Coordinate Control of Host Centrosome Position, Organelle Distribution, and Migratory Response by Toxoplasma gondii via Host mTORC2

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The invasion of host cells by Toxoplasma gondii is accompanied by a reorganization of host cell structure, in which the host centrosome and Golgi apparatus are localized to the vacuole, and mitochondria, microtubules, and endolysosomes are recruited to the vacuole perimeter. The mechanism and functional significance of this process have not been well defined. Here, we report that the centrosome-vacuole association was abolished in mammalian target of rapamycin complex 2 (mTORC2)-deficient cells, which also displayed a disordered distribution of perivacuolar host mitochondria and lysosomes. Infection of fibroblasts led to stable, mTORC2-dependent activation of Akt, and Akt inhibition mimicked the effect of mTORC2 ablation on centrosome, mitochondria, and lysosome localization. Mobilization of the centrosome by Akt inhibition was abrogated by inhibitors of glycogen synthase kinase 3 (GSK3), implying that the centrosome is constrained to the vacuole through an mTORC2-Akt-GSK3 pathway. Infected cells were incapable of migration in a wounded monolayer model, and this effect was associated with the inability of centrosomes to reorient in the direction of migration. Both migration and centrosome reorientation were fully restored upon ablation of mTORC2. These findings provide the first linkage of host signals that govern the polarization response, resulting in a stable, intrinsically polarized state. Multiple signal transduction pathways have been implicated in centrosome and microtubule polarization. Signaling via the Ser/Thr kinase Akt has been implicated in polarization during migratory responses in fibroblasts (14), and we have previously demonstrated that T. gondii infection of primary fibroblasts results in stable activation of host Akt (15). The upstream activators of Akt include phosphatidylinositol 3-kinase (PI3K) and mTOR complex 2 (mTORC2) (16). TORC2 has been implicated in the control of cell polarity during migration in Dictyostelium (17, 18), but the function of this complex in mammalian cells has not been well defined. We have noted a localization of host mTOR to the vicinity of the PV in T. gondii-infected cells and have observed up-regulation of mTORC1 function in these cells (15). In the current study, we have investigated the role of mTORC2-Akt signaling in host cell reorganization. Our findings demonstrate that this pathway is critical for control of centrosome position and organelle distribution in parasitized cells and indicate a link between centrosome constraint and regulation of migratory response.

EXPERIMENTAL PROCEDURES

Parasite Preparation and Cell Culture—T. gondii RH strain expressing yellow fluorescent protein (YFP) (a gift from Dr. Boris Striepen, University of Georgia) was maintained in human foreskin fibroblasts (HFFs). This strain is designated YFP-RH. Rictor-deficient and wild-type control MEFs were gifts from Dr. Mark Magnuson (Vanderbilt University).
mLST8-deficient and wild-type control MEFs were gifts from Dr. David Sabatini (Whitehead Institute). HFFs and MEFs were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (HyClone). To reduce basal Akt signaling, experiments were conducted in serum-free medium beginning at the time of infection. In some experiments, cells were treated with PL3K inhibitors LY294002 (LC Laboratories), wortmannin (EMD Biosciences), or PIK-90 (Axon Medchem), with Torin1 (gift from Dr. Nathanael Gray, Harvard Medical School), with Akt inhibitor VIII (AKTI-1/2; EMD Biosciences), with GSK3 inhibitors LiCl or SB216763 (EMD Biosciences), or with the microtubule-depolymerizing agent nocodazole (Sigma).

Immunoblotting Analysis—After washing with cold phosphate-buffered saline, cells were lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Ten to twenty micrograms of protein extracts were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with the indicated antibodies. Antibodies were obtained from Cell Signaling Technology for detection of pAkt (Ser473) (9271), pAkt (Thr308) (2965), total Akt (9272), pGSK3β (Ser21) (9336), phospho-S6 kinase-1 (Thr421) (9234), total S6 kinase-1 (9202), pS6 (Ser235/Ser236) (2211), total S6 (2217), and total GSK3β (9317). Anti-YFP was from Becton Dickinson.

