Localization of mTORC2 activity inside cells

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Activation of protein kinase Akt via its direct phosphorylation by mammalian target of rapamycin (mTOR) complex 2 (mTORC2) couples extracellular growth and survival cues with pathways controlling cell growth and proliferation, yet how growth factors target the activity of mTORC2 toward Akt is unknown. In this study, we examine the localization of the obligatory mTORC2 component, mSin1, inside cells and report the development of a reporter to examine intracellular localization and regulation by growth factors of the endogenous mTORC2 activity. Using a combination of imaging and biochemical approaches, we demonstrate that inside cells, mTORC2 activity localizes to the plasma membrane, mitochondria, and a subpopulation of endosomal vesicles. We show that unlike the endosomal pool, the activity and localization of mTORC2 via the Sin1 pleckstrin homology domain at the plasma membrane is PI3K and growth factor independent. Furthermore, we show that membrane recruitment is sufficient for Akt phosphorylation in response to growth factors. Our results indicate the existence of spatially separated mTORC2 populations with distinct sensitivity to PI3K inside cells and suggest that intracellular localization could contribute to regulation of mTORC2 activity toward Akt.

Introduction

Mammalian target of rapamycin (mTOR) couples signaling from growth factor receptors with intracellular nutrient-sensing pathways, and thereby is critically involved in the control of cellular anabolic functions, growth, survival, and proliferation (Bracho-Valdés et al., 2011; Laplante and Sabatini, 2012). In eukaryotic cells, mTOR exists in two distinct multisubunit complexes with nonoverlapping functions: mTORC1 and mTORC2. Both complexes share their namesake kinase, mTOR, and its constitutive binding partner, mLST8. mTORC2 additionally contains the obligatory scaffold mRictor and mSin1 and, unlike mTORC1, is insensitive to inhibition by rapamycin. mTORC1 functions as a sensor of intracellular nutrient and energy levels and controls the balance between cellular anabolic and catabolic processes. mTORC2 couples extracellular signals (such as growth factors and cytokines) to mTORC1 activation, cell proliferation, and survival through direct phosphorylation of the AGC family protein kinase Akt on Ser473 in its hydrophobic C-tail, which is required for its maximum activation (Sarbassov et al., 2005).

To integrate extracellular signals with intracellular nutrient availability, the activity and regulation of mTORC2 complexes must be tightly coupled to its intracellular localization. Indeed, activation of mTORC1 by amino acids is dependent on its association with the lysosomal membranes (Sancak et al., 2008; Bar-Peled et al., 2013). Yet, unlike mTORC1, the precise localization of mTORC2 inside cells is debatable. Thus, in mammalian cells, the obligatory mTORC2 components mRictor and mSin1 displayed reticular immunofluorescent staining patterns, with little or no staining at the plasma membrane (Oh et al., 2010; Boulbès et al., 2011; Betz et al., 2013). Furthermore, mTORC2 activity physically associated with a subpopulation of ribosomes and mitochondria (Zinzalla et al., 2011; Betz et al., 2013), thereby implicating cellular endomembranes as potential sites of mTORC2 activity. However, constitutive localization of myristoylated Akt at the plasma membrane results in its hyperphosphorylation (Andjelković et al., 1997), suggesting that mTORC2 could additionally localize to the plasma membrane. Consistent with this notion, mTORC2 was shown to be targeted to the plasma membrane by syndecan-4 (Partovian et al., 2008), and yeast TORC2 also localizes to the plasma membrane and membrane-proximal vesicles and is required for cell survival (Berchtold and Walther, 2009). However, which of these membranes display mTORC2 activity in the cellular context and to which extent these pools could contribute to phosphorylation of mTORC2 substrates, such as Akt, remains unclear.

Akt phosphorylation by mTORC2 requires the product of PI3K, the membrane lipid phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3), which accumulates in response to PI3K stimulation by many growth factor receptors. This led some authors to propose that the mTOR kinase is directly activated by...
PI(3,4,5)P₃, Thus, Liu et al. (2015) observed that the isolated lipid-binding pleckstrin homology (PH) domain of mSin1 binds to the mTOR kinase domain and proposed that in the absence of PI(3,4,5)P₃, the PH domain of mSin1 suppresses mTOR kinase activity. In their model, binding of the mSin1 PH domain to PI(3,4,5)P₃ both localizes mTORC2 to the membrane and relieves inhibition of mTOR, leading to Akt phosphorylation. An alternative mechanism of growth factor–induced Akt phosphorylation would be a PI(3,4,5)P₃–dependent recruitment of the substrate (Akt) to constitutively active membrane–associated mTORC2. In line with this scenario, myristoylated Akt is hyperphosphorylated in the absence of growth factor stimulation (Burgering and Coffer, 1995; Andjelković et al., 1997). It is therefore unknown whether and to which extent growth factors could directly modulate mTORC2 activity.

