds to the sperm plasma membrane overlying the acrosome, where it plays a role in the binding of the sperm to proteins on the zonal pellucida of unfertilized oocytes. Interestingly, this binding does not involve the RGD motif of SED1.

Since RPE cells are highly phagocytic, and use some of the same phagocytic pathways used by macrophages (viz Merkt and Gas6/PS), we have investigated whether MFG-E8 is present in the retina and/or RPE, and whether this molecule may play a similar role in RPE cells during the phagocytosis of shed POS, as it does in the phagocytosis of apoptotic cells by macrophages and other phagocytic cells.

METHODS

Isolation and growth of retinal pigment epithelium cells: Pigmented retinal dystrophy rats (rdy/rdy, p+, p+; RCS), (UCSF) and normal pigmented Long Evans (LE) rats were maintained on a 12 h light:12 h dark cycle. Postnatal day 12-15 rats were sacrificed by CO₂ inhalation and the eyes were removed. RPE cells were isolated as previously described [35] and seeded on 13 mm glass coverslips at a density of 22,000 cells per 75 cm². The care and treatment of animals adhered to the NIH Guide for the Care and Use of Laboratory Animals and to the UCLA Animal Research Committee Policies and Procedures.

Isolation and feeding of photoreceptor outer segments: POS were isolated by sucrose density gradient centrifugation of homogenized retinas from rats older than 30 days [13]. The final pellet was suspended in GM at a concentration of 10⁷ POS/ml. Confluent monolayers of RPE cells on glass coverslips were transferred to individual wells of 24 well tissue culture plates. A 0.5 ml aliquot of the POS suspension was added to each well and incubated for 1 h at 37 °C in 95% air, 5% CO₂.

Double immunofluorescence labeling and quantitation of photoreceptor outer segments phagocytosis: After 1 h of incubation with POS, coverslips were washed twice by vigorous agitation in PBS containing 1 mM CaCl₂ and 1 mM MgSO₄ to remove unbound POS and fixed in 3.7% formaldehyde in PBS, pH 7.4. Bound POS were stained with rabbit anti-bovine POS antisera and FITC-labeled secondary antibody. After cell permeabilization with 47.5% ethanol, ingested POS were stained with rabbit anti-bovine POS antibody and Texas Red-labeled secondary antibody. Bound and ingested POS were quantitated as previously described [13,36,37]. POS were counted at 360x magnification using a 1 cm² (10x10) ocular grid. Each treatment was done in duplicate and at least 5 grid areas were counted on each coverslip. Each experiment was repeated at least twice. Results are expressed as the mean of the pooled data, plus or minus the SD.

Antibodies, SDS-PAGE, Western blotting, and immunodetection: An antisera (204) to a common C-terminal peptide sequence of rat and mouse MFG-E8 (NSHKKNIFEKPF) conjugated to KLH was generated in rabbits (In vitrogen, Carlsbad, CA). An IgG fraction was prepared by adsorption of the antisera to a protein A column (Amersham-Pharmacia, Piscataway, NY) and elution with 0.1 M citric acid, pH 3.0. A peptide affinity column was prepared by coupling the peptide antigen to an agarose matrix using the AminoLink Plus Immobilization Kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. MFG-E8 specific antibody was purified by adsorption of the IgG fraction to the peptide column and elution with 0.1 M glycine-HCl, pH 2.75. The eluted antibody (204AP) was immediately neutralized and dialyzed against PBS, pH 7.4. The IgG concentration of the MFG-specific antibody was determined, assuming an E₂₈₀ of 1.35. Monoclonal antibody 18A2-G10 to mouse MFG-E8 was generated in hamster [29].

Freshly isolated and cultured rat RPE cells, freshly isolated rat and mouse retina were solubilized in RIPA buffer for 60 min on ice. Insoluble material was removed by centrifugation at 16 000 x g for 30 min at 4 °C. The protein concentration of the clarified supernatant was determined using the MicroBCA kit (Pierce). 50 μg of each tissue were separated on 7.5% SDS-minigels (BioRad, Hercules, CA) and transblotted [9]. The presence of MFG-E8 was detected using the two antibodies listed above. Gels were stripped for 30 min at 55 °C in 62.5 mM Tris, pH 6.7, 2% SDS, 100 mM βME between probing with different antibodies.

