Cleavage of Mer Tyrosine Kinase (MerTK) from the Cell Surface Contributes to the Regulation of Retinal Phagocytosis

Ah-Lai Law, Célia Parinot, Jonathan Chatagnon, Basile Gravez, José-Alain Sahel, Shomi S. Bhattacharya, Emeline F. Nandrot

To cite this version:

Ah-Lai Law, Célia Parinot, Jonathan Chatagnon, Basile Gravez, José-Alain Sahel, et al.. Cleavage of Mer Tyrosine Kinase (MerTK) from the Cell Surface Contributes to the Regulation of Retinal Phagocytosis. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2015, 290 (8), pp.4941-4952. 10.1074/jbc.M114.628297. hal-03086745

HAL Id: hal-03086745
https://hal.archives-ouvertes.fr/hal-03086745

Submitted on 22 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Cleavage of Mer Tyrosine Kinase (MerTK) from the Cell Surface Contributes to the Regulation of Retinal Phagocytosis*

Ah-Lai Law1,2, Célia Parinot1,2, Jonathan Chatagnon1, Basile Gravez1, José-Alain Sahel1, Shomi S. Bhattacharya1,2, and Emeline F. Nandrot1,2

From the 1INSERM, U968, 2Sorbonne Universités, UPMC Univ Paris 06, UMR_S968, Institut de la Vision, 3CNRS, UMR_7210, 17 Rue Moreau, Paris, F-75012, France, the 1Centre Hospitalier National d’Ophthalmologie des Quinze-Vingts, INSERM-Direction de l’Hôpitalisation et de l’Offre de Soins Centre d’Investigation Clinique 1423, Paris, F-75012, France, the 2**Institute of Ophthalmology, University College London, WC1E 6BT London, United Kingdom, and the Andalusian Center of Molecular Biology and Regenerative Medicine (CABIMER), 41092 Sevilla, Spain

Phagocytosis of apoptotic cells by macrophages and spent photoreceptor outer segments (POS) by retinal pigment epithelial (RPE) cells requires several proteins, including MerTK receptors and associated Gas6 and protein S ligands. In the retina, POS phagocytosis is rhythmic, and MerTK is activated promptly after light onset via the αvβ5 integrin receptor and its ligand MFG-E8, thus generating a phagocytic peak. The phagocytic burst is limited in time, suggesting a down-regulation mechanism that limits its duration. Our previous data showed that MerTK helps control POS binding of integrin receptors at the RPE cell surface as a negative feedback loop. Our present results show that a soluble form of MerTK (sMerTK) is released in the conditioned media of RPE-J cells during phagocytosis and in the interphotoreceptor matrix of the mouse retina during the morning phagocytic peak. In contrast to macrophages, the two cognate MerTK ligands have an opposite effect on phagocytosis and sMerTK release, whereas the integrin ligand MFG-E8 markedly increases both phagocytosis and sMerTK levels. sMerTK acts as a decoy receptor blocking the effect of both MerTK ligands. Interestingly, stimulation of sMerTK release decreases POS binding. Conversely, blocking MerTK cleavage increased mostly POS binding by RPE cells. Therefore, our data suggest that MerTK cleavage contributes to the acute regulation of RPE phagocytosis by limiting POS binding to the cell surface.

Phagocytosis is a general process that permits the elimination of apoptotic cells and foreign bodies by professional phagocytes such as macrophages (1). Other efficient phagocytes exist in the body, including retinal pigment epithelial (RPE)3 cells that lie adjacent to photoreceptors at the back of the eye (2). Constant light illumination of photosensitive outer segment of photoreceptor cells for vision generates oxidative damage. In parallel, photoreceptors continuously renew their outer segments and shed their aged tips (POS), which are then phagocytosed daily by neighboring RPE cells (3, 4). This circadian-regulated process is indispensable for vision (5, 6), and the synchronized internalization of POS is dependent on the activation of cell surface receptors and associated downstream signaling molecules.

RPE phagocytosis uses many of the molecular components employed by macrophages to clear apoptotic cells. Specific recognition of cells to be eliminated is triggered by the exposure of phosphatidylinerseines (PS) on the external membrane leaflet (7–9). Normal RPE cells specifically bind and internalize PS-containing liposomes (10). Milk fat globule-EGF factor 8 protein (MFG-E8), a soluble bridging molecule secreted by macrophages and dendritic cells, links the PS-containing apoptotic cells to αvβ3 and αvβ5 integrin receptors via its RGD motif (11). MFG-E8 is secreted by RPE cells (12) and mediates PS binding to αvβ5 integrin receptors that are uniquely expressed on the apical surface of RPE cells (13). The αvβ5 integrin-MFG-E8 couple is essential for the daily burst of phagocytosis observed 1–2 h after light onset as shown by knock-out animal models (12, 14).

Another series of soluble ligands able to bind PS for apoptotic cell elimination are Gas6 and protein S (15, 16), vitamin K-dependent proteins that are common ligands for the TAM (Tyro3, Axl, and MerTK) family of tyrosine kinase receptors (17). Both ligands can independently activate MerTK in macrophages and RPE cells (18, 19) and stimulate POS phagocytosis by RPE cells in vitro (20, 21). Although they circulate in the bloodstream, they are also synthesized in the retina and

* This work was supported by Fondation Voir et Entendre and Fondation Bettencourt Schueller, young investigator grants (to E. F. N.), Agence Nationale de la Recherche, Jeunes Chercheurs/Jeunes Chercheuses Grant ANR-12-JSV1–0003 (to E. F. N.), and Chaire d’Excellence (to S. S. B.), CNRS.

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Institut de la Vision, 17 Rue Moreau, Paris, F-75012, France. Tel.: 33-1-5346-2541; Fax: 33-1-5346-2602; E-mail: emeline.nandrot@inserm.fr.

3 The abbreviations used are: RPE, retinal pigment epithelium; IRBP, interphotoreceptor matrix; POS, photoreceptor outer segment; ANOVA, analysis of variance; CM, conditioned medium; MerTK, soluble MerTK; CHX, cycloheximide; qPCR, quantitative PCR; PS, phosphatidylserine; rMerTK, recombinant mouse MerTK; HBSS, Hanks’ buffered saline solution.
MerTK Cleavage Regulates RPE Phagocytosis

secreted in the interphotoreceptor space (19). Single and double knock-out mice showed that these two ligands can compensate for each other to some extent but are both required for POS phagocytosis (22). Indeed, double knock-out mice recapitulated the fast retinal degeneration phenotype observed in MerTK-deficient animals (23–25). MerTK-deficient animals carry more widespread defects, as general phagocytosis of apoptotic cells is also impaired (26). MerTK has been shown to be the indispensable receptor for POS internalization (4, 27, 28).

