Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2

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Tubersclerosis is an autosomal dominant disorder characterized by the development of aberrant growths in many tissues and organs. Linkage analysis revealed two disease-determining genes on chromosome 9 and chromosome 16. The tuberous sclerosis complex-2 gene (TSC2) on chromosome 16 encodes the tumor suppressor protein tuberin. We have shown earlier that loss of TSC2 is sufficient to induce quiescent cells to enter the cell cycle. Here we show that TSC2-negative fibroblasts exhibit a shortened G1 phase. Although the expression of cyclinE, cyclinA, p21, or Cdc25A is unaffected, TSC2-negative cells express much lower amounts of the cyclin-dependent kinase (CDK) inhibitor p27 because of decreased protein stability. In TSC2 mutant cells the amount of p27 bound to CDK2 is diminished, accompanied with elevated kinase activity. Ectopic expression studies revealed that the aforementioned effects can be reverted by transfecting TSC2 in TSC2-negative cells. High ectopic levels of p27 have cell cycle inhibitory effects in TSC2-positive cells but not in TSC2-negative counterparts, although the latter still depend on CDK2 activity. Loss of TSC2 induces soft agar growth of fibroblasts, a process that cannot be inhibited by high levels of p27. Both phenotypes of TSC2-negative cells, their resistance to the activity of ectopic p27, and the instability of endogenous p27, could be explained by our observation that the nucleoprotein p27 is mislocated into the cytoplasm upon loss of TSC2. These findings provide insights into the molecular mechanism of how loss of TSC2 induces cell cycle entry and allow a better understanding of its tumor suppressor function.

MATERIALS AND METHODS

Cells, Cell Culture, Flow Cytometry, and Centrifugal Elutriation. EEF4 (TSC2-positive) and EEF8 (TSC2-negative) cells were derived from Eker rat embryos homozygous for the wild-type and the Eker-mutant TSC2 gene, respectively. Whole embryos were removed on day 10.5 before in utero deaths of the Eker homozygous mutants have occurred. Sam-

Abbreviations: TSC: tuberous sclerosis complex; CDK, cyclin-dependent kinase; GFP, green fluorescence protein.

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cultures were kept at 37°C and 7% CO2 and routinely screened. All cells were grown either in DMEM or RPMI 1640 medium, both supplemented with 10% calf serum and antibiotics (30 mg/liter of penicillin, 50 mg/liter of streptomycin sulfate). All cultures were kept at 37°C and 7% CO2 and routinely screened for mycoplasma.

For cytofluorometric analyses, cells were harvested by trypsinization and fixed by rapid submersion in 5 ml of ice-cold 85% ethanol. After at least 1-hr fixation at -20°C, cells were pelleted and stained in 1 ml of staining solution (0.25 mg/ml propidium iodide/0.05 mg/ml RNase/0.1% Triton X-100 in citrate buffer, pH 7.8). Stained cells were analyzed on a Becton-Dickinson FACScan. Separation of logaritmically growing cells into distinct cell cycle phases was accomplished by centrifugal elutriation as described (32).

Western Blot Analysis, Immunoprecipitation, and Immunocomplex Kinase Assays. 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 phenylmethylsulfonyl fluoride, 0.5 mM NaF, 0.5 mM Na3VO4, 0.02 μg/ml leupeptin, 0.02 μg/ml aprotinin, 0.003 μg/ml benzamidinchloride, 0.1 μg/ml trypsin inhibitor, and 0.5 mM DTT. Cells were lysed by freeze and thaw. After 20 min on ice, the extracts were centrifuged, and supernatants were stored at -70°C. Protein concentrations were determined by using the Bio-Rad protein assay reagent with BSA as a standard. A total of 100 μg of protein was run on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to confirm equal loaded amounts of protein. Immunodetection was performed by using specific antibodies, and the signals were developed by using the enhanced chemiluminescence method (Amersham). Immunoprecipitations and analyses of CDK activities by using glutathione S-transferase-retinoblastoma protein or histone H1 as substrate, respectively. Immunoprecipitates (IP) performed with antituberin or anti-CDK2 antibodies were investigated for p27, cyclin E, and cyclin A-associated kinase activities by using glutathione S-transferase-retinoblastoma protein or histone H1 as substrate, respectively.