Immunofluorescence Assay—Cells were seeded on coverslips and placed in a 24-well plate prior to infection. Cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% fetal bovine serum in phosphate-buffered saline, and then incubated overnight at 4 °C with primary antibodies, diluted in 1% bovine serum albumin in phosphate-buffered saline, against either α-tubulin (Abcam), γ-tubulin (Santa Cruz), cytochrome c (Abcam), phosphatidylinositol triphosphate (PIP₃; Echelon), or LAMP1 (Becton Dickinson). Cells were washed and incubated with Cy5-conjugated secondary antibodies for 1 h at room temperature. After extensive washing, the samples were dried and mounted with ProLong Gold antifade reagent (Invitrogen). Images were collected on a fluorescence microscope (Olympus 1X81) in the Analytical Imaging Facility of the Albert Einstein College of Medicine and analyzed using ImageJ.

RESULTS

Parasite-mediated Host Centrosome Polarization Requires Host mTORC2—To examine the effect of host mTORC2 on centrosome orientation in parasitized cells, centrosome polarization to the PV was assessed in wild-type and mTORC2-deficient MEFs that were infected for 24 h with a fluorescent strain of T. gondii. Immunostaining for the centrosome marker γ-tubulin revealed a close association between the host cell centrosome and the PV in wild-type cells (Fig. 1, A and B, upper panels), consistent with earlier studies (6, 7). In contrast, in MEFs deficient in either of two mTORC2 components, Rictor and mLST8, this association was disrupted, and centrosome...
position was observed to be distributed around the periphery of the host nucleus in an apparently random orientation with respect to the PV (Fig. 1, A and B, lower panels). The loss of constraint in centrosome position was associated with altered microtubule distribution. Although microtubule wrapping of the PV was observed in all genotypes, as previously observed for wild-type cells (6, 7), ~50% of mTORC2-deficient MEFs displayed an additional concentration of microtubules adjacent to the host nucleus at some distance from the PV (Fig. 1C). No cytoskeletal alteration was detected in mTORC2-deficient cells in the absence of parasite (supplemental Fig. S1). Infection had no effect on host actin organization (data not shown), consistent with previous studies (6). These data indicate that host mTORC2 signaling is an essential mediator of parasite control of host centrosome position.

mTORC2-dependent Activation of Host Akt by T. gondii—
The best characterized effector of mTORC2 is Akt. Akt can be activated by mTORC2-mediated phosphorylation in the hydrophobic motif at Ser473 (19–23), coupled with a requisite phosphorylation in the activation loop at Thr308, an event that is mediated by -3-phosphoinositide-dependent kinase-1 and is dependent on both Ser473 phosphorylation (24) and the PI3K-dependent generation of plasma membrane-bound PIP3, to which Akt and PDK-1 each bind (25). We examined the relationship between mTORC2 and Akt signaling in parasitized HFFs, in which we had previously demonstrated the stable activation of Akt by T. gondii (15). Activation of Akt in these cells, as well as phosphorylation of the Akt substrate GSK3β, was comparable in intensity to insulin treatment (Fig. 2A) and was detectable within 1 h of infection (supplemental Fig. S2A). Activation was mTOR-dependent because it was eliminated by treatment with the specific mTOR inhibitor Torin1 (26) (Fig. 2B) as well as by treatment with the nonspecific PI3K/mTOR inhibitors LY294002 and wortmannin (data not shown). Parasitized HFFs also displayed mTORC1 activation, as seen by phosphorylation of ribosomal protein S6 (Fig. 2B) as well as S6 kinase-1 (supplemental Fig. S2A). The latter event is not observed in all cell lines (15). As expected, treatment with the mTORC1 inhibitor, rapamycin, had no effect on Akt signaling (Fig. 2B) unless treatment was extended to time points at which mTORC2 is affected (27) (supplemental Fig. S2B), supporting the mTORC2 dependence of Akt activation in parasitized cells. Consistently, Akt activation and GSK3β phosphorylation were not observed upon infection of Rictor−/− or mLST8−/− MEFs (Fig. 2C and supplemental Fig. S3). Note that although mLST8 is a component of both mTORC1 and mTORC2, mLST8−/− MEFs are not defective in mTORC1 function (21). These data demonstrate the activation of mTORC2-Akt signaling in parasitized fibroblasts. The completion of Akt activation by phosphorylation at Thr308 is likely to occur via plasma membrane-localized -3-phosphoinositide-dependent kinase-1 because we observe a marked accumulation of PIP3 at the plasma membrane of parasitized cells (Fig. 2D) and an abrogation of Akt activation by the specific PI3K inhibitor PIK-90 (28) (supplemental Fig. S2C).

