

Mammalian Septins Are Required for Phagosome Formation

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Septins are members of a highly conserved family of filamentous proteins that are required in many organisms for the completion of cytokinesis. In addition, septins have been implicated in a number of important cellular processes and have been suggested to have roles in regulating membrane traffic. Given the proposed role of septins in cell membrane dynamics, we investigated the function of septins during Fc γ R-mediated phagocytosis. We show that several septins are expressed in RAW264.7 and J774 mouse macrophage cell lines and that SEPT2 and SEPT11 are colocalized with submembranous actin-rich structures during the early stages of Fc γ R-mediated phagocytosis. In addition, SEPT2 accumulation is seen in primary human neutrophils and in nonprofessional phagocytes. The time course of septin accumulation mirrors actin accumulation and is inhibited by latrunculin and genistein, but not other inhibitors of phagocytosis. Inhibition of septin function by transient expression of the BD3 domain of BORG3, known to cause septin aggregation, or depletion of SEPT2 or SEPT11 by RNAi, significantly inhibited Fc γ R-mediated phagocytosis of IgG-coated latex beads. Interestingly, this occurred without affecting the accumulation of actin or the actin-associated protein coronin-1. These observations show that, although not necessary for actin recruitment, septins are required for efficient Fc γ R-mediated phagocytosis.

INTRODUCTION

Phagocytosis of invading microorganisms is an important immune response orchestrated by neutrophils and cells of the monocyte/macrophage lineage. These specialized cells, termed “professional” phagocytes, recognize foreign microbes using specific cell surface receptors with the aid of host proteins called opsonins. Immunoglobulin G antibodies (IgG) are one of the major opsonins found within serum, and receptors that recognize IgG-opsonized particles (Fc γ R) do so via a highly conserved extracellular Fc-binding domain (Ravetch and Kinet, 1991). A number of key events take place between the time of initial binding of IgG-coated particles to the cell surface and the eventual degradation of these pathogens within intracellular phagosomes. The first notable event is the clustering and subsequent tyrosine phosphorylation of Fc γ Rs. Although the receptors themselves do not contain any intrinsic tyrosine kinase activity, several protein tyrosine kinases are known to be activated upon the clustering of Fc γ Rs (Greenberg *et al.*, 1994; Indik *et al.*, 1995b). Phosphorylation of tyrosine residues within the conserved immunoreceptor tyrosine activation motif (ITAM) provides important docking sites for SH2-containing molecules, most notably the tyrosine kinase Syk (Greenberg *et al.*, 1994, 1996; Indik *et al.*, 1995a; Crowley *et al.*, 1997; Kiefer *et al.*, 1998). Ultimately these early signaling events lead to local remodeling of the submembranous actin cytoskeleton

(Greenberg *et al.*, 1990), whose intracellular force-generating capabilities contribute, along with the targeted delivery of new membrane (Bajno *et al.*, 2000; Niedergang *et al.*, 2003), to the growth of the plasma membrane around the particle. Interestingly, expression of the Fc γ RIIA receptor in nonphagocytic cells, such as COS or Chinese hamster ovary (CHO) cells, is sufficient to confer on them phagocytic properties (Indik *et al.*, 1995a).

Although Fc γ R-mediated phagocytosis is an actin-dependent process, the role of other cytoskeletal elements is not particularly well understood. Several members of the septin family have been shown to be colocalized with filamentous actin (Kinoshita *et al.*, 1997, 2002; Xie *et al.*, 1999), possibly by their binding to myosin (Joo *et al.*, 2007). Septins are a highly conserved family of filamentous GTPases that were originally identified in *Saccharomyces cerevisiae* as factors controlling bud-site selection (for reviews see Gladfelter *et al.*, 2001; Longtine and Bi, 2003). However, since their discovery as factors that control cytokinesis in animal cells (Neufeld and Rubin, 1994; Kinoshita *et al.*, 1997; Surka *et al.*, 2002), numerous observations suggest that septins may also regulate other important cellular functions including cell membrane dynamics and vesicle fusion events (Fares *et al.*, 1995, 1996; DeMarini *et al.*, 1997; Hsu *et al.*, 1998; Beites *et al.*, 1999; Joo *et al.*, 2005).

In this study we investigated the role of mammalian septins during Fc γ R-mediated phagocytosis. We find that the murine macrophage cell lines J774 and RAW264.7 express a subset of septin gene products. Antibodies to two of these, SEPT2 and SEPT11, reveal that these proteins transiently accumulate at the phagocytic cup in J774, RAW264.7, and human neutrophils and in CHO or HeLa cells engineered to phagocytose. Inhibition of septin function, either by the use

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of a dominant inhibitor of septins, or by RNA interference (RNAi)-mediated depletion, significantly inhibits Fc γ R-mediated phagocytosis without affecting the signaling events required for actin accumulation.

MATERIALS AND METHODS

Reagents and Antibodies

Tissue culture supplies were purchased from Wisent (St. Bruno, QC, Canada). Unconjugated latex beads were from Bangs Laboratories (Fisher, IN). Fluorescein isothiocyanate (FITC), Cy3- and Cy5-conjugated donkey anti-rabbit antibodies, and Cy5-conjugated donkey anti-human IgG, were from Jackson ImmunoResearch (West Grove, PA). Human IgG was from Sigma-Aldrich (St-Louis, MO). Mouse anti-phosphotyrosine mAb was from Upstate (Charlottesville, VA). Alexa 488-, Rhodamine-conjugated donkey anti-mouse secondary antibodies, Rhodamine-phalloidin, and Alexa 488-phalloidin were from Molecular Probes (Eugene, OR). Rabbit anti-human septin 2 and 11 antibodies were raised against a synthetic peptides corresponding to the first 12 amino acids of human septin 2 and 11, respectively, and purified on columns containing the covalently bound peptide as described previously (Surka *et al.*, 2002). psiRNA-hH1GFPzeo and psiRNA-hH1gz-Scr (a plasmid containing a control sequence specific for luciferase) vectors were from InvivoGen (San Diego, Ca). All restriction enzymes were from New England Biolabs (Beverly, MA). Wortmannin, latrunculin B, and genistein were from EMD Biosciences (San Diego, CA). HeLa cells were obtained from the ATCC (Manassas, VA), and CHO-IIA cells were kindly provided by Dr. Alan Schreiber (University of Pennsylvania, Philadelphia, PA). HP Genome-wide small interfering RNA (siRNA) for human SEPT11 was obtained from Qiagen (Mississauga, ON, Canada) for the following target sequences: hs_SEPT11_2 HP, hereafter called SEPT11 siRNA1, 5'CAAGAGG-AATTGAAGATTTAAA3' and hs_SEPT11_5 HP, hereafter called SEPT11 siRNA2, 5'ACGGCTGAAGT-TAACCATTGT3'.

