A peptide inhibitor derived from p55PIK phosphatidylinositol 3-kinase regulatory subunit: a novel cancer therapy

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Abstract

p55PIK, a regulatory subunit of phosphatidylinositol 3-kinase (PI3K), specifically interacts with retinoblastoma protein (Rb) through the unique NH2 terminus of p55PIK, N24. This interaction is critical for cell proliferation and cell cycle progression. To examine p55PIK as a potential target for cancer therapy, we generated an adenovirus expressing N24 (Ad-N24-GFP) and studied its effects on the proliferation of cultured cancer cells, including human colon (HT29) and thyroid (FTC236) cancer cells. Ad-N24-GFP blocked cell proliferation and induced cell cycle arrest in all cancer cell lines tested. N24 induced cell cycle arrest at G0-G1 phase in cell lines that expressed Rb. Interestingly, N24 inhibited cell proliferation by blocking cell cycle transition at both S and G2-M phases in FTC236 cells, which did not express Rb. When Rb was knocked down by short hairpin RNA in HT29 cells, N24 also inhibited cell cycle progression at S and G2-M phases, suggesting that p55PIK regulates cell cycle progression by Rb-dependent and Rb-independent mechanisms. Finally, Ad-N24-GFP markedly decreased the growth of xenograft tumors derived from HT29 and FTC236 cancer cells in athymic nude mice. Our data strongly suggest that N24 peptide is an effective anticancer therapy, which specifically inhibits PI3K signaling pathways mediated by p55PIK. Moreover, they show that the regulatory subunit of an enzyme, in addition to its catalytic subunit, can be an important target for drug development. [Mol Cancer Ther 2008;7(12):3719–28]

Introduction

Class IA phosphatidylinositol 3-kinase (PI3K) signaling is critical for tumor cell proliferation and cell cycle progression. In human cancers, genetic dysregulation of its signaling components can occur at several levels, including deletion and mutation of PTEN, which dephosphorylates PIP3 to PIP2, as well as gene amplification of PIK3CA and Akt, a major downstream effector (1). Additionally, somatic mutations of PIK3CA have been reported in >25% of colorectal gastric, breast, and brain tumors, making it one of the most commonly mutated oncogenes in human cancer (2).

PI3Ks are heterodimers that have lipid and serine/threonine kinase activities and are composed of p110 catalytic and regulatory subunits (3, 4). Three genes encode the p110 catalytic subunits (α, β, and δ). The δ isoform is mainly present in leukocytes, whereas the α and β isoforms are expressed in a broad tissue distribution. There are three genes encoding regulatory subunits (PIK3R1, PIK3R2, and PIK3R3), which give rise to at least six products: p85α, p55α, and p50α (splicing variants from PIK3R1), p85β (encoded by PIK3R2); and p55PIK (p55γ) and p50PIK (p50γ; alternative translation initiation products from PIK3R3 mRNA). The three p110 catalytic subunit isoforms all can interact with each of the regulatory subunits, suggesting that different regulatory subunits may mediate signaling of specific PI3K pathways (3, 4). Currently, the signaling mechanisms for specific pathways are not well understood and few functions mediated by specific regulatory subunits of PI3K have been identified.

Given the prominent role of PI3K signaling in cell proliferation and its activation in many cancers, the PI3Ks have become important targets for the development of new anticancer drugs. Indeed, recent studies with PI3K catalytic subunit inhibitors have shown promise against a variety of cancers (5). However, the clinical application of these inhibitors has been limited due to their broad effects on PI3K signaling pathways and metabolic side effects such as hyperglycemia. Therefore, the development of more specific therapies against PI3K that increase both clinical efficacy and safety is urgently needed.

We found previously that the p55PIK subunit of PI3K is associated with the important cell cycle regulator, retinoblastoma protein (Rb), via the NH2-terminal 24-amino acid residues (N24) of p55PIK (6). This p55PIK/Rb interaction

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appeared to be essential for cell proliferation. In support of this notion, ectopically expressed N24 peptide, which competes with endogenous p55PIK binding to Rb, inhibited cell proliferation in several cancer cell lines (6). Recently, gene expression profiling studies showed increased p55PIK mRNA expression in ovarian cancers, and immunohistochemical studies showed strong expression of p55PIK protein in 88% of ovarian cancer specimens examined (7). Additionally, inhibition of p55PIK expression by small interfering RNA decreased cell proliferation in ovarian cancer cell lines (7). Taken together, these findings suggest that p55PIK may have a critical role in tumor cell proliferation and oncogenesis.