Akt-GSK3β Signaling Mediates Host Centrosome Constraint in Infected Cells—To examine the role of Akt signaling in centrosome polarity, we treated parasitized HFFs with Akt inhibitor VIII (AKTII), an allosteric inhibitor that targets Akt1 and Akt2
Roles of Akt and GSK3 in mTORC2-dependent centrosome polarization. A, HFFs were infected overnight with YFP-RH (pseudocolored red) and treated for 2 h with vehicle or 10 μM Akt inhibitor VIII (AKTi) followed by immunostaining for γ-tubulin. Centrosome positions are indicated by arrowheads. Scale bar, 5 μm. B, illustration of distance calculation. The shortest distance between the PV (red) and the centrosome (green) is measured using ImageJ software. C, GSK3 inhibition reverses Akt inhibitor-induced centrosome depolarization. HFFs were infected with YFP-RH overnight and treated for 2 h with 10 μM Akt inhibitor VIII, either alone or together with 10 μM SB216763 (SB), 20 μM LiCl, or 10 μM nocodazole (NOC). Cells were then subjected to immunostaining using anti-γ-tubulin. *, p < 0.01 compared with AKTi alone by Student's t test. Error bars, S.E. D, GSK3 inhibition enhances centrosome polarization in mTORC2-deficient cells. MEFs of the indicated genotypes were infected with YFP-RH overnight and treated with 10 μM SB216763 or vehicle (Con) for 2 h prior to immunostaining. Between 32 and 40 cells were examined in each group. The data in the figure are representative of two to three similar experiments. WT, wild type.

Treatment with AKTi for 2 h was sufficient to mobilize the centrosome, resulting in a randomized distribution indistinguishable from that observed in mTORC2-deficient cells (Fig. 3A and supplemental Fig. S4). Similar data were obtained using the PI3K/mTOR inhibitors LY294002 andwortmannin (data not shown). These results indicate that the mTORC2-dependent restriction of centrosome position is mediated by Akt and demonstrate that continual signaling is required to maintain PV-centrosome association. The Akt substrate GSK3β has recently been proposed as a master regulator of the microtubule cytoskeleton (30) as a consequence of its ability to phosphorylate multiple substrates, including APC, CLASP, CRMP-2, pVHL, and MAP1B, which associate with microtubule plus ends and promote microtubule stability and polarization (30–34). The stability and polarity-promoting effects of these substrates are negatively regulated by GSK3β activity, which is locally suppressed near the leading edge of migrating cells (35). GSK3β-regulated APC-microtubule association was shown to be required for centrosome polarization during astrocyte migration in a wound model (35). It was therefore possible that PV-centrosome association was impaired by mTORC2-Akt activation of polarity signals that act via GSK3β. In support of this idea, we found that inhibition of GSK3, using either LiCl or SB216763, restored PV-centrosome association in Akt-inhibited HFFs (Fig. 3C). Similarly, the GSK3 inhibitor SB216763 restored PV-centrosome association in mLST8−/− deficient MEFs (Fig. 3D). Notably, GSK3 inhibition increased PV-centrosome association even in wild-type cells (Fig. 3D), suggesting that the parasitized cell maintains a dynamic balance between polarizing and depolarizing signals.