DNA Constructs

The construction and utilization of mammalian expression vectors pEGFP-PLC δ PH, pRFP-coronin-1, and pEGFP-PM have been described elsewhere (Botelho *et al.*, 2000; Scott *et al.*, 2005; Yan *et al.*, 2005). GFP₃-BD3 plasmid (Joberty *et al.*, 2001) was kindly provided by I. G. Macara (University of Virginia, Charlottesville, VA). pmRFP-PLC δ PH was generated by cutting the PH domain from pEGFP-PLC δ PH vector using XhoI and BamHI and subcloning the fragment into the corresponding sites of pDsRed-Monomer C1 vector (Clontech, Mountain View, CA). To generate siRNA specific for nucleotides 524–544 relative to the start codon of the human septin 2, the following complementary pair of nucleotides for this region including a hairpin sequence containing a BamHI site were generated to include Acc65I and HindIII at the ends: 5'GTACCTCGAATATTGTGCTGTCATTGGGATCCCAATGACAGGCACACAATATTCTTTTTGGAAA3' and 5'AGCTTTTCCAAAAAGAATATTGTGCTGCTGTCATTGGGATCCCAATGACAGGCACACAATATTGAG3'. These were cloned into the corresponding sites of psiRNA-hH1GFPzeo vector, expressing short hairpin RNA (shRNA) and green fluorescent protein (GFP) from H1 and CMV promoters, respectively.

Cell Culture and Transfection with DNA or shRNA

Culture conditions for mouse macrophage cell lines RAW 264.7, J774, and CHO cells stably transfected with Fc γ RIIA receptor (CHO-IIA) have been described elsewhere (Hackam *et al.*, 1997; Vieira *et al.*, 2003). CHO-IIA cells and RAW 264.7 cells were transiently transfected by using FuGene 6 (Roche, Nutley, NJ) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as suggested by the manufacturers. Cells were transfected either with GFP₃-BD3 using GFP as a control for 36–48 h or with shRNA-septin 2 using shRNA-control as control for 84–96 h. All other plasmids were transfected for 24 h.

Isolation of Human Neutrophils

Human blood was obtained from healthy volunteers. The neutrophils were purified using Polymorphprep (Accurate Chemical and Scientific, Westbury, NY) gradient separation procedure according to the manufacturer's instructions. Isolated neutrophils were resuspended in PBS containing 1 mM calcium chloride, 1 mM magnesium chloride, and 10 mM glucose at $\sim 1 \times 10^6$ cells/ml and used within 6 h of isolation.

Phagocytosis Assays and Inhibitors Treatment

Latex beads were opsonized with 0.8 mg/ml human IgG, incubated for 1 h at 37°C, and washed with phosphate-buffered saline (PBS) 3 times. When noted, the cells were treated with 250 nM wortmannin or 5 μ M latrunculin B for 30 min or 100 μ g/ml genistein for 3 h before phagocytosis. Opsonized beads were allowed to attach to the cells for 10 min on ice in cold HEPES-buffered RPMI. Unbound beads were washed away with cold PBS. Phagocytosis was started by replacing cold medium with HEPES-buffered RPMI prewarmed to

37°C and incubated at 37°C for the designated time. When necessary, the beads were stained by incubating with Cy5-labeled donkey anti-human IgG on ice for 15 min. Cells were then fixed with 4% paraformaldehyde and permeabilized as described previously (Yan *et al.*, 2005). Endogenous SEPT2 was detected by immunostaining with rabbit anti-human septin 2 primary antibody and Cy3-, FITC-, or Cy5-labeled donkey anti-rabbit secondary antibodies. In the case of SEPT11, cells were fixed for 10 min in 1% paraformaldehyde at 37°C, as above, stained with Cy5 donkey anti-human antibody to detect external beads, and then permeabilized with MeOH at -20°C for 10 min.

For phagocytic index assays, RAW 264.7 or CHO-IIA cells transfected either with GFP-BD3 or with shRNA-septin 2 were treated as above. HeLa cells were transfected with siRNA for 2 d and then retransfected with a plasmid containing the Fc γ IIA-GFP receptor, and phagocytosis was performed on the third day. The phagocytic index was calculated by counting the average numbers of internalized beads per cell and normalized to that of the controls performed the same day. The binding index was calculated by counting the average numbers of bound beads on the cell surface without performing phagocytosis and normalized as per the phagocytic index.

Microscopy

Laser-scanning confocal microscopy was performed using an LSA 510 laser scanning confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) with 63 \times or 100 \times oil immersion objective and analyzed using Photoshop 6.0 (Adobe, San Jose, CA). Spinning disk images were captured on a Zeiss Axiovert 200M equipped with a Quorum confocal spinning disk head (Quorum Technologies, Guelph, ON, Canada).

RESULTS

Septin Expression in Phagocytic Cells

To determine if any septin proteins might play a role in phagocytosis, we first performed Western blotting to examine their expression in two different murine macrophage cell lines, RAW264.7 and J774. We have previously found that almost all septin genes are expressed in brain (unpublished observations) so brain tissue was used as a positive control in these experiments. In addition, we examined the septin expression profile in a CHO cell line that has been engineered to phagocytose by stable expression of the Fc γ IIA receptor. As can be seen in Figure 1, SEPT2, SEPT6, SEPT10, and SEPT11 were abundantly expressed in all four cell types. SEPT9 was detectable in CHO cells but much less so in brain and not at all in RAW 264.7 or J774 cells. SEPT7 and was not studied here because of the lack of a suitable antibody, and SEPT12 is not shown because it is only abundantly expressed in testes and was not present in the brain or any of the cell lines (Steels *et al.*, 2007). Because septins typically associate in coimmunoprecipitating complexes (for example, see Kinoshita *et al.*, 2002) and because our antibodies to SEPT2 and SEPT11 are superior to anti-SEPT6 and SEPT10 for immunocytochemistry, we chose to focus on SEPT2 and SEPT11 for the remaining studies.

