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ORIGINAL ARTICLE

Tuberin, p27 and mTOR in different cells

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Abstract Mutations in the genes TSC1 or TSC2 cause the autosomal dominantly inherited tumor suppressor syndrome tuberous sclerosis, which is characterized by the development of tumors, named hamartomas, in different organs. The TSC gene products, hamartin and tuberin, form a complex, of which tuberin is assumed to be the functional component. Both, hamartin and tuberin have been implicated in the control of the cell cycle by activating the cyclin-dependent kinase inhibitor p27 and in cell size regulation by inhibiting the mammalian target of rapamycin (mTOR) a regulator of the p70 ribosomal protein S6 kinase (p70S6K) and its target the ribosomal protein S6. The tuberin/hamartin complex was shown to protect p27 from protein degradation. Within the mTOR signaling pathway tuberin harbors GTPase activating (GAP) potential toward Rheb, which is a potent regulator of mTOR. In this study, we have analyzed the protein levels of tuberin, p27, cyclin D1, mTOR and phospho mTOR Ser2448 (activated mTOR), S6 and phospho S6 Ser240/244 (activated S6) and as controls α-tubulin and topoisomerase IIβ, in ten different cells, including primary normal cells, immortalized and transformed cell lines.

Keywords Tuberin · p27 · mTOR · S6

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Introduction

The tumor suppressor gene TSC1 encodes hamartin (The TSC1 Consortium 1997) and TSC2 encodes tuberin (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). Inactivation of TSC1 or TSC2 causes tuberous sclerosis (TSC) with similar phenotype. TSC is an autosomal dominant tumor syndrome (affecting approximately 1 in 6,000 individuals), which is characterized by the development of hamartomas in the kidneys, heart, skin and brain. The latter often cause seizures, mental retardation and developmental disorders, including autism. TSC patients carry a mutant TSC1 or TSC2 gene in each of their somatic cells and loss of heterozygosity has been documented in a wide variety of TSC tumors (Pan et al. 2004; Astrinidis and Henske 2005; Crino et al. 2006).

Tuberin and hamartin form a heterodimer. Tuberin, which is assumed to be the functional component of this protein complex, is a multifunctional protein, which is involved in the regulation of cell size, cell cycle, translation, transcription, apoptosis and cell differentiation. A wide variety of proteins, implicated in different regulations, have been demonstrated to interact with tuberin (Rosner et al. 2004, 2008). A major function of the TSC1/TSC2 complex is its role as a GTPase activating protein against Rheb (Ras homolog enriched in brain), which in turn regulates mTOR (mammalian target of rapamycin) signaling. One major function of mTOR is to phosphorylate and activate p70S6K (p70 ribosomal protein S6 kinase) leading to activation of the ribosomal protein S6 via phosphorylation at Ser240/244. The mTOR signaling network has a central role in the regulation of cell growth in response to growth factors, cellular energy and nutrient levels (Corradetti and Guan 2006; Guertin and Sabatini 2007). Tuberin is localized to both the nucleus and the

cytoplasm, and Akt phosphorylation of tuberin induces cytoplasmic tuberin localization (Rosner et al. 2007b). In fact, tuberin is phosphorylated by several kinases, including Akt, AMP-activated protein kinase (AMPK), extracellular signal-regulated kinase (ERK), RSK and glycogen synthase kinase 3β (GSK3β), which regulate its activity (Pan et al. 2004; Wullschleger et al. 2006; Corradetti and Guan 2006; Guertin and Sabatini 2007; Rosner et al. 2008).

The cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ (p27) accumulates in G0/G1 cells and is localized in the nucleus where it regulates CDKs. During the transition to S phase p27 is translocated to the cytoplasm and degraded by the ubiquitin–proteasome pathway. Accordingly, p27 is a major regulator of the mammalian cell cycle progression (reviewed in Blain et al. 2003; Nho and Sheaff 2003; Sicinski et al. 2007). Besides its functions in the regulation of endocytosis (Xiao et al. 1997), transcription (Henry et al. 1998), neuronal differentiation (Soucek et al. 1998a) or apoptosis (Inoki et al. 2003; Shah et al. 2004; Freilinger et al. 2006a, b, 2008), tuberin also regulates cell cycle progression. Downregulation of tuberin expression induces quiescent fibroblasts to enter the cell cycle and TSC2−/− fibroblasts exhibit a shortened G1 phase. Overexpression of TSC1 or TSC2 triggers an increase in G1 cells and p27 protein levels (Soucek et al. 1997, 1998b; Miloloza et al. 2000). Tuberin negatively regulates the activity of CDK2. Tuberin binds to the cyclin-dependent kinase inhibitor p27 and, thereby, prevents p27 degradation via its SCF-type E3 ubiquitin ligase complex. Tuberin also triggers an upregulation of the amount of p27 bound to CDK2 (Rosner and Hengstschläger 2004). Furthermore, in tuberin negative cells nuclear p27 is delocalized into the cytoplasm. Recently, it was reported that tuberin induces nuclear p27 localization by inhibiting its 14-3-3-mediated cytoplasmic retention. Akt-mediated phosphorylation of p27, but not of tuberin, negatively regulates tuberin's potential to trigger p27 nuclear localization, demonstrating that p27 localization during the mammalian cell cycle is under the control of tuberin (Rosner et al. 2007a).

Considering the knowledge described above, it is of theoretical and practical importance to compare the protein expression levels of tuberin, p27, cyclin D1, mTOR and phospho mTOR Ser2448 (activated mTOR), S6 and phospho S6 Ser240/244 (activated S6) and as controls α-tubulin and topoisomerase IIβ in different cells. Theoretically, it is interesting to study putative correlations of tuberin's major downstream targets. Practically, it forms an important basis for further studies on tuberin's effects on cell cycle regulation and cell size control to know which proteins of the mentioned cascades are expressed at which levels in commonly used cells.

In this report, we now present an analysis of the expression levels of the proteins mentioned above in ten different commonly used human cells, including primary normal cells, immortalized and transformed cell lines.

Materials and methods

Cells and cell culture

All cells used for this study are of human origin and have been obtained from the American Type Culture Collection (ATCC). For detailed description of the cells and of the used growth conditions see Table 1. All cells were grown in medium supplemented with antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulphate) at 37°C and 5% CO_2 and were routinely screened for mycoplasma.

Immunoblotting

Total protein was extracted of logarithmically growing cells. For preparing lysates cells were washed with PBS, collected by scraping and lysed in buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM

Table 1 Cells used in this study

	ATCC#	Organ/tissue		Morphology	Growth medium
WI-38	CCL-75	Fetal lung	Normal	Fibroblast	DMEM/10%FCS
MRC-5	CCL-171	Fetal lung	Normal	Fibroblast	DMEM/10%FCS
IMR-90	CCL-186	Fetal lung	Normal	Fibroblast	DMEM/10%FCS
MDA-MB-468	HTB-132	Mammary gland, breast	Adenocarcinoma	Epithelial	McCoy's/10%FCS
MCF7	HTB-22	Mammary gland, breast	Adenocarcinoma	Epithelial	DMEM/10%FCS
SK-N-SH	HTB-11	Brain	Neuroblastoma	Epithelial	RPMI/20%FCS
SAOS-2	HTB-85	Bone	Osteosarcoma	Epithelial	RPMI/20%FCS
HT-1080	CCL-121	Connective tissue	Fibrosarcoma	Epithelial	DMEM/10%FCS
HeLa	CCL-2	Cervix	Adenocarcinoma	Epithelial	DMEM/10%FCS
293	CRL1573	Embryonic kidney	Virus transformed	Epithelial	DMEM/10%FCS

