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Unraveling the Molecular Mystery of Retinal Pigment Epithelium Phagocytosis

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88.1 Introduction

Photoreceptor outer segments (POS) in the retina are susceptible to photodamage. The clearance of damaged molecules and retinoid cycle require the shedding of POS at the tip of the outer segments in a diurnal rhythm, and the phagocytosis of shed POS by retinal pigment epithelium (RPE) cells (Strauss 2005). Defects in RPE phagocytosis signaling, such as MerTK phagocytic receptor, cause retinal degeneration. Our understanding of RPE phagocytosis is relatively limited. Only a handful of phagocytosis ligands and receptors of RPE cells were identified and characterized on case-by-case basis with daunting challenges. The barrier is how to identify unknown signaling molecules in an unbiased manner. Here we summarize a unique strategy of phagocytosis-based functional cloning for unbiased identification of phagocytosis ligands, which can be used as molecular probes to further delineate phagocytosis receptors, signaling cascades, and interactions with POS.

88.2 Materials and Methods

88.2.1 Open Reading Frame Phage Display

Open reading frame (ORF) phage display cDNA library of adult mouse eyes was used for phagocytosis-based functional selection in ARPE19 cells, as described (Fig. 88.1a) (Caberoy et al. 2010a). After three rounds of phage selection, individual clones were randomly picked from plates of enriched phages and analyzed for their phagocytosis activity in the same cells. Positive clones were identified by DNA sequencing.

88.2.2 RPE Phagocytosis Assay

Membrane vesicles were prepared from the retina of wild-type or Tulp1^{-/-} mice, as described and labeled with CFSE (Caberoy et al. 2010a). Alternatively, membrane vesicles were prepared from Neuro-2a cells expressing membrane-targeted green fluorescent protein (mGFP). Vesicles were used for ARPE19 phagocytosis in the presence or absence of FLAG-tagged Tulp1, and phagocytosed fluorescence signals were analyzed by confocal microscopy (Caberoy et al. 2010a).

88.2.3 MerTK Ligand Studies

For co-immunoprecipitation (Co-IP) study, FLAG-tagged Tulp1 was expressed in HEK293 cells. Cell lysates were prepared and incubated with Mer-Fc (MerTK extracellular domain fused to human IgG1 Fc domain, R&D Systems) at 4°C, followed by protein A resin. The resin was washed and analyzed by Western blot using anti-FLAG mAb. For MerTK autophosphorylation, D407 RPE cells were stimulated with Tulp1 for 30 min at 37°C. The cells were lysed and analyzed by Western blot using anti-phospho-MerTK, as described previously (Caberoy et al. 2010c). For intracellular signaling study, ARPE19 cell phagocytosis of mGFP-labeled Neuro-2a vesicles was stimulated by Tulp1 in the presence or absence of excessive Mer-Fc. The cells were fixed, permeabilized, and incubated with antibodies against nonmuscle myosin II-A heavy chain (NMMII-A), followed by Texas Red-labeled secondary antibody and confocal microscopy analysis. For phagocytosis prey binding, Jurkat cells were induced for apoptosis by etoposide (40 µM) for 16 h and washed (Caberoy et al. 2010c). Healthy and apoptotic Jurkat cells were incubated with FLAG-tagged Tulp1, followed by FITC-labeled anti-FLAG antibody and flow cytometry analysis.

88.3 Results

88.3.1 Unbiased Identification of Tulp1 as a Phagocytosis Ligand

We recently characterized the feasibility to enrich phagocytosis ligands by phage display (Caberoy et al. 2009). The question is whether this approach can be used for identification of unknown RPE phagocytosis ligands. Owing to uncontrollable protein reading frame, phage display with conventional cDNA library identified high percentage of non-ORF clones encoding short unnatural peptides (Li and Caberoy 2010), which have minimal implication in protein interaction networks. To address this problem, we constructed an ORF phage display cDNA library from adult mouse eye with minimal reading frame problem (Caberoy et al. 2010b). We designed a strategy of phagocytosis-based functional cloning for unbiased identification of RPE phagocytosis ligands (Fig. 88.1a) (Caberoy et al. 2010a). After three rounds of phage selection, functional analysis of enriched phage clones identified Tulp1 as a new ligand. Tulp1 as a ligand for RPE phagocytosis was independently validated by reduced phagocytosis with membrane vesicles prepared from Tulp1^{-/-} mouse retina (Fig. 88.1b). Recombinant FLAG-tagged Tulp1 was also capable of stimulating RPE phagocytosis (Fig. 88.1c). Tulp1 highly expressed in photoreceptor inner segments (Milam et al. 2000) has no classical signal peptide, but was characterized for its unconventional secretion (Caberoy and Li 2009), suggesting that it has the physiological access to its receptor on RPE surface.