Fc γ -Receptor-mediated Phagocytosis: SEPT2 and SEPT11 associate with forming phagosomes

Although the full range of functions of septins remains unknown, these filamentous GTPases are required for cytokinesis and accumulate along with actin at the mitotic cleavage furrow in animal cells (Neufeld and Rubin, 1994; Kinoshita *et al.*, 1997; Xie *et al.*, 1999; Surka *et al.*, 2002). To determine whether septins may play a role in Fc γ R-mediated phagocytosis, another actin-dependent cellular process, we first examined their distribution in cells phagocytosing latex beads via Fc γ RIIA receptors (Figure 2). Robust accumulation of SEPT2 was observed in the murine macrophage cell lines J774 and RAW 264.7, although a stronger accumulation was observed in the former. In J774 cells SEPT2 accumulated all around the particle (Figure 2, a and b), whereas in RAW264.7 cells the accumulation was most typically seen at the base of the phagosome pedestal (Figure 2, c and d).

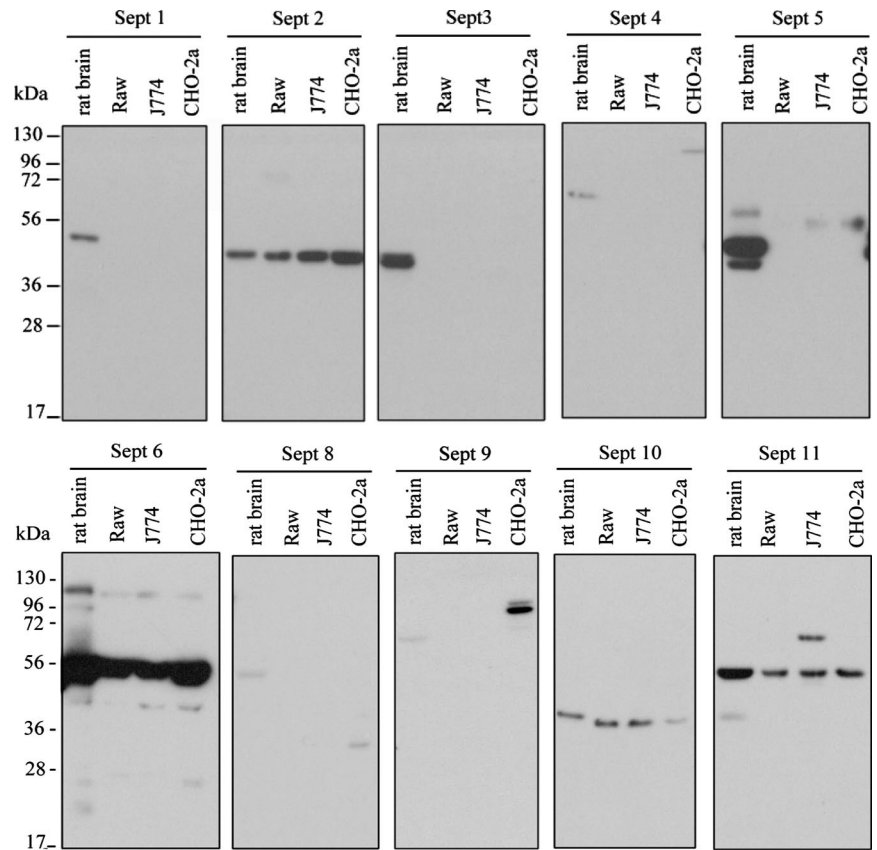


Figure 1. Western blot results of septins in phagocytic cell lines. RAW 264.7, J774, and CHO-IIA cell were harvested, and 10 μ g of each cell lysate with 10 μ g of rat brain as control were separated by 12% SDS-PAGE before immunoblotting. The blots were probed with rabbit polyclonal antibodies specific to septins 1–6 and 8–11.

Strong accumulation was also observed in primary human neutrophils (Figure 2, e and f) and in CHO-IIA cells stably expressing the Fc γ IIA receptor (Figure 2, g and h). It should be noted that the time course of phagocytosis in each of these models is quite different, with neutrophils being the fastest and CHO-IIA being the slowest. To determine if this was a specific accumulation of SEPT2, or involved multiple septins, likely in a complex, we also stained for SEPT11 (Figure 2, i and j). Although immunocytochemistry with this antibody is inferior to that of SEPT2, a clear accumulation of SEPT11 could be seen associated with the nascent phagosome in CHO-IIA cells.

Having established the presence of SEPT2 and SEPT11 in cells capable of Fc γ R-mediated phagocytosis, we next explored the time course of their appearance on the phagosome. Given the slower kinetics of CHO-IIA phagocytosis, these cells were chosen to monitor the internalization process. CHO-IIA cells were challenged with human IgG-opsonized latex beads for different periods of time at 37°C, fixed, and then stained for filamentous actin using Alexa488-labeled phalloidin and SEPT2. Within 3–5 min, opsonized latex beads are seen engaged in phagocytosis. Unsealed phagosomes were identified by staining for external human IgG after light fixation in the absence of detergent (not shown) and are indicated with open arrows (Figure 3a). The accumulation of filamentous actin in collar-like structures is characteristic of nascent phagosomes (Kolotila and Diamond, 1988; Greenberg *et al.*, 1990). Fully internalized particles not detected by immunostaining for human IgG in nonpermeabilized cells (filled arrows, Figure 3a) appeared to concurrently lose both actin and SEPT2.

We have previously shown that septins possess polybasic sequences that interact with phosphatidylinositol lipids

(Zhang *et al.*, 1999), and these lipids are also known to influence actin polymerization. We therefore examined the timing of PIP₂ accumulation at the phagosome relative to that of SEPT2. In this case CHO-IIA cells were transiently transfected with pEGFP-PLC δ PH (Scott *et al.*, 2005) and examined for 10 min after initiation of phagocytosis. As seen in Figure 3b, SEPT2 accumulates within 3 min to unsealed nascent phagosomes (open arrows) in concert with PIP₂ accumulations detected by pEGFP-PLC δ PH. As with actin, the SEPT2 and PIP₂ levels decrease on sealed phagosomes with a similar time course (solid arrows, 10 min).