The centrosome mobilization that we observe in Akt-inhibited cells might reflect random migration following release from the PV, or alternatively it might represent a polarization response to microenvironmental signals that act via an mTORC2-Akt-independent pathway. Consistent with the latter interpretation, centrosome mobilization following Akt inhibition was prevented by microtubule destabilization with nocodazole (Fig. 3C), suggesting that mobilization is an active microtubule-guided process.

Host mTORC2-Akt Signaling Is Required to Maintain Organelle Distribution at the PV Perimeter—Because lysosomes and mitochondria can associate with and traffic via microtubules (36–38), we next asked whether the distribution of these host organelles near the PV was dependent on mTORC2-Akt signals. In untreated, wild-type parasitized MEFs, the PV was observed to be uniformly lined with host mitochondria around the entire vacuolar circumference (Fig. 4A, WT), consistent with previous reports (8). In mLST8−/− or Rictor−/− MEFs, this association, although still evident, was no longer uniform. Instead, large portions of the vacuolar circumference were now devoid of host mitochondria, which appeared in clumps distributed asymetrically around the vacuole at irregular intervals (Fig. 4A). Comparable data were obtained upon treatment of wild-type infected cells with Akt inhibitor VIII, and this effect could be reversed by simultaneous inhibition of GSK3 (Fig. 4B). Equivalent results were obtained using either YFP-RH or wild-type RH strain (data not shown). Similarly, host lysosomes displayed a uniform punctate distribution surrounding the PV in wild-type MEFs, whereas a clumped, asymmetric distribution occurred in mLST8−/− cells and Rictor−/− cells (Fig. 5A) as well as in wild-type cells treated with Akt inhibitor VIII (Fig. 5B). No effect of mTORC2 depletion was observed on the distribution of mitochondria or lysosomes in uninfected MEFs (Figs. 4C and 5C). These results demonstrate a correlation between parasite regulation of the host centrosome/microtubule network and the maintenance of an ordered distribution of mitochondria and lysosomes around the PV, indicating a potential mechanistic linkage between these events.

Migratory Suppression in Parasitized Cells Correlates with Host Centrosome Constraint—The migratory response in fibroblasts is correlated with centrosome reorientation toward the leading edge (39). We reasoned that if centrosome orientation were necessary for directed migration, then constraint of the centrosome in parasitized cells might create a “frozen polarity” that would impair the reorientation required for directional migratory responses. To test this idea, we examined fibroblast migration in a wound response model. Parasitized HFFs were severely impaired in migration into the wound, in accord with the frozen polarity model (Fig. 6A). Treatment with Torin1, but not rapamycin, substantially restored migration (Fig. 6A), implying that the inhibitory effect of the parasite is dependent on mTORC2 signaling. These findings are consistent with the idea that centrosome mobility is a prerequisite for directed
migration in these cells. Similarly, migration was impaired by parasitization in wild-type MEFs, but was completely restored in mLST8−/− MEFs (Fig. 6, B–E). Neither Torin1 treatment nor mLST8 ablation significantly affected migration in unparasitized cells (Fig. 6, A and E). These results demonstrate that host mTORC2 signaling leads to migratory suppression in T. gondii-infected fibroblasts.

The suppression of migration by T. gondii suggested that parasite-constrained host centrosomes might be incapable of reorientation toward the wound. To assess reorientation, we measured the angle between the direction to the wound and the axis connecting the centrosome and nuclear center (Fig. 7A). This angle was small in both wild-type and mLST8−/− uninfected MEFs (Fig. 7A), indicating efficient centrosome reorientation in these cells, consistent with the lack of effect of mLST8 ablation on migration (Fig. 6, B and E).

