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Cytoplasmic/nuclear localization of tuberin in different cell lines

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Summary. Tuberous sclerosis (TSC) is an autosomal dominantly inherited disease affecting 1 in 6000 individuals. The TSC gene products, hamartin and tuberin, form a complex, of which tuberin is assumed to be the functional component being involved in a wide variety of different cellular processes. Tuberin has been demonstrated to be localized to both, the cytoplasm and the nucleus. The cytoplasmic/nuclear localization of tuberin is known to be regulated by the serine/threonine protein kinase Akt. Akt also regulates the cytoplasmic/nuclear localization of the cyclin-dependent kinase inhibitor p27. In this study the localization of these two Akt-regulated proteins was analysed in different cell lines.

Keywords: Tuberin – p27 – Localization – Nucleus – Cytoplasm

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant tumor syndrome that affects ~1 in 6000 individuals. It 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 (Astrinidis and Henske, 2005). The tumor suppressor gene TSC1 encodes hamartin (The TSC1 Consortium, 1997) and TSC2 encodes tuberin (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993). 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. Inactivation of TSC1 and TSC2 causes a similar phenotype and tuberin and hamartin form a heterodimer, of which tuberin is assumed to be the functional component (Pan et al., 2004; Astrinidis and Henske, 2005).

Tuberin 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). 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. The mTOR signaling network has a central role in the regulation of cell growth in response to growth factors, cellular energy and nutrient levels. Tuberin is phosphorylated by several kinases, including the AMP-activated protein kinase (AMPK), Akt, extracellular signal-regulated kinase (ERK) and RSK, which regulate its activity (Pan et al., 2004; Corradetti and Guan, 2006; Wullschleger et al., 2006).

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 prorgession (reviewed in Blain et al., 2003; Nho and Sheaff, 2003). 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., 2006), 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. 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. Furthermore,

in tuberin negative cells nuclear p27 is delocalized into the cytoplasm (Soucek et al., 1997, 1998b; Miloloza et al., 2000; Rosner and Hengstschläger, 2004; Rosner et al., manuscript in preparation).

We recently reported that high amounts of ectopic activated Akt increase the phosphorylation of tuberin S939 and T1462 and downregulate nuclear localization of endogenous tuberin as well as of ectopic tuberin. Downregulation of phosphorylation of tuberin via a dominant negative Akt mutant mediated the opposite effects on the localization of endogenous tuberin. A TSC2 mutant, which cannot be phosphorylated on S939 and T1462, showed increased nuclear localization. Taken together, these findings demonstrated that tuberin is localized to both, the nucleus and the cytoplasm, and that Akt phosphorylation of tuberin induces cytoplasmic tuberin localization (Rosner et al., 2007). In this report we now present the cytoplasmic/nuclear distribution of tuberin in cell lines, which have repeatedly been used to study mammalian TSC gene functions in the past.

Materials and methods

Cells, cell culture

HEK293 cells (human embryonic kidney), HeLa cells (human cervical carcinoma), NIH3T3 cells (mouse fibroblasts) and Rat1 cells (rat fibroblasts) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulphate). The cultures were kept at 37° C and 5% CO_2 and routinely screened for mycoplasma.

Nuclear and cytoplasmic fractionation

Adherent cells were washed twice with PBS, collected by scraping and pelleted by centrifugation. Cell pellets were lysed in 5 packed cell volume buffer F1 containing 20 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchlorid, 10 μg/ml trypsininhibitor) for 2 min at room temperature and subsequent incubation on ice for 10 min. Thereafter NP-40 was added at a final concentration of 1% (v/v) and lysates were homogenized by passing through a 20-gauge needle for three times. Nuclei were pelleted by centrifugation at 600 *g* for 5 min at 4 °C and supernatant containing cytoplasmic proteins was collected and stored at −80 °C. Remaining nuclei were washed three times in buffer F1 containing 1% NP-40. During the last wash nuclei were stained with trypan blue and microscopically examined for number, purity and integrity. The nucleic pellets were lysed in buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, supplemented with protease inhibitors (see above) by repeated freezing and thawing. Supernatants containing soluble nucleic proteins were collected by centrifugation at 25000 *g* for 20 min and stored at −80 °C (Rosner et al., 2007).

