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Identification of TOR Signaling Complexes: More TORC for the Cell Growth Engine

Minireview

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The Target of Rapamycin (TOR) proteins function in signaling pathways that promote protein synthesis and cell growth. In yeast, TOR signaling is regulated by nutrient availability, whereas in metazoan cells TOR activities may be controlled by both nutrients and growth factors. The recent identification of novel TOR-interacting proteins has provided crucial insights into TOR regulation and function.

A recent strategy, termed "chemical genetics," involves the use of drugs or other bioactive compounds as probes for the dissection of signaling pathways. The application of small molecules to intact cells has uncovered regulatory mechanisms that would have been difficult to elucidate with traditional genetic techniques, particularly in mammalian systems. To appreciate the investigative power of chemical genetics, one need only examine the history of rapamycin, a bacterially derived drug that holds great promise in several clinical settings.

In the late 1970's, rapamycin was identified as a potent antifungal and immunosuppressive agent (Abraham and Wiederrecht, 1996). The drug was clinically approved for use in kidney transplant patients in 1999, and additional applications in autoimmune and chronic inflammatory diseases are at various stages of testing. Two rapamycin-related drugs, CCI-779 (Wyeth-Ayerst) and RAD001 (Novartis), have also generated considerable excitement in both the clinical and basic research cancer communities. In addition, CCI-779 displays remarkable efficacy in the prevention of restenosis after coronary artery interventions with stent implantation. Thus rapamycin and its analogs have clinical potential in three major therapeutic arenas: organ transplantation, cancer, and cardiovascular disease.

Seminal insights into rapamycin's mechanism of action emerged from studies in *Saccharomyces cerevisiae* (for review, see Schmelzle and Hall, 2000). Screens for mutations that rendered *S. cerevisiae* resistant to the growth-inhibitory effects of rapamycin identified two highly homologous Target of Rapamycin (TOR) proteins, TOR1 and TOR2. The interaction between the drug and each TOR protein was contingent on the formation of a pharmacologically active complex between rapamycin and its intracellular receptor, *FK*506-*b*inding *p*rotein of *12* kDa (FKBP12). The identification of this complex as the proximate TOR ligand enabled purification of mammalian TOR (mTOR) from tissue extracts. The amino acid sequence of mTOR (also known as FRAP or RAFT1) is remarkably homologous to those of yeast TOR1 and TOR2, suggesting that the functions of the rapamycin-sensitive regulatory pathway(s) governed by TOR were highly conserved in eukaryotic cells.

The TOR proteins are members of the phosphoinositide (PI) 3-kinase related kinase (PIKK) family, which includes mammalian ATM, ATR, and DNA-PK (Tibbetts and Abraham, 2000). Like other PIKK family members, the TORs are large polypeptides (280–300 kDa) that bear a carboxy-terminal region with sequence similarity to the catalytic domains of PI3-kinases. In spite of the similarity to lipid kinases, the PIKK family members that possess active kinase domains phosphorylate proteins on Ser or Thr residues. The consensus phosphorylation site for all PIKK family members (except the TORs) is Ser/Thr followed by Gln at the +1 position. The preferred sequence motif for the TORs remains unclear; however, known in vitro substrates contain Ser/Thr followed by Pro or a hydrophobic amino acid at the +1 position. The lack of a consensus motif for substrate recognition by TOR kinases hints that these PIKK family members may rely on an alternative mechanism for substrate identification or regulation in intact cells.

In yeast, *TOR* gene depletion or rapamycin exposure triggers a stress response program that strongly resembles the nutrient starvation phenotype. These observations provided the first indication that TOR signaling coordinates nutrient availability with cell growth and proliferation. TOR1 and TOR2 are functionally redundant in this nutrient response pathway, but TOR2 carries out an additional, rapamycin-insensitive function related to the control of the actin cytoskeleton that is essential for yeast cell viability (Schmelzle and Hall, 2000). Investigators using rapamycin as a research tool should take heed of these observations, which indicate that rapamycin is a selective, rather than global, inhibitor of TOR function in eukaryotic cells.

