Mammalian target of rapamycin: Master regulator of cell growth in the nervous system

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Summary. The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase that regulates a number of diverse biologic processes important for cell growth and proliferation, including ribosomal biogenesis and protein translation. In this regard, hyperactivation of the mTOR signaling pathway has been demonstrated in numerous human cancers, including a number of inherited cancer syndromes in which individuals have an increased risk of developing benign and malignant tumors. Three of these inherited cancer syndromes (Lhermitte-Duclos disease, neurofibromatosis type 1, and tuberous sclerosis complex) are characterized by significant central nervous system dysfunction and brain tumor formation. Each of these disorders is caused by a genetic mutation that disrupts the expression of proteins which negatively regulate mTOR signaling, indicating that the mTOR signaling pathway is critical for appropriate brain development and function. In this review, we discuss our current understanding of the mTOR signaling pathway and its role in promoting ribosome biogenesis and cell growth. We suggest that studies of this pathway may prove useful in identifying molecular targets for biologically-based therapies of brain tumors associated with these inherited cancer syndromes as well as sporadic central nervous system tumors.

Key words: mTOR, NF1, TSC, PEN, Rapamycin

Introduction

The protein kinase mammalian target of rapamycin (mTOR) is an important regulator of cell growth and proliferation in a variety of organisms ranging from yeast to mammals. Its importance in controlling metabolism and growth in single cell eukaryotes is well established; however, its relevance to human cancer has only recently been explored. In this regard, inappropriate activation of mTOR signaling has been demonstrated in a number of inherited cancer syndromes that predispose affected individuals to develop benign and malignant tumors (Inoki et al., 2005). Strikingly, a number of these disorders affect the central nervous system, including Lhermitte-Duclos disease (LDD), tuberous sclerosis complex (TSC), and neurofibromatosis type 1 (NF1). Each of these disorders is caused by mutations of genes that encode negative regulators of the mTOR signaling pathway. The observation that these three single gene disorders all converge on a common signaling pathway suggests that mTOR signaling plays a central role in brain tumor biology.

The mTOR signaling pathway

Eukaryotic TORs are large proteins (~280 kDa) that exhibit 40-60% sequence homology at the amino acid level and are members of the phosphotidylinositol-3-kinase (PI3K)-related protein kinase (PIKK) family (Abraham, 2004). Unlike PI3K, which possesses lipid kinase activity, PIKK family members function as serine/threonine protein kinases. TOR was first identified by genetic screens in yeast aimed at identifying the protein target of rapamycin, a potent immunosuppressive macrolide produced by Streptomyces hygroscopicus (Heitman et al., 1991). The mechanism by which rapamycin and its analogs inhibit TOR is unique: rapamycin first binds to its cellular receptor, the FK506 binding protein (FKBP12) and it is the rapamycin-FKBP12 complex that inhibits TOR function. This mechanism of action is conserved in eukaryotes, with single TOR orthologs identified in C. elegans (Long et al., 2002), D. melanogaster (Oldham et al., 2000; Zhang et al., 2000), and mammals (Brown et al., 1994; Sabatini et al., 1994).

Although the exact mechanism by which the rapamycin-FKBP12 complex inhibits TOR kinase
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activity is unclear, recent evidence suggests that rather than inhibiting the TOR kinase domain directly, the rapamycin-FKBP12 complex may block protein-protein interactions critical for appropriate TOR kinase activity. In yeast, two distinct TOR genes interact with unique protein partners to form TOR complexes that have differential sensitivities to rapamycin and mediate distinct biological effects (Loewith et al., 2002). Although only one TOR gene exists in mammals, mTOR exists in two distinct multi-protein signaling complexes, only one of which binds to rapamycin-FKBP12 (Fig. 1).

