Annexin A2 Regulates Phagocytosis of Photoreceptor Outer Segments in the Mouse Retina

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Submitted December 15, 2008; Revised June 24, 2009; Accepted June 25, 2009

Monitoring Editor: Jean E. Gruenberg

The daily phagocytosis of shed photoreceptor outer segments by pigment epithelial cells is critical for the maintenance of the retina. In a subtractive polymerase chain reaction analysis, we found that functional differentiation of human ARPE19 retinal pigment epithelial (RPE) cells is accompanied by up-regulation of annexin (anx) A2, a major Src substrate and regulator of membrane–cytoskeleton dynamics. Here, we show that anx A2 is recruited to the nascent phagocytic cup *in vitro* and *in vivo* and that it fully dissociates once the phagosome is internalized. In ARPE19 cells depleted of anx A2 by using small interfering RNA and in *ANX A2*^{−/−} mice the phagocytosis of outer segments was impaired, and in *ANX A2*^{−/−} mice there was an accumulation of phagocytosed outer segments in the RPE apical processes, indicative of retarded phagosome transport. We show that anx A2 is tyrosine phosphorylated at the onset of phagocytosis and that the synchronized activation of focal adhesion kinase and c-Src is abnormal in *ANX A2*^{−/−} mice. These findings reveal that anx A2 is involved in the circadian regulation of outer segment phagocytosis, and they provide new insight into the protein machinery that regulates phagocytic function in RPE cells.

INTRODUCTION

The retinal pigment epithelium (RPE) performs a range of functions that directly support the retina (Strauss, 2005), including the daily phagocytosis and digestion of shed photoreceptor outer segments (POS). The importance of this activity is exemplified in the dystrophic Royal College of Surgeons rat, which due to a failure in phagocytosis undergoes a progressive and rapid retinal degeneration characterized by accumulation of cellular debris and photoreceptor cell death. The gene responsible for this pathology was identified by positional cloning as the Mer tyrosine kinase (MerTK), a key signaling intermediate that is expressed in RPE cells and plays an essential role in the internalization of bound POS (Chaitin and Hall, 1983; D'Cruz *et al.*, 2000). Recent studies have provided insight into other RPE cell proteins that have distinct roles either in the binding or the internalization of shed POS. For example, the apically expressed $\alpha\beta 5$ integrin is required for efficient binding of POS, and RPE cells from $\beta 5$ ^{−/−} mice show markedly reduced binding of POS *in vitro* (Nandrot *et al.*, 2004). Intriguingly, engagement of the $\alpha\beta 5$ integrin by its cognate ligand, milk fat globule-EGF8 (MFG-E8), is essential for the circadian

synchronization of phagocytosis (Nandrot and Finnemann, 2006; Nandrot *et al.*, 2007), and although phagocytosis of POS occurs in *MFG-E8*^{−/−} and $\beta 5$ ^{−/−} mice, in both mutants the daily rhythm is absent. In contrast, focal adhesion kinase (FAK) and MerTK are not required for the initial binding of POS to the apical surface of the RPE, but they are sequentially activated by $\alpha\beta 5$ receptors and are essential for phagosome internalization (Feng *et al.*, 2002; Finnemann, 2003).

Between the pigment epithelium and neuroretina RPE cells extend apical processes into the interphotoreceptor matrix in which they interdigitate with the terminal POS. Because the apical processes are rich in actin, closure of the nascent phagocytic cup depends upon the reorganization of actin in conjunction with membrane fusion. Proteomic analyses of RPE and RPE apical processes have identified many actin modulators that may mediate these activities (West *et al.*, 2003; Bonilha *et al.*, 2004), including several members of the annexin family of Ca²⁺ and phospholipid-binding proteins. Consistent with these reports, we observed up-regulation of annexins A2 and A4 concomitant with acquisition of phagocytic competence in functionally differentiated ARPE19 cells (Turowski *et al.*, 2004). Although the function of annexin (anx) A4 is not known, anx A2 has roles in endocytosis (Emans *et al.*, 1993; Harder and Gerke, 1993; Jost *et al.*, 1997; Morel *et al.*, 2009) and pinocytosis (Merrifield *et al.*, 2001), and in macrophages anx A2 associates with phagosomes (Diakonova *et al.*, 1997). These observations suggested to us that up-regulation of annexin A2 in differentiated RPE cells may be required for the development of phagocytic capability. Here, we show that anx A2 is highly enriched on newly formed phagosomes in RPE cells and that siRNA-mediated depletion of anx A2 results in impairment

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-12-1204>) on July 8, 2009.

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Abbreviations used: anx, annexin; FAK, focal adhesion kinase; POS, photoreceptor outer segments; PtdIns4,5P₂, phosphatidylinositol 4,5-bisphosphate; MerTK, Mer tyrosine kinase; RPE, retinal pigment epithelium.

of POS internalization. We also show that anx A2 is rapidly phosphorylated on tyrosine upon POS binding to ARPE19 cells and at or shortly before light onset in the RPE of normal mice. However, in *ANX A2^{-/-}* mice, the activation of FAK and c-Src is markedly delayed and phagosomes accumulate in the RPE apical processes. These findings provide direct evidence that anx A2 is necessary for the normal circadian phagocytosis of POS by RPE cells.

