A Novel Function for the Tumor Suppressor p16^{INK4a}: Induction of Anoikis via Upregulation of the $\alpha_5\beta_1$ Fibronectin Receptor

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Abstract. The tumor suppressor gene p16^{INK4a} inhibits the kinase activity of the cyclin-dependent kinase 4–6/cyclin D complexes and subsequent phosphorylation of critical substrates necessary for transit through the G1 phase of the cell cycle. Recent studies suggested that control of the G1/S boundary might not be the sole biological function of p16^{INK4a}. We hypothesized that p16^{INK4a} might influence hitherto unknown critical features of a malignant epithelial phenotype, such as anchorage dependence. Here we provide evidence that stable transfection of p16^{INK4a} restitutes apoptosis induction upon loss of anchorage (anoikis) in a variety of human cancer cells. Anoikis in p16^{INK4a}-transfected cells was evidenced by DNA fragmentation and poly(ADP-ribose) polymerase cleavage upon cultivation on polyhydroxyethylmethacrylate-coated dishes and was associated with suppression of anchorage-independent growth as well as complete loss of tumorigenicity. p16^{INK4a}-mediated anoikis was due to selective transcriptional upregulation of the α_5 integrin chain of the $\alpha_5\beta_1$ fibronectin receptor as detected by FACS® analysis, immunoprecipitation, Northern blotting, and nuclear run-on assays. Addition of soluble fibronectin and inhibitory α_5 antibodies to nonadherent cells completely abolished p16^{INK4a}-mediated anoikis, whereas laminin was ineffective. Furthermore, antisense-induced downregulation of the α_5 integrin chain in p16^{INK4a}-transfected cells restored resistance to anoikis. These data suggest a novel functional interference between a cell cycle–regulating tumor suppressor gene and membrane-bound integrins, thus regulating a hallmark feature of an epithelial transformed phenotype: susceptibility to anoikis.

Key words: tumor suppressor p16^{INK4a} • anoikis • fibronectin • integrin • tumorigenicity

Introduction

The tumor suppressor p16^{INK4a}, inhibitor of cyclin-dependent kinase (Cdk)[1] 4, represents a major target in human carcinogenesis (Okamoto et al., 1994). Functional inactivation of p16^{INK4a} has been described in a wide variety of human malignancies and transformed cell lines (Serrano, 1997). Underlying mechanisms involved in p16^{INK4a} inactivation comprise homozygous deletions, intragenic mutations, and transcriptional silencing due to promoter hypermethylation (Yang et al., 1995; Liggett and Sidransky, 1998; Caldas et al., 1994). In contrast to p16^{INK4a}, other members of the INK4 family (p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}) play minor roles in tumorigenesis (Schwaller et al., 1997). The p16^{INK4a} gene, which is located on chromosome 9p21, encodes a protein of 156 amino acids comprising 4 ankyrin repeats. The main biological function of p16^{INK4a} involves regulation of cell cycle progression at the G1/S boundary (for review see Ruas and Peters, 1998). Progression through G1 requires Cdk4–6/cyclin D–dependent phosphorylation of the retinoblastoma protein (Rb) and the related "pocket proteins," p107 and p130, resulting in liberation of associated transcription factors necessary for DNA replication during S phase of the cell cycle. p16^{INK4a} acts in the so termed p16^{INK4a}/Cdk4/cyclin D1/Rb pathway by selectively binding to and inhibiting Cdk4–6, thus preventing pRb phosphorylation and resulting in cell cycle arrest at the G1 checkpoint (Sherr, 1996; Serrano, 1997).

Recent experimental evidence suggested additional biological functions for p16^{INK4a}. Apart from cell cycle con-

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[1]*Abbreviations used in this paper:* Cdk, cyclin-dependent kinase; GAPDH, glyceraldehyde 3-phospate dehydrogenase; MDCK, Madin-Darby canine kidney; PARP, poly(ADP-ribose) polymerase; polyHEMA, polyhydroxyethylmethacrylate; Rb, retinoblastoma protein; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP biotin nick end labeling.

trol, $p16^{INK4a}$ has been implicated in other fundamental cellular processes, such as angiogenesis (Harada et al., 1999), cell senescence (Votja and Barrett, 1995), tumor invasion (Chintala et al., 1997), cell spreading (Fåhraeus and Lane, 1999), and apoptosis (Sandig et al., 1997; Naruse et al., 1998). In part, $p16^{INK4a}$ elicits such pleiotropic effects by modulating the expression or function of distinct key target molecules like vascular endothethial growth factor (Harada et al., 1999), matrix metalloproteinase 2 (Chintala et al., 1997), nuclear factor κB (Wolff and Naumann, 1999), β1,4-galactosyltransferase (Zhang et al., 1999), integrin $\alpha_v\beta_3$ (Fåhraeus and Lane, 1999), or pRb (Fang et al., 1998).

In contrast to their malignant counterparts, nontransformed epithelial cells will undergo apoptosis when they lose contact with their underlying matrix. In a physiological context, this phenomenon is observed in normal skin (Polakowska et al., 1994), colonic epithelium (Hall et al., 1994), and in the involuting mammary gland (Boudreau et al., 1995). Induction of apoptosis upon loss of anchorage has been termed anoikis (Greek for homelessness) (Frisch and Ruoslahti, 1997) and has been shown to occur in epithelial (Frisch and Francis, 1994) and endothelial cells (Meredith et al., 1993). Anoikis can be considered as a safety program for the organism, which prevents survival and reattachment of detached cells to new matrices at inadequate locations. During malignant transformation, most epithelial cells will become resistant to anoikis, thereby gaining an important growth advantage and providing the basis for tumor spread, growth at distant places, and formation of metastasis.

Integrins, which are large glycoproteins that function as transmembrane receptors, mediate cell adhesion to extracellular matrix and participate in signal transduction. They are involved in the regulation of complex biological processes such as angiogenesis, tumor cell migration, invasion, and metastasis (Hynes, 1992; Schwartz et al., 1995). Furthermore, they were identified as central mediators of anoikis in several cell types (Varner and Cheresh, 1996; Frisch and Ruoslahti, 1997). Currently, 8 β and 17 α integrin subunits have been characterized that associate with heterodimers and generate >20 different integrins (Lafrenie and Yamada, 1996). The integrin $\alpha_5\beta_1$ represents a specific fibronectin receptor which plays a central role in anchorage-dependent survival and apoptosis and is frequently lost or down-regulated in transformed cells. Conversely, ectopic expression of integrin $\alpha_5\beta_1$ has been demonstrated to regulate tumor cell proliferation in vitro as well as tumor growth in vivo (Giancotti and Ruoslahti, 1990; Symington, 1990; Varner et al., 1995).

We now provide evidence that the tumor suppressor $p16^{INK4a}$ induces anoikis via a novel mechanism involving upregulation of the fibronectin receptor $\alpha_5\beta_1$.

Materials and Methods

Cell Culture

The human pancreatic carcinoma cell line Capan-1 (HTB 79; American Type Culture Collection) was grown in monolayer cultures in RPMI medium (Life Technologies) supplemented with 15% fetal calf serum (Biochrom), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. The human melanoma cell line NKI4, donated by Dr. J. Eberle (Freie Universität Berlin, Universitätsklinikum der Freien Universität Berlin, Berlin, Germany), and Madin-Darby canine kidney (MDCK, CCL-34; American Type Culture Collection) cells were cultivated in DMEM medium (Life Technologies) supplemented with 10% fetal calf serum. The human hepatocellular carcinoma cell line SKHep1, provided by Dr. D. Schuppan (University of Erlangen, Erlangen, Germany), was cultivated in RPMI medium supplemented with 20% fetal calf serum.

