14 Roles of Integrin Receptors in the Daily Phagocytosis of Photoreceptor Outer Segment Fragments by the RPE

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Advances in genomics and molecular biology have led, over the last decade, to the identification of numerous molecules that form the phagocytic machinery of the retinal pigment epithelium (RPE). This research has also shown that the RPE phagocytic mechanism belongs to a group of related clearance mechanisms that share common phagocytic receptors. However, the precise roles of any of these receptors in the daily phagocytic burst characteristic of the RPE remain to be explored. Here, we focus on contributions of the integrin receptor αvβ5 to outer segment phagocytosis by RPE cells. *In vitro* functional assays and developmental expression studies indicate an important function for αvβ5 receptors in phagocytosis by human, rat, and mouse RPE. We discuss recent evidence that signaling pathways via αvβ5 integrin in both directions across the RPE plasma membrane may promote synchronized, timely, and repeated phagocytosis unique to RPE. αvβ5 integrin engagement initiates a downstream signaling response that may serve to coordinate the activity of multiple components of the RPE phagocytic machinery. Furthermore, regulation of activities of αvβ5 integrin receptors themselves by RPE signaling mechanisms may prevent phagocytosis by RPE cells upon completion of their daily clearance of shed photoreceptor outer segment fragments.

1. Introduction

The retinal pigment epithelium (RPE) is a highly specialized monolayer epithelium that forms the outermost layer of the retina. Function and integrity of the neural retina strictly depend on activities of the RPE. Polarized RPE cells adhere tightly to the underlying Bruch's membrane at their basal surface. The tight junctions of RPE cells effectively separate RPE basal from apical membrane domains and choroidal from retinal tissue, forming the blood-retina permeability barrier. The specialized microvilli of the RPE apical surface do not face a lumen like those of most other epithelia. Rather, they reach into the subretinal space where they make contacts with the interphotoreceptor matrix and the outer segment portions of photoreceptor rods and cones. Adhesive contacts and functional interactions between RPE and photoreceptor outer segments are likely dynamic since photoreceptor activity is regulated by light and circadian mechanisms. This is particularly evident in the contributions of RPE and photoreceptors cells to the synchronized renewal of photoreceptor outer segments.

In order to ensure lifelong function, both photoreceptor rod and cone neurons continuously replace their outer segments (Young, 1967). This renewal process is initiated in the connecting cilium. New membranous disks carrying photosensitive opsin pigments are inserted into the proximal end of the outer segment from the base of the inner segment. With further disk synthesis, disks progressively move towards the distal end of the outer segments. To maintain constant outer segment length, the distal, most aged outer segment tip detaches once daily in a process called photoreceptor disk shedding. Shedding precedes a burst of phagocytic activity by the RPE that efficiently removes shed OS from the retina and recycles their components (Young and Bok, 1969).

The phagocytic clearance of shed photoreceptor outer segment fragments (OS) by RPE cells is crucial to maintain photoreceptor health. Failure of RPE cells to engulf OS causes accumulation of OS debris in the subretinal space followed by rapid photoreceptor degeneration and blindness in the Royal College of Surgeons (RCS) rat strain (Dowling and Sidman, 1962; Mullen and LaVail, 1976). As discussed in chapter 13, a single gene defect, in the *mer* gene encoding Mer receptor tyrosine

kinase (MerTK), abolishes efficient OS phagocytosis by RCS RPE cells (D'Cruz *et al*., 2000; Nandrot *et al*., 2000). The catastrophic effect of the RCS mutation illustrates that photoreceptor function and, consequently, vision, strictly depend on RPE phagocytosis of spent OS.

RPE cells are the most active phagocytes known in nature. In the mammalian eye, one RPE cell serves approximately 30 photoreceptor cells, each of which sheds ~7% of its outer segment mass per day (Young, 1967; Young, 1971). Therefore, each RPE cell must completely dispose of 25,000 to 30,000 OS disks before its next phagocytic challenge (Besharse and Defoe, 1998). RPE cells do not normally divide in the adult mammalian eye. As continuous OS renewal involves daily RPE phagocytosis, each individual RPE cell phagocytoses more material over a lifetime than any other cell type in the body. The continuous nature of photoreceptor outer segment renewal implies that even minor delays or inefficiencies in OS clearance by RPE cells will gradually cause undigested OS components to accumulate. Indeed, lipofuscin storage bodies containing a complex mix of proteins and lipids accumulate in human RPE cells over time and may be associated with incomplete turnover of shed OS material (Feeney, 1978). Recent *in vitro* studies have shown that lipofuscin components may directly impair RPE function and viability (Finnemann *et al*., 2002b; Holz *et al*., 1999; Sparrow *et al*., 1999). This suggests that impaired RPE phagocytosis of OS may contribute to the development or progression of age-related retinal disease such as age-related macular degeneration.

OS renewal in higher vertebrates is synchronized by circadian rhythms that are influenced by the daily dark-light cycle (Goldman *et al*., 1980). While rods shed their outer segments primarily at the onset of light, cones shed at the onset of dark. For example, RPE cells in the rod-dominant rat retina contain peak numbers of phagosomes at ~2 hours after the onset of light in the morning (LaVail, 1976). In contrast, RPE cells of the cone rich lizard retina exhibit increased number of phagosomes ~2 hours after dusk (Young, 1977). Thus, RPE cells phagocytose once or twice daily depending on whether they serve rods, cones or both. There is no evidence that the phagocytic activity of the RPE is directly regulated by circadian rhythms. However, RPE cells in the eye face circadian OS shedding to which they react with rhythmic phagocytosis. It has long

been proposed that RPE cells may downregulate their uptake activity following a phagocytic burst since untimely and potentially damaging engulfment of photoreceptor tips has never been observed. We have yet to understand how inactivation of molecular components of the phagocytic machinery may serve to control RPE phagocytic function.

