Reduction of Cytosolic p27<sup>kip1</sup> Inhibits Cancer Cell Motility, Survival, and Tumorigenicity

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Abstract

We generated a p27<sup>kip1</sup> mutant (p27ΔNLS) that localized exclusively in cell cytosol. Expression of p27ΔNLS in MCF7 breast cancer cells down-regulated RhoA and increased motility, survival, and Akt levels, in the absence of an effect on cell cycle distribution. RNA interference of p27 in U87 glioma cells, which express p27 predominantly in the cytoplasm, inhibited motility and survival. Conversely, knockdown of p27 in COS7 cells, with >95% nuclear p27 expression, accelerated proliferation but had no effect on motility or survival. U87 cells in which p27 had been eliminated by RNA interference exhibited lower Akt levels, shorter Akt turnover, and markedly impaired tumorigenicity in vivo. These xenografts were less invasive and exhibited increased apoptosis compared with p27-expressing tumors. Expression of cytosolic p27 in primary human breast carcinomas correlated linearly with Akt content as measured by immunohistochemistry. These data suggest that cytoplasmic p27 can exert oncogenic functions by modulating Akt stability, cell survival, and tumorigenicity.

Introduction

p27<sup>kip1</sup> is a member of the Kip family of cyclin-dependent kinase (Cdk) inhibitors initially discovered as having a Cdk-inhibitory activity induced by extracellular antimitogenic signals (1, 2). It accumulates in serum-starved and density-arrested cells and its overexpression results in cell cycle arrest in G<sub>1</sub> and/or apoptosis. Consistent with its role as a tumor suppressor, p27-null mice develop multiorgan hyperplasia and increased susceptibility to cancer (3). Loss of a single allele of p27 confers increased susceptibility to carcinogen-induced tumors in mice (4) and low levels of p27 protein have been associated with poor prognosis in several human cancers (5, 6). Inactivation of the cell cycle inhibitory activity of p27 is commonly observed in several cancers by mechanisms that mainly involve accelerated proteolysis, sequestration by cyclin D-Cdk complexes, and posttranslational modifications leading to nuclear export and/or retention in the cytosol (7).

Cytoplasmic translocation of p27 has been increasingly recognized in primary human tumors associated with poor survival whereas nuclear expression confers a more favorable outcome (5, 6). The oncogenic kinase Akt and the growth factor–dependent human kinase-interacting stathmin have been causally associated with cytoplasmic retention and nuclear export of p27 (8–11), respectively, further implying a link between mislocalized p27 and enhanced transformation. A simple explanation as to how the cytoplasmic redistribution of p27 may contribute to transformation is derepression and activation of nuclear Cdk2. Interestingly, p27 does not fit Knudson’s “two-hit” criterion for most tumor suppressor genes in that inactivating mutations or homozygous deletions of both p27 alleles are exceedingly rare (3). This suggests lack of a selective pressure to lose p27 completely perhaps as the result of “gain-of-function” effects of low levels of mislocalized p27.

Several mechanistic data suggest that cytosolic p27 has functions opposite to its tumor suppressor role. In the cytosol, p27 assembles cyclin D/Cdk4 complexes and promotes their import into the nucleus (12, 13). Transduction of TAT-p27 fusion protein into HepG2 hepatocellular cancer cells results in enhanced cell migration (14). Treatment with hepatocyte growth factor induces Ser<sup>10</sup> phosphorylation-dependent nuclear export of p27. Once in the cytoplasm, p27 stimulates Rac-dependent migration of HepG2 cells and embryonic fibroblasts (15). In this study, a region in the COOH-terminal domain of p27, independent of its Cdk-inhibitory activity, was required for growth factor–stimulated migration. In a more recent report, Besson et al. (16) showed that p27 regulates cell migration by titrating the function of the RhoA GTPase as a result of direct binding to RhoA and inhibition of activation by its guanine nucleotide exchange factors. Thus, by decreasing actin stress fiber formation and adhesion, p27 alters the balance between RhoA and Rac and thus allows cell movement. Taken together, these data suggest that p27 may act as a tumor suppressor or as an oncogene depending on its subcellular localization.

Therefore, to study gain-of-function effects of cytoplasmic p27 on transformation, we engineered a p27 mutant that exclusively localized in the cytosol. Expression of this mutant in MCF7 cells increased cell motility and survival while up-regulating Akt protein stability. To extend these observations, we hypothesized that elimination of p27 by RNA interference would have opposite effects in cells with predominant nuclear versus cytosolic localization. Knockdown of p27 in PTEN-null U87 tumor cells with predominant cytosolic p27 resulted in reduced cell motility, enhanced apoptosis, and lower levels and stability of Akt in vitro, as well as reduced tumorigenicity, tumor cell viability, and invasiveness in vivo. However, in COS7 cells with >95% nuclear p27, RNA interference of p27 only accelerated cell cycle progression without affecting cell survival or motility. These data support the oncogenic role of cytoplasmic mislocalized p27 and its potential use as a therapeutic target. The results also suggest that this approach would have opposite consequences depending as to where in the tumor cell p27 is predominantly localized.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell lines, plasmids, and retroviruses. All cell lines were from the American Type Culture Collection (Rockville, MD). MCF-7, human embryonic kidney cells 293T, African green monkey fibroblast cells COS7, Phoenix-Ampho cells, and U87-MG glioma cells were grown in DMEM (Cambrex, Walkersville, MD) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) in a humidified 5% CO2 incubator at 37°C. The p27 (−/−) mouse embryonic fibroblasts were generated as described previously (17) and were maintained in DMEM/10% FBS.