Generation of Stably Transfected $p16^{INK4a}$-expressing Cell Clones

Full-length human-$p16^{INK4a}$ cDNA was subcloned into the eukaryotic expression vector pRC/CMV (Invitrogen), where $p16^{INK4a}$ is under control of the CMV promoter and can be selected by neomycin resistance. Transfections were done using lipofectamine (Life Technologies), which generated liposome complexes. For stable transfections, 5 μg plasmid and 30 μl lipofectamine were incubated in 1.6 ml medium for 45 min at room temperature. 10^6 cells plated on a 10-cm dish were exposed to the formed liposome complexes diluted in 8 ml medium for 6 h. Cells were washed and maintained in fresh medium overnight. After trypsinization, resuspended cells were portioned into three 20-cm dishes and selection was done with G418 (Life Technologies; 1 mg/ml for Capan-1 and MDCK, 0.4 mg/ml for NKI4, and 1.5 mg/ml for SKHep1) for 3 wk. Independent stable clones were isolated and cultivated in the presence of G418. The vector pRC/CMV without insert was stably transfected in parallel to generate mock-transfected control clones. Downregulation of the α_5 integrin chain in $p16^{INK4a}$-expressing cells was done by cotransfecting the plasmid pCMV5′ α_5 antisense (provided by B. von Lampe, Freie Universität Berlin, Universitätsklinikum der Freien Universität Berlin, Berlin, Germany), which contains human α_5 antisense cDNA and the expression vector pIRES1-hyg (CLONTECH Laboratories, Inc.), and can be selected by hygromycin resistance. 12 μg of each plasmid and 120 μl lipofectamine were incubated for 45 min at room temperature in 6 ml media. Liposome complexes were diluted in 20 ml media and added to 1.5×10^7 cells plated the day before on a 20-cm dish. Transfected cells were selected in the presence of 0.1 mg/ml hygromycin (Life Technologies) and 1 mg/ml G418.

Immunoprecipitation and Western Blot Analysis

Cells grown to subconfluency were lysed in 50 mM Hepes, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween 20, 1 mM DTT, 1 mM sodium fluoride, 10 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 3 μg/ml aprotinin, and 2 μM leupeptin. Cell lysates were immunoprecipitated with rabbit anti–human Cdk4 pAb (Santa Cruz Biotechnology, Inc.), and immune complexes were recovered with protein A–Sepharose beads (Sigma-Aldrich) overnight at 4°C. Equal amounts of immunoprecipitated proteins were separated on SDS-PAGE gels and electroblotted to PVDF membranes (NEN Life Science Products). Membranes were sequentially probed with a monospecific mouse anti–human anti-$p16^{INK4a}$ antibody (final dilution 1:1,000; Neomarkers) or anti-Cdk4 antibody (1:1,000; BD PharMingen) overnight at 4°C. After exposure for 2 h at room temperature to secondary goat anti–mouse horseradish peroxidase–conjugated antibody (1:20,000; Dako), protein bands were visualized using the ECL system (Amersham Pharmacia Biotech).

Anchorage-independent Growth Assays

For evaluation of anchorage-independent growth, cells were analyzed in a human tumor clonogenic assay. 10^3 cells were diluted in 1 ml methylcellulose/agar mixture resolved in Iscove's medium (Life Technologies) supplemented with 27% fetal bovine serum (HyClone). Cells were plated in triplicates on 3-cm dishes and incubated for 12 d. Only cell aggregates of >20 cells were evaluated, and vital colonies were counted with an inverted light microscope.

Tumor Growth in Nude Mice

Female inbred Swiss *nu/nu* mice weighing ~25 g were obtained from Charles River Laboratories. Animals were purchased as specific pathogen free and received water and food ad libitum. Mice were subcutaneously injected with 1.5×10^6 Capan-1 cells into the right inguinal fat pad. Each experimental group consisted of at least seven animals. Tumor-bearing mice were examined for palpable primary tumors twice weekly. Growth

of tumors was determined by caliper measurements of the largest diameter and its perpendicular. Tumor size was calculated as: tumor volume (mm^3) = $0.5 \times a \times b^2$, where a is the largest diameter and b is its perpendicular. Two independent experiments were performed to ensure the reproducibility of the results. Tumors were embedded in paraffin. The lung, the liver, and the spleen were removed and fixed in Bouin's solution to identify potential metastatic tumor spread.

In Situ Apoptosis Detection

Detection of apoptotic cells in mouse xenografts was performed using the TACS in situ apoptosis detection kit (Genzyme), following the manufacturer's instructions. In brief, after deparaffinization of tissues, apoptotically generated DNA fragments were labeled by incorporation of biotinylated nucleotides using terminal deoxynucleotidyl transferase in a buffer including manganese. Incorporated biotinylated nucleotides were detected using a streptavidin–horseradish peroxidase conjugate with DAB as a substrate, which produces a brown color in apoptotic cells. Intact cells were counterstained with methyl green.

Analysis of Integrin Expression by Flow Cytometry

10^6 cells were resuspended in 100 μl PBS containing the appropriate dilutions of subunit-specific integrin antibodies (α_1 [1:20; T-Cell Sciences], α_2 [1:100; dianova], α_3 [1:20; Oncogene], α_4 [1:100; dianova], α_5 [1:50; Dako; clone P1D6], α_6 [1:25; dianova], α_v [1:100; dianova], β_1 [1:50; dianova], β_3 [1:75; dianova], β_4 [1:100; Telios], and β_5 [1:100; dianova]). After incubation for 15 min at 4°C, cells were washed with PBS and incubated with a secondary FITC-conjugated antibody (goat anti–mouse FITC, 1:50; Sigma-Aldrich) or for α_6 integrin subunit with rabbit anti–rat FITC (1:50; Sigma-Aldrich). Integrin expression levels compared with mouse Ig control antibody (1:100; Dako) were then determined by flow cytometry using a FACSCalibur™ (Becton Dickinson). Evaluation was done using the CellQUEST™ program (Becton Dickinson).

α_5 Integrin Immunoprecipitation

10^7 cells were incubated with 1 μg/μl biotin-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer) for 5 min at room temperature in labeling buffer (137 mM NaCl, 5 mM KCl, 6 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 g/liter glucose) for 5 min at room temperature and lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM $CaCl_2$, 2 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, and 1 mM pepstatin A. Biotinylated protein lysate (100 μg) was immunoprecipitated by integrin anti-α5 antibody P1D6 (Dako) using rabbit anti–mouse IgG conjugated to protein A–Sepharose (Sigma-Aldrich). Extensively washed immunoprecipitates were separated on SDS-PAGE gel and electroblotted to PVDF membranes. Biotinylated protein bands were detected by streptavidin–horseradish peroxidase (Sigma-Aldrich) and visualized using the ECL system (Amersham Pharmacia Biotech).