Other evidence suggests that mTOR activity is also regulated by nutrient availability. Transfer of cultured mammalian cells from standard growth medium into amino acid- and/or glucose-free medium leads to rapid dephosphorylation of two known mTOR substrates, 4E-BP1 (PHAS-I) and S6K1 (p70 S6 kinase) (Gingras et al., 2001). In the dephosphorylated state, 4E-BP1 binds avidly to eIF-4E, thereby suppressing cap-dependent protein synthesis. Restoration of nutrients provokes multisite phosphorylation of 4E-BP1 by mTOR (and possibly other protein kinases), release of eIF-4E, and resumption of cap-dependent translation. Similarly, S6K1 activity is repressed in starved cells, and nutrient stimulation leads to its phosphorylation and activation. Activated S6K1 stimulates ribosome biogenesis, which upregulates the translational capacity of the cell. Thus, mTOR is a central component of a rapamycin-sensitive signaling pathway that coordinates protein synthesis with glucose and amino acid availability.

In contrast to yeast, mammalian cell proliferation in tissues may be controlled by the supply of growth factors rather than nutrients, because body metabolism strives to maintain nutrient homeostasis in tissues. Some investigators argue that mTOR is strictly tied to

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the nutrient-sensing pathway and that mitogenic signals emanating from growth factor receptors provide parallel, independent inputs into both the translational and cell cycle progression machinery. This unidirectional model for afferent signaling to mTOR is far from proven. For example, the biochemical pathway through which changes in glucose and amino acid concentrations communicate with the TOR proteins has not been defined. Second, the strongest experiments supporting this model typically involve starvation in amino acid- and/or glucose-free medium, followed by acute readdition of nutrients. Although mTOR-dependent signaling events are then observed, the conditions do not mimic the more moderate fluctuations in nutrient levels that occur physiologically. Perhaps mTOR activity is constitutively active in tissue cells unless there is a pathological insult, such as loss of blood supply due to vascular occlusion or hemorrhage.

An alternative model for mTOR regulation posits that metazoan evolution has linked mTOR function to hormone-dependent mitogenic signals, in addition to a primordial nutrient response pathway. Indeed, PI3-kinase and its downstream target, AKT, have emerged as solid candidates for the regulation of mTOR function in growth factor-stimulated cells (Mills et al., 2001; Nave et al., 1999; Sekulic et al., 2000). However, critics of the growth factor model for mTOR regulation rightfully argue that the supporting evidence is purely correlative, and indeed is contradicted by genetic studies in *Drosophila*, which placed PI3-kinase and dTOR in parallel pathways (Montagne et al., 1999; Radimerski et al., 2002).

The controversies surrounding mTOR regulation and function stem in part from the paucity of information regarding the proteins that communicate directly with mTOR (and yeast mTORs, for that matter). This situation has now been addressed in dramatic fashion with the publication of three landmark reports that unveil a series of TOR-interacting proteins in yeast and mammalian cells. The notion that the TORs function in multiprotein complexes was founded in part on structural homology predictions. The large amino-terminal regions ($\sim$2000 amino acids) of these proteins contain at least 20 HEAT repeats, which form α-helical structures and provide hydrophobic surfaces favorable for protein-protein interactions.

The authors of all three studies used mass spectrometry to identify proteins that coprecipitated with TORs from yeast or mammalian cell extracts. Crucial to their success was the method of sample preparation prior to immunoprecipitation. Sabatini and coworkers surmised that mTOR-containing complexes might be prone to dissociate in buffer containing nonionic detergent. To counter this tendency, they added a reversible chemical cross-linker to their lysis buffer to stabilize interactions between mTOR and putative partner proteins. This strategy yielded a novel 150 kDa polypeptide, which they termed *r*egulatory *a*ssociated *p*rotein of m*TOR* (raptor).

On the other hand, it was known that the in vitro kinase activity of mTOR toward 4E-BP1 and S6K1 was unusually sensitive to nonionic detergents such Triton X-100 or NP-40 (Brunn et al., 1997; Isotani et al., 1999), although significant substrate phosphorylation was observed in the presence of Tween-20. Accordingly, Yonezawa and colleagues prepared putative "active" mTOR immunoprecipitates in Tween-20-containing buffer and "inactivated" (dissociated) the immunoprecipitates by washing with NP-40-containing buffer. Their NP-40 eluate contained a protein whose primary sequence was identical to that of raptor.