Secondary antibodies used were HRP conjugated rabbit anti hamster IgG (ICN Biomedicals, Aurora, OH) and HRP conjugated donkey anti-rabbit IgG, (Amersham Pharmacia), both at a dilution of 1:10 000. Immunoreactive bands were visualized using a chemiluminescent detection system (Amersham Pharmacia). For surface staining of cultured RPE cells, we used antibody 204AP followed by biotinylated goat anti rabbit IgG (1:50) and fluorescein labeled streptavidin (1:100) both from Amersham-Pharmacia.

Identification of short- and long-forms of MFG-E8: RNA isolated from Long Evans rat testis, retina, RPE (fresh and cultured), brain, and liver and from mouse retina was reverse transcribed to cDNA, amplified by PCR and analyzed by agarose electrophoresis. Two PCR products were observed in the PCR fragments, indicating the presence of both the long and short forms of MFG-E8.
transcribed to produce cDNA using oligo dT Superscript First Strand Kit (Invitrogen). Based on the rat MFG-E8 sequence [38] (Genbank accession number D84068), the following synthetic oligonucleotide primers were designed to encompass the full length coding region of the sequence starting at base 39 and extending to base 1366 (sense: 5'-AGC ATG CAG TTC TCC CGT GTG-3', Primer 1, base 39; antisense: 5'-CCT CTG GCC GTC CTC GTC ACT TG-3', Primer 2, base 1366). PCR was conducted using a Robocycler (Stratagene, La Jolla, CA) for 35 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. Depending on the starting tissue, one or two bands were produced. The two bands obtained from rat testis cDNA (1328 bp and 1496 bp) were excised. Each product was subcloned into pCR 2.1 TOPO (Invitrogen). Based on the rat MFG-E8 sequence (bases 864-1366). Primers used to generate this probe were:

- sense: 5'-CCC CTG GGC CTG AAG AA T AAC AC-3' (Primer 6, base 1366).
- antisense: 5'-CCT CTG GCC GTC CTC GTC ACT TG-3', Primer 2, base 1366).

The expected products from the short and long forms of MFG-E8 are 102 and 270 bp, respectively.

**MFG-E8 riboprobes:** Three Digoxygenin (dig)-labeled riboprobes for Northern analysis were synthesized from rat testis first strand cDNA. The first probe was a 503 base probe for the 3′-region common to MFG-E8 long and short forms (bases 864-1366). Primers used to generate this probe were:

- sense: 5'-GGG CTA CTC GGG CAT CCA CT-3', Primer 3, base 341; antisense: 5'-ATA TAC ACA GAC GAG GCA GAA TTC-3', Primer 4, base 442.

These primers, which span the splice region containing the long form insert, are situated in the third and fifth exons. The expected products from the short and long forms of MFG-E8 are 102 and 270 bp, respectively.

To total RNA was isolated from Long Evans rat testis, retina, RPE (fresh and cultured), brain, liver and mouse retina using Tri Reagent (MRC, Inc., Cincinnati, OH) and further purified using an RNeasy MiniElute Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA was quantitated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). 15 µg (retina, testis, liver, brain), 30 µg (freshly isolated RPE), or 2 µg (cultured RPE) of total RNA were separated on 1% agarose-formaldehyde gels and transferred to Hybond-N+ nylon membrane (Amersham Pharmacia) by downward transfer. After UV irradiation, membranes were prehybridized for 2 h and hybridized overnight in Ultrasahv (Ambion, Austin, TX) containing 100 ng/ml of either the MFG-E8 common (503 bases), MFG-E8L (270 bases) or MFG-E8S (102 bases) dig-labeled probe. Bands were visualized using anti-digoxigenin-AP (Roche) and Duolux chemiluminescent substrate (Vector Laboratories, Burlingame, CA).