To examine p55PIK as a potential target for cancer therapy, we generated a N24 peptide expression system in replication-deficient adenovirus (Ad-N24-GFP). Ad-N24-GFP inhibited cell proliferation and cell cycle progression G_{0/1}-G_{1} phase in HT29 human colon cancer cells as well as in numerous other cell lines, which express Rb. Interestingly, Ad-N24-GFP strongly inhibited cell proliferation of FTC236 human thyroid cancer cells, which lack Rb, by blocking its cell cycle progression at S and G_{2/3}-M phases. Similarly, when Rb was knocked down by short hairpin RNA in HT29 cells, Ad-N24-GFP caused cell cycle arrest at S and G_{2/3}-M phases, suggesting that N24 blocks cell proliferation by Rb-dependent and Rb-independent mechanisms. Furthermore, Ad-N24-GFP markedly decreased the growth of HT29 and FTC236 xenograft tumors in athymic nude mice. These studies on N24 inhibition of the growth of HT29 and FTC236 xenograft tumors in athymic nude mice. These studies on N24 inhibition of cell proliferation in several cancer cell lines (7). Taken together, these findings suggest that p55PIK may have a critical role in tumor cell proliferation and oncogenesis.

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**Materials and Methods**

**Reagents and Antibodies**

Antibodies to Akt, pAkt (Ser^{473}), and green fluorescent protein (GFP) were from Cell Signaling. Antibody to gliceraldehyde 3-phosphate dehydrogenase was from Trevigen. Antibodies to Ki-67, Rb (IF8), and proliferating cell nuclear antigen were from Santa Cruz Biotechnology. Antibody to cyclin B1 was from Calbiochem. Antibody to mitotic protein monoclonal-2 was from Chemicon. Antibodies to bromodeoxyuridine (BrdU)- and FITC-labeled anti-mouse IgG antibodies were from Sigma. Propidium iodide (PI) was from Sigma. Primers used in this study were synthesized by IDT.

**Cell Culture**

Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_{2} in air. Two follicular thyroid carcinomas cell lines, FTC133 and FTC236, were derived from cultures obtained from the primary tumor (FTC133) and a nodal metastasis (FTC236) of a follicular thyroid carcinoma (8) and were cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies). HT29 cells, obtained from the American Type Culture Collection, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. HEK293, obtained from the American Type Culture Collection, were routinely cultured in DMEM supplemented with 15% fetal bovine serum.

**Preparation of Adenovirus Constructs**

The pShuttle-CMV, AdEasy1, and *Escherichia coli* bacteria BJ5183 were gifts from R. Vogelstein’s laboratory (Johns Hopkins Medical Institutes; ref. 9). The cDNA encoding the first NH_{2}-terminal 24 amino acids of p55PIK fused to the COOH-terminal end of GFP was obtained by PCR amplification from a construct described previously (6) and the cDNA encoding GFP was amplified from pEGFP-N1 (Clontech) using primers (forward primer 5'-TTTTGATATCAGTGAACCGTCAGATCCGCTA and reverse primer 5'-TTTTGATATCCTACAAATGTGG-GTATGGCCTGA) and cloned into the EcoRV site of pShuttle-CMV vectors. The Pmel1-linearized shuttle plasmids, mixed with adenoviral backbone AdEasy1, were used to cotransform *E. coli* BJ5183 bacteria. Recombinant adenovirus vectors expressing N24-GFP or GFP were amplified and linearized with PacI and purified for the transfection of HEK293 cells. The virus DNA with inserts was transfected with HEK293 cells and the successful infection of adenovirus particles was confirmed after infection of HEK293 cells by examining the expression of GFP signal in cells. The amplification of recombinant virus was done in HEK293 cells according to standard methods published. For virus transductions, HT29 and FTC236 cells were plated and cultured overnight at 37°C. The cells were incubated with adenovirus at a multiplicity of infection (MOI) of 50 overnight, medium with adenovirus was discarded, and cells were grown in fresh medium for another 48 h or as indicated.

**Western Blotting and Immunofluorescence**

Cells were washed in PBS twice and lysed in SDS sample buffer. Protein concentration was determined. Protein was separated on 4% to 15% premade Tris-HCl SDS-PAGE and transferred to a polyvinylidene difluoride membrane (BioRad). Immunoblot assays were done by the procedure described previously (6).