Importantly, mutations in MERTK have been shown to cause different types of retinal dystrophies in humans (29–31) and are associated with more widespread diseases such as cancer (32), lupus-like autoimmunity (33, 34), and atherosclerosis (35).

Phagocytosis is a powerful process strictly regulated at several cellular levels. In addition to the sequential receptor-ligand activation steps undertaken by the αvβ3 integrin receptor, MerTK also controls the number of particles to be engulfed (36, 37), thus acting as a negative feedback regulator. However, in contrast to macrophages, RPE cell phagocytosis follows a diurnal cyclic rhythm despite the permanent contact between POS and RPE cells (5), suggesting that a more sophisticated regulatory mechanism exists in RPE cells.

There are numerous ways by which ligand/receptor function can be regulated, such as the control of cell surface receptor numbers, their ligand affinities, or the production of a soluble form of the receptor by proteolytic cleavage of the extracellular domain (38). Soluble receptors can have more than one occupation (39); they may function as binding proteins to stabilize ligands in the extracellular matrix and enhance interaction with the membrane-bound receptors, can down-regulate ligand-related activation and associated downstream signaling, or might act as decoy receptors by competing with full-length membrane receptors for ligand binding.

Recently, both Axl and MerTK have been shown to be cleaved and released as soluble proteins by fibroblasts and macrophages, respectively (40, 41). The soluble form of MerTK (sMerTK) was found to function as a decoy receptor for Gas6 and inhibit activation of full-length membrane-bound receptors (41). In this study, we show that sMerTK is released by RPE cells in vitro, and MerTK cleavage is potentiated by MFG-E8. In addition, sMerTK contributes to the down-regulation of POS binding and subsequent internalization by acting as a decoy receptor as well as by down-regulating directly the phagocytic capacity of RPE cells. Furthermore, sMerTK release is rhythmic in vivo and thus may help limit the phagocytic peak duration.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Reagents were from Invitrogen, unless otherwise stated. Recombinant proteins and antibodies against mouse MerTK, mouse MFG-E8, and mouse Gas6 were from R & D Systems, and recombinant protein S was from MP Biomedicals.

Other antibodies used against various proteins were as follows: PEDF (Abcam); mouse CD51 (ov integrin) and mouse Tyro3 (BD Transduction Laboratories); rabbit β5 integrin H-96, MerTK E-20, and IRBP C-18 (Santa Cruz Biotechnology); rabbit tubulin (Sigma); rabbit ZO-1 (Invitrogen); and rabbit phospho-MerTK (FabGennix Inc. International).

Cell Culture—J774 macrophage cells were cultured at 37 °C with 5% CO₂ in DMEM containing 10% FCS, supplemented with 1% nonessential amino acids and 1% sodium pyruvate. The rat RPE-J cell line (ATCC) was maintained at 32 °C and 5% CO₂ in DMEM with 4% CELLect Gold FCS (ICN), supplemented with 10 mM HEPES and 1% nonessential amino acids. For experiments, J774 cells were used 3 days after splitting. RPE cells were plated on Alcian blue-coated 24- or 96-well plates and allowed to polarize for 6 days before use.

Animals—Wild-type mice (129T2/SvEmsl) were housed under cyclic 12-h light/12-h dark conditions (light onset at 8:00 h) and fed ad libitum. Animals were handled according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. For experiments, mice were killed by CO₂ asphyxiation at different times during the day. The 11 time points analyzed were as follows: 6:00, 7:00, 8:00 (light onset), 9:00, 10:00 (phagocytosis peak), 11:00, 12:00, 16:00, 20:00 (light offset), 22:00, and 24:00. Eyeballs were carefully enucleated and rinsed in HBSS without Ca²⁺ and Mg²⁺. The lens and vitreous humor were dissected out. Retinas were delicately separated from the eyecups containing the RPE/choroid layers and quickly frozen in independent tubes in liquid nitrogen. For each animal, one eye was used for RNA extraction and gene expression testing and the second eye for protein expression level assessment (see below).

POS Isolation—POS were isolated according to established protocols (42) from porcine eyes fresh from the slaughterhouse. Briefly, in the dark under dim red light, eyeballs were separated into anterior and posterior halves, and retinas were collected in homogenization buffer (20% sucrose, 20 mM Tris acetate, pH 7.2, 2 mM MgCl₂, 10 mM glucose, 5 mM taurine). Following collection, the retina suspension was shaken to disrupt the different cell layers and filtered three times through gauze to remove large tissue fragments. The resulting crude retina suspensions were layered onto continuous 25–60% sucrose gradients (in Tris acetate, pH 7.2, 10 mM glucose, 5 mM taurine) and centrifuged at 25,000 rpm for 50 min at 4 °C (Beckman SW32-Ti swing rotor). The resulting orange bands were collected and diluted in 4–5 volumes of buffer containing 20 mM Tris acetate, pH 7.2, 5 mM taurine, followed by centrifugation at 5,000 rpm for 10 min at 4 °C (Beckman JA25.50 rotor). Pellets were washed in buffer containing 10% sucrose, 20 mM Tris acetate, pH 7.2, 5 mM taurine. The suspensions were spun at 5,000 rpm for 10 min at 4 °C before resuspending in 10% sucrose, 20 mM sodium phosphate, pH 7.2, and 5 mM taurine and centrifugation at 5,000 rpm for 10 min at 4 °C. Isolated POS were resuspended, counted, aliquoted, and frozen in DMEM containing 2.5% sucrose.

For labeling, POS were resuspended in 10% sucrose, 20 mM sodium phosphate, pH 7.2, and 5 mM taurine and incubated with 1 mg/ml fluorescein isothiocyanate (FITC) (Molecular Probes) for 1.5 h at room temperature by rotating. Labeled POS were then washed and frozen as described above.

POS Phagocytosis—Cells were challenged with ~10 POS per RPE cell resuspended in the culture medium without FCS for durations ranging from 1 h to overnight depending on the experiment. In some assays, recombinant integrin or MerTK
MerTK Cleavage Regulates RPE Phagocytosis

Cells were incubated in serum-free medium or POS for various lengths of time to obtain MerTK release in the culture medium. Soluble proteins present in the conditioned media (CM) of cultured cells were collected and concentrated ~6-fold using Amicon ultrafiltration filter units (Millipore) with a cutoff limit of 30 kDa. When CM were used to resuspend POS, the cutoff limit of the columns was at 100 kDa, to remove growth factors and ligands secreted by RPE cells from the CM, and the concentration factor was ~50-fold. Soluble proteins from the interphotoreceptor matrix of mouse eyes were washed from each separated cup (RPE/choroid) and corresponding retina in HBSS without Ca\(^{2+}\) and Mg\(^{2+}\) for 20 min on a shaker at 4 °C. Samples were centrifuged at 14,000 g for 5 min at 4 °C. Supernatants were collected and ultrafiltered at 110,000 × g for 30 min at 4 °C for further clean-up (Sorvall M120 S.E. Discovery, S120-A T2 rotor).