SEPT2 Accumulation Depends on Actin

To begin to examine the signals that are responsible for septin accumulation at the nascent phagosome, CHO-IIA cells were treated with inhibitors capable of blocking phagocytosis. Wortmannin, a potent inhibitor of type I and III PI3 kinases, partially inhibits phagocytosis of large particles (Araki *et al.*, 1996; Cox *et al.*, 1999). After confirming that wortmannin had inhibited phagocytosis under the conditions used (not shown), there remains robust accumulation of actin at the base of bound opsonized beads, and SEPT2 can be seen to accumulate at these sites (Figure 4, a and b), indicating that PI3-kinase activity is not required for SEPT2 accumulation. We therefore examined an upstream event in the signaling process, the activation of src-family tyrosine kinases (Greenberg *et al.*, 1993). Using the general inhibitor of tyrosine kinases, genistein, we found that in the majority of the cells actin accumulation at the phagosome was inhibited. In these cells the accumulation of SEPT2 was also significantly reduced (Figure 4, c and d), consistent with the role of tyrosine kinases in early stages of actin remodeling.

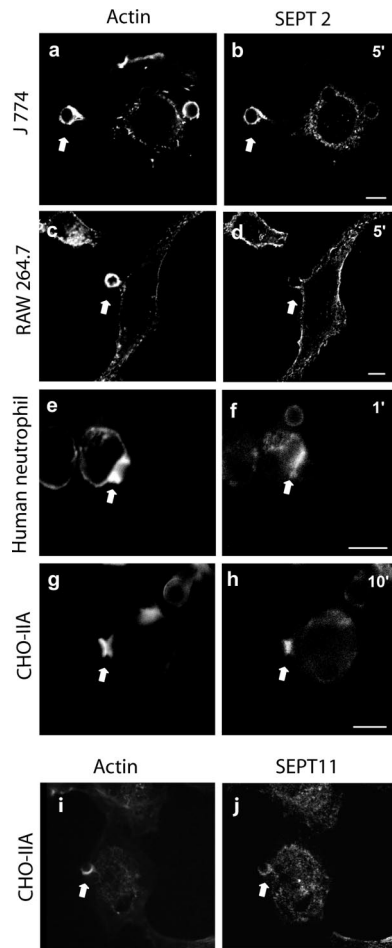


Figure 2. Endogenous SEPT2 and SEPT11 accumulate on phagosomes in different phagocytic cells. Opsonized beads were allowed to attach to the cells for 10 min on ice and then RAW 264.7 (a and b), J 774 (c and d), human neutrophil (e and f), and CHO-IIA cells (g–j) were warmed to 37°C to start phagocytosis for 5, 5, 1, and 10 min, respectively, at 37°C. Cells were then fixed and endogenous SEPT2 was stained with rabbit polyclonal anti-human SEPT2 antibody (b, d, f, and h) or SEPT11 with anti-SEPT11 (j) and detected with Cy3-conjugated donkey anti-rabbit antibody. Actin was stained with Alexa 488-phalloidin (a, c, e, g, and i). The arrows indicate accumulated actin and septin at sites of interaction with opsonized beads in each cell line. Bars, 5 μm.

Similarly, direct inhibition of actin accumulation at the phagosome by pretreatment of the cells with latrunculin B resulted in an inhibition of both actin and septin accumulation at the phagocytic cup (Figure 4, e and f). Together, these results suggest that the accumulation of septins at the phagosome depends on the accumulation of actin. These results are quantified in Figure 4g.

SEPT2 Function Is Required for Efficient Phagocytosis

The transient accumulation of SEPT2 at the phagosome raised the possibility that it may provide a necessary function for phagocytosis. To determine if septins are required for phagocytosis, we used two approaches to inhibit their function. First, we took advantage of a dominant inhibitor known to inhibit septins by causing their aggregation. Borg proteins are Cdc42 effectors, and one of the Borg isoforms, Borg3, was shown to bind to septins via its third Borg

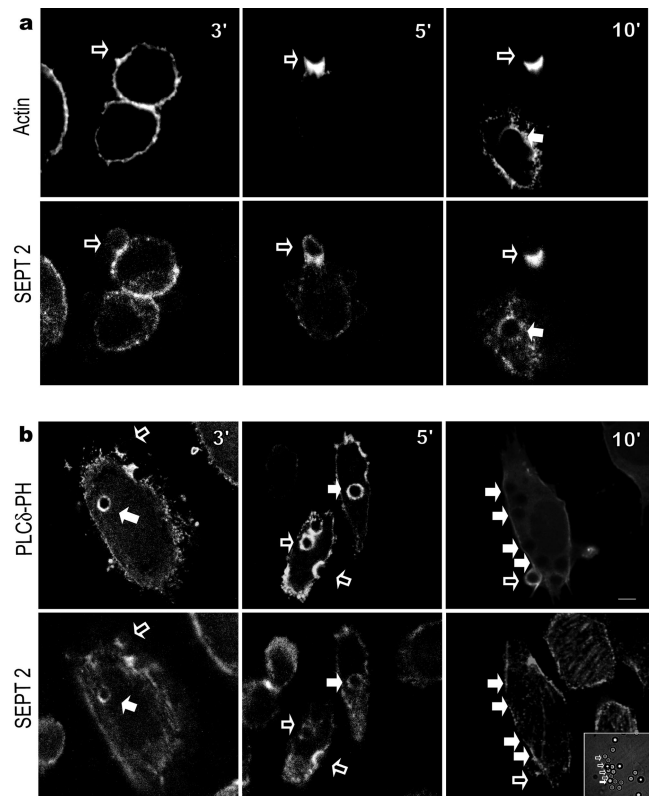


Figure 3. Time course of SEPT2, actin and PLCδ-PH accumulation on phagosomes during phagocytosis in CHO-IIA cells. (a) Opsonized beads were allowed to attach to CHO-IIA cells for 10 min on ice and then warmed up to 37°C to start phagocytosis. At the designated time interval, cells were cooled down on ice to stop phagocytosis. External beads were stained by Cy5-conjugated donkey anti-human IgG antibody for 15 min on ice. Cells were then fixed and stained with rabbit polyclonal anti-human SEPT2 antibody and Cy3-conjugated donkey anti-rabbit secondary antibody for endogenous SEPT2 and Alexa488-phalloidin for actin. (b) CHO-IIA cells were transfected with RFP-PLCδ-PH for 24 h. Phagocytosis was performed and cells were stained as described above. Top panels in a and b are actin and RFP-PLCδ-PH, respectively, and bottom panels are SEPT2. Open arrows point to unsealed phagosomes and solid arrows point to sealed phagosomes. Bars, 5 μm.