To generate a p27 mutant with aspartic acid substitutions at Ser10 and Thr157 (referred herein as Flag-p27DD), we used the pcDNA 5 Flag-p27 plasmid (11) as the parent vector and the Stratagene QuickChange Multi Site-Directed Mutagenesis kit with two primers, namely (a) Ser10 mutant-F: 5′-phospho-GAGTGTCTAACGGGGACCCTAGCCTGGAGC-3′ and (b) Thr157 mutant-F: 5′-phospho-AATAAGGAAGGACCTGCAGACGAGCGAGTTCTT-CTACTCAA-3′, according to the Stratagene Mutagenesis Protocol. To generate a mutant p27 with neutral hydrophobic residues replacing positive residues in the nuclear localization sequence (Flag-p27DNLS: 5′-KKRR to ALA and 18KR to LA), four primers were generated and two of them contained a specific nucleotide mutations: (a) FYW3: 5′-GCGGAAATCTTACGTGTTTCAAGAGATTTCATATCCCGGCAGTGCGGG-3′, (b) FYW4: 5′-GCCGGAATTCTTACGTGTTTCAAGAGATTTCATATCCCGGCAGTGCGGG-3′, (c) FYW5: 5′-CCTGCAACCGCAGCTTCATCTACFCAAAACCAGCAGCAACAAATGTTTCA-3′, and (d) FYW6: 5′-GTATTGAGAGAACTGTGCCTGGTTGCGGTGCTAACGC-CATTTCCTGAGATTGTCTGATACCC-3′. First, using pcDNA-p27 as template, two different overlapping fragments of p27 genes were obtained by PCR amplifications with primers FYW3 and FYW6, FYW5, and FYW4, respectively. Second, 1 L of each of the two fragments was pooled together and a second round PCR was conducted with primers FYW3 and FYW4 to generate the full-length p27DNLS cDNA fragment, which was cloned into the pcDNA-Flag vector to generate pcDNA-Flag-p27DNLS. Subsequently, the wild-type and mutant fragments were excised by BglII/EcoRI restriction enzyme digestion and cloned into the pBMN-Ires-EGFP retroviral vector (18) to generate pBMN-Ires-EGFP-Flag-p27 plasmids. These were transduced into Phoenix-Ampho packaging cells (19) to generate retroviruses that express short hairpin RNAs (shRNA) against mouse p27, two complementary oligonucleotides: 5′-phospho-6GTTTTGGAAT and 5′-phospho-AATAAGGAAGGACCTGCAGACGAGCGAGTTC TCTACTCAA-3′ were annealed and inserted into the same vector, which is specific against mouse p27 [multiplicity of infection (MOI) 50:1] and selected in 1 ng/mL puromycin. p27-shRNA were isolated and expanded in puromycin-containing media. p27-shRNA plasmids were transduced with retroviruses encoding shRNAs for mouse and human p27 (MOI 50:1) as described (22). After sorting GFP-positive cells by flow cytometry, stably transduced cells were selected in medium containing 1 mg/mL G418 (Research Products International Corp., Mt. Prospect, IL). U87 and COS7 cells were transduced with retroviruses encoding shRNAs for mouse and human p27 and selected in 1 mg/mL puromycin (Calbiochem, San Diego, CA). Drug-resistant single cell colonies expressing p27-shRNA were isolated and expanded in puromycin-containing media. Expression of p27 was confirmed by immunoblot analysis of cell lysates.

