TOR complex 2 (TORC2) in *Dictyostelium* suppresses phagocytic nutrient capture independently of TORC1-mediated nutrient sensing

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**SUMMARY**

The TOR protein kinase functions in two distinct complexes, TOR complex 1 (TORC1) and 2 (TORC2). TORC1 is required for growth in response to growth factors, nutrients and the cellular energy state; TORC2 regulates AKT signaling, which can modulate cytoskeletal polarization. In its ecological niche, *Dictyostelium* engulf bacteria and yeast for nutrient capture. Despite the essential role of TORC1 in control of cellular growth, we show that nutrient particle capture (phagocytosis) in *Dictyostelium* is independent of TORC1-mediated nutrient sensing and growth regulation. However, loss of *Dictyostelium* TORC2 components Rictor/Pia, SIN1/RIP3 and Lst8 promotes nutrient particle uptake; inactivation of TORC2 leads to increased efficiency and speed of phagocytosis. In contrast to phagocytosis, we show that macropinocytosis, an AKT-dependent process for cellular uptake of fluid phase nutrients, is not regulated by either of the TOR complexes. The integrated and balanced regulation of TORC1 and TORC2 might be crucial in *Dictyostelium* to coordinate growth and energy needs with other essential TOR-regulated processes.

**Key words:** Rapamycin, Raptor, Actin, FKBP12, 4E-BP, *Dictyostelium*

**INTRODUCTION**

The protein kinase TOR functions within (at least) two distinct multiprotein complexes (Laplante and Sabatini, 2009; Lee et al., 2005; Loewith et al., 2002; Sarbassov et al., 2004; Soulard et al., 2009; Yang and Guan, 2007). TOR complexes 1 (TORC1) and 2 (TORC2), that integrate signals arising from nutrients, the cellular energy state, and other stimuli to regulate growth and cytoskeletal response.

TORC1 is comprised of TOR, Raptor and Lst8 (mammalian GbL), and is required for cellular growth. The phosphorylation targets of TORC1 include the protein translation regulators 86 kinase (S6K) and 4E-BP1 (Brunn et al., 1997; Price et al., 1992). The activated phosphorylated form of SK6 phosphorylates and activates ribosomal protein S6, which stimulates protein synthesis. Conversely, 4E-BP, an inhibitor of eukaryotic (protein translation) initiation factor eIF4E, is inactivated by phosphorylation by TOR. TOR–Raptor interactions are essential for TORC1 function, and genetic inactivation of either TOR or Raptor in yeast, *Drosophila*, *Caenorhabditis elegans* or mammals is lethal (Gangloff et al., 2004; Hara et al., 2002; Kim et al., 2002; Kunz et al., 1993; Long et al., 2002; Murakami et al., 2004; Oldham et al., 2000). TORC1 is sensitive to inactivation by the anti-fungal, anti-neoplastic and immunosuppressant agent rapamycin (Abraham and Wiederrecht, 1996; Laplante and Sabatini, 2009; Loewith et al., 2002; Sarbassov et al., 2006; Sarbassov et al., 2005; Soulard et al., 2009; Yang and Guan, 2007). Rapamycin in complex with the cellular protein FKBP12 (Heitman et al., 1991; Kim et al., 2002) disrupts TOR–Raptor interactions and suppresses TOR phosphorylation of S6K and 4E-BP1, protein synthesis and cell growth.

TOR and Lst8 are also constituents of a separate protein complex, TORC2. Unlike TORC1, TORC2 does not contain Raptor and does not exhibit direct sensitivity to inactivation by rapamycin (Loewith et al., 2002; Sarbassov et al., 2006; Sarbassov et al., 2005). In addition to TOR and Lst8, the TORC2 of mammals, *Dictyostelium* and *Saccharomyces cerevisiae* contain Rictor-like ([Sarbassov et al., 2004], *Dictyostelium* PIA (Chen et al., 1997), *S. cerevisiae* AVO3 (Loewith et al., 2002)) and SIN1-like ([Frias et al., 2006; Jacinto et al., 2006; Yang et al., 2006], *Dictyostelium* RIP3 (Lee et al., 1999), *S. cerevisiae* AVO1 (Loewith et al., 2002)) subunits. Although Lst8 is required for full activity of TORC1 (Kim et al., 2003; Roberg et al., 1997), it might primarily function within TORC2; Lst8-null mouse embryos more resemble rictor-null embryos than raptor-null embryos (Guetin et al., 2006).

Many TORC2 functions are mediated by effects on the AKT protein kinase (Huang and Manning, 2009; Laplante and Sabatini, 2009; Soulard et al., 2009; Yang and Guan, 2007). AKT is activated by phosphorylation within the kinase activation loop by phosphoinositide-dependent protein kinase 1 (PDK1) and within a C-terminal hydrophobic (HM) motif by TORC2. In mammalian cells, AKT activity does not require HM phosphorylation by TORC2; TORC2 phosphorylation increases the substrate target range of AKT. Also in mammalian cells, TORC1 can inhibit...
phosphoinositide 3-kinase (PI3K) activity and suppress phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] accumulation (Shah et al., 2004). This action blocks recruitment of PDK1 and AKT to membranes and suppresses AKT phosphorylation by PDK1. Unregulated TORC1 can, thus, inhibit AKT activity, even in TORC2-activated cells, indicating antagonistic cross-talk between the TOR complexes. But, these regulatory interactions are not universal. In Dictyostelium, the activities of both PDK1 and TORC2 are completely independent of PI3K (Kamimura and Devreotes, 2010; Liao et al., 2010) and, thus, antagonistic cross-talk between TOR complexes 1 and 2 in the context of PI3K and AKT is not observed.