homology domain, BD3. Overexpression of BD3 fused to GFP was shown to result in the coaggregation of GFP-BD3 and septins into cytoplasmic structures in the majority of cells (Joberty *et al.*, 2001). The aggregation depletes the available septins, thereby inhibiting their function. We therefore overexpressed GFP-BD3 in CHO-IIA cells and found cytoplasmic aggregates in which SEPT2 and GFP colocalized in ~50% of transfected cells (data not shown), similar to that previously reported (Joberty *et al.*, 2001), but for all of these studies we limited our analysis to those cells exhibiting a clear cytoplasmic aggregation of GFP. Transfected cells were exposed to opsonized latex beads, allowed to phagocytose at 37°C for 30 min, and unbound beads were washed away. After fixation without permeabilization, the cells were stained with anti-human IgG antibodies to detect external particles. As can be seen in Figure 5a, cells overexpressing GFP-BD3 (arrowhead) were unable to phagocytose particles, whereas those transfected with GFP alone (Figure 5b, arrowhead) were fully able to internalize numerous beads. To ensure that this was not an artifact of the CHO-IIA model system,

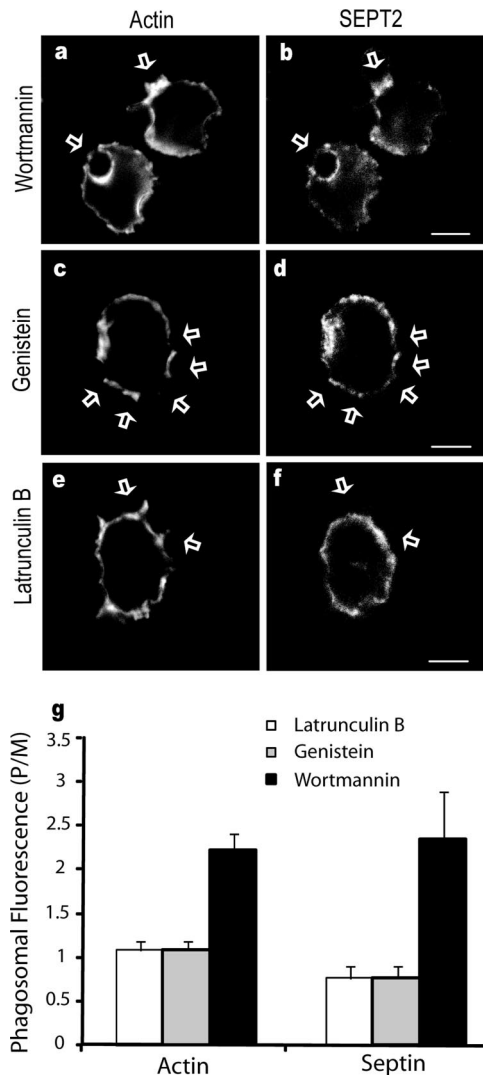


Figure 4. Effects of inhibitors on endogenous SEPT2. CHO-IIA cells were treated with 250 nM wortmannin, or 5 μ M latrunculin B for 30 min or 100 μ g/ml genistein for 3 h before phagocytosis. Opsonized beads were allowed to attach to the cells for 10 min on ice and then warmed to 37°C to start phagocytosis for 15 min. External beads were stained with Cy5-conjugated donkey anti-human antibody on ice for 15 min. The cells were then fixed and immunostained with Alexa 488-phalloidin for actin and SEPT2 antibody, followed by Cy3-conjugated donkey anti-rabbit antibody for endogenous SEPT2. (a and b) wortmannin, (c and d) genistein, and (e and f) latrunculin treatment. Arrows point to adherent beads. Bars, 5 μ m. (g) Quantitation of actin and SEPT2 accumulation after treatment with inhibitors. To quantify the accumulation of actin and SEPT2 at the phagosomal membrane, lines were drawn through the intersections of the phagocytic cup and the contralateral plasma membrane on the colocalized images. The fluorescence intensity of individual pixels was determined using Image J and presented as the ratio of that on the phagosome (P) to that of the normalized plasma membrane (M), hence P/M. In all cases subthreshold intensities were used to ensure that the signals were not saturated. Data are mean \pm SE of more than three independent experiments.

we repeated the same experiments in RAW 264.7 cells. As shown in Figure 5c, transfected RAW 264.7 cells (arrowhead) were unable to ingest bound particles (open arrow), whereas neighboring untransfected cells phagocytosed particles completely (filled arrow). Again, control GFP-trans-

fected cells, like their untransfected neighbors, were fully capable of robust phagocytosis (Figure 5d).

Although Borg3 is known to bind and influence septin organization in cells, it is not known how it does this or is it certain if it is specific for septins. We therefore sought an additional approach to inhibit septin function and used RNAi to deplete SEPT2 levels in phagocytic cells. To do so, we used a plasmid-based shRNA approach in which a previously characterized sequence was used to deplete SEPT2 (Kinoshita *et al.*, 2002). In cultures transfected with the SEPT2 shRNA vector containing a GFP reporter ~70% of the cells were effectively transfected, and by Western blotting we see an ~50% decrease in SEPT2 levels (Figure 6d). In SEPT2-shRNA-transfected cells (in Figure 5e, GFP-positive cells, indicated with a white dotted line) there were few internalized beads and many more that could be detected by antibodies in nonpermeabilized cells (open arrow). In contrast, neighboring GFP-negative cells were capable of ingesting particles efficiently (filled arrows). Transfection of the vector containing a scrambled sequence had no effect on phagocytosis efficiency (Figure 5f). Similar results were obtained when RAW 264.7 cells were used, although the low efficiency of transfection precluded their quantification.

Analogous experiments were also performed for SEPT11. In the first study we transiently transfected the Fc γ IIA receptor along with GFP-BD3 into CHOIIA cells and found that, like SEPT2, SEPT11 accumulates in the BD3 aggregate and phagocytosis is inhibited in these cells (Figure 5g). This inhibition of phagocytosis could be due solely to the depletion of SEPT2, which is also found in the aggregate, so to determine if SEPT11 is also required for phagocytosis we set out to deplete it by siRNA. Unfortunately, the sequence of SEPT11 in Chinese hamster is not known, and available rodent SEPT11 siRNA sequences did not appear to work in this cell line (data not shown). However, HeLa cells, like CHO cells, become phagocytic when forced to express the Fc γ IIA receptor. We therefore tested two commercial siRNA reagents specific for distinct target sequences and found that they were efficient at depletion of human SEPT11 protein from HeLa cells (Figure 6f). We therefore transfected HeLa cells with siRNA specific for human SEPT11, subsequently transiently transfected a GFP-tagged version of the Fc γ IIA receptor, and measured phagocytosis in the GFP-positive cells. As can be seen in Figure 5h, Fc γ IIA receptor-GFP-positive cells, which were depleted of SEPT11, failed to internalize beads, in contrast to the negative control siRNA. The same results were obtained with both siRNA sequences (not shown).

