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Tuberin negatively affects BCL-2's cell survival function

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Summary. Uncontrolled cell cycle progression and cell growth are key properties of tumor cells. The tumor suppressor genes responsible for the autosomal dominantly inherited disease tuberous sclerosis (TSC) have been demonstrated to control both, cell cycle and cell size regulation. Hamartin, encoded by TSC1, and tuberin, encoded by TSC2, form a complex, of which tuberin is assumed to be the functional component. Loss of TSC genes function triggers hamartoma development in TSC patients. However, in vivo mostly tumor cell development is rapidly terminated via apoptosis. BCL-2, the founding member of the BCL-2 family of proteins, is well known for its anti-apoptotic properties. Here we show that pro-apoptotic actinomycin D cannot interfere with BCL-2's cell survival functions. However, we found tuberin to negatively regulate BCL-2's anti-apoptotic effects on low serum-induced apoptosis. These findings warrant further investigations to elucidate the molecular mechanism underlying tuberin's negative effects on cell survival.

Keywords: Tuberous sclerosis – Tuberin – Hamartin – Cell survival – BCL-2

Introduction

A mutation in a tumor suppressor gene is analogous to a dysfunctional brake in an automobile – the car doesn't stop even when the driver attempts to engage it. Tumor suppressor gene mutations drive the neoplastic process by increasing tumor cell number through the stimulation of cell birth or the inhibition of cell death or cell-cycle arrest (Vogelstein and Kinzler, 2004). A tumor suppressor could mediate its anti-neoplastic effects by negatively regulating cell cycle progression, cell growth and/or cell survival.

In mammalians the ~20 BCL-2 family members fall into at least three interacting groups. All share at least one of four relatively conserved BCL-2 homology domains. The founding member BCL-2 and its close homologs BCL-X_L, BCL-w, A1 and Mcl-1 inhibit apoptosis, while other members of the BCL-2 family, such as e.g. BAD or BAX, are pro-apoptotic. BAD heterodimerizes with BCL-2 and blocks its cell survival function. BCL-2 expression increases the propensity for tumorigenesis. Extensive studies have established that the cell survival function of BCL-2 plays an important role in oncogenesis (Zha et al., 1996; Adams, 2003; Danial and Korsmeyer, 2004).

Tuberous sclerosis complex (TSC) is a common genetic disorder in which affected individuals develop mental retardation, developmental brain defects and seizures. This autosomal dominant tumor syndrome affects ~1 in 6,000 individuals. The severity of TSC and its impact on the quality of life is extremely variable. A variety of tumors, named hamartomas, characteristically occur in different organs of TSC patients, such as kidney, heart, skin and brain (Kwiatkowski, 2003). Two genes have been shown to be responsible for TSC: the tumor suppressor gene TSC1 on chromosome 9q34 encodes hamartin (The TSC1 Consortium, 1997) and TSC2 on chromosome 16p13.3 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, such as hamartomas, giant cell astrocytomas or renal carcinomas. Tumor development is assumed to be the result of such somatic ''second hit'' mutations according to Knudson's tumor suppressor model. Progression of TSC hamartomas to malignancy is very rare. Inactivation of TSC1 and TSC2 causes a similar phenotype and tuberin and hamartin form a heterodimer, suggesting they might affect the same processes. Hamartin can bind tuberin and, thereby, prevent tuberin

ubiquitination and degradation. Tuberin is assumed to be the functional component of the complex and has been implicated in the regulation of different cellular functions, such as endocytosis (Xiao et al., 1997) or transcription (Henry et al., 1998). A major function of the TSC1/TSC2 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) signalling. The TSC1/TSC2 complex antagonizes the mTOR signalling network – which has a central role in the regulation of cell growth in response to growth factors, cellular energy and nutrient levels (Li et al., 2003; Pan et al., 2004).