During growth in the wild, Dictyostelium engulf bacteria and yeast for nutrient particle capture by phagocytosis, an actin-dependent process. TORC1 in Dictyostelium has an essential role in control of cellular growth, and although TORC2 will phosphorylate AKT (Kamimura and Devreotes, 2010; Lee et al., 2005; Liao et al., 2010), the mechanistic interconnections of PDK1 and TOR in control of AKT in Dictyostelium differ significantly from those in mammalian cells. In Dictyostelium, phosphorylation of AKT by PDK1 and TORC2 are very tightly coordinated (Liao et al., 2010). PDK1 is unable to phosphorylate AKT in the absence of phosphorylation at the HM site, but PDK1 phosphorylation of AKT is absolutely required for activity in vivo. Furthermore, HM site phosphorylation is sufficient for AKT activity in vivo (Liao et al., 2010). Thus, in vivo, AKT requires phosphorylation at both the kinase and HM domains. In addition, PDK1 in Dictyostelium does not interact with PtdIns(3,4,5)P_3 and functions independently of PI3K (Liao et al., 2010). Finally, we have shown that phosphorylation of AKT at both the kinase and HM sites in growing Dictyostelium is unaffected by loss of TORC2 components Rictor/Pia, Lst8 or SIN1/RIP3 (Liao et al., 2010).

Despite the essential role of TORC1 in the control of cellular growth, we show that inhibition of TORC1 activity by depletion of nutrients from media, by genetic depletion of Raptor or by short-term treatment with rapamycin does not alter phagocytosis. Because TORC2 is implicated in the regulation of cell polarization and chemotaxis via its effects on actin function in Dictyostelium (Kamimura and Devreotes, 2010; Lee et al., 2005; Liao et al., 2010), we examined whether TORC2 is similarly required for regulation of actin assembly to promote nutrient particle capture. However, rather than suppressing yeast engulfment as might have been expected from previous hypotheses, we show that loss of TORC2 components promotes nutrient uptake via the upregulation of phagocytosis, supporting a negative role for TORC2 in nutrient particle capture.

We suggest that nutrient particle capture in Dictyostelium is negatively regulated by TORC2, but that particle capture is independent of TORC1-mediated nutrient sensing and growth regulation and of TORC1- or TORC2-dependent feedback loops that act on AKT. The parallel regulations of TORC1 and TORC2 might balance growth rates and regulate the growth-to-development transition. In this context, activation of TORC2 during early development might promote leading edge function of chemotaxing cells, but also inhibit phagocytosis, a potentially antagonistic pathway during Dictyostelium aggregation.

Results
Phagocytosis does not correlate with TORC1 activity
In the wild, Dictyostelium cells acquire nutrients through phagocytosis (Cardelli, 2001). Because TORC1 signaling promotes growth in response to nutrients, we examined whether TORC1 also regulates nutrient particle capture during Dictyostelium growth.

Dictyostelium grow logarithmically in nutrient-rich media provided that nutrients are not depleted and that cells are at moderate density (<5×10^6 cells/ml). Upon transfer of logarithmically growing Dictyostelium from growth medium to starvation (e.g. into 12 mM phosphate buffer), cells undergo growth arrest and a growth-to-development transition (Kimmel and Firtel, 2004). To determine whether TORC1 in Dictyostelium impacted nutrient particle capture, we quantified phagocytic rates in cells with fully activated or suppressed TORC1. To assay relative in vivo TORC1 activity, we monitored phosphorylation levels of the TORC1 substrate 4E-BP.

Logarithmically growing Dictyostelium showed persistent phosphorylation of 4E-BP as monitored with antibodies against human 4E-BP1 phosphorylated at T37 and/or T46 (Fig. 1A), or at T70 (data not shown) by using immunoblots. However, within 10 minutes of transfer from growth medium to starvation buffer there was rapid dephosphorylation of 4E-BP. Levels of phosphorylated 4E-BP did not change further when cells were maintained in starvation buffer for 2 hours (Fig. 1A) or more (data not shown). These data match those of mammalian cells cultured in serum-replete or serum-deprived medium and indicate that in vivo TORC1 activity is rapidly suppressed if cells are removed from an exogenous nutrient source. Re-exposure of starved Dictyostelium to fresh nutrients promoted re-phosphorylation of 4E-BP and cell proliferation (data not shown).

To determine whether there is a functional correlation between in vivo TORC1 activity and phagocytic rates, growing Dictyostelium were mixed with yeast labeled with tetramethyl rhodamine isothiocyanate (TRITC), and phagocytic uptake was monitored over an extended time course by quantifying internalized fluorescent yeast (Khurana et al., 2005). We simultaneously assayed in vivo TORC1 activity (i.e. 4E-BP phosphorylation) and quantified phagocytosis over 2 hours using growing cells or cells that had been nutrient-starved for 2 hours and maintained in starvation buffer (Fig. 1B,C). As shown, phosphorylated 4E-BP levels from growing cells in nutrient-rich medium or from cells starved in buffer differed significantly in phosphorylated 4E-BP levels and remained so for the first hour of the phagocytosis time-course assay (Fig. 1B). Nonetheless, phagocytic rates were identical between the two cell populations (Fig. 1C), which is consistent with work of others (Kato et al., 2007; Khurana et al., 2005). Eventually, incubation of Dictyostelium with yeast particles caused a moderate activation of TORC1 and 4E-BP phosphorylation. Nonetheless, phagocytic rates remained unaltered. 4E-BP re-phosphorylation could be similarly induced in starved cells by the addition of either glucose (80 mM) or the essential amino acid leucine (5 mM), but not glycine (5 mM), without an effect on phagocytosis (data not shown). Thus, nutrient particle capture efficiency does not correlate with in vivo TORC1 activity.