The results of three independent experiments were quantified and are shown in Figure 6. As can be seen in Figure 6, a–c and e, overexpression of Borg3 GFP-BD3 or shRNA depletion of SEPT2 and siRNA depletion of SEPT11 resulted in a significant decrease of between 50 and 70% of the phagocytosis without affecting the ability of the particles to bind to the phagocytic cells, for both the engineered phagocytic CHO-IIA and HeLa cells and the macrophage cell line RAW 264.7.

SEPT2 Depletion Does Not Affect Actin Accumulation

Septins and actin are closely associated in cells (Kinoshita *et al.*, 1997; Xie *et al.*, 1999) and functional interdependence has been suggested to exist between them. Specifically, depletion of septins results in loss of stress fibers, suggesting that they stabilize actin filaments, and actin filaments appear to serve as templates for septin filament assembly (Kinoshita *et al.*, 2002; Schmidt and Nichols, 2004). We therefore examined the effects of septin inhibition on actin accumulation at the

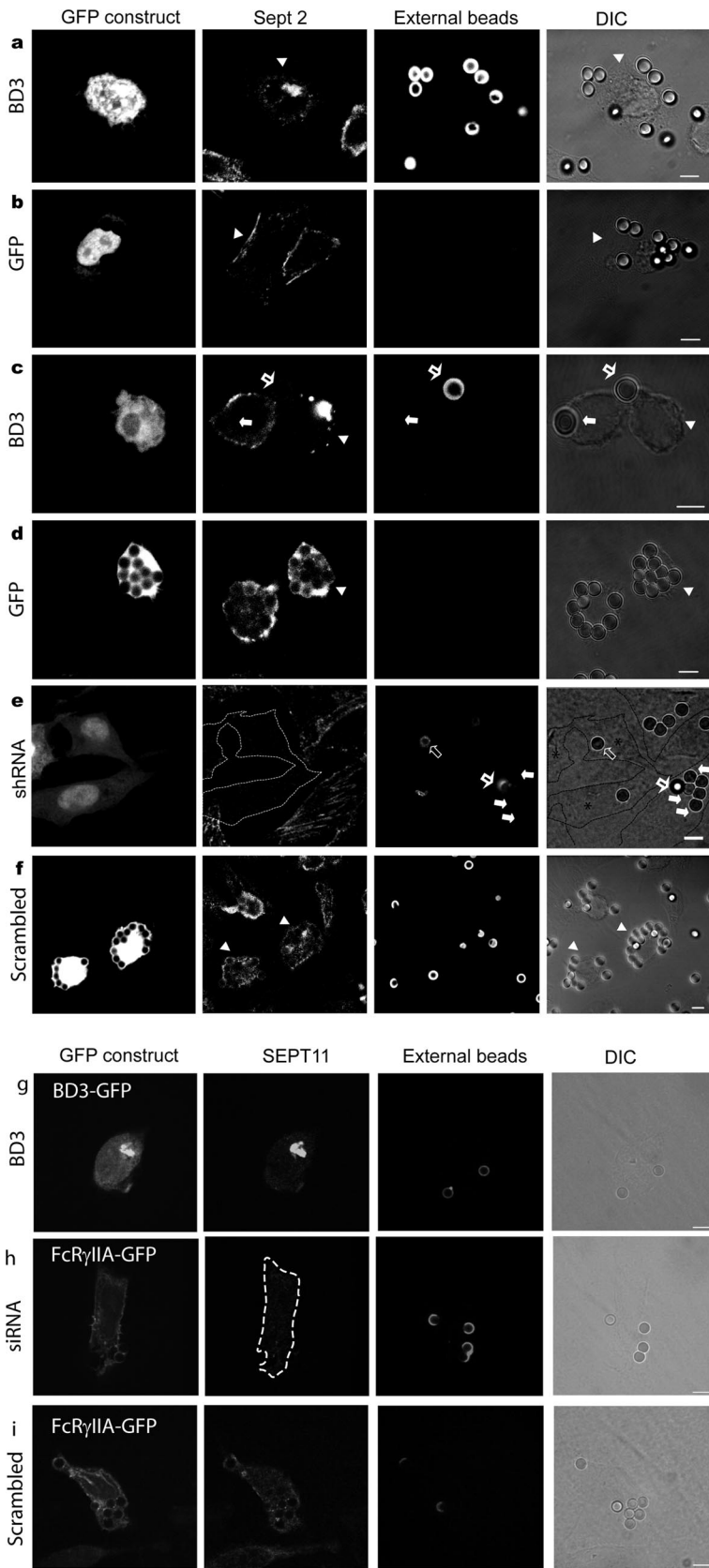


Figure 5. Effects of BD3 and RNAi on endogenous SEPT2 and SEPT11. CHO-IIA cells (a and b) or RAW cells (c and d) were either transfected with GFP-BD3 (a and c) or with GFP as control (b and d) for 36 h. The cells were allowed to ingest IgG opsonized beads for 45 or 30 min at 37°C for CHO-IIA cells or RAW cells, respectively. External beads were stained with Cy5-donkey anti-human IgG for 15 min on ice before immunostaining of the endogenous SEPT2. CHO-IIA cells were transfected with SEPT2 shRNA (e) or with scramble shRNA as control (f) for 84 h. The cells were allowed to ingest IgG opsonized beads for 45 min at 37°C. External beads and endogenous SEPT2 were immunostained as above. Panels from left to right are GFP constructs, endogenous SEPT2, external beads and differential interference contrast (DIC). Open arrows, unsealed phagosomes; solid arrows, sealed phagosomes. Arrowheads point to the transfected cells. (g) As in panel a, CHO-IIA cells were transfected with GFP-BD3 and in this case were stained for SEPT11. (h and i) siRNA specific for SEPT11 (SEPT11 siRNA2) or a scrambled control sequence were transfected into HeLa cells 72 h before the phagocytosis assay, and Fc γ IIA-GFP was transfected 1 d before the phagocytosis assay. In e and h, transfected cells are also indicated by white dotted lines. Bars, 5 μ m.

nascent phagosome. As shown in Figure 7, a–c, in CHO-IIA cells expressing GFP-BD3 (Figure 7a), when particles bind to

these cells there is robust accumulation of actin (arrows, Figure 7c) despite the failure to detect SEPT2 accumulation