Human raptor contains a unique amino-terminal "raptor N-conserved" (RNC) region (Kim et al., 2002) followed by three HEAT repeats and seven WD domains, the latter spanning the carboxy-terminal one-third of the protein. Thus, the domain structure of raptor is consistent with a role as an adaptor in multiprotein complexes. Kim et al. found that raptor is stoichiometrically associated with mTOR, suggesting that it is an obligate subunit of all mTOR complexes.

To show that raptor is a critical regulator of mTOR function, Kim et al. (2002) used small-interfering RNA (siRNA) technology to knock down raptor expression in human cells; the phenotypic consequences of raptor deficiency (i.e., inhibition of amino acid-induced S6K1 activation and a reduction in cell size) were qualitatively similar to those induced by either siRNA-mediated mTOR depletion or treatment with rapamycin. Hara et al. (2002) used an immune complex kinase assay as the readout for mTOR function and found that raptor binds directly to 4E-BP1 and S6K1. Additional results support a model in which raptor functions as a bridging protein that presents 4E-BP1 and S6K1 (and possibly additional substrates) to the mTOR kinase domain for phosphorylation. The substrate-scaffolding model nicely explains the earlier finding that nonionic detergents inhibit 4E-BP1 phosphorylation by mTOR; since these detergents dissociate raptor, they block the raptor-dependent presentation of 4E-BP1 to mTOR.

The common message from the *Cell* papers on mammalian raptor is that its association with mTOR is required for the optimal phosphorylation of 4E-BP1 and S6K1 in nutrient-stimulated cells. That raptor and TOR reside in the same signaling pathway is supported by genetic analyses in *C. elegans* (Hara et al., 2002). In other respects, however, these studies have some significant discrepancies. Sabatini and coworkers found that the stability of the raptor-mTOR complex was increased when cells were amino acid or energy starved (Kim et al., 2002). The transition to this "tight" complex correlated with the inhibition of mTOR-dependent signaling in cells and with the repression of mTOR kinase activity in vitro. Notably, the stability of the mTOR-raptor complex was not modulated by polypeptide growth factors, which suggests that raptor is selectively involved in the relay of nutrient-derived signals to mTOR. From these observations, Kim et al. concluded that raptor is a bidirectional modulator, inhibiting mTOR under nutrient-poor conditions and activating it when adequate supplies of amino acids and carbohydrates are available.

Although this model of interplay between mTOR and raptor is appealing, Yonezawa and colleagues, studying the same protein-protein interaction, obtained no evidence for changes in raptor-mTOR complex stability when cells were shifted between nutrient-rich and nutrient-poor media (Hara et al., 2002). The reason for this discrepancy may be linked to the different detergent conditions used by the two laboratories in preparing the cellular extracts; the buffer used by Sabatini may have distinguished two affinity states for the mTOR-raptor