α_5 Integrin Northern Blot

mRNA was isolated from Capan-1 cells with a Poly A+ tract kit (Promega) according to the manufacturer's protocol. Purified RNA was quantified by spectrophotometric measurement. 5 μg of each sample was separated on a 1% agarose/MOPS/formaldehyde gel. After electrophoresis, gels were capillary blotted overnight to Hybond-N filters (Amersham Pharmacia Biotech). RNA was then immobilized by UV cross-linking. The vector pcDNA3/α_5 containing full-length human α_5 cDNA, was provided by Dr. K. Schramm (Freie Universität Berlin, Universitätsklinikum der Freien Universität Berlin, Berlin, Germany). The α_5 insert was released by HindIII digestion, purified by gel extraction, and randomly labeled with [α-^{32}P]dCTP using a Megaprime DNA labeling kit (Amersham Pharmacia Biotech). The cDNA probe was purified on a Sephadex G-50 column to remove unincorporated nucleotides. Hybridization was carried out at 65°C with Quick-Hyb buffer (Stratagene). Membranes were washed at 65°C using a stringency of 0.1× SSC, 0.1% SDS, and exposed to x-ray films at −80°C.

α_5 Integrin Nuclear Run-on

Nuclear run-on transcription was performed as described previously by Rosewicz et al. (1994). In brief, nuclei from 10^8 cells were purified by centrifugation through a sucrose cushion. Transcribed RNA products labeled through [α-^{32}P]UTP incorporation were purified by DNase I treatment, proteinase K digestion, phenol/chloroform extraction, and propanol precipitations. Unincorporated nucleotides were removed using the RNeasy mini kit (QIAGEN). Equal counts (5×10^6 cpm) of run-on probe were hybridized at 42°C for 48 h to cDNAs of $p16^{INK4a}$, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and α_5 integrin subunit (5 μg/slot) denatured at 65°C in 0.2 M NaOH before slot blotting on nitrocellulose membranes and immobilization by UV cross-linking. Supplementation of α-amanitin (2 μg/ml) was used as control for transcription specificity. After hybridization, membranes were washed with 2× SSC at 60°C for 1 h, then 2× SSC with RNase A (100 μg/ml) at 37°C for 30 min, and a final wash with 2× SSC and 0.5% SDS at 37°C for 1 h. Membranes were exposed to x-ray films at −80°C.

Induction of Anoikis

To prevent cell adhesion, 6-well plates were covered with a solution of polyhydroxyethylmethacrylate (polyHEMA; Sigma-Aldrich), dissolved at 10 mg/ml in ethanol. To coat 6-well plates, 3 ml of polyHEMA solution was added to each well. Plates were kept at 37°C for at least 3 d until the solvent had completely evaporated. To induce anoikis, 5×10^5 resuspended cells were cultured in RPMI medium containing 15% fetal calf serum for 20 h on polyHEMA-coated dishes at 37°C and 5% CO_2. Subsequently, cells were gently recovered and submitted to apoptosis detection assays.

Determination of Apoptotic Cells by Flow Cytometry

Cells were fixed with ice-cold ethanol (70% final concentration), resuspended in 0.5 ml solution of 20 μg/ml propidium iodide and 200 μg/ml DNase-free RNase in PBS, and incubated for 30 min at 37°C. For flow cytometric analysis, at least 10,000 cells were evaluated using a FACSCalibur™. Cell cycle distribution and pre-G_1 fraction were determined and quantitated using the CellQUEST™ program.

Poly(ADP-Ribose) Polymerase Western Blot

10^6 Capan-1 cells were lysed in 60 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromphenol blue, sonicated for 15 s, and incubated at 65°C for 15 min. Whole cell lysates were separated on a 10% SDS-PAGE gel. Full-length poly(ADP-ribose) polymerase (PARP) and the apoptosis-related cleavage fragment were detected by Western blotting with a monospecific mouse anti–human PARP antibody (1:100; Calbiochem) and visualized as described above (see Western blot analysis).

DNA Fragmentation

10^6 Capan-1 cells were lysed in 10 mM Tris-HCl, pH 8.2, 400 mM sodium chloride, 2 mM EDTA containing 1% SDS, and 50 μg/ml proteinase K. DNA was precipitated with ethanol, washed, dried, and resolved in TE buffer containing 50 μg/ml RNase A. Equal amounts of DNA (1 μg) were electrophoresed in a 1% agarose gel. Fragmented DNA was stained with ethidium bromide, visualized under UV light, and photographed. A 100-bp DNA ladder was electrophoresed in parallel for size determination.

Anoikis Protection Assay

To verify the specificity of the α_5 integrin–mediated effect on anoikis, 5×10^5 cells plated on polyHEMA-coated dishes were treated with various concentrations of soluble fibronectin, laminin (Sigma-Aldrich), or an inhibitory α_5 integrin antibody (10 mg/ml; Biomol). After 20 h incubation, anoikis was examined by FACS® analysis.

Results

Stable Transfection of $p16^{INK4a}$ Results in Binding to Cdk4

After stable transfection of human $p16^{INK4a}$ cDNA into the human pancreatic cell line Capan-1, 20 independently isolated $p16^{INK4a}$-positive clones were generated. All clones showed similar $p16^{INK4a}$ protein expression levels in Western blot analysis (data not shown). Therefore, three

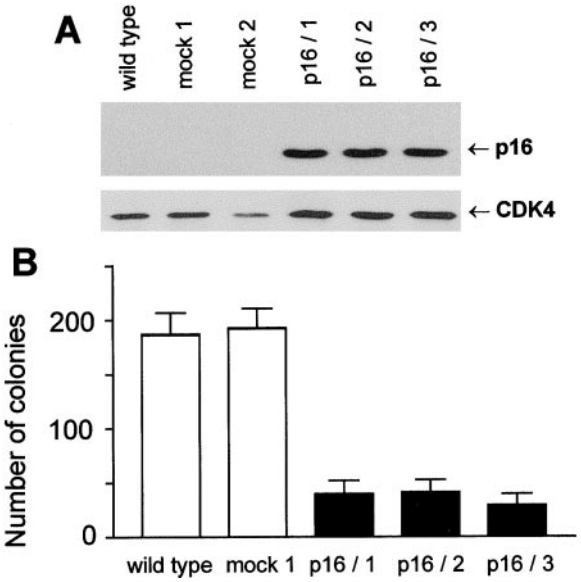

Figure 1. p16^{INK4a} inhibits anchorage-independent growth. (A) Human full-length p16^{INK4a} cDNA was cloned into the eukaryotic expression vector pRC/CMV and stably transfected in Capan-1 cells. Three randomly selected p16^{INK4a}-positive clones (p16/1,2,3) were chosen for further experiments. Cdk4 was immunoprecipitated from whole cell lysates of either control or p16^{INK4}-expressing clones. Immunoprecipitates were then analyzed by Western blotting using monospecific antibodies against p16^{INK4a} and Cdk4. (B) Anchorage-independent growth was examined using a soft agar colony formation assay. 10^3 cells were seeded in a methylcellulose/agar mixture and incubated for 12 d. Vital colonies of >20 cells were counted using a microscope. Shown are the mean ± SEM of three independent experiments, each performed in triplicates.