Expression of p27 was confirmed by immunoblot analysis of cell lysates. Immunoprecipitation, immunoblotting, and Rho/Rac assays. MCF-7, COS7, or 293T cells were washed twice with ice-cold PBS and lysed in NP40 lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.1 mmol/L EDTA, plus protease inhibitors and phosphatase inhibitors]. After sonication for 10 seconds at the minimal setting and centrifugation, the supernatant was collected and total protein concentration in it was measured using the BCA assay reagent (Pierce, Rockford, IL). For immunoprecipitation, 500 μg protein sample were diluted in immunoprecipitation buffer [50 mmol/L Tris-Cl (pH 7.9), 50 mmol/L Nacl, 0.1 mmol/L EDTA, 1% glycerol, 0.2% NP40, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride] to make a 500 μl final volume, and incubated with 2 μg antibodies overnight at 4°C. Fifty microliters of a 50% slurry of 1:1 mixed protein A/G-Sepharose beads were added to each sample and incubated for 1 hour at 4°C. The beads were then washed thrice with cold immunoprecipitation buffer at 15-minute intervals, resuspended in 2× SDS-gel loading buffer, and boiled for 5 minutes before SDS-PAGE. For immunoblotting, 30 μg protein extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary and horseradish peroxidase–conjugated secondary antibodies (Promega, Madison, WI) as described (21). Primary antibodies included Flag monoclonal antibody (mAb), Flag rabbit polyclonal antibody (pAb), actin mAb, and human vinculin mAb (Sigma, St. Louis, MO); p27 mAb (Transduction Laboratories, San Diego, CA); phosphotyrosine, RhoA, FAK rabbit pAb (Santa Cruz Biotechnology, Santa Cruz, CA); total Akt, S473 phospho-Akt, phospho-GSK3β/β rabbit pAb, and total GSK3β mAb (Cell Signaling, Beverly, MA); Rac mAb (BD Biosciences, San Diego, CA); proliferating cell nuclear antigen (PCNA) mAb (Pharmingen, San Diego, CA); total and T202/Y204 phospho-mitogen-activated protein kinase (MAPK) rabbit pAb (Promega). To pull down GTP-bound form of Rho or Rac, a glutathione S-transferase (GST) fusion protein with Biotin-Rho

Figure 1. Cellular localization of p27 mutants. A, schema showing site-specific mutagenesis of p27. In p27-DD, S10 and T157 residues were replaced with aspartic acid (D) to mimic phosphorylation. In p27DNLS, positive amino acids within the NLS of p27 were replaced with neutral hydrophobic residues. B, immunofluorescence assay showing subcellular localization of p27 wild-type and mutants in COS7 cells. Transfected p27 was visualized with Flag mAb and FITC-labeled anti-mouse IgG as described in Materials and Methods. Hoechst, nuclear staining. The p27DNLS mutant was completely cytoplasmic whereas p27-DD retained nuclear localization.
binding domain (RBD) or a GST-Pak binding domain (PBD) fusion, respectively, each precoupled to agarose-glutathione beads (Cytoskeleton, Inc., Denver, CO) were used as described previously (22, 23). In brief, 20 μg beads were added to 250 μg cleared cell lysates and incubated for 30 minutes at 4°C.

Cell cycle analysis. Cells were harvested by trypsinization, washed twice with ice-cold PBS, resuspended in cold PBS, and fixed by adding absolute ethanol (while vortexing) to a final ethanol concentration of 67%. After fixing overnight at -20°C and rehydrating in PBS for 30 minutes on ice, cell nuclei were stained for 30 minutes in the dark with 30 μg/mL propidium iodide (Sigma) containing 125 units/mL protease-free RNase (Calbiochem), both diluted in PBS. Cells were filtered through a 5 μm pore size nylon mesh (Small Parts, Inc., Miami Lakes, FL). A total of 15,000 stained nuclei were analyzed using the BD CellQuest Pro software package coupled to a FACSCalibur flow cytometer (Becton Dickinson, Mansfield, MA) as described (21).

Cell motility and adhesion. Transwell motility assays were done using 5 μm pore, 6.5-mm polycarbonate transwell filters (Corning Costar Corp., Cambridge, MA). Cells (1.5 × 105) suspended in medium containing 0.1% FBS were seeded on the upper surface of the filters and allowed to migrate toward 0.1% FBS or 10% FBS-containing media in the bottom compartment for 20 hours. The cells remaining on the upper surface were wiped off with a cotton swab and the cells that had migrated to the underside of the transwell filters were fixed, stained with Diff-Quick kit (Dudingen, Switzerland), and counted by bright field microscopy at ×200 in five random fields. For wound closure assay, stably transfected p27(−/−) mouse embryonic fibroblasts in full medium were allowed to reach >90% confluence on six-well plates and then switched to serum-free medium for 30 hours. The monolayers were next scraped with a plastic pipette tip as described (24). Phase-contrast images were photographed at 0, 12, and 24 hours after wounding. To measure adhesion, cells were trypsinized and resuspended in DMEM/10% FBS. About 2 × 105 cells per well were added to uncoated six-well tissue culture plates and allowed to attach at 37°C. After 1, 2, or 4 hours, cells that had not attached were removed and the attached cells were trypsinized and measured using a Coulter counter. Cell adhesion was calculated as the percentage of attached cells over the total input per well.

Apopotis and cell viability. Five hundred thousand cells per well in six-well plates were incubated in serum-free DMEM for 72 hours. Both attached and suspended cells were harvested and washed with PBS, before being subjected to APO-BRDU analysis with the use of an apo-BrdUrd assay kit (Phoenix Flow Systems, San Diego, CA) according to the protocol of the manufacturer. FITC-positive apoptotic cells were quantitated in a FACScalibur Flow Cytometer (BD Biosciences). Assessment of DNA fragmentation in serum-starved cells was done as described previously (25). DNA fragments were separated in 1.5% agarose gels. Cell viability was also assessed by trypan blue exclusion. For this, after 3 days in serum-free medium, floating and adherent cells were collected and incubated in 0.2% trypan blue for 2 minutes at room temperature. Trypan blue was removed manually and their percentage calculated over the total cell input.