Two methods were used in these studies to measure the DNA synthesis in cells by the immunostaining of BrdUrd incorporation into DNA. FTC236 cells were plated onto coverslips in 12-well plate and cultured in 10% fetal bovine serum-DMEM overnight. Cells cultured on coverslips were incubated with BrdUrd (10 μg/mL) for 3 h after cells were infected for with Ad-GFP or Ad-N24-GFP adenovirus. Some cells were treated with LY294002 (20 μmol/L) for 48 h. The coverslips were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Immunostaining with anti-BrdUrd antibodies and the nuclear staining with PI were done as described previously (10). Images were acquired by confocal laser scanning microscopy (LSM-410; Carl Zeiss). After immunostaining against BrdUrd and counterstaining of cell nuclei with PI, DNA synthesis was determined by counting the percentage of BrdUrd\(^{+}\) cells in total cells. The images were merged using Adobe Photoshop software.
In some set of experiments, for immunostaining BrdUrd incorporation into DNA, cells cultured in dishes were incubated with BrdUrd (10 μg/mL) for 3 h. The cells were detached, washed twice with PBS, and fixed in 4% formaldehyde for 15 min at room temperature. Immunostaining with anti-BrdUrd antibodies and nuclear staining with PI were done as described previously. After immunostaining against BrdUrd and counterstaining cell nuclei with PI, DNA synthesis was determined by flow cytometry analysis (6).

Immunohistochemical staining of tumor sections with anti-pRb and Ki-67 antibodies (Santa Cruz Biotechnology) was done as described previously (11).

**Cell Number Counting and Flow Cytometry for Cell Cycle Analysis**

Cells were detached, washed, and resuspended in PBS. The cells were counted. For the flow cytometry analysis, cells were detached by trypsinization, washed twice with cold PBS, and resuspended in 80% ethanol for at least 30 min at −20°C. Then, cells were washed once with PBS and incubated on ice for 10 min with PI (50 μg/mL). Finally, the stained cells were analyzed using FACSCalibur from BD Biosciences. Aggregated cells revealed by forward scattering were filtered out of the data set before analysis. To determine G0-G1, S, and G2-M populations, the settings scattering were filtered out of the data set before analysis. After immuno- staining with anti-BrdUrd antibodies and nuclear staining with formaldehyde for 15 min at room temperature. Immunohistochemical staining of tumor sections with anti-pRb and Ki-67 antibodies (Santa Cruz Biotechnology) was done as described previously (11).

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**RNA Isolation and Analyses**

Total RNA was isolated from FTC236 and two human breast cancer cells with wild-type Rb, MCF-7 and T47D, using TRIzol reagent obtained from Invitrogen. The isolated RNA was reverse transcribed using a kit from Qiagen (One-Step PCR) primed by random oligo primers and then subjected to PCR analysis. Primers used included for Rb (forward 5′-TGGTGAATCATTCGGGACTTC and reverse 5′-GGACTCTCCTGGGAGATGTTT) and glyceraldehyde-3-phosphate dehydrogenase (forward 5′-TGTTGACCATGAGAAGTATGACAAC and reverse 5′-GGTGTG-CCAGT-¶-¶CCAGT). The condition for the PCR was 94°C for 5 min (94°C for 45 s, 55°C for 1 min, and 72°C for 45 s), 35 cycles, 72°C for 5 min.

**Generation of Rb-Deficient HT29 Cells**

HT29 human colon cancer cells (expressing Rb) were transfected with pMSCVpuro-Rb3C, a plasmid containing a short-hairpin sequence targeted to human Rb or control vector (12). Transfectants harboring the construct or control vector (pMSCV) were selected with puromycin and the loss of Rb expression in selected clones was determined by Western blotting (12). Clones in which the expression of Rb was significantly decreased were expanded and used in experiments.

**Animal Studies**

Athymic male nude mice at age 8 to 10 weeks weighing 22 to 24 g, obtained from the Wuhan Laboratory Animal Center, were used in this study. Mice were housed under specific pathogen-free conditions in a temperature- and humidity-controlled environment and given unlimited access to water and food. Mice were acclimatized for 7 days before initiation of experiments. All animal experiments were done in accordance with institutional animal research guidelines approved by the local ethics committee.