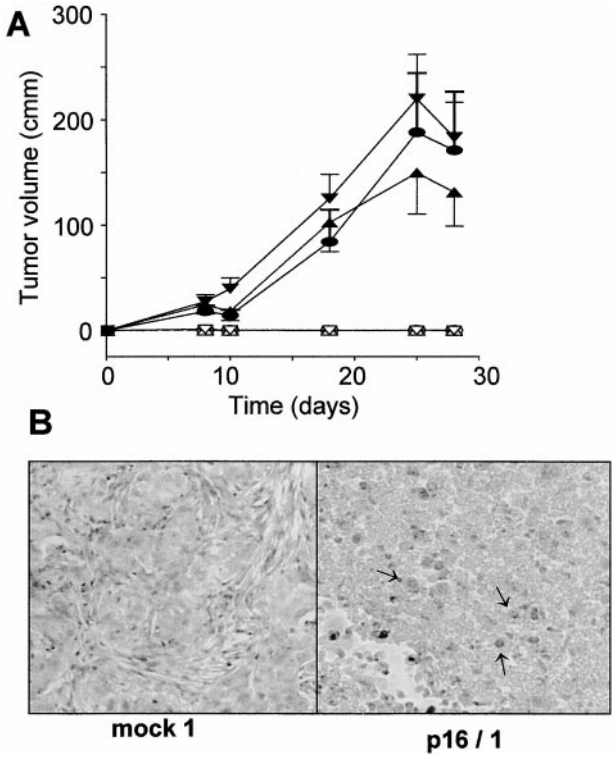

Figure 2. Tumorigenicity in vivo. (A) Nude mice were subcutaneously injected with 2×10^6 Capan-1 cells from either control clones (●, wild-type; ▲, mock 1; and ▼, mock 2) or p16^{INK4}-transfected clones (○, p16/1; ▽, p16/2; and △, p16/3). Each clone was injected in at least six animals. Tumor growth was then monitored at the indicated time points. Shown is a representative (mean ± SEM) of two independent experiments yielding identical results. (B) In situ apoptosis detection. Tissue sections of xenografts were prepared at day 2 after cell injection. Cellular apoptosis was detected using the TUNEL assay as described in Materials and Methods. Arrows indicate representative apoptotic cells characterized by a brown color (magnification, ×100).

p16^{INK4a}-positive clones, designated p16/1, p16/2 and p16/3, were randomly selected for further investigations. As shown in Fig. 1 A, the functional integrity of the expressed protein was confirmed based on its ability to bind Cdk4 by using Cdk4 immunoprecipitation followed by Western blot detection with a monospecific p16^{INK4a} antibody.

p16^{INK4a} Inhibits Anchorage-independent Growth

Cell–cell and cell–matrix interactions impose stringent control on normal, but not transformed, epithelial cells. Colony formation assays exploit the disruption of these contacts by isolation and immobilization of cells in soft agar to specifically monitor properties of the transformed phenotype. Therefore, effects of p16^{INK4a} reexpression on anchorage-independent cell growth were evaluated by monitoring colony formation in soft agar. Reexpression of p16^{INK4a} reduced the number of viable colonies to 20–25% compared with controls (Fig. 1 B). At microscopic examination, a large fraction of p16^{INK4a}-transfected cells shrank and displayed fragmented nuclei after few days, suggesting induction of apoptosis rather than prolonged cell cycle arrest.

p16^{INK4a} Confers Loss of Tumorigenicity in Nude Mice

To directly evaluate the role of p16^{INK4a} for tumor formation in vivo, we xenotransplanted p16^{INK4a}-transfected and p16^{INK4a}-negative cells into nude mice. Mice treated with parental and mock-transfected cells developed solid tumors with a take rate of 100% (n = 42), and an average tumor volume of 150 cmm 3 wk after injection. In striking contrast, no measurable tumor formation occurred in mice that received p16^{INK4a}-expressing tumor cells (n = 40) (Fig. 2 A). These results were confirmed in two independent experiments.

To follow up the fate of p16^{INK4a}-expressing cells, we investigated the subcutaneously injected cells for the presence of apoptosis 2 d after xenotransplantation. Paraffin-embedded tissues were screened for apoptotic cells using the terminal deoxynucleotidyl transferase–mediated dUTP biotin nick end labeling (TUNEL) technique (Fig. 2 B). Whereas p16^{INK4a}-negative control cells formed adenocarcinomas without evidence of relevant apoptosis, p16^{INK4a}-positive cells failed to organize into tumor structures but instead underwent massive cell death due to apoptosis.

p16^{INK4a} Induces Anoikis In Vitro

Since matrix detachment represented a potential trigger for the apoptosis induction observed in vivo, this hypothe-

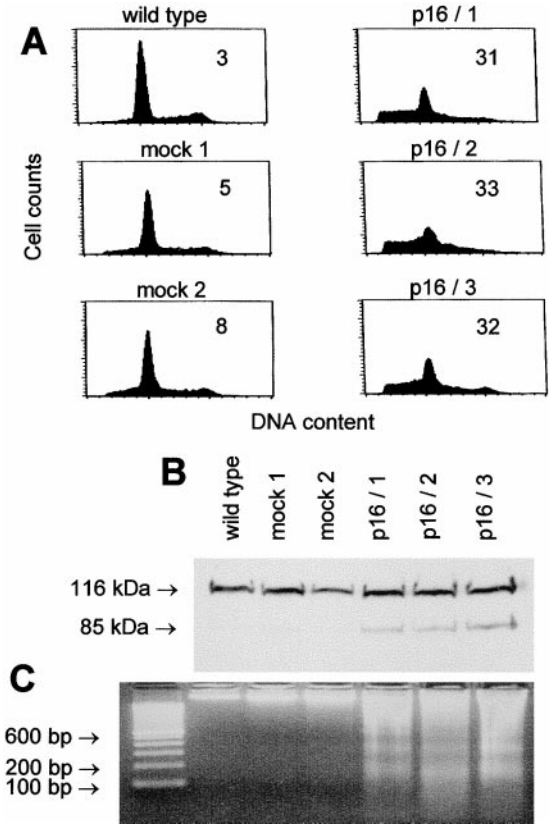

Figure 3. Analysis of anoikis. Anoikis was examined by three different experimental procedures. For all experiments, 5×10^5 resuspended cells were plated on polyHEMA-coated dishes. After 20 h, cells were harvested and subsequently analyzed. A representative of three independent experiments yielding identical results is shown. (A) FACS® analysis. Cells were ethanol-fixed, stained with propidium iodide, and analyzed by flow cytometry. The percentage of cells containing subdiploid DNA is indicated in each panel. (B) PARP cleavage. Cell lysates were prepared and immunoblotted with an mAb to PARP. The indicated molecular mass of the full-length PARP protein and its apoptotic cleavage fragment were deduced from a molecular size marker electrophoresed in parallel. (C) DNA laddering. From each clone, genomic DNA was extracted and 1 μg was electrophoresed on a 1% agarose gel. Fragment sizes of the 100-bp marker are indicated.

sis was tested using an in vitro anoikis assay. PolyHEMA, due to its uniformly nonionic nature, prevents cell attachment and deposition of matrix components, thus maintaining cells in suspension (Folkman and Moscona, 1978). Therefore, we examined anoikis by culturing cells on nonadhesive polyHEMA-coated dishes and subsequently analyzed cell cycle distribution by flow cytometry. A five- to sixfold increase of apoptosis (subdiploid DNA content in pre-G_1) was detected in $p16^{INK4a}$-transfected cells (p16/1, 31%; p16/2, 33%; and p16/3, 32%) compared with controls (wild-type, 3%, mock 1, 5%, mock 2, 8%) (Fig. 3 A) at 20 h. Induction of anoikis was detectable as early as 3 h and increased time-dependently with a maximum at 20 h after plating on polyHEMA (data not shown). $p16^{INK4a}$-mediated induction of anoikis was further confirmed by apoptosis-specific cleavage of the mitochondrial PARP protein (Fig. 3 B) and appearance of oligonucleosomal DNA fragmentation (DNA laddering) (Fig. 3 C), both of which were virtually absent in $p16^{INK4a}$-negative controls.