This complex consists of mTOR, mLST8 (also known as GβL), and raptor (regulatory associated protein of mTOR) and mediates rapamycin-sensitive coupling of nutrient and mitogen signals to the regulation of cell growth and proliferation. In the rapamycin-insensitive complex, raptor is replaced by rictor (rapamycin insensitive companion of mTOR) (Sarbassov et al., 2004). Unlike other proteins in mTOR complex, rictor is poorly conserved in multicellular organisms. Because mTOR-dependent biologic effects have generally been assessed by rapamycin sensitivity, the upstream regulators and downstream targets of the rapamycin-insensitive mTOR-rictor complex remain poorly understood. Currently, the major known function of the rapamycin-insensitive mTOR-rictor complex is regulation of actin cytoskeleton organization (Jacinto et al., 2004; Sarbassov et al., 2004)

TOR is an essential protein, as loss of TOR expression results in early embryonic death in C. elegans (Long et al., 2002), D. melanogaster (Zhang et al., 2000) and mice (Hentges et al., 2001; Gangloff et al., 2004; Murakami et al., 2004). Genetic studies in Drosophila first revealed the role of dTOR in regulating cell size and proliferation. dTOR-deficient flies exhibit cell-autonomous decreases in both cell size and cell proliferation (Zhang et al., 2000). This phenotype closely recapitulates that of flies in which positive regulators of the PI3K signaling pathway are inactivated (Chen et al., 1996; Leevers et al., 1996; Bohni et al., 1999; Weinkove et al., 1999), suggesting that TOR may function within this signaling pathway. When activated by receptor tyrosine kinases, PI3K phosphorylates membrane phospholipids, generating lipid second messengers. These second messengers recruit and activate a second kinase, Akt (also known as protein kinase B). Current models suggest that Akt activates mTOR either by direct phosphorylation (Nave et al., 1999) or by inactivating the protein complex encoded by the TSC1 and TSC2 genes (Dan et al., 2002; Inoki et al., 2002; Potter et al., 2002). Inactivation of the TSC1/2 protein complex, as occurs in tuberous sclerosis complex (see below), results in hyperactivation of the small GTPase Rheb (Ras homolog enriched in brain), another direct activator of mTOR (Long et al., 2005). In addition to regulation by the PI3K pathway, mTOR is also regulated by a number of other inputs, including nutrient, energy, and oxygen availability. Though these factors will not be discussed in this review, together they help to coordinate protein synthesis rates to ensure that cell growth and proliferation are tightly coupled with environmental cues relevant to the overall health of the cell.

mTOR signaling has been shown to regulate numerous biological processes, including ribosomal biogenesis, protein translation, cell size regulation, and cell proliferation. mTOR regulates protein translation through two parallel pathways: activation of ribosomal S6 kinase-1 (S6K1) and suppression of the elongation factor 4E binding protein 1 (4E BP1). Phosphorylation of S6K1 on Thr389 and Ser371 residues is required for its activation and both sites are phosphorylated by mTOR in vitro (Pearson et al., 1995; Saitoh et al., 2002). Active S6K1 then phosphorylates the 40S ribosomal protein S6. It was believed that phosphorylation of S6 increases the association of mRNAs that contain a 5'-terminal oligopyrimidine (5'-TOP) sequence with polysomes. These mRNAs include those encoding all known ribosomal proteins and several elongation factors. S6K-dependent increases in these proteins result in increased

Fig. 1. mTOR functions in two distinct protein complexes to regulate diverse biologic processes. The two mTOR complexes are distinguished by the binding of either raptor or rictor. The mTOR-raptor complex is sensitive to the mTOR inhibitor rapamycin and is thought to regulate mTOR-dependent control of protein translation and ribosome biogenesis. The mTOR-rictor complex is insensitive to rapamycin. The only known function of the mTOR-rictor complex is regulation of actin cytoskeleton organization, possibly through Rac1 and PKGα.
ribosome biogenesis and an overall increase in protein translation (Jefferies et al., 1994, 1997; Terada et al., 1994). However, recent work has suggested alternative mechanisms for regulating the translation of 5'-TOP mRNAs that do not require S6K1 activity or S6 phosphorylation (Pende et al., 2004). Therefore, although S6K1 activity and S6 phosphorylation temporally correlate with translation of 5'-TOP mRNAs, the exact mechanism by which mTOR controls 5'-TOP mRNA translation has yet to be fully elucidated.