DISCUSSION

The findings of this study provide the first evidence for the involvement of a host signaling pathway in the structural reorganization of cells invaded by Toxoplasma. The data show that
Host mTORC2-Akt signaling is required for multiple aspects of this reorganization, including the localization of the host centrosome, the organization of microtubules, and the distribution of mitochondria and lysosomes that surround the PV. Furthermore, continual signaling through this pathway is required to maintain these effects, which are reversible by inhibitor treatment.

A role for TORC2 complexes in cellular polarization has been suggested by studies in yeast and Dictyostelium. In Saccharomyces cerevisiae, TORC2 mutants are defective in actin polarization (40). In Dictyostelium, chemotaxis is dependent on TORC2, which is required for the localized phosphorylation of an Akt homologue (PKBR1) near the leading edge of the cell (17, 18). The directional persistence of migration in this organism is dependent on centrosome orientation (41). Our results indicate that mTORC2 can control both centrosome position and migratory response in mammalian cells, suggesting that a signaling sequence connecting TORC2, Akt, centrosome orientation, and directional migration may be conserved in evolution. Consistent with this idea, Akt activity has been found to promote growth factor-induced microtubule stability and motility in fibroblasts (14, 42). In mammals, the function of mTORC2 has not been well defined. Mice lacking mTORC2 components die in midgestation with developmental abnormalities that are apparently not due to dysregulated cell growth or apoptosis (20, 21). Conceivably, migratory or other polarity-based functions involved in embryonic morphogenesis are mTORC2-dependent and employ a signaling pathway similar to that which is exploited by the parasite.

Whereas the association of the centrosome with the PV is mTORC2-dependent, polarized migration in the wound model was independent of mTORC2. This mechanistic distinction between the responses of the centrosome to wound compared with parasite-initiated signals suggests that multiple polarization pathways may coexist in a single cell. Furthermore, our data indicate that these pathways can act competitively so that abrogation of one pathway can be a prerequisite for the action of another. The ability to assess alternative polarization pathways in a competitive setting, as in our migration studies, provides a new approach to the identification of components that limit the polarizing response. We interpret our findings as supporting the view that centrosome mobility is a limiting component and that the frozen polarity of the centrosome underlies the migratory suppression mediated by the parasite. Alternative possibilities, however, warrant investigation. For example, the orientation of the Golgi apparatus may be important because this organelle, which also associates with the PV, can provide microtubule nucleation function (11). The importance of centrosome orientation to the leading edge for directed migration has been controversial because it is not observed in some experimental systems (43–46). Future studies will be directed to the use of the novel system we have described to elucidate further the mechanistic linkage between centrosome orientation and migratory response.

In the parasitized fibroblast, our results show that mTORC2-Akt signaling controls centrosome localization via GSK3. This finding contrasts with the study of Gundersen and co-workers showing that during the migratory response of fibroblasts in a

![FIGURE 6. Role of mTORC2 in infection-induced suppression of cell migration.](image-url)
wound model, centrosome reorientation is independent of GSK3β, whereas microtubule stabilization was GSK3β-mediated (47). Earlier work from this group had shown that centrosome reorientation in the fibroblast wound model depends on signaling through a cdc42-Par6/protein kinase Cζ axis (39). This pathway performs a similar function in the astrocyte wound model; however, in the latter model GSK3β (a substrate of protein kinase Cζ) promotes centrosome polarity (35). A more recent study in the astrocyte model demonstrated that protein kinase Cζ can signal to microtubule-associated effectors via both GSK3-dependent and -independent mechanisms (48). It is possible that both GSK3-dependent and -independent polarization pathways exist and that the former are selectively activated via mTORC2. A large number of cell surface receptors are capable of initiating polarized migratory responses in mammalian cells, but in many cases the downstream effectors required for centrosome/microtubule polarization and directional persistence been not been defined. It will be of interest to determine whether the mTORC2-Akt-GSK3 pathway exploited by T. gondii functions in physiological responses to such receptors in uninfected cells.