$p16^{INK4a}$ Mediates Anoikis in Different Cell Types

We next investigated whether $p16^{INK4a}$-mediated induction of anoikis was cell type specific. We therefore stably transfected $p16^{INK4a}$ in cell lines lacking endogenous $p16^{INK4a}$ from different origins such as liver (SKHep1), skin (NKI4), and kidney (MDCK). Expression of $p16^{INK4a}$ in transfected clones was confirmed by Western blotting using $p16^{INK4a}$-specific antibodies (Fig. 4 A). Upon reintroduction of $p16^{INK4a}$, anoikis increased five- to sixfold in Capan-1 cells. In NKI4 and SKHep1 cells, $p16^{INK4a}$ reexpression resulted in a four- to fivefold increase of anoikis in two independent transfected cell clones compared with parental and mock-transfected controls (Fig. 4 B). Moreover, stable transfection of $p16^{INK4a}$ in two MDCK cell clones resulted in an increase of anoikis of 354 ± 64%

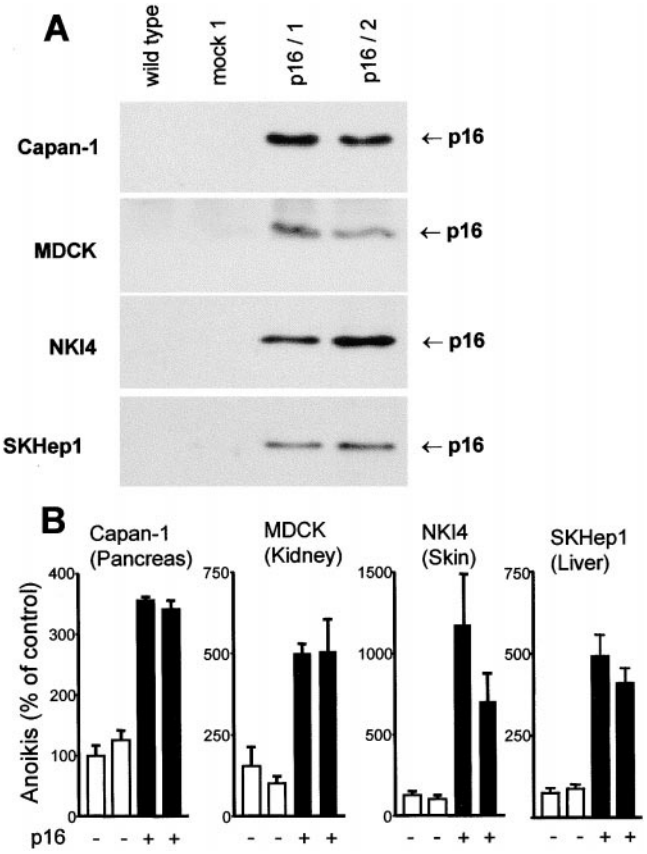

Figure 4. $p16^{INK4a}$ induces anoikis in cell lines from different origin. (A) $p16^{INK4a}$ was stably transfected into pancreatic tumor cell line Capan-1, melanoma cell line NKI4, hepatocellular carcinoma cell line SKHep1, and kidney cell line MDCK. Expression of $p16^{INK4a}$ was confirmed by Western blotting using monospecific $p16^{INK4a}$ antibodies. (B) For determination of anoikis, 5×10^5 cells of each control or $p16^{INK4a}$-positive clone were plated on polyHEMA-coated dishes and incubated for 6 h (MDCK), 20 h (Capan-1 and SKHep1), or 72 h (NKI4). The amount of apoptotic cells was subsequently determined by FACS® analysis. Shown are the mean ± SEM of three independent experiments, each performed in triplicates.

compared with p16^{INK4a}-negative mock-transfected controls (Fig. 4 B).

Effects of p16^{INK4a} on Integrin Expression

Because cell–matrix interactions are critically determined by integrins, we hypothesized that p16^{INK4a} regulates anoikis via modulation of integrin expression. Using a broad panel of monospecific antibodies against various integrins in flow cytometry analysis, we compared the integrin expression pattern in relation to the p16^{INK4a} status. Compared with isotype-matched control antibodies, Capan-1 cells expressed α_1, α_2, α_3, α_5, α_6, α_v and β_1, β_4, β_5 integrins, whereas no expression of the α_4 and β_3 integrin subunits could be detected (Fig. 5 A). Most integrins were equally expressed in both cell populations regardless of the p16^{INK4a} status. In contrast, we observed a selective and significant increase of the α_5 integrin subunit in all p16^{INK4a}-expressing cell clones compared with controls (Fig. 5 A). It is noteworthy that the expression level of the β_1 subunit, which is the unique partner for α_5 to form the $\alpha_5\beta_1$ fibronectin receptor and also forms heterodimers with eight other α subunits, was not influenced by p16^{INK4a} (Fig. 5 A).

To confirm the increased expression of the α_5 integrin subunit observed by flow cytometry, we examined α_5 expression in more detail. As revealed by immunoprecipitation, Northern blotting, and nuclear run-on assays, expression of the $\alpha_5$integrin subunit was significantly increased upon reintroduction of p16^{INK4a}. Using α_5 immunoprecipitation of cell surface biotinylated proteins, we observed a three- to fivefold increase of the α_5 integrin chain in p16^{INK4a}-transfected cells compared with p16^{INK4a}-negative controls (Fig. 5 B). As expected, the β_1 integrin subunit of the $\alpha_5\beta_1$ fibronectin receptor was coimmunoprecipitated by the α_5 antibody, reflecting the assembly of intact fibronectin receptor in the transfected clones (Fig. 5 B). Similarly, Northern blot analysis (Fig. 5 C) and nuclear run-on experiments (Fig. 5 D) revealed three- to fourfold increased α_5 mRNA concentrations in p16^{INK4a}-expressing cells, indicating that reexpression of p16^{INK4a} results in transcriptional upregulation of the α_5 integrin subunit.

Fibronectin Selectively Inhibits p16^{INK4a}-mediated Anoikis

To further support the hypothesis that p16^{INK4a} induces anoikis via upregulation of the unligated $\alpha_5\beta_1$ fibronectin receptor, the effects of fibronectin ligand on induction of anoikis were examined. Fibronectin is the only known ligand for integrin $\alpha_5\beta_1$, but it is also recognized by other integrins (Akiyama et al., 1995). Soluble fibronectin added to cells plated on polyHEMA-coated dishes completely inhibited p16^{INK4a}-mediated anoikis, whereas it had no effect on wild-type or mock-transfected control cells (Fig. 6 A). Inhibition of anoikis by soluble fibronectin occurred in a dose-dependent manner with a maximal and complete inhibition at 0.3 mg/ml of soluble fibronectin (Fig. 6 B). Furthermore, the anoikis inhibitory effects were specific for fibronectin, as other extracellular matrix components, such as collagen type IV (data not shown) or laminin, had no influence on anoikis (Fig. 6 A). Therefore, soluble fibronectin blocked the integrin $\alpha_5\beta_1$–mediated apoptotic signals in nonadherent cells.

