Chapter 95
Live Imaging of LysoTracker-Labelled Phagolysosomes Tracks Diurnal Phagocytosis of Photoreceptor Outer Segment Fragments in Rat RPE Tissue *Ex Vivo*

Yingyu Mao and Silvia C. Finnemann

Abstract Renewal of rod photoreceptor outer segments in the mammalian eye involves synchronized diurnal shedding after light onset of spent distal outer segment fragments (POS) linked to swift clearance of shed POS from the subretinal space by the adjacent retinal pigment epithelium (RPE). Engulfed POS phagosomes in RPE cells mature to acidified phagolysosomes, which accomplish enzymatic degradation of POS macromolecules. Here, we used an acidophilic fluorophore LysoTracker to label acidic organelles in freshly dissected, live rat RPE tissue flat mounts. We observed that all RPE cells imaged contained numerous acidified POS phagolysosomes whose abundance per cell was dramatically increased 2 h after light onset as compared to either 1 h before or 4 h after light onset. Lack of organelles of similar diameter (of 1–2 μm) in phagocytosis-defective mutant RCS rat RPE confirmed that LysoTracker live imaging detected POS phagolysosomes. Lack of increase in lysosomal membrane protein LAMP-1 in RPE/choroid during the diurnal phagocytic burst suggests that formation of POS phagolysosomes in RPE *in situ* may not involve extra lysosome membrane biogenesis. Taken together, we report a new imaging approach that directly detects POS phagosome acidification and allows rapid tracking and quantification of POS phagocytosis by live RPE tissue *ex situ*.

Keywords Acidification · LAMP-1 · Lysosomes · LysoTracker · Phagolysosomes · Phagosomes · Phagocytosis · Photoreceptor outer segments · RCS · RPE

Abbreviations

POS	Photoreceptor outer segment fragments
RPE	Retinal pigment epithelium

S. C. Finnemann (✉) · Y. Mao
Department of Biological Sciences, Center for Cancer, Genetic Diseases and Gene Regulation, Fordham University, Larkin Hall, 441 East Fordham Road, Bronx, NY 10458, USA
e-mail: finnemann@fordham.edu

C. Bowes Rickman et al. (eds.), *Retinal Degenerative Diseases,* Advances in Experimental Medicine and Biology 854, DOI 10.1007/978-3-319-17121-0_95

95.1 Introduction

Diurnal shedding and clearance of photoreceptor outer segment fragments (POS) by the retinal pigment epithelium (RPE) promotes continuous outer segment renewal that is important for long-term viability and function of vertebrate photoreceptors (Young 1967; Young and Bok 1969). In the mammalian eye, POS shedding and engulfment are precisely synchronized by light and circadian regulation to take place immediately after light onset (LaVail 1976). As a result, numbers of phagosome organelles containing engulfed POS in the RPE *in situ* of wild-type mice and rats reach a daily peak 1–2 h after light onset (LaVail 1976; Nandrot et al. 2004; Nandrot et al. 2007).

The steep decline of detectable POS phagosomes in the RPE after the daily burst of POS uptake implies that phagosomes rapidly mature to acidified phagolysosomes, in which digestive hydrolases efficiently degrade POS components. The daily maturation process of POS phagosomes to phagolysosomes remains incompletely understood. Fusion as well as "kiss-and-run" connections with *bona fide* lysosomes likely both contribute to the acidification of phagolysosomes, which carry the lysosomal membrane marker protein LAMP-1 (Bosch et al. 1993). We hypothesized that digestive organelles of the RPE *in situ* may differ at times of active POS clearance as compared to other times with respect to size, distribution, abundance, or extent of acidification to accomplish timely POS degradation. Labeling with LysoTracker biosensor acidified organelles in live rat RPE tissue in freshly dissected flat mounted eyecups, we observed acidified POS phagosomes in wild-type (but not phagocytosis-defective RCS) rat RPE that dramatically increased in abundance 2 h after light onset. This formation of acidified POS phagolysosomes in wild-type RPE did not correlate with a detectable increase in LAMP-1.

95.2 Materials and Methods

95.2.1 Animals

All procedures involving animals were performed following the ARVO statement for the "Use of Animals in Ophthalmic and Vision Research", and reviewed and approved by the Fordham University Institutional Animal Care and Use Committee. Sprague-Dawley and pink-eyed, tan-hooded RCS rats were raised and housed in 12-h light:12-h dark light conditions and fed *ad libitum*. 28–35-day-old rats were sacrificed by CO_2 asphyxiation following updated AVMA guidelines followed by immediate dissection of posterior eyecups and removal of neural retina.

95.2.2 LysoTracker Live Staining and Imaging

Freshly dissected eyecups were incubated in FluoroBrite™ DMEM with 0.4 µM LysoTracker Green DND-26 and 5 µM DAPI nuclei stain (all Life Technologies) at 37 °C for 15 min, flat-mounted and imaged on a Leica TSP5 confocal microcopy system. Images were compiled and processed using Adobe Photoshop CS4.

95.2.3 Immunoblotting Protein Quantification

Posterior eyecups containing RPE and choroid (R/Ch) and neural retinas (NR) were lysed in 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1% Triton X-100 supplemented with protease and phosphatase inhibitor cocktails. Lysates were analyzed by SDS-PAGE and immunoblotting for LAMP-1, PSD95 (both Cell Signaling), and RPE65 (Genetex). Bands were quantified by densitometry using GE ImageQuant TL 7.0.

95.3 Results

95.3.1 Live Imaging of LysoTracker Reveals POS Phagolysosomes and their Diurnal Peak in Abundance After Light Onset in Wild-type But Not Phagocytosis-Defective RCS Rat RPE in Eyecups Ex Vivo

To examine acidified cytoplasmic organelles in the RPE, we used a fluorescent acidophilic biosensor, LysoTracker, to stain and image live RPE tissue in freshly dissected, flat mounted eyecups from rats sacrificed 1 h before, 2 or 4 h after the onset of light. At all time points, we observed that the brightest LysoTracker positive compartments shared a diameter of 1–1.6 µm (Fig. 95.1a–c), which is similar to the size of early phagocytosed POS, suggesting that they are POS phagolysosomes. These acidified compartments were by far most abundant 2 h after light onset matching the diurnal burst of POS engulfment. In contrast, phagocytosis-defective RCS RPE contained almost no phagosomes but numerous small-size acidic compartments that are likely *bona fide* lysosomes (Fig. 95.1d)

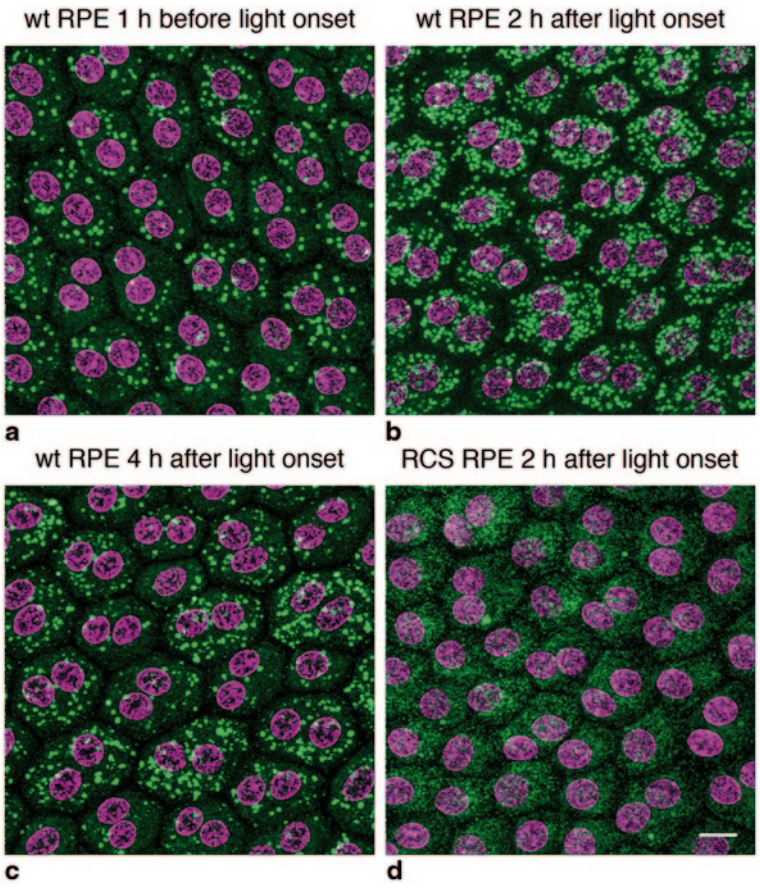

Fig. 95.1 Live imaging reveals diurnal increase of abundance of acidified phagolysosomes in live wild-type but not phagocytosis-defective RCS mutant rat RPE *ex vivo*. LysoTracker (*green*) and nuclei (*pink*) live staining of wild-type (wt) RPE in eyecup flat-mounts harvested at times as indicated, 1 h before (**a**), 2 h (**b**), or 4 h (**c**), after light onset or of RCS RPE 2 h after light onset (**d**). Representative fields of three independent experiments are shown. Images are maximum projections of z-stacks obtained using identical imaging parameters. Scale bar: 10 μm

95.3.2 Levels of Mature LAMP-1 in RPE/Choroid Do Not Change with Light Onset

LAMP-1 is a heavily glycosylated membrane protein that is primarily targeted to lysosomal membranes (Carlsson et al. 1988; Harter and Mellman 1992). The molecular size of rat LAMP-1 polypeptide is ~49 kDa. *N*- and *O*- glycosylation in the Golgi apparatus yields numerous forms of mouse LAMP-1 of 92–140 kDa (Andrejewski et al. 1999). In immunoblots, we detected 95–125 kDa forms in RPE/choroid and 75–95 kDa forms as well as a 49 kDa form (likely the immature precursor)

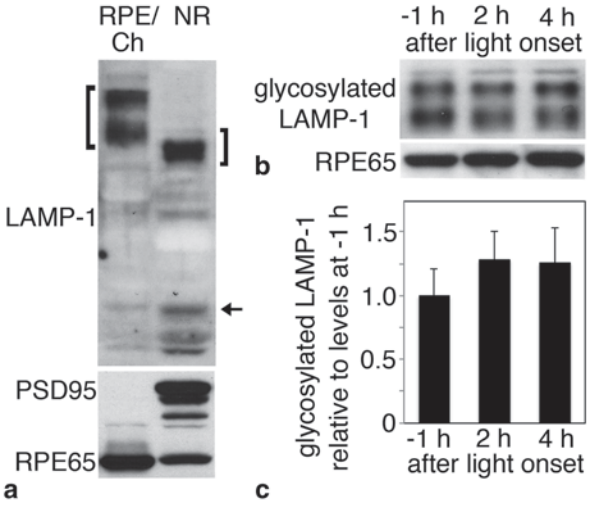

Fig. 95.2 Levels of lysosomal marker protein LAMP-1 do not change after light onset in rat posterior eyecups containing RPE and choroid. **a** Immunoblotting detects LAMP-1 in both RPE/choroid (RPE/Ch) and neural retina (NR) but higher molecular weight bands differ in size indicating differential glycosylation. *Open bracket* indicates glycosylated LAMP-1 in RPE lysate; *close bracket* indicates glycosylated LAMP-1 in retina lysate. *Arrow* indicates unglycosylated LAMP-1. RPE65 and PSD95 were detected on the same blot membrane to indicate enrichment of RPE and neural retina in rat eye fractions, respectively. **b** and **c** Levels of glycosylated LAMP-1 in RPE/choroid do not differ between 1 h before (−1 h), 2 or 4 h after (+2 h, +4 h) light onset. RPE65 detection of the same membrane is shown as loading control. *Bars* indicate relative level of all forms of glycosylated LAMP-1 normalized to RPE65. *Blots* show representative results (**a** and **b**). *Bars* show mean ± SD, of three independent experiments (**c**).

in neural retina (Fig. 95.2a). Levels of glycosylated LAMP-1 are similar in RPE/choroid samples collected before and after light onset (Fig. 95.2b, c), suggesting that POS phagolysosome formation is unlikely to involve a burst of lysosome membrane formation.

95.4 Discussion

In this study, we use LysoTracker biosensor to detect acidified phagosomes in live rat RPE in freshly dissected posterior eyecup flat mounts. To our knowledge, we report the first experimental approach that allows observing acidified phagolysosomes in live RPE tissue. It provides a rapid, simple, and direct assessment of the RPE's phagocytic load that is an ideal complement to established methods analyzing POS phagosomes in RPE tissue after fixation and processing (Young and Bok 1969; Gibbs et al. 2003; Sethna and Finnemann 2013).

We classify the LysoTracker-labelled compartments we observe in wild-type rat RPE as POS phagolysosomes based on (1) their similarity in size to POS phagosomes (Bosch et al. 1993); (2) their increased abundance at the time POS phagosomes peak in the RPE (LaVail 1976; Nandrot et al. 2004); and (3) their absence in phagocytosis-defective RCS RPE (Bok and Hall 1971; Mullen and LaVail 1976). Co-staining of these LysoTracker-positive phagosomes with antibodies specific to either opsin N- or C-terminus, known to differ in stability to RPE lysosomal processing (Esteve-Rudd et al. 2014; Wavre-Shapton et al. 2014), will allow in the future further specification of the content of acidified phagolysosomes and the POS digestion process of the RPE *in situ*.

We found that levels of glycosylated LAMP-1 in tissue extracts enriched in RPE do not increase at the diurnal peak in POS phagosome content in the RPE. Only glycosylated LAMP-1 reaches lysosomes. Thus, levels of glycosylated LAMP-1 are an indirect indicator of the overall quantity of intracellular membranes of lysosomal origin (assuming constant LAMP-1 membrane concentration). Further experiments are ongoing to confirm the preliminary implication of this finding that POS phagolysosomal membranes form largely at the expense of free lysosomal membranes.

Acknowledgments This study was supported by NIH grant EY13295.

References

Andrejewski N, Punnonen EL, Guhde G et al (1999) Normal lysosomal morphology and function in LAMP-1-deficient mice. J Biol Chem 274:12692–12701

Bok D, Hall MO (1971) The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. J Cell Biol 49:664–682

Bosch E, Horwitz J, Bok D (1993) Phagocytosis of outer segments by retinal pigment epithelium: phagosome–lysosome interaction. J Histochem Cytochem 41:253–263

Carlsson SR, Roth J, Piller F et al (1988) Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylactosaminoglycan. J Biol Chem 263:18911–18919

Esteve-Rudd J, Lopes VS, Jiang M et al (2014) *In vivo* and *in vitro* monitoring of phagosome maturation in retinal pigment epithelium cells. Adv Exp Med Biol 801:85–90

Gibbs D, Kitamoto J, Williams DS (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

Harter C, Mellman I (1992) Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. J Cell Biol 117:311–325

LaVail MM (1976) Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. Science 194:1071–1074

Mullen RJ, LaVail MM (1976) Inherited retinal dystrophy: primary defect in pigment epithelium determined with experimental rat chimeras. Science 192:799–801

Nandrot EF, Kim Y, Brodie SE et al (2004) Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking avb5 integrin. J Exp Med 200:1539–1545

Nandrot EF, Anand M, Almeida D et al (2007) Essential role for MFG-E8 as ligand for avb5 integrin in diurnal retinal phagocytosis. Proc Natl Acad Sci U S A 104:12005–12010

Sethna S, Finnemann SC (2013) Analysis of photoreceptor rod outer segment phagocytosis by RPE cells *in situ*. Methods Mol Biol 935:245–254

Wavre-Shapton ST, Meschede IP, Seabra MC et al (2014) Phagosome maturation during endosome interaction revealed by partial rhodopsin processing in retinal pigment epithelium. J Cell Sci 127(17):3852-3861. doi:10.1242/jcs.154757

Young RW (1967) The renewal of photoreceptor cell outer segments. J Cell Biol 33:61–72

Young RW, Bok D (1969) Participation of the retinal pigment epithelium in the rod outer segment renewal process. J Cell Biol 42:392–403