In this study, we examined the intracellular localization of the activity of endogenous mTORC2 and its regulation by growth factors. To date, there are only a few tools that allow the resolution of enzymatic activity within a cellular context. Existing approaches use either biochemical fractionation (Zinzalla et al., 2011; Betz et al., 2013), immunofluorescent staining (Boulbés et al., 2011), or fluorescence resonance energy transfer–based reporters (Zhou et al., 2015). These methods either lack the resolution to discriminate between intracellular subpopulations of the enzyme, or they assume that localization of individual components in fixed cells serves as a proxy for enzymatic activity of the complex. To overcome these limitations and examine the intracellular localization and regulation of the endogenous mTORC2 activity, we have developed a compartment-specific biochemical reporter. Using this reporter, we demonstrate that in cultured mammalian cells, an mTOR–dependent, rapamycin–independent activity is present at the plasma membrane, the outer mitochondrial membrane, and a subpopulation of early and late endosomes. We demonstrate that neither growth factors nor PI3K inhibition has an effect on mTORC2 activity at the plasma membrane, and growth factors induce Akt phosphorylation solely through its recruitment to the membrane. Unlike the plasma membrane and mitochondria, early and late endosomes displayed a PI3K–dependent mTORC2 activity, suggesting the existence of mTORC2 subpopulations with distinct sensitivity to PI3K. Furthermore, we show that mTORC2 is targeted to cellular membranes by the PH domain of mSin1 in a PI(3,4,5)P₃–independent manner and that subcellular partitioning of mTORC2 and counteracting phosphatases helps limit Akt phosphorylation to cellular membrane compartments. Collectively, our results demonstrate that, similar to mTORC1, mTORC2 activity is associated with cellular membrane compartments and also highlight the role of subcellular partitioning as a general mechanism of regulating signaling efficiency inside cells.

Results and discussion
Development and characterization of the mTORC2 activity reporter
To examine the localization of endogenous mTORC2 activity in cells, we developed a reporter system based on the mTORC2 substrate Akt. Earlier studies demonstrated that depletion or knockout of mSin1 or mRictor dramatically reduced phosphorylation on Ser473 in the hydrophobic C-terminal motif of Akt (Frias et al., 2006; Guerin et al., 2006; Jacinto et al., 2006), demonstrating that phosphorylation of this site is a good proxy of mTORC2 activity. Subunits of mTORC2 were found to localize to several intracellular membrane compartments (Betz and Hall, 2013). To probe mTORC2 activity at cellular membranes, we used the FRB:T2098L:FKBP chemically induced heterodimerization system with the nonimmunosuppressive rapamycin analogue AP21967 (Fig. 1 A). This approach allowed us to compare the activity of endogenous mTORC2 at specific subcellular compartments.

To recruit FRB:Akt2 to various membrane compartments, we generated a panel of FKBP:mCherry fusions with the C-terminal 30 aa of KRas4B, full-length Rab5, Rab11, Rab7, the C-terminal 33 aa of Bcl-Xₐ, or the transmembrane ER cargo protein TcR-β. These constructs correctly localized to the plasma membrane, early, recycling, and late endosomes, outer mitochondrial membrane, and ER membrane, respectively (Fig. 1 B). The addition of 250 nM AP21967 resulted in the efficient recruitment of cytosolic FRB:Akt2-citrine to the respective compartments (Fig. 1 B and Videos 1, 2, 3, 4, 5, and 6), with the half-time of recruitment between 13.8 ± 1.0 min for the ER and 26.9 ± 9.0 min for the plasma membrane (Fig. 1 C).

An earlier study (Edwards and Wandless, 2007) indicated that AP21967 could interfere with the activity of mTORC1 and therefore with the phosphorylation of S6 kinase (S6K), which could potentially augment mTORC2 activity via the proposed S6K–dependent feedback loop (Xie and Proud, 2013; Liu et al., 2014b). We tested this by monitoring the phosphorylation of S6K and the mTORC2 endogenous substrate Akt. Treatment with 250 nM AP21967 did not decrease phosphorylation of endogenous S6K nor had any significant effect on endogenous Akt (Fig. S1, A and B), demonstrating that the concentration of rapalog used in our study did not interfere with the activities of mTORC1 or mTORC2. Furthermore, because of the presence of the T2098L mutation in the FRB fragment, AP21967 did not recruit endogenous mTOR (Fig. S1 C). We noticed that the C-terminal fluorescent tag interfered with basal FRB:Akt2 phosphorylation on Ser473 (not depicted). N-terminal fusion of Akt2 with FRB, however, had no effect on basal–, AP21967–, or growth factor–induced phosphorylation of the FRB:Akt2 reporter and was therefore used throughout the study. We dubbed this reporter LocaTOR2 (localization of mTOR complex 2).