Indirect immunofluorescence assay. Cells grown on coverslips were fixed in methanol for 20 minutes at -20°C and then rehydrated in PBS for 30 minutes on ice. After blocking with 3% nonfat milk in PBS for 30 minutes, cells were incubated for 60 minutes with primary antibodies (1:500) diluted in 1% milk, and then with fluorescent secondary antibodies or phallolidin (1:500) for 30 minutes at room temperature. Cell nuclei were stained with 1 μg/mL Hoechst for 10 minutes at room temperature. Coverslips were mounted on 25 × 75 mm microslides (VWR Scientific, Atlanta, GA) using AquaPolyMount (Polysciences, Warrington, PA). Fluorescent images were captured using a Princeton Instruments cooled charge-coupled device digital camera from a Zeiss Axioskop upright microscope. Fluorescent antibodies were as follows: Oregon green-α-mouse IgG, Texas red-α-rabbit IgG and Texas red-phallolidin (Molecular Probes, Eugene, OR).

35S metabolic labeling and pulse-chase. Cells grown on six-well plates were washed and incubated with methionine- and cysteine-free DMEM (Life Technologies Invitrogen, Carlsbad, CA) at 37°C for 30 minutes. Cells were next labeled with 200 μCi/mL Tras35S-Label (MP Biomedical, Irvine, CA) in 0.5 mL methionine/cysteine-free DMEM at 37°C for 30 minutes, washed twice with fresh DMEM, and incubated with DMEM/10% FBS. At variable times after the addition of serum-containing medium, cells were washed with ice-cold PBS and harvested in 200 μL NP40 lysis buffer (above).
Three hundred micrograms of protein were precipitated with 2 µg of an Akt antibody (Cell Signaling). The 35S-labeled immune complexes were resolved by SDS-PAGE and visualized by autoradiography.

Xenograft studies. U87 cells (5 × 10⁶) in a 0.25 mL volume were injected s.c. via a 22-gauge needle in the dorsal space of 4- to 5-week-old athymic mice (Harlan Sprague-Dawley, Madison, WI). Mice were examined thrice a week for tumor development. Tumor diameters were measured with calipers and volume calculated by the formula: \[ \text{volume} = \text{width}^2 \times \text{length} / 2 \]. Apoptosis in the xenografts was measured by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) analysis with the Apoptag Detection kit (Serologicals Corp., Norcross, GA) as described previously (17). p27 content in tumors was assessed by immunohistochemistry using a p27 monoclonal antibody (Transduction Laboratories) following previously published methods (11).

Immunohistochemistry of primary breast cancers. Sections (4 µm) from formalin-fixed, paraffin-embedded blocks of 100 invasive breast cancers were subjected to immunohistochemistry as described previously (11). Akt, S473 P-Akt, and MAPK protein content was expressed as a Histo-score, which was calculated as a combination of intensity and percentage of antibody-stained cancer cells in 10 high-power fields: score = (% cells with weak staining) \times 1 + (% cells with medium staining) \times 2 + (% cells with high staining) \times 3. By this analysis, four groups were generated: negative, 1 to 100 (weak), 101 to 200 (medium), and >200 (intense). Tumors with p27 in both cytosol and nucleus were scored as cytosolic p27, whereas exclusive nuclear staining was scored as nuclear. A minimum of 1,000 cells were scored in each specimen.

Results

p27ΔNLS localizes exclusively in the cytosol. We sought to generate a mutant of p27 with exclusive cytosolic localization. p27 is phosphorylated in S10 by human kinase-interacting stathmin and this modification promotes its binding to exportin...
CRM1 (26, 27). Subsequently, in mid-G1 phase of the cell cycle, Akt has been shown to phosphorylate p27 in T157; this modification in the NLS of p27 inhibits complex formation with importin α, thus resulting in retention of p27 in the cytosol (8, 28). Therefore, we generated a phospho-mimicking mutant at S10 and T157 by replacing these two residues with aspartic acid (Flag-p27-DD). Similar to wild-type p27, p27-DD localized predominantly in the nucleus of COS7 cells (Fig. 1B), suggesting that phosphorylation at S10 and T157 are not sufficient for cytosolic retention. In a second mutant, we replaced the positive residues 152RKR and 165KR in the bipartite NLS with neutral hydrophobic residues ALA and LA, respectively (Fig. 1A). As measured by Flag immunofluorescence assay, p27ΔNLS in COS7 cells was exclusively cytosolic (Fig. 1B). Similar results were observed in MCF-7 (Fig. 2A) and 293T cells (Supplementary Fig. 1A). Based on these data, we used p27ΔNLS in subsequent studies addressing the role of cytoplasmic p27.