Immunoblotting

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 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 benzamidinchlorid, 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 and Hengstschläger, 2004). For immunodetection antibodies specific for the following proteins were used: tuberin (C-20, Santa Cruz), p27 (57, Transduction Laboratories), α-tubulin (Ab-1, Calbiochem), topoisomerase IIβ Transduction Laboratories), c-jun (H-79, Santa Cruz). Signals were detected with appropriate HRP-conjugated secondary antibodies and the enhanced chemiluminescence method.

Results and discussion

Western blot analyses of total protein lysates of the different cell lines demonstrated that the logarithmically growing human cell lines, HEK293 and HeLa, and the rodent cell lines, NIH3T3 and Rat-1, express relatively comparable levels of endogenous tuberin. 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). Accordingly, we also analysed the protein levels of p27 in total lysates of these cell lines. The two rodent cell lines, NIH3T3 and Rat-1, express high amounts of endogenous p27. Compared to these rodent cell lines HEK293 cells express significantly lower levels of p27 protein. In the human cervix carcinoma cell line HeLa we detected the lowest p27 expression level. Whereas the tuberin protein size is comparable in all cell lines, it is known that human, mouse and rat p27 are of different size (Fig. 1A). Two aspects are important for the interpretation of these data: 1) From a wide variety of investigations in the past we know that the here used antibodies specific for tuberin and for p27 detect human and rodent proteins with very comparable efficiencies (Soucek et al., 1997, 1998b; Rosner et al., 2003; Rosner and Hengstschläger, 2004). 2) Although tuberin is well known to be a major regulator of p27 activity (Soucek et al., 1998b; Rosner and Hengstschläger, 2004), correlations between tuberin protein amounts and p27 protein amounts can not necessarily be expected. The amounts of tuberin proteins do not represent p27-regulating tuberin activity, because tuberin is activated and inactivated via a wide variety of different phosphorylation events (Pan et al., 2004; Astrinidis and Henske, 2005; Corradetti and Guan, 2006). In addition, tuberin is not the only regulator of p27. For example, the p27 degradation cascade could be differently active in these cells (Blain et al., 2003; Nho and Sheaff, 2003).

The major aim of this study was to investigate the cytoplasmic/nuclear localization of tuberin in different