MATERIALS AND METHODS

RNA Extraction and Northern Hybridization

mRNA was isolated from ARPE19 cells grown for 5 wk on either porcine lens capsule or on porous polyester inserts (Corning Life Sciences, Lowell, MA), by using a QuickPrep MicromRNA purification kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) according to the manufacturer's protocol. For Northern blotting, 2 μ g of RNA from ARPE19 cells grown on either lens capsule grown or porous polyester were resolved on a 1.2% agarose-formaldehyde gel and electroblotted onto nylon membrane (Hybond XL; GE Healthcare). A probe containing a fragment of the anx A2 cDNA in pADV (cloned from the original subtracted library) that was PCR generated using vector primers and a 1-kb PstI fragment of glyceraldehyde-3-phosphate dehydrogenase were labeled with [γ -³²P]dCTP by using random hexamers. Membranes were prehybridized at 72°C for 60 min with continuous agitation in ExpressHyb solution (Clontech, Basingstoke, Hampshire, United Kingdom) followed by hybridization with denatured radiolabeled cDNA probes at 72°C overnight in ExpressHyb. Membranes were washed for 20 min four times in 2 $\times$ SSC/0.5% SDS at 68°C followed by two washes for 20 min in 0.2 $\times$ SSC/0.5% SDS at 68°C. Specific hybridization signals were detected by exposing the membranes to x-ray film overnight with an intensifying screen at -70°C.

Cell Culture and Mice

All tissue culture reagents were purchased from Invitrogen (Paisley, United Kingdom) unless otherwise stated. ARPE19 cells were cultured on 75-cm² culture flasks (Nalge Nunc International, Rochester, NY) in high-glucose DMEM, containing 10% fetal bovine serum and used after 1 wk at 100% confluence. RPE-J cells were cultured as described previously (Finnemann, 2003). Wild-type and annexin A2 null mutant mice (Ling *et al.*, 2004) were maintained on a regular diet of food and water ad libitum, in accordance with UK Home Office regulations. Mice were killed by cervical dislocation and immediately enucleated.

Annexin A2 Small Interfering RNA (siRNA) Treatment

ARPE19 cells were cultured for 2 d in the presence of the anx A2-specific mRNA target constructs 5'-GUGCAUAUGGGUCUGAA-3' and 5'-AAC-CUGGUUCAGUGCAUUGAG-3' (Dharmacon RNA Technologies, Lafayette, CO) with Oligofectamine reagent (Invitrogen) at 40–60% confluence and treated again 3 d before experiments.

Photoreceptor Outer Segment (OS) Isolation and Labeling

POS were isolated from fresh porcine eyes and labeled with Alexa Fluor 488/555 (Invitrogen) as described previously (Molday *et al.*, 1987). The harvested rod outer segments were fed to cells at a concentration of 10⁷/ml.

Immunoprecipitation, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Western Blotting

Immunoprecipitation of tyrosine phosphorylated annexin 2, FAK and c-Src was performed as described previously (Deora *et al.*, 2004). In brief, control and POS-stimulated ARPE19 cells were washed twice with ice-cold phosphate-buffered saline (PBS) followed by incubation on ice with 500 μ l of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 10 min. Lysates were centrifuged for 15 min at 13,000 $\times$ g at 4°C to pellet insoluble material. Supernatants were aspirated and incubated with 25 μ l of immobilized anti-phosphotyrosine monoclonal immunoglobulin G (IgG) (p-Tyr; Cell Signaling Technology, Danvers, MA) at 4°C overnight. For immunoprecipitation of tyrosine-phosphorylated annexin 2, Src, and FAK from primary mouse RPE, eye cups were trimmed and cut along the ora-serrata to separate the anterior and posterior segments of the eye. The vitreous and lens were removed from the posterior segment, and the neuroretina was carefully peeled away and removed. The remaining posterior segment of the eye cup was incubated in 500 μ l of lysis buffer for 10 min and centrifuged for 1 min at 13,000 $\times$ g to pellet insoluble material. The supernatant was aspirated and incubated with 25 μ l of immobilized anti-phosphotyrosine monoclonal IgG at 4°C overnight.

For Western blot analysis, ARPE19 cells were seeded onto 12-well plates (Nalge Nunc International) before treatment with anx A2 siRNA as described above. Cells were washed twice with ice-cold PBS before lysing with reducing sample buffer and denaturing at 95°C for 5 min. Samples were resolved by 10% SDS-PAGE and transferred onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Antibodies to c-Src (Cell Signaling Technology) and FAK (Santa Cruz Biotechnology, Santa Cruz, CA) were used to probe for tyrosine phosphorylated annexin 2. The anx A2 was detected using either a monoclonal antibody (mAb) to annexin 2 (BD Biosciences, San Jose, CA) or the HH7 antibody (gift from Volker Gerke, Muenster, Germany) at a dilution of 1:500. Blots were incubated with polyclonal goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibody (Dako UK, Ely, Cambridgeshire, United Kingdom) and protein bands detected using ECL Western blotting detection reagent (GE Healthcare).