Cytosolic p27 down-regulates RhoA and cell adhesion but increases cell motility and survival. We stably transduced MCF-7 human breast cancer cells with retroviruses expressing wild-type and p27ΔNLS. Both ectopic proteins were expressed at equal levels but Flag immunofluorescence assay showed predominantly nuclear localization of wild-type p27 whereas p27ΔNLS was only detectable in the cytosol (Fig. 2A and B). MCF-7/p27 cells exhibited a reduced S and increased G1 phase fractions compared with vector controls. On the other hand, MCF-7/p27ΔNLS cells exhibited a cell cycle profile identical to control cells (Fig. 3A), supporting the absence of p27ΔNLS in the nucleus and, hence, its inability to inhibit Cdk2 and delay cell proliferation.

**Figure 4.** Knockdown of p27 inhibits U87 cell motility. A, detection of endogenous p27 in U87 and COS7 cells by immunofluorescence assay using p27 mAb and Oregon green anti-mouse IgG. U87 cells exhibit >50% of p27 in the cytosol whereas COS7 cells express >95% of p27 in the nucleus. Hoechst, nuclear staining. B, puromycin-resistant single-cell colonies of U87 and COS7 cells transduced with human and mouse (control) p27shRNA retroviruses were expanded and subjected to p27 immunoblot. Clones 7 and 10 were selected for subsequent studies. C, cells were seeded on 5 μm pore size transwell filters and allowed to migrate toward 10% FBS. After 20 hours, cells on the underside of the filters were fixed, stained, and counted as indicated in Materials and Methods. Data are quantitated in bar graph at bottom. Column, mean of three wells; bars, SD. D, GTP-bound RhoA in the indicated cell lysates was eluted from GST-RBD coupled to agarose-glutathione beads. Eluates were immunoblotted with a RhoA antibody (top). Middle graph, RhoA activity from three independent experiments quantitated by densitometry. Total cell lysates (50 μg/lane) were separated by SDS-PAGE and subjected to immunoblot analysis with RhoA, PCNA, and vinculin antibodies. E, focal adhesions were visualized with human vinculin (hVin-1) mAb and Oregon green anti-mouse IgG. Knockdown of p27 resulted in a reduction of focal adhesions (arrows) in U87 but not COS7 cells.
Because p27 has been shown to inhibit RhoA activation and induce cell motility, we next examined if the cytosolic mutant was more potent than wild-type p27 on regulating these functions. RhoA activity was 15-fold lower in MCF-7/p27<sub>DNLS</sub> and 5-fold lower in MCF-7/p27 compared with vector controls. Rac activity did not change (Fig. 2C). This difference may reflect the increased availability of p27<sub>DNLS</sub> in the cytosol where it titrates RhoA. Similar results were obtained in 293T cells transfected with wild-type and DNLS p27 (Supplementary Fig. IC). Time-dependent adhesion was impaired by stable expression of cytosolic p27 with MCF-7/p27ΔNLS cells exhibiting a 40% reduction in adhesion at 4 hours compared with cells transfected with wild-type p27 or vector alone (Fig. 2D). p27ΔNLS-expressing MCF-7 cells exhibited a >3-fold increased migration through transwells toward 10% FBS compared with cells transfected with wild-type p27 (Fig. 2E). Similar results were obtained in wound closure assays with p27-null mouse embryonic fibroblasts. Fibroblasts transfected with p27ΔNLS migrated into and closed the wounded area within

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**Figure 5.** RNA interference of p27 inhibits U87 cell survival without altering cell cycle progression. A, DNA histograms of propidium iodide–labeled U87 and COS7 cells stably transduced with the indicated vectors. The percentage of gated cells in G1, S, and G2–M phases are indicated. Mean and SD values are representative of three independent experiments. B, cells (5 × 10<sup>5</sup> per well in six-well plates) were incubated in serum-free DMEM for 72 hours and then subjected to APO-BRDU assay as indicated in Materials and Methods. Percent indicates the proportion of (gated) FITC+ apoptotic cells. U87 cells transfected with the human p27 siRNA retrovirus exhibited a 3.5-fold greater proportion of apoptotic cells compared with controls. C, cells were incubated in serum-free DMEM for 72 hours. Adherent and floated cells were then pooled, their DNA was collected, and evaluated for evidence of internucleosomal fragmentation in 1.5% agarose gels as indicated in Materials and Methods. D, cells incubated in serum-free DMEM for 72 were stained with 0.2% trypan blue. Columns, mean percentage of trypan blue+ cells of three wells; bars, SD.
20 hours whereas in monolayers of mouse embryonic fibroblasts trans vectored with wild-type p27 or vector alone the wound remained open (Supplementary Fig. IIB).

Despite their reduced adhesion, cells expressing p27ΔNLS did not display an impaired viability. Because cytoplasmic localization of p27 has been associated with anchorage-independent growth of tumor cells and more virulent tumor behavior (5), we speculated on an antiapoptotic effect of the cytosolic mutant. After 72 hours under serum-free conditions, there were 40% to 50% less MCF-7/p27ΔNLS apoptotic cells compared with cells transfected with wild-type p27 or vector alone (Fig. 3B). Immunoblot analysis of signaling molecules involved in cell survival showed marked up-regulation of total Akt, P-Akt, and P-GSK3β in cells expressing the cytosolic mutant of p27. Total MAPK and P-MAPK did not change (Fig. 3C). Although to a lesser degree, similar results were obtained in COS7 cells transfected with the same vectors (Supplementary Fig. IIIA). Consistent with the cell cycle inhibitory effect of wild-type p27, MCF-7/p27 cells contained reduced PCNA levels compared with MCF-7/p27ΔNLS cells (Fig. 3C).