**In situ hybridization:** In situ hybridization was carried out according to Braissant and Wahl [39] with the exception that hybridization and washing were performed at 47 °C. All reagents were purchased from Roche. Detection of MFG-E8 mRNA was carried out using the Dig-labeled 503 base probe that recognizes the c-terminal sequence common to both long and short forms of MFG-E8 mRNA. Eyes were removed from adult Wistar rats and BALB-C mice. The anterior segment and lens were removed and the eyecups were fixed in 4% formaldehyde in PBS, pH 7.4 for 6 h at 4 °C. Eyecups were cryoprotected in 30% sucrose/PBS at 4 °C, embedded in OCT (Sakura Finetek USA, Torrance, CA) and stored at -80 °C. Frozen sections were cut at 12 µm and used immediately. Sections were mounted in Vectashield Mounting Medium (Vector Laboratories).

**Immunohistochemistry:** Frozen sections were cut from OCT-embedded albino rat and mouse eyes, dried for 15 min at 37 °C, washed 2x15 min in PBS, permeabilized for 10 min in PBS/0.25% Triton X-100 (PBST) and blocked for 1 h at RT in blocking buffer (PBST+10% normal goat serum). Sections were incubated for 1 h at RT with primary antibody 204 AP at 2 µg/ml in PBST+3% normal goat serum, washed 3x10 min in PBS/0.1% Tween 20 and incubated for 1 h in the dark at RT with Alexa 488-labeled goat anti rabbit IgG (1:1000, Molecular Probes, Eugene OR). Since 18A2 is a hamster monoclonal antibody, after incubating with this antibody at 1:100 dilution, the sections were washed 3 x 10 min in PBS/0.1% Tween 20 and incubated for 1 h with a rabbit anti-hamster antibody (1:100), washed and incubated with Alexa 488 labeled antibody as above. After washing 3 x 10 min in PBS/0.1% Tween 20, slides were mounted in Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories), and examined under epifluorescence using a Nikon Eclipse E 8000 microscope.

**siRNA experiments:** All siRNA reagents were purchased from Dharmacon Inc., (Lafayette, CO). Long Evans RPE cells were grown on 13 mm (for POS phagocytosis) or 18 mm (for RNA isolation) disks as described above. Four days after seeding, the disks were transferred to antibiotic-free GM and transfected with siRNA SMARTpool reagent to rat MFG-E8 using Dharmafect 1 transfection reagent. Controls consisted of non-transfected cells, cells transfected with Dharmafect 1 only (mock transfection), cells transfected with siGlo cyclophilin and transfected with siRNA SMARTpool reagent to rat MFG-E8 using Dharmafect 1 transfection reagent. Controls consisted of non-transfected cells, cells transfected with Dharmafect 1 only (mock transfection), cells transfected with siGlo cyclophilin.
B siRNA or with an siControl Non-Targeting siRNA Pool. After 48 h, 72 h, and 96 h, the transfected cells were washed free of the transfection medium and fed POS for 1 h in complete GM. Bound and ingested POS were stained and quantitated as described above. Total RNA was isolated from duplicate 18 mm disks treated with siRNA as above. The integrity of the RNA was ascertained by running 1 µg on a 1% agarose/formaldehyde gel and staining with ethidium bromide. Each treatment was done in duplicate and each experiment was repeated at least three times. In every experiment, a separate disk was transfected with siGlo cyclophilin B siRNA. This disk, which was not fed POS, was fixed for 1 h with 4% formaldehyde in PBS and examined under epifluorescence using a rhodamine filter set to qualitatively ascertain the efficiency of transfection.

Surface staining of cultured RPE cells: RPE cells were grown on glass disks and transfected for 48 h, 72 h, and 96 h with MFG-siRNA, or were mock transfected as described above. Control cells were untreated. Disks were washed in PBS and fixed for 30 min in 4% formaldehyde at RT. All disks were blocked in PBS/1% BSA and incubated for 1 h with 2 µg/ml of antibody 204AP in PBS/1% BSA, washed 3x10 min in PBS/0.1% BSA and incubated with biotinylated donkey anti-rabbit IgG (1:100, Amersham) for 30 min at RT. After washing 3x10 min with PBS/0.1% BSA, disks were reacted with fluorescein labeled streptavidin (1:50, Amersham) for 1 h. Control disks eliminated the primary antibody. Disks were mounted in Vectashield Mounting Medium for Fluorescence (Vector Laboratories) and photographed under epifluorescence illumination.