Originally, work on mammalian cells has implicated the TSC proteins in cell cycle regulation. In the mammalian cell cycle, the transition from the G0/G1 phase to S phase, in which DNA replication occurs, has been shown to be regulated by cdks. Mitogen-dependent D-type cyclins are expressed first during early G1. They associate with cdk4 or cdk6 to form an active kinase complex that phosphorylates the retinoblastoma protein and thereby activates E2F transcriptional activity. Among the genes activated via members of the E2F protein family are cyclin E, which complexed with cdk2 promotes the G1/S transition by phosphorylation of different substrates, and cyclin A, which activates cdk2 to further initiate DNA replication. The transition from G2 to M phase is regulated by cyclin B/cdc2 and cyclinA/cdc2. In addition, two families of cdk inhibitors are known: the INK4 family (p15, p16, p18, and p19) regulating cdk4 and cdk6, and the Cip/Kip family (p21, p27, and p57) inhibiting a broader range of cdks (Sherr, 2000). Antisense inhibition of TSC2 expression induces quiescent fibroblasts to enter the cell cycle and TSC2-negative cells 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 and p27 stability is decreased in tuberin-negative cells (Soucek et al., 1997, 1998; Miloloza et al., 2000). Recently, tuberin was found to bind p27. Tuberin inhibits p27 degradation via sequestering p27 from Skp2. Tuberin also triggers an upregulation of the amount of p27 bound to CDK2 (Rosner and Hengstschläger, 2004).

As described above, a tumor suppressor could mediate its anti-neoplastic effects by negatively regulating cell cycle progression, cell growth and/or cell survival. To its effects on cell cycle and cell growth regulation, we here add the influence on BCL-2-mediated cell survival regulation to the spectrum of tuberin's functions.

Materials and methods

Cells, tissue culture and actinomycin D treatment

Rat 6 (R6) fibroblasts, stably transfected with empty vector control (R6 control cells) or BCL-2 (R6 BCL-2 cells), were kindly provided by Dr. C. Borner (Borner, 1996). The cells 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). Cultures were kept at 37 °C and 5% CO_2 and routinely screened for mycoplasma. Apoptosis was induced by incubation with 40 μM actinomycin D (Sigma) for 16 h.

Transfections

For transfections the following plasmids were used: the empty pcDNA3 vector; pcDNA3 harboring full-length human wild-type TSC1 or TSC2 (already used in Miloloza et al., 2000; Rosner et al., 2004); pcDNA3 harboring full-length BAD (Harada et al., 2001). Cell transfections were performed by the calcium phosphate method as described (Soucek et al., 1997).

Western blot analyses

Protein extracts were prepared in buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 0.5 mM NaF, 0.5 mM Na_3VO_4, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.3 μg/ml benzamidinchloride, 10 μg/ml trypsin inhibitor and 0.5 mM DTT. Cells were lysed by freezing and thawing. After 20 min on ice, the extracts were centrifuged and the supernatants were stored at −70 °C. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay reagent with bovine serum albumin as the standard. Proteins were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the loaded protein. Immunodetection was performed using anti-BCL-2 antibody (N-19, St. Cruz), anti-tuberin antibody (C-20, St. Cruz), anti-hamartin antibody (2197; kindly provided by M. Nellist), or anti-BAD antibody (Cell Signaling). Signals were detected using the enhanced chemiluminescence method (Amersham, Little Chalfont, UK).

Luciferase assays

Cells were transfected as described above and co-transfected with a luciferase reporter plasmid. Cell pellets were lysed in reporter lysis buffer (Promega), and to achieve complete cell lysis, the cell suspensions were immediately frozen at −80 °C and then thawed rapidly. Subsequently the lysates were centrifuged at 12000 *g* for 1 min at room temperature and the supernatant was assayed for luciferase activity. 20 μl of supernatant were mixed with 100 μl luciferase assay reagent (Promega), and luminescence was measured with a luminometer (FLUOstar, BMG Labtechnologies, Germany). The luciferase activity was recorded as relative light units. The reduction of luciferase activity is a consequence of cell death (Harada et al., 2001).

Statistical analyses

The significance of the observed differences was determined by Student's *t*-test (paired, two-tails) using GraphPad INSTAT software. $P > 0.05$ are defined as not significant.

Results

To investigate the role of BCL-2 in this study we made use of Rat 6 fibroblasts, stably transfected with empty vector control (R6 control cells) or with ectopic BCL-2 (R6 BCL-2 cells) (Borner, 1996). Western blot analysis revealed that R6 control cells express BCL-2 protein amounts typical for fibroblasts. The high expression level of the ectopic BCL-2, which is of the same size as endogenous BCL-2, was confirmed by immunoblotting (Fig. 1A).