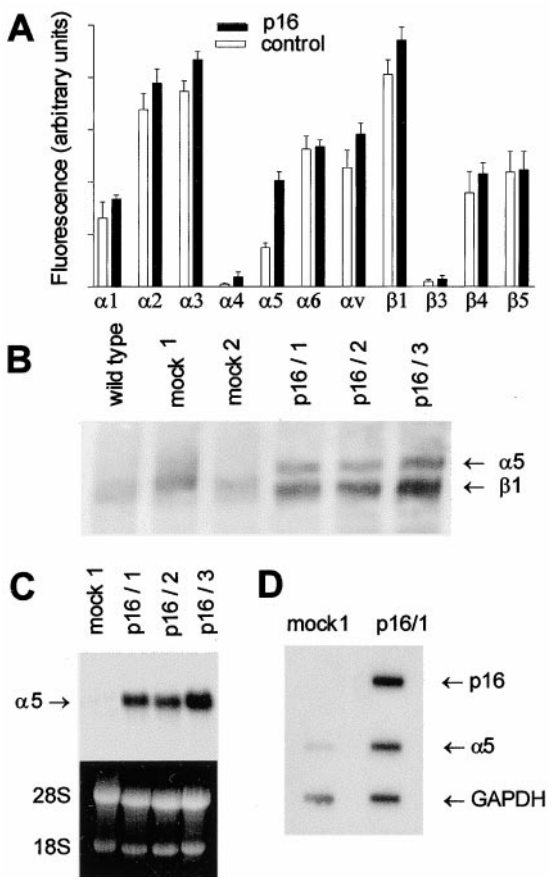

Figure 5. Analysis of integrin expression. (A) Capan-1 cells were incubated with the indicated mAb against various α and β integrin subunits and subsequently analyzed by flow cytometry. Results from three control (wild-type, mock 1/2) and three p16 (1/2/3) clones are summarized and the mean ± SEM of two independent experiments is shown. (B) Biotinylated cell surface proteins were immunoprecipitated with an α_5 integrin mAb and detected using a streptavidin-peroxidase conjugate in ECL visualization. The size of α_5 and β_1 integrin subunits was deduced from markers electrophoresed in parallel. (C) Poly A+ purified mRNA (5 μg/lane) of the indicated cell clones was analyzed by Northern blotting using a [^{32}P]dCTP-labeled α_5 integrin cDNA probe. Bands reflecting α_5 integrin mRNA (4.2 kb) and rRNAs are indicated. As loading control, samples were stained with ethidium bromide before blotting (bottom). (D) Nuclei were prepared from a mock-transfected and a p16^{INK4a}-positive clone. After in vitro transcription in the presence of [α-^{32}P]UTP, ^{32}P-labeled RNA (5×10^6 cpm) was hybridized for 48 h to cDNAs of p16^{INK4a}, GAPDH, and α_5 integrin subunit immobilized on a nitrocellulose membrane. No hybridization signal was detectable when α-amanitin was added to the transcription reaction, confirming the specificity of the observed hybridization signals (not shown). One representative autoradiograph out of three independent experiments yielding identical results is shown.

To further investigate the role of $\alpha_5\beta_1$ integrin in p16^{INK4a}-mediated anoikis, we disturbed the $\alpha_5\beta_1$ integrin–fibronectin interaction by the addition of an inhibitory anti-α_5 mAb, which completely abolished soluble fibronectin-mediated inhibition of anoikis (Fig. 6 B). Anti-

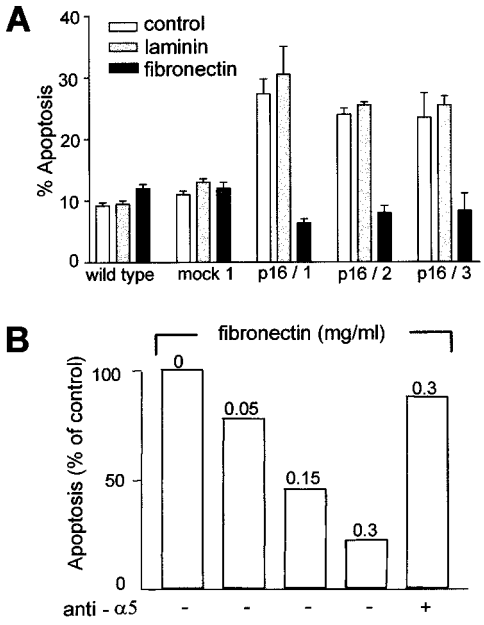

Figure 6. Effects of fibronectin on anoikis. (A) 5×10^5 cells were plated on polyHEMA dishes and incubated for 20 h with soluble fibronectin (0.3 mg/ml) or laminin (0.15 mg/ml). Apoptotic cells were subsequently quantitated by flow cytometry. Shown is the mean ± SEM of three independent experiments. (B) 5×10^5 cells of Capan-1 clone p16/1 were plated on polyHEMA dishes and incubated with the indicated concentrations of soluble fibronectin and inhibitory anti-α_5 antibody for 20 h. Apoptosis was detected and quantitated by flow cytometry measuring the apoptotic pre-G1 peaks. One representative of three experiments is shown.

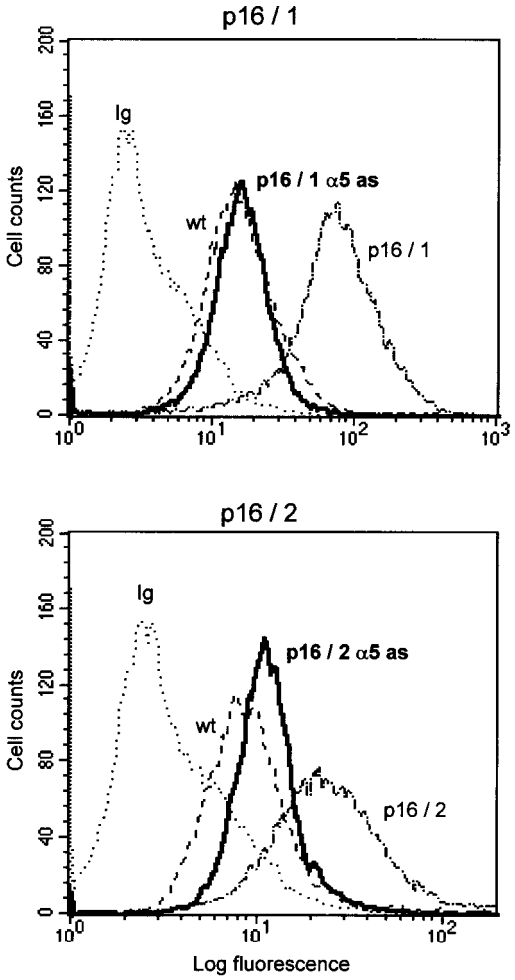

Figure 7. Antisense-induced downregulation of the α_5 integrin chain. 1.5×10^7 cells of Capan-1 clone p16/1 and p16/2 were cotransfected using lipofectamine with the vectors pCMV-5′ α_5/antisense and pIRES1-hyg. After 4 d of selection in the presence of 0.1 mg/ml hygromycin, cells were pooled and α_5 expression was detected by FACS® analysis using a monospecific antibody against α_5 integrin chain and immunoglobulins (Ig) as isotype control. Demonstrated are the α_5 expression levels of Capan-1 wild-type (wt), $p16^{INK4a}$-transfected (p16/1), and $p16^{INK4a}$ plus α_5 antisense cotransfected (p16/α_5 as) Capan-1 clones. A representative out of three experiments yielding identical results is shown.

body-mediated impairment of α_5–fibronectin interaction dose-dependently restituted $p16^{INK4a}$-dependent anoikis (not shown). These results provide further support that $p16^{INK4a}$-mediated anoikis is directly correlated with overexpression of the unligated fibronectin receptor $\alpha_5\beta_1$.