Treatment of isolated photoreceptor cells with antibody 204AP: If MFG-E8 functions as a ligand for the binding of POS to RPE cells, it should be possible to inhibit such binding with an MFG-E8 specific antibody, such as 204AP. Since RCS-RPE cells are able to bind POS, but show very little ingestion, we used these cells to determine whether this antibody was able to inhibit POS binding, and also to see if the antibody stained isolated POS, thus indicating the presence of MFG-E8 on these organelles.

Outer segments were isolated as described above and fed to RCS-RPE cells for 1 h at RT. After washing and fixing as described above, the disks, with attached POS, were stained with antibody 204AP, followed by biotinylated secondary antibody and fluorescein streptavidin.

In order to determine whether antibody 204AP inhibits binding of POS to RPE cells, outer segments were isolated as described above and incubated for 30 min at RT with 5 µg/ml of antibody 204AP in GM. The POS were fed to RCS RPE cells growing on glass disks in the presence of the antibody for 1 h at RT. Bound POS were stained and quantitated as described above.

Quantitative PCR: Real-time PCR was performed using Full Velocity SYBR Green QPCR Master Mix (Stratagene) containing cDNA transcribed from 33 ng of total RNA and a primer concentration of 100 nM. Primers 3 and 4 were used for specific MFG-E8 quantitation. Cyclophilin B primers (sense, 5'-ATC GTG GGC TCC GTT GTC TTC CTT-3' and antisense, 5'-CAT CCG GGC CCA TAG TGC TTC AG-3') were used as a control for template content and off-target interference. All reactions were prepared in triplicate in a 25 µl volume and were run and analyzed in an Mx3000P cycler (Stratagene) according to the manufacturer’s instructions. Mx3000P analysis software was used to evaluate Ct relative to control samples in order to determine relative knockdown of MFG-E8 message concentration.

RESULTS

MFG-E8 long and short forms are present in rat tissues: PCR: Although MFG-E8S has been shown to be present in a number of tissues (boar, bovine, mouse, rat, human) [20] the long form of MFG-E8 has been shown only in mouse mammary glands [27] macrophages [23] and epidermal keratinocytes [30]. In the current experiments, PCR of cDNA prepared from rat testis RNA, using

![Figure 1. Rat tissues produce both MFG-E8L and MFG-E8S. A: PCR of rat testis cDNA using MFG-E8-specific primers (1 and 2) produces two bands of 1328 bp and 1496 bp. B: PCR of rat tissue cDNA using nested primers (3 and 4) designed to span the region of the long form insert, produces two bands of 270 bp and 102 bp. Ms represents mouse; L represents liver; B represents brain; TC represents tissue culture; T represents testis.](image-url)
primers designed to amplify the full length short form of rat MFG-E8, resulted in the production of two bands of 1328 bp and 1496 bp (Figure 1A). Subsequent PCR amplification of cDNA from rat testis, brain and RPE (fresh and cultured), using stepped in primers 3 and 4 (situated in exons 3 and 5, see Figure 2A), resulted in two bands of 102 bp and 270 bp (Figure 1B). The 270 bp product was absent in retina and present in only small amounts in liver. In all of these tissues, the 102 bp product predominates. Sequencing of this 102 bp product yielded the expected sequence for rat MFG-E8 [38]. Sequencing of the 270 bp transcript revealed a 168 bp splice variant with the sequence shown in Figure 2B inserted after base 370 of the rat MFG short form sequence. Translation of this nucleotide sequence results in a 56 amino acid insert that is rich in proline and threonine (Figure 2C). This insert is similar to that found in mouse MFG-E8L, but is longer by 19 amino acids.
acids and contains six AV/IPPT/P motifs, as compared to 2 such motifs in mouse MFG-E8L (Figure 2C). Additionally the rat insert contains 41% proline (10 residues) plus threonine (13 residues), as compared to 30% (5 proline and 6 threonine residues) in the mouse. Identical results were obtained from rat RPE cDNA (data not shown). Examination of the published nucleotide sequence of rat chromosome 1, on which the MFG-E8 gene is located, gives the intron/exon pattern for this gene shown in Figure 2A, where exon 4 codes for the 168 bp insert. Alternative splicing of this gene is responsible for generating the long and short forms of this protein. The complete mRNA sequence for rat MFG-E8L has been submitted (Genbank accession number DQ455823).