Immunofluorescence Imaging

ARPE19 and RPE-J cells were seeded onto MatTek dishes (MatTek, Ashland, MA) before treatment with anx A2 siRNA. Cells were washed twice with ice-cold PBS and fixed with 4% paraformaldehyde for 20 min. After fixation, cells were washed twice with PBS, and primary antibody containing 0.2% Triton was added overnight. Cells were washed a further three times with copious amounts of PBS followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) and Alexa Fluor 660 Phalloidin (Invitrogen). For immunofluorescent imaging of OS phagocytosis, cells were seeded as described above and then treated with anx A2 siRNA or a nonspecific oligonucleotide. Media were aspirated, and OS were fed to the RPE cells at a concentration of 1 $\times$ 10⁷/ml. Cells were washed twice with warm PBS,

Figure 1. anx A2 is required for phagocytosis of photoreceptor outer segments. (A) Northern blots of anx A2 and GAPDH (loading control) from ARPE19 cells grown for 5 wk on polyester filters (P) or porcine lens capsule (L) showing up-regulation of anx A2 expression. (B) Western blot showing increased expression of anx A2 protein (36-kDa band) on lens capsule versus polyester. (C) ARPE19 cells were cultured on glass coverslips for 2 wk, treated with control or anx A2-specific siRNA and then immunostained for F-actin and anx A2. Under these conditions, anx A2 depletion is partial, with some cells (arrowheads) retaining normal anx A2 expression. (D) Whole cell lysates were prepared from cells treated as described above and Western blotted for anx A2 and α -tubulin. (E) ARPE19 cells were cultured as described in C and then incubated with fluorescently labeled POS before fixation at the times indicated. Surface-bound fluorescence was quenched, and the internalized POS fluorescence quantified on a plate reader. Error bars are $\pm$ SEM, $n = 3$. p values were calculated using Student's t test as follows: $p^* < 0.5$, $p^{**} < 0.05$, and $p^{***} < 0.005$.

somes at different stages of internalization. (C) RPE-J cells were incubated with fluorescently labeled POS, processed as described in B, and imaged by confocal microscopy. The z-sections show that anx A2 is enriched at the apical cell surface and associates with forming and newly formed phagosomes. (D) z-sections of RPE-J cells showing the enrichment of anx A2 (green) and F-actin (blue) with newly formed and early phagosomes (red). (E) Primary porcine RPE cells were microinjected with a plasmid encoding anx A2-GFP, returned to culture overnight, and then incubated with fluorescently tagged POS before fixation and confocal microscopy. The panels to the left show marked enrichment of anx A2-GFP on a partially internalized phagosome (yellow arrowhead) but none on a fully internalized phagosome (white arrowhead). The z-sections of this cell reveal enrichment of anx A2-GFP in the apical processes around the phagosome.

followed by fixation and primary antibody incubation as described above. The anx A2-conjugated cells were then incubated with Alexa Fluor 546-conjugated anti-mouse IgG (Invitrogen) and Alexa Fluor 660 Phalloidin (Invitrogen). All imaging was performed using a confocal system (SP2 AOBIS; Leica, Wetzlar, Germany) and a DMIRB IRE 2 inverted microscope.

Quantification of POS Binding and Internalization

ARPE19 cells were seeded onto 48-well plates. Cells were incubated with anx A2 siRNA or a control oligonucleotide as described above. Cells were fed with labeled POS at a concentration of 1×10^7 /ml at left for 15-, 30-, 90-, and 150-min time points followed by washing twice with ice-cold PBS and fixing with 4% paraformaldehyde (PFA). In some cases, ice-cold 0.2% trypan blue was added to cells for 10 min after washing to quench fluorescence derived from externally bound particles. This method allows bound POS to be distinguished from bound and internalized POS (Finnemann *et al.*, 1997). Trypan blue was washed off with ice-cold PBS before fixing. POS binding and internalization was quantified using a Safire plate reader (SAFIRE; Firmware version 2.00 03/02 Safire; XFLUOR4 version V 4.20 Tecan, Reading, United Kingdom).

Electron Microscopy

Wild-type and ANX A2^{-/-} mice were killed at 0800 h, and eyes were immediately removed into fixative. For conventional electron microscopy, mouse eyes were embedded as described previously, except eyes were post-fixed in 1% osmium tetroxide alone (Futter *et al.*, 2004). After embedding in Epon, 70- to 80-nm sections were cut and stained with lead citrate. For cryo-immuno-electron microscopy (immunoEM), mouse eyes were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde (for anx A2 staining) or 4% paraformaldehyde (for 1D4 staining) and then dissected, embedded in gelatin, infiltrated in sucrose, mounted on pins, and frozen in liquid nitrogen as described previously (Futter *et al.*, 2004). Thawed 60- to 70-nm cryosections were labeled with affinity-purified rabbit anti-anx A2 polyclonal antibody (kindly provided by Dr. J. Ayala-Sanmartin) followed by 10-nm protein A gold, or 1D4 anti-rhodopsin mAb (Abcam, Cambridge, United Kingdom) followed by a rabbit anti-mouse bridging antibody and 15-nm protein A gold as described previously (Slot *et al.*, 1991). All specimens were viewed on a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan). Images were gathered with either Kodak electron microscope film 4489 (the conventional EM) or a Gatan OriusSC100B charge-coupled device camera (the cryo-immunoEM). In quantitative experiments, phagosomes were identified as approximately round structures exhibiting at least one of the following characteristics: 1) the presence of membranous stacks; 2) a mean diameter of at least 75% that of the rod outer segments; and 3) for apical phagosomes, surface membranes enclosed and segregated from the apical membranes of the RPE. The entire RPE length of each eye was used for phagosome counting with the location of each phagosome mapped by measuring its distance from Bruch's membrane.