Expression of LocaTOR2 was equal to or less than that of endogenous Akt (Fig. S1 D). In agreement with a previous study (Thoreen et al., 2009), treatment with the mTOR–specific inhibitor Torin1 dramatically reduced phosphorylation of both LocaTOR2 and endogenous Akt (Fig. 1 D). Conversely, rapamycin did not impair phosphorylation of the hydrophobic motif of LocaTOR2 or endogenous Akt (Fig. 1 D), demonstrating that this site (Ser473 in endogenous Akt and Ser474 in LocaTOR2) is specifically targeted by mTORC2, but not mTORC1, activity.

Finally, we tested whether AP21967–induced LocaTOR2 phosphorylation was dependent on the expression level of the recruiter constructs. Rapalog–induced phosphorylation of the reporter was dependent on the identity of the recruiter but did not correlate with the relative expression of the FKBP:mCherry constructs (R² = 0.18; Fig. S1 E). Collectively, these data demonstrate that LocaTOR2 could serve as a faithful reporter of the endogenous mTORC2 activity.

Intracellular localization of mTORC2 activity
Next, we examined which intracellular membranes display mTORC2 activity. Rapalog–induced recruitment of LocaTOR2 to the plasma, early and late endosomal, and outer
mitochondrial membranes resulted in a significant increase of its phosphorylation on Ser474 in the absence of growth factor stimulation (Figs. 2 A and 3, A and B). Phosphorylation was most prominent at the plasma membrane and mitochondria, followed by late and early endosomes. No significant increase in phosphorylation was observed for recycling endosomes or ER, suggesting that mTORC2 activity at these compartments is either scarce or that counteracting phosphatases efficiently dephosphorylate the reporter. Treatment with the mTOR-specific inhibitor Torin1 either before or after AP21967-induced recruitment to the plasma membrane, late endosomes, and mitochondria potently reduced LocaTOR2 phosphorylation on Ser474 (Fig. 2 A), demonstrating that its phosphorylation at these compartments was indeed mediated by mTORC2. AP21967 treatment had no effect on the phosphorylation of Thr450 in the Akt turn motif (Fig. S2 B), consistent with the proposed role of mTORC2-mediated cotranslational phosphorylation of this site required for Akt stability (Oh et al., 2010). Therefore, our data demonstrate that mTORC2 activity toward Akt is restricted to specific membrane compartments.

In agreement with the Western blot (WB) results, LocaTOR2 phosphorylation was restricted to subcellular compartments containing the respective recruiter constructs and was most prominent at the plasma membrane and mitochondria, as demonstrated by immunofluorescent staining using a Ser473 phosphospecific antibody (Fig. 2, B and C). Furthermore, immunoprecipitation of the recruited LocaTOR2 showed enrichment of phosphorylated reporter in the pull-down fractions (Fig. S1 D), indicating that the observed increase in reporter phosphorylation was caused by its recruitment to the specific membrane compartments.

Marked increase of the reporter phosphorylation upon its recruitment to the plasma membrane, mitochondria, and late endosomes suggested that mTORC2 activity is predominantly localized to these membrane compartments. Alternatively, the reporter could be phosphorylated elsewhere in the cell, and accumulation of the phosphorylated LocaTOR2 at these sites could have resulted from its reduced dephosphorylation by cytosolic phosphatases upon rapalog-induced membrane recruitment. To discriminate between these two scenarios, we treated the cells with the phosphatase inhibitor calyculin A after AP21967-induced recruitment of the reporter. Calyculin A induced a further increase in LocaTOR2 phosphorylation (Fig. S2 A), indicating that phosphatase activity efficiently counteracts mTORC2 kinase activity at these compartments. Furthermore, treatment with Torin1 after reporter recruitment reduced LocaTOR2 phosphorylation (Fig. 2 A), demonstrating that membrane binding does not protect the reporter from dephosphorylation. Collectively, these results demonstrate that the plasma membrane, mitochondria, and vesicles of the
endosomal system display an endogenous mTOR-dependent activity similar to that of mTORC2.

**Regulation of mTORC2 activity by PI3K**

Activity of PI3K is required for growth factor–induced Akt phosphorylation. A recent study by Liu et al. (2015) proposed that the PH domain of mSin1 suppresses mTOR kinase activity and argued that PI(3,4,5)P₃ binding to the mSin1 PH domain could relieve mTOR inhibition and lead to the activation of mTORC2 in response to growth factors. The authors tested this hypothesis by monitoring Akt Ser473 phosphorylation in response to PI3K inhibitors and depletion of endogenous PI3K by shRNA. PI(3,4,5)P₃, however, is also required for Akt membrane binding, thus making it impossible to discriminate between PI(3,4,5)P₃-induced mTORC2 activation and PI(3,4,5)P₃-dependent Akt recruitment using PI3K inhibitors.