Downregulation of the α_5 Integrin Subunit Inhibits $p16^{INK4a}$-mediated Anoikis

To more directly evaluate the relationship between $p16^{INK4}$-mediated $\alpha_5\beta_1$ upregulation and restoration of susceptibility to anoikis, we inhibited the expression of the α_5 chain in two independent $p16^{INK4a}$-transfected clones by transfecting an antisense α_5 cDNA construct. As shown by FACS® analysis, overexpression of α_5 antisense cDNA resulted in inhibition of α_5 protein expression comparable to $p16^{INK4}$-negative wild-type or mock-transfected controls (Fig. 7). Subsequent cell cycle distribution (Fig. 8, A and B) and PARP cleavage analysis (Fig. 8 C) revealed that downregulation of the α_5 subunit by antisense transfection resulted in almost complete inhibition of anoikis compared with $p16^{INK4a}$-positive controls. These data provide direct evidence that $p16^{INK4a}$-mediated upregulation of the $\alpha_5\beta_1$ fibronectin receptor is required for induction of anoikis.

Discussion

Recent experimental evidence suggested additional functions of $p16^{INK4a}$, other than controlling the G1 checkpoint of the cell cycle. (Chintala et al., 1997; Fåhraeus and Lane, 1999; Harada et al., 1999; Wolff and Naumann, 1999; Zhang et al., 1999). The picture emerging from these studies envisions $p16^{INK4a}$ as a more general modulator of the cellular phenotype via interactions with multiple cellular targets. This notion is supported by some biochemical features of $p16^{INK4a}$, which imply biological functions in addition to cell cycle control: (a) the binary $p16^{INK4a}$–Cdk4 complex exists in a 1:1 stoichiometry, but not all $p16^{INK4a}$ molecules are complexed with Cdks, and a considerable amount of free $p16^{INK4a}$ seems to be present in cells during the entire cell cycle (Della Ragione et al., 1996); (b) in addition to nuclear localization, $p16^{INK4a}$ is also distributed in the cytoplasm (Sano et al., 1998); (c) due to the extremely

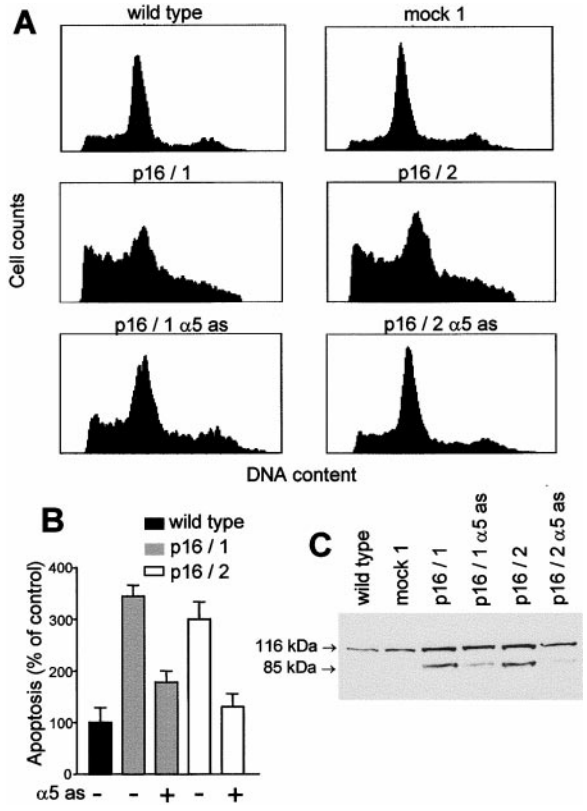

Figure 8. Effects of α_5 integrin downregulation on $p16^{INK4a}$-mediated anoikis. 5×10^5 cells of control, $p16^{INK4a}$-positive, or $p16^{INK4a}$-positive plus α_5 antisense–transfected clones generated as described in the legend to Fig. 7, were plated on polyHEMA-coated dishes for 20 h and subsequently analyzed for cell cycle distribution by flow cytometry. Shown is a representative cell cycle distribution (A) and the mean ± SEM of three independent experiments (B). (C) PARP cleavage. After cultivation on polyHEMA, cell lysates were prepared and immunoblotted with an mAb against PARP.

stable $p16^{INK4a}$ mRNA (half-life > 24 h) and protein (half-life 8–18 h) (Serrano, 1997), $p16^{INK4a}$ is expressed constantly during progression through the cell cycle, whereas expression of other regulators of the G1 checkpoint, like cyclin D1, is mainly restricted to the G1 phase (Soucek et al., 1995).

In this study, we provide experimental evidence for yet another novel biological function of the tumor suppressor gene $p16^{INK4a}$. Reconstituted expression of $p16^{INK4a}$ in human pancreatic cancer and other tumor cell lines confers the capability to undergo anoikis, a central attribute specifically lost during malignant transformation of most epithelial malignancies.

We chose pancreatic cancer cells as a suitable model to investigate our hypothesis that $p16^{INK4a}$ might influence anoikis for two reasons: (1) pancreatic cancer has the highest frequency of $p16^{INK4a}$ alterations of all human malignancies (>90%) (Schutte et al., 1997) and (2) this tumor is characterized by particular early and aggressive local growth and metastasis (Rosewicz and Wiedenmann, 1997), suggesting that failure to undergo anoikis might play a crucial role in its tumor biology. The majority of pancreatic cancers contain multiple genetic defects such as activation of the oncogene K-ras and inactivation of the tumor suppressor genes DPC4, p53, and $p16^{INK4a}$ (Rozenblum et al., 1997; Mangray and King, 1998). The human pancreatic cancer cell line Capan-1 represents a suitable in vitro model because the genetic background of Capan-1 cells is similar, if not identical, to human pancreatic cancer. Capan-1 cells express activated K-ras (Tsuchida et al., 1998), mutated DPC4 (Grau et al., 1997), and p53 (Bouvet et al., 1998), whereas $p16^{INK4a}$ is lost due to homozygous deletion (Huang et al., 1996; Schutte et al., 1997).

Reintroduction of $p16^{INK4a}$ in Capan-1 cells significantly inhibited anchorage-independent growth in soft agar. These results, which can be explained either by $p16^{INK4a}$-induced G1 arrest or induction of apoptosis, are in agreement with previous findings where ectopic $p16^{INK4a}$ expression was shown to suppress colony formation in vitro in glioblastoma cells (Higashi et al., 1997) or in lung cancer cells (Lee et al., 1998). Similar results were reported for $p16^{INK4a}$-mediated effects in vivo. Growth of $p16^{INK4a}$ xenografts was inhibited in xenotransplants derived from colon carcinoma (Spillare et al., 1996), human head and neck squamous cancer (Rocco et al., 1998), and mesothelioma (Frizelle et al., 1998). Surprisingly, and in contrast to previously published studies (Serrano, 1997), we observed a complete loss of tumorigenicity in $p16^{INK4a}$-transfected human pancreatic cancer cells. This phenomenon was at least in part due to initiation of apoptosis upon xenotransplantation in nude mice as detected by in situ TUNEL staining. Since $p16^{INK4a}$ did not alter the rate of apoptosis in adherent Capan-1 cells (data not shown), one possible trigger for induction of apoptosis in $p16^{INK4a}$-expressing cells might be based on their loss of attachment to extracellular matrix when injected into nude mice. Based on these results, we further pursued the hypothesis that $p16^{INK4a}$ specifically affected cellular anchorage dependence. Cellular responses to loss of matrix contact were studied in detail using the polyHEMA system as an in vitro approach to study anoikis. In $p16^{INK4a}$- transfected Capan-1 cells we demonstrated a time-dependent induction of programmed cell death after matrix detachment, i.e., anoikis, as evidenced by subdiploid DNA content, DNA fragmentation, and cleavage of the mitochondrial PARP protein. In contrast, $p16^{INK4a}$-negative parental or mock-transfected controls revealed negligible amounts of anoikis.