Inactivation of TORC1 through Raptor depletion suppresses growth but does not alter phagocytosis
Although our data indicate that nutrient sensing via TORC1 does not regulate phagocytosis, it is possible that nutrient withdrawal might not fully suppress TORC1 function. Therefore, we inactivated TORC1 by depleting Raptor protein, an essential
subunit of TORC1. We were unable to disrupt the Raptor gene in Dictyostelium using multiple targeting vectors and various selection conditions that optimized cell growth. Raptor is probably an essential gene in Dictyostelium, as it is in mammals, C. elegans, Drosophila and yeast (Hara et al., 2002; Kim et al., 2002). Therefore, we chose to deplete Raptor using a tetracycline repression (Tet-off) system that directs the conditional expression of an interfering RNA, based upon a long-hairpin (lhRNA) structure (Rosel and Kimmel, 2006).

For RNA interference (RNAi) experiments, cells were cotransformed with a raport (Tet-off) expression vector and a vector that encodes the tetracycline-dependent transrepressor (Blaauw et al., 2000). Cells grown in the presence of tetracycline did not express the engineered raptor RNAi; however, upon removal of tetracycline, raport RNAi was induced and, with time, endogenous raptor mRNA was depleted (Fig. 2A; to ~75%). Cell growth was also slowed and largely repressed by 24–30 hours (Fig. 2B). Similarly, phosphorylated 4E-BP levels in Raptor-depleted cells (raport RNAi cells) were reduced to approximately the level observed in wild-type cells starved of nutrients (data not shown).

We next quantified phagocytosis in control and raport RNAi-expressing cells after 34–40 hours of induction. Untransformed wild-type cells and mock (Blaauw et al., 2000) cells (wild-type cells containing the parental vectors that lacked the raport RNAi sequence) grown with or without tetracycline served as additional standards. No differences in phagocytosis were observed among any of these various cell lines, regardless of exposure to tetracycline (Fig. 2C; data not shown). Thus, inactivation of TORC1 through depletion of Raptor does not regulate phagocytosis. These data suggest that despite the role of TORC1 as a nutrient sensor and growth regulator, phagocytosis functions independently of changes in TORC1 activity.

**Long-term treatment with rapamycin stimulates phagocytosis in a Raptor-dependent manner**

To further address the role of TORC1 in the control of phagocytosis, we examined the effects of the TORC1 inhibitor rapamycin. To control for potential non-specific actions of rapamycin, we identified and genetically inactivated the rapamycin sensor FKBP12. Growth of Dictyostelium cells was strongly inhibited by 500 nM rapamycin (Fig. 3A) (Lee et al., 2005), whereas cells mutated for FKBP12 were insensitive to rapamycin (Fig. 3A). To determine the rapidity of the rapamycin response, we examined its effect on the phosphorylation of 4E-BP. The addition of 500 nM rapamycin to enriched growth medium rapidly (<10 minutes) reduced phosphorylated 4E-BP levels to that of starved cells (Fig. 3B).

We next quantified the effects of 500 nM rapamycin on phagocytosis. Cells were pre-incubated with rapamycin for 30 minutes, and phagocytosis was monitored for another 120 minutes in the continued presence of rapamycin. There was no initial effect of rapamycin on phagocytosis, but with extended treatment times (>90 minutes) rapamycin exerted a mild, but reproducible stimulatory effect (Fig. 3C; supplementary material Table S1, Fig. S1). This stimulation was greatly enhanced when pretreatment with rapamycin was extended to 5 hours prior to the phagocytosis assay (Fig. 3C; supplementary material Table S1, Fig. S1). Phagocytic rates in fkbp12-null cells were unaffected by rapamycin regardless of treatment times or
RNAi cells grown in the absence of tetracycline; see Fig. 2A,B) were largely refractory to rapamycin-stimulated phagocytosis (Fig. 3D; supplementary material Fig. S1); the small differences were not statistically significant ($P>0.1$), and probably reflect residual Raptor levels. Because phagocytic rates did not correlate with TORC1 activity (Fig. 1B,C and Fig. 3B,C), the long-term effects of nutrient particle capture through TOR–Raptor destabilization by rapamycin suggest interplay with another pathway.

**TORC2 suppresses phagocytosis**

Long-term rapamycin treatment can reduce TORC2 activity through inhibition of TORC2 assembly in mammalian cells (Sarbassov et al., 2006) and can partially inhibit TORC2 phosphorylation of AGC kinases AKT and PKBR1 in *Dictyostelium* (supplementary material Fig. S2). This raised the possibility that TORC2 might be an inhibitory target for the action of rapamycin on phagocytosis in *Dictyostelium*. We, thus, compared phagocytosis by *Dictyostelium* cells mutated for various TORC2 component genes with that of wild-type cells. For consistency and clarity, we chose to unify *Dictyostelium* and mammalian nomenclatures (see Materials and Methods) and henceforth refer to TORC2 components in *Dictyostelium* as Rictor(Pia), Sin1(RIP3) and Lst8.

Wild-type cells exhibited linear unsaturated particle uptake during the time-course of the experiment. However, cells lacking Rictor(Pia), Sin1(RIP3) or Lst8, central members of the TORC2 complex, had significantly elevated rates of phagocytosis for both yeast particles (Fig. 4A, Table 1) and latex beads (supplementary material Fig. S3) in comparison with wild-type controls. The initial rates (i.e. at 30 minutes) of yeast particle uptake for the various mutant strains exceeded that of wild-type cells by about sixfold.

Because actin polymerization is an essential determinant for phagocytosis and because TORC2 is implicated in actin regulation, we examined filamentous (F)-actin cytoskeletal changes in real-time during phagocytosis by wild-type cells and by cells lacking TORC2 components. F-actin was monitored by expression of the F-actin binding domain (ABD) of ABP-120/ filamin fused with GFP (GFP–ABD) (Pang et al., 1998). During phagocytosis there is an initial polymerization of actin at the site of particle attachment (see Fig. 4B, Table 2). Then, the phagocytic cup forms, with the yeast particle surrounded by F-actin- associated membrane (see Fig. 4B, Table 2). As the particle becomes fully internalized, F-actin is rapidly depolymerized. Actin polymerization and depolymerization at the phagocytic cups was not morphologically different in the various TORC2 mutant cell lines in comparison with wild-type (Fig. 4B).