Treatment with actinomycin D induces apoptosis in fibroblasts (Hengstschläger et al., 1999). Luciferase assays revealed that treatment with actinomycin D for 16 h induced cell death in R6 control cells. The luciferase activity of cells transfected with the luciferase reporter plasmid but not treated with actinomycin D were set to 100%. Upon actinomycin D treatment the amount of luciferase activity was reduced from 100 to <60%. Over 40% of the transfected cells died due to the treatment with actinomycin D (Fig. 1B). Overexpression of BCL-2 in R6 cells totally inhibited actinomycin D-mediated apoptosis. Student's *t* test analyses demonstrated both, the apoptotic effects of actinomycin D and the cell survival effects of BCL-2, to be statistically significant (Fig. 1B). These data demonstrate that the ectopic BCL-2 protein in R6 BCL-2 cells is functional in mediating cell survival effects. Furthermore, these findings allow the conclusion that luciferase assays represent a reproducible experimental approach to detect apoptosis as well as the cell survival effects of BCL-2 in R6 fibroblasts.

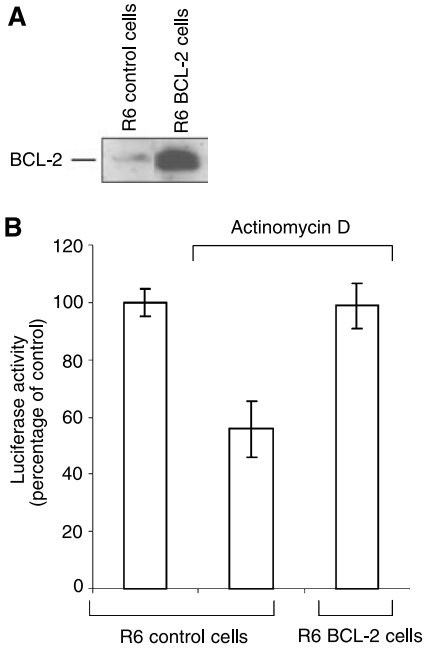

Fig. 1. BCL-2's cell survival effects on actinomycin D-induced apotosis. **A** Rat 6 (R6) fibroblasts, stably transfected with empty vector control or with ectopic BCL-2, were investigated for BCL-2 expression by Western blot analysis. **B** R6 control cells and R6 BCL-2 cells were transfected with a luciferase reporter plasmid and FCS was reduced to 0.1% for 24 h. Luciferase assays revealed that treatment with actinomycin D for 16 h induced cell death in R6 control cells, but not in R6 BCL-2 cells ($P < 0.05$; Student's *t* test)

BAD is known to counteract BCL-2's cell survival functions (see introduction). To test the effects of BAD on BCL-2 mediated cell survival in our here used cell system we ectopically overexpressed BAD. In mouse cells a 23-kDa BAD short protein (BAD_S) and a 28-kDa BAD long protein (BAD_L) can be detected. Most murine tissues express BAD_S, whereas BAD_L levels are more variable. In human cells only the BAD_S protein is detectable (Ranger et al., 2003). The here ectopically overexpressed BAD is the long protein BAD_L. Western blot analyses confirmed the ectopic overexpression of BAD in R6 control cells and in R6 BCL-2 cells (Fig. 2A, B). Luciferase assays after transient overexpression of BAD revealed that (after 24 h in 0.1% FCS) BAD can induce similar effects in both, R6 cells and R6 BCL-2 cells (compare Fig. 2C, D). These data demonstrate that under these experimental conditions ectopic BAD overcomes/titrates the anti-apoptotic function of BCL-2. This is in perfect agreement with the knowledge that BAD heterodimerizes with BCL-2 and blocks its cell survival function (Zha et al., 1996; Adams, 2003; Danial and Korsmeyer, 2004).

We next wanted to investigate whether the TSC tumor suppressor proteins tuberin and/or hamartin also negatively regulate BCL-2's cell survival effects. Ectopic overexpression of tuberin and hamartin in R6 control cells and in R6 BCL-2 cells was confirmed by Western blot analyses (Fig. 2A, B). Luciferase assays demonstrated that indeed transient overexpression of TSC1 or TSC2 also negatively regulates BCL-2's cell survival effects (compare Fig. 2C, D). This finding cannot be explained by different overexpression levels of tuberin or hamartin in R6 control cells compared to R6 BCL-2 cells. In fact, we found tuberin and hamartin to inhibit BCL-2's cell survival effects in R6 BCL-2 cells although these proteins were lower overexpressed in these cells compare to R6 control cells (compare Fig. 2A with Fig. 2B). Taken together, these data show that the TSC proteins harbor the potential to interfere with BCL-2's cell survival effects comparable to the well known apoptotic molecule BAD.