2. Experimental Approaches to RPE Phagocytosis

The traditional animal model to study RPE phagocytosis is the rat. Phenotype-oriented research has long compared retina and RPE of rat strains with wild-type RPE phagocytic function (e.g. Long Evans, Sprague Dawley) with those of the mutant RCS rat strain, the naturally occurring animal model of non-phagocytic RPE (Bourne *et al*., 1938; Dowling and Sidman, 1962; Edwards, 1977; Mullen and LaVail, 1976). More recently, knockout and transgenic animal technologies have begun to provide powerful tools for the generation of new animal models of altered RPE function, producing targeted mutations in specific genes known to be expressed in the RPE (Duncan *et al*., 2003; Gibbs *et al*., 2003). Because of our better knowledge of mouse genetics, the mouse is the preferred animal model for such genotype-oriented research that analyzes the role of a specific gene product in particular RPE functions.

Both rats and mice have in common that they are born blind. Early postnatal photoreceptor and RPE cells in these animals are immature. Maturation occurs during the first two postnatal weeks. It comprises the formation of outer segments by photoreceptor rods and cones, as well as the development of highly elongated apical microvilli and changes in protein expression and cell polarity by RPE. Tight adhesion of murine RPE and neural retina does not exist until postnatal day 8 (PN8) (Mayerson *et al*., 1985). Daily shedding of OS and their phagocytosis by RPE commence in rats at ~PN12 (Ratto *et al*., 1991). Upregulation of expression or apical relocalization of RPE proteins coinciding with the onset of RPE adhesion or phagocytosis suggests that these proteins may play a role in these activities (Marmorstein *et al*., 1998). Therefore, rat and mouse eyes provide ideal model systems to identify molecules involved in RPE-neural retina interactions.

The phagocytic uptake of spent OS by RPE cells *in vivo* can be studied by analyzing eyecup cross-sections obtained from eyes of experimental animals or, more rarely, human donors postmortem. Evaluating the histology of eyecup sections recorded by light or transmission electron microscopy provides a powerful tool to qualitatively observe phagocytosis by RPE *in vivo.* Quantification of RPE phagocytosis *in vivo* based on counting the number of OS phagosomes present in RPE cells is laborious and time consuming. Importantly, each sample only represents a snap shot of this continuous RPE activity. To record the entire phagocytic process multiple samples harvested at different time points of the circadian rhythm must therefore be analyzed. Variability between individual animals and small sample sizes restricted by the amount of effort required to analyze each sample may thus obscure minor differences in RPE phagocytic activity between experimental groups of animals. Furthermore, the different size and appearance of early and late phagosomes can render identification of phagocytosed OS difficult. Accurate quantification of phagocytosed material in RPE *in vivo* remains therefore largely restricted to large phagosomes at early time points after engulfment that can still be unequivocally recognized by the presence of stacked outer segment membrane disks. Finally, RPE phagocytosis *in situ* cannot easily be analyzed separately from burst of photoreceptor disk shedding. Thus, an *in vivo* phenotype of altered RPE phagocytosis may be a consequence of a primary defect in aging or shedding of distal OS tips.

Two types of *in vitro* RPE cell model systems allow studying OS phagocytosis by RPE cells separately from photoreceptor outer segment shedding. First, RPE cells can be isolated and maintained as a primary culture (Edwards, 1977). Primary, unpassaged RPE cells in culture retain many characteristics of RPE cells *in situ*: they keep their original pigment granules, they form a monolayer of hexagonal epithelial cells with extended apical microvilli (Bonilha *et al*., 1999). Although primary RPE cells lose some of the polarized protein distribution of RPE *in situ*, they recognize, bind, and internalize isolated photoreceptor outer segment fragments (Edwards and Szamier, 1977; Marmorstein *et al*., 1998). Notably, primary RPE can be prepared from wild-type and mutant donor animals thus allowing to directly assess the effect of a particular genotype on RPE phenotype and phagocytic function.

Permanent RPE cell lines provide a second type of *in vitro* RPE model system. Several different cell lines originally derived from human [e.g. ARPE-19 (Dunn *et al*., 1996) and d407 (Davis *et al*., 1995)] or rat RPE [e.g. RPE-J, (Nabi *et al*., 1993)] are freely available to the research community and have been characterized by numerous investigators. These immortalized RPE cells lack pigmentation and exhibit protein expression, surface polarity, and growth behavior characteristic to each cell line but different from that of primary RPE. The OS binding and engulfment kinetics of stable RPE cell lines are generally slower than those of primary RPE cells, which may be due to lower levels of expression or due to delayed activation of any constituent of the RPE phagocytic machinery. However, the high phagocytic capacity of the three RPE derived cell lines listed above and the selectivity of their phagocytic activity towards OS suggest that their RPE-specific phagocytic mechanisms remain largely in place (Finnemann *et al*., 1997, Finnemann and Rodriguez-Boulan, 1999).

These *in vitro* models of RPE have different advantages and disadvantages. Primary cells resemble RPE *in vivo* more closely, but variability between donors or preparations, as well as their limited availability restricts their studies largely to small-scale experiments. They are ideal for phagocytosis analysis by microscopy that requires few cells in an epithelial patch per sample. In contrast, homogeneous RPE cell lines offer virtually unlimited numbers of RPE cells for highly reproducible experiments with multiple parallel samples of any size. They are uniquely suited for biochemical analysis of phagocytosis involving protein purification and activity assays that require many thousands of synchronized cells for detection.

