Silencing of the *CHM* Gene Alters Phagocytic and Secretory Pathways in the Retinal Pigment Epithelium

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PURPOSE. Choroideremia (CHM) is an X-linked progressive degeneration of the retinal pigment epithelium (RPE), photoreceptors, and choroid caused by mutations in the *CHM* gene, which encodes Rab escort-protein-1 (REP-1). REP-1 enables posttranslational isoprenyl modification of Rab GTPases, proteins that control vesicle formation, movement, docking, and fusion. The aim of this study was to determine the effect of REP-1 depletion on vesicular trafficking in phagocytic and secretory pathways of human RPE.

METHODS. In vitro, REP-1 expression was inhibited in human fetal RPE (hfrPE) cells by siRNA knockdown and its effects measured on the uptake of bovine photoreceptor outer segments (POS), proteolysis of POS rhodopsin, phagosomal pH, phagosome fusion with early and late endosomes/lysosomes, and polarized secretion of cytokines.

RESULTS. Depletion of REP-1 in human RPE cells did not affect POS internalization but reduced phagosomal acidification and delayed POS protein clearance. REP-1 depletion also caused a decrease in the association of POS-containing phagosomes with late endosomal markers (Rab7, LAMP-1) and increases in the secretion of monocyte chemotactic protein (MCP-1) and interleukin (IL)-8 by hfrPE cells.

CONCLUSIONS. Lack of REP-1 protein expression in hfrPE cells leads to reduced degradation of POS most likely because of the inhibition of phagosome-lysosome fusion events and increased constitutive secretion of MCP-1 and IL-8. These observations may explain the accumulation of unprocessed outer segments within the phagolysosomes of RPE cells and the presence of inflammatory cells in the choroid of patients with CHM. (*Invest Ophthalmol Vis Sci.* 2010;51:1143–1150) DOI:10.1167/iov.09-4117

The mechanism of degeneration of the retinal pigment epithelium (RPE), photoreceptors, and choroid in choroide-remia (CHM) remains largely unknown. This X-linked, monogenic disorder is caused by mutations in the ubiquitously expressed *CHM* gene, which encodes Rab escort-protein-1 (REP-1).^{1,2} REP-1 participates in the geranylgeranylation of ras-related GTPases, Rab proteins, that are integral to the trafficking of vesicles in endocytic and exocytic pathways.^{3–6} Patients

with CHM have a relative or complete lack of REP-1,^{7–9} which conceivably cannot be compensated for in the eye by REP-2, a product of the CHM-like gene and the only other REP.¹⁰ Alternatively, the relative affinity of some Rab/REP-2 complexes for geranylgeranyl transferase may be less than that of Rab/REP-1.¹¹ The lack of REP-1 within cells of the eye has been hypothesized to affect the transport of opsin to the photoreceptor outer segment, the apical distribution of melanosomes in the RPE, and the phagocytosis of photoreceptor outer segments by the RPE.¹² For example, the RPE in the zebrafish *chm* model fails to process outer segments within the phagolysosomes.¹³

The RPE is a monolayer of polarized pigmented cells that lies between the neuroretina and the choroid and that plays a crucial role in the function and survival of the retina by performing a number of essential functions such as the phagocytosis of photoreceptor outer segments and the maintenance of immune privilege within the eye by polarized balanced secretion of anti-inflammatory and proinflammatory cytokines, among them interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1.^{14,15} Various lines of evidence point to the RPE as having a central role in the pathogenesis of CHM. Rodrigues et al.,¹⁶ studying the eye of a 19-year-old CHM affected male, identified a few pigment-filled macrophages within the retina that had attached outer segment structures, phagosomes, occasional melanin granules, and “curvilinear rod-like profiles.” The authors suggested the possibility of a defect in outer segment phagocytosis. Bonilha et al.,¹⁷ reporting the pathology on a 91-year-old female CHM carrier, noted the absence of RPE apical microvilli and basal infoldings. Moreover, the RPE donor basal surface was dominated by the presence of banded fibers composed of clumps of widely spaced collagen. Bruch’s membrane and the space between the basal membrane of the RPE contained many smooth and bristle-like-coated vesicles. RPE ultrastructural changes were consistent with cells that could not carry out several nurturing functions.

The aim of our study was to determine the effect of REP-1 depletion on cellular trafficking in endocytic and exocytic pathways in human RPE cells. Here we show that lack of REP-1 expression leads to reduced degradation of POS by RPE cells, most likely because of the inhibition of the phagosome-lysosome fusion events, and increased constitutive secretion of MCP-1 and IL-8 by RPE cells. These observations may explain the accumulation of unprocessed outer segments within the phagolysosomes of RPE cells and the finding of inflammatory cells in pathologic eye specimens from patients with CHM.

METHODS

Primary Culture and Transfections

Human fetal RPE (hfrPE) cells were generously provided by the laboratory of Sheldon Miller (National Eye Institute, National Institutes of Health, Bethesda, MD). Cells were cultured in MEM- α modified medium with additional supplements and 5% fetal bovine serum, as described previously.¹⁸ For all the experiments, hfrPE cells were used at passage 1. Before the experiments, cells were seeded at high density

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($1 \times 10^5/\text{cm}^2$) on 96- or 6-well plates or chamber slides and allowed to differentiate for 2 weeks. To study the polarized secretion of cytokines, hRPE cells were seeded onto 0.4- μm pore polyester transwells (Transwell; Corning Inc., Corning, NY)¹⁵ and were maintained for 3 to 4 weeks before experiments.

CHM gene expression was knocked down by transfecting hRPE with *CHM* (REP-1) siRNA using an siRNA pool (ONTARGETplus; Dharmacon, Lafayette, CO) and transfection reagent (DharmaFECT 4; Dharmacon). Cells transfected with nontarget siRNA were used as a control. The efficiency of silencing was determined by Western blot analysis of REP-1 expression up to 7 days, including 96 hours after transfection, to verify that the knockdown was present and persisted throughout the experiment.

Preparation of Photoreceptor Outer Segments

Photoreceptor outer segments (POS) were isolated on 25% to 60% sucrose gradients from fresh bovine eyes according to the methods of Finnemann et al.¹⁹ and were covalently labeled with pH-sensitive rhodamine-based dye (pHrodo; Invitrogen, Carlsbad, CA) or pH-insensitive Alexa Fluor 488 dye (Invitrogen). Briefly, POS were resuspended in 10% sucrose, 100 mM sodium bicarbonate buffer, pH 8.5, and then incubated with either rhodamine-based succinimidyl ester (pHrodo; Invitrogen) or Alexa Fluor 488 at a final concentration of 1 mg dye/10 mg protein during 1 hour in the dark at room temperature. Labeled POS were washed twice in 10% sucrose, 20 mM Na-phosphate, pH 7.2, and twice in 2.5% sucrose in DMEM and then were resuspended in 2.5% sucrose in DMEM.

Phagocytosis Assays

hRPE cells were challenged with rhodamine-based dye-labeled POS at a ratio of 10 POS/cell for 2 hours (pulse) followed by up to 25 hours of chase time. Phagocytic activity was evaluated by confocal microscopy and an automated fluorescence microplate reader. Two factors were used to validate the assay: cytochalasin D, a toxin that promotes net depolymerization during actin filament turnover²⁰ and causes near complete abrogation of POS internalization, and bafilomycin A1, a specific inhibitor of vacuolar-type H⁺-ATPase, that increases intraphagosomal pH.²¹ Data were presented as fluorescence intensity ratios (FIR), which were calculated by dividing rhodamine-based dye fluorescence counts by DAPI (nuclei) counts, thereby normalizing for the number of hRPE cells.

Assay for Degradation of Phagocytosed POS Proteins

Confluent hRPE cells were challenged with unlabeled POS for 2 hours (pulse) and chased up to 23 hours. Cells were lysed in RIPA buffer (Pierce, Rockford, IL) containing a cocktail of protease inhibitors (Complete; Roche, Indianapolis, IN). Degradation of phagocytosed POS opsin was determined by Western blot analysis of hRPE cell protein extracts using a mouse monoclonal anti-rhodopsin antibody (Millipore/Chemicon, Temecula, CA). Integrated optical density (IOD) of the bands corresponding to the rhodopsin monomer were quantified, normalized to β -actin expression (IOD norm), and compared with the normalized IOD at 2-hour pulse/0-hour chase, which was set as 100%. Results were expressed as follows: % Degradation = $100\% - \text{IOD (norm) 23-hour chase} / \text{IOD (norm) 0-hour chase} \times 100\%$.

Measurement of Phagosomal pH

Phagosomal pH was measured according to Savina et al.²² with modifications. Briefly, POS were labeled with rhodamine-based dye (pH sensitive) and Alexa Fluor 488 (pH insensitive). The hRPE cells, plated on 96-well plates and transfected with nontarget or *CHM* siRNA, were pulsed with labeled POS for 2 hours, chased for 25 hours, and analyzed with an automated fluorescence microplate reader. The ratio of the fluorescence intensity between the two dyes was determined. Values were compared with a standard curve obtained by resuspending the

cells that had phagocytosed POS for 2 hours at a fixed pH (ranging from pH 4.5 to pH 7) and containing 0.1% Triton X-100. In some cases, RPE cells were incubated for 1 hour before a pulse with 5 μM cytochalasin D or 400 nM bafilomycin A1.

Immunofluorescence Staining and Confocal Microscopy

hRPE cells plated in eight-well chamber slides (Laboratory-Tek; Nalge Nunc International, Naperville, IL) were continuously fed with POS over 6 hours, then were fixed in 4% paraformaldehyde-PBS, permeabilized, and blocked with normal goat serum in 0.05% Tween-20/PBS (PBST). The cells were double stained with antibodies to rhodopsin and LAMP-1 or Rab7A. Nuclei were stained with 4',6'-diamino-2-phenylindole (DAPI; 1 $\mu\text{g}/\text{mL}$ in PBS). Secondary antibodies used were conjugated with Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen). Confocal microscopy was performed with a laser scanning confocal microscope (SP2, with TCS software version 11.04; Leica, Exton, PA) and 40 $\times$ or 63 $\times$ oil objectives. Magnifications varied, and scale bars are therefore digitally included in some of the pictures.

Image Analysis

Rhodamine-based dye POS particle counting and quantitative colocalization analysis of POS rhodopsin and late endosomal markers, LAMP1, and Rab7A in hRPE cells were performed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Pearson's correlation coefficient was calculated using JACoP Plugin.²³ Pearson's correlation coefficient²⁴ depends on the amount of colocalized signals in both channels; values range from 1 to -1 , with 1 representing complete positive correlation, -1 representing negative correlation, and zero representing no correlation.

Phagosome Isolation

Phagosomes were isolated according to Hoppe et al.²⁵ Briefly, after transfection of hRPE cells with either *CHM* or nontarget siRNA in 6-well plates, phagocytosis was initiated by adding 40 μL of a 10-mg/mL suspension of opsonized carboxylated paramagnetic latex beads (1 μm in diameter; Dynal; Invitrogen, Oslo, Norway). After 6 hours or 24 hours, cells were collected by gentle scraping and centrifugation at 200g. Cells were then homogenized in buffer containing 250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES, pH 7, and protease inhibitors (Complete; Roche). Phagosomes were isolated using magnet extraction (Dynal; Invitrogen). Laemmli buffer (2 $\times$) was used to elute hRPE proteins associated with latex beads.

Western Blot and Band Densitometry Analyses

Proteins, separated by SDS-PAGE on 4% to 20% gradient mini-gels and transferred onto a 0.45- μm nitrocellulose membrane (Invitrogen), were probed with one of the following antibodies: rabbit anti-human LAMP-1, rabbit anti-human EEA-1, rabbit anti-Rab5A, rabbit anti-Rab 7A (all from Cell Signaling, Danvers, MA), or mouse anti-REP-1, clone 2F1 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected with a corresponding second anti-IgG antibody conjugated with horseradish peroxidase (Zymed Laboratory, Inc., San Francisco, CA) and imaged on x-ray film using chemiluminescent substrates (SuperSignal West Pico or SuperSignal West Dura; Pierce). Densitometric analysis was performed with ImageJ software. Results are presented as IOD normalized to β -actin expression.

Secretion of Cytokines by hRPE Cells

For the cytokine secretion assays, the hRPE cells were seeded onto 0.4- μm pore polyester transwells (Transwell; Corning Inc., Corning, NY) as described by Maminishkis et al.,¹⁸ maintained for 3 to 4 weeks before experiments, and transfected with *CHM* either or nontarget siRNA. Cells were incubated for 24 hours in serum-free medium containing glutamine-penicillin-streptomycin and nonessential amino ac-

FIGURE 1. Depletion of REP-1 expression in hRPE cells. (A) Whole cell lysate representing equal amounts of total protein were analyzed by Western blotting with antibodies, as indicated. Silencing of the *CHM* gene did not affect the expression levels of REP-2 and Rab. (B) Quantification of Western blot analysis shows that the REP-1 protein level was reduced to 80% to 90% by *CHM* siRNA transfection compared with nontarget siRNA control (mean $\pm$ SD; $n = 3$).

ids. The supernatants from both apical and basal compartments were then assayed for cytokine levels with a commercial technology (SearchLight; Pierce Biotechnology) and by using commercially available ELISA for human MCP-1, IL-8, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN). The transwells were constructed with unequal volumes in the apical and basal baths (0.5 and 1.5 mL, respectively).¹⁵ The amounts of secreted cytokines were corrected for this volume difference and expressed in nanograms or picograms per milliliter.

Statistical Analysis

Unless otherwise noted, all experiments were repeated at least three times, and data are presented as the mean $\pm$ SEM. Statistical comparisons were made using the Student's *t*-test (unpaired, two tailed, unless otherwise specified; Excel; Microsoft, Redmond, WA). Differences were considered to be significant if $P < 0.05$.

RESULTS

Effect of REP-1 Deficiency on Phagocytosis of POS by hRPE Cells

Human fetal RPE cells (hRPE)¹⁸ were selected as a model to study the role of REP-1 in the phagocytosis of POS. Western blot analysis of lysates from hRPE cells (Figs. 1A, B) showed that the expression level of REP-1 was reduced by 80% to 90% in cells transfected with *CHM* siRNA compared with nontargeting siRNA. This effect persisted for at least 7 days. The expression levels of REP-2 and Rab GTPases did not change, confirming the specificity of silencing (Fig. 1A).

To analyze whether depletion of REP-1 would alter POS phagocytosis by human RPE cells, we labeled POS with pH-sensitive rhodamine-based dye, which fluoresces only in an acidic environment and allowed us to track any changes in the internalization of particles by cells. Cells transfected with *CHM* or nontarget siRNA or treated with 400 nM Bafilomycin A1 or 5 μ M cytochalasin D were challenged with rhodamine-based dye-POS for 2 hours (pulse) followed by up to 25 hours of chase time (Figs. 2A, 2A2). Cytochalasin D, an inhibitor of POS internalization, served as a negative control. Bafilomycin A1 was selected as a control for intraphagosomal pH elevation. Phagocytosis by hRPE cells in response to *CHM* siRNA, bafilomycin, or cytochalasin D treatment was expressed as FIR (see Methods). Depletion of REP-1 expression resulted in a $31.2\% \pm 1.9\%$ ($n = 3$) reduction in FIR of internalized rhodamine-based dye-POS particles after 21 hours of chase compared with nontarget siRNA (Fig. 2A1). In the presence of bafilomycin A1 (400 nM), a $30.0\% \pm 9.6\%$ ($n = 3$) decrease in FIR was noticeable after 2 hours of chase (Fig. 2A2). Cytochalasin D (5

μ M) totally abolished internalization of POS and caused a dramatic decrease in the intensity of rhodamine-based dye fluorescence ($83.13\% \pm 0.66\%$; $n = 3$) after 6 hours of chase compared with control.

The lower intensity of rhodamine-based dye fluorescence in REP-1-depleted cells could be the result of either a lower number of internalized particles or an elevated pH of POS-containing phagosomes. To test this hypothesis, we first counted the numbers of POS particles internalized by hRPE cells using confocal microscopy. Analysis of REP-1-depleted hRPE cells, after phagocytic challenge with rhodamine-based dye-POS, showed a trend toward fewer rhodamine-based dye-POS particles internalized by the cells compared with nontarget siRNA control (Fig. 2B). Analysis of maximal projections of multiple areas of the samples using ImageJ software showed however, that, the differences in the numbers of internalized particles were not statistically significant (Student's *t*-test; $P > 0.05$; $n = 5$).

To measure phagosomal pH in RPE cells, we labeled POS with pH-sensitive (rhodamine-based [pHrodo]) and pH-insensitive (Alexa Fluor 488) fluorescent dyes. The ratio in fluorescence intensity between the two dyes reflected the pH in the phagosomal environment. In *CHM* siRNA-transfected hRPE cells, phagosomal pH dropped from $\text{pH } 6.78 \pm 0.02$ ($n = 4$) after 2 hours of pulse with POS and 0 hours of chase time to $\text{pH } 5.96 \pm 0.05$ ($n = 4$) after 25 hours of chase. Phagosomal pH in REP-1-depleted cells after 25 hours of chase was approximately 0.2 pH units higher than in nontarget siRNA control in which phagosomal pH was 5.78 ± 0.03 ($n = 4$) after 25 hours of chase. The differences were statistically significant (Student's *t*-test; $P < 0.05$; Fig. 2C). The effect of bafilomycin A1 on phagosomal acidification was more pronounced after 4 hours of chase time. Thus, in the presence of bafilomycin A1 (400 nM), phagosomal pH was on average 0.41 pH units higher than the nontarget siRNA control. In the presence of cytochalasin D, phagosomal pH was approximately 1.08 pH units higher after 25 hours of chase compared with nontarget siRNA control. Thus, the lower intensity of rhodamine-based dye fluorescence in REP-1-depleted cells was likely the result of a relative failure of POS-containing phagosomes to properly acidify.

Given that a moderate elevation of phagosomal pH could result in decreased rates of degradation of POS rhodopsin in REP-1-depleted cells, we assessed the proteolysis of 41-kDa monomeric rhodopsin by hRPE cells. Cells transfected with nontarget siRNA degraded $73\% \pm 7\%$ ($n = 4$) POS rhodopsin in 2 hours of pulse and 23 hours of chase. Depletion of REP-1 in hRPE cells significantly delayed POS protein clearance (only $44\% \pm 8\%$ [$n = 4$] of POS rhodopsin was degraded in 23 hours of chase). Degradation of POS rhodopsin by the hRPE cells

FIGURE 2. Phagocytosis of pH-sensitive rhodamine-based dye-labeled POS and changes in phagosomal pH of hRPE cells transfected with CHM siRNA. **(A1, A2)** Fluorescence intensity of pH-sensitive rhodamine-based dye-POS internalized by hRPE cells after 2 hours (pulse) and the indicated periods of chase time. Fluorescence of pH-sensitive rhodamine-based dye-POS was normalized to DAPI (nuclei). Values given are mean $\pm$ SEM ($n = 3$). Representative results from three independent experiments are shown. **(A1)** Depletion of REP-1 expression resulted in a reduction of fluorescence intensity of internalized pH-sensitive rhodamine-based dye-POS compared with nontarget siRNA (asterisks; Student's t -test; $P < 0.05$). **(A2)** Fluorescence intensity of internalized pH-sensitive rhodamine-based dye-POS in the presence of bafilomycin A1 or cytochalasin D compared with nontarget siRNA control. **(B)** Confocal images of transfected hRPE cells challenged with pH-sensitive rhodamine-based dye-POS (red) for 2 hours (pulse) and 0 hour and 25 hours of chase. Cell nuclei were labeled with DAPI (blue). Maximal projections of

representative whole cell x - y scans acquired at 1- μ m intervals are shown. Scale bar, 20 μ m. The graph represents the analysis of the numbers of internalized pH-sensitive rhodamine-based dye-POS particles using ImageJ software. Bars represent mean $\pm$ SEM ($n = 5$), internalized POS per 100 RPE cells as indicated (black bars, nontarget siRNA; gray bars, CHM siRNA). Differences in the numbers of internalized particles were not statistically significant (Student's t -test; $P > 0.05$). **(C)** Changes in phagosomal pH of hRPE cells challenged with POS labeled with pH-sensitive rhodamine-based dye (pH-sensitive dye) or Alexa Fluor 488 (pH-insensitive dye). The ratio in fluorescence intensity between the two dyes reflected the pH in the phagosomal environment. Phagosomal pH in REP-1-depleted cells was elevated after 25 hours of chase compared with nontarget siRNA control (asterisks; Student's t -test; $P < 0.05$). Bafilomycin A1 (400 nM) and cytochalasin D were used as controls. Values given are averages $\pm$ SEM ($n = 4$). Results of a representative experiment of two independent experiments are shown.

treated with cytochalasin D was used as a negative control (6% $\pm$ 10%; $n = 5$; Figs. 3A, B).

Next, we sought to determine whether REP-1 knockdown modified the composition of phagosomes as a result of altered fusion events with endosomes and lysosomes. Phagosomes from hRPE cells were isolated using serum-opsonized magnetic latex beads, and phagosome-associated proteins were subjected to Western blot analysis for the presence of markers for early (EEA1, Rab 5A) and late (LAMP-1 and Rab 7A) endosomes. Although magnetic latex beads are not a natural target for RPE cells, they are a widely accepted tool for studying phagocytosis because they yield the best purity of phagosomes, surpassing the sucrose density flotation procedure.²⁵ After REP-1 knockdown in hRPE cells and after 24 hours of phagocytosis, the association of LAMP-1 with latex-containing phagosomes decreased to 66.2% $\pm$ 0.7% ($n = 3$), and the association of Rab7A decreased to 50.1% $\pm$ 11.6% ($n = 3$) compared with nontarget siRNA control, which was set as 100%. At the same time, the amounts of Rab5A and EEA1 proteins in CHM siRNA-transfected cells associated with latex bead-containing phagosomes were elevated in the first 6 hours (Rab5A, 142.4% $\pm$ 16.4%, $n = 3$; EEA1, 149.2% $\pm$ 12.2%, $n = 3$) and declined after 24 hours (Rab5A, 81.5% $\pm$ 4%, $n = 3$; EEA1, 19.6% $\pm$ 2%, $n = 3$) of phagocytosis compared with nontarget siRNA control (Figs. 4A, B).

To define whether the association of late endosomal markers with phagosomes containing POS would also be affected in REP-1-deficient RPE cells, we continuously fed the cells with POS during 6 hours and double stained them with antibodies to rhodopsin (POS) and markers for the late endosomes (LAMP-1 and Rab7A). Confocal microscopy and colocalization analysis

revealed significantly fewer POS-containing phagosomes colocalized with LAMP-1 (Fig. 5A, overlay and colocalized points) and Rab7A (Fig. 5B, overlay and colocalized points) in REP-1-deficient RPE cells. For quantitative analysis of colocalization, we calculated Pearson's correlation coefficient using JACoP Plugin of ImageJ software. This coefficient varied from 0 to 1, the former corresponding to nonoverlapping images and the latter reflecting 100% colocalization between both images. We found that, in CHM siRNA-transfected cells, Pearson's correlation coefficient for LAMP1 and POS rhodopsin dropped to 0.143 compared with control, for which Pearson's correlation coefficient was 0.420 (Fig. 5A). For Rab7A and POS rhodopsin proteins, Pearson's correlation coefficient decreased to 0.151 in REP-1-depleted cells compared with control, for which Pearson's correlation coefficient was 0.186 (Fig. 5B).

Silencing of CHM Gene Affects Secretion of Chemokines by Human Fetal RPE Cells but Not Growth Factors

We demonstrated that silencing of the CHM gene caused an increase in the secretion of IL-8 and MCP-1 by hRPE cells to both the apical and the basal compartments compared with nontarget siRNA-transfected cells. The IL-8 concentration in the apical bath changed from 2.26 $\pm$ 0.26 ng/mL ($n = 4$) for control cells to 6.92 $\pm$ 1.39 ng/mL ($n = 4$) for REP-1 depleted RPE cells. The IL-8 concentration numbers in the basal compartments were 0.98 $\pm$ 0.08 ng/mL and 4.03 $\pm$ 1.01 ng/mL ($n = 4$) for control and REP-1-depleted cells, respectively. The concentrations of MCP-1 in the apical compartment were 43.36 $\pm$ 1.35 ng/mL for control cells and 51.50 $\pm$ 0.63 ng/mL

FIGURE 3. Silencing of *CHM* (REP-1) impairs degradation of POS rhodopsin by hRPE cells. Confluent hRPE cells transfected with either *CHM* siRNA or nontarget siRNA or treated with cytochalasin D were challenged with unlabeled POS for 2 hours and chased for 23 hours. Then samples were lysed in RIPA buffer, and (A) Western blot analysis of the levels of rhodopsin in the protein extracts was performed. (B) IOD of the bands corresponding to the rhodopsin monomer were quantified, normalized to that of β -actin, and compared with the IOD at 2 hours, which was set as 100%. All values are mean $\pm$ SEM, with $n = 4$. Representative results from three independent experiments are shown. Depletion of REP-1 in hRPE cells significantly delayed POS protein clearance (asterisks; Student's *t*-test; $P < 0.05$). Degradation of POS rhodopsin by the hRPE cells treated with cytochalasin D provided a negative control.

($n = 4$) for REP-1-depleted RPE cells. The concentration of MCP-1 in the basal bath changed from 10.20 ± 0.09 ng/mL ($n = 4$) for control cells to 14.93 ± 0.94 ng/mL ($n = 4$) for REP-1-depleted RPE cells. The secretion of growth factors (VEGF and bFGF) did not change significantly (Figs. 6).

DISCUSSION

In the present study, we relied on a model of human fetal RPE that exhibits many of the physiological characteristics of adult

RPE.¹⁸ Knockdown experiments in the RPE offer the opportunity to address questions regarding whether the phagocytosis of outer segments might be affected by the relative lack of REP-1. Knockdown may also allow the opportunity to understand at what stage—surface binding, internalization (phagosome formation), fusion with endosomes and lysosomes (phagosome maturation), or degradation of the phagosomal content—the process of phagocytosis could be altered. Furthermore, knockdown experiments offer the opportunity to study the effect not only on phagocytic pathways but also on secretory pathways in the RPE.

Here we demonstrate that lack of REP-1 protein expression leads to reduced degradation of POS by RPE cells, most likely because of an inhibition of the phagosome-lysosome fusion events, and increased constitutive secretion of MCP-1 and IL-8 by RPE cells.

Effect of REP-1 Deficiency on Phagocytosis of POS by hRPE Cells

Phagocytosis of the POS consists of multiple processes such as POS recognition by RPE cells, binding, ingestion, phagosome maturation, and degradation of phagocytosed material. Interruption of any of these steps could lead to the accumulation of unprocessed material inside or outside RPE cells and eventually to retinal degeneration.

Here we show that lack of REP-1 expression results in altered degradation of POS by RPE cells or at least in the degradation of the major protein of rod outer segments, rhodopsin. Our data provide possible explanations for the observations of others studying clinical cases of CHM^{16,17} and animal models¹³ which have suggested a defect in phagocytosis.

To elucidate at what stage the process of phagocytosis is altered in REP-1-deficient cells, we first studied the internalization of POS labeled with rhodamine-based dye, which fluoresces only in an acidic environment. Depletion of REP-1 expression caused an average 20% reduction in fluorescence intensity of internalized rhodamine-based dye-POS particles compared with nontarget siRNA, likely as a result of the failure of POS-containing phagosomes to acidify properly. Quantitative analysis revealed that the differences in the numbers of rhodamine-based dye-POS ingested particles were not statistically significant compared with nontarget siRNA-treated cells. We found that the transfection of RPE cells with CHM siRNA resulted in an average 0.2 pH increase of phagosomal pH compared with nontarget siRNA-trans-

FIGURE 4. Association of early and late endosome/lysosome markers with paramagnetic latex bead-containing phagosomes altered after silencing of *CHM* (REP-1). hRPE cells were transfected either with *CHM* siRNA or nontarget siRNA and challenged with fetal bovine serum-coated magnetic latex beads during indicated periods of time, after which phagosomal fractions were obtained from cell homogenates with a magnet. (A) RPE proteins were then subjected to Western blot analysis using specific antibodies recognizing EEA1, Rab5A-early endosome markers, LAMP1, and Rab7A-late endosome/lysosome markers. (B) IOD of the immunoreactive bands was analyzed by densitometry using ImageJ software. Results are presented as relative optical density of phagosome-associated markers normalized to that of β -actin as the loading control. All values are mean $\pm$ SEM, with $n = 3$ (asterisks; Student's *t*-test; $P < 0.05$). Representative results from two independent experiments are shown.

FIGURE 5. Silencing of the *CHM* gene decreased the association of the late endosome markers LAMP-1 and Rab7A with POS-containing phagosomes in hRPE cells. hRPE cells transfected with either *CHM* siRNA or nontarget siRNA were continuously fed with POS over 6 hours and double stained with antibodies to rhodopsin (POS, red) and (A) LAMP-1 (green) or (B) Rab7A (green). Nuclei were stained with DAPI (blue). Maximal projections of representative whole cell *x-y* scans acquired at 0.2- μ m intervals are shown. Scale bars, 20 μ m. Overlays of the channels and the images showing the points of colocalization between (A) LAMP1 or (B) Rab7A and POS are shown, with the labeled proteins indicated above the pictures. Boxed images were magnified. Pearson's correlation coefficient revealed significantly fewer POS-containing phagosomes, which colocalized with LAMP-1 or Rab7A (overlay and colocalized points) in REP-1-deficient RPE cells.

fected cells. Several investigators have shown that acidification is a critical determinant in phagosome maturation.^{26,27} In addition, most lysosomal proteases have an optimal proteolytic activity between pH 5.5 and 6.5,²⁸ and phagocytosed particles can begin to be degraded only when the

phagosome reaches a sufficiently low pH. Deguchi et al.²¹ demonstrated in vivo that bafilomycin A1, a specific inhibitor of vacuolar-type H⁺-ATPase, which increases intraphagosomal pH on average by 0.6 pH units, markedly inhibited the degradation of POS in the phagolysosomes.

FIGURE 6. Silencing of the *CHM* gene affects the secretion of chemokines but not growth factors by hRPE cells. Values given are the concentrations of MCP-1, IL-8, and VEGF in ng/mL or bFGF in pg/mL in the serum-free media secreted by the cells in 24 hours to the apical or basal baths. All values are mean $\pm$ SEM, with $n = 4$. Representative results from two independent experiments are shown (asterisks; Student's *t*-test; $P < 0.05$).

Phagosomes are known to fuse first with early and late endosomes and then with lysosomes, thus progressively acquiring different proteins, such as Rab GTPases, the acidification machinery (V-type ATPase), and the lysosomal proteases from the endocytic pathway.^{26,28,29}

Rab GTPases have been implicated principally in the control of vesicle docking and fusion.^{3,30} In one of the earliest known maturation events, Rab5 is recruited to phagosomes and fuses with early endosomes. Bridging between the two compartments is thought to be the role of early endosome antigen 1 (EEA1), the Rab5 effector.^{26,31,32} Rab7 is essential for phagosome fusion with late endosomes or lysosomes.^{30,33} Impairment of Rab7 function limits the degree of acidification of the phagosomal lumen. LAMP1 is a glycoprotein that is specifically localized to acidic lysosome structure and essential for the recruitment of Rab7 to the phagosome.³²

We hypothesize that under-prenylation of multiple Rab proteins resulting from REP-1 deficiency in the *CHM* knockdown human RPE cells could cause alteration in the fusion of phagosomes with endosomes and lysosomes and, as a result, the elevation of phagosomal pH and a deficiency in the degradation of POS.

In our experiments, silencing of *CHM* in human RPE cells resulted in a distribution of Rab5A and EEA1 consistent with delayed phagosomal maturation. The amount of Rab5A and EEA1 protein associated with latex bead-containing phagosomes was elevated in the first 6 hours and declined after 24 hours of phagocytosis compared with nontarget siRNA-transfected cells. At the same time, the association of late endosomal markers, LAMP-1 and Rab7A, with latex-containing and POS-containing phagosomes was diminished at 6 and 24 hours of phagocytosis, indicating reduced fusion of late endosomes and lysosomes with newly internalized targets.

Our observations suggest that a lack of REP-1 protein expression leads to a deficiency in the degradation of POS by RPE cells, most likely because of the failure of POS-containing phagosomes to acidify properly as a result of inhibition of the phagosome-lysosome fusion events. Decreased rates of POS degradation by RPE cells could lead to an accumulation of unprocessed material inside the cells and to gradual functional decline over many years. According to Burke,³⁴ even subtle modifications of cell shape or subcellular organization could have profound consequences for the ability of the RPE to support the survival of adjacent photoreceptors over time.

Effect of REP-1 Deficiency on Secretion of Chemokines and Growth Factors by hRPE Cells

RPE cells are thought to play an important role in immune responses and may help maintain immune privilege within the eye by the secretion of anti-inflammatory and proinflammatory cytokines in a polarized manner. Some cytokines (chemokines IL-8 and MCP-1) and growth factors (VEGF, PEDF, bFGF) are constitutively secreted by RPE cells.^{14,15} In a similar fashion, we showed that significant levels of IL-8, MCP-1, bFGF, and VEGF were constitutively secreted by hRPE cells in vitro. IL-8 belongs to the CXC chemokine family and has strong chemotactic and activating properties for neutrophils. MCP-1 is a member of the CC chemokine family and is primarily chemotactic for monocytes and lymphocytes. The polar secretion of IL-8 and MCP-1 by human RPE cells may contribute to the regulation of immune and inflammatory responses in the posterior segment of the eye.^{14,15}

Cytokines are secreted through a variety of pathways and by the actions of specific regulatory molecules.³⁵ Secretory vesicle traffic is thought to be regulated by a family of Rab GTPases. To date, 11 Rab isoforms (Rab3A/B/C/D, Rab4A, Rab8B, Rab11B, Rab26, Rab27A/B, and Rab37) have been implicated

in regulated secretion by certain cells^{36,37} and can either stimulate or inhibit the secretory activity.^{36,38} Rab27A is of particular interest because Rab27A is expressed in the RPE and choroid, the two cell layers that degenerate in CHM, and Rab27A is found to be under-prenylated in CHM lymphoblasts.^{39,40} Izumi et al.⁴⁰ showed that the Rab27 subfamily regulates various exocytotic pathways using multiple organelle-specific effector proteins. In addition, Oynebraten et al. demonstrated⁴¹ that IL-8/CXCL8 can be stored in the Weibel-Palade body of endothelial cells, which are Rab27-positive, in contrast to GRO/MCP-1-containing granules, which are Rab27-negative. We hypothesize that the under-prenylation of certain Rabs, such as Rab27A, in the RPE of patients with CHM may alter the constitutive level of secretion of chemokines, among them IL-8 and MCP-1.

In our experiments, silencing of the *CHM* gene caused an increase in the secretion of MCP-1 and IL-8 in hRPE cells. REP-1 depletion in the eyes of patients with CHM and carriers may then result in elevated amounts of MCP-1 and IL-8 and could partially explain the presence of inflammatory cells within the choroid of the eye of a 91-year-old CHM carrier.¹⁷ A similar explanation may apply to the T-lymphocytic infiltration found in the choroid of a 30-year-old man with CHM.⁴² The increased secretion of the proangiogenic chemokines MCP-1 and IL-8 as a result of REP-1 depletion may also underlie rare clinical cases of intraretinal and choroidal neovascularization seen in patients with CHM.^{43–46}

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