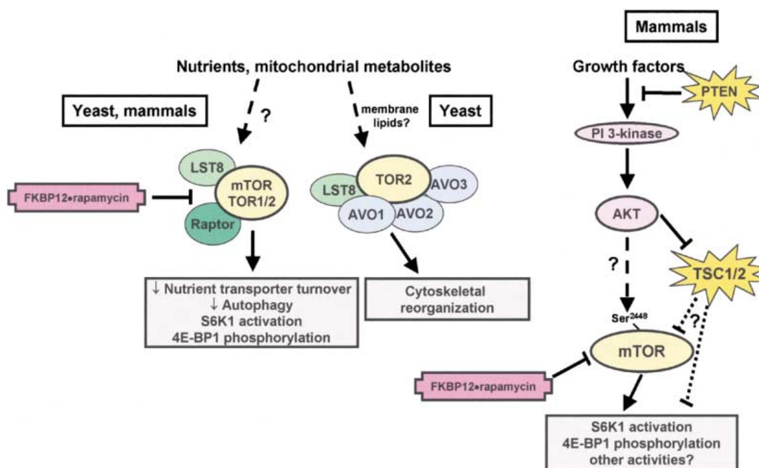

Figure 1. TOR Signaling Complexes in Eukaryotic Cells

The left side of the figure depicts the TORC1 and TORC2 complexes, which mediate cellular responses to nutrient availability and energy production. In mammalian TORC1, raptor may serve as a scaffolding subunit that binds S6K1 and 4E-BP1 and facilitates their phosphorylation by the mTOR kinase domain. The TORC2 complex plays an essential role in actin polarization and cell viability in budding yeast, but may have no direct mammalian homolog. The right side outlines a tentative pathway that links mitogen-dependent AKT activation to the stimulation of TOR-dependent responses in *Drosophila* and mammalian cells. The TSC complex serves as a negative modulator of this pathway and may function via direct interactions with dTOR/mTOR or by blocking the access of these TORs to their downstream target proteins.

complex, whereas that employed by Yonezawa could not do so. Furthermore, Hara et al. performed assays largely with transiently transfected cells that overexpressed raptor and/or mTOR, whereas Kim et al. studied endogenous mTOR-raptor complexes in human cells. Overexpression of raptor and mTOR could be a significant issue, because additional mTOR-interacting proteins may contribute to the stability of the endogenous mTOR-raptor complexes in untransfected cells (see below; Loewith et al., 2002). Obviously, important technical differences need to be addressed before firm conclusions regarding the impact of nutrient status on the stability of the mTOR-raptor complex in mammalian cells can be drawn.

Similar uncertainty surrounds the effect of rapamycin on the mTOR-raptor interaction. Given that rapamycin treatment induces a starvation-like phenotype, one might expect that the drug would increase the stability of the mTOR-raptor complex in nutrient-replete cells. Instead, Kim et al. (2002) found that cellular exposure to rapamycin severely weakened this complex, more so in the presence of nutrients than in their absence. However, Hara et al. (2002) again found no effect of rapamycin on the stability of the complex. Loewith et al. (2002) also examined this issue and concurred with the negative result of the Yonezawa group. Although it is tempting to go with the majority opinion (i.e., FKBP12•rapamycin binding does *not* alter the raptor-mTOR interaction), additional experimentation is needed before the model proposed by Sabatini and coworkers is accepted or overturned.

A final fundamental discrepancy between the two *Cell* reports concerns the role of raptor as a regulator of mTOR kinase activity: dual activator/repressor or activator only? Once again, the different conclusions of the two laboratories may stem from seemingly minor differences in their experimental approach. Hara et al. (2002) observed that coprecipitation of raptor with mTOR was essential for the phosphorylation of 4E-BP1 and S6K1 in immune complex kinase assays. In contrast, Kim et al. (2002) found that mTOR kinase activity was inversely related to the amount of coprecipitating raptor, which indicated that raptor could interfere with substrate phosphorylation by the mTOR catalytic domain. The key difference here may be that Kim et al. used a fragment of S6K1 as the phosphoacceptor in their kinase assays, whereas Hara et al. (2002) used full-length S6K1 or 4E-BP1. If the assertion that raptor functions as a substrate scaffold for mTOR is true, then it is possible that the S6K1 fragment used by the Sabatini group simply lacks the putative raptor binding region. Consequently, phosphorylation of the S6K1 fragment by mTOR would appear to be raptor independent, and might depend instead on passive diffusion of the substrate into the active site of the mTOR kinase domain. In this setting, the presence of bound raptor might actually interfere with the access of GST-S6K1 fragment to the mTOR active site. Indeed, this model accommodates Kim et al.'s observation that exposure of mTOR to Triton X-100, which effectively strips raptor from mTOR, strongly activates mTOR kinase activity toward the GST-S6K1 substrate. Kim et al. also reported that raptor overexpression inhibits mTOR function in intact cells. Although these findings are consistent with the idea that raptor dampens mTOR signaling in vivo, the overexpression of a putative substrate-scaffolding protein could suppress mTOR function indirectly by sequestering its intracellular substrates in nonproductive complexes.