Northern blotting: Using a 503 base dig-labeled RNA probe to the c-terminal end of rat MFG-E8S, we detected a broad band of mRNA of 2.1-2.5 kilobases on Northern blots of various rat tissues (data not shown). In order to determine whether specific mRNA’s exist for the long and short forms of MFG-E8, we designed dig-labeled RNA probes (102 and 270 bases) which would span the inserted sequence. Using these probes, we were able to distinguish mRNA transcripts for both the short form (2.2 kilobases) and long form (2.4 kilobases) of MFG-E8 on Northern blots (Figure 3). The short form of MFG-E8 is present in fresh and cultured RPE, retina, brain, liver and testis (Figure 3A). However the long form is present only in fresh and cultured RPE and testis (Figure 3B, lanes 1, 2 and 5). The 270 base dig-long probe showed some affinity for the short form of MFG-E8 mRNA as evidenced by the presence of a 2.2 kilobase band in RNA from rat and mouse retina (Figure 3B, lanes 3 and 4). In both testis and fresh RPE, the short form mRNA predominates. When RPE cells are grown in tissue culture, both long and short form mRNA’s are significantly upregulated.

Western blotting: Immunoblotting of RIPA extracts of rat and mouse retina, and freshly isolated rat RPE, showed only the short form of MFG-E8. Surprisingly the size of MFG-E8 (76 kDa) in fresh RPE is greater than that in fresh retina (67 kDa; Figure 3C). Since retina does not contain the message for the long form of MFG-E8, we conclude that the protein seen in this tissue is MFG-E8S. While the 76 kDa form seen in fresh RPE might represent the long form, we do not consider this likely since Northern blots of fresh RPE contained only very small amounts of the long form mRNA and much larger amounts of the short form mRNA (see Figure 3A).

Immunohistochemistry: Immunohistochemical localization of MFG-E8 has been studied in both mouse and rat eyes. While the results were qualitatively similar, mouse eye sections gave more intense staining using two different antibodies, and are thus reported here. Since these antibodies gave slightly different staining patterns, results are reported according to the antibody used.

204AP: This affinity purified antibody to a c-terminal peptide that is common to rat and mouse MFG-E8 gave intense staining of the inner segment layer of photoreceptor cells and lighter staining of the RPE cells, with slightly heavier staining of the basolateral surfaces (Figure 4A). The inner plexiform layer and the outer layer of the OPL were also stained.

Light staining of the nerve fiber layer was observed.

18A2: Which is a monoclonal antibody to mouse rMFG-E8, also stained the photoreceptor inner segment region, but with a punctate pattern, as opposed to the uniform staining of the inner segment layer that is seen with antibody 204AP. Interestingly, the staining appears to be localized to the junction between the inner and outer segments (Figure 4B). Additionally, 18A2 gave light staining of the Müller cell end feet.

Since both PCR and Northern blotting clearly showed that only the short form of MFG is present in the neural retina, the staining observed with these two antibodies must represent the distribution of MFG-E8S in the retina. It is also likely that the staining observed in the RPE is primarily due to MFG-E8S, since the mRNA for this form of MFG greatly predominates and we have been able to show only a single band on Western blots of extracts of freshly isolated RPE, corresponding to MFG-E8S.

Staining of cultured retinal pigment epithelium cells: Cells stained with antibody 204AP and Alexa 488 secondary antibody gave discrete but light staining of the cell surface. In order to amplify the staining intensity we utilized a sandwich technique with a biotinylated second antibody, followed by fluorescein-labeled streptavidin. Cultured rat RPE cells, stained by this method, showed a punctate staining of the entire cell surface (Figure 5). This pattern suggests that it may be microvilli that are stained. An identical staining pattern was seen with LE or RCS RPE cells (data not shown). No staining of any structures is seen when the primary antibody is eliminated (Figure 5C).

