Mertk Triggers Uptake of Photoreceptor Outer Segments during Phagocytosis by Cultured Retinal Pigment Epithelial Cells*

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The RCS rat is a widely studied model of recessively inherited retinal degeneration. The genetic defect, known as rdy (retinal dystrophy), results in failure of the retinal pigment epithelium (RPE) to phagocytize shed photoreceptor outer segment membranes. We previously used positional cloning and in vivo genetic complementation to demonstrate that Mertk is the gene for rdy. We have now used a rat primary RPE cell culture system to demonstrate that the RPE is the site of action of Mertk and to obtain functional evidence for a key role of Mertk in RPE phagocytosis. We found that Mertk protein is absent from RCS, but not wild-type, tissues and cultured RPE cells. Delivery of rat Mertk to cultured RCS RPE cells by means of a recombinant adenovirus restored the cells to complete phagocytic competency. Infected RCS RPE cells ingested exogenous outer segments to the same extent as wild-type RPE cells, but outer segment binding was unaffected. Mertk protein progressively co-localized with outer segment material during phagocytosis by primary RPE cells, and activated Mertk accumulated during the early stages of phagocytosis by RPE-J cells. We conclude that Mertk likely functions directly in the RPE phagocytic process as a signaling molecule triggering outer segment ingestion.

Phagocytosis is a process by which large particles are internalized by cells to form phagosomes. The process can be divided into three phases: binding, ingestion, and digestion. Retinal pigment epithelial (RPE)1 cells, which form a polarized epithelium between the photoreceptor cells and the choroid in the outer retina, phagocytize more biomass than any other mammalian cell type (1). The RPE phagocytizes photoreceptor outer segment (OS) membranes (2) that are shed as part of the normal ongoing process of photoreceptor OS renewal (3). Failure of OS membrane uptake leads to photoreceptor cell death (4), as illustrated by the RCS rat, a widely used model for recessively inherited retinal degeneration. The RCS mutation rdy (retinal dystrophy) causes, either directly or indirectly, a defect in RPE phagocytosis (4). This defect leads to an accumulation of shed OS membranes in the subretinal space (4) and a rapid and progressive degeneration of photoreceptor cells (5).

The molecular mechanisms of RPE phagocytosis are unclear. Studies of the internalization of exogenous OS by cultured primary RPE cells suggested a receptor-mediated process (6–8). Inhibition of the RPE cell culture phagocytic assay by anti-receptor antibodies or competitive ligands suggested several specific proteins that might play a role in the process, including the mannose receptor (9, 10), CD36 (11), and αvβ3 integrin (12–14). Inhibition of αvβ3 integrin function disrupts the OS binding phase of RPE phagocytosis, whereas the mannose receptor and CD36 have been implicated in both OS binding and ingestion. Cultured RCS RPE cells bind exogenous OS at wild-type levels. However, only a small percentage of bound OS are ingested by RCS RPE cells (15), indicating that the protein encoded by the rdy locus is critical, directly or indirectly, for OS uptake.

The gene corresponding to rdy remained unknown until recently. The mannose receptor protein and messenger RNA are present in the RPE of both wild-type and RCS rats from postnatal day (P)5 to adult (16). CD36 null mice have been reported to have normal electrotretinography and retinal histology (17). These data suggest that neither the mannose receptor nor CD36 is the gene mutated in the RCS rat. We used positional cloning to identify a mutation in the receptor tyrosine kinase gene Mertk in the RCS rat. A deletion of RCS genomic DNA results in expression of an aberrant Mertk transcript with a translation termination signal after codon 20 (18), likely a complete loss-of-function, or null, allele. Mertk was an appropriate candidate for rdy in light of evidence that a signaling defect might underlie the RCS RPE phagocytic phenotype (19–21). The discovery of mutations in the human ortholog, MERTK, in individuals with retinitis pigmentosa indicated that Mertk is essential for maintenance of the mammalian retina (22). Subsequently, in vivo genetic complementation of the RCS phenotype by viral mediated gene transfer conclusively demonstrated that Mertk is the gene for rdy (23).

