Chapter 11
In Vivo and in Vitro Monitoring of Phagosome Maturation in Retinal Pigment Epithelium Cells

Julian Esteve-Rudd, Vanda S. Lopes, Mei Jiang and David S. Williams

Abstract The ingestion and degradation of photoreceptor disk membranes is a critical and major role for the retinal pigment epithelium (RPE). To help elucidate the cellular events involved in this role, functional in vivo and in vitro assays need to be developed further. Here we propose a method to help monitor phagosome maturation, using antibodies against different epitopes of opsin. We show that antibodies specific for the C-terminus of opsin label only immature phagosomes located in the apical region of the RPE. In contrast, antibodies recognizing the N-terminus also label more mature phagosomes, located more basally. The combined use of antibodies against different opsin epitopes thus provides a valuable tool in the study of phagosome maturation in the RPE.

Keywords RPE · Phagocytosis · Opsin · Protein degradation · Phagosome

11.1 Introduction

Each day the tips of the photoreceptor outer segments (POSs) are ingested and degraded by the adjacent retinal pigment epithelium (RPE) cells [1]. A defect at any stage of this process can lead to photoreceptor impairment and degeneration. Photoreceptor degeneration in the RCS rat and *Mertk* knockout mouse is caused by

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a failure at the ingestion stage [2, 3]. After ingestion, phagosomes are transported from the apical RPE towards the basal side and undergo degradation by fusion with lysosomes [4–6]. Defects in degradation lead to the accumulation of undigested debris in the RPE, the formation of sub-RPE deposits, and photoreceptor degeneration, as shown in transgenic mice overexpressing mutant Cathepsin D, the main proteolytic enzyme involved in opsin degradation [7]. Phagosome degradation can also be impaired by defects in the transport of phagosomes and/or lysosomes, causing a delay in the fusion of the two organelles. Lack of MYO7A retards the transition of POSs from the apical to the basal RPE, resulting in a delay in phagosome digestion [8].

We have developed a method for measuring POS digestion in cultured mouse primary RPE cells [9, 10], based on previously described assays [2]. Here, we describe a more detailed method for the monitoring of POS maturation both in vivo and in cell culture, based on the detection of different epitopes by different opsin antibodies. This approach was suggested by previous immunoelectron microscopy (immunoEM) studies [11; and C.E. Futter, personnal communication]. The method allows us to differentiate early phagosomes from late-stage phagosomes and can be used to identify photoreceptor or RPE defects in mutant mice.

11.2 In Vivo Assessment of Phagosome Maturation in the RPE

Ultrathin retinal sections were obtained as described previously [12]. They were double-labeled with mAb1D4, which was generated in a mouse and specifically recognizes the C-terminus of opsin [13, 14], and pAb01 made in a rabbit against bovine opsin [15]. ImmunoEM of RPE showed that the mAb1D4 antibody labeled apical phagosomes, while the pAb01 antibody-labeled apical, central, and basal phagosomes (Fig. 11.1). Given the apical to basal migration of phagosomes during their maturation [5, 6], this result suggests that the C-terminus epitope of opsin Opsin is lost quickly during the phagosome maturation process.

11.3 Phagocytosis of POSs by Cultured Mouse RPE Cells

Primary cultures of RPE from wild-type mice were prepared, and the phagocytosis assay was performed as described [8, 9], with the following modifications. Cells, grown on transwell filters for 7–10 days, were incubated for 20 min with a suspension of 1 to 5×10^6 gradient-purified POSs. Unbound POSs were then removed by extensive washing with PBS, and cells were either fixed or incubated for an additional period ("chase" following the POS "pulse"), and then fixed (Fig. 11.2a). Immunofluorescence labeling of bound and total POSs was performed using a combination of mAb1D4 and pAb01 (Fig. 11.2). POSs that were only bound to the