Because rapamycin and FKBP12 specifically alter the interaction of TOR and Raptor within TORC1, we utilized Raptor-depleted cells to determine whether the longer-term sensitivity of phagocytosis to rapamycin required functional TORC1. Phagocytosis is stimulated by long-term rapamycin treatment in the various control cells, which express normal levels of Raptor, regardless of exposure to tetracycline (Fig. 3D; data not shown). However, Raptor-depleted cells (i.e. *raptor* conditions (Fig. 3C; supplementary material Table S1, Fig. S1), confirming the specificity of the rapamycin response.

Because rapamycin and FKBP12 specifically alter the interaction of TOR and Raptor within TORC1, we utilized Raptor-depleted cells to determine whether the longer-term sensitivity of phagocytosis to rapamycin required functional TORC1. Phagocytosis is stimulated by long-term rapamycin treatment in the various control cells, which express normal levels of Raptor, regardless of exposure to tetracycline (Fig. 3D; data not shown). However, Raptor-depleted cells (i.e. *raptor*
wild-type cells (Fig. 4B, Table 2). Thus, TORC2-deficient cells were significantly more efficient than wild-type cells in the phagocytic process, ultimately defining the increased rates observed during the quantitative, time-course phagocytosis assay (Fig. 4A).

**TSC2 pathway regulation of phagocytosis**

We next evaluated the potential role of other components of the TOR signaling pathway during phagocytosis. TSC2 is suggested to play a dual role in TOR signaling (Huang and Manning, 2009). As a Rheb–GAP, TSC2 serves to antagonize the TORC1 activator Rheb, but TSC2 has also been shown to activate TORC2 in mammalian cells (Huang et al., 2008).

We have identified TSC2 in the *Dictyostelium* genome by bioinformatic comparison (supplementary material Fig. S4A). *Dictyostelium* TSC2, of ∼325 kDa, has the characteristic tuberin and GAP domains and a putative AKT target at Ser2428 within the interdomain region (supplementary material Fig. S4A). TSC1 is less well-defined by annotation and we have not identified a *Dictyostelium* ortholog. Currently, we do not know if a functionally equivalent *Dictyostelium* TSC1 is too diverged or absent. The TSC1-interacting region of TSC2 lies within in its N-terminus, a region in *Dictyostelium* TSC2 that is both highly diverged in sequence and interrupted by homopolymeric asparagine repeats (supplementary material Fig. S4A).

Fig. 3. Differential effects of rapamycin on growth, 4E-BP phosphorylation and phagocytosis. (A) FKBP12 is required for rapamycin to inhibit *Dictyostelium* growth. Wild-type (WT) and *fkbp12*-null cells were grown to log phase (<2 × 10^6 cells/ml) and diluted into fresh media containing DMSO alone (control) or with 500 nM rapamycin. Aliquots were taken at various times to evaluate relative growth rate. (B) Rapamycin induces the rapid dephosphorylation of 4E-BP. Wild-type cells were grown to log phase (<2 × 10^6 cells/ml) and rapamycin was added to 500 nM. Whole cell protein samples were taken at times indicated and immunoblotted using antibodies against human 4E-BP phosphorylated at T37 and/or T46 (p4E-BP) and actin. Experiments were repeated three times. (C) Long-term, but not short-term, treatment with rapamycin stimulates phagocytosis. Wild-type or *fkbp12*-null cells were untreated (Control) or pretreated with 500 nM rapamycin for 30 minutes (Rap) or 5 hours (Rap, 5 hr), and mixed with TRITC-labeled, heat-killed yeast particles in the continued presence (or absence) of 500 nM rapamycin. At the times indicated, samples were removed and monitored by fluorimetric analyses. Arbitrary fluorescence units were used to normalize each strain relative to the maximum obtained for untreated control cells within the same experiment. Each of the treatments was compared at least three times; values indicate means ± s.d. (see supplementary material Table S1, Fig. S1). (D) Raptor is required for long-term stimulation of phagocytosis by rapamycin. raptor RNAi cells were cultured with or without tetracycline (see Fig. 2) for 34–40 hours, and then either untreated or pretreated with 500 nM rapamycin for 5 hours or not treated. The various cells were then mixed with TRITC-labeled, heat-killed yeast particles in the continued presence (or absence) of rapamycin and/or rapamycin. At the times indicated, samples were removed and monitored by fluorimetric analyses. Arbitrary fluorescence units were used to normalize each strain relative to the maximum obtained for control cells (tetracycline; – rapamycin) within the same experiment. Each of the treatments was compared at least three times; values indicate means ± s.d. For each experiment, we first confirmed growth inhibition of raptor-depleted cells after removal of tetracycline (see Fig. 2B). Data are from cells that also displayed suppression of Raptor expression. Statistical analysis did not reveal significant differences (P<0.05) between raptor RNAi cells and raptor RNAi cells treated with rapamycin (see supplementary material Fig. S1).
rates of phagocytosis (Fig. 5, Table 3) and, remarkably, cells lacking Rheb exhibited an increase in phagocytosis (Fig. 5, Table 3; supplementary material Fig. S5). The data suggest that AKT, TSC2 and Rheb function coordinately during phagocytosis, with the potential for AKT to function upstream during phagocytosis as a negative regulator of TSC2 signaling.