Table 2 Antibodies used in this study

Tuberin	Tuberin (C-20)/Santa Cruz
p27	p27 (clone 57)/BD Transduction Laboratories
Cyclin D1	Cyclin D1 (M-20)/Santa Cruz
Phospho-mTOR (Ser2448)	mTOR (S2448)/cell signaling
mTOR	mTOR/cell signaling
Phospho-S6 (Ser240/244)	S6 (S240/244)/cell signaling
S6	S6/cell signaling
α-Tubulin	α-Tubulin (Ab-1)/Calbiochem
Topoisomerase IIβ	topoIIβ (clone 40)/BD Transduction Laboratories

EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4 supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchloride, 10 μg/ml trypsin inhibitor by repeated freezing and thawing. Supernatants were collected by centrifugation and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumine as the standard. Proteins were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein (Rosner et al. 2003). The antibodies used for immunodedection are described in detail in Table 2. Signals were detected with appropriate horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence method (Rosner et al. 2007a).

Western blot densitometry

The blots were densitometrically scanned. The signals of phospho mTOR (Ser2448) have been normalized to the according mTOR signals. The signals of phospho S6 (Ser240/244) have been normalized according to the S6 signals. The results are given in relation to the highest value set as 1.

Results and discussion

It was the aim of this study to compare the protein expression levels of tuberin, p27, cyclin D1, mTOR and phospho mTOR Ser2448 (activated mTOR), S6 and phospho S6 Ser240/244 (activated S6) in different cells. As normal non-transformed human counterparts we included three different lines of normal fetal lung fibroblasts (WI-38, MRC-5, IMR-90), all obtained from the ATCC. We compared the protein expression levels of these normal cells with the expression patterns of seven human cell lines derived from adenocarincomas, either of mammary gland origin (MDA-MB-468 and MCF7) or of cervical origin (HeLa), from neuroblastoma (SK-N-SH), from osteosarcoma (SAOS-2), from fibrosarcoma (HT-1080) and from adenovirus transformed embryonic kidney cells (293) (Table 1).

As proven by cell doubling studies and flowcytometric DNA analyses (data not shown) all cells were growing logarithmically at the time point of protein extraction. After determination of the protein concentration using the Bio-Rad protein assay with bovine serum albumin as the standard, for each cell line exactly the same amount of total protein was run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein. Ponceau-S staining proved that the amount of protein loaded on each lane was equal in the western blot presented in Fig. 1. We co-analyzed two proteins, α-tubulin and topoisomerase IIβ, which are not involved in tuberin-dependent biochemical pathways. These control analyses revealed that observed differences in protein expression levels are not caused by loading differences. We found specific cells to express high levels of α-tubulin, but low levels of topoisomerase IIβ (all relative to the other analyzed cells), and other cells harboring vice versa expression levels. The three normal cells (WI-38, MRC-5, IMR-90) and MDA-MB-468, MCF-7, SK-N-SH and HT-1080 cells express relatively high levels of α-tubulin, of which WI-38 express very low levels of topoisomerase IIβ and SK-N-SH high levels of topoisomerase IIβ. Interestingly, the osteosarcoma-derived cell line SAOS showed the lowest level of α-tubulin and the highest level of topoisomerase IIβ of all analyzed cell lines (Fig. 1a).

Tuberin is a tumor suppressor protein, which is believed to be regulated via its phosphorylation by a variety of different kinases (see "Introduction") rather than by the control of its protein expression levels (Pan et al. 2004; Wullschleger et al. 2006; Corradetti and Guan 2006; Guertin and Sabatini 2007; Rosner et al. 2008). However, it was interesting to see that the three normal cell lines exhibit higher endogenous tuberin protein levels than the seven transformed cell types. Within these seven transformed cell lines MCF-7 and SK-N-SH appeared to express higher levels of tuberin protein levels than the others (Fig. 1a).

One major target of tuberin is the cyclin-dependent kinase inhibitor p27 (Soucek et al. 1997, 1998b; Miloloza et al. 2000; Rosner and Hengstschläger 2004; Rosner et al. 2007a). Accordingly, it was of interest to also analyze the protein levels of p27 in total lysates of these cell lines. Tuberin was shown to stabilize p27 by protecting it from degradation via its SCF-type E3 ubiquitin ligase complex (Rosner and Hengstschläger 2004).