Another well-studied mTOR target, 4EBP1, also regulates protein translation. In nutrient- and growth factor-depleted cells, 4EBP1 binds tightly to the mRNA cap binding protein eIF4E (elongation initiation factor 4E), and prevents eIF4E from binding to the 5'-cap or other components of the translation initiation complex (Pause et al., 1994). mTOR-dependent 4EBP1 phosphorylation disrupts the 4EBP1-eIF4E interaction, allowing eIF4E binding to eIF4G, and facilitates the formation of an active translation initiation complex. Consistent with this mechanism of action, expression of a 4EBP1 mutant that is refractive to mTOR-mediated phosphorylation inhibits protein synthesis and cell cycle progression (Fingar et al., 2004).

In addition to its role in regulating translation initiation, mTOR is also a key player in the process of ribosomal biogenesis. In this fashion, mTOR regulates protein synthesis rates at multiple levels (Fig. 2). Studies in both yeast and mammalian cells have demonstrated that rapamycin blocks the biosynthesis of ribosomes by inhibiting transcription of RNA polymerase I (Pol I)-dependent rRNA genes, Pol II-dependent ribosomal protein genes, and Pol III-dependent tRNA genes (Zaragoza et al., 1998). TOR inhibition results in inactivation of the Pol I-associated essential transcription factor, TIF1A, to block transcription initiation and control rRNA transcription (Mayer et al., 2004). In yeast, Pol II-dependent genes are regulated by TOR by multiple mechanisms, including the zinc finger transcription factor SFP1 that binds and regulates ribosomal protein genes in a TOR-dependent manner as well as histone-modifying factors that serve as TOR effectors (Marion et al., 2004). Thus, TOR links growth factor status and nutrient availability to both the synthesis of new ribosomes and the initiation of protein translation.

Interestingly, we have recently demonstrated that TOR exerts control over ribosome biogenesis, not only translation initiation. Although the exact mechanism by which mTOR/S6K1 controls 5'-TOP mRNAs remains unclear, it is well established that phosphorylation of 4E-BP1 by mTOR releases eIF4E, which is then free to associate with eIF4G to stimulate translation initiation.
by regulating rRNA and ribosomal protein gene transcription, but also by regulating the export of newly synthesized ribosomal subunits from the nucleolus to the cytoplasm (Pelletier et al., 2007). The export process is regulated by a nucleolar protein termed nucleophosmin (NPM). NPM is highly expressed in proliferating cells and is overexpressed in a variety of neoplasms, including prostate, ovarian and colon cancers. Consistent with these correlations, Npm-deficient fibroblasts exhibit decreased proliferation (Grisendi et al., 2005). NPM is an essential protein, as two recent reports have shown that Npm is required for embryonic development (Colombo et al., 2005; Grisendi et al., 2005). NPM interacts with nucleolar components of newly synthesized ribosomes and shuttles from the nucleolus/nucleus to the cytoplasm in a CRM1-dependent manner (Yu et al., 2006). We have recently demonstrated a role for NPM in ribosome biogenesis, as overexpression of NPM enhances the export of ribosomal subunits from the nucleolus to the cytoplasm and results in increased rates of protein synthesis (Yu et al., 2006; Maggi and Weber, unpublished results). Overexpression of a nucleophosmin mutant protein incapable of nuclear/cytoplasmic shuttling blocks ribosomal export and leads to cell cycle arrest. Importantly, we have shown that treatment with rapamycin inhibits NPM protein expression, demonstrating a role for mTOR in regulating cellular levels of NPM and therefore export of newly synthesized ribosomes to the cytoplasm where translation initiation occurs (Pelletier et al., 2007).

**Insights from inherited cancer syndromes**

Recent studies of mTOR regulation have identified that several proteins which converge on mTOR signaling are encoded by genes mutated in inherited tumor predisposition syndromes. A number of these disorders are characterized by significant neurologic complications, including brain tumor formation (Fig. 3). This suggests that mTOR signaling plays a particularly important role in regulating normal brain development and neural/epithelial cell growth.

**Lhermitte-Duclos disease**

Lhermitte-Duclos Disorder (LDD), or dysplastic gangliocytoma of the cerebellum, is a rare, benign tumor of the cerebellar cortex. Typically, patients present with headaches, gait disturbances, and cranial nerve dysfunction. LDD frequently occurs in association with Cowden disease, an autosomal dominant tumor...
TSC is the cortical tuber, a benign growth of immature neuroglial cells in the brain that forms early in development. The number and location of these tubers correlates with the neurologic complications associated with the disease, including epilepsy, autism, and mental retardation (Goodman et al., 1997; Asato and Hardin, 2004; Wiznitzer, 2004). Individuals with TSC are predisposed to the development of dysplastic growths called subependymal nodules that arise from cells lining the lateral ventricle. While often asymptomatic, subependymal nodules may progress to form subependymal giant cell astrocytomas (SEGAs).