88.3.2 Characterization of MerTK as a Tulp1 Receptor

To identify the receptor of Tulp1, we analyzed its binding to several known RPE phagocytosis receptors by Co-IP. The results showed that Tulp1 was a MerTK-binding protein (Fig. 88.2a). However, proteins with binding activity to a receptor may not always be real ligands, because proteins could bind to the receptor simply through nonligand binding sites without receptor activation and signaling cascade. A genuine ligand should be able to activate the cognate receptor and elicit receptor-specific signaling cascade. MerTK autophosphorylation has been widely used as a surrogate marker of its activation. The results showed that Tulp1 induced MerTK autophosphorylation in RPE cells (Fig. 88.2b). Moreover, Tulp1 induced rearrangement of NMMII-A (Fig. 88.2c), which was previously described as a MerTK-dependent signaling process (Strick et al. 2009). Excessive Mer-Fc blocked Tulp1-induced NMMII rearrangement (Fig. 88.2c).

Phagocytosis ligands, such as Gas6 and protein S of the only two unknown MerTK ligands, discriminatively bind to apoptotic cells, but not healthy cells, for selective phagocytic clearance of apoptotic cells. Likewise, these ligands in theory should specifically bind to shed POS vesicles, but not to unshed POS, so that only shed POS will be phagocytosed by RPE cells. However, it is technically difficult to prepare unshed POS for the analysis. Thus, we analyzed Tulp1 binding to apoptotic and healthy Jurkat cells. The results showed that Tulp1 selectively bound to apoptotic cells, but not healthy cells (Fig. 88.2d). Deletion of Tulp1 C-terminal end of 44 amino acids causes retinal degeneration with an unknown mechanism (Banerjee et al. 1998). This deletion also drastically reduced Tulp1 binding to apoptotic cells. Thus, these results indicated that the C-terminal domain of Tulp1 is essential for binding to phagocytosis preys.

88.4 Discussion

RPE phagocytosis is critical for the clearance of shed POS and the maintenance of the precise length of POS for its viability and photoexcitability. A handful of phagocytosis ligands, including Gas6, protein S, MFG-E8, were previously identified for a limited number of RPE phagocytosis receptors, such as MerTK, $\alpha_v \beta_5$ integrin and CD36. Most of these ligands and receptors were originally identified in macrophage phagocytosis on case-by-case basis with challenges and subsequently verified in RPE phagocytosis. The problem is that we really do not know the relative contribution of these ligands and receptors to RPE phagocytosis unless we thoroughly map and characterize all of them. However, identification of unknown signaling pathways for RPE phagocytosis is daunting in the absence of any molecular probe to begin with. By exploiting its unique functional character of phagocytosis, we identified Tulp1 as a new RPE ligand in the absence of receptor information by ORF phage display coupled with phagocytosis-based functional selection. This further led to identification of its receptor MerTK and characterization of the related intra-cellular signaling cascade. The C-terminal domain of Tulp1 with phagocytosis prey binding activity could be used as a bait to further identify its binding partners on apoptotic cells and POS vesicles in the future by yeast two hybrid system, mass spectrometry or even ORF phage display (Caberoy et al. 2010b). In summary, this study illustrated that the combination of ORF phage display and phagocytosis-based functional selection is capable

of identifying phagocytosis ligands in the absence of receptor information (Fig. 88.2e). The intriguing part is that the heterogeneous ORF phage display cDNA library, when mixing with unknown heterogeneous receptors on phagocyte surface, is able to pull out new ligands with specific function. Identified ligands could be used as molecular probes to delineate their receptors, intracellular signaling cascades and binding partners on phagocytosis preys. By exploiting the only common functional characteristic of all phagocytes, this new strategy is able to unravel the mystery of molecular phagocyte biology in the absence of any molecular information. Conceivably, we should be able to thoroughly map all the ligands and receptors for RPE and other phagocytes by this new strategy to improve our capacity to modulate phagocytosis activity for disease therapy.

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Fig. 88.1.

Unbiased identification of phagocytosis ligands by ORF phage display. **(a)** The selection scheme of ORF phage display. **(b)** Membrane vesicles prepared from Tulp1^{-/-} mouse retina have reduced phagocytosis activity in ARPE19 cells. **(c)** FLAG-Tulp1 facilitates ARPE19 cell phagocytosis with membrane vesicles prepared from Neuro-2a cells. (Caberoy et al. 2010a with permission from *Exp. Cell Res.*)

Fig. 88.2.

Tulp1 as a new MerTK ligand. **(a)** Co-IP of Tulp1 and Mer-Fc. **(b)** MerTK autophosphorylation induced by Tulp1. Gas6 (50 nM) was included as a positive control. Western blots were analyzed using antibodies against phospho-MerTK, MerTK, or control RPE65 protein. **(c)** Tulp1-induced NMII-A rearrangement is blocked by excessive Mer-Fc. Red signal for NMII-A; green signal for GFP-labeled phagocytosed cargos; yellow signal, overlapping of the red and green signals. **(d)** Tulp1 binds to apoptotic cells, but not healthy cells. Deletion of C-terminal 44 amino acids abolished Tulp1 binding to apoptotic cells. (Caberoy et al. 2010c with permission from *EMBO J.* for a–d). **(E)** Unbiased mapping of phagocytosis ligands, receptors, intracellular signaling cascades and binding partners on preys by ORF phage display with phagocytosis-based functional selection