The identification of Mertk provides an initial focus for elucidating molecular mechanisms of RPE phagocytosis. In the present study, we sought to determine whether the site of action of Mertk was indeed the RPE, as suggested by genetic chimera experiments (24) and, if so, whether Mertk protein was directly involved in the ingestion step of OS phagocytosis. We tested whether viral mediated gene transfer of Mertk to

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1 The abbreviations used are: RPE, retinal pigment epithelium; OS, outer segment; P, postnatal day; m.o.i., multiplicity of infection; WGA, wheat germ agglutinin; DMEM, Dulbecco’s modified Eagle’s medium; Endo H, endo-β-N-acetylgalactosaminidase H; PNGase F, peptide N-glycosidase F; GFP, green fluorescent protein.
were dissociated from the beads in 1× denaturing buffer (New England Biolabs, Beverly, MA) and treated according to the manufacturer's instructions with either PNGase F (New England Biolabs), to remove all forms of the N-linked oligosaccharides, or with Endo H (New England Biolabs), to cleave the chito-biose core of high mannose and hybrid forms of N-linked oligosaccharides.

**Phalloloid Labeling for F-actin in RPE Culture**—Confluent wild-type RPE primary cells were washed with DMEM and fixed in 4% paraformaldehyde for 10 min at room temperature. Cell membranes were permeabilized with 0.5% Triton X-100, and cells were incubated for 30 min with fluorescein phallolidin (Molecular Probes, Eugene, OR) to detect F-actin by fluorescence microscopy.

**Phagocytic Assay**—The ability of RPE cells to phagocytize OS was measured as reported previously (27). OS were suspended in DMEM containing 5% fetal bovine serum and 5% sucrose at a concentration of 1 × 10^6 OS per ml. One ml of OS was added to each well of a 4-well chamber slide and incubated with adenovirus-infected or uninfected cells for 4 h at 37°C. Then unbound OS were washed away with DMEM, and cells were fixed for 15 min with 4% paraformaldehyde in phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 5 mM NaHPO_4, 2 mM KHPO_4, pH 7.4). To distinguish total and bound OS, samples were divided into two groups. Each group contained 2 wells of cells. Group 1 was permeabilized with 0.5% Triton X-100 and group 2 remained unpermeabilized. OS were immunolabeled with anti-rhodopsin monoclonal antibody Rho 4D2 (1:200 dilution) followed by Texas Red-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) as described (26), and NRK-49F cells were maintained as recommended by the company.

Generation of a Polyclonal Antibody against Rat Merkt—A DNA fragment encoding the 103 C-terminal amino acids of rat Merkt was inserted into pGEX-1 (AMRAD Corp. Ltd., Australia) and transformed into bacterial strain BL21(DE3)pLysS (Invitrogen, Carlsbad, CA) to express the protein. The fusion protein was purified from bacterial lysates by affinity chromatography on immobilized glutathione (Sigma) and injected into rabbits to generate polyclonal antibodies.

Recombinant Adenovirus Infection of Primary RPE, RPE-J, and NRK-49F Cells—A recombinant adenovirus containing the complete open reading frame of rat Merkt driven by a cytomegalovirus promoter was constructed as described (23). To deliver Merkt into RPE cells, Ad-Merkt was added to confluent RPE cultures at various multiplicities of infection (m.o.i.) and incubated for 42 h at 37°C. A recombinant Ad-GFP (a gift from Dr. Yongjian Wu, Stanford University) was used as a control. RPE-J cells were grown on Matrigel (Fisher) for 6 days and then infected with Ad-Merkt at an m.o.i. of 2 for 42 h at 37°C. NRK-49F cells were incubated with Ad-Merkt or Ad-GFP at an m.o.i. of 10 for 42 h at 37°C.

**Immunoblotting**—Wheat germ agglutinin (WGA)-enriched samples, deglycosylated samples, immunoprecipitation samples, and whole cell lysates were separated by 6 or 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride (Millipore, Bedford, MA) for 2 h at 100 V. The blots were incubated with WGA-Sepharose beads (Sigma) for 3 h at 4°C with rotation. Beads were washed five times with 1% Nonidet P-40 lysis buffer, and glycoproteins were dissociated from the beads by boiling for 5 min in 2× loading buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.2% dithiothreitol, 0.02% bromphenol blue, pH 6.8).

**Protein Deglycosylation**—Glycoproteins enriched with WGA beads were dissociated from the beads in 1× denaturing buffer (New England Biolabs, Beverly, MA) and treated according to the manufacturer's instructions with either PNGase F (New England Biolabs), to remove all forms of the N-linked oligosaccharides, or with Endo H (New England Biolabs), to cleave the chito-biose core of high mannose and hybrid forms of N-linked oligosaccharides.