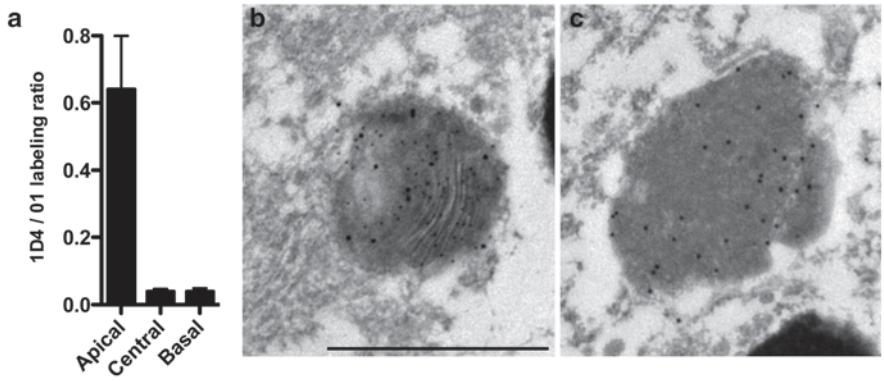

Fig. 11.1 ImmunoEM opsin labeling of RPE phagosomes. **a** Quantification of gold particles corresponding to mAb1D4 or pAb01 labeling on phagosomes located in the apical, central, and basal regions of the RPE. The concentration of immunogold label per area of all the phagosomes in each of the three regions of the RPE was measured for each antibody on double-labeled sections. A phagosome was identified by the presence of pAb01 labeling. Significant mAb1D4 labeling was present only on phagosomes in the apical region. **b**, **c** Electromicroscopies (EMs) showing labeled phagosomes from the apical (**b**) and basal (**c**) regions; mAb1D4 is indicated by the smaller 12-nm *gold* particles and pAb01 by the larger 15-nm particles. Scale bar: 1 μm

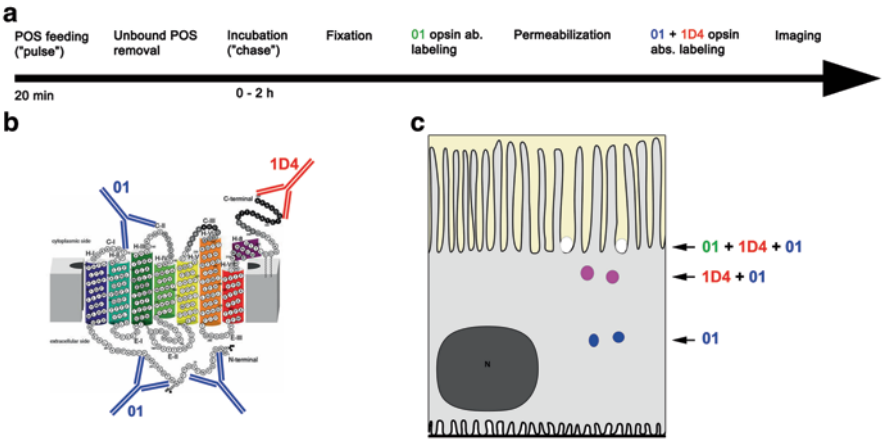

Fig. 11.2 Method for phagocytosis assay on RPE cells in culture using a combination of opsin antibodies. **a** Time course of phagocytosis assay and immunolabeling. Color-coded 01 and 1D4 correspond to secondary antibodies that were used to detect primary antibodies in each case (*green* corresponds to Alexa-488, *red* to Alexa-568 and *blue* to Alexa-647). **b** Scheme of opsin structure (From [16]. Permission pending) with different epitopes for pAb01 and mAb1D4 indicated. **c** Scheme illustrating possible combinations of opsin immunolabeling of bound and ingested POSs, after merging the three acquisition channels. POSs that are bound to the external cell surface (i.e., not ingested) are labeled with pAb01 before permeabilization, and mAb1D4 and pAb01 after permeabilization, and appear *white*. Phagosomes labeled with mAb1D4 and pAb01 after permeabilization appear in *magenta*. Phagosomes labeled only with pAb01 after permeabilization appear in *blue*

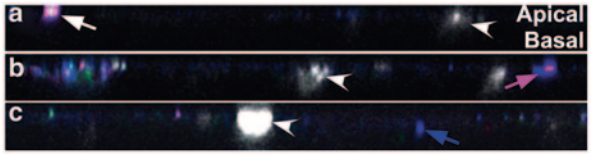

Fig. 11.3 Phagosome localization at different digestion stages in cultured RPE cells. **a, b, c** Orthogonal projections from a stack of confocal slices of a single RPE cell from a culture fed with POSs and immunolabeled as described. **a** POS that is bound to the external cell surface and labeled by all three secondary antibodies (*white arrow*). **b** Phagosome labeled with mAb1D4 and pAb01 after permeabilization (*magenta arrow*) in the apical region of the RPE cell. **c** Phagosome labeled with pAb01 only after permeabilization (*blue arrow*), located in the central soma region of the cell. *Arrowheads* indicate RPE cell nuclei stained with DAPI (shown in *white*). Scale bar: 20 μm

cell surface were labeled first, and then, after cell permeabilization by treatment with 50% ethanol, all POSs were labeled with mAb1D4 and pAb01, followed by Alexa-568 and Alexa-647 secondary antibodies. Ingested POSs were identified by subtracting the bound-only POSs (white) from the total POSs. POSs labeled by mAb1D4 are shown in magenta, while POSs labeled by pAb01 but not mAb1D4 are in blue (Fig. 11.2c). POSs at least 1 μm in diameter were counted at × 400 magnification in all cells within five fields of view chosen randomly. Images were collected with a Fluoview FV1000 confocal microscope (Olympus) and analyzed on ImageJ.