Flat Mounting and Staining

Eyes were harvested at various times before and after light onset. Each eye was trimmed, and the surface of the cornea was pierced before immersing in 2% PFA in 2× PBS for 2 min, followed by incising along the ora serrata to separate the eye into anterior and posterior segments. The lens and vitreous were carefully removed from the posterior segment of the eye, and the neuroretina was peeled away to expose the RPE. Incisions were made from the peripheral edge of the eye cup toward the optic nerve head to open the eye cup into a "flower." This was followed by fixing further with 4% PFA in 2× PBS for 4 min before immersing briefly in 2× PBS and then for at least 2 h

Figure 3. Outer segment phagocytosis stimulates tyrosine phosphorylation of anx A2. (A) ARPE19 cells were exposed to purified POS for times indicated, lysed, and immunoprecipitated (IP) with PY100 anti-phosphotyrosine antibodies (p-Tyr) coupled to beads. Immunoprecipitates were washed, resolved by SDS-PAGE, transferred to filters by Western blotting (WB), and probed with antibodies to anx A2 and c-Src. The blots show rapid and transient tyrosine phosphorylation of anx A2 and c-Src upon incubation with POS. (B) Before incubation with PY100 antibodies described above, 50 μ l of whole cell lysate (WCL) was removed from the sample and resolved by SDS-PAGE. After Western blotting, samples were probed with antibodies to anx A2 and c-Src. For both experiments, results are also presented as mean -fold change in band intensity. Error bars are $\pm$ SEM, $n = 3$.

in blocking solution (3% Triton X-100, 0.5% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide). The flowers were then incubated with zona occludens (ZO)-1 (1:400; gift from Karl Matter) and 1D4 rhodopsin (1:1000; Abcam) antibodies made up in blocking solution overnight at room temperature. After primary antibody incubation, eyes were washed three times in blocking solution followed by 1-h incubation with fluorescent-conjugated secondary antibodies at room temperature and washing three times in blocking solution. Eyes were then mounted onto glass slides in Mowiol and covered with glass coverslips. Flat mounts were imaged on a Image Capture fluorescence microscope (Leica). Positively stained phagosomes and RPE cells (distinguished by ZO-1 staining) were counted for four to six areas of view. Data are represented as average number of phagosomes per cell for each eye.

Measurement of F/G Actin Ratio in Annexin 2-depleted Cells

Annexin 2 was depleted from ARPE19 cells grown in six-well plates by 5-d treatment with siRNA as described above. Control cells were treated with a green fluorescent protein (GFP)-specific siRNA. Cells were scraped off the plate with a rubber policeman and resuspended in 3 volumes of lysis buffer (3 mM imidazole/HCl, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 1 mM dithiothreitol, 0.2 mM ATP, and 2 μ M phalloidin). The cells were passed through a 22-gauge needle seven times and then spun briefly (1000 rpm for 10 min at 4°C in a benchtop centrifuge) to give a postnuclear supernatant. This was then centrifuged at 200,000 $\times$ g for 40 min on a RC M150 ultracentrifuge (Sorvall, Newton, CT) to provide a F-actin-containing pellet. The supernatant (containing the G-actin) was removed, and the pellet resuspended in an equal volume of lysis buffer containing 1% Triton X-100. Equal volumes of both supernatant and pellet were resolved by SDS-PAGE gel, and proteins were visualized by Western blotting. Images of the resulting blots were analyzed in MetaMorph (Molecular Devices, Sunnyvale, CA). Data represent the mean of three independent experiments.

Microinjection of Primary Porcine RPE Cells

Primary porcine RPE cells were prepared as described above. After maintenance in culture for 10 d, they were microinjected with a FemtoJet micro-manipulator (Pi 145, Pc 20, 0.2 s; Eppendorf, Hamburg, Germany). DNA was injected at 0.2 mg/ml in double-distilled H₂O in the presence of tetramethylrhodamine B isothiocyanate dextran (10,000 mol. wt., lysine fixable; Invitrogen). DNA was centrifuged at 80,000 rpm for 30 min in an RC M150 ultracentrifuge (Sorvall) before use to ensure there were no particulates to block the needle.

RESULTS

Annexin A2 Is Required for Normal Phagocytosis of POS by RPE Cells

Having identified anx A2 as a marker of ARPE19 differentiation in a previous subtractive PCR analysis (Turowski *et al.*, 2004), we first validated the results of the genetic screen by Western and Northern blotting. mRNA from ARPE19 cells grown for 5 wk on porcine lens capsule or transwell polyester tissue culture filters was subject to Northern blot analysis to investigate the abundance of transcripts for anx A2. The anx A2 mRNA levels were higher in cells cultured on lens capsule than on plastic, compared with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control (Figure 1A). Consistent with this result, elevated expression of anx A2 protein in cells cultured on lens capsule was observed by Western blotting (Figure 1B).