The first insights into the function of the Tsc1 and Tsc2 gene products came from genetic studies in Drosophila. Mutation of the Tsc1 or Tsc2 homologues in flies resulted in a striking increase in organ size highly reminiscent of flies that lack expression of dPten (Huang et al., 1999; Ito and Rubin, 1999; Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). These observations suggested that the Tsc1/2 protein complex, like Pten, negatively regulates growth factor signaling. Genetic epistasis experiments revealed that the Tsc1/2 complex functions downstream of Akt in the PI3K signaling pathway (Potter et al., 2001). Further studies have demonstrated that tuberin functions as a guanosine triphosphate activating protein (GAP) towards the small GTPase Rheb (Ras homolog enriched in brain), a direct mTOR activator (Long et al., 2005) to inhibit mTOR signaling (Castro et al., 2003; Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003).

Like LDD, important insights into the role of mTOR signaling in TSC-associated neurologic disease have been gleaned from studies of murine models. Mice homozygous for loss of Tsc1 or Tsc2 die during mid-embryogenesis (Kobayashi et al., 1999, 2001; Kwiatkowski et al., 2002). Tsc1+/− and Tsc2+/− mice are viable but the number of brain astrocytes in the central nervous system is increased (Uhlmann et al., 2002a). Together, these observations support the role of tuberin and hamartin as regulators of cell growth and proliferation in the central nervous system. To study this further, our laboratory has used a conditional knockout approach to inactivate Tsc1 in astrocytes (Tsc1 CKO). Tsc1 CKO mice exhibit an increase in brain size, an increase in the number of brain astrocytes, and abnormal neuronal organization in the hippocampus (Uhlmann et al., 2002b). This alteration of brain organization is associated with seizure activity and early death. Astrocytes from Tsc1 CKO mice grown in vitro exhibit increased cell growth and cell size associated with hyperactivation of the mTOR signaling pathway (Uhlmann et al., 2004). Treatment with rapamycin blocks mTOR pathway hyperactivation and restores normal cell size in Tsc1−/− astrocytes, indicating that mTOR signaling is required for this biological effect. Consistent with the results in Tsc1−/− astrocytes, we and others have demonstrated that mouse embryonic fibroblasts lacking either Tsc1 or Tsc2 exhibit mTOR hyperactivation (Kwiatkowski et al., 2002; Uhlmann et
NF1 is the most common of the autosomal dominant tumor predisposition syndromes, affecting approximately 1 in 3000 individuals worldwide. NF1 is classified as a neurocutaneous syndrome, as children with this disorder typically present with characteristic skin pigmentation abnormalities and are prone to develop nervous system tumors. Neurofibromas of the peripheral nervous system are the most common tumor associated with NF1, affecting more than 95% of affected individuals (Reynolds et al., 2003). Dermal neurofibromas are benign tumors associated with peripheral nerves that contain a variety of cell types, including Schwann cells, fibroblasts, and mast cells. Unlike dermal neurofibromas, plexiform neurofibromas are diffuse, infiltrative tumors that occur in 30% NF1 patients. These tumors can transform into malignant peripheral nerve sheath tumors, highly aggressive cancers associated with poor patient survival (Korf, 1999).

The central nervous system complications associated with NF1 are often the most disabling for patients. Children with NF1 are prone to develop low-grade (World Health Organization grade I) pilocytic astrocytomas, including optic gliomas that arise within the visual pathway (optic nerve, optic chiasm, and hypothalamus). While these tumors represent only 2-5% of childhood brain tumors, greater than 70% are associated with NF1 (Listernick et al., 1997). Optic glioma in the context of NF1 arise in young children (median age of onset: 4.9 years) and can result in significant morbidity, including vision loss and precocious puberty due to hypothalamic dysfunction. In addition, adults with NF1 are at increased risk for developing higher grade brain tumors (Gutmann et al., 2003).