Therefore, we next tested whether mTORC2 activity on different membrane compartments is indeed PI3K dependent. To do so, we used LocaTOR2, which can be recruited to the plasma membrane independently from PI(3,4,5)P₃. Neither serum withdrawal nor pretreatment with the specific PI3K inhibitor GDC-0941 had a significant effect on LocaTOR2 phosphorylation upon its recruitment to the plasma membrane (Fig. 3, A–C). Importantly, inhibition of PI3K abrogated phosphorylation of endogenous Akt, as expected. Furthermore, treatment with insulin subsequent to LocaTOR2 recruitment to the plasma membrane did not further increase its phosphorylation on Ser474, yet resulted in robust phosphorylation of endogenous Akt (Fig. 3 D). This is consistent with earlier studies.
mSin1 targets mTORC2 to cellular membranes independent of PI(3,4,5)P₃

Our results indicate that several intracellular membrane compartments display mTORC2 activity and are distinctly regulated by PI3K. Next, we asked how mTORC2 is targeted to these compartments and whether this targeting depended on PI3K activity.

The only obligate mTORC2 subunit with a distinct lipid-binding domain is mSin1, which contains a PH domain (Schroder et al., 2007; Berchtold and Walther, 2009). Therefore, we examined the localization of mSin1-GFP in cells. Alternative splicing generates six mSin1 isoforms, of which isoforms 1, 2, and 5 could incorporate into mTORC2 (Fig. 4 A; Frias et al., 2006). First, we tested whether C-terminal GFP fusions of isoforms 1, 2, and 5 retain the capacity to interact with the endogenous components of mTORC2. In agreement with a previous study (Frias et al., 2006), pull-down experiments using anti-GFP single-chain antibody (scAb) demonstrated that isoforms 1, 2, and 5 all coimmunoprecipitated with endogenous mTOR and mRictor (Fig. 4 B). Immunoprecipitated mTORC2 was able to phosphorylate purified full-length Akt on Ser473 and was sensitive to inhibition by the mTOR inhibitor Torin1, indicating that the complex is catalytically active (Fig. 4 C). Collectively, these results indicate that mSin1-GFP is efficiently incorporated into mTORC2 and that the GFP tag does not interfere with the formation of the functional mTORC2 complex, thus allowing us to examine membrane targeting of mTORC2 using mSin1-GFP.
Figure 4. PH domain targets mSin1/mTORC2 to cellular membranes. (A) Schematic representation of mSin1 isoforms 1, 2, and 5. Yellow boxes represent the PH domain. [B] Isoforms 1, 2, and 5 of mSin1-GFP are incorporated into mTORC2. HEK293T cells were transiently transfected with GFP or the corresponding mSin1-GFP isoform, and lysed, and GFP-tagged proteins were pulled down using GFP-Trap beads. Shown is a representative pull-down result. n = 2 for mSin1.1 and mSin1.5; n = 3 for mSin1.2. (C) In vitro kinase assay with immunoprecipitated mTORC2. HEK293T cells transiently transfected with GFP or mSin1.2WT-GFP were stimulated with 100 ng/ml insulin for 10 min and lysed, and GFP-tagged proteins were pulled down using GFP-Trap beads. The beads were preincubated with or without 100 nM Torin1 and incubated for 1 h at 37°C with purified dephosphorylated recombinant full-length human Akt1 with or without 0.5 mM ATP, and pSer473 phosphorylation was determined using PAGE/WB. Shown is a representative result of two independent experiments. (D–F) Intracellular localization of isoforms 1, 2, and 5 of mSin1-GFP in HeLa cells. (D) The PH domain targets mSin1 to cellular membranes. HeLa cells were transiently transfected with mSin1.2WT-GFP or mSin1ΔPH-GFP. (E) mSin1 localizes to the plasma membrane and endosomal vesicles. HeLa cells were transiently cotransfected with mSin1.2-GFP and markers of the plasma membrane (PM; mCherry-KRas4BΔ30) along with early (mCherry-Rab5) and late endosomes (mCherry-Rab7). (F) The PH domain targets mSin1 to cellular membranes. HeLa cells were transiently transfected with mSin1.2WT-GFP or mSin1ΔPH-GFP. [D–F] Bars, 10 µm. (G) The PH domain is dispensable for mSin1 incorporation into mTORC2. HeLa cells were transiently
Both isoforms 1 and 2 of mSin1-GFP localized to the cytosol, nucleoplasm, plasma membrane, and highly dynamic endomembrane vesicles in HEK293T, HeLa, and MCF7 cells (Figs. 4 D and S3, A and B; and Video 7), consistent with the localization of the mSin1 yeast orthologue Avo1 (Berchtold and Walther, 2009). Isoform 2 displayed stronger vesicular localization. To investigate the identity of these mSin1-containing vesicles, we coexpressed mSin1-GFP with resident markers of various endosomal subpopulations. In addition to the plasma membrane, mSin1 prominently colocalized with Rab5- and Rab7-positive early and late endosomes (Figs. 4 E and S3 B). To discriminate whether mSin1 localized at the membrane or inside the lumen of the endosomes, we used structured illumination microscopy (SIM). SIM generated high-resolution (full width at half-maximum: ~100 nm) images, confirming that mSin1 localizes at the plasma membrane and on the surface, but not inside the lumen of late endosomes (Fig. S3 C). Earlier studies using antibodies against mTORC2 components reported staining of the ER and ER-associated mitochondrial membranes (Boulbês et al., 2011; Betz et al., 2013). We did not observe any reticular and/or mitochondrial staining in cells overexpressing mSin1-GFP using confocal microscopy or SIM, which otherwise generated high-resolution images of the resident ER and mitochondrial markers (Fig. S3 C). No appreciable reticular structures could be detected even when we amplified the mSin1-GFP signal using Atto488-conjugated anti-GFP scAb. Collectively, these imaging results show that mSin1-GFP localization, with the notable lack of expected mitochondrial staining, is consistent with our biochemical evidence for mTORC2 activity at the plasma membrane and endosomes.