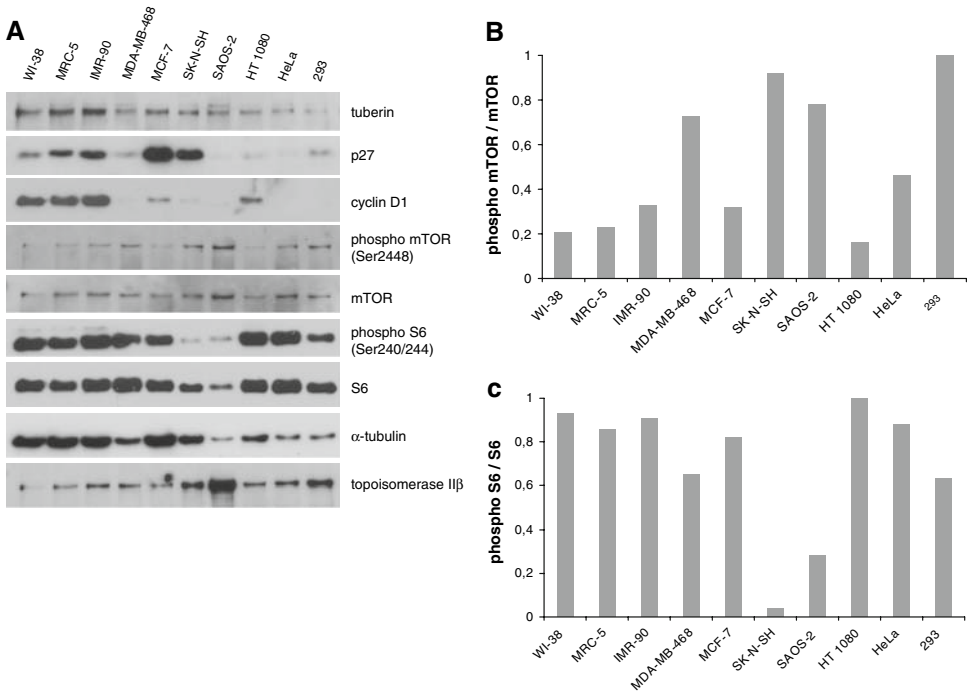

Fig. 1 Protein levels in different cells. **a** Total protein of logarithmically growing WI-38, MRC-5, IMR-90, MDA-MB-468, MCF-7, SK-N-SH, SAOS-2, HT-1080, HeLa and 293 cells (for details of the different cells see Table 1 and the text) was extracted. Western blot analyses of tuberin, p27, cyclin D1, phospho mTOR (Ser2448), mTOR, phospho S6 (Ser240/244), S6, α-tubulin and topoisomerase IIβ protein levels were performed (for details of the used antibodies see Table 2 and the text). **b** In addition, western blot results were densitometrically analyzed. The signals of phospho mTOR have been normalized to the according mTOR signals (highest level set as 1). **c** The signals of phospho S6 have been normalized to the according S6 signals (highest level set as 1)

Accordingly, one could speculate that cells expressing high levels of tuberin should also exhibit high p27 protein levels. Still, such clear correlations between tuberin protein amounts and p27 protein amounts cannot necessarily be expected due to different reasons. (1) The amount of tuberin protein does not represent p27-regulating tuberin activity, because tuberin is activated and inactivated via a wide variety of different phosphorylation events (Pan et al. 2004; Wullschleger et al. 2006; Corradetti and Guan 2006; Guertin and Sabatini 2007; Rosner et al. 2008). (2) Tuberin is clearly not the only regulator of p27 (Blain et al. 2003; Nho and Sheaff 2003; Sicinski et al. 2007). (3) Last but not least, it has recently been shown that besides regulating p27 degradation, tuberin also affects the cytoplasmic/nuclear localization of p27. It was reported that tuberin, which is a nuclear and cytoplasmic protein, induces nuclear p27 localization by inhibiting its 14-3-3-mediated cytoplasmic retention (Rosner et al. 2007a; Rosner and Hengstschläger 2007). These findings are of special interest considering that for p27 a duality of function in tumorigenesis has recently been identified. p27 acts as a tumor suppressor by inhibiting CDK activities in the nucleus. However, in human malignancies mutation/inactivation of p27 is only rarely detectable. Instead, p27 is degraded or relocalized to the cytoplasm. Several data provide evidence that cytoplasmic p27 might harbor oncogenic potential via regulating migration (Besson et al. 2004, 2007; Sicinski et al. 2007; Denicourt et al. 2007).