Sample Lysis and Immunoblotting—Cultured cells were solubilized in 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl\(_2\), and 1% Triton X-100, pH 7.4, buffer with 1 mM PMSF and 1% each of protease and phosphatase inhibitor mixtures (Sigma). Tissues were solubilized in 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl\(_2\), 1 mM EGTA, and 1% each of protease and phosphatase inhibitor mixtures. Whole cell lysates representing 8% of one well of a 24-well plate and 7.5% of one eye cup/retina were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membrane (Whatman). For soluble extracellular proteins, ~30% of concentrated conditioned media and 25% of interphotoreceptor matrix proteins mixed 1:1 between separated cups and matched retina samples were used. Immunoblots were probed with primary antibodies overnight and secondary antibodies for 2 h, washed four times in 1× TBS, 0.05–1% Tween 20 at room temperature followed by chemiluminescence detection (PerkinElmer Life Sciences). Chemiluminescence films (Amer sham Biosciences) were scanned, and signals were quantified using ImageJ 1.45s (National Institutes of Health).

Immunofluorescence Microscopy—After incubation with unlabeled POS, cells were washed twice with HBSS-CM (with Ca\(^{2+}\) and Mg\(^{2+}\)) or PBS-CM for surface or intracellular labeling, respectively. Briefly, for live labeling of cell surface proteins, antibodies were diluted in HBSS-CM, and cells were incubated for 45 min on ice. Cells were washed twice with PBS-CM before TCA fixation on ice for 15 min. Fixed cells were re-hydrated with PBS-CM and treated with 30 mM glycine in PBS-CM, and nonspecific sites were blocked with 1% BSA in PBS-CM. Primary antibody incubation for intracellular labeling were performed overnight at 4 °C or for 2 h at room temperature. Cells were washed three times with 1% BSA in PBS-CM. AlexaFluor secondary antibodies (Molecular Probes) were incubated on cells for 2 h at room temperature, followed by washing with PBS-CM, labeling cell nuclei with DAPI, and mounting onto glass slides with Aqua-Poly/Mount (Polysciences, Inc.).

All fluorescent images were acquired with an upright Olympus FV1000 confocal microscope using the Fluoview 2.1c software. Similar stacks of images were compiled and further treated equally for signal output levels using Adobe Photoshop CS6. Relative surface fluorescence levels were quantified with Image J 1.45s.

Quantification of MerTK Gene Expression—Total RNAs were extracted from separated retina and RPE/choroid according to the manufacturer’s protocol using two DNase steps (Illustra RNAspin Mini, GE Healthcare). RNAs were verified on 1% agarose gels and yields assessed using a spectrophotometer. 500 ng of RNAs were converted to cDNAs in a 50-μl volume following the instructions provided for 1 h at 42 °C (Reverse Transcription System, Promega). qPCRs using the SYBR Green PCR Master Mix were processed as follows on a 7500 Fast Real Time PCR System apparatus (Applied Biosystems): 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The ribosomal protein Rho0 (Rplp0) gene was used as internal control. Oligonucleotides with the following sequences were designed to obtain 150-bp amplicons: Rplp0, forward 5′-CCTGAAATGCTGACATCAC and reverse 5′-TGCGAGGACGGCTTGTAAC; MerTK, forward 5′-CGTCTGTCCTAACCCTGCACCT and reverse 5′-GTACTGTGGAGATTGGACT. Relative amounts of MerTK were calculated using the 2^-ΔΔCt method evaluating differences in fold expression with the internal control and amounts at 8 a.m. (8:00, light onset) being set as 1.

Studying the Effects of MerTK Ectodomain Cleavage—To investigate the possible role of shed MerTK receptors on POS tethering and internalization, CM were collected after the overnight incubation of RPE-J or J774 cells on 10-cm dishes and concentrated on filter columns with a cutoff at 100 kDa to eliminate ligands and growth factors. Each CM from one dish was used for 20 wells of a 96-well plate. FITC-POS were resuspended either in medium or in each CM, and naive cells were challenged for 3 h and their phagocytic capabilities quantified.
Alternatively, recombinant mouse MerTK, constituted from the extracellular domain fused with a human Fc domain, was mixed with the POS resuspension in the presence or absence of phagocytosis ligands before POS challenge.

To study the effects of MerTK cleavage on cells' further phagocytic activity, RPE-J cells were plated on 48-well plates, and cells were either pre-stimulated with unlabeled POS or medium for 3 h to initiate receptor cleavage in the presence or absence of 100 μg/ml cycloheximide to stop protein neosynthesis. Control wells were stimulated with medium only or left untouched. Cells were then stimulated with labeled POS with or without cycloheximide for phagocytosis assays.

**Blockade of Protease Function**—To test the effect of blocking protease function on MerTK cleavage and phagocytosis, cells were washed with DMEM and were pretreated for 3 h with medium or POS, with the addition of the nonspecific inhibitor TAPI-0 (Peptides International) at 200 μM (41) or DMSO as control. In the first series of experiments, CM were collected and concentrated as described above for immunoblotting. In the second series of experiments, phagocytosis assays were performed, again with TAPI-0 or DMSO.

**Statistical Analysis**—All experiments were repeated between 3 and 8 times. Obvious outliers were removed from the calculations on the basis of their highest difference to the calculated mean when including all samples. Significance of results was assessed using either the Student's t test in one-to-one comparisons or nonparametric one-way ANOVA with a Tukey post test when comparing multiple samples. In all cases, significance levels are depicted as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**RESULTS**

*MerTK Cleavage Regulates RPE Phagocytosis*

**FIGURE 1.** Cleavage of MerTK is potentiated by POS and decreases amounts of available surface receptors. A and B, representative immunoblots and corresponding quantifications showing that sMerTK is cleaved from the cell surface with time and is present in increasing amounts in the CM from J774 (A) and RPE-J (B) cells incubated with either medium (med) or POS for 1, 3, and 6 h or overnight (N) at a smaller size than the full-length MerTK (MerTK) detected in cell lysates. Concomitant decrease of total lysate amounts is only detectable for J774 cells. Sizes are in kDa. Relative levels with the levels in wells subjected to medium change and immediate retrieval are set as 1, mean ± S.D., n = 3, Student's t test, *, p < 0.05; **, p < 0.01; ***, p < 0.001. C, representative control RPE-J immunoblots for various proteins as indicated. PEDF serves as positive control, and Tyro3, uv, and β5 integrin as negative controls for CM release detection, and tubulin corresponds to a lysate loading control. D, surface amounts of full-length MerTK decrease after 3 h of POS incubation as shown by immunofluorescence labeling (left panels, green labeling) and corresponding quantification of relative MerTK levels (right panel) at the surface of RPE-J cells when compared with nontreated cells (I) as indicated. ZO-1 (purple) is used as cell junction marker. Nuclei (blue) are marked with DAPI. Scale bar, 20 μm. Means ± S.D., n = 2 independent experiments, Student's t test, *, p = 0.009.
POS compared with medium alone. However, very little variation in total MerTK levels was detected in cells lysates. Interestingly, there was much more of a production of sMerTK by macrophages than by RPE cells.