In our previous study, we also noted that elevation of anx A2 levels correlated with a marked improvement in phagocytic function in differentiated RPE cells (Turowski *et al.*, 2004). To gain insight into a possible role for anx A2 in POS phagocytosis, we examined the effects of siRNA-mediated anx A2 depletion on subconfluent cultures of ARPE19 cells grown on plastic (Figure 1C). The variable patterns of F-actin staining in these cells are a function both of the selected confocal plane and differences in the differentiation status of individual cells and are not due to changes in the G- to F-actin ratio, because these were not significantly altered by depletion of anx A2 (Supplemental Figure 1). We observed partial depletion of anx A2 (Figure 1D), and a quantitative kinetic analysis of internalized POS revealed a significant delay in POS phagocytosis in the anx A2-depleted cell population throughout the duration of the experiment (Figure 1E). Binding of POS to ARPE19 cells was not inhibited by depletion of anx A2 (data not shown).

Figure 4. The anx A2 is enriched on early phagosomes in the normal mouse retina. Wild-type (A–C) or *ANX A2*^{−/−} (D) mice were killed 1–2 h after light onset, eyes were processed for cryo-immunoEM and stained for anx A2. Gold particles are indicated by arrows and the boxed areas are shown at higher magnification in the insets for easier visualization. Small numbers of gold particles on the photoreceptor discs within the lumen of the phagosome are non-specific because they are present in both wild-type and knockout mice. Specific anx A2 staining is present on the perimeter membrane of phagosomes found in the most apical region of the cell, suggesting that they are newly internalized (A and B), but it is largely absent from the perimeter membrane of phagosomes found deeper in the cell (C) or from phagosomes from *ANX A2*^{−/−} mice (D). Bar, 0.5 μ m.

Anx A2 Is Recruited to the Forming Phagosome

We next investigated the subcellular localization of anx A2 in primary porcine RPE, ARPE19, and RPE-J cells during POS binding and internalization. For these experiments, confluent cultures were maintained at high density for 2 wk on glass coverslips before exposure to fluorescently labeled POS and subsequent fixation at defined time points. Cells were immunostained for anx A2 and also F-actin, because reorganization of the actin cytoskeleton is a key early event in phagocytosis, and in previous work we have shown that anx A2 is an important regulator of actin dynamics (Hayes *et al.*, 2006). Confocal microscopy revealed enrichment of anx A2 and F-actin at the point of contact between POS aggregates and the apical RPE cell surface (arrowhead in Figure 2A). In some instances, cells contained both partially and fully internalized POS, revealing that although anx A2 is recruited to newly formed phagosomes, mature internalized POS lack any associated anx A2 or F-actin (Figure 2, A and B). These findings were confirmed in z-sections of many cells, such as those exemplified in Figure 2B in which early but not late phagosomes are enriched in anx A2. To investigate the generality of this observation we also investigated the localization of anx A2 and F-actin in polarized rat RPE-J cells undergoing phagocytosis. Confocal images revealed that anx A2 is enriched at the apical cell surface in RPE-J cells, and as in ARPE19 cells and primary porcine RPE, F-actin and anx A2 were indeed both recruited to newly internalized and nascent phagosomes (z-sections in Figure 2C and merged images in Figure 2D). Finally, we microinjected an anx A2-GFP expression construct into primary porcine RPE before incubation with POS and confocal imaging. In these experiments, we observed enrichment of anx A2-GFP on forming but not mature phagosomes (Figure 2E), which when taken with the data mentioned above strongly suggests that a role for anx A2 in POS phagocytosis is most likely to be at the time of POS engagement and initial internalization.

It is established that binding of POS to RPE cells activates a hierarchy of tyrosine kinases that are essential for phagosome internalization, and that FAK and MerTK play prominent roles in this process (Feng *et al.*, 2002; Finnemann, 2003). Because FAK and anx A2 are substrates for several tyrosine kinases, including Src (Bellagamba *et al.*, 1997), and because we have recently shown that anx A2 is required for the plasma membrane targeting and activation of Src (Hayes and Moss, 2009), we next tested whether anx A2 and c-Src become tyrosine-phosphorylated during POS phagocytosis by ARPE19 cells. Within 15 min of adding POS to ARPE19 cells, tyrosine phosphorylation of both anx A2 and c-Src was observed, in the latter case probably implying activation of the kinase (Figure 3, A and B). The kinetics of tyrosine phosphorylation of anx A2 and c-Src were virtually identical and temporally coincident with the initial phase of POS binding. Together, the data presented in Figures 1–3 suggest that in cultured RPE cells anx A2 has a role in the internalization of newly formed phagosomes and that its activity in this context may be as a regulator of Src, effector of Src, or both.