Unlike isoforms 1 and 2, isoform 5 of mSin1 lacks the PH domain. Correspondingly, mSin1.5-GFP did not show any membrane localization but accumulated in the nucleus and cytosol (Fig. 4 D). Importantly, mSin1.5 still communoprecipitated with mTOR and mRictor (Fig. 4 B) in agreement with an earlier study (Frias et al., 2006). Similarly, truncation of the PH domain abrogated mSin1.2 membrane localization, resulting in accumulation of mSin1.2ΔPH-GFP in the nucleus, but did not affect its interaction with mRictor and mTOR (Fig. 4, F and G). This is consistent with earlier studies (Frias et al., 2006; Liu et al., 2013) and with a recent EM study demonstrating that the PH domain of yeast Avo1 is dispensable for TORC2 assembly (Gaubitz et al., 2015). Collectively, these data suggest that whereas isoforms 1, 2, and 5 all incorporate into the cytosol, nucleoplasm, plasma membrane, and highly dynamic endosomal vesicles.

Because we found mTORC2 activity at the plasma membrane to be independent of PI(3,4,5)P₃, we next asked whether mSin1 localization was influenced by PI(3,4,5)P₃ levels. PH domains bind to various phosphoinositide lipids, including PI(3,4,5)P₃, both in vitro and in vivo. Therefore, we examined whether PI3K activity had any effect on the membrane binding of full-length mSin1 by comparing the localization dynamics of mSin1-GFP and the mCherry-tagged PH domain of Akt, which binds PI(3,4,5)P₃ (Thomas et al., 2002; Zoncu et al., 2009).

Treatment with insulin induced rapid and robust translocation of mCherry-PHₐkt from the cytosol to the plasma membrane (Fig. 4 H). Conversely, application of the specific PI3K inhibitor GDC-0941 resulted in rapid dissociation of mCherry-PHₐkt from the membrane into the cytosol. Unlike mCherry-PHₐkt, mSin1 did not change its localization upon growth factor stimulation or treatment with the PI3K inhibitor (Fig. 4 H), indicating that mSin1 binds to cellular membranes independent of PI(3,4,5)P₃. This finding is consistent with earlier studies, which indicated that purified PH domains of both mSin1 and Avo1 bound 3- and 4-phosphorylated phosphoinositides in vitro, showing no preference for PI3K products (Schroder et al., 2007; Berchtold and Walther, 2009). Collectively, our results indicate that, contrary to the model proposed by Liu et al. (2015), mTORC2 localization and activity at the plasma membrane are PI3K independent.

Regulation of Akt phosphorylation by intracellular kinase-phosphatase partitioning

Our results demonstrate that the GFP-tagged mSin1 isoform 5 incorporates into mTORC2, consistent with earlier studies (Frias et al., 2006; Yuan et al., 2015), and could localize mTORC2 to the cytoplasm or the nucleus. We next asked whether mTORC2 activity is restricted to cellular membranes or whether the cytosolic pool could also contribute to the phosphorylation of the reporter.