RESULTS

Loss of TSC2 Affects Cell Cycle Regulation. To explore potential effects of loss of TSC2 on cell cycle regulation, we generated immortalized cell lines derived from Eker rat embryos homozygous for the wild-type and the Eker-mutant TSC2 gene, respectively. Exponentially growing EEF4 cells (TSC2-positive) and their TSC2-negative counterparts (EEF8) were cytofluorometrically analyzed for DNA content. Both cell types showed a cell cycle distribution characteristic of growing cells. Quantitation of multiple experiments showed that loss of TSC2 caused a decrease in the number of G1 cells (from 67% ± 4% to 56% ± 3%) with a concomitant increase in the number of S phase cells (Fig. 1A). In parallel, determination of the cell doubling time by cell counting showed a reduction in the doubling time upon loss of TSC2 of 4.1 ± 0.3 hr mainly because of a shortened G1 phase (Fig. 1B). Next, we asked whether loss of TSC2 triggers deregulation of cell cycle-regulating molecules. Western blot analyses revealed 10% FCS and assayed for focus formation at different time points.

Immunocytochemistry. For immunocytochemical detection of p27, EEF4 and EEF8 cells were grown on glass coverslips and fixed in cold methanol/acetone (1:1). Cells were incubated with anti-p27 antibody overnight at 4°C. Thereafter cells were washed, incubated with a biotinylated secondary antibody, washed again, and incubated with fluorescein isothiocyanate-conjugated streptavidin (details of the method are described in ref. 18).

Soft Agar Growth Assay. Cells (100) were mixed with 0.8% agar and poured onto a bed of 1.4% agar in culture plates. Both top and bottom agar were prepared in DMEM/10% fetal calf serum (FCS). Cells were fed every second day with DMEM/
that the expression of cyclin E, cyclin A, and p21 was unaffected and that the CDK-activating phosphatase Cdc25A was even slightly down-regulated in TSC2-negative cells. Loss of TSC2 caused a remarkable down-regulation of endogenous p27 levels (Fig. 1C). Analyses of cyclin E- and cyclin A-associated CDK2 activities showed that this down-regulation of the CDK-inhibitor p27 was accompanied by induced CDK2 activity. CDK4-associated kinase activity was not affected by loss of TSC2 (Fig. 1D). p27 inhibits CDK2 activity by directly binding the kinase. Accordingly, the amount of p27 bound to CDK2 reflects the pool of inactive CDK2 in the cells (36). Western blot analysis of CDK2 immunoprecipitates revealed that the amount of p27 bound to the kinase decreases upon loss of TSC2. The levels of CDK2-bound cyclin E or cyclin A and of CDK4-bound p27 were not affected upon loss of TSC2 (Fig. 1E). These data suggest that induction of CDK2 activity in TSC2-negative cells is a result of deregulated p27 expression. In addition, the results showed that under the conditions of these experiments tuberin does not bind to CDK2, cyclin E, cyclin A, or p27. (Fig. 1E).

Abundance of p27 Depends on TSC2. To exclude the possibility that the effects of TSC2 on cell cycle distribution and on p27 expression were caused by clonal effects of EEF4 and EEF8 cells, we transfected EEF8 cells with TSC2. Ectopic overexpression of TSC2 in TSC2-negative cells reverted the aforementioned effects, resulting in elongated G1 phase and up-regulated p27 levels (Fig. 2A). To further confirm that secondary, undefined changes associated with cell immortalization are not necessary for TSC2-negative EEF8 cells to exhibit altered cell cycle control we examined early passages of primary embryonic fibroblasts derived from TSC2-positive (EEF-TSC2+/+) or TSC2-negative (TSC2−/−) Eker rats. Also in these primary cells loss of tuberin triggered a decrease in G1 cells and down-regulation of p27 expression (Fig. 2A). These data allow the conclusion that the differences in cell cycle regulation between EEF4 and EEF8 cells are caused by the loss of TSC2. To determine whether altered levels of TSC2 lead to deregulation of cell cycle distribution and p27 expression in other cells, we transfected Rat1 immortalized fibroblasts and SKNSH human neuroblastoma cells with TSC2. In both cellular systems high levels of TSC2 triggered an elongation of the G1 phase and up-regulation of p27 expression (Fig. 2B). We recently have established the conditions to specifically down-regulate TSC2 in Rat1 cells via antisense oligonucleotides (18, 30). Now we found that TSC2-antisense treatment resulted in a shortened G1 phase and decreased levels of p27 (Fig. 2B). Taken together, these data demonstrate that cell cycle distribution and p27 expression depend on TSC2 expression in different cells.