In situ hybridization: In agreement with the above immunohistochemical results, a 503 base dig-labeled RNA antisense probe common to both the long and short forms of rat and mouse MFG-E8, showed robust staining of the photoreceptor inner segments and of rat RPE (Figure 6B). The outer segments were completely devoid of staining. Cell bodies of the INL showed moderate staining, with some cells adjacent to the inner and outer plexiform layers showing more intense staining (Figure 6B arrowheads). It may be relevant that the entire OPL, and the outermost layer of the IPL are stained with the MFG-E8 antibody 204AP (see Figure 4B). MFG-E8 has been shown to be secreted by some cells, and it is possible that horizontal cells and amacrine cells, whose cell bodies are located in these areas, secrete MFG-E8 into the inner and outer plexiform layers, where it performs some as yet unknown function. The perikarya of some ganglion cells are intensely stained with the riboprobe, while others show no staining at all. Interestingly, ganglion cell bodies show no staining with antibodies 204AP or 18A2; however some staining of the nerve fiber layer/Muller cell end feet is seen with these antibodies (see Figure 4), which may be due to MFG-E8 that is synthesized and secreted by the ganglion cells. A sense probe showed no staining of any retinal cells or retinal layers (Figure 6A). Mouse eyes showed an identical staining pattern with the riboprobe (data not shown).

Antibody 204AP does not inhibit POS binding to RCS-RPE cells: In order to determine whether MFG-E8 specific antibody 204AP was able to bind to POS and to inhibit bind-
Figure 4. Immunofluorescence staining of MFG-E8 in the mouse eye. Immunofluorescence staining of MFG-E8 in mouse eyes using antibodies 204AP (A, B, C), and 18A2 (D, E, F). A represents brightfield, B shows 204AP staining, C shows DAPI staining of the same section. In the absence of primary antibody, no staining was observed (not shown). D is brightfield, E is 18A2 staining, and F is DAPI staining of the same section. G shows stains with no primary antibody and H is DAPI staining of G. In both sections shown, the POS layer has torn with the result that a thin layer of POS is attached to the apical surface of the RPE. Original magnification = 40x.

Figure 5. Immunofluorescence staining of cultured rat RPE cells with antibody 204AP. (A) shows staining with 204AP, (B) is brightfield, and (C) is staining with no primary antibody. Original magnification equals to 40x.
ing to cultured RPE cells, POS were preincubated with the antibody and fed to cultured RCS-RPE cells in the presence of the antibody. However, POS binding was not reduced by this treatment, nor were the POS stained by the antibody (data not shown). This result is in agreement with the complete lack of staining of POS by antibodies 204AP and 18A2 (see Figure 4).

**RNA knockdown and POS phagocytosis:** Treatment of cultured rat RPE cells with an siRNA pool to rat MFG-E8 resulted in an 85% decrease in the amount of MFG-E8 mRNA within 48 h; continued incubation for 72 or 96 h did not further decrease the amount of specific mRNA (Figure 7A). Expression of Cyclophilin B message was unaffected by treatment with the siRNA pool to MFG-E8, supporting the specific targeting of MFG-E8 mRNA by the siRNA pool (data not shown). Mock transfected cells, or cells incubated with a non-targeting siRNA pool showed no significant increase in MFG-E8 mRNA. Despite the almost complete depletion of MFG-E8 specific mRNA, POS phagocytosis was unaffected by this treatment (Figure 7B).

Immunoblots of extracts of cultured RPE cells (either untreated or siRNA treated) showed the presence of a major band with a MW of 76 kDa (Figure 7C), corresponding to MFG-E8 found in fresh RPE cells. In addition, a smaller amount of a band with a MW of 67 kDa is seen, which corresponds to the major MFG-E8S band found in the retina, as well as a band with a MW of 58 kDa. None of these bands are affected by siRNA treatment. However, untreated cultured cells also show a major band with a MW of 90 kDa, which is almost completely eliminated after siRNA treatment for 96 h.

Thus it appears from these results, that MFG-E8 (short or long form) does not play a role in POS phagocytosis by cultured RPE cells. It is however, possible that RPE cells over very slowly in these cultured RPE cells, and that sufficient MFG-E8S may remain on the cell surface after the above treatment with siRNA to fulfill a potential role in the binding of POS to the RPE. The efficiency of transfection, as measured by the uptake of fluorescent siGlo-labeled cyclophilin-specific siRNA, was close to 100%. All of the transfected RPE cells show a uniform fluorescence due to the siGlo, with the majority of cells also showing brightly staining inclusions which may represent siGlo-cyclophilin siRNA that has been taken up into lysosomes (Figure 7D).