Taken together, no culture model exists that fully reconstitutes the apical interactions of RPE. RPE tissue *in situ* as well as primary and stable RPE cells *in vitro* each allow unique insight into the RPE phagocytic activity. Further understanding of the RPE phagocytosis mechanism may greatly benefit from studying these different experimental model systems in parallel.

3. The Receptor-Mediated Phagocytosis Mechanism of the RPE

Phagocytic mechanisms require the coordinated function of multiple phagocyte surface receptors. Three plasma membrane receptors have been assigned distinct roles in OS phagocytosis by RPE cells. These are MerTK (D'Cruz *et al*., 2000; Nandrot *et al*., 2000), the scavenger receptor CD36 (Ryeom *et al*., 1996) and the adhesion receptor αvβ5 integrin (Finnemann *et al*., 1997; Lin and Clegg, 1998; Miceli *et al*., 1997). As discussed in chapter 13, MerTK deficiency abolishes the RPE's ability to internalize OS *in vitro* and *in vivo*, causing retinal degeneration in transgenic mice and in the RCS rat (Bok and Hall, 1971; D'Cruz *et al*., 2000; Duncan *et al*., 2003; Mullen and LaVail, 1976; Nandrot *et al*., 2000; Scott *et al*., 2001; Vollrath *et al*., 2001). The impaired ability of macrophages derived from these animals to phagocytose apoptotic cells confirms earlier findings that the RPE phagocytosis mechanism for OS is similar to the macrophage uptake mechanism for apoptotic cells (Finnemann and Rodriguez-Boulan, 1999; Scott *et al*., 2001).

Like MerTK, the scavenger receptor CD36 participates in clearance phagocytosis by RPE cells and by macrophages. *In vitro* experiments suggest that CD36 does not act during the OS recognition/binding phase of RPE phagocytosis (Finnemann and Silverstein, 2001). Instead, CD36 ligation by antibodies or ligands, such as lipoproteins or unidentified serum proteins, regulates the rate with which RPE cells internalize OS following their recognition and binding by other RPE surface receptors. As CD36 clustering at the basal RPE surface affects OS engulfment at the apical RPE surface, CD36 likely functions primarily as a signaling molecule during RPE phagocytosis.

In contrast to MerTK and CD36, the adhesion receptor αvβ5 integrin plays a key role in the binding step of OS phagocytosis by RPE cells in culture. Binding and internalization of OS by RPE cells *in vitro* can be distinguished by their different temperature sensitivity (Finnemann and Rodriguez-Boulan, 1999). Stable binding of isolated OS to the RPE surface in a largely αvβ5 integrin dependent manner occurs at temperatures of 17°C or higher. OS remain tethered at 17°C but will be rapidly engulfed at any time if the cells are shifted to 32°C. *In vitro*, apoptotic cells and

OS compete for integrin-dependent recognition by RPE cells as well as by macrophages (Finnemann and Rodriguez-Boulan, 1999).

These RPE receptors have in common that they have similar if not identical functions in phagocytic mechanisms of other cell types, especially of professional phagocytes such as macrophages and dendritic cells. Furthermore, RPE cells express additional surface receptors that mediate uptake by macrophages and may be relevant to OS clearance by the RPE. These include glycosylation pattern recognition receptors such as the mannose receptor (Boyle *et al*., 1991; Shepherd *et al*., 1991) and CD14 (Elner *et al*., 2003), as well as scavenger receptors, such as SRA (E. Dufour and S.C.F., unpublished observation), MARCO (Dufour *et al*., 2003), SR-BI and SR-BII (Duncan *et al*., 2002; Provost *et al*., 2003).

The competition of apoptotic cells with experimental photoreceptor OS for OS phagocytosis and the overlap of elements of RPE and macrophage phagocytosis machineries suggest that these two cell types employ the same uptake mechanism in response to a one-time phagocytic challenge. This indicates that the RPE phagocytic pathway belongs to a group of conserved phagocytosis mechanisms that have evolved to provide efficient, non-inflammatory clearance of spent cells (undergoing apoptosis) or spent photoreceptor OS fragments (shed daily in the retina). This is a major conceptual shift from the long-standing but never proven hypothesis that the preference of the RPE phagocytic mechanism for OS over many other particles has its basis in RPE-specific expression of molecules uniquely suited to phagocytose OS. However, only RPE cells phagocytose with a daily rhythm once a day and every day for life. A prompt burst of phagocytosis in response to OS challenge followed by long periods of time during which the RPE phagocytosis mechanism must not be active may be the primary feature that distinguishes phagocytosis by RPE from phagocytosis by any other cell type. The apical surface of the RPE remains in permanent contact with photoreceptor outer segments in the retina. Synchronized phagocytosis of spent OS may thus require the RPE to coordinate functions of numerous proteins to ensure the remarkable consistency of its phagocytic rhythm. Receptor proteins that reside at the apical, phagocytic surface of the RPE, such as $\alpha v\beta 5$ integrin, are likely targets for functional regulation that will directly affect the RPE phagocytic activity.

4. Evidence for a Role of αvβ5 Integrin in RPE Phagocytosis

Integrins form a large family of heterodimeric plasma membrane receptors αvβ5 that mediate cell adhesion to extracellular substrates. Many integrins recognize the peptide motif RGD in ligand proteins. A number of cell types *in vivo* and in culture express the integrin receptor αvβ5. Dendritic cells and activated macrophages employ αvβ5 receptors to phagocytose apoptotic cells (Albert *et al*., 1998; Finnemann and Rodriguez-Boulan, 1999). Other functions of αvβ5 integrin include angiogenic signaling upon endothelial cell adhesion to vitronectin and osteopontin (Friedlander *et al*., 1995; Friedlander *et al*., 1996), vitronectin endocytosis (Memmo and McKeown-Longo, 1998) and adenovirus entry (Wickham *et al*., 1994; Wickham *et al*., 1993).

αvβ5 is the only integrin family receptor that localizes to the apical, phagocytic surface of RPE cells in the eye. Based on this expression pattern and polarity, Anderson and colleagues first proposed a role for αvβ5 integrin in human retina in adhesive interactions of apical microvilli of the RPE with ligands either exposed on photoreceptor outer segments or residing in the interphotoreceptor matrix in the subretinal space (Anderson *et al*., 1995). As in human retina, RPE cells in the mature rat retina express high levels of αvβ5 integrin at their apical surface (Finnemann *et al*., 1997). Numerous other integrin receptors also expressed by the RPE in the eye are found exclusively at the basal plasma membrane where they are thought to mediate adhesion of RPE to Bruch's membrane substrates. Onset of β5 protein expression in rat RPE precisely coincides with postnatal establishment of mature interactions between RPE and photoreceptors that include daily OS shedding and phagocytosis: β5 is absent from rat RPE at birth but reaches adult protein levels around postnatal day 11 (Finnemann *et al*., 1997). Taken together, these *in vivo* data strongly suggest that αvβ5 integrin receptors contribute to functional interactions of the apical surface of RPE with photoreceptor outer segments in the eye.

A number of plasma membrane proteins are known to exhibit 'reversed polarity' in the RPE in the eye in that they primarily localize to the apical surface of the RPE but to the basolateral surface in most other epithelia.

These proteins include the immunoglobulin family members N-CAM and EMMPRIN, the sodium pump, and αvβ5 integrin. N-CAM, EMMPRIN, and the sodium pump lose their apical polarity in most models of RPE in culture and assume a basolateral or non-polar plasma membrane distribution (Gundersen *et al*., 1993; Marmorstein *et al*., 1996). This suggests that activities of RPE cells that depend on the special apical localization of these proteins are probably not retained by RPE in culture. In contrast, αvβ5 integrin receptors retain a primarily apical polarity in stable and primary RPE cells in culture (Finnemann, 2003a, and Fig. 1). This persistent apical distribution of αvβ5 integrin *in vitro* correlates well with the maintenance of phagocytic activity of RPE in culture towards isolated photoreceptor OS.

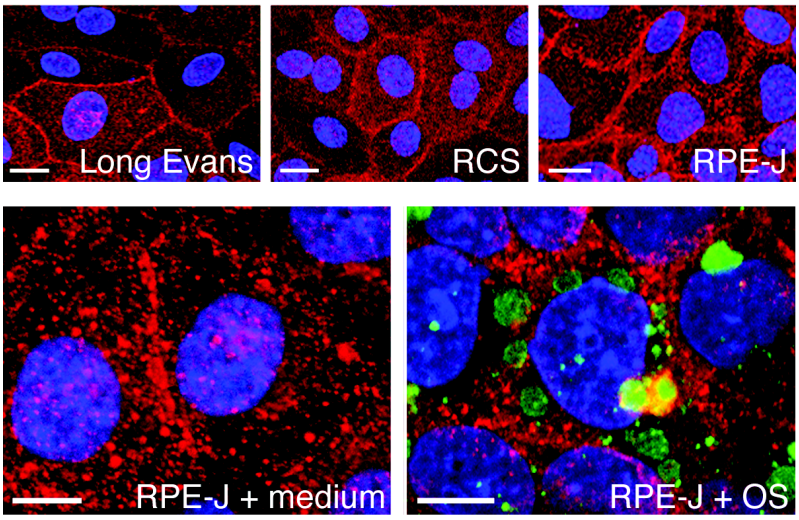

Fig. 1. Correlation of αvβ5 integrin receptor localization in RPE with OS phagocytosis. Primary, unpassaged RPE derived from Long Evans or RCS rats, and stable RPE-J cells, as indicated in the panels, were labeled live on ice for 45 minutes with 2 μg/ml αvβ5 receptor antibody P1F6 before fixation and incubation with AlexaFluor594-anti-mouse IgG (shown in red). Nuclei were labeled with DAPI (shown in purple). Fields show laser confocal 3D projections. Scale bars: 8 mm. Top panels: Primary and stable rat RPE cells in culture possess αvβ5 integrin receptors at their apical, phagocytic surface. Bottom panels: αvβ5 integrin receptor re-distributes to the site of surface-bound OS (OS shown in green) after 1 hour of phagocytic challenge of RPE-J cells.

Several independent studies have demonstrated that primary RPE in culture derived from adult rat, fetal or adult human RPE as well as the stable RPE derived cell lines rat RPE-J and human ARPE-19 employ αvβ5 integrin receptors during the recognition/binding phase of OS phagocytosis (Finnemann *et al*., 1997; Lin and Clegg, 1998; Miceli *et al*., 1997). αvβ5 inhibition using antibodies or RGD peptides strongly reduces OS binding by RPE in culture but does not affect internalization of surface-tethered OS (Finnemann *et al*., 1997). The downregulation of αvβ5 integrin expression in response to vitreous incubation of cultured porcine RPE cells correlates with a diminished OS binding capacity of these cells (Feng *et al*., 2003).

Taken together, developmental expression studies and *in vitro* functional assays suggest a conserved role for the integrin receptor αvβ5 in OS phagocytosis by human and rodent RPE. RPE cells in culture employ αvβ5 receptors in an early step of OS uptake since inhibition or lack of αvβ5 surface receptors strongly reduces tethering of isolated OS to the RPE surface. β5 integrin knockout mice provide the opportunity to study RPE that permanently lacks αvβ5 receptors. β5 knockout mice are viable and fertile (Huang *et al*., 2000) and, at a young age, have normal retina morphology and function (our unpublished results). Ongoing experiments in our lab compare OS uptake by primary RPE cells isolated from β5 knockout mice and from control mice of the same genetic background. These studies will allow us to directly assess the function of αvβ5 integrin receptors in OS phagocytosis by RPE cells.

It is important to note that the nature of OS contact differs significantly between the *in vitro* phagocytosis assay and the phagocytic challenge of RPE cells with shed OS in the retina. In *in vitro* assays, tethering of particles to the RPE surface must be mechanically stable enough to withstand the shearing forces during stringent washes aimed at removing excess unbound OS before fixation and quantification of bound particles. In contrast, separate assessment of OS binding and of engulfment of bound OS is impossible in the intact retina. *In vivo*, the apical RPE surface is in permanent contact with photoreceptor cone and rod outer segments, intact tips or shed OS and shearing forces are absent. It is therefore possible that OS engulfment by RPE cells *in situ* may not require an initial stable binding by RPE surface receptors. We propose a

dual function for αvβ5 integrin in OS phagocytosis: (1) to mediate robust surface tethering of shed OS to the RPE phagocytic surface (this structural function may be mainly relevant for the uptake of particles by RPE cells in culture), and (2) to initiate RPE signaling pathways upon interaction with OS that activate the RPE's machinery for particle internalization (this signaling function is likely important in both *in vitro* and *in vivo* uptake of OS and may be the primary role for αvβ5 receptors at the apical surface of the RPE in the retina).

5. αvβ5 Integrin Signaling During RPE Phagocytosis

5.1. *Activation of OS internalization by αvβ5 integrin signaling*

Outside-in signaling pathways relay signals from ligand-occupied integrin receptors to cytoplasmic signaling cascades. The final targets of these signaling pathways are often components of the cytoskeleton whose re-arrangement promotes cellular activities such as cell attachment and spreading, cell migration, and phagocytosis. Integrin receptors do not possess intrinsic enzymatic activity that could initiate a signaling cascade. However, integrin cytoplasmic domains assemble cytosolic proteins that serve to transduce integrin signals and to link plasma membrane integrin complexes with cytoskeletal elements. Ligand binding to the integrin receptor extracellular domain may alter the affinity of the receptor intracellular domain for cytosolic integrin binding proteins. Thus, integrins often bind different sets of proteins depending on receptor occupancy.

We have recently utilized this integrin characteristic to begin to unravel the signaling cascade activated by OS interaction with αvβ5 integrin receptor at the apical surface of RPE cells in culture (Finnemann, 2003a). Phagocytic challenge with isolated photoreceptor OS rapidly increased the presence of multiple tyrosine phosphorylated proteins associated with αvβ5 integrin in RPE cells (Fig. 2A). Taking clues from the molecular size of complexed proteins, we were able to identify one of these proteins as focal adhesion kinase (FAK). FAK is a cytoplasmic non-receptor tyrosine kinase that transduces signaling pathways downstream of activated integrins in many cell types (for a recent review on FAK see Parsons, 2003).

The dramatic reduction of OS internalization by RPE cells in which FAK function was specifically inhibited demonstrated that FAK is an important mediator of a signaling response to OS challenge (Finnemann, 2003a). Interestingly, FAK resided in a complex with apical αvβ5 integrin in RPE cells even before phagocytic challenge with OS (Fig. 2B). OS challenge caused a rapid increase in FAK association with αvβ5 followed by dissociation of FAK from αvβ5 complexes (Fig. 2B).

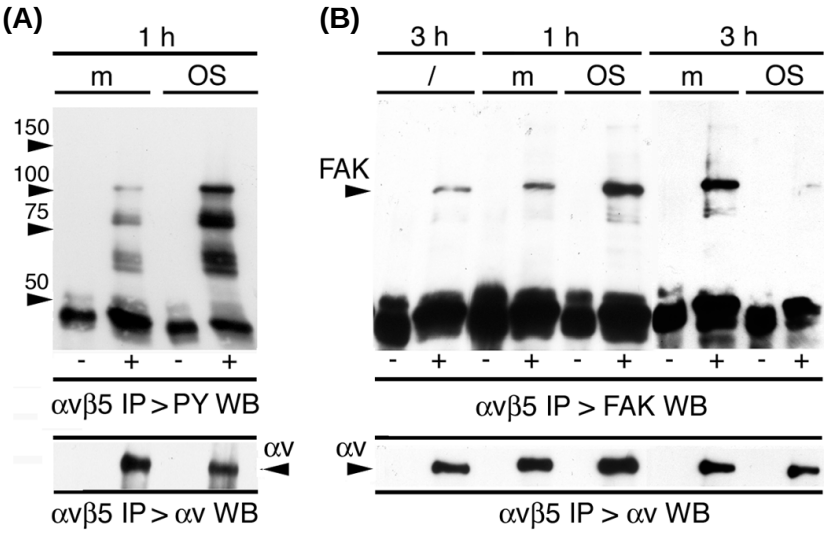

Fig. 2. Increased tyrosine phosphorylation of αvβ5 integrin complexes and dissociation of FAK from αvβ5 integrin in RPE cells challenged with OS. **(A)** αvβ5 antibody (+ lanes) isolated proteins associated with αvβ5 integrin receptors from RPE-J cell lysates that increased in tyrosine phosphorylation in response to OS incubation (OS) as compared to incubation with assay medium alone (m). Molecular masses are in kDa. **(B)** αvβ5 antibody (+ lanes) immunoprecipitated αvβ5 complexes from lysates of RPE-J cells challenged for different periods of time with medium (m) or OS (OS). Precipitated proteins were separated by SDS-PAGE and immunoblotted for FAK (upper panels). Before and 1 hour after OS addition, αvβ5 antibody co-precipitated FAK. Three hours after challenge with OS, but not with medium alone, FAK no longer resided in the αvβ5 complex. αv antibody reprobing of blots showed that P1F6 immunoprecipitated similar amounts of αvβ5 from all samples (A and B, lower panels). Bands corresponding to FAK and αv are indicated. Non-immune IgG did not immunoprecipitate FAK or αv (A and B, – lanes). Modified from Finnemann (2003a), with permission from Oxford University Press.

Furthermore, FAK showed increased phosphorylation at multiple tyrosine residues in response to OS phagocytic challenge (Finnemann, 2003a). While OS challenge caused persistent phosphorylation of tyrosine residues 397 (FAK's sole autophosphorylation site) and 576 (located in FAK's kinase domain and indicative of kinase activity), it resulted in a transient increase in FAK phosphorylation at tyrosine 861. Phosphorylation at FAK tyrosine 861 can mediate direct binding of FAK to the β5 integrin cytoplasmic tail (Eliceiri *et al*., 2002). Indeed, the time course of elevated phosphorylation at tyrosine 861 was in good agreement with the recruitment and subsequent dissociation of FAK from the αvβ5 complex in RPE cells: both FAK levels in αvβ5 complexes and tyrosine 861 phosphorylation levels increased ~3-fold during the first hour of OS challenge. After 2 hours of OS challenge, FAK tyrosine 861 levels decreased significantly and FAK no longer bound to the αvβ5 complex. This precise temporal correlation of residue specific phosphorylation and FAK-αvβ5 association suggests that FAK binding to αvβ5 complexes may be direct, using the same mechanism previously discovered by Eliceiri and colleagues. In their model system, Src kinase activity is required for stimulation of tyrosine 861 phosphorylation and FAK binding to αvβ5. The functional relationship of Src family kinases with FAK activity in RPE cells and with OS phagocytosis remains to be elucidated.

In contrast to tyrosine residue 861, tyrosine phosphorylation of FAK residues 397 and 576 are usually related to increased enzymatic activity of FAK (Calalb *et al*., 1995; Maa and Leu, 1998). During RPE phagocytosis, duration of FAK activation as shown by elevated tyrosine phosphorylation of residues 397 and 576, by far exceeds FAK's time of residence in the αvβ5 complex. This suggests that activated FAK may interact with and phosphorylate downstream targets subsequent to its release and at sites unrelated to αvβ5 and OS. At this time, we are only beginning to unravel the signaling cascade downstream of FAK that is elicited by OS-αvβ5 interaction.

FAK signaling downstream of αvβ5 integrin primarily affects the MerTK-dependent engulfment of surface–tethered OS by RPE in culture (Finnemann, 2003a). Engagement of MerTK by soluble low-affinity ligands can activate OS phagocytosis by RPE cells *in vitro* (Hall *et al*.,

2001). However, the relevance of these experiments to MerTK activation in the retina and MerTK's precise function in promoting internalization have yet to be determined. Tyrosine phosphorylation of MerTK during OS uptake is thought to reflect MerTK activity. Strikingly, expression of FAK mutants that specifically silences FAK signaling in RPE cells abolished the increase in tyrosine phosphorylation of MerTK induced by OS phagocytic challenge. These data suggest that MerTK engagement by soluble or OS-bound ligands in *in vitro* phagocytosis assays is not sufficient to increase tyrosine phosphorylation of MerTK. Rather, MerTK phosphorylation may occur as part of a complex signaling cascade, which involves FAK activity upstream of MerTK as well as receptor ligation. Taken together, these results provide the first evidence that FAK provides a functional link between αvβ5 integrin receptors and MerTK in RPE cells.

Another important target of signaling processes emanating from the cytoplasmic face of phagocyte plasma membrane receptors is the actin cytoskeleton. Proper cytoskeletal recruitment is critical in several steps of phagocytosis such as the accumulation of filamentous actin beneath and partially surrounding surface-bound particles (formation of the phagocytic cup), the closure of the phagosome and its traction inside the cell to complete the phagocytosis process (Castellano *et al.*, 2001). Small GTPases of the Rho family mediate such actin microfilament re-organization and have been implicated in several forms of phagocytosis (Castellano *et al.*, 2001; Chimini and Chavrier, 2000). αMβ2 integrin-dependent uptake of complement opsonized beads is mediated by RhoA (Caron and Hall, 1998). In contrast, apoptotic cell uptake by dendritic cells via αvβ5 requires Rac1 (Albert *et al.*, 2000). Direct substrates of FAK and Src, adaptor proteins CrkII and $p130^{CAS}$ are induced to form a complex in RPE cells upon OS challenge (Finnemann, 2003a). Specific inhibition of FAK blocks CrkII-$p130^{CAS}$ complex formation in RPE cells. Since CrkII/$p130^{CAS}$ signaling promotes Rac1 activation in integrin-dependent phagocytosis by dendritic cells and FAK activation promotes CrkII/$p130^{CAS}$ signaling in integrin-dependent phagocytosis by RPE cells, FAK may regulate Rho family GTPases in RPE cells to activate engulfment.

5.2. *Regulation of αvβ5 integrin in RPE cells*

Regulation of integrin receptor function can occur via regulation of receptor expression at different levels: (1) transcription and/or translation of integrin subunits, (2) α and β integrin subunit dimer assembly, (3) trafficking to the cell surface (usually as pre-assembled αβ heterodimers), and (4) recruitment to plasma membrane sites of activity (in polarized cells). During developmental maturation of murine RPE cells *in vivo*, these mechanisms ensure that αvβ5 integrin receptors are expressed at the apical, phagocytic surface of the RPE precisely in time for the onset of daily phagocytosis (Finnemann *et al*., 1997).

In addition to the regulation of integrin receptor expression, integrin receptor function can be controlled by mechanisms that regulate the activities of existing surface integrin heterodimers. Inside-out signaling mechanisms regulate integrin receptor function independent of integrin-ligand binding (Giancotti, 2003). They control integrin receptor function by modifying either integrin affinity (binding activity due to active conformation of individual receptors) or integrin avidity (binding activity due to clustering or cooperation of multiple receptors). Like outside-in signaling pathways, they may involve dynamic assembly of cytosolic protein complexes with integrin cytoplasmic domains at the plasma membrane and linkage of such complexes with cytoskeletal elements (Giancotti and Ruoslahti, 1999).

In the eye, circadian rhythms influenced by the dark-light cycle regulate photoreceptor shedding, thus initiating daily RPE phagocytosis. RPE cells react to their daily challenge of shed OS with prompt and efficient uptake suggesting that their phagocytic machinery is active at the time of phagocytic challenge (LaVail, 1976). At other times, regulatory mechanisms of RPE cells likely inhibit the RPE phagocytic function. Untimely attack of intact outer segment tips does not occur although these always remain in immediate proximity to RPE apical projections (Besharse and Defoe, 1998). We therefore hypothesized that timely regulation of phagocytic receptor activity is utilized by RPE cells to control their phagocytic function independently of photoreceptors.

Indeed, the OS binding activity of RPE cells that employs αvβ5 integrin receptors is not constitutively active but subject to regulation by RPE signaling mechanisms. OS binding by RPE cells requires the activity of

signaling pathways involving protein kinase C (PKC) even before OS challenge (Finnemann and Rodriguez-Boulan, 1999). PKC inhibition in monocytes and in RPE cells severs the cytoskeletal linkage of αvβ5 receptors and blocks the OS binding activity of both phagocytes. These results suggest that, in RPE cells in culture, inside-out signaling pathways involving PKC may control the binding phase of OS phagocytosis by regulating the activity of αvβ5 integrin.

To directly distinguish activated from inactive integrin heterodimers, Pampori and colleagues generated and tested a recombinant protein, termed Wow-1, that mimics a monovalent ligand of αvβ3 and αvβ5 integrin receptors fused to the Fc portion of mouse Ig heavy chain (Pampori *et al*., 1999). Wow-1 binds only to activated integrin receptors and Wow-1 labeling therefore allows the direct observation of activated integrins in live and fixed cells by indirect fluorescence microscopy. Interestingly, OS phagocytosis but not assay medium alone significantly reduced Wow-1 labeling in polarized RPE-J cells (Fig. 3). This demonstrates, for the first time, that RPE cells *in vitro* downregulate the

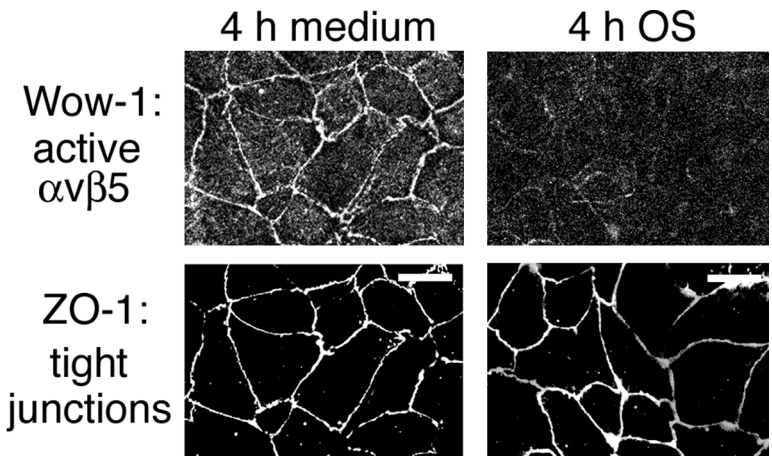

Fig. 3. Downregulation of high-affinity αvβ5 receptors in RPE in response to OS challenge. RPE-J cells received assay medium or OS as indicated before fixation and double labeling with Wow-1 and ZO-1. Scale bars: 10 μm. Printed with permission from Finnemann (2003b).

levels of active αvβ5 integrin receptors following OS phagocytosis.

Finally, is there a refractory period following OS phagocytosis by experimental models of RPE cells that may directly indicate that RPE cells intrinsically regulate their phagocytic activity? To test this, we subjected RPE cells in culture to a second challenge with isolated OS at different periods of time after an initial challenge. Strikingly, RPE cells in culture bind only 10%–25% as many OS as during the first challenge if they receive a second challenge with isolated OS within ~10 hours of an initial one (Fig. 4).

OS binding is completely restored if the interval between challenges is at least 12 hours. Inhibition of OS binding to cell surface receptors of RPE cells in culture is not solely due to surface receptor occupation as surface-bound OS are internalized within 3–5 hours of an initial challenge (Finnemann *et al*., 1997). These experiments suggest that RPE cells in culture downregulate their ability to phagocytose additional phagocytic particles through temporary inhibition of OS binding. Importantly, stable RPE cell lines of human and of rat origin as well as primary, unpassaged

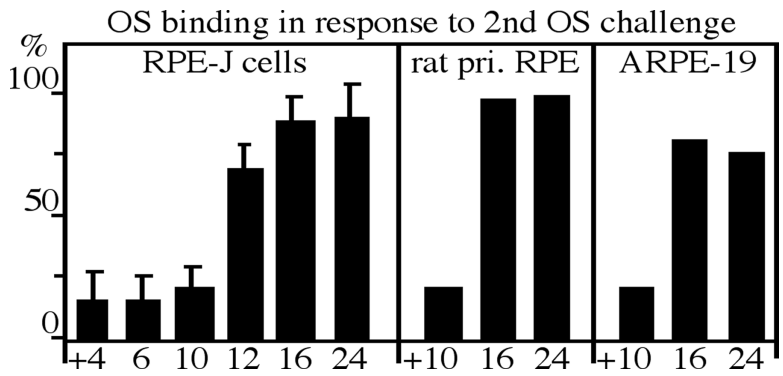

Fig. 4. Lack of OS binding by primary and stable RPE cells in culture within up to 10 hours of an initial phagocytic 'meal.' At different times up to 24 hours following an initial OS challenge with unlabeled OS (as indicated), RPE-J cells, primary Long Evans rat RPE (rat pri. RPE), and ARPE-19 cells received a second phagocytic challenge with FITC-OS. Uptake values are given as percent of OS binding during the first OS challenge. Values are averages ± s.d., n = 4, for RPE-J, and mean values, n = 2, for rat primary RPE and ARPE-19. Printed with permission from Finnemann (2003b).

rat RPE share this regulatory mechanism.

Taken together, these experiments studying experimental OS challenge of RPE in culture provide initial evidence that regulation of αvβ5 integrin receptors may contribute to the apparent downregulation of RPE phagocytic activity following a phagocytic meal. Refusal of RPE cells to bind OS in re-challenge experiments coincides with absence of active αvβ5 integrin receptors at the apical surface of RPE, as indicated by loss of Wow-1 labeling. These *in vitro* data fit a working hypothesis of αvβ5 integrin receptor activation linked to an approximate 12 : 12 hours of light : dark rhythm with which RPE cells phagocytose rod and cone OS. They will facilitate the design of experiments to unravel integrin phagocytic receptor regulation in response to the daily phagocytic burst of the RPE in the eye.

6. Conclusion

During the previous decade, research by many investigators led to the identification of numerous molecules that build the RPE phagocytosis machinery. Unexpectedly, phagocytic receptors exclusive to RPE or OS specific ligands have not been found. Receptors of RPE that participate in OS uptake, MerTK, CD36, and αvβ5 integrin, are not restricted in their expression to phagocytic cells. They participate in other forms of phagocytosis as well as in cellular activities unrelated to phagocytosis in cell types other than RPE. Further mechanistic studies are therefore needed to unravel how activities of these common proteins during OS phagocytosis contribute to the distinct phagocytic function of the RPE. Signaling pathways in RPE that regulate activities of RPE receptors, their downstream effectors, and their cross-talk to promote recurrent phagocytosis of OS are largely unknown. We have discussed initial evidence obtained from studies of RPE in culture suggesting that functional interactions of αvβ5 integrin with other components of the phagocytic machinery may contribute to the synchronized, timely, and repeated phagocytic clearance of OS that is unique to the RPE. These findings are summarized in Fig. 5. Such emerging knowledge of signaling during phagocytosis by RPE in culture provides a basis for future experiments

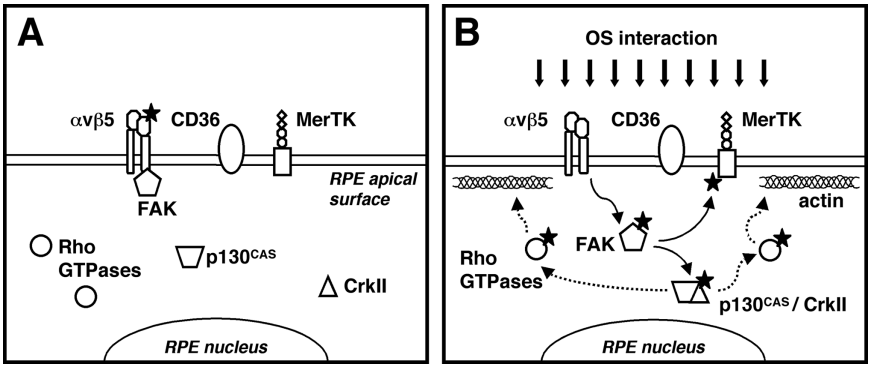

Fig. 5. αvβ5 integrin-dependent signaling response to OS phagocytic challenge of RPE cells. Stars indicate proteins activated by tyrosine phosphorylation.

to unravel the mechanism of phagocytosis by RPE *in vivo*.

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