**TSC2 Affects the Regulation of p27 Stability.** It had been shown earlier that the abundance of p27 is mainly controlled by regulation of protein stability. It has been reported that the half-life of p27 is long in G0/G1 cells and sharply decreases when cells enter S phase. This cell cycle regulation of p27 has been suggested to occur via the ubiquitin-proteasome pathway and, compared with S phase cells, G0/G1 cells contain a far lower amount of p27 ubiquitinating activity (36). If p27 stability is affected in TSC2-negative cells, this observation would predict that the elimination of p27 during the G1/S transition would be deregulated in these cells and that the half-life of p27 is shortened upon loss of TSC2. We first separated EEF4 and EEF8 cells according to their different cell cycle phases by centrifugal elutriation and analyzed the fractions representative for the G1/S transition for p27 expression. This experiment revealed that in TSC2-negative cells p27 is degraded earlier compared with their TSC2-positive counterparts (Fig. 3A). We next inhibited translation by cycloheximide treatment of EEF4 and EEF8 cells and analyzed degradation of p27. In agreement with earlier reports on the

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**Fig. 2.** p27 protein levels depend on the TSC2 status. (A) TSC2-negative cells (EEF8) were transfected with the empty control vector or with an expression vector containing TSC2 cDNA. After 14 days of antibiotic selection, cells were analyzed for DNA distribution on the flow cytometer and for tuberin and p27 protein expression by Western blotting. Early passages of primary embryonic fibroblasts derived from TSC2-positive (EEF-TSC2+/+) or TSC2-negative (TSC2−/−) Eker rats were analyzed for DNA distribution and tuberin and p27 expression. (B) Rat1 immortalized fibroblasts and SKNSH human neuroblastoma cells were transfected with the empty control vector or with an expression vector containing TSC2 cDNA. After 14 days of antibiotic selection cells were analyzed for DNA distribution and for tuberin and p27 protein expression. Rat1 cells also were treated with TSC2 antisense oligonucleotides for 24 hr and analyzed as described above.

**Fig. 3.** TSC2 affects the regulation of p27 stability. (A) Logarithmically growing EEF4 and EEF8 cells were separated according to the different cell cycle phases by centrifugal elutriation. The obtained cell fractions were cytofluorometrically analyzed for DNA distribution after staining DNA with propidium iodide (Upper). Protein extracts of the obtained fractions were analyzed for p27 protein expression by Western blot analysis. (B) Logarithmically growing EEF4 and EEF8 cells were incubated in 100 μg/ml of cycloheximide for the indicated time periods. p27 protein levels were compared by Western blot analysis. (C) Logarithmically growing EEF4 and EEF8 cells were incubated for 4 hr with [35S]methionine and then chased in medium containing unlabeled methionine for the indicated times. Radiolabeled p27 was immunoprecipitated, separated by gel electrophoresis, and detected by autoradiography. To be able to detect p27 degradation in EEF8 cells on the same Western blot with EEF4 cells it was necessary to perform long exposures (compare with Fig. 1C). The presented blots are overexposed in respect to p27 expression in EEF4 cells. p27 protein started to decrease after 3-hr cycloheximide treatment of EEF4 cells (data not shown).
half-life of p27 (reviewed in ref. 36), p27 started to decrease at 3 hr of cycloheximide treatment of EEF4 cells (data not shown). In EEF8 cells p27 levels already decreased after 1 hr (Fig. 3B). These results were confirmed by analyzing p27 stability via a pulse–chase experiment (Fig. 3C). These findings provide strong evidence that TSC2 affects the process of p27 degradation.