RNA interference of p27 inhibits tumor cell motility and survival. If cytosolic p27 exerts oncogenic functions, we hypothesized that elimination of endogenous p27 in cells with predominant expression of p27 in the cytosol will inhibit the transformed phenotype. On the other hand, in cells with p27 predominantly localized in the nucleus, where its Cdk-inhibitory and tumor suppressor functions are dominant, elimination of p27 would have an oncogenic effect. Therefore, we stably transduced a retroviral vector expressing short hairpin (sh) human or mouse (control) p27 RNAs in U87 and COS7 cells. U87 are PTEN-null human glioma cells with high levels of active phosphatidylinositol 3-kinase (PI3K) and Akt (29) and with >50% expression of p27 in the cytosol. COS7 are monkey kidney cells transformed with SV40 large T antigen and with >95% of p27 detectable in the nucleus even under logarithmic growth conditions (Fig. 4A). Cells expressing human p27 shRNAs (reflected as knock-down or kd) but not mouse (control) p27 shRNAs exhibited variable levels of p27 down-regulation (Fig. 4B). U87 clone 7 and COS7 clone 10 were selected and expanded for further experiments.

Cells were seeded on transwells and allowed to migrate toward 10% FBS. After 20 hours, the U87 hp27-kd cells showed markedly impaired motility compared with U87 mp27-kd and wild-type cells. In contrast, COS7 hp27-kd cell motility was minimally inhibited compared with controls (Fig. 4C). Consistent with inhibitory effect of cytosolic p27 on RhoA, U87 hp27-kd cells exhibited higher RhoA activity compared with controls but this was not observed in COS7 hp27-kd cells (Fig. 4D). The down-regulation of RhoA suggested an increase in cell adhesiveness. Indeed, U87 hp27-kd cells exhibited extensive staining with a vinculin antibody along the cell edge, whereas, in control cells, vinculin was mainly detectable at membrane ruffles and lamellipodia, suggestive of increased adhesion in knockdown cells. Conversely, COS7 hp27-kd cells exhibited the same pattern of vinculin staining than control cells.
RNA interference of p27 in U87 cells did not alter their cell cycle distribution (Fig. 5A). Opposite to this, knockdown of p27 in COS7 cells resulted in an approximate 50% reduction in cells in G1 and close to a doubling of the proportion of cells in S phase (Fig. 5A). Finally, we examined survival in U87 cells. By three different assays, APO-BRDU (TUNEL), DNA laddering in agarose gels, and trypan blue exclusion, a higher level of apoptosis was detectable in serum-starved U87 hp27-kd cells compared with controls (Fig. 5B and C). The proportion of apoptotic COS7 hp27-kd, COS7 mp27-kd, and vector controls was similar (data not shown). Finally, we compared three U87 clones (1, 3, and 7) with different levels of p27 as a result of RNA interference. RhoA activity, total Akt, P-Akt, P-GSK3β, migration through transwells, and survival under serum-free conditions were different than controls and this difference correlated with the endogenous level of p27 (Supplementary Fig. IV).

**Cytosolic p27 stabilizes Akt.** The effect of p27 knockdown on U87 cell survival and the up-regulation of Akt levels in MCF-7 cells transduced with p27ΔNLS (Fig. 3C) prompted us to examine Akt levels in U87 hp27-kd cells. In these cells, protein levels of total Akt, P-Akt, and P-GSK3β were lower than in controls (Fig. 6A). Because p27 has been shown to couple to Akt (11), we next examined if the cytosolic mutant associated better than wild-type p27 with Akt. As determined by Flag immunoblot, precipitation of Akt in MCF-7 cells with a subsaturating concentration of Akt antibody pulled down a higher amount of Flag-tagged p27ΔNLS that wild-type p27 per the amount of precipitated Akt (Fig. 6B). Thus, we speculated that cytosolic p27 could stabilize Akt. Hence, we examined the turnover of metabolically labeled, newly synthesized endogenous Akt in U87 and MCF-7 cells. In U87 hp27-kd cells, the half-life of 35S-labeled Akt was <1 hour whereas in controls it was ~4 hours (Fig. 6D). Conversely, expression of p27ΔNLS in MCF-7 cells prolonged Akt half-life to ~8 hours compared with 1 to 2 hours in MCF-7/vector cells (Fig. 6D).