To discriminate between membrane-bound and cytosolic LocaTOR2, we modified the system so that FKBP₃₇₃₅₉-mCherry-Akt₂K₁₄₄A could be recruited to the plasma membrane through the Escherichia coli dihydrofolate reductase (DHFRR)-tagged TagBFP-CAAX construct using the bifunctional reagent SLF-TMP. In this system, membrane recruitment could be reversed by administration of the competitor TMP (Fig. 5, A and B; and Video 8; Liu et al., 2014a). Because of a K14A mutation in the Akt PH domain, which prevents its binding to PI(3,4,5)P₃, basal phosphorylation of FKBP₃₇₃₅₉-mCherry-Akt₂K₁₄₄A in cells grown in 10% serum was negligible, as expected (Bellacosa et al., 1998; Thomas et al., 2002). In agreement with the results obtained using LocaTOR2, recruitment of FKBP₃₇₃₅₉-mCherry-Akt₂K₁₄₄A to the plasma membrane resulted in its robust phosphorylation on Ser474. Addition of the competitor TMP induced FKBP₃₇₃₅₉-mCherry-Akt₂K₁₄₄A dissociation from the membrane and was accompanied by a loss of Ser474 phosphorylation (Fig. 5, B and C), demonstrating that phosphorylation of the construct was exclusively driven by its recruitment to mTORC2-containing membranes. Notably, neither SLF-TMP nor TMP affected phosphorylation of endogenous Akt, demonstrating that this reversible recruitment system is orthogonal to PI3K activity. Furthermore, efficient dephosphorylation of the construct upon addition of TMP suggests that cytosolic phosphatases, such as PP2A or PHLPP, dominate over mTORC2 activity (if any) in the cytosol. Therefore, these data indicate that inside cells, the activity of mTORC2 is primarily associated with membrane compartments. This intracellular partitioning of the mTORC2 kinase to membranes and phosphatases (PHLPP and/or PP2A) in the cytosol could control the extent of Akt phosphorylation inside cells.
In summary, using imaging and biochemical approaches, we demonstrate that in cells, endogenous mTORC2 activity localizes to the plasma membrane, outer mitochondrial membranes, and a subpopulation of endosomal vesicles. Activity of endogenous mTORC2 at the plasma membrane is independent from PI3K activity and growth factors, suggesting that growth factors could induce Akt phosphorylation exclusively through its recruitment to the plasma membrane, where mTORC2 localizes by means of PH domain–containing mSin1 isoforms. Our results suggest that dynamic partitioning of mTORC2 and active Akt could serve to regulate the localization and extent of Akt phosphorylation and signaling in response to growth factors.

In this study, we have examined the localization of mTORC2 activity and its regulation in cultured mammalian cells. Previous studies using indirect immunofluorescence and biochemical fractionation assigned ER and mitochondria as the major mTORC2 sites (Boulbès et al., 2011; Zinzalla et al., 2011; Betz et al., 2013). Discrimination between diffuse cytosolic, vesicular, and true reticular staining using IF and conventional, diffraction-limited microscopy, however, is at best nontrivial (Schnell et al., 2012). More importantly, intracellular localization is not necessarily a proxy for enzymatic activity. Even isolation of mTORC2 by biochemical fractionation or coimmunoprecipitation experiments does not warrant conclusions about the localization of mTORC2 activity in the cellular context, as inside cells the purified activity could be efficiently outcompeted by phosphatases catalyzing the reserve reaction. These limitations motivated us to develop a reporter for probing endogenous mTORC2 activity in living cells.

Using a newly developed reporter, we have localized mTORC2 activity to cellular membrane compartments, including the plasma membrane, outer mitochondrial membrane, and a subpopulation of endosomal vesicles. This is in agreement with earlier indirect evidence of mTORC2 activity at these compartments (Schenck et al., 2008; Chaturvedi et al., 2011; Huynh et al., 2012; Betz et al., 2013) and suggests that the intracellular localization of mTORC2 could be physiologically relevant. We did not observe significant mTORC2 activity on recycling endosomes and the ER, indicating that mTORC2 activity is localized to specific subcellular compartments. Importantly, our data do not rule out reticular localization of mTORC2 as proposed previously (Oh et al., 2010; Boulbès et al., 2011); the lack of efficient phosphorylation of LocaTOR2 at Ser473 could be accounted for by stronger phosphatase activity at the ER. Alternatively, the ER pool of mTORC2 could be responsible for cotranslational phosphorylation of the Akt and PKC turn motifs, which were shown to be insensitive to dephosphorylation (Oh et al., 2010), suggesting that inside cells, mTORC2 could exist as functionally distinct subpopulations.

Our imaging results demonstrate that the mTORC2-targeting subunit mSin1 localizes both at membranes and in the cytosol and that its association with the complex is independent from its lipid-binding PH domain. This suggests that mTORC2 could reside both in the cytosol and on membranes. Using reversible chemically induced dimerization, we show...
that the activity of the cytosolic mTORC2 pool toward its substrates is efficiently outcompeted by the counteracting phosphatases, indicating that, similar to mTORC1, mTORC2 activity inside cells is primarily restricted to membrane compartments. This subcellular partitioning could help ensure activation of mTORC2 substrates, such as Akt, PKC, or serum/glucocorticoid-regulated kinase, in the vicinity of downstream effectors and thus provide a generic mechanism for the control of signaling fidelity inside cells.