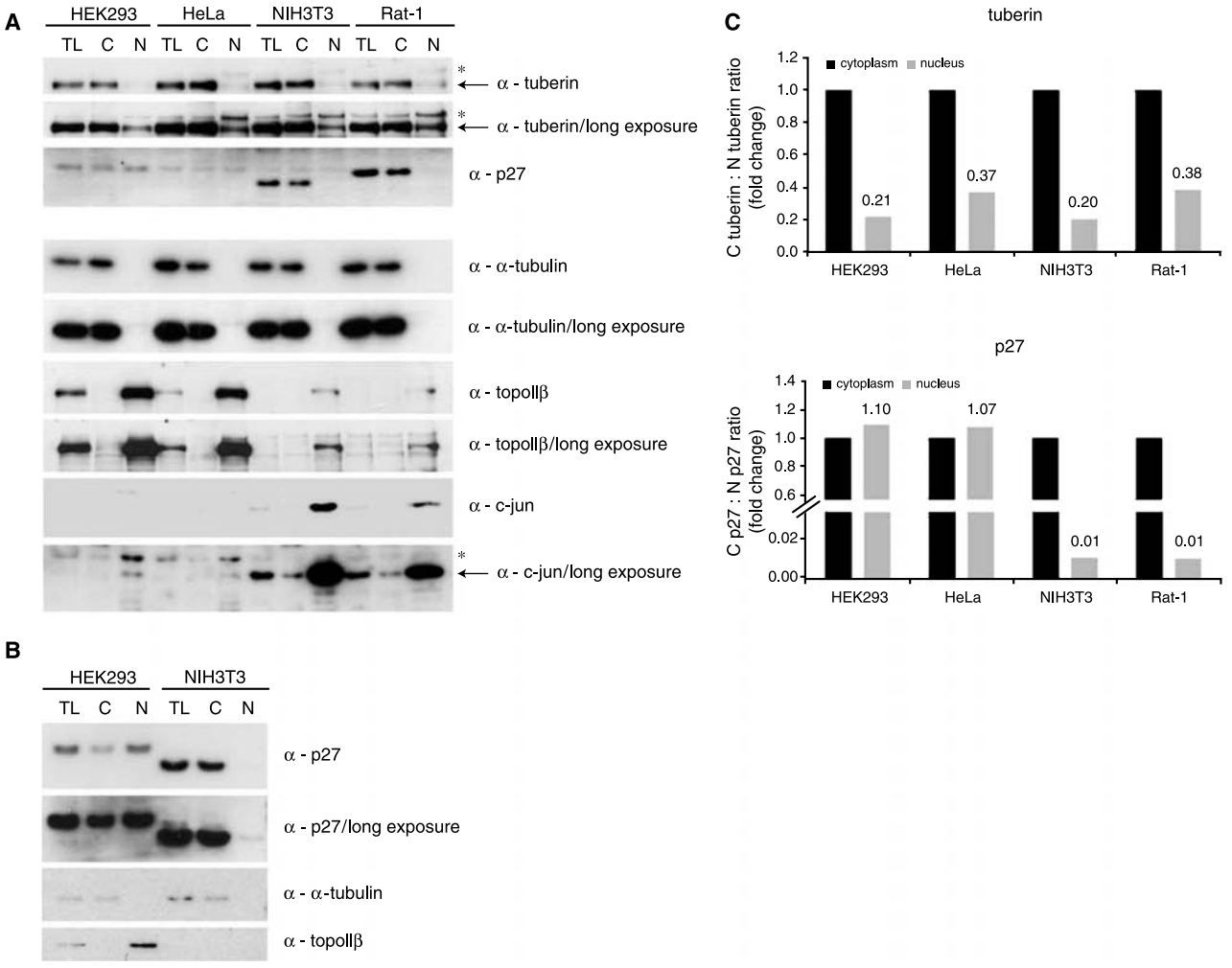

Fig. 1. Tuberin localization in different cell lines. **A** Protein extracts of total lysates (*TL*), cytoplasmic (*C*) and nuclear (*N*) fractions of logarithmically growing HEK293, HeLa, NIH3T3 and Rat-1 cells were analysed for tuberin and p27 protein levels via Western blotting. Shorter and longer exposures are presented to allow a better visualization of the different nuclear and cytoplasmic protein levels (the asterisks indicate unspecific bands). Purity of fractions was proven by co-analysing α-tubulin (cytoplasmic), topoisomerase IIβ (nuclear) and c-jun (nuclear). The anti-topoisomerase IIβ antibody recognizes human proteins with higher affinity, the anti-c-jun antibody has higher affinity to rodent proteins. **B** The experiments described in **A** were repeated using experimental conditions allowing a better visualization and comparison of nuclear p27 protein levels. **C** The Western blot results were densitometrically analysed. The nuclear signals are presented in relation to the cytoplasmic signals set to 1

cell lines. We recently described that tuberin is localized to both, the nucleus and the cytoplasm, and that Akt phosphorylation of tuberin induces cytoplasmic tuberin localization (Rosner et al., 2007). Here we wanted to further investigate two questions: 1) Is tuberin a cytoplasmic and nuclear protein in different cells, or is it exclusively cytoplasmic in one cell line and exclusively nuclear in another cell line? 2) Given tuberin can be found in both compartments in different cell lines, how is the distribution between nuclear and cytoplasmic tuberin in these lines?