**Suppression of p27 impairs tumorigenicity.** To examine the effect of p27 knockdown in U87 tumorigenicity, we implanted cells in athymic nude mice. All mice injected with parental cells (seven of seven) or with control cells transduced with mouse p27 shRNAs (seven of seven) developed tumors of >3 mm in minimal diameter. However, only three of eight (37%) mice injected with U87 hp27-kd cells formed palpable tumors within 15 days after tumor cell inoculation. Expression of p27 by immunohistochemistry was almost undetectable in U87 hp27-kd tumors arguing against loss of the shRNAs in vivo (Fig. 7E and F). U87 hp27-kd tumors were encapsulated and exhibited a lower histologic grade

**Figure 7.** RNA interference of p27 inhibits tumor cell invasiveness and viability in vivo. A to D, H&E sections of U87-hp27 shRNA and U87-mp27 shRNA (control) xenografts. Xenografts in which p27 had been knocked down were encapsulated and contained more necrotic areas than control tumors. E to F, immunohistochemical detection of p27 as described in Materials and Methods. G to H, detection of TUNEL+ (apoptotic) cells in U87 tumor sections by immunohistochemistry. I, total number of TUNEL+ tumor cell nuclei in twenty 40X fields from a total of three individual tumor tissue sections per group (P < 0.05; Student’s t test).

(Fig. 4E). In all cells, vinculin content as measured by immunoblot was similar (Fig. 4D). Supporting a functional significance to these findings, time-dependent adhesion was increased in U87 hp27-kd cells with ≥90% of cells attached at 2 hours compared with <80% at 4 hours in both U87 m27-kd controls and wild-type cells (Supplementary Fig. IIIIB).

**Table 1.** U87 cell tumorigenicity

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tumors formed</th>
<th>Volume (mm³)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>7 of 7 (100%)</td>
<td>5,853 ± 4,849</td>
</tr>
<tr>
<td>hp27 shRNA</td>
<td>3 of 8 (37%)</td>
<td>757 ± 395</td>
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<tr>
<td>mp27 shRNA</td>
<td>7 of 7 (100%)</td>
<td>2,661 ± 867</td>
</tr>
</tbody>
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NOTE: The indicated cells were injected s.c. in athymic nude mice as described in Materials and Methods and measured serially with calipers once palpable tumors developed. Tumor incidence and mean tumor volumes ± SE at 21 days postinoculation are shown.
and volume as well as a higher degree of TUNEL+ apoptotic cells (Fig. 7B, D, H, and I; and Table 1). On the other hand, control tumors were larger, of high grade, locally invasive, and with significantly less TUNEL+ cells (Fig. 7A, C, G, and I; Table 1).

**Cytosolic p27 correlates with total Akt content in primary breast cancers.** Activated Akt phosphorylates p27 in T157 and T198, resulting in binding to 14.3.3. These modifications prevent p27 from binding to importin α, thus blocking nuclear import (28, 30, 31). Therefore, activated Akt has been shown to correlate with presence of p27 in tumor cell cytosol. This does not rule out the possibility that cytosolic p27 may, in turn, stabilize and correlate with total Akt protein levels. Thus, we subjected 100 primary breast cancers to immunohistochemistry using antibodies against p27 and total and S473 P-Akt. Tumors were divided in four groups (negative, weak, intermediate, and intense) according to the level of Akt and P-Akt expression. Only four specimens did not stain for p27. Only 26, 33, and 16 tumors were negative for Akt, P-Akt, and total MAPK, respectively. All levels of total Akt correlated linearly with the proportion of specimens expressing p27 in tumor cell cytosol (68.8%, 45.8%, 30%, and 0% in the intense, intermediate, weak, and negative Akt groups, respectively; \( P < 0.05 \), Pearson \( \chi^2 \)). Conversely, levels of p42/44 MAPK (Erk) did not change as a function of presence of p27 in the cytosol (\( P > 0.05 \)). Levels of P-Akt staining also correlated with presence of p27 in tumor cell cytosol, although this association was not as linear as with total Akt expression (Fig. 8).

**Discussion**

We have examined the oncogenic effects of the tumor suppressor p27. A phospho-mimicking mutant of p27 in which S10 and T157 had been replaced with aspartic acid localized...
predominantly in the cell nucleus. Like for T157, Akt-dependent phosphorylation of T198 also results in cytoplasmic retention of p27 (31). Indeed, in Akt-transfected HEK293 cells, single T157A or T198A mutants of p27 localize in the cytoplasm, whereas a double p27 T157A/T198A mutant is completely resistant to Akt-mediated cytoplasmic relocalization (32). In agreement with these data, phosphorylation of both T157 and T198 have been shown to be required for binding 14.3.3, the anchor proteins that keep p27 in the cytoplasmic compartment (30, 31). These data suggest that T198 phosphorylation is required for cytoplasmic localization and potentially explain the inability of the p27 S10A/T157A mutant to be retained in the cytosol. However, p27ΔNLS with neutral hydrophobic residues replacing positive residues in the NLS (Fig. 1), localized exclusively in the cytosol and increased motility and survival with no effect on cell cycle distribution. These data agree with a previous report by McAllister et al. (15), in which deletions or mutations in the COOH-terminal region of p27 affected cell migration but not cell cycle arrest. Further, a truncated form of p27 containing amino acids 1 to 103 binds Cdk2 and induces G1 arrest without affecting cell scattering or filopodia formation (14), implying that p27ΔNLS had not lost Cdk2 binding.