Secretion of the PEDF growth factor by RPE cells was used as a positive control of protein detection in CM samples and tubulin as a control for equal protein loading of lysate samples (Fig. 1C). The cleavage process was specific to the MerTK receptor, as neither Tyro3 (belonging to the TAM family) nor αv nor β5 integrin subunits (jointly forming the αvβ5 binding receptor) were found in the CM of RPE-J cells.

We also evaluated the amounts of full-length MerTK receptors present at the surface of RPE-J cells by immunofluorescence labeling of surface MerTK (Fig. 1D). Decrease in surface MerTK was concomitant to the release of sMerTK in the CM. After 3 h of incubation, the amount of intact receptors detected at the cell surface was decreased in POS-stimulated cells, when compared with nontreated cells.

sMerTK Release into the Interphotoreceptor Space in Vivo Varies over Time—Given that MerTK activation by phosphorylation is synchronized with the phagocytic peak in vivo (14), it would be interesting to see whether sMerTK release into the interphotoreceptor matrix (IPM) changes over the light/dark cycle. We sacrificed animals at 11 different time points during the day and isolated soluble proteins from the interphotoreceptor space. We detected different amounts of sMerTK in the IPM of wild-type mice depending on the time of day (Fig. 2A). We used IRBP, a protein constitutively present in the IPM, and PEDF, a growth factor secreted by RPE cells on their apical side in vivo, as positive controls to validate our IPM protein retrieval technique. Quantifications of replicate experiments show that sMerTK levels seem to follow a bimodal profile of release, with levels of sMerTK augmenting just before light onset and offset (8 a.m. and 8 p.m. in our facility, respectively). Maximum MerTK cleavage was observed at the light offset (8 p.m., 20:00). Interestingly, levels of sMerTK decrease at 9 a.m., just before and increase at the time of the phagocytic peak (2 h after light onset, 10 a.m. in our facility) (Fig. 2B). After peak phagocytosis time, levels of sMerTK slowly rise after 12 p.m. until 8 p.m. Levels of IRBP do not seem to vary along the light/dark cycle, although levels of secreted PEDF appear to increase slightly after or at each light switch (9 a.m. and 8–9 p.m.).

We then investigated whether MerTK gene expression was modified to replenish cleaved MerTK receptors. With the second eye from each animal, we quantified MerTK gene expression levels by qPCR in the RPE/choroid fraction. Interestingly, the gene transcription pattern appears to be somewhat complementary to the sMerTK cleavage profile (Fig. 2C). Indeed, MerTK synthesis reached its minimum at the time of both peak phagocytosis and sMerTK detection in the IPM. Moreover, maximal gene expression occurred at 11 a.m. right after the phagocytic peak.

Different Roles of the Phagocytic Ligands in sMerTK Release—In vivo, phagocytosis is rhythmically activated by the receptor-ligand couple αvβ5 integrin-MFG-E8 (12, 14). In addition, MerTK ligands Gas6 and protein S are also expressed in the retina, and both are required for photoreceptor survival (19, 22). Thus, we set out to examine the possible implications of these different ligands on sMerTK cleavage and their role in POS phagocytosis. RPE cells were stimulated with POS in the presence or absence of 2 μg/ml Gas6, protein S, or MFG-E8 (Fig. 3A) (20, 21). The integrin ligand MFG-E8 had more impact on sMerTK release than MerTK’s own ligands. Gas6 seems to slightly increase sMerTK release, although protein S signifi-
cantly reduces it, especially in the presence of POS. The 3-h phagocytosis assays using the same conditions showed a similar trend with MFG-E8 strongly augmenting total phagocytosis, corresponding to the sum of bound and internalized POS, whereas the impact of MerTK ligands, Gas6 and protein S, was more restricted, with Gas6 limiting internalization, although protein S appeared to increase it slightly (Fig. 3).

All three ligands are present in the IPM in vivo and may contribute to the timely daily activation of the RPE phagocytic machinery. Thus, to examine their role further, we carried out...
similar assays using 1–10 μg/ml ligand concentrations either alone or in combination to test whether we could see more marked differences. J774 macrophages responded with an equal increase in total POS phagocytosis in all conditions, regardless of the dose and combination of ligands tested compared with POS alone (on average × 1.36 ± 0.05; Fig. 3C). In contrast, these ligands had distinctive effects on total POS phagocytosis by RPE-J cells. As showed in Fig. 3B, MFG-E8 (× 1.7–1.8 ± 0.2) dramatically affected total POS phagocytosis compared with Gas6 (× 0.8 ± 0.1) or protein S (× 1.1 ± 0.1) (Fig. 3D). Increasing MFG-E8 concentrations improves RPE-J phagocytosis above 5 μg/ml. Increasing Gas6 concentrations resulted in slightly decreased phagocytosis, although protein S doses had a small stimulatory effect above 2 μg/ml. Stimulating RPE cells with POS in the presence of both Gas6 and protein S at increasing concentrations had similar effects on total phagocytosis as Gas6 alone. However, when all three ligands were combined, the phagocytic profile was similar to cells stimulated with MFG-E8 alone at 1–2 μg/ml, although levels show a decrease at 5–10 μg/ml.