**DISCUSSION**

In the experiments reported here, we have investigated a possible role for MFG-E8 in the binding of POS to the surface of cultured RPE cells. However our results do not support such a role.

We have shown that the short form of MFG-E8 is present in rat RPE and retina, as well as in testis, brain and liver. However, the long form of this protein is present only in RPE and testis. This is the first demonstration that MFG-E8L is present in any species other than the mouse. Additionally, the insert in rat MFG-E8L is 19 amino acids longer than the insert found in mouse MFG-E8L, while the short forms of the proteins show 94% amino acid identity.

Immunohistochemical staining, using two different, but highly specific antibodies to MFG-E8 showed that it is present in the RPE and the inner segments of the POS. While antibody 204AP showed uniform staining of the inner segments, antibody 18A2 displayed a punctate staining localized between the inner and outers segments. This punctate staining pattern, and its localization, suggests that it might represent MFG-E8 that has been secreted from the inner segments and is localized in the IPM. MFG-E8 has been shown to be secreted as exosomes in other cell types [23,29].

Because MFG-E8 contains both a PS-binding motif, as well as an RGD motif, it has been thought to function as an opsonin in the phagocytosis of apoptotic cells. Indeed, a number of studies have showed that MFG-E8, in conjunction with either αvβ3 or αvβ5 integrins on the phagocyte surface, promotes the ingestion of apoptotic lymphocytes [23,25,29] and sickle red blood cells [31]. Ennslin and Shur [33] have demonstrated that SED1, a homolog of MFG-E8, which is secreted by the cells of the caput epididymis, is required for the binding of sperm to the zona pellucida of unfertilized oocytes, but this binding is not RGD-dependent. Thus the bridging function of MFG-E8 has been shown to occur by two different mechanisms.

By analogy with mouse macrophages, we show that cultured rat RPE cells produce both the long and short forms of MFG-E8. Histochemical staining of cultured rat RPE cells using an affinity purified antibody showed that MFG-E8 is present on the cell surface, where it might be expected to be available to interact with PS on the surface of shed POS. MFG-E8 is not present on POS, nor does the MFG-E8 specific antibody 204AP inhibit POS binding. Treatment of cultured RPE cells with an siRNA pool that resulted in an 85% reduction in MFG-E8 specific mRNA, failed to reduce POS phagocytosis or to deplete the cells of MFG-E8S. Such treatment did, however, significantly reduce the amount of a 90 kDa band, which may be MFG-E8L, but again without reducing POS phagocytosis. As observed on both Northern and Western blots, cultured RPE cells produce increased amounts of MFG-E8 long and short form message and protein. In fact we have been unable to detect MFG-E8L protein in fresh RPE cells, but it is present in moderate amounts in cultured RPE cells.

Immunostaining of sectioned eyes and cultured RPE cells gives what appears to be conflicting results. Fresh sectioned material shows uniform staining of the RPE cell layer with some slight increase in staining of the basal membrane, but with no apparent staining of the apical surface or microvilli of these cells (Figure 4). By contrast, cultured RPE cells show punctate staining of the cell surface, which we attribute to staining of the microvilli (Figure 5). As shown in Figure 7C, MFG-E8 is greatly upregulated in cultured RPE cells with a number of isoforms that are not seen in fresh RPE (Figure 3E). It is possible that one or more of these isoforms is targeted to the plasma membrane in cultured cells. Nakatani et al. [32] have shown that MFG-E8 is produced and targeted to a membrane fraction at a time when the mammary glands are undergoing involution and when there is a substantial increase in the rate of epithelial cell apoptosis. This suggests that MFG-E8 might...
only be produced when it is needed, and is targeted to an appropriate compartment, such as the plasma membrane.

Of interest is the pattern of MFG-E8 reactive bands obtained on growing RPE cells in tissue culture. In addition to the major 67 kDa MFG-E8S band that is found in fresh tissues (see Figure 3C), a 74 kDa band and a 58 kDa band are observed (Figure 7C). Due to the high specificity of the affinity purified 204AP antibody, we consider it very probable that

Figure 6. In situ hybridization staining for MFG-E8 in the rat eye. In situ hybridization staining of rat eye with sense (A) and antisense (B) dig-labeled 503 base RNA probes to MFG-E8. The RPE, photoreceptor cell inner segments (IS) and some ganglion cells (GCL) are heavily stained. Cells on the inner and outer layers of the INL also show staining (arrowheads). Outer segments are present in (B) but show no staining with the riboprobe. Original magnification equals to 40x.

Figure 7. The effect of siRNA treatment on POS phagocytosis by cultured rat RPE cells. A: MFG-E8 mRNA knockdown by treatment with: (a) control, (b) mock transfection (transfection reagent only), (c) non-targeting siRNA, (d) MFG-E8-specific targeting siRNA for 48 h, (e) 72 h, (f) 96 h. B: POS phagocytosis by cultured RPE cells after treatment with: (a) control, (b) mock transfection, (c) non-targeting siRNA, (d) 48 h, (e) 72 h, (f) 96 h incubation with MFG-E8-specific targeting siRNA. C: Western blot of RIPA extracts of cultured RPE cells after (a) no treatment, (b) 96 h MFG-E8-specific targeting siRNA. D, E: Fluorescence image of cultured RPE cells transfected for 96 h with siGlo-labeled cyclophilin siRNA; Panel E is a brightfield image of D. Original magnification 20x.
all of these bands represent MFG-E8. While the 74 kDa band has the MW attributed to MFG-E8L found in mouse tissue [28] we consider it likely that this and the 58 kDa band represent differentially glycosylated forms of MFG-E8S that are accumulated intracellularly in vitro, but not in vivo. As mentioned above, the messages for both the long and short forms are greatly upregulated in cultured RPE cells, and this is reflected in the amount of a putative long form protein (90 kDa) that is produced by these cells and which is sensitive to knockdown by siRNA treatment. Because of the number of proline and threonine residues in the insert found in rat MFG-E8L, this form has the potential for heavy glycosylation, and thus a significant increase in MW over MFG-E8S. Differentially glycosylated forms of MFG-E8 have previously been shown [27].

In support of a potential role for MFG-E8 in POS phagocytosis is the observation that oxidized PS “represents a significantly more favorable site for MFG-E8 interaction with lipid bilayers as compared to other phospholipids” [24], coupled with the report [40] that “retinal lipids recovered from dark adapted rats following physiological light exposure demonstrate in vivo formation of specific oxidized phosphatidylcholine (PC) molecular species”. Although Sun et al. [40] suggest that oxidized PC may function as a ligand for CD36, it is possible that other molecular species, such as PS are also subject to light-induced oxidation in the retina, and may serve as potent ligands for MFG-E8.

Another potential role for MFG-E8 is to aid in the adherence of the retina to the RPE through interaction with αvβ5 integrin on the apical surface of the RPE [16]. Nandrot et al. [41] have shown that retinal adhesion is markedly reduced in β5−/− mice and suggest that the “IPM ensheathing apical RPE microvilli contains ligand proteins for αvβ5 integrin that remain to be identified.” MFG-E8 is a strong candidate for one of these ligands, based on its demonstrated binding to this integrin and to PS.

Although the studies reported here do not demonstrate a role for MFG-E8 in POS phagocytosis, the localization of this molecule in the RPE and retina, and the preponderance of evidence from numerous studies showing its function in supporting/mediating the phagocytosis of apoptotic cells by macrophages and immature dendritic cells, and its role in the binding of sperm to proteins in the zona pellucida of the oocyte, suggest that it could function in a similar role in the RPE. Additionally, the specific localization of MFG-E8L to the RPE, suggests that this macromolecule may perform a crucial function in this tissue, and thus may be a target for disease if mutated. The MFG-E8 gene has been mapped in mouse (chr 7D2) rat (chr 1q31), human (chr 15q25) and dog (chr 3, locus not determined). It will be interesting to determine whether any known retinal degenerations map to these chromosomal locations. The future availability of an MFG null mouse may demonstrate whether lack of this gene leads to retinal degeneration, or to more subtle retinal abnormalities.

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