To find out whether the results of our studies using RPE cell lines would be recapitulated *in vivo*, we performed cryo-immuno-electron microscopy on retinal sections from C57/B6 mice and *ANX A2*^{-/-} mice. Sections were cut from eyes taken shortly after lights on, at which time phagocytosis is expected to be at or near its diurnal peak. In control C57/B6 mice, newly formed phagosomes (as judged by their apical localization and clearly defined membranous discs)

exhibited anx A2 expression as revealed by gold particles in close association with the limiting membrane of the phagosome (Figure 4, A and B). In contrast, gold labeling was absent from more mature phagosomes (Figure 4C), although with the exception in all instances of a few gold particles within the phagosome. This latter labeling is nonspecific because photoreceptors do not express anx A2, and it was also observed in both early and late phagosomes in sections from the *ANX A2*^{-/-} mouse (Figure 4D). These results are therefore consistent with the findings reported in Figure 2, and confirm that *in vivo*, as in cultured cells, anx A2 is specifically associated with newly formed phagosomes.

Phagosome Internalization Is Impaired in the Annexin A2 Knockout Mouse

Next, we investigated whether the requirement for annexin A2 in POS internalization described in Figures 1 and 2

Figure 5. Phagosome processing is impaired in the *ANX A2*^{-/-} mouse. (A) Electron micrograph showing an *ANX A2*^{-/-} mouse RPE cell and a partially phagocytosed fragment of POS (Phag). The location of Bruch's membrane (BM), pigment granules (PG), and photoreceptor OS are indicated. (B) The positions of phagosomes were mapped according to their distance from Bruch's membrane, by using electron micrographs of retinal sections from control and *ANX A2*^{-/-} mice. The graph shows the relative distribution of phagosomes, with the shaded area highlighting the accumulation of apical phagosome in the *ANX A2*^{-/-} mice. *n* = 121 for control and *n* = 78 for *ANX A2*^{-/-} mice. (C) High-power magnification of the phagosome in A, showing the apical processes of the RPE cell in close apposition (asterisks).

Figure 6. Diurnal phagocytosis of photoreceptor outer segments is retarded in mice lacking annexin A2. Eyes were processed for cryo-immunoEM and stained using the 1D4 anti-rhodopsin antibody (A). The boxed areas are shown in higher magnification in B–D. Abundant gold particles were observed both on the photoreceptor outer segments adjacent to the apical RPE (B) and on apical phagosomes (C), but they were absent from mature phagosomes (D). To measure the diurnal shedding and uptake of spent outer segments, retinas were flat mounted (E), stained using the 1D4 antibody and an anti-ZO-1 antibody, and images of regions of interest (red circles and high-magnification zoom) were captured for morphometric analysis. (F) Average numbers of 1D4-positive phagosomes per cell were counted from four to six regions of interest for both control and ANX A2^{-/-} retinas at times before and after lights on as indicated ($n = 3$ eyes per time point for control and $n = 4$ eyes per time point for knockouts). p values were calculated using Student's t test as follows: $p^* < 0.05$.

would lead to a discernible defect in phagocytosis in the ANX A2^{-/-} mouse. Retinal sections were prepared as described above from ANX A2^{-/-} and wild-type eyes harvested 30 min after lights on, and the numbers and positions of phagosomes were mapped according to their relative distance from Bruch's membrane (Figure 5A). In control mice, the phagosomes partitioned into two major peaks, one peak consisting of early phagosomes at the apical RPE cell surface roughly coincident with the photoreceptor interface, and the other peak comprising more mature phagosomes in the basal compartment of the cell. However, in the ANX A2^{-/-} mice, ~15% of the total phagosome population was retarded in the apical processes (shaded area in Figure 5B), a domain in which we did not observe any phagosomes in the control mice. The total number of phagosomes per unit RPE length was not significantly different in the ANX A2^{-/-} and wild-type mice. Collectively, these findings suggest that shedding of outer segments occurs normally in the absence of annexin A2 but that the rapid engulfment and internalization of POS are defective. The reasons for this are not clear, but some electron micrographs of shed outer segments in the ANX A2^{-/-} mice revealed that although the apical microvilli lie in close apposition, the membranes were apparently unfused (Figure 5C), providing a possible explanation for the delay in transport toward the apical surface of the RPE cell.

The delay in POS internalization observed *in vivo* is consistent with the findings reported in cultured cells (Figure 1) but does not provide information about the kinetics of POS phagocytosis during the diurnal peak. To address this question we performed a quantitative analysis of phagosome internalization over an approximately 5-h period around

lights on. In these experiments, we used the 1D4 anti-rhodopsin antibody, which we show here to specifically recognize only early phagosomes. Thus, immunoelectron microscopy revealed prominent gold labeling in the photoreceptor outer segments and in apical phagosomes, but none in more basal phagosomes, despite these structures still clearly containing the membranous discs characteristic of photoreceptors (Figure 6, A–D; for more examples, see Supplemental Figure 2). Using this antibody together with antibodies to ZO-1, we probed RPE flat mounts from control and ANX A2^{-/-} mice prepared from eyes at time points before and after lights on. Regions of interest were defined for each flat mount and the numbers of 1D4-positive phagosomes and cells were counted in order to obtain an average for each retina (Figure 6E). The numbers of phagosomes per cell were then plotted as a function of time, relative to lights on, for both control and mutant mice (Figure 6F). These data show a transient increase in 1D4-positive phagosome numbers in control mice shortly after lights on, consistent with the well documented diurnal burst of outer segment shedding, but a delayed and more persistent accumulation of phagosomes in the ANX A2^{-/-} mice. Analysis of 1D4-positive phagosome numbers revealed significantly more ($p = 0.05$) phagosomes in the ANX A2^{-/-} mice than the control mice at ~1 h after lights on, corresponding to the time of maximal shedding and uptake (Figure 6G).