Still, in this study we found that the five cell lines with the highest levels of tuberin, the three normal counterparts and MCF-7 and SK-N-SH, also exhibit much higher levels of p27 compared to the other analyzed lines (Fig. 1a). This correlation is not exclusively due to negative effects of the tumor suppressor tuberin on cell proliferation commonly associated with elevated p27 levels, that can be concluded from the cyclin D1 protein level data. The expression levels of cyclin D1, a positive marker for cell proliferation (Musgrove 2006), did not negatively correlate with the levels of tuberin and p27 (Fig. 1a).

Tuberin negatively regulates mTOR via its role as a GTPase activating protein against Rheb (Corradetti and Guan 2006; Guertin and Sabatini 2007; see also "Introduction"). Accordingly, one would expect cells expressing high levels of endogenous tuberin to harbor low mTOR activity. Representative for active mTOR, we analyzed phospho mTOR (Ser2448) and densitometrically normalized the levels to the according western blot bands of total mTOR protein (Fig. 1a, b). In agreement with the assumptions described above, we found that the three normal cell lines exhibiting high levels of endogenous tuberin also show low levels of phosphorylated mTOR. Analyzing the tumor derived cells also revealed a clear trend that cells expressing low tuberin levels also have high endogenous amounts of phosphorylated mTOR. In addition, within the transformed cell panel MCF-7 cells exhibit the highest level of tuberin accompanied by the lowest level of phospho mTOR. The only real exception of this rule is the cell line HT-1080, with low amounts of both, phospho mTOR and tuberin levels (Fig. 1a, b).

Here, it is important to note that phospho mTOR Ser 2448 (this phosphorylation is under the control of tuberin)

represents activated mTOR, but that full activation of this kinase depends on several other factors. Recent advances have identified two structurally and functionally distinct mTOR-containing complexes. mTORC1 contains raptor, mLST8 and PRAS40, whereas mTORC2 consists of mLST8, rictor, sin1 and protor. The only extensively described substrates of mTORC1 are the eukaryotic initiation factor 4E binding protein-1 and the already mentioned p70S6K. Study of mTORC2 is in its infancy, but an important recent advance was the finding that mTORC2 phosphorylates the oncogenic kinase Akt at the stimulatory Ser473 residue. Since p70S6K Thr389 and Akt Ser473 are exclusively phosphorylated by mTORC1 and mTORC2, respectively, the regulation is analyzed to evaluate mTORC1 and mTORC2 activities in vivo (Corradetti and Guan 2006; Guertin and Sabatini 2007; Bhaskar and Hay 2007; Manning and Cantley 2007).

As already described, one major function of mTORC1 is to phosphorylate and activate p70S6K leading to the activation of the ribosomal protein S6 via phosphorylation at Ser240/244 (Bhaskar and Hay 2007; Manning and Cantley 2007; see also "Introduction"). With our additional analysis of phospho S6 Ser 240/244 (normalized to total S6 protein levels), we just wanted to test whether the levels of phospho mTOR Ser2448 and of phospho S6 Ser 240/244 correlate. We clearly found this not to be the case (Fig. 1a, c), providing further support for the widely accepted notion that mTOR Ser2448 phosphorylation is only one of several mechanisms regulating mTOR activity toward its downstream targets.

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