Multiple data suggest that partial loss and/or mislocalization of p27 is associated with enhanced transformation and, second, that complete loss of p27 might oppose proliferation and maintenance of the transformed phenotype. For example, homozygous deletions of the p27 gene are exceedingly rare in human cancers (3). Loss of a single allele of p27 confers increased susceptibility to carcinogen-induced lung, intestine, and pituitary tumors in mice (4) as well as tumors induced by oncogenes or by loss of tumor suppressor genes in genetically engineered mice (17, 33). In the latter group, tumors occurring in p27+/− mice exhibit higher levels of nuclear and/or total cyclin D1, consistent with the role of p27 in the assembly of cyclin D1/Cdk4 complexes and facilitation of cyclin D1 translocation to the nucleus (12, 34). Furthermore, the wild-type p27 allele is retained in the p27+/− tumors. Consistent with this, p27-null mammary glands display low proliferation, severely altered morphogenesis, and low cyclin D1 levels (13). A second study did not confirm these last findings although enhanced mammary gland apoptosis was observed during pregnancy (35). Finally, malignant behavior and cyclin D1 levels are inhibited in p27−/− transgenic tumors compared with tumors with wild-type p27 (17, 33). Taken together, these data suggest that cancers with low p27 levels or with p27 haploinsufficiency lack a selective pressure to lose p27 function completely, as this would result in loss of cyclin D1/Cdk4 function.

Cytoplasmic localization of p27 has been associated with anchorage-independent growth of tumor cells, higher tumor stage, and poor patient outcome in some cancer types (9, 36–40). Oncogenic effects of cytoplasmic p27, other than cyclin D1/Cdk4 assembly, would further abrogate any selective pressure to eliminate p27 completely. Data presented herein with PTEN-mutant U87 glioma cells support an oncogenic role for cytoplasmic p27 in that its elimination by RNA interference resulted in reduced tumorigenicity, cancer cell viability, and invasiveness in vivo. Stable expression of h27 shRNAs, which should also interfere with nuclear p27 in these cells and hence increase growth, did not accelerate U87 cell proliferation. This results suggests that U87 cell proliferation might be under maximal stimulation by alternative signaling pathways that do not require p27-mediated inhibition of Cdk2.

Oncogenic Function of Cytoplasmic p27

There are few other examples of a prosurvival role of p27. p27−/− mammary glands exhibit increased postlactational involution and apoptosis (13). Mouse mesangial cells and rat fibroblasts that are deficient in p27 undergo apoptosis upon serum starvation. In these cells, inhibition of Cdk2 protected these cells from apoptosis (41). In leukemic cells, proteolytic fragments of overexpressed p27 prevent apoptosis, possibly through inhibition of cytochrome c release (42). In experimental glomerulonephritis and in small-cell lung cancer cells, there is clear evidence that p27 protects against inflammatory injury and/or conditions of nutrient and oxygen deprivation (43, 44). Finally, in T98G glioma cells, down-regulation of both p27 and its ubiquitin ligase Skp2 using siRNA induces apoptosis (45). In patients with breast cancer, high levels of p27 are associated with axillary lymph node metastases and shorter survival (46, 47). High p27 expression has been reported in virtually all cases of small-cell lung cancer (43). Although these associations would seem to be paradoxical, it seems, from data shown in these reports, that the expression of p27 was not limited to tumor cell nuclei, thus raising the likely possibility of cytoplasmic p27 is present in the more metastatic tumors.

In COS7 cells with p27 predominantly in the nucleus, where its tumor suppressive and Cdk-inhibitory effects predominate, knockdown of p27 derepressed cell cycle progression without detectable induction of apoptosis. Thus, cytoplasmic p27 can be construed as a therapeutic target and/or a biomarker of aberrant PE3K/Akt signaling in human cancer cells. However, as suggested by the results with COS7 cells, therapeutic inhibition of nuclear p27 in cells with mostly nuclear expression of the Cdk inhibitor might accelerate tumor growth. Viral vector-mediated overexpression of p27 has been shown to induce cell cycle arrest and/or induce apoptosis of tumor cells in culture (48–54). The purpose of this approach has been at least in part to restore Cdk inhibition in tumors with low p27 expression. Recent data as well as results presented herein suggest reexamination of reconstitution of p27 as a therapeutic strategy in cancer. First, it is unlikely that with current viral vector approaches, the transduction efficiency and levels of p27 observed in tumor cells in culture will be achieved in vivo. Second, Cdk2 has been found to be dispensable for tumor cell growth (55). Third, in cancer cells with aberrant oncogene signals that retain p27 in the cytosol, this strategy may amplify oncogenic functions of the “tumor suppressor” and be clinically counterproductive.

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References
Reduction of Cytosolic p27^Kip1 Inhibits Cancer Cell Motility, Survival, and Tumorigenicity

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