To exclude a cell type–specific phenomenon, we investigated whether $p16^{INK4a}$-mediated anoikis could also be observed in other epithelial cell systems derived from a different tissue context. Therefore, we stably transfected $p16^{INK4a}$ into $p16^{INK4a}$-negative cell lines from human liver (SKHep1), human skin (NKI4), and canine kidney (MDCK). In NKI4 cells the $p16^{INK4a}$ gene contains a point mutation inducing a stop codon (Castellano et al., 1997). Like Capan-1 cells, SKHep1, MDCK, and NKI4 cells were all negative in $p16^{INK4a}$ Western blot analyses using monospecific human $p16^{INK4a}$ antibodies (Fig. 4 A). In all three cell lines, we consistently observed induction of anoikis upon reconstitution of $p16^{INK4a}$ expression. Therefore, the $p16^{INK4a}$-mediated induction of anoikis is not restricted to pancreatic cancer cells, but appears to be operative in a variety of cell types of different origin.

Because integrins have been implicated in regulation of

anoikis, we speculated that $p16^{INK4a}$ might regulate anoikis via alteration of the cellular integrin composition. FACS® analysis revealed expression of six different α and three different β integrins in Capan-1 cells. However, the integrin expression pattern between $p16^{INK4a}$ negative controls and $p16^{INK4a}$-transfected cells was very similar, with the exception of α_5, which was consistently overexpressed in all $p16^{INK4a}$ independently transfected cell clones examined.

The α_5 integrin subunit selectively associates with β_1 to form the $\alpha_5\beta_1$ fibronectin receptor. Although regulatory effects on $\alpha_5\beta_1$ fibronectin receptor expression have been described for cytokines (Roberts et al., 1988; Huang and Chakrabarty, 1994; Dedhar, 1989), hormones (Nakamura et al., 1998), pharmacological substances (Symington et al., 1989), and other integrins (Tomatis et al., 1999), the upregulation of $\alpha_5\beta_1$ by a cell cycle–regulating tumor suppressor gene observed in this study represents a novel and unexpected finding. Even though incompletely understood, $\alpha_5\beta_1$ expression is generally regulated by various molecular mechanisms such as transcriptional activation (Delcommenne and Streuli, 1995), mRNA stability (Xu and Clark, 1996), and translational control (Harwood et al., 1999). Using a combined approach of immunoprecipitation, Northern blotting, and nuclear run-on analysis, we observed transcriptional induction of α_5 upon reexpression of $p16^{INK4a}$ as a major cause for increased surface membrane expression of the fibronectin receptor. As there is currently no evidence for binding of $p16^{INK4a}$ to DNA, a physical contact with the α_5 promoter appears unlikely. However, the α_5 promoter contains consensus binding sites for different transcription factors such as SP1, AP1, AP2, and ets (Birkenmeier et al., 1991). Therefore, it appears possible, though speculative, that $p16^{INK4a}$-mediated transcriptional activation of α_5 is transduced indirectly through interaction of $p16^{INK4a}$ with other transcription factors, as has recently been shown for interaction with nuclear factor κB (Wolff and Naumann, 1999).

What is the biological significance of $p16^{INK4a}$-mediated upregulation of the $\alpha_5\beta_1$ integrin? The $\alpha_5\beta_1$ fibronectin receptor plays an important role for regulation of tumor growth. Compared with their nontransformed counterparts, $\alpha_5\beta_1$ expression is frequently lost during malignant transformation (Plantefaber and Hynes, 1989), a phenomenon that has been observed in human colonic (Stallmach et al., 1992), mammary (Zutter et al., 1990), and pancreatic cancer (Hall et al., 1991; Shimoyama et al., 1995). Moreover, loss of $\alpha_5\beta_1$ integrin expression in CHO cells increased tumorigenicity (Schreiner et al., 1991) in vivo. In analogy, de novo expression of the $\alpha_5\beta_1$ integrin has been shown to reduce tumor formation as well as metastasis in CHO cells (Giancotti and Ruoslahti, 1990), in K562 erythroleukemia cells (Symington, 1990), and in HT29 colon carcinoma cells (Stallmach et al., 1994; Schirner et al., 1998; Schmidt et al., 1998). Independent of the in vitro system examined, the amount of cellular $\alpha_5\beta_1$ expression has been consistently found to be of critical importance because it determines the extent of growth inhibition (Ruoslahti, 1994–95).

The $\alpha_5\beta_1$ integrin, when not bound to fibronectin, transmits a negative growth signal to the cell, which can be reverted by attachment to fibronectin (Juliano and Varner, 1993). For example, CHO cells do not undergo apoptosis upon serum withdrawal when cells are attached to fibronectin through $\alpha_5\beta_1$ (Zhang et al., 1995). Moreover, the fibronectin receptor was shown to convert survival signals into death signals when dissociated from its ligand (Varner et al., 1995, Frisch and Ruoslahti, 1997), a process that is reflected when tumor cells lose contact with their original tissue context. In this context, the amount of unligated cellular $\alpha_5\beta_1$ integrin expression is of critical importance because it determines the susceptibility to cell growth regulation (Giancotti and Ruoslahti, 1990; Schreiner et al., 1991; Varner et al., 1995). We demonstrated that restoration of susceptibility to anoikis occurred in cells, which upregulate the $\alpha_5\beta_1$ integrin upon $p16^{INK4a}$ reexpression. Furthermore, subsequent antisense-induced downregulation of the α_5 subunit almost completely abolished $p16^{INK4a}$-mediated anoikis. Thus, upregulation of the fibronectin receptor represents the crucial key for induction of anoikis upon reconstitution of $p16^{INK4a}$. Accordingly, we observed that cells that upregulate the fibronectin receptor upon $p16^{INK4a}$ reexpression were protected from anoikis when supplemented with the soluble form of the specific ligand fibronectin under nonadherent conditions. This finding is in agreement with previous reports showing antiapoptotic effects of soluble fibronectin and other soluble extracellular matrix proteins on suspended cells (Bozzo et al., 1997; Fukai et al., 1998). We provided three independent lines of evidence to support the conclusion that $p16^{INK4a}$-mediated upregulation of unligated $\alpha_5\beta_1$ accounts for the extent of anoikis: (1) the anoikis- protective effect was specific for fibronectin, whereas other ECM components, such as laminin and collagen type IV, were ineffective; (2) the anoikis-inhibitory effects of fibronectin were dose dependent, with a maximal effect in the physiological range of soluble fibronectin present in human plasma at ~30 mg/dl (Wu et al., 1993); and (3) treatment with an inhibitory anti-α_5 antibody, which disrupts the $\alpha_5\beta_1$ integrin–fibronectin interaction, abolished the anoikis-protective effect of fibronectin. Therefore, the current results underline the significance of fibronectin receptor occupation for cell survival and extend previous reports by demonstrating a novel functional link between a nuclear tumor suppressor gene and a membrane-bound integrin.

In conclusion, we presented evidence suggesting that $p16^{INK4a}$ can restitute a less malignant phenotype by providing the protective machinery to control cell death upon loss of cell–extracellular matrix contact. Aside from its inhibitory effects on cell cycle progression, these observations provide further rationale for ongoing strategies to exploit reconstitution of $p16^{INK4a}$ as a therapeutic modality for epithelial malignancies (Sandig et al., 1997; Rocco et al., 1998).

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