For the tumor growth studies, cultured HT29 cells were incubated with Ad-GFP or Ad-N24-GFP adenovirus (MOI = 50) overnight. The cells were collected, washed, and resuspended in culture medium (5 × 10^6/mL). Cell suspensions (100 μL; 5 × 10^6 cells) were injected into mice. Mice were sacrificed at indicated times after injection, and the tumors were removed and analyzed.

To observe the inhibitory effects of N24 on the tumor growth in nude mice, cultured HT29 or FTC236 cells were collected, washed, and resuspended in culture medium (5 × 10^6/mL). Cell suspensions (100 μL) were injected into nude mice; when tumor sizes reached ~15 mm^3 (1 week after inoculation), mice were randomly assigned to three experimental groups: control (intratumoral injection of PBS), Ad-GFP, and Ad-N24-GFP (intratumoral injection of 50 μL containing 5 × 10^10 plaque-forming virus). The injection of adenovirus or PBS was repeated weekly until 1 week after the third injection, when the animals were sacrificed and their tumors were collected for analysis.

**Statistical Analysis**

Data are presented as mean ± SD. P values were calculated using an unpaired Student’s t test when only two groups were compared.

**Results**

**Generation of Adenovirus Expressing N24**

We generated the adenovirus construct expressing N24 peptide fused to GFP, Ad-N24-GFP, as described in Materials and Methods. We then transformed HT29 human colon cancer cells that express Rb (13, 14) with Ad-N24-GFP and confirmed intracellular N24-GFP expression by Western blotting. Next, we determined the N24 expression efficiency in cells infected with a series of MOIs ranging from 1 to 100 by measuring the percentage of cells expressing GFP 48 h after infection. Approximately 90% cells expressed significant amounts of N24-GFP or GFP when MOI was >50 (data not shown). Accordingly, a MOI of 50 was used in all subsequent experiments studying the effects of N24 in cell culture.

**Ad-N24-GFP Induces the Cell Cycle Arrest at G0-G1 Phase in HT29 Human Colon Cancer Cells**

We initially examined the effects of N24 on cellular proliferation by transforming HT29 cells with Ad-N24-GFP or control adenovirus only expressing GFP (Ad-GFP) (Fig. 1A). After infection, cells were counted daily for the next 4 days. Ad-GFP slightly decreased cell number after 4 days (13%) compared with uninfected cells. However, Ad-N24-GFP significantly decreased cell proliferation by 74% after 4 days when compared with Ad-GFP (2.8 × 10^4 versus 1.1 × 10^5 cells per well). Addition of LY294002 (20 μmol/L), a PI3K inhibitor that blocks the enzyme
activity of the p110 catalytic subunits, decreased cell proliferation of HT29 cells to a similar extent as Ad-N24-GFP (data not shown). Next, we analyzed the effect of N24 on the DNA synthesis of transformed cells (Fig. 1B). Cells were cultured for 48 h after adenovirus infection and incubated with BrdUrd for 3 h. The incorporation of BrdUrd into DNA was determined by flow cytometry analysis. Representative data seen in three independent experiments. Note that the percentage of cells in active DNA synthesis is defined as BrdUrd- cells/total cells (the events in R2 area/total events) and the average BrdUrd incorporation into DNA (mean BrdUrd signal intensities in R2 events) is shown in arbitrary units with the mean of untransformed controls defined as 100%. C, Ad-N24-GFP effects on cell cycle progression in HT29 cells. Cell cycle analysis was done on HT29 cells that were uninfected or infected with either Ad-N24-GFP or Ad-GFP as described in Materials and Methods. Representative histograms from an individual experiment. Similar results were obtained in three independent experiments. D, effect of Ad-N24-GFP on apoptosis in HT29 cells. Annexin V binding in HT29 cells treated under the same conditions as in Fig. 2B. Cell number and staining of PI with DNA were measured using flow cytometry analysis. Representative data seen in three independent experiments and the percentage of cells in early-phase apoptosis (bottom right) or later-phase apoptosis (top right) in control, Ad-GFP-infected, or Ad-N24-GFP-infected cells.