Neither TORC1 nor TORC2 regulate macropinocytosis or cell adhesion

AKT is an essential regulator of nutrient uptake through macropinocytosis (Rupper et al., 2001), a fluid phase uptake process that differs from phagocytosis (Cardelli, 2001). Because activated TORC2 suppresses nutrient particle uptake and because TORC2 can also target AKT, we wished to determine whether TOR regulation was also involved in nutrient uptake through macropinocytosis. First, utilizing a previously characterized akt

null strain (Meili et al., 1999), we showed that, as expected, macropinocytosis was suppressed in

null cells (Fig. 6A). Nonetheless, we also showed that macropinocytosis was unaffected by the loss of TORC2 components Rictor(Pia), Sin1(rip3) or Lst8 (Fig. 6A; data not shown). We further defined the specificity of TORC2 and TORC1 action by the inability of Raptor depletion or of long- (or short-) term 500 nM rapamycin treatment to affect macropinocytosis (Fig. 6A; data not shown). Thus, neither TORC2 nor TORC1 appear to regulate macropinocytosis.

To test whether the effect of TORC2 suppression of phagocytosis resulted from altered cellular adhesion, we quantified cell association of the various strains with surface matrices. We found that inactivation of TORC2 or TORC1, or

Table 1. Relative phagocytosis in TORC2 mutant cells compared with wild-type cells

| Cell line | At 30 minutes | At 120 minutes |
|-----------|---------------|----------------|
| rictor(pia) | 9.5±2.0 (P<0.0001) | 3.3±0.7 (P<0.0001) |
| sin1(rip3) | 6.6±1.1 (P<0.0001) | 2.7±0.5 (P<0.0001) |
| lst8 | 5.2±2.1 (P<0.003) | 2.4±0.2 (P<0.0001) |

Phagocytosis was compared at 30 minutes or at 120 minutes and relative values normalized to wild-type cells at the same time points (see Fig. 4A). Phagocytosis of rictor(pia), sin1(rip3) and lst8" cells reached an effective plateau by 60 minutes, whereas wild-type cells exhibited linear activity through 120 minutes. Hence, comparative rates of phagocytosis in the mutant cells are maximal by 30 minutes. Results are shown ± s.d. The two-tailed Student’s t-test was used for statistical analysis.

fig. 4. TORC2 suppresses phagocytosis. (A) Wild-type (WT) and mutant strains of Dictyostelium were mixed with TRITC-labeled, heat-killed yeast particles. At the times indicated, samples were removed and monitored by fluorimetric analyses. Arbitrary fluorescence units were used to normalize each strain relative to the maximum obtained for wild-type within the same experiment. Each of the cell lines was examined at least four times; values indicate means ± s.d. (see Table 1). Differences to wild-type were statistically significant (P<0.05). (B) Actin cytoskeletal associations at the phagocytic cup do not require TORC2. The various cell lines were marked with the fluorescence F-actin binding marker GFP–ABD (green) and mixed with TRITC-labeled, heat-killed yeast particles (red). Individual cells were imaged simultaneously for GFP and rhodamine fluorescence over time using confocal microscopy. Polymerization and depolymerization of actin (green) is observed around a yeast particle (red), marked by an arrow. Although expression of GFP–ABD has a slight deleterious effect on phagocytosis, it reduces particle engulfment rates equivalently in all cell lines. Thus, even in the absence of GFP–ABD, the rictor(pia)- and sin1(rip3)-null cells exhibit proportionately more efficient phagocytosis than do wild-type controls (see Table 2). The white-to-yellow arrow transition marks the time of particle internalization, which occurs more rapidly in the sin1(rip3)- and rictor(pia)-null cells than in wild-type cells (see also Table 2).
Table 2. Loss of TORC2 components increases the efficiency of phagocytosis

| Cell line | F-actin contacts* | Phagocytic cups† (%) | Engulfed yeast‡ (%) | Relative engulfment speed§ |
|-----------|-------------------|----------------------|---------------------|---------------------------|
| Wild-type | 40                | 55 (n=22)            | 35 (n=14)           | 1.0±0.1                   |
| rictor(pia) | 24                | 92 (n=22)            | 88 (n=21)           | 1.8±0.2 (P<0.001)         |
| sin1(rip3) | 28                | 93 (n=26)            | 89 (n=25)           | 2.0±0.2 (P<0.001)         |

*Number of yeast–Dictyostelium interactions assayed that induced F-actin polymerization (see Fig. 4B).
†Percentage number of yeast particles that initiated F-actin contacts and became partially surrounded by F-actin-associated membrane (see Fig. 4B).
‡Percentage number of yeast particles that initiated F-actin contacts and became fully internalized and no longer surrounded by F-actin-associated membrane (see Fig. 4B).
§Engulfment time of individual cell lines was assessed from a minimum of 14 engulfment events, for times from initial F-actin contact to full internalization.

The relative engulfment speed was calculated as an average of individual engulfment events in comparison to wild-type. Mean value for wild-type cells was arbitrarily set to 1.0; 2.0 indicates that mutant cells fully engulf particles twice as quickly as do wild-type cells (see Fig. 4B). Results are shown ± s.d. The two-tailed Student’s t-test was used for statistical analysis.

Rheb inhibits phagocytosis in a TORC2-dependent, but AKT-independent, pathway

Data obtained using sin1(rip3)-, rictor(pia)- and lst8-null cells indicate that TORC2 is a major regulator of phagocytosis (see Fig. 4). Although nutrient regulation of TORC1 is minimally involved in phagocytosis (see Figs 1, 2), AKT, TSC2 and Rheb also have a significant function (Fig. 5). Because AKT is an upstream inhibitor of TSC2, as well as a potential downstream positive target of TORC2, its genetic position in the regulation of phagocytosis is not clear.

To investigate the genetic connections among the various components further, we examined strains carrying additional mutations in the pathway. First, we expressed mutated forms of Rheb that exist in preferentially GTP-bound (RhebQ64L) and non-GTP-bound (RhebG106S) states (Long et al., 2005; Tabancay et al., 2003) and that function, respectively, as dominant-active and dominant-negative variants in other systems (supplementary material Fig. S4B). The Rheb constructs were N-terminally tagged with GFP, and expression of each variant was confirmed in cell populations by fluorescence and immunoblotting.