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**Immunofluorescence Microscopy**—Whole-cell RPE-J and NRK-49F cells were incubated with OS (3 × 10^7 per ml) or control medium for 1–3 h at 32°C. The cells were washed twice with cold phosphate-buffered saline containing 1 mM sodium orthovanadate (Sigma) and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) containing 10 mM sodium orthovanadate and the protease inhibitor mixture. The cell lysate was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was incubated with 20 μl of the Merkt C-terminal antibody (1:100) followed by Texas Red-conjugated anti-mouse IgG and Oregon Green-conjugated anti-rabbit IgG (Molecular Probes). Fluorescent labeling was observed under a Zeiss fluorescence microscope, and images were taken from 10–15 random fields (0.036 mm² per field) for each group. OS with an estimated diameter of 1 μm or larger were manually counted. The total number of OS (bound plus ingested) was obtained from group 1 samples, and the number of bound OS was obtained from group 2 samples. The number of ingested OS was obtained by subtracting bound OS from total OS. For each experimental condition, the assay was repeated at least three times. Results for each condition were presented as a means ± S.E. A Student's t test was used for statistical evaluation.

**Double Immunolabeling for OS and Merkt**—Confluent wild-type primary RPE cells were incubated with OS for 1–3 h, and unbound OS were removed by washing with DMEM. Cells were fixed in 100% ethanol for 5 min at −20°C. Localization of the remaining OS and Merkt was examined by immunolabeling with the Rho 4D2 (1:100) and the C-terminal antibody (1:100) followed by Texas Red-conjugated anti-mouse IgG and Oregon Green-conjugated anti-rabbit IgG (Molecular Probes).

**Immunoprecipitation**—RPE-J cells were incubated with OS (3 × 10^7 per ml) or control medium for 1–3 h at 32°C. The cells were washed twice with cold phosphate-buffered saline containing 1 mM sodium orthovanadate (Sigma) and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) containing 10 mM sodium orthovanadate and the protease inhibitor mixture. The cell lysate was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was incubated with 20 μl of the Merkt C-terminal antibody overnight at 4°C. The antigen-antibody complex was precipitated by protein A-Sepharose beads (Amersham Biosciences AB), and proteins were dissociated from the beads by boiling for 5 min in 2× loading buffer.

**RESULTS**

**Merkt Protein Is Absent from the RCS Rat**—Our molecular genetic data predict that only a 20-aa amino acid N-terminal peptide of Merkt can be synthesized in the RCS rat (18). It is therefore likely that most or all of the protein is missing from RCS tissues. To test this prediction, we generated a polyclonal antibody directed against the C terminus of rat Merkt and assessed its specificity by immunoblotting. As shown in Fig. 1A, anti-Merkt recognized two bands of ~200 and 150 kDa from rat kidney fibroblasts infected with a recombinant adenovirus, Ad-Merkt, which expresses rat Merkt (23). These bands were absent from Ad-GFP infected or uninfected cells, indicating that the antibody specifically recognizes Merkt. Pre-immune serum did not detect bands in any of the samples (data not shown).

The C-terminal antibody was used to assess Merkt expression in primary RPE cultures from RCS or a wild-type, congenic strain, RCS-ryd+. As expected, anti-Merkt recognized a
cells were resolved by 7.5% SDS-PAGE and analyzed as in A lysates of cultured wild-type RCS-rdy (lane 1) and RCS (lane 2) RPE cells were resolved by 7.5% SDS-PAGE and analyzed as in A.

full-length Mertk protein is absent from RCS tissues. Equal amounts of WGA-enriched glycoproteins isolated from various wild-type RCS-rdy* (odd numbered lanes) and RCS (even numbered lanes) tissues were resolved by 6% SDS-PAGE and analyzed by immunoblot with the C-terminal antibody (A) or a polyclonal antiserum directed against the C terminus of Mertk (B). A single membrane was probed twice. B, lane 1, two bands are clearly, but faintly, visible on the original film. The band in lane 6 is probably an artifact because it is not detected by the C-terminal antibody and is too large to be the predicted truncated protein.

We assessed the effect of Mertk expression on the phagocytic ability of RCS RPE by a cell culture assay. RPE monolayers were incubated with purified rat OS for 4 h. The cells were then washed, fixed, and either permeabilized with Triton X-100 or not, and OS were detected with a monoclonal antibody directed against rhodopsin to determine the number of bound and ingested OS. Bound and ingested OS in RCS RPE, virus-infected RCS RPE, and wild-type RPE cells were quantified (Fig. 5A). Consistent with the results of Chaitin and Hall (15), RCS RPE cells bound similar numbers of OS as did wild-type RPE (p =
0.70) but could not ingest any of the bound OS. By contrast, wild-type RPE cells ingested 73% of total OS. Introduction of Mertk into RCS RPE dramatically altered the difference in OS ingestion between these two types of RPE cells (Fig. 5 A).