Defective Tyrosine Kinase Signaling during Phagocytosis in the Absence of annexin A2

Finally, we examined tyrosine kinase signaling during retinal phagocytosis in normal and ANX A2^{-/-} mice, specifically to find out whether annexin A2 is tyrosine phosphorylated

Figure 7. Tyrosine kinase signaling is defective in anx A2-deficient retinas. Eye cup lysates were prepared from control (A) and ANX A2^{-/-} (B) mice at intervals indicated, from before “lights on” in the animal house to approximately midmorning. The shaded bar shows the time of artificial daybreak (a 15-min ramp) in each experiment. Using the lysate of one eye, anti-phospho-tyrosine (p-Tyr) antibodies were used to immunoprecipitate (IP) tyrosine-phosphorylated proteins that were then probed on Western blots by using antisera to FAK, c-Src, and anx A2. Relative changes in band intensities are also quantified $\pm$ SEM, where $n = 3$ for FAK and c-Src in control animals, and $n = 4$ for FAK and c-Src in knock-outs. The data show that anx A2 is tyrosine phosphorylated with similar kinetics to FAK and c-Src in control mice but that tyrosine phosphorylation of FAK and c-Src is significantly delayed in the ANX A2^{-/-} mice. Western blots (WB) of whole cell lysates prepared from the other eye were probed using antisera to FAK, c-Src, and anx A2, revealing no significant change in expression of any of these proteins during the period of the experiment.

during phagocytosis *in vivo* and also to ask whether the absence of anx A2 would lead to changes in FAK activation. We therefore probed anx A2, c-Src, and FAK in phosphotyrosine immunoprecipitates prepared from eye cup lysates from age-matched control and ANX A2^{-/-} mice at intervals from just before lights on, which occurs as a 15-min ramp in our animal facility, to late morning. In normal mice, we observed tyrosine phosphorylation of FAK, c-Src, and anx A2 from shortly before light onset to a peak after ~105 min, which then declined to baseline during the next 90 min (Figure 7A). There was no significant underlying change in the expression of FAK, c-Src, and anx A2 at the protein level during this time. The time course adopted for parallel experiments in the ANX A2^{-/-} mice was shifted forward by 1 h because preliminary data revealed a delay in signaling events. In sharp contrast to the observations in normal mice in which FAK and c-Src tyrosine phosphorylation were first detectable just before light onset, tyrosine phosphorylation of both FAK and c-Src did not commence until some 2 h later in the absence of anx A2 (Figure 7B). The peaks of FAK and c-Src tyrosine phosphorylation were also correspondingly delayed, although the expression of both kinases was constant at the protein level. Together, these results show that anx A2 is required for the prompt rhythmic activation of FAK and c-Src during phagocytosis of POS by RPE cells.

DISCUSSION

In this study, we have provided the first direct evidence of a role for anx A2 in phagocytosis, the first evidence that anx A2 participates in a circadian-regulated process *in vivo*, and established that in RPE cells anx A2 functions as an early signaling intermediate in POS uptake. RPE cells are among the most phagocytically active of all cells in the body and several members of the annexin family have been identified

in mature human pigment epithelium (Alge *et al.*, 2003; West *et al.*, 2003). However, in a subtractive PCR analysis only annexins A2 and A4 were up-regulated when ARPE19 cells were allowed to differentiate on porcine lens capsule, used here as a surrogate for Bruch's membrane (Turowski *et al.*, 2004). Our data show that anx A2 is apically expressed in RPE-J cells and primary porcine RPE, whereas in ARPE19 cells it is expressed both apically and in the cytoplasm. Nevertheless, in all cell types analyzed, anx A2 was recruited to the intracellular apical surface-binding sites of POS and to newly formed phagosomes, suggesting that the apical pool of anx A2 may be more functionally relevant during retinal phagocytosis. Our findings in cultured cells are supported by immunogold labeling experiments on normal mouse retinas and are consistent with proteomic studies that identified anx A2 as a component of RPE apical microvilli (Bonilha *et al.*, 2004).