As phagocytosis proceeds in two sequential and distinct steps, binding and internalization (43), we repeated phagocytosis assays under the same conditions with the different ligands to test whether their doses and/or combinations affected binding and/or internalization during RPE phagocytosis. POS binding profiles were less modified by the addition of ligands compared with POS alone than total phagocytosis profiles (data not shown). MFG-E8 slightly stimulated binding (× 1.3 ± 0.2); Gas6 and protein S inhibited binding alone or in combination (× 0.8 ± 0.2, × 0.8 ± 0.1, × 0.8 ± 0.1, respectively). Combination of all three ligands had no effect (× 1.1 ± 0.3). Interestingly, the effect on POS internalization followed a similar trend as the one observed for total phagocytosis, with the effects being even more marked (compare Fig. 3, E with D). We confirmed a strong stimulatory effect with the addition of MFG-E8 to POS (on average × 2.3 ± 0.1) compared with POS alone. The dose-dependent inhibitory effect of Gas6 was significant at all doses tested (on average × 0.7 ± 0.04). In contrast, protein S had a slight dose-dependent stimulatory role ranging from × 1 to 1.3 ± 0.1. Combining both Gas6 and protein S at equal doses quenched the stimulatory effects observed with protein S alone, with levels comparable with POS alone (× 1–1.2 ± 0.3). However, when added to MFG-E8, Gas6 and protein S appeared to enhance the stimulatory effect of MFG-E8 (× 2.7 ± 0.5).

siMerTK Acts as a Decoy Receptor—It was previously shown that siMerTK prevents the phagocytosis of apoptotic cells by J774 macrophages by acting as a decoy receptor (41). To test the hypothesis that MerTK could act as a decoy receptor and abolish POS uptake by RPE cells, we incubated RPE-J cells overnight with POS to stimulate siMerTK cleavage. CM were collected and concentrated on columns with a cutoff size of 100 kDa to dispose of the ligands secreted by RPE cells and other small molecules such as growth factors that could interfere with the experiment. Concentrated CM containing siMerTK were used to resuspend and allowed to bind POS before challenging non-stimulated RPE-J cells for 3 h (Fig. 4A). CM from both RPE-J and J774 macrophages significantly block POS binding. CM from RPE-J seem to increase slightly POS internalization, suggesting a potential faster uptake of bound POS. We confirmed this result by using a recombinant mouse MerTK (rMerTK) that only contained the extracellular domain of MerTK (Fig. 4B). rMerTK also blocked POS phagocytosis. In the same con-
MerTK Cleavage Regulates RPE Phagocytosis

FIGURE 5. Release of sMerTK down-regulates mostly the binding step of RPE phagocytosis. A–C, stimulating cleavage of MerTK using a preincubation step with either medium (m, gray bars) or POS (P, black bars) with (+) or without (−) the addition of CHX to block protein neosynthesis inhibits relative POS internalization to a greater extent than relative POS binding for J774 macrophages (A, compare − and + bars) when compared with cells not subjected to a preincubation step (−, white bars). In contrast, the binding step is slightly more affected than the internalization step for RPE-J cells. B, RPE, compare gray/black bars to white bars in + panel) in the presence of CHX as indicated. A and B, mean ± S.D., n = 3−5, *, p < 0.05; **, p < 0.01; ***, p < 0.001. Statistical significance was assessed using a one-way nonparametric ANOVA test with a Tukey post-test using nontreated cells (−) as reference. C, immunofluorescence assay showing that, when protein replacement was inhibited using CHX (with CHX), less full-length MerTK receptors (green) were seen at the cell surface when compared with cells not treated with CHX (no CHX) as indicated. ZO-1 (purple) is used as a cell junction marker, and nuclei (blue) are marked with DAPI. Scale bar 20 μm. D, cleavage of MerTK can be inhibited by using the protease inhibitor TAPI-0 as observed on immunoblots where sMerTK levels decrease in the CM of RPE-J cells with TAPI-0 (+) treatment compared with control DMSO (−) as indicated. Levels of active phosphorylated MerTK (P-MerTK) seem to increase concomitantly to the sMerTK decrease (compare − versus + lanes). Corresponding lysates show that full-length MerTK (MerTK) or control tubulin levels do not vary. Sizes are in kDa. E, quantification of relative phagocytosis levels in the same conditions as in D shows that inhibition of MerTK cleavage increases significantly POS binding, although POS internalization was less affected (compare white bars to black bars). Nontreated cells (−) are used as reference. Mean ± S.D., n = 3, *, p < 0.05; **, p < 0.01. Statistical significance was assessed using the Student’s t test with nontreated cells (−) as reference for each bar pair.

Conditions, we added each of the three phagocytic ligands previously tested. Although rMerTK had no effect on the MFG-E8-related stimulation of POS phagocytosis, it was able to counteract the effect of both MerTK ligands on phagocytosis. Indeed, the lowest levels of phagocytosis were reached when rMerTK was present concomitantly with Gas6 and protein S. Our data show that the presence of sMerTK has an inhibitory effect and blocks POS from being recognized and phagocyted by RPE cells via functional full-length MerTK receptors.

Cleavage of MerTK Modulates POS Binding by RPE Cells—To further study the effect of MerTK receptor cleavage during POS phagocytosis, we performed a two-step experiment on J774 macrophages and RPE-J cells. First, cells were pre-stimulated with medium or unlabeled POS for 3 h to enable receptor cleavage as observed in Figs. 1 and 3 in the absence or presence of cycloheximide (CHX) to inhibit protein neosynthesis and thus avoid reconstitution of the full-length MerTK pool of receptors at the cell surface. Second, phagocytosis assays were performed with or without cycloheximide as before. Blocking protein neosynthesis reduced both binding and internalization by non-pre-stimulated J774 cells by 60 ± 4 and 30 ± 4%, respectively (Fig. 5A, compare − and + bars). Reducing the amounts of full-length MerTK receptors at the J774 cell surface by pre-incubating the cells with medium or POS did not modify extensively their binding but significantly altered their internalization capacity by 75 ± 5% in both conditions (p < 0.0005, compare gray/black bars to white bar). Internalization percentages were not modified when cells could resynthesize proteins (on average + 32 ± 2% for medium or POS pretreated cells versus + 33 ± 2% for non-pretreated cells, − cycloheximide). However, internalization percentage lowered to 25 ± 10% when cells could not produce new proteins (+ cycloheximide).

In contrast, RPE-J cells responded differently, as binding was more affected when full-length membrane-bound MerTK receptors could not be replenished at the cell surface (Fig. 5B, compare − and + bars). In non-pretreated cells, cycloheximide only impacted RPE-J binding and internalization by 17 ± 5 and 8 ± 2%, respectively (Fig. 5B, compare white bars). Interestingly, cells pretreated with medium or POS to cleave MerTK
had their binding significantly impaired by 37 ± 4% (p < 0.005) and 38 ± 5% (p < 0.001), when internalization was diminished only by 29 ± 2 and 22 ± 2% (p < 0.001), respectively (Fig. 5B, compare gray and black bars, respectively). Internalization percentages were not affected between the different conditions (with or without pretreatment and with or without protein neosynthesis inhibition), staying at an average of 30 ± 2% in all samples, showing that differences were not due to impaired internalization in RPE-J cells. Levels of available full-length receptors at the cell surface were indeed decreased after 3 h of POS stimulation when treated with CHX compared with POS stimulation alone (Fig. 5C).