Infection of RCS RPE by Ad-Mertk at an m.o.i. of 2 caused the infected cells to ingest as many OS as wild-type RPE (p = 0.84). The number of bound OS was not altered in comparison to uninfected RCS (p = 0.47) or wild-type RCS-rdy+ cells (p = 0.70). The functional rescue that we observed is specifically due to Mertk because infection with Ad-GFP did not result in an increase in OS ingestion (p = 0.86).

Additional Mertk did not increase OS ingestion in wild-type RPE (Fig. 5A). Cells infected with Ad-Mertk at an m.o.i. of 2 ingested an equal amount of OS as compared with uninfected cells (p = 0.88), despite the higher level of Mertk in virus-infected cells (Fig. 5B). The slight increase in the number of bound OS (Fig. 5A) was not significant (p = 0.34).

**Mertk Co-localizes with OS**—The preceding data demonstrate that the RPE is indeed the site of action of Mertk with respect to OS phagocytosis. To assess whether Mertk is directly involved in OS ingestion, we examined the subcellular localization of the protein during a time course of RPE phagocytosis. Double immunolabeling of uninfected wild-type RPE cells during the first 3 h of phagocytosis revealed progressive co-localization of Mertk with OS (Fig. 6). A small number of OS co-localized with punctate Mertk signals after 1 h of incubation (Fig. 6, A–C, arrowheads). The number OS with accompanying Mertk increased by 2 h (Fig. 6, D–F), and by 3 h, the patterns of OS staining and punctate Mertk staining were almost identical (Fig. 6, G–I). These data indicate that Mertk gradually accumulates around OS with a time course that is similar to the progressive ingestion of OS (7, 15).

**Mertk Phosphorylation during RPE Phagocytosis**—If Mertk is directly involved in OS ingestion, as suggested by the co-localization data, then the receptor should be increasingly activated after addition of OS. To test this hypothesis, we examined the activation state of the receptor by monitoring the extent of phosphorylated tyrosine residues in Mertk during the first 3 h of OS phagocytosis. To obtain a sufficient number of cells, we used RPE-J, a well characterized rat RPE cell line with the ability to phagocytize OS (12, 26), and infected them with Ad-Mertk (m.o.i. = 2) to enhance detection of tyrosine-phosphorylated receptor molecules. After 1 h of OS incubation, we did not detect activated Mertk by immunoprecipitation with anti-Mertk and immunoblotting with an anti-phosphotyrosine monoclonal antibody (Fig. 7). However, after 3 h, two tyrosine-phosphorylated forms of Mertk were readily detected (Fig. 7). Activated Mertk was not observed in the absence of added OS (Fig. 7). A parallel experiment with uninfected RPE-J cells yielded similar results, except the signals were much weaker (data not shown).
DISCUSSION

We have transferred wild-type Mertk to RCS RPE cells, which we showed lacked Mertk protein, and completely corrected the phagocytic defect of the cells. These results definitively establish the RPE as the site of action of Mertk with respect to OS phagocytosis, as suggested previously (24). Moreover, the fact that Ad-Mertk-infected RCS cells bound and ingested OS at wild-type levels demonstrates that the rest of the phagocytic machinery in RCS RPE is normal and that RCS RPE cells have the same potential as wild-type RPE for OS internalization. Reported biochemical abnormalities of RCS RPE, such as increased calcium membrane conductance and altered cAMP and inositol phosphate second messenger metabolism (31), are likely secondary to the loss of Mertk function.

Mertk did not affect the binding phase of phagocytosis in primary RPE cell culture; modest overexpression of the protein (m.o.i. = 2) in RCS RPE and wild-type RPE cells did not significantly increase OS binding. These results are consistent with previous reports indicating that $\alpha_\beta\gamma$ integrin is a major

**FIG. 6.** Mertk co-localizes with OS during phagocytosis. Primary wild-type RPE cells were incubated with OS for 1–3 h. An anti-rhodopsin monoclonal antibody combined with Texas Red-conjugated anti-mouse IgG was used to label OS (A, D, G, and J). An anti-Mertk polyclonal antibody combined with Oregon Green-conjugated anti-rabbit IgG was used to label Mertk (B, E, and H). Pre-immune serum was used as a negative control to demonstrate the specificity of anti-Mertk labeling (E). Single color images from the same field were merged (C, F, I, and L). Arrowheads indicate selected examples of co-localization sites A–F. Bar, 10 μm.

**FIG. 7.** Outer segments activate Mertk. RPE-J cells were infected with Ad-Mertk, and 2 days later cells were incubated with or without exogenous rat OS for 1–3 h. Equal amounts of immunoprecipitated samples were resolved by 6% SDS-PAGE and analyzed by immunoblotting with an anti-phosphotyrosine monoclonal antibody.
OS binding receptor for RPE in cell culture (12, 14). However, αβ2 integrin cannot be essential for retinal structure and function because mice with a targeted disruption of the β2 gene have normal retinal anatomy and electroretinography at 1 and 4 months of age,2 despite the fact that disc shedding and phagocytosis begin around P12. The apparent discrepancy between the role of αβ2 integrin in cell culture and in vivo may result from physical differences in the process of RPE phagocytosis in these two settings. In vivo, OS are closely apposed to RPE microvilli, whereas in cell culture, purified OS are suspended in culture medium and added to cells. Thus, OS binding in cell culture may not be relevant to, or may be substantially different from, the normal in vivo OS phagocytic process. By contrast, Merk is required for RPE phagocytosis of OS both in vivo (23) and in cell culture (the present study).

We generated a polyclonal antibody suitable for immunoblotting and demonstrated that the antibody specifically recognizes 190–200- and 150–160-kDa forms of Merk in the RPE and assorted other tissues. These sizes are significantly larger than the molecular weight predicted on the basis of the rat primary amino acid sequence. We found that a large majority of the excess molecular weight is due to the presence of N-linked oligosaccharides and that the two forms arise from differential glycosylation. Both forms of the receptor present in RPE-J cells can be activated by OS (Fig. 7). It therefore appears that both forms are functional and probably localize to the plasma membrane.

Merk appears to be an integral component of the phagocytic machinery. During the early stages of RPE phagocytosis, Merk progressively co-localized with OS. The time course of co-localization matched that of the activation of Merk, as measured by tyrosine phosphorylation, suggesting that a close association with OS may be required to activate the receptor. Because Merk only stimulated OS internalization and not binding, the protein must be critical for the ingestion phase. The delayed activation of Merk is consistent with the observed initial delay in the kinetics of OS ingestion by cultured RPE cells (7, 12, 15). By 3 h of incubation, however, substantial OS ingestion has occurred (7), and about 70% of total OS are ingested by 4 h (Fig. 5A). The fact that nearly all OS were accompanied by punctate Merk signals at 3 h (Fig. 6) suggests that the receptor becomes internalized with OS as part of the phagosome. Further studies are required to address the turnover of Merk.

The requirement for Merk in both RPE phagocytosis of OS in the RCS rat and macrophage phagocytosis of apoptotic cells in the Mertk mouse (32), combined with the general similarities between RPE phagocytosis and the uptake of apoptotic cells by macrophages and other professional phagocytes, suggests that the two processes may share mechanistic features. Activation of Merk could trigger an intracellular signaling pathway that controls rearrangement of cytoskeletal components necessary for OS or apoptotic cell ingestion.

Phosphotyrosine accumulates within actin cups that form immediately beneath the site of apoptotic cell ingestion during macrophage phagocytosis (33). Moreover, apoptotic cell uptake by professional and non-professional phagocytes requires a tyrosine kinase signaling pathway to activate CrkII and Rac (33, 34). The Caenorhabditis elegans homologs CED-2 (CrkII) and CED-10 (Rac) are required for engulfment of apoptotic cells, demonstrating an ancient origin to at least part of this pathway (35). This signaling pathway may also be activated during RPE phagocytosis of OS.

It is not yet known whether phosphatidylserine plays a key role in the recognition of OS by RPE cells, as it does in the recognition of apoptotic cells by macrophages (36). It is interesting that annexin V binds avidly to purified rat OS, indicating that phosphatidylserine is exposed on the outside. A secreted ligand of Merk, Gas6 (28, 37), binds phosphatidylserine (38) and may serve as a bridge between OS and Merk at the RPE plasma membrane during phagocytosis, as we suggested previously (18). Consistent with this model, Hall and colleagues (39) recently reported that Gas6 stimulates phagocytosis of exogenous OS by rat primary RPE cells. It will be of great interest to determine whether Gas6 plays a key role in RPE phagocytosis and/or internalization of apoptotic cells in vivo.

In summary, we have demonstrated that Merk is an integral component of the RPE phagocytic process in cell culture, in which it probably functions to trigger ingestion of bound OS. Future studies on the interaction of Merk with upstream and downstream proteins will help to elaborate the molecular mechanism of RPE phagocytosis. The common requirement for Merk in uptake of apoptotic cells by professional phagocytes and OS phagocytosis by RPE indicates that elucidation of this mechanism may have general implications.

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