The NF1 gene is located on chromosome 17q11 and encodes a 220-250 kDa cytoplasmic protein termed neurofibromin. Neurofibromin is highly expressed in neurons and glial cells in the brain, but is also highly expressed in the kidney, thymus, spleen, and testis (Daston et al., 1992; Nordlund et al., 1993; Gutmann et al., 1995). Like the TSC1/2 genes, NF1 functions as a tumor suppressor, requiring inactivation of both copies of NF1 for tumor formation. Neurofibromin is a Ras GTPase activating protein (GAP), converting the proto-oncogene Ras from a GTP-bound active form to a GDP-bound inactive form (DeClue et al., 1992). Re-expression of the NF1 GAP domain or pharmacologic inhibition of Ras rescues the increased proliferation observed in cells lacking a functional copy of NF1 (DeClue et al., 1992; Hiatt et al., 2001), indicating that Ras is an important mediator of neurofibromin-dependent cell proliferation.

To better understand the molecular mechanism of NF1-associated astrocytoma formation, our laboratories used an unbiased proteomics approach to identify neurofibromin protein targets in mouse astrocytes. Many of the proteins identified by this method were proteins involved in ribosome biogenesis, including NPM (Dasgupta et al., 2005). Consequently, protein translation was increased nearly 8-fold in NF1-deficient astrocytes. In NF1-deficient astrocytes, we found that the mTOR signaling pathway was hyperactivated, an effect that was blocked by rapamycin (Dasgupta et al., 2005). Similarly, NF1-deficient fibroblasts exhibit mTOR pathway activation that is rescued by expression of the neurofibromin GAP-related protein domain, suggesting that Ras-dependent signaling mediates mTOR hyperactivation downstream of neurofibromin (Johannessen et al., 2005). The increase in protein synthesis characteristic of NF1−/− astrocytes was inhibited by rapamycin, indicating that mTOR is required for increased protein translation in these cells. Moreover, both NF1−/− astrocytes and NF1−/− Schwann cells exhibit increased cell proliferation that is sensitive to rapamycin (Dasgupta et al., 2005; Johannessen et al., 2005). Collectively, these studies identify mTOR as an important effector of Ras signaling important for neurofibromin-dependent cell proliferation.

**mTOR as a therapeutic target in brain tumors**

The essential role of mTOR in brain development and its hyperactivation in several inherited brain tumor syndromes raise the exciting possibility that inhibition of mTOR or its downstream targets may have therapeutic applications in patients with brain tumors. Rapamycin is currently being studies for its anti-tumor effects in a number of different organ systems. In murine models of breast cancer (Namba et al., 2006) and acute lymphoblastic leukemia (Teachey et al., 2006), rapamycin or its analog CCI-779 have been shown to inhibit tumor growth, suggesting that rapamycin, either alone or in combination with other chemotherapeutic agents may prove to be an efficacious treatment for patients. In brain tumors, a small molecular inhibitor of PI3K and mTOR was shown to block the proliferation of glioma xenografts in mice, highlighting the importance of PI3K/mTOR signaling in glioma formation and growth (Fan et al., 2006). Furthermore, in a small study on patients with TSC and SEGAs, oral rapamycin treatment resulted in partial tumor regression and was well tolerated (Franz et al., 2006).

While the functions of the mTOR targets S6K and 4EBP1 have been, and continue to be, explored, it is expected that other downstream targets of mTOR signaling will be identified that are important for tumor formation and growth. Two recent studies identified PKCα and Rac1 as important targets of mTOR signaling (Jacinto et al., 2004; Sarbassov et al., 2004). Importantly, mTOR-dependent regulation of these proteins has been shown to alter actin cytoskeleton dynamics, suggesting that mTOR may play roles in other aspects of tumor biology which are dependent on the actin cytoskeleton,
including cell motility and invasion. Furthermore, the identification of NPM, an important regulator of ribosomal biogenesis and cell proliferation, as an mTOR signaling target raises the possibility that inhibitors of NPM function may impair cell growth in tumors characterized by mTOR hyperactivation. Further molecular dissection of the mTOR signaling pathway has great potential for identifying additional pharmacologic targets that, in addition to rapamycin, hold promise for patients suffering from brain tumors with deregulated mTOR signaling.

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