Finally, our study sheds light on the regulation of mTORC2 activity by growth factors. We found that inside cells, mTORC2 appears to exist as at least two subpopulations with distinct sensitivity to PI3K. Although the functional significance of these subpopulations remains unclear, it is tempting to speculate that the plasma membrane–localized, constitutively active mTORC2 could help couple Akt activation with the stimulation of growth factor receptors. Conversely, Akt-mediated phosphorylation of mSin1 on Thr86, which was shown to result in increased mTORC2 activity (Humphrey et al., 2013; Yang et al., 2015), could account for PI3K sensitivity of the endosomal mTORC2 pool so that intracellular trafficking would deliver mTORC2 to specific Akt substrates located at subcellular membrane compartments, such as lysosomal Tsc2 (Schenck et al., 2008; Menon et al., 2014) or glial fibrillary acidic protein (GFAP) (Arias et al., 2015). In line with this model, lysosomal subpopulations of mTORC2 and Akt were shown to control the efficiency of chaperone-mediated autophagy by regulating the assembly of the chaperone-mediated autophagy translocation complex (Arias et al., 2015). Furthermore, inhibition of membrane trafficking disrupted phosphorylation of Akt and its substrates (Schenck et al., 2008; Nazarewicz et al., 2011; Gao et al., 2012) and resulted in deregulated gene expression in response to B cell receptor activation (Chaturvedi et al., 2011). Similarly, mTORC2 at mitochondria could help ensure efficient coupling of Akt phosphorylation to downstream targets like Bad, Bim, or Bcl-XL, which are themselves located at the mitochondria, thereby directly linking mTORC2 with cell survival as proposed earlier (Betz and Hall, 2013; Betz et al., 2013). Although further studies will be necessary to address the potential physiological relevance of PI3K-dependent and independent mTORC2 pools and to which extent they contribute to the phosphorylation of various mTORC2 substrates, our results suggest that, similar to mTORC1, the activity and function of mTORC2 are tightly coupled to its intracellular localization.

Materials and methods

Reagents and antibodies

AP21967 (or its equivalent A/C dimerizer, 635057) was from Ariad Pharmaceuticals and Takara Bio Inc., respectively. GDC-0941 (G9252) was from LC Labs. Rabbit AktSer473 (193H12) (4058), rabbit pan-Akt 11E7 (4685), rabbit S6KThr389 108D2 (9234), rabbit mTOR 7C10 (2983), rabbit GAPDH D16H11 (5174), and rabbit γ-tubulin (5886) antibodies were from Cell Signaling Technology. Mouse RICTOR 7B3 (MA-5-15681) antibody was from Thermo Fisher Scientific. Mouse Sin1 1C7.2 (05-1044) antibody was from EMD Millipore. Mouse mCherry (632543) antibody was from Takara Bio Inc. GFP-Trap_A (gta-20), RFP-Trap_A (rta-10), and Atto488-conjugated GFP booster (gba-488) scAb were from ChromoTek. Recombinant human insulin (12643) was from Sigma-Aldrich.

The constructs used in the study were generated using standard molecular biology protocols and will be deposited to the AddGene repository (72899–72909). pmCherry-PHα46 was a gift from T.A. Leonard (Max F. Perutz Laboratories, Vienna, Austria), and pTagBFP-2×DHFR-CaaX was a gift from Y. Wu (Max Planck Institute for Molecular Physiology, Dortmund, Germany).

Cell culture

HEK293T cells were from ATCC; HeLa and MCF7cells were gifts from R. Foisner (Max F. Perutz Laboratories, Vienna, Austria) and N. Huttary (Medical University of Vienna, Vienna, Austria), respectively. All cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 1 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell transfections were performed using transfection reagent (Turbofect) or 1 mg/ml polyethyleneimine solution at a ratio of 1 µg DNA per 3 µl polyethyleneimine. When indicated, the cells were serum starved for 18 h in serum-free DMEM.

Immunoblotting and immunoprecipitation

For immunoblotting, the cells were rinsed in ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (20 mM Tris-Cl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate) or 0.5% NP-40 buffer (20 mM Tris-Cl, pH 8.0, 140 mM NaCl, and 0.5% NP-40) containing Complete MINI/PhoStop phosphatase inhibitors cocktail (Roche). Cell debris was removed by centrifugation at 14,000 g at 4°C for 10 min, and the cleared cell lysates were used in 8% SDs-PAGE. Separated proteins were electroblotted onto nitrocellulose membrane, and the membranes were blocked in 5% BSA/Tween 20–TBS buffer. Immune complexes were visualized using HRP-conjugated secondary antibodies and ECL. Select WB detection reagent (GE Healthcare). ECL chemiluminescence was recorded using the FusionFX imaging system (Pqelab), and band intensity quantitation was performed using ImageJ GelAnalyzer script (National Institutes of Health). For immunoprecipitation experiments, the cells were lysed in 0.3% CHAPS buffer (40 mM Hepes, pH 7.4, 120 mM NaCl, 1 mM EDTA, and 0.3% CHAPS; Complete MINI/PhoStop inhibitor cocktail) according to Cameron et al. (2011), and GFP fusion proteins from the cleared cell lysates were pulled down using GFP-Trap beads (ChromoTek) according to the manufacturer’s protocol. The kinase activity of the immunoprecipitated mTORC2 complex was assayed using dephosphorylated full-length human recombinant Akt1 as a substrate (a gift from I. Lučić, Max F. Perutz Laboratories, Vienna, Austria).