Direct experimentation is required to rationalize the discrepant results obtained by the two teams. However, the scaffolding model for raptor function is made even more attractive by the recent identification of a common, 5 amino acid TOR-signaling (TOS) motif in 4E-BP1 and S6K1 (Schalm and Blenis, 2002). An intact TOS sequence is required for mTOR-dependent phosphorylation of both proteins following nutrient readdition to starved cells. It will be interesting to learn whether the TOS motif serves as a docking site for raptor in mammalian cells.

In a spectacular report that followed on the heels of the *Cell* papers, Michael Hall's laboratory has identified *five* proteins that coprecipitate with *S. cerevisiae* TOR1 and/or TOR2; one of these proteins, named KOG1 (*K*ontroller *o*f *G*rowth-1), is the budding yeast homolog of raptor.

Loewith et al. (2002) resolved two structurally and

functionally distinct TOR complexes (TORCs) (see Figure 1). TORC1 contains either TOR1 or TOR2, together with KOG1, and binds FKBP12•rapamycin. TORC2 contains TOR2 exclusively and has four additional subunits, one of which (LST8) is shared with TORC1, whereas the others (AVO1, AVO2, and AVO3) are uniquely present in TORC2. Strikingly, TOR2 fails to bind FKBP12•rapamycin in the context of TORC2. Hence, TORC2 is a strong candidate for the transmission of rapamycin-insensitive signals leading to actin cytoskeleton reorganization in budding yeast. Similar cytoskeletal defects are observed in yeast mutants that fail to express TOR2, AVO1, or LST8.

The evolutionary conservation of KOG1 (human raptor), LST8, and AVO1 (human SIN1) in mammalian cells hints that mTOR may also reside in at least two TORCs. The evidence for a human TORC1 complex is strong, given that both raptor and human LST8 can be coimmunoprecipitated with mTOR (Loewith et al., 2002). The existence of a human TORC2-like complex is considerably more tenuous, given that an association between human SIN1 and mTOR could not be documented. Furthermore, inhibition of mTOR function in mammalian cells has not been associated with significant defects in the actin cytoskeleton. However, the phrase "absence of evidence is not evidence of absence" is relevant here, because our knowledge of mTOR function is largely derived from studies with rapamycin-treated cells, and the yeast studies have taught us that TORC2 signaling to actin filaments is not affected by rapamycin. One possibility is that a mammalian TORC2-like complex assembles around another protein kinase, perhaps a member of the MAP kinase family, in place of mTOR.

In summary, a decade-long investigation that began with a clinically promising drug led to the discovery of the TOR kinases, and now to the identification of a multiprotein complex that plays important roles in cell growth, proliferation, and tumorigenesis. The identification of raptor and other TOR partner proteins offers pivotal insights into the signal transduction network that surrounds the TOR proteins and may yield new drug targets as well. A working model for the TOR signaling pathways in yeast and mammals is presented in Figure 1. Based on the available evidence, KOG1/raptor is proposed to transduce nutrient-related signals to yeast and mammalian TORs. The upstream regulatory pathway linked to TORC2 is unknown, but may involve lipid-derived second messengers (Loewith et al., 2002).

Additional studies will be required to define the linkage (if any) between the PI3-kinase-AKT signaling pathway and raptor. However, the notion that this pathway couples growth factor receptor occupancy to mTOR has received a boost from an unexpected source. Recent studies in both *D. melanogaster* and mammalian cells suggest that the tuberous sclerosis (TSC) complex interacts with mTOR and represses mTOR-dependent S6K1 activation (Gao et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). Tuberous sclerosis is an autosomal dominant disease that is characterized by the growth of multiple benign tumors and mental retardation. Patients with tuberous sclerosis have mutations in either of the two TSC complex subunits, TSC1 (hamartin) or TSC2 (tuberin). In response to growth factor stimulation (or oncogenic insults such as loss of PTEN), AKT phosphorylates TSC2, and this modification relieves the mTOR-repressive function of the TSC complex. Whether TSC binds to the raptor-mTOR complex (i.e., mammalian TORC1) or yet another TORC in mammalian cells remains an important but unanswered question. With many new TOR partners now in hand, we should see a quick resolution to some long-standing questions and controversies surrounding TOR signaling, and undoubtedly a few more surprises as well.

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