Ad-N24-GFP Induces Cell Cycle Arrest at S and G2-M Phases in Rb-Deficient FTC236 Human Thyroid Cancer Cells

We next examined the effects of N24 on a metastatic thyroid cell line, FTC236 cells (Fig. 2A). We observed that cell cycle distribution of cells infected with Ad-GFP when compared with uninfected control cells. In contrast, cells infected with Ad-N24-GFP exhibited cell cycle arrest at the G0-G1 phase. Of note, we did not observe increased apoptosis in HT29 cells transformed by Ad-N24-GFP using Annexin V binding, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining, or caspase cleavage assays (Fig. 1D; data not shown). These findings suggest that N24 had a cytostatic, rather than cytotoxic, effect by blocking cell cycle progression in these cells. We also examined N24 effects on cell proliferation in cancer cell lines from cervical (Hela), thyroid (WRO, ARO, FTC133, and NPO), liver (HepG2), and prostate (PC3, DU145, and LNCaP) as well as two breast cancer cell lines (MCF-7 and T47D) and observed similar inhibition as seen in HT29 cells (data not shown).

Figure 1. Effects of Ad-N24-GFP on cell proliferation, DNA synthesis, cell cycle progression, and apoptosis. A, effects of Ad-N24-GFP on cell proliferation of HT29 cells. HT29 cells were plated on 12-well plates and cell number was counted 0 to 4 d after infection with Ad-N24-GFP or Ad-GFP. Mean ± SD of triplicate samples. Similar results were seen in three independent experiments. B, Ad-N24-GFP effects on DNA synthesis. HT29 cells were infected with the Ad-N24-GFP or Ad-GFP or not infected. BrdUrd was added 48 h after infection, and cells were cultured for an additional 3 h. Cells then were collected and immunostained for BrdUrd and counterstained for nuclei acid with PI. BrdUrd incorporation into DNA and DNA content in nuclei were determined by flow cytometry analysis. Representative data seen in three independent experiments. Note that the percentage of cells in active DNA synthesis is defined as BrdUrd- cells/total cells (the events in R2 area/total events) and the average BrdUrd incorporation into DNA (mean BrdUrd signal intensities in R2 events) is shown in arbitrary units with the mean of untransformed controls defined as 100%. C, Ad-N24-GFP effects on cell cycle progression in HT29 cells. Cell cycle analysis was done on HT29 cells that were uninfected or infected with either Ad-N24-GFP or Ad-GFP as described in Materials and Methods. Representative histograms from an individual experiment. Similar results were obtained in three independent experiments. D, effect of Ad-N24-GFP on apoptosis in HT29 cells. Annexin V binding in HT29 cells treated under the same conditions as in Fig. 2B. Cell number and staining of PI with DNA were measured using flow cytometry analysis. Representative data seen in three independent experiments and the percentage of cells in early-phase apoptosis (bottom right) or later-phase apoptosis (top right) in control, Ad-GFP-infected, or Ad-N24-GFP-infected cells.
N24-GFP expression significantly decreased the FTC236 cell proliferation by 80% after 4 days when compared with GFP alone (1.9 x 10^4 versus 1.2 x 10^5 cells per well). We examined BrdUrd incorporation in transformed cells and found that N24 decreased the percentage of cells actively synthesizing DNA by 84% (32.1% in Ad-GFP infected cells versus 5.2% in Ad-N24-GFP infected cells; Fig. 2B). Next, we used flow cytometry analysis to examine the effects of LY294002 and N24 on cell cycle progression (Fig. 2C). LY294002 increased the number of cells in G1 phase while concomitantly decreasing their number in S and G2-M phases, consistent with the notion that PI3K is important for cell cycle progression, especially at the cell cycle entry phase. Surprisingly, in contrast to the effects of N24 on HT29, MCF-7, and T47D cells, N24 decreased the cell population of FTC236 cells in G0-G1 phase while increasing the number of cells in S and G2-M phases. These findings suggest that N24 blocked cell cycle progression in FTC236 cells at the S and G2-M phases rather than at the G0-G1 phase.

We showed previously that N24 inhibited cell cycle progression and induced G0-G1 arrest by interrupting p55PIK/Rb interaction (6). Our observations of the effects of N24 on cell cycle progression of FTC236 cells prompted us to examine Rb expression in FTC236 cells. Interestingly, neither Rb protein nor mRNA expression was detected in FTC236 cells in contrast to T47D and MCF-7 cells (Fig. 2D and E). These findings suggest that decreased transcription of Rb mRNA is the main mechanism for the lack of Rb protein expression in FTC236 cells.

Figure 2. Effects of Ad-N24-GFP on cell proliferation and cell cycle progression in Rb-deficient FTC236 cells. A, Ad-N24-GFP effects on cell proliferation. FTC236 cells were plated on 12-well