Image acquisition and analysis

Laser-scanning confocal microscopy of fixed samples was performed using LSM700 or LSM710 systems equipped with 63x/NA 1.4 oil immersion objectives and Zen software (ZEISS). Live-cell imaging was performed using a LIVE spinning-disk system (Visitron Systems) equipped with an electron-multiplying charge-coupled device camera (Evolve; Photometrics), an environmental control (5% CO2, 37°C) unit, a 63x/NA 1.4 oil immersion objective, and VisiView software (Visitron Systems). Imaging medium was DMEM supplemented with 1 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, and supplemented with 10% FBS where indicated in the text. Image enhancement (contrast stretching and channel overlays) and analysis were done using ImageJ (v. 2.0.0-re-14; Fiji build). All adjustments were equally applied across the entire image; no nonlinear adjustments were used. In immunofluorescent and live-cell imaging experiments, cells that appeared to contain >10% overexpressed (saturated) pixels were excluded from quantitative analyses. For SIM, the cells were grown on 170 ± 5-µm glass coverslips (HighPrecision; Sigma-Aldrich), fixed, permeabilized with 0.1% Triton X-100, blocked using 10% goat serum
in 1× PBS, stained using Atto488-conjugated anti-GFP scAb (ChromoTek), and mounted using Mowiol or VectaShield media. 3D SIM images were acquired using an OMX system (Del outlaw; GE Healthcare) equipped with a 60x/NA 1.42 PlanApo N objective (Olympus), 488- and 568-nm laser lines, and a pco.edge front-illuminated sCMOS 15-bit camera and were reconstructed using a SoftWoRx software suite (v. 6.5.1; GE Healthcare). Lateral and axial SIM image registration was performed using Image Registration Target Slide (52-852833-000; GE Healthcare) and microspheres (TerraSpec; Thermo Fischer Scientific), respectively.

Online supplemental material
Fig. S1 shows WBs demonstrating no effect of AP21967 on endogenous Akt, S6K, and mTOR and shows the independence of LocaTOR2 phosphorylation on the expression level of the recruiter constructs. Fig. S2 shows WBs demonstrating that membrane recruitment does not protect LocaTOR2 from dephosphorylation and has no effect on Thr450 phosphorylation. Fig. S3 shows colocalization analysis of mSin1-GFP and various membrane markers using SIM. Videos 1, 2, 3, 4, 5, and 6 are time-lapse videos demonstrating recruitment of the LocaTOR2 probe to the plasma membrane, outer mitochondrial membranes, ER, and early, recycling, and late endosomal subpopulations upon addition of 250 nM AP21967 in live cells. Video 7 is a time-lapse video showing intracellular dynamics of mSin1-phosphorylated GFP in a live cell. Video 8 shows SLF-TMP–induced recruitment and TMP-induced dissociation of FKBPs/mCherry–Akt2.

References


Acknowledgments
The authors acknowledge Dr. Lijuan Zhang and Dr. Kareem Elsayad of the Advanced Microscopy Facility of the Vienna BioCenter Core Facilities, member of the Vienna BioCenter Austria, for assistance with acquisition and analysis of 3D SIM data. The authors are grateful to Dr. Roland Foisner and Nicolette Huttary for providing HeLa and MCF7 cells, respectively, and Iva Lučić for providing purified dephosphorylated recombinant full-length human Akt1 for mTORC2 in vitro kinase assays. The TagBP2:2xDHFR-CAAX construct and SLF-TMP were kindly provided by Dr. Yaowen Wu and Dr. Xi Chen, and pmCherry-PH was a gift from Thomas A. Leonard. The authors are grateful to Dr. Philippe Bastiaens for intense discussions of the manuscript and proposing the idea of the reversible recruitment experiments (Fig. 5).

The authors declare no competing financial interests.

Author contributions: All authors performed experiments and evaluated results. I. Yudushkin conceived the study, and M. Ebner and I. Yudushkin proposed and designed experiments. I. Yudushkin wrote the manuscript. All authors contributed to the discussion and evaluation of the manuscript.

Submitted: 18 October 2016
Revised: 19 December 2016
Accepted: 4 January 2017
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