The suppression of host cell migration by T. gondii is a novel finding of our study. This result provides direct evidence for a functional impact on the host cell of the structural reorganization induced by the parasite. Alternatively, it cannot be ruled out that mTORC2 signaling affects migration independent of its effects on reorganization. Further mechanistic studies will be required to settle this question. It also remains to be determined whether the effect of the parasite is on directional persistence, consistent with the observed effect on centrosome orientation, or on the speed of migration (motility). Toxoplasma has been shown to enhance the motility of dendritic cells (49); directional persistence was not examined by these authors. A specific suppression of directionality, rather than speed, might represent a logical adaptation for the parasite because the generation of T cell immune responses may depend on the homing of infected antigen-presenting cells to lymphatics and to T cell areas in lymphoid tissues. The extension of our findings to cell types that participate in immune responses represents an important subject for further study.

In addition to its role in mediating parasite effects on polarity and migration, mTORC2-Akt signaling also had a pronounced effect on the distribution of host mitochondria and lysosomes at the PV surface. The simplest explanation for this observation is that the distribution of these organelles may be governed by interaction with the centrosome/microtubule network. Mitochondria and lysosomes can associate with and traffic via microtubules (36–38). A possible scenario is that is that the initial recruitment of the organelles is mediated by PV-associated host microtubules and that association is then stabilized by tethering factors, such as ROP2 in the case of mitochondria. Disruption of host centrosome sequestration, following the ablation of mTORC2-Akt-GSK3 signaling, might then perturb the distribution of PV-associated microtubules while leaving the tethering function intact, resulting in organelles that are PV-associated but no longer homogeneously distributed. Our observation that parasite-regulated mitochondrial distribution is GSK3-dependent is consistent with this idea. More detailed microscopic studies of organelle and microtubule distribution, as well as mechanistic analysis of the role of mTORC2-Akt effectors, will be required to evaluate this hypothesis. Alternatively, organelle distribution may reflect other functions of the mTORC2-Akt pathway; for example, mitochondrial homeostasis may be adversely affected by reduced Akt-GSK3 signaling (50).

It remains to be determined whether the maintenance of an orderly distribution of host organelles at the PV perimeter has functional significance for parasite growth and pathogenesis. We did not observe a parasite growth defect in mTORC2-deficient MEFs (data not shown). However, growth was only examined in the presence of abundant nutrients and the absence of stress. We have previously shown that host structures, identified as autophagosomes by the presence of LC3, cluster near the PV and that host cell autophagy, up-regulated upon T. gondii infection, becomes essential for optimal parasite growth when environmental nutrients are reduced to physiological levels (9). It is possible, then, that under conditions that are suboptimal but physiologically relevant, homogeneous organelle distribution becomes critical for the efficient delivery of host resources to the PV.

In summary, the major conclusions from our study are as follows. First, continual signaling through host mTORC2-Akt is required for the retention of the host centrosome and the homogeneous distribution of lysosomes and mitochondria near the PV. This finding provides the first evidence that mTORC2 can function to control centrosome position in mammalian cells. Second, Akt control of host centrosome position and
mitochondrial distribution in parasitized fibroblasts is mediated via GSK3. Third, *T. gondii* suppresses both centrosome reorientation and migratory response in host fibroblasts. Finally, host cell migration and centrosome mobility are coordinate regulated by mTORC2, consistent with the view that centrosome orientation is an important component of directed migration. Overall, our findings provide an initial mechanistic insight into *T. gondii*-induced host cell reorganization and indicate a potential consequence of this process for host cell function. Further exploration of the pathways controlling these events should illuminate parasite manipulation of the host cell as well as provide insight into the regulation of polarity and architecture in mammalian cells.

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