Expression of (non-GTP-bound) RhebG106S in wild-type cells enhanced phagocytosis, mimicking the rheb-null phenotype (Fig. 7A, Table 3). Conversely, (GTP-bound) RhebQ64L repressed phagocytosis when expressed in wild-type cells (Fig. 7A, Table 3), similar to the repression in tsc2-null cells (Fig. 5). We then examined genetically the epistatic relationship between Rheb and AKT and between Rheb and TORC2 by comparing rates of phagocytosis among wild-type and akt-, rip3- and lst8-null cells that expressed or did not express the dominant-active GTP-bound Rheb variant RhebQ64L. Whereas wild-type and akt-null cells expressing RhebQ64L were suppressed for phagocytosis (Fig. 7B), expression of RhebQ64L in either the rip3- or lst8-null background

Table 3. Relative phagocytosis in TORC2 regulatory mutant cells compared with wild-type cells

| Cell line | At 90 minutes | At 120 minutes |
|-----------|---------------|----------------|
| akt       | 2.9±0.39 (P<0.0001) | 2.5±0.17 (P<0.0001) |
| tsc2      | 0.7±0.19 (P<0.01)    | 0.6±0.20 (P<0.001) |
| rheb      | 2.0±0.40 (P<0.001)   | 2.0±0.20 (P<0.0001) |
| RhebQ64L  | 1.9±0.73 (P<0.01)    | 1.6±0.19 (P<0.001) |
| RhebG106S | 0.4±0.38 (P<0.01)    | 0.3±0.05 (P<0.001) |

Phagocytosis was measured at 90 minutes or at 120 minutes and relative values normalized to wild-type cells at the same time point (see Figs 6, 7). Results are shown ± s.d. The two-tailed Student’s t-test was used for statistical analysis.
had no effect on phagocytosis (Fig. 7C). Yeast particle uptake remained elevated in these cells compared with akt-null or wild-type cells that express RhebQ64L. TORC2 in Dictyostelium also phosphorylates PKBR1, an AGC kinase related to, but distinct from, AKT (Meili et al., 1999). Although AKT is the more active kinase during growth phase, TORC2 regulation of PKBR1 could still mediate regulation of phagocytosis. Nonetheless, we showed that pkbr1-null cells expressing RhebQ64L were suppressed for phagocytosis to the same degree as wild-type cells expressing RhebQ64L (supplementary material Fig. S6). These results also indicate that akt/pkbr1-null cells expressing RhebQ64L would have defects in both macropinocytosis and phagocytosis and, thus, would grow extremely poorly under all conditions. Consistent with this prediction, we were unable to isolate for study akt/pkbr1-null cells expressing RhebQ64L.

Collectively, the data indicate that Rheb-mediated suppression of phagocytosis requires TORC2, but that Rheb–GTP functions independently of AKT (or of PKBR1) as a downstream target, but potentially within an AKT–TSC2–Rheb circuit.

Discussion

In its ecological niche, Dictyostelium phagocytose bacteria and yeast to capture nutrients. We have shown that cells deficient in TORC2 components are more effective throughout the phagocytic pathway during quantitative, time-course measurements (see Fig. 4A). The initial rates (i.e. at 30 minutes) for the various TORC2-deficient strains exceeded wild-type rates by six- to tenfold, positioning TORC2 as a major negative regulator of phagocytosis in Dictyostelium.

In contrast to phagocytosis, macropinocytosis, the process of cellular uptake of fluid phase nutrients, is unaffected by the loss of TORC2 components or by the inhibition of TORC1 by rapamycin. We recognize that the laboratory strains used were selected for growth in liquid media (Maniak, 2001), and that the independence of macropinocytosis from TOR regulation might reflect second-site optimization for macropinocytosis in these strains.

Nonetheless, we have established phagocytosis in Dictyostelium as a downstream target of TORC2 signaling (see Fig. 8). This suggests that in addition to TORC1, TORC2 in Dictyostelium is also involved in nutrient-related signaling. In contrast to TORC1, which activates growth in response to high nutrients, TORC2 inhibits nutrient uptake. Consistent with these conclusions, we observed an inverse relationship (supplementary material Fig. S7) between in vivo TORC2 activation during development (Lee et al., 2005; Liao et al. 2010; Meili et al., 1999) and the relative ability of cells to undergo phagocytosis (Katoh et al., 2007). The local activation of TORC2 during chemotaxis might possibly serve to both activate the leading edge but also suppress phagocytosis, a potentially antagonistic pathway.
It should also be noted that phagocytic processes in Dictyostelium and mammalian macrophages are mechanistically conserved (Cardelli, 2001). Conceivably, TORC2 plays a crucial role during innate immune response and microbial defense (Sinclair et al., 2008), as for nutrient particle capture in Dictyostelium. The interrelationships of the TOR complexes might define new modes for balancing cell proliferation with growth arrest and other cellular processes.

The defined role of TORC2 in phagocytosis in Dictyostelium has permitted development of a novel and quantifiable assay for analyzing additional components in TOR signaling. This unique approach has allowed us to explore genetic interactions that had not been previously appreciated in Dictyostelium. Expression of GTP-bound Rheb suppresses phagocytosis in wild-type and akt-null cells, but it is unable to do so in either lst8- or rip3-null cells. Thus, activated Rheb regulates phagocytosis in a pathway that is independent of AKT, but integrated with TORC2.

A mechanism consistent with the genetic and known biochemical data would place AKT–TSC2–Rheb in a single functional regulatory pathway, with activation of Rheb antagonistic to nutrient particle capture in Dictyostelium (Fig. 8). Thus, akt- and rheb-null cells exhibit elevated rates of phagocytosis, whereas tsc2-null cells are suppressed. In addition, loss of PTEN, an antagonist of PI3K–AKT signaling, represses phagocytosis in Dictyostelium (Dormann et al., 2004).