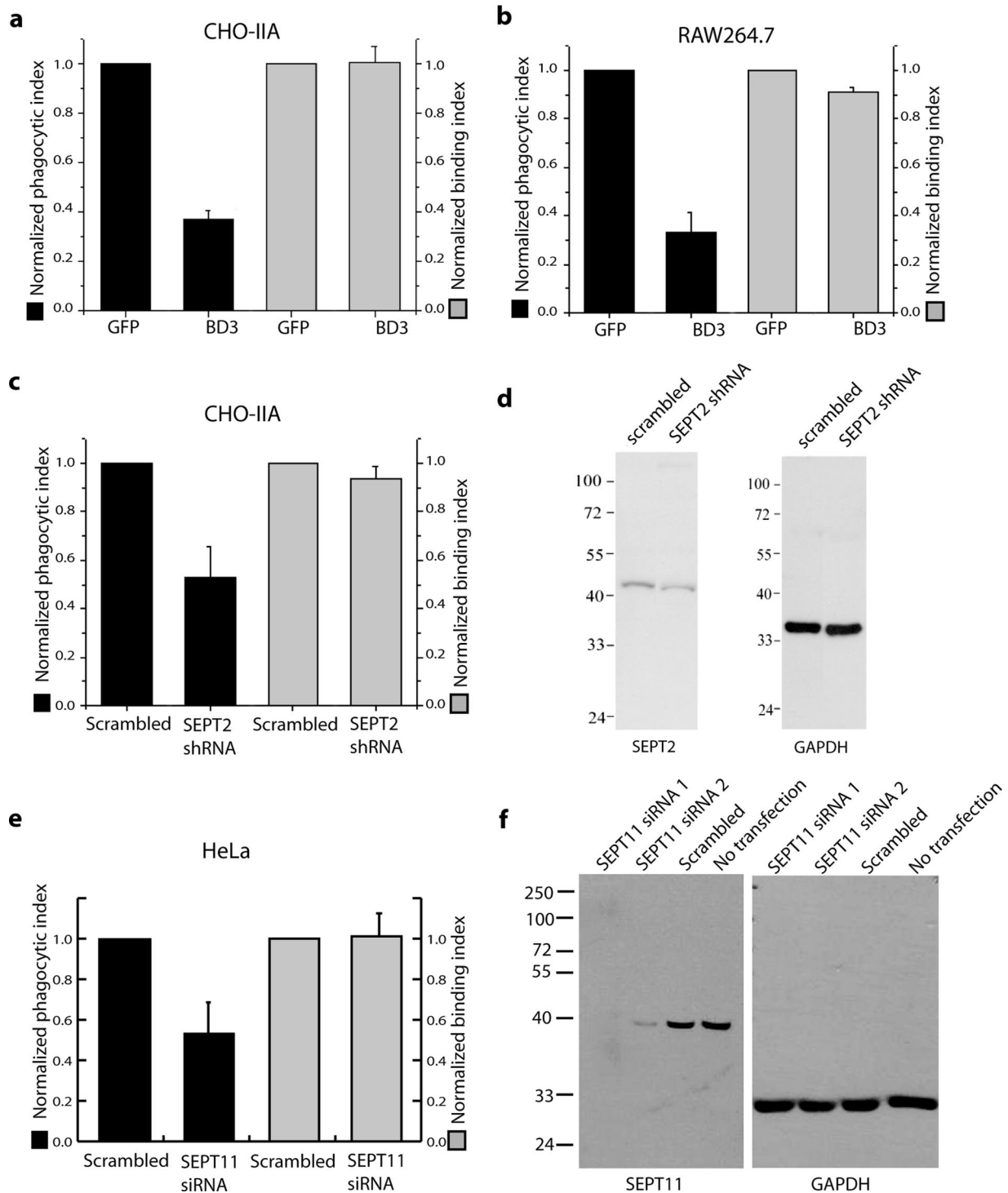


Figure 6. BD3 or Sept2 shRNA inhibit Fcγ-IIA receptor mediated phagocytosis. (a and b) Quantification of phagocytic and binding properties in cells transfected with either GFP (control) or with GFP-BD3. Phagocytosis was processed at 37°C for 45 min for CHO-IIA cells (a) and 30 min for RAW cells (b). (c) Quantification of phagocytic and binding properties in CHO-IIA cells transfected with either scramble (control) or SEPT2 shRNA. External and internalized beads were discerned by staining external beads with cy5-donkey anti-human IgG staining for 15 min on ice before permeabilization and immunostaining of the endogenous SEPT2 and internal beads. Phagocytic index and binding index were normalized to control. Data are mean ± SE of at least three independent experiments with at least 50 cells being counted in each case; *p < 0.05 and **p < 0.01. (d) Lysates from CHO-IIA cells transfected with scramble DNA or SEPT2 shRNA were run in 12% SDS-PAGE. The blots were probed with either SEPT2 antibody (left) or GAPDH antibody as loading control (right). (e) Quantification of phagocytosis in HeLa cells depleted of SEPT11. Experiments were performed as in c except that SEPT11 siRNA2 was transfected for 3 d before phagocytosis assay and FcγIIA-GFP was transfected in the same culture 1 d before assay. GFP-positive cells were counted for presence of internal and total beads. Similar results were obtained with SEPT11 siRNA1. (f) Lysates from HeLa cells transfected with SEPT11 siRNA1 or 2 or a scrambled control were run in 12% SDS-PAGE. The blots were probed with either SEPT11 antibody (left) or GAPDH antibody as loading control (right).