**Cell Cycle Inhibitory Effects of p27 Are Inactivated in TSC2-Negative Cells.** In all normal cells, forced expression of p27 causes the cell cycle to arrest in G1, an effect that can be visualized by an increase of G1 cells upon ectopic p27 expression in logarithmically growing cells. We confirmed this potency of p27 by overexpressing this CDK inhibitor in TSC2-positive EEF4 cells. Quantitation of multiple experiments revealed that overexpression of p27 triggers an increase of G1 cells of 18% ± 3% (Fig. 4A). Strikingly, no effect on cell cycle distribution was observed when p27 was overexpressed in EEF8 cells to the same levels as in EEF4 cells (Fig. 4A). High levels of ectopic p21 arrested both EEF4 and EEF8 cells in G1 (data not shown). From our data on p27 stability in TSC2-negative cells (described above) one could speculate that p27 cannot trigger cell cycle inhibitory effects because of its increased degradation in these cells. However, our observation that p27 was expressible to the same levels in EEF8 cells as in EEF4 cells made that unlikely (Fig. 4A). It had been shown earlier that degradation of p27 is induced by phosphorylation on Thr-187 in the p27 C terminus via CDK2 (34, 36, 37). To address the role of CDK2-dependent p27 degradation in growth rescue by loss of TSC2, we overexpressed the nonphosphorylatable p27VPKK mutant (mutation of Thr-187 to valine, compare to ref. 34) in TSC2-negative EEF8 cells. p27VPKK behaved exactly as wild-type p27 in the transfection assay, indicating that CDK2-dependent p27 degradation is not the mechanism used by TSC2-negative cells to overcome p27-dependent growth arrest (Fig. 4B). It is noteworthy that we found the p27VPKK mutant to be expressible to the same amounts in both EEF4 cells and EEF8 cells without mediating cell cycle effects (data not shown). The resistance of TSC2-negative cells against p27-dependent arrest could be explained by two different mechanisms: p27 could be inactive in inhibiting CDKs in EEF8 cells, or loss of TSC2 could trigger cell proliferation independently of CDK2 activity (proliferation despite inactive CDK2). To further investigate this issue we tested whether TSC2-negative cells can grow independently of active CDK2. Overexpression of a dominant-negative mutant of CDK2, which was shown earlier to inhibit CDK2 activity (35), clearly arrested TSC2-negative fibroblasts (Fig. 4B). These data show that loss of TSC2 does not mediate CDK2-independent cell proliferation and strongly suggest that p27 cannot efficiently inhibit CDK2 in EEF8 cells.

Transformation of primary cells by oncogenes is multistep, requiring the cooperation of two genes. c-Myc alone, however, is capable of transforming specific rat cell lines, associated with anchorage-independent growth (reviewed in ref. 38) as confirmed by the experiment presented in Fig. 4C. Soft agar assays further demonstrated that loss of TSC2 induces growth independent of cell adhesion, a process that could not be inhibited by high ectopic levels of p27 (Fig. 4C). Taken together, these data demonstrate that loss of TSC2 mediates growth advantage and that p27 cannot exert its cell cycle inhibitory function in cells, which lost TSC2 expression.

**Loss of TSC2 Affects p27 Localization.** So far we have described two phenotypes induced by loss of TSC2: (i) p27 is unstable in TSC2-negative cells, leading to elevated CDK2 activity and shortened G1 phase, and (ii) TSC2 mutant cells are resistant to ectopic p27. Our finding, that these cells depend on functional CDK2, strongly suggests that p27 cannot inhibit CDK2 in these cells. To further elucidate the mechanism of the affects of TSC2 on p27 we first tested whether the CDK2 complex is, per se, resistant to p27 in TSC2-negative cell extracts. Kinase assays revealed that CDK2 precipitated from EEF8 cells can be inhibited in vitro by the addition of recombinant p27 protein (Fig. 5A). These data strongly suggest that p27 is functionally disabled in vivo in TSC2-negative cells. We next investigated the subcellular localization of p27 in EEF4 and in EEF8 cells. p27 has been reported to exclusively localize into the nucleus (reviewed in ref. 28). While EEF4 cells exhibit nuclear localization of p27, this protein is mislocated into the cytoplasm upon loss of TSC2 (Fig. 5B). Although it appeared that the nuclei of EEF8 cells also weakly stained p27 positive, a major portion of p27 was detectable in the cytoplasm. This mislocation of p27 upon loss of TSC2 could be an explanation for both the instability of endogenous p27 in TSC2-negative cells and the resistance of TSC2-mutant cells to the cell cycle inhibitory function of ectopic p27.

**DISCUSSION**

Forced expression of p27 causes the cell cycle to arrest in G1 (39, 40), and conversely, inhibition of p27 expression by antisense oligonucleotides drives quiescent cells into the cell cycle (41). It is widely accepted that the latter is the result of activation of G1-CDKs upon loss of p27 (27–29). In this report we have shown that loss of TSC2 inactivates p27’s property to inhibit cell cycle progression. We further found that loss of TSC2 triggers down-regulation of p27 expression caused by...
Nuclei were identified by 4 signals in EEF8 cells have been enhanced to visualize p27 localization.

Our finding that the nucleoprotein p27 becomes mislocated into the cytoplasm upon loss of TSC2-, affect p27 stability? Two different pathways could explain the resistance of TSC2-negative cells, and (ii) by using different approaches we have shown that the stability of p27 decreases upon loss of TSC2. The former phenotype is independent of CDK2-dependent p27 degradation, because the nonphosphorylatable p27VPKK mutant also was unable to affect cell cycle progression of TSC2-negative cells. On the other hand, it could be that the elevated degradation of p27 in TSC2-negative cells is a secondary effect associated with the inhibition of p27’s activity in these cells.

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