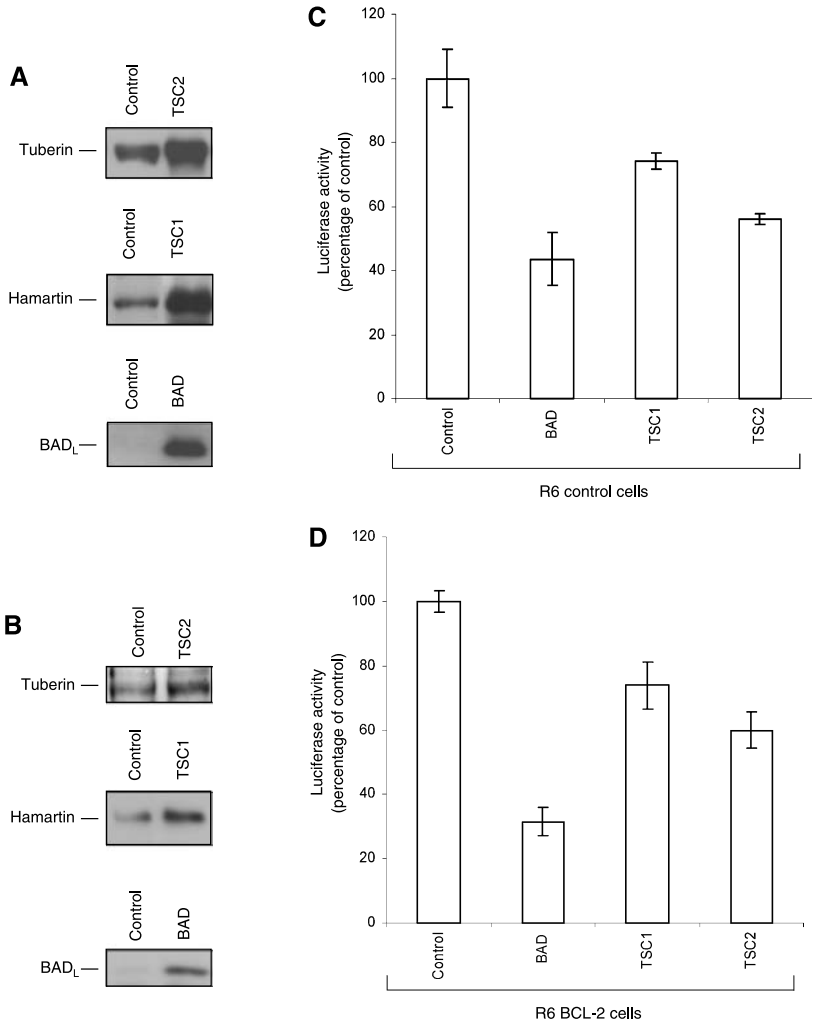

Fig. 2. Tuberin negatively affects BCL-2's cell survival function. **A** R6 control cells were transiently transfected with TSC2, TSC1, or BAD. Immunoblot analyses of tuberin, hamartin and BAD were performed. **B** R6 BCL-2 cells were transiently transfected with TSC2, TSC1, or BAD. Immunoblot analyses of tuberin, hamartin and BAD were performed. R6 control cells (**C**) and R6 BCL-2 cells (**D**) were transiently transfected with BAD, TSC1, or TSC2 as described above. Cells were co-transfected with a luciferase reporter plasmid. After 24 h in 0.1% FCS the viability of the cells was analysed via luciferase activity. In both, R6 control cells and R6 BCL-2 cells, cell death significantly increased upon overexpression of BAD, TSC1, or TSC2 compared with cells transfected with the control vector, set as 100% ($P < 0.05$; Student's t test)

Discussion

Tuberin has been demonstrated to negatively control cell cycle progression and cell size control (see Introduction). Deregulation of cell cycle and/or cell size control is a hallmark of tumor cell development. However, under physiological conditions, neoplastic cell development should rapidly be terminated via apoptosis. Survival and further progression of only rare neoplastic cells is allowed by defects in the regulation of apoptosis. Accordingly, a tumor suppressor could mediate its anti-neoplastic effects by negatively regulating cell cycle progression, cell growth and/or cell survival. Disease causing mutations within a tumor suppressor gene could trigger increased cell cycle progression and cell size regulation and could lead to a block of the apoptotic machinery, which is necessary to terminate the development of cells with neoplastic features.