An axis involving AKT–TSC2–Rheb would seem to implicate TORC1 in the regulation of phagocytosis in Dictyostelium. However, other aspects of TORC1 regulation are not consistent with TORC1 as the primary regulator. Manipulation of in vivo TORC1 activity by withdrawal of nutrients or short-term treatment with rapamycin does not alter phagocytosis, but reduces 4E-BP phosphorylation. In addition, depletion of Raptor causes growth arrest without affecting phagocytosis. Although long-term treatment with rapamycin will induce growth arrest and stimulate phagocytosis, the latter event will not occur in Raptor-depleted cells. Unlike mammalian cells, Rheb is not required in Dictyostelium for growth or for TORC1 activity in growth media. A Rheb–TORC1 connection with phagocytosis would be separate from a TORC1 dependency for cell growth and phosphorylation of 4E-BP.

Long-term effects of rapamycin on phagocytosis could result from secondary transcriptional changes. Although we cannot exclude this completely, several observations are not simply consistent with this as the major response. Rates of phagocytosis in cells deficient for Raptor, Rictor(Pia) or SIN1(RIP3) were not altered upon treatment with rapamycin (supplementary material Table S1, Fig. S1). If the primary response to rapamycin were transcriptional, we might have detected some changes in phagocytosis. We also suggest that if a major effect on phagocytosis were transcriptional, we would have observed differences in the rates of phagocytosis between growing and 2-hour starved cells, in which TORC1 activity was also rapidly repressed (Fig. 1) and major transcriptional changes ensued.

Loss of TORC2 components in Dictyostelium clearly activates phagocytosis, and genetic data indicate that Rheb suppression of phagocytosis cannot occur in the absence of productive TORC2. Although these might be unlinked events, data from Schizosaccharomyces pombe and mammalian cells indicate a potential function for Rheb that is independent of TORC1 (Aspuria et al., 2007; Karbowniczek et al., 2004; Otsubo and Yamamoto, 2008; Urano et al., 2007; Weisman et al., 2005).

During growth of Dictyostelium, PDK1 and HM site phosphorylations of AKT and AKT in vivo enzymatic activity

Fig. 7. Activated Rheb requires TORC2, but not AKT, to suppress phagocytosis. Wild-type (WT) and mutant strains of Dictyostelium were mixed with TRITC-labeled, heat-killed yeast particles. At the times indicated, samples were removed and monitored by fluorimetric analyses. Arbitrary fluorescence units were used to normalize each strain relative to the maximum obtained for wild-type within the same experiment. Each of the cell lines was examined at least three times; values indicate means ± s.d. Differences to wild-type for all stains were statistically significant (P<0.05). (A) Expression of the GTP-bound, Rheb\textsuperscript{Q64L} and the non-GTP-bound Rheb\textsuperscript{D60I} variants have contrasting effects on phagocytosis (see Table 3). (B) Expression of the GTP-bound Rheb variant Rheb\textsuperscript{Q64L} suppressed phagocytosis of akt-null cells. (C) Expression of the GTP-bound Rheb variant Rheb\textsuperscript{Q64L} is not able to suppress phagocytosis rip3- or lst8-null cells.
are unaffected by loss of TORC2 components (Liao et al., 2010). The cross-dependencies of PDK1 and TORC2 on AKT phosphorylation (Liao et al., 2010) and the independence of PDK1 from PI3K and TORC1 regulation (Kaminura and Devreotes, 2010; Liao et al., 2010) further demonstrate that AKT, or the related kinase PKBR1, do not function as downstream targets of either TORC2 or TORC1 in control of phagocytosis. Although during development there is a dependency of actin polarization on a TORC2–AKT pathway (Lee et al., 2005), during growth these components are unlinked for phagocytic uptake.

**Materials and Methods**

**Growth, development and transformation of Dictyostelium discoideum**

*Dictyostelium* AX3 cells were grown axenically in D3T medium (KD Medical) at 21°C (Kim et al., 1999). For transgene expression, *Dictyostelium* cells were transformed with 5–10 µg of circular plasmid DNA by electroporation. Linearized plasmids were used for targeted gene disruptions. Depending upon the antibiotic resistance cassette used, the medium was supplemented with 20 µg/ml G418 (Invitrogen) and/or 10 µg/ml blasticidin S (Fisher).

For Tet-off regulated expression (Blaauw et al., 2000), parental AX3 cells were co-transformed with the transactivator plasmid (MB35) and response plasmid (MB38) with or without the RNAi insert that was generated by linking a 760-nucleotide (nt) hairpin *Raptor* sequence separated by 372 nt of unpaired sequence to the tetracycline-repressible promoter (see below). Transformed cells were selected in the presence of tetracycline. To silence gene expression, media were supplemented with 30 µg/ml of tetracycline. To induce expression, exponentially growing cultures were washed twice with D3T medium and resuspended at 2 × 10^6 cells/ml in fresh D3T lacking tetracycline. Cells in media with or without tetracycline were grown in shaking culture and counted twice daily to assess growth. Samples were also withdrawn for RNA analysis.

For rapamycin growth curve analysis, log phase cells were diluted to ~2 × 10^6 cells/ml. Rapamycin was added to a concentration of 500 nM in DMSO, and DMSO was added to control cells. Shaking cultures were counted twice daily to assess growth.

**Cloning and molecular analyses**

Null mutants of FKBP12, Lst8, TSC2 and Rheb were generated by homologous recombination using targeting constructs disrupted by insertion of a blasticidin resistance cassette. All the transformants were screened by PCR and further confirmed by DNA and RNA blot hybridization. All of the constructs were verified by sequence analyses.