The kinetics of phagosome binding by anx A2, and its subsequent dissociation during phagosome maturation, were indistinguishable from those of F-actin. Actin polymerization drives phagosome formation, and in other phagocytic cell types it has been reported that F-actin begins to depolymerize at or before the point at which phagosome closure is complete (May and Machesky, 2001). It has been proposed that phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P₂) at the site of formation of the phagocytic cup and its subsequent hydrolysis as the cup closes are the key signaling events regulating the cycle of actin polymerization and depolymerization (Botelho *et al.*, 2000; Scott *et al.*, 2005). Because anx A2 is a potent regulator of actin dynamics (Merrifield *et al.*, 2001; Hayes *et al.*, 2006) and binds both cholesterol-rich membranes, Ca²⁺, and PtdIns4,5P₂ (Hayes *et al.*, 2004; Rescher *et al.*, 2004; Gerke *et al.*, 2005), it is well placed to function as a sensor of these second messengers and signaling intermediates, linking

(physically or indirectly) the phagosome membrane and the actin cytoskeleton. In support of this idea, we recently demonstrated that anx A2 can nucleate actin polymerization in vitro on beads coated with PtdIns4,5P₂ (Hayes *et al.*, 2009), and previous studies have shown that anx A2 nucleates actin patches on early endosomes (Morel *et al.*, 2009).

The activity of anx A2 as a regulator of actin dynamics is now known to be modulated by tyrosine phosphorylation. Thus, recent studies have shown that phosphomimetic mutants of anx A2 (anx A2^{Y23E} or anx A2^{Y23D}) can directly elicit changes in the F-actin cytoskeleton (Rescher *et al.*, 2008; de Graauw *et al.*, 2008) and drive endosome internalization (Morel and Gruenberg, 2009). Here, we microinjected GFP fusions of such mutants into primary porcine RPE cells and analyzed their subcellular localization after the addition of fluorescently labeled POS (Supplemental Figure 3). Wild-type anx A2, as well as the Y23E phosphomimetic and Y23F nonphosphorylatable anx A2 mutants, all localized to the apical cell surface and became enriched on forming phagosomes. Thus, recruitment of anx A2 to forming phagosomes is probably driven by factors such as elevation of intracellular Ca²⁺ and PtdIns4,5P₂ binding, with tyrosine phosphorylation occurring after phagosome association. This would be consistent with our recent finding that anx A2 is required for the plasma membrane targeting and activation of Src (Hayes and Moss, 2009), and the observations here that Src activation is markedly delayed in *ANX A2*^{-/-} mice.

In experiments in which anx A2 was depleted using siRNA, and in mice lacking anx A2, we observed significant retardation in POS internalization rather than an absolute failure. Unsurprisingly, given the intracellular localization of anx A2 in these cells, there was no effect on POS binding, and internalized phagosomes seemed structurally normal in retinal sections taken from *ANX A2*^{-/-} mice. However, consistent with data obtained in ARPE19 and RPE-J cells, the *ANX A2*^{-/-} mice exhibited defective uptake of shed POS, and as mentioned above, these mice also demonstrated a marked delay in the activation of FAK and c-Src. Although c-Src has not been shown previously to be activated during POS phagocytosis, its involvement is to be expected given the close interplay of this kinase with FAK (Mitra and Schlaepfer, 2006) and the participation of both kinases in phagocytosis in other cell types (Finnemann, 2003; Abram and Lowell, 2008). FAK has been shown to lie upstream of MerTK in the hierarchy of signaling molecules that regulate POS phagocytosis in RPE cells, and our observation that FAK activation is delayed in the *ANX A2*^{-/-} mouse suggests a model in which recruitment of anx A2 to the forming phagosome leads to activation of c-Src, which in turn is required for the activation of FAK.

At the cellular level, the phenotype of the *ANX A2*^{-/-} mice has parallels with that of mice lacking the Usher syndrome 1B gene myosin VIIa (Gibbs *et al.*, 2003). In these mice, the initial retrieval of outer segments is relatively normal, but newly formed phagosomes are retarded in the apical zone of the RPE cell body. Therefore, in terms of the kinetics of phagosome transport, anx A2 would seem to act upstream of myosin VIIa. The longer term consequences of *ANX A2* gene knockout on the health and function of the retina are not known. *ANX A2*^{-/-} animals at age 12 mo have normal retinal histology (unpublished observations) as was reported for *Myo VIIa*^{-/-} mice, although the association of myosin VIIa with Usher syndrome exemplifies the point that even a relatively subtle phagosome transport defect may have pathological consequences in species with sufficient longevity. Although little has been published on annexins in the retina, anx A2 expression has been reported

to increase with ageing in human RPE/choroid (Ida *et al.*, 2003) and has also been identified in drusen (Crabb *et al.*, 2002). The significance of these observations is not clear, but anx A2 is acutely sensitive to systemic stress factors associated with ageing, and covalent modification of anx A2 by glutathionylation, peroxynitrosylation, or glycation alters the properties of the protein (Hayes *et al.*, 2007). In future studies, examination of the effects of stress-associated modifications of anx A2 on its role in phagocytosis may provide insight into the mechanisms responsible for declining RPE cell function with age.

ACKNOWLEDGMENTS

We are grateful to Keith Morris and Matthew Pearson for technical support and to Volker Gerke (University of Muenster, Muenster, Germany), Karl Matter (University College London, London, United Kingdom), and Jesus Ayala-Sanmartin (Université Pierre et Marie Curie, Paris, France) for generously providing antibodies. This work was supported by grants from The Wellcome Trust (ref: GR072694MF), the Medical Research Council and Fight for Sight. QL and KAH were supported by grants from the NIH (HL 42493, HL 67939, and HL 46403).

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