To confirm the effect of sMerTK on the respective phagocytosis steps in RPE-J cells, we used a pharmacological inhibitor of some metalloproteinases, TAPI-0, which has been shown to block sMerTK shedding in macrophages (41). As done in the previous experiment, RPE-J cells were treated with TAPI-0 with or without preincubation with medium or POS to stimulate sMerTK release. Western blot analysis revealed that cleavage of MerTK was diminished in the presence of TAPI-0 in comparison with controls after both medium and POS stimulation (Fig. 5D). Inhibition of metalloproteases, which decreased MerTK cleavage, concomitantly increased the number of MerTK receptors that were activated by phosphorylation, although no modification of the total amounts of MerTK in full cell lysates could be observed as already described in Fig. 1B. When phagocytosis assays were performed with the same TAPI-0 treatment conditions, the amount of POS bound by RPE cells was increased by 26 ± 2 and 57 ± 3% when preincubated with medium and POS, respectively (Fig. 5E, compare white versus black bars). However, in the same conditions POS internalization was affected to a lesser extent and diminished by 9 ± 2% after medium and 23 ± 2% after POS pre-stimulation of sMerTK release.

**DISCUSSION**

RPE cells are different from macrophages in that they are in continuous contact with POS, and phagocytosis occurs at a specific time, although macrophages must dispose of apoptotic cells as soon as they encounter them (5). Thus, activation of the POS is a continuous contact with POS, and phagocytosis occurs at a specific time (5). Thus, activation of the reaction requires rapid and effective control of POS internalization in RPE-J cells. Levels of available full-length receptors at the cell surface were indeed decreased after 3 h of POS stimulation when treated with CHX compared with POS stimulation alone (Fig. 5C).

To confirm the effect of sMerTK on the respective phagocytosis steps in RPE-J cells, we used a pharmacological inhibitor of some metalloproteinases, TAPI-0, which has been shown to block sMerTK shedding in macrophages (41). In our study, two approaches allowed us to show that sMerTK also acts as a decoy receptor in the retina. In RPE cells, both ligand functions are impacted by the presence of sMerTK, leading to low levels of phagocytosis. However, another effect of sMerTK release could be that proteolysis of the full-length receptor might prevent further activation of MerTK in vivo. In intracellular domains and thus block stimulation of downstream internalization signaling pathways. Indeed, we show that when RPE cells pre-stimulated with medium or POS to generate receptor cleavage were challenged with POS in the presence of cycloheximide to inhibit protein neosynthesis, and thus replenishment of surface-bound full-length MerTK receptors, phagocytosis was significantly reduced. This effect was also observed in cells pre-stimulated with medium alone, implying that the observed decrease in phagocytic activity of RPE cells is not due to the depletion of membrane receptors that would normally be internalized during phagocytosis but instead to the reduced availability of functional full-length MerTK receptors at the cell surface after cleavage of their ectodomain. The more pronounced effect observed after the POS preincubation step could be due to an additive effect of increased sMerTK shedding obtained as well as usage of full-length MerTK receptors during the preincubation step. Conversely, blocking cleavage of MerTK with a pharmacological protease inhibitor approach increased POS phagocytosis, especially at the binding step level, strengthening this hypothesis. Given that MerTK receptors dimerize which triggers downstream auto-phosphorylation (49, 50), it is possible thatMerTK activity is controlled to restrict the duration of the phagocytic peak, crucial in avoiding too many POS being internalized, is still unclear. We know that proper control of all steps of phagocytosis is necessary as deregulation of its completion can lead to various retinal disease phenotypes, some of them resembling age-related macular degeneration (14, 44–46). In addition, we recently showed that the MerTK receptor also bears a role in controlling POS binding aside from its indispensable role for POS internalization (37). This study provides evidence of a negative feedback mechanism that down-regulates MerTK activity during RPE phagocytosis by generation of a soluble form of the receptor.

Macrophages have been previously shown to generate sMerTK from the full-length membrane-bound receptor through a proteolytic cleavage (41). Here, we find that sMerTK is cleaved from RPE cells, and this phenomenon seems to be slightly increased in the presence of POS compared with medium change alone, an effect not observed with J774 macrophages that equally recognize and interact with apoptotic cells and POS (47). These results suggest that cleavage and release of sMerTK is intrinsic to RPE cells and is in part stimulated by POS phagocytosis. Levels of full-length receptors in cell lysates were decreased accordingly in J774 macrophages, whereas no gross difference is observed for RPE-J cells. Interestingly, RPE-J cells appear to have much less MerTK cleaved compared with macrophages, which could explain why we cannot detect any significant change in cell lysate levels. RPE-J seems to also release full-length MerTK receptors, possibly via the exocytosis of microvesicles or exosomes as was shown for some cell lines (48).

MerTK proteolysis can limit the cell phagocytic activity in different ways. In macrophages, the cleaved sMerTK receptor has a direct antagonistic role on the activity of full-length membrane-bound MerTK via its interaction with Gas6, and thus it acts as a decoy receptor (41). In our study, two approaches allowed us to show that sMerTK also acts as a decoy receptor in the retina. In RPE cells, both ligand functions are impacted by the presence of sMerTK, leading to low levels of phagocytosis. However, another effect of sMerTK release could be that proteolysis of the full-length receptor might prevent further activation of MerTK in intracellular domains and thus block stimulation of downstream internalization signaling pathways. Indeed, we show that when RPE cells pre-stimulated with medium or POS to generate receptor cleavage were challenged with POS in the presence of cycloheximide to inhibit protein neosynthesis, and thus replenishment of surface-bound full-length MerTK receptors, phagocytosis was significantly reduced. This effect was also observed in cells pre-stimulated with medium alone, implying that the observed decrease in phagocytic activity of RPE cells is not due to the depletion of membrane receptors that would normally be internalized during phagocytosis but instead to the reduced availability of functional full-length MerTK receptors at the cell surface after cleavage of their ectodomain. The more pronounced effect observed after the POS preincubation step could be due to an additive effect of increased sMerTK shedding obtained as well as usage of full-length MerTK receptors during the preincubation step. Conversely, blocking cleavage of MerTK with a pharmacological protease inhibitor approach increased POS phagocytosis, especially at the binding step level, strengthening this hypothesis. Given that MerTK receptors dimerize which triggers downstream auto-phosphorylation (49, 50), it is possible that MerTK activity is controlled to restrict the duration of the phagocytic peak, crucial in avoiding too many POS being internalized, is still unclear. We know that proper control of all steps of phagocytosis is necessary as deregulation of its completion can lead to various retinal disease phenotypes, some of them resembling age-related macular degeneration (14, 44–46). In addition, we recently showed that the MerTK receptor also bears a role in controlling POS binding aside from its indispensable role for POS internalization (37). This study provides evidence of a negative feedback mechanism that down-regulates MerTK activity during RPE phagocytosis by generation of a soluble form of the receptor.
MerTK Cleavage Regulates RPE Phagocytosis

ously been shown to inhibit MerTK shedding in macrophages (41). Very recently, the cleavage site in mouse macrophages has been located to proline 485 in MerTK, and it has been shown that sMerTK cleavage requires the metalloprotease ADAM17 (51). Given the differences in the molecular regulation of phagocytosis and MerTK cleavage between macrophages and RPE cells, it would be interesting to investigate whether ADAM17 also participates in sMerTK release in the retina. Other candidates bearing a tissue-specific action that can be inhibited by TAPI-0 would also be candidates for investigation.