The here presented finding that tuberin negatively affects BCL-2's cell survival function makes it tempting to speculate that loss of this function via natural occurring disease causing mutations within TSC2 may underlie the development of inappropriate growths (hamartomas) – the hallmark of the disease TSC (Kwiatkowski, 2003).

However, phosphoryation of tuberin by the AMP-activated protein kinase (AMPK) has been demonstrated to protect cells from energy deprivation-induced apoptosis. Recently, tuberin has been shown to be regulated by cellular energy levels and to play an essential role in the cellular energy response pathway. Under energy starvation conditions, AMPK phophorylates tuberin. This phosphorylation by AMPK activates tuberin and is required for the regulation of cell size control in response to energy deprivation (Inoki et al., 2003).

Recent data also suggest that inappropriate activation of the Rheb/mTOR/p70S6K cassette imposes a negative feedback program to attenuate insulin receptor sub-

strates (IRSs) – dependent processes such as cell survival. The Rheb/mTOR/p70S6K pathway induces depletion of IRS1 and IRS2. Overactivation of this cassette, whether by genetic inactivation of TSC1 or TSC2 or by ectopic high levels of Rheb, is sufficient to induce insulin resistance (Shah et al., 2004).

On the other hand, since tuberin is known to be involved in the regulation of p70S6K, this tumor suppressor could be speculated to negatively affect cell survival. Growth factors activate AKT to phosphorylate and inhibit tuberin's functions leading to upregulation of p70S6K activity (Li et al., 2003; Pan et al., 2004). The activities of AKT and p70S6K promote cell survival (Franke et al., 2003). For example, rapamycin, which is known to block p70S6K activity via affecting mTOR, was found to block cell survival induced by insulin-like growth factor-1 (Harada et al., 2001). Tuberin also blocks p70S6K activity via regulating mTOR (Li et al., 2003; Pan et al., 2004) and could therefore also (analogous to rapamycin) mediate negative effects on cell survival regulation. In fact recent findings of our laboratory support this notion. We found that tuberin harbors the potential to activate BAD (Freilinger et al., 2006).

In fact, first preliminary evidence that tuberin could negatively regulate cell survival was provided by overexpression experiments in NIH3T3 cells. Cells transfected with the empty vector control or with a TSC2 expression plasmid were processed for in situ end-labeling reactivity, which provides a sensitive assay for DNA degradation. Quantitative analysis of the cultures revealed that cells over-expressing TSC2 were prone to apoptosis (Pasumarthi et al., 2000).

In addition, very recently reported data also provide evidence that tuberin might mediate negative effects on cell survival regulation. Ectopic TSC2 expression was found to increase the susceptibility of the TSC2-null renal tumor cell line ERC-18 to apoptosis induced by okadaic acid and the phosphatidylinositol 3-kinase inhibitor, LY294002. Although the precise mechanism by which TSC2 promotes apoptotic susceptibility to these two compounds remains unclear, the authors speculated that alterations in the mTOR signalling pathway are not required (Kolb et al., 2005).

Taken together all the findings on the role of TSC2 in cell survival regulation described above suggest that depending on the exogenous triggers of apoptosis tuberin might fulfil different functions.

We here report for the first time that tuberin negatively affects BCL-2's cell survival function. We found ectopic tuberin expression to mediate negative effects on BCL-2's anti-apoptotic function in low serum-induced apoptosis. Interestingly, we also found TSC1 to harbour comparable potentials as TSC2. Since for most of the described functions of the TSC1/TSC2 complex tuberin has been shown to be the functional component, one could speculate that hamartin triggers these effects indirectly by regulating tuberin. Hamartin can bind tuberin and, thereby, prevent tuberin ubiquitination and degradation (Li et al., 2003; Pan et al., 2004). To test this and other hypotheses it is essential to further elucidate the molecular mechanism how tuberin mediates its negative effects on cell survival. This study warrants further investigations into this direction.

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