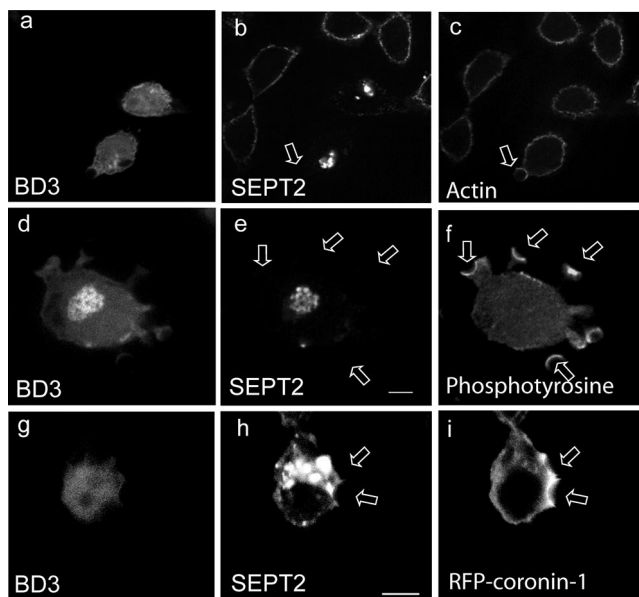


Figure 7. Effects of BD3 on part of the proteins related to Fc receptor signal pathway of CHO-IIA cells. CHO-IIA cells transfected with GFP-BD3 (a, d, and g) or cotransfected with GFP-BD3 and RFP-coronin-1 (i) were allowed to undergo for phagocytosis for 10 min before fixation. Endogenous SEPT2 was immunostained by SEPT2 antibody (b, e, and h), actin by Rhodamine-phalloidin (c) and phosphotyrosine by mouse anti-phosphotyrosine mAb (f). Open arrows, polystyrene beads at unsealed phagocytic cups; Bars, 5 μ m.

(Figure 7b). Similar observations are made with phosphotyrosine accumulation (Figure 7, d–f). This indicates that although septins may influence actin stability to some extent, they are not required for the accumulation of F-actin at the phagocytic cup.

We have previously shown that coronin-1 is an actin-associated protein necessary for phagosome formation, and one of its functions may be to recruit Arp2/3 to the nascent phagosome (Yan *et al.*, 2005). We therefore examined the accumulation of coronin-1 by transient transfection of RFP-coronin-1 in CHO-IIA cells expressing GFP-BD3. As seen in Figure 7, g–i, cells exhibiting aggregates of SEPT2 (Figure 7h) still had bound particles to which RFP-coronin-1 was recruited (Figure 7i, arrows). Hence, both the accumulation of F-actin and the recruitment of actin assembly factors appear to occur normally in the absence of functional septins.

DISCUSSION

Septins are filamentous GTPases conserved from yeast to man that have been implicated in a variety of cellular processes including cytokinesis, signal transduction, and membrane trafficking. It has also been speculated that they could function as membrane diffusion barriers. Given their many possible functions, we investigated their role in phagocytosis. We have shown here that phagocytic cells express a variety of septins, including septins 2, 6, 10, and 11. Using specific reagents to SEPT2 and SEPT11, we showed that these proteins transiently accumulated on phagosomes produced by murine macrophage cell lines, primary human neutrophils, and even in a CHO cell line engineered to be phagocytic. The time course of septin accumulation at phagosomes was consistent with that of PIP₂ and actin. Inhibition of septins, either by causing their aggregation by

overexpression of the BD3 domain of Borg3 (Joberty *et al.*, 2001) or by depletion of SEPT2 or SEPT11 by RNAi, decreased the efficiency of phagocytosis between 50 and 70%. This occurred despite the apparently normal recruitment of actin and actin-associated proteins to the nascent phagosome. Moreover, using a phagocytosis-competent cell line, CHO-IIA and transiently transfected HeLa cells, we were able to show that SEPT2 and SEPT11 are involved in the signaling events initiated by a single class of immunoglobulin receptors, Fc γ RIIA.

The mechanisms recruiting septins to the phagosome are not yet clear, but we and others have previously shown that septins are capable of binding to phosphatidylinositol lipids (Zhang *et al.*, 1999; Casamayor and Snyder, 2003; Rodriguez-Escudero *et al.*, 2005). Septins possess a conserved polybasic region adjacent to the GTPase domain that appears to be responsible for this interaction (Zhang *et al.*, 1999). In light of the importance of PIP₂ production (Coppolino *et al.*, 2002; Defacque *et al.*, 2002) and phosphoinositide 3-kinase signaling (Araki *et al.*, 1996) to Fc γ R-mediated phagocytosis, it is possible that one of roles of these lipids is in recruitment of the septins. Alternatively, others have shown that septins can be recruited to actin bundles by adaptor proteins such as anillin (Kinoshita *et al.*, 2002) and myosin (Joo *et al.*, 2007), raising the possibility that the accumulation of septins at the phagosome may be due to their assembly on actin-rich structures. However, anillin resides in the nucleus of interphase cells and would not be anticipated to be available as a linker. Hence, if septins are recruited to the phagosome by the presence of actin bundles, other scaffolding proteins such as myosin may be necessary to provide the linkage.

The exact role of septins in phagocytosis is unclear, but it does not appear to involve the recruitment or assembly of actin filaments in the vicinity of the nascent phagosome. This is somewhat surprising because mutations in yeast septins have been shown to affect the organization of filamentous actin (Adams and Pringle, 1984). In addition, depletion of septins in mammalian cells results in a reduction of actin stress fibers (Kinoshita *et al.*, 2002; Joo *et al.*, 2007), suggesting that septins may play a role in stabilizing these structures. However, we observed significant actin accumulation at the site of Fc γ RIIA receptor engagement, even in the absence of SEPT2 accumulation. In the case of SEPT2 or SEPT11 RNAi treatment, this could occur if other septins provided redundant roles in this process, but in cells transfected with GFP-BD3, all of the septins in the cell are aggregated together and therefore unavailable (unpublished observations and Joberty *et al.*, 2001).

Septins have also been implicated in regulating the stability of the microtubule network by binding to the microtubule-stabilizing protein MAP4 (Kremer *et al.*, 2005), and microtubule dynamics have been implicated in phagocytosis (Araki, 2006; Khandani *et al.*, 2007). However, we were unable to see changes in microtubule clustering, MTOC reorientation toward the phagosome, or changes in the levels of acetylated tubulin following SEPT2 knockdown or BD3 transfection (data not shown), as would be expected if septins regulated microtubule dynamics during this process. Another possible role of septins in phagocytosis could stem from their ability to anchor multiprotein complexes, as they do at the yeast bud neck during cytokinesis (Gladfelter *et al.*, 2001). In mammalian cells the recruitment of filamentous septins may aid in recruiting other associated multiprotein complexes (Joo *et al.*, 2007) to forming phagosomes on the plasma membrane and disruption of filaments would diminish the efficiency of the phagosomal machinery of the

cell. Evidence suggests that mammalian septins also participate in vesicle trafficking (Hsu *et al.*, 1998; Beites *et al.*, 2001), a process important for efficient phagosome formation (Bajno *et al.*, 2000; Niedergang *et al.*, 2003). It will therefore be interesting to determine whether depletion of septins in phagocytic cells inhibits membrane/vesicle trafficking during Fc γ R mediated phagocytosis.

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