Null mutants of *FKBP12*, *Lst8*, *TSC2* and *Rheb* were generated by homologous recombination using targeting constructs disrupted by insertion of a blasticidin resistance cassette. All the transformants were screened by PCR and further confirmed by DNA and RNA blot hybridization. All of the constructs were verified by sequence analyses. The *rip3*-, *akt*-, *crac*- and *tra*-null cells were generously provided by Rick Firtel (University of California, San Diego, CA), Carol Parent (Centre for Cancer Research, Bethesda, MD) and Peter Devreotes (John Hopkins University School of Medicine, Baltimore, MD).

To generate regulated RNAi expression of *Raptor*, we constructed a long-hairpin RNA construct directed to a central region of *Dictyostelium Raptor* as part of the Tet-off system (Blaauw et al., 2000, Rosel and Kimmel, 2006). The region corresponding to 1860–2999 nt of *Raptor* complementary DNA (cDNA) was PCR-amplified from genomic DNA using the forward primer 5’-AGATCTATGAAAGTTAAAATTGGTGATCTTTG-3’ and the reverse primer 5’-GACGTCGAGTAATATTTTTGCAATTGGATTAG-3’ and ligated in the antisense orientation into the BglII/AatII sites of the Tet-off MB38 response plasmid to express antisense *Raptor*. Sense *Raptor* sequence 2239–2999 nt was PCR amplified from genomic DMSO and DMSO was added to control cells. Shaking cultures were counted twice daily to assess growth.
DNA using the forward primer 5'-GACGCTGCGTTGCGAGAA-GTG-3' and reverse primer 5'-GCCGGTGGTGGAAGAAT-3' and ligated in the sense orientation at the AatII/MluI sites of MB38 immediately 3' to the antisense insert. In both cases, genomic DNA was amplified from an intronless region of Raptor. All of the constructs were verified by sequence analyses.

Full-length Rheb cDNA was amplified by RT-PCR from total Dictyostelium vegetative RNA using the forward primer 5'-GGATCCATGCAAGGAC-3' and reverse primer 5'-TCTAGATGTCACCATC-3'. The Qiang OneStep RT-PCR Kit. To generate Gfp-tagged, TSC2 (DDB0214908), Raptor (DDB0191024), FKBP12 (DDB0201329), Lsh (DDB0232310), Pi3 (DDB0185005), RIP3 (DDB021662), TSC2 (DDB021824) and Rheb (DDB0229441), TORC2 components Pia and RIP3 have functional equivalents in the yeasts, Drosophila and mammals, and although the identification of the Dictyostelium genes (Chen et al., 1997; Lee et al., 1999) predate that of other systems (Frias et al., 2006; Jacinto et al., 2006; Löweth et al., 2002; Sarbassov et al., 2004; Yang et al., 2006), there has been near-universal agreement on a common nomenclature. As we believe consistency and clarity are essential for communication among workers in diverse systems, we have chosen to modify nomenclature of the Dictyostelium genes by additionally incorporating mammalian terminology. Hence, we refer to Pia as Rictor(Pia) and RIP3 as Sin1(RIP3).

Quantitative phagocytosis

Quantitative phagocytosis using heat-killed yeast cells was performed as described (Khurana et al., 2005). Dictyostelium were washed and resuspended in 17 mM phosphate buffer, pH 6.2 (PB) at 2 x 10^6 cells/ml. After recovery for 15 minutes in shaking suspension at room temperature, TRITC-labeled, heat-killed yeast cells were added to the cell suspension. Samples of 1 ml were withdrawn at indicated times and added to Trypan Blue solution (120 mg/ml dissolved in 20 mM sodium citrate containing 150 mM NaCl) to quench the fluorescence of non-integrated yeast. Cell pellets were collected and resuspended in PB, and fluorescence measured in a luminescence spectrometer LS50B (Perkin-Elmer) using 544 nm excitation of TRITC. Cell pellets were collected by centrifugation at 1600 r.p.m. Unattached cells were removed and counted using a hemocytometer.

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TORC2 suppresses phagocytosis

F-actin analyses by fluorescence microscopy

Polymerized (filamentous) F-actin was monitored by expression of Gfp–ABD, the F-actin binding domain (ABD) of ABD–GFP fused with GFP (Pang et al., 1998). Cells expressing Gfp–ABD fusion proteins were harvested in log phase, washed and resuspended in PB. Cells were plated on chambered cover glasses (Nalge Nunc International) and allowed to settle for 15 minutes. In vivo phagocytosis, using TRITC-labeled yeast, was performed as described previously (Khurana et al., 2005). Live cells were observed using an inverted microscope (Axiovert 100M) equipped with a confocal laser system (LSM 510, Carl Zeiss) and an oil immersion 63 x NA 1.4 objective lens. An argon laser (488 nm) was used for excitation of Gfp, and HeNe1 (535 nm) was used for excitation of TRITC.

Quantitative macropinocytosis

Log-phase cells were incubated with TRITC–dextran at room temperature, with shaking at 160 r.p.m. (Khurana et al., 2005). At selected times, 1-ml samples were withdrawn and quenched with Trypan Blue. Cell pellets were collected and resuspended in PB, and intracellular fluorescence measured; excitation was at 544 nm and emission at 574 nm. Arbitrary fluorescence units were normalized to the maximal value obtained for wild-type cells within the same experiment. All experiments were performed three to four times.

Cell adhesion

Adhesion of cells to a solid matrix was performed as described (Khurana et al., 2005). Briefly, log-phase cells were plated on tissue culture dishes and allowed to settle for 2 hours. The dishes were then shaken for 60 minutes at varying speeds, from 0 to 90 r.p.m. Unattached cells were removed and counted using a hemocytometer.
which is rapamycin sensitive, have distinct roles in cell growth control.

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protein required for chemotaxis and cyclic adenosine monophosphate signal relay in

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