To investigate these issues we biochemically isolated extracts enriched either for nuclear proteins or for cytoplasmic proteins from all four cell lines. The purity of the obtained nuclear and cytoplasmic protein extracts was investigated by analysing the expression of α-tubulin (cytoplasmic), topoisomerase IIβ (nuclear) and c-jun (nuclear). As nuclear markers we analysed both, topoisomerase IIβ and c-jun, because the anti-topoisomerase IIβ antibody recognizes human proteins with higher affinity and the anti-c-jun antibody has higher affinity to rodent proteins (Fig. 1A). These analyses revealed that the obtained biochemical fractionations were clearly of the quality to allow the investigation of cytoplasmic and nuclear protein distributions.

By Western blot analyses we found the levels of cytoplasmic tuberin to be much higher than those of nuclear

origin. To allow a better visualization of the different nuclear and cytoplasmic protein distributions longer and shorter exposures are presented (Fig. 1). These data clearly demonstrate that tuberin is predominantly cytoplasmic in all cells tested, independent whether the cell is of human or of rodent origin and also independent whether a fibroblast, a kidney cell or a transformed cervix carcinoma cell is tested. HEK293 cells and NIH3T3 cells express slightly lower amounts of nuclear tuberin compared to HeLa cells and Rat-1 cells. This distribution did not correlate with the total tuberin protein amounts in these cells. Rat-1 and HEK293 cells harbor slightly lower total tuberin compared to HeLa and NIH3T3 cells (Fig. 1).

To allow comparison of tuberin's cytoplasmic/nuclear protein distributions with the distribution of another cytoplasmic/nuclear protein we co-analysed p27. However, in contrast to tuberin the p27 protein distribution varied from cell line to cell line. Whereas in HEK293 and HeLa cells cytoplasmic and nuclear p27 protein amounts were very comparable, we found p27 to be predominantly cytoplasmic in NIH3T3 and Rat-1 cells. In fact, in the latter nuclear p27 could only be detected on very long exposures (compare Fig. 1A and B). We found a correlation between high amounts of total p27 protein and the predominant cytoplasmic localization of this CDK inhibitor. Further investigations are warranted to test, whether this holds true in other cell lines.

Taken together, our here reported findings demonstrate that tuberin and p27 are differently distributed between the cytoplasm and the nucleus of different cell lines. This is of particular interest, since the nuclear/cytoplasmic localization of both proteins has recently been shown to be regulated by Akt. The serine/threonine protein kinase Akt plays a role in the control of cell cycle, cell growth, apoptosis and cell energy metabolism. Hyperactivation of Akt is a hallmark of a wide variety of different human cancers. Akt can potentially phosphorylate over 9000 substrates in mammalian cells including typical cytoplasmic as well as nuclear proteins (Furuya et al., 2006; Martelli et al., 2006; Wullschleger et al., 2006). Phosphorylation by Akt has also been shown to be able to regulate protein localization via affecting either nuclear import or nuclear export. For example, Akt has been demonstrated to regulate the nuclear/cytoplasmic localization of the cyclin-dependent kinase inhibitor p27 (Fujita et al., 2002; Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002). Tuberin 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 and Hengstschläger, 2004). Beside typical cytoplasmic proteins, such as e.g. all isoforms of the mammalian 14-3-3 protein family, tuberin also was shown to bind to nuclear proteins, such as e.g. the cell cycle molecules cyclin B1 or cyclin-dependent kinase 1 (Wienecke et al., 1996; Lou et al., 2001; Rosner and Hengstschläger, 2004; Astrinidis and Henske, 2005; Corradetti and Guan, 2006). Accordingly, it was interesting to investigate the cytoplasmic/nuclear localization of tuberin and the role of Akt phosphorylation in tuberin localization. Very recently, we reported that ectopic activated Akt increase the phosphorylation of tuberin and downregulate its nuclear localization. Downregulation of phosphorylation of tuberin via a dominant negative Akt mutant mediated the opposite effects. These findings demonstrated Akt phosphorylation to trigger cytoplasmic tuberin localization (Rosner et al., 2007).

In this report we describe for the first time that although the cytoplasmic/nuclear localization of both, tuberin and p27, is regulated by the same kinase, Akt, there are significant differences regarding their cytoplasmic/nuclear distributions in different cell lines. These findings allow the conclusion that Akt can affect the localization of different substrates differently in the same cell.

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