In vivo, we show that the release of sMerTK follows a circadian profile, decreasing slightly just before and increasing at peak phagocytosis time and during the following hour. Interestingly, peak sMerTK levels were detected at light offset, which could help keep phagocytosis down when light levels change. These results suggest that ectodomain cleavage of MerTK might function to avoid prolonged engulfment of POS and thus limit the duration of the phagocytosis peak to the narrow window that occurs just after light onset. Combined with our previous results showing the punctual intracellular stimulation of MerTK via αvβ5 integrin–related signaling cues (12, 14), it appears that multiple pathways participate in the phagocytic burst in the retina, both through intra- and extracellular molecules. In the absence of MFG-E8 or αvβ5 integrin, phagocytosis still occurs but on a steady-state basis, and so it is therefore important to explore the diurnal status of sMerTK in the retina of animals depleted of MFG-E8 or αvβ5 integrin.

To study the possible contributions of both αvβ5 integrin and MerTK ligands in MerTK ectodomain cleavage, cells were challenged with POS in the presence of MFG-E8, Gas6, and protein S, and the levels of sMerTK present in the CM and corresponding phagocytic activity were quantified. Our results show that the integrin ligand MFG-E8 increases the release of sMerTK, whereas both MerTK ligands have limited effects in comparison with POS alone. Interestingly, these results were observed even in the absence of POS, suggesting that ligand binding to both phagocytic receptors regulates MerTK cleavage even in the absence of phagocytosis. Concomitantly, the phagocytic activity profile matched the sMerTK release profile. When different doses and potential additive effects were tested in both macrophages and RPE cells, we observed very different responses between the two cell types. Macrophages reacted almost equally to all ligands at most doses, either alone or in combination, with an average 36% increase in total phagocytosis. In contrast, RPE cell total phagocytosis was markedly augmented upon increasing MFG-E8 stimulation alone or combined with Gas6 and protein S at 1–2 μg/ml concentrations (+80%). At higher doses, the MFG-E8-positive effect was counterbalanced to some extent by Gas6 and protein S: Gas6 alone bears an inhibitory role (−20%) whereas protein S alone is slightly stimulatory (+10%) with increasing concentrations, and their effects compensate each other when mixed at a 1:1 ratio. Ligand combinations and their various concentrations acted mostly on internalization, as all effects were more pronounced than on POS binding.

The important stimulatory effect of MFG-E8 on both phagocytosis and sMerTK cleavage in RPE cells points out that integrin signaling pathways synchronizing phagocytosis in vivo may contribute to the release of sMerTK from the apical surface, either directly or indirectly, to help down-regulate phagocytosis. Furthermore, our results indicate that MerTK ligands Gas6 and protein S appear to have opposite and more subtle roles in the regulation of POS phagocytosis and MerTK cleavage by the RPE, which appear to be dose-dependent, at least in vitro. Gas6 seems to act as a negative regulator, although protein S appears to stimulate phagocytosis. As both ligands are not required for the rhythm of phagocytosis, although they are essential for phagocytosis completion (12, 22), they may contribute to the fine-tuning of MerTK activation. Thus, we postulate that ligands in the IPM may cooperate to tightly regulate numbers of full-length MerTK receptors present at the RPE cell surface that can be activated. This mechanism would allow for the precise control of POS engulfment to limit peak duration. To date, concentrations of individual ligands in the IPM in vivo are still unknown. Therefore, further studies that will assess the bioavailability of these three ligands throughout the light/dark cycle will be crucial for understanding the respective contribution of each ligand during POS phagocytosis in vivo.

In conclusion, our data show that RPE phagocytosis in vitro and in vivo is partly regulated by the proteolytic cleavage of the full-length membrane-bound MerTK receptor, which is critical for the uptake of POS. Receptor ectodomain cleavage provides a negative feedback loop in controlling timely MerTK activity. It is not uncommon that soluble receptors carry multiple roles in fine-tuning receptor function (39). This is exciting, and further work will elucidate the precise mechanisms of MerTK receptor shedding and how it modulates its own biological activity in vivo.

Acknowledgments—We thank Stéphane Fouquet (Institut de la Vision) and Quentin Riou for help with confocal microscopy. The Institut de la Vision is funded by INSERM, Université Pierre et Marie Curie-Paris, CNRS, and Département de Paris.

REFERENCES

1. Rabinovitch, M. (1995) Professional and non-professional phagocytes: an introduction. Trends Cell Biol. 5, 85–87
2. Strauss, O. (2005) The retinal pigment epithelium in visual function. Physiol. Rev. 85, 845–881
3. Young, R. W., and Bok, D. (1969) Participation of the retinal pigment epithelium in the rod outer segment renewal process. J. Cell Biol. 42, 392–403
4. Bok, D., and Hall, M. O. (1971) The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. J. Cell Biol. 49, 664–682
5. LaVail, M. M. (1976) Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. Science 194, 1071–1074
6. Young, R. W. (1977) The daily rhythm of shedding and degradation of cone outer segment membranes in the lizard retina. J. Ultrastruct. Res. 61, 172–185
7. Savill, J., Dransfield, I., Hogg, N., and Haslett, C. (1990) Vitrinectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature 343, 170–173
8. Boesze-Battaglia, K., and Albert, A. D. (1992) Phospholipid distribution among bovine rod outer segment plasma membrane and disk membranes. Exp. Eye Res. 54, 821–823
9. Ruggiero, L., Connor, M. P., Chen, J., Langen, R., and Finnemann, S. C. (2012) Diurnal, localized exposure of phosphatidylserine by rod outer segment tips in wild-type but not ligh5+/− or Mfge8+/− mouse retina. Proc.
10. Ryeom, S. W., Silverstein, R. L., Scotto, A., and Sparrow, J. R. (1996) Binding of anionic phospholipids to retinal pigment epithelium may be mediated by the scavenger receptor CD36. *J. Biol. Chem.* **271**, 20356–20359

11. Hanayama, R., Tanaka, M., Miwa, K., Shinohora, A., Iwamatsu, A., and Nagata, S. (2002) Identification of a factor that links apoptotic cells to phagocytes. *Nature* **417**, 182–187

12. Nandrot, E. F., Anand, M., Almeida, D., Atabai, K., Sheppard, D., and Finnemann, S. C. (2007) Essential role for MFG-E8 as ligand for αvβ5 integrin in diurnal retinal phagocytosis. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 12005–12010

13. Finnemann, S. C., Bonilha, V. L., Marmorstein, A. D., and Rodriguez-Boulan, E. (1997) Phagocytosis of rod outer segments by retinal pigment epithelial cells requires αvβ5 integrin for binding but not for internalization. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12932–12937

14. Nandrot, E. F., Kim, Y., Brodie, S. E., Huang, X., Sheppard, D., and Finnemann, S. C. (2004) Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking αvβ5 integrin. *J. Exp. Med.* **200**, 1539–1545

15. Ishimoto, Y., Ohashi, K., Mizuno, K., and Nakano, T. (2000) Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. *J. Biochem.* **127**, 411–417

16. Manfioletti, G., Brancolini, C., Avanzi, G., and Schneider, C. (1993) The c-mer gene in RCS rats unravels general mechanisms of physiological cell adhesion and apoptosis. *Cell. Mol. Biol.* **13**, 4976–4985

17. Hafizi, S., and Dahlbäck, B. (2006) Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. *FEBS J.* **273**, 5231–5244

18. Chen, J., Carey, K., and Godowski, P. J. (1997) Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. *Oncogene* **14**, 2033–2039

19. Prasad, D., Rothlin, C. V., Burroll, P., Bursyn-Cohen, T., Lu, Q., Garcia de Frutos, P., and Lemke, G. (2006) TAM receptor function in the retinal pigment epithelium. *Mol. Cell. Neurosci.* **33**, 96–108

20. Hall, M. O., Prieto, A. L., Ohin, M. S., Abrams, T. A., Burgess, B. L., Heeb, M. J., and Agnew, B. J. (2001) Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. *Exp. Eye Res.* **73**, 509–520

21. Hall, M. O., Ohin, M. S., Heeb, M. J., Burgess, B. L., and Abrams, T. A. (2005) Both protein S and Gas6 stimulate outer segment phagocytosis by cultured rat retinal pigment epithelial cells. *Exp. Eye Res.* **81**, 581–591

22. Bursyn-Cohen, T., Lew, E. D., Través, P. G., Burroll, P. G., Hash, J. C., and Lemke, G. (2012) Genetic dissection of TAM receptor-ligand interaction in retinal pigment epithelial cell phagocytosis. *Neuron* **76**, 1123–1132

23. Duncan, J. L., LaVail, M. M., Yasmurna, D., Mathes, M. T., Yang, H., Trautmann, N., Chappelow, A. V., Feng, W., Earp, H. S., Matsushima, G. K., and Vollrath, D. (2003) An RCLS-like retinal dystrophy phenotype in mer knockout mice. *Invest. Ophthalmol. Vis. Sci.* **44**, 826–838

24. Nandrot, E., Dufour, E. M., Provost, A. C., Péquignot, M. O., Bonnel, S., Gogat, K., Marchant, D., Rouillac, C., Sélucule de Conde, B., Bihoreau, M. T., Shaver, C., Dufier, J. L., Marsac, C., Lathrop, M., Menasché, M., and Abitbol, M. (2000) Homozygous deletion in the coding sequence of the αv5 integrin gene causes age-related changes in mouse retinal pigment epithelium. *Arterioscler. Thromb. Vasc. Biol.* **20**, 135–140

25. Sather, S., Kenyon, K. D., Lefkowitz, J. B., Liang, X., Varnum, B. C., Henson, P. M., and Graham, D. K. (2007) A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. *Blood* **109**, 1026–1033

26. Parinot, C., Rieu, Q., Chatagnon, J., Finnemann, S. C., and Nandrot, E. F. (2014) Large-scale purification of porcine or bovine photoreceptor outer segments for phagocytosis assays on retinal pigment epithelial cells. *J. Vis. Exp.* **94**, e52100

27. Hall, M. O., and Abrams, T. (1987) Kinetic studies of rod outer segment binding and ingestion by cultured rat RPE cells. *Exp. Eye Res.* **45**, 907–922

28. Am. J. Pathol. **161**, 1515–1524

29. Gibbs, D., Kitamoto, J., and Williams, D. S. (2003) Abnormal phagocytosis by retinal pigmented epithelium that lacks myosin VIIa, the Usher syndrome 1B protein. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6481–6486

30. Wavre-Shapton, S. T., Tolmachova, T., Lopes da Silva, M., da Silva, M., Futter, C. E., and Seabra, M. C. (2013) Conditional ablation of the choroidal epithelial gene causes age-related changes in mouse retinal pigment epithelium. *Am. J. Pathol.* **182–187

31. Tschernutter, M., Jenkins, S. A., Waseem, N. H., Saihan, Z., Holder, G. E., Bird, A. C., Bhattacharya, S. S., Ali, R. R., and Webster, A. R. (2006) Clinical characterisation of a family with retinal dystrophy caused by mutation in the Mertk gene. *Br. J. Ophthalmol.* **90**, 718–723

32. Wavre-Shapton, S. T., Tolmachova, T., Lopes da Silva, M., da Silva, M., Futter, C. E., and Seabra, M. C. (2013) Conditional ablation of the choroidal epithelial gene causes age-related changes in mouse retinal pigment epithelium. *Am. J. Pathol.* **182–187

33. Finnemann, S. C., and Rodriguez-Boulan, E. (1999) Macrophage and retinal pigment epithelial phagocytosis: apoptotic cells and photoreceptors compete for αvβ3 and αvβ5 integrins, and protein kinase C regulates αvβ5 binding and cytosekeletal linkage. *J. Exp. Med.* **190**, 861–874
48. Raposo, G., and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* **200**, 373–383

49. Ling, L., Templeton, D., and Kung, H. J. (1996) Identification of the major autophosphorylation sites of Nyk/Mer, an NCAM-related receptor tyrosine kinase. *J. Biol. Chem.* **271**, 18355–18362

50. Tibrewal, N., Wu, Y., D’mello, V., Akakura, R., George, T. C., Varnum, B., and Birge, R. B. (2008) Autophosphorylation docking site Tyr-867 in Mer receptor tyrosine kinase allows for dissociation of multiple signaling pathways for phagocytosis of apoptotic cells and down-modulation of lipopolysaccharide-inducible NF-κB transcriptional activation. *J. Biol. Chem.* **283**, 3618–3627

51. Thorp, E., Vaisar, T., Subramanian, M., Mautner, L., Blobel, C., and Tabas, I. (2011) Shedding of the Mer tyrosine kinase receptor is mediated by ADAM17 protein through a pathway involving reactive oxygen species, protein kinase Cδ, and p38 mitogen-activated protein kinase (MAPK). *J. Biol. Chem.* **286**, 33335–33344