11.4 Assessment of Phagosome Maturation in Cultured RPE

To determine the localization of phagosomes on the apical-basal plane of RPE cells grown on filters, stacks of confocal slices separated by 0.3 μm were projected orthogonally (Fig. 11.3). Our results are consistent with our observations in vivo. Bound POSs appear at the apical surface of RPE cells. POSs labeled with both mAb1D4 and pAb01 after permeabilization are ingested but reside within the apical region, while POSs labeled only with pAb01 after permeabilization are located more basally.

We also performed quantitative assays to evaluate the digestion rate of phagosomes, by assessing the decay rate of different opsin epitopes. We observed that the mAb1D4 labeling disappeared more quickly than the pAb01 labeling during the chase period. Figure 11.4 illustrates an experiment with a chase period of 2 h. These experiments indicate that loss of a C-terminal epitope of opsin constitutes an early step in phagosome digestion.

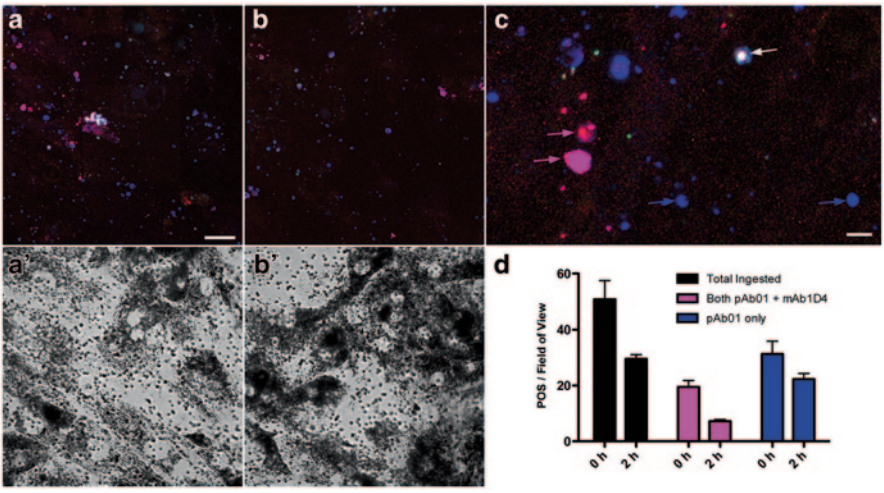

Fig. 11.4 Time-course of POS digestion in cultured RPE cells. **a, b** Micrographs showing immunofluorescence labeling of POSs with mAb1D4 + pAb01, as described, immediately after removal of the POSs (end of pulse, 0 h) (**a**) and after 2 h subsequent incubation (chase) (**b**). **a'**, **b'** Bright field images of the same areas shown in **a** and **b**, respectively. **c** High-magnification detail of **a**, showing phagosomes at different stages of maturation. **d** Quantification of POSs in cultured RPE cells after 0 h (pulse only) and 2 h chase periods. Graph shows total ingested POSs (*black*), ingested POSs that were labeled with both pAb01 and mAb1D4 (*magenta*), and ingested POSs that were labeled with pAb01 only—no mAb1D4 labeling (*blue*). The overall reduction in the number of phagosomes during the 2-h chase was 42 %. The number of POSs labeled with both mAb1D4 and pAb01 decreased by 63 %, while the number of POSs labeled with pAb01 alone decreased by 29 %. Scale bars: **a**, **b**: 20 µm; **c**: 5 µm

11.5 Conclusions

Observations of phagosome location and the rate of phagosome degradation indicate that the mAb1D4, which binds to a C-terminal epitope on opsin, recognizes only early phagosomes. Thus, in comparison with an opsin antibody that binds to epitopes elsewhere, different stages of phagosome maturation can be identified. This assay could be used to help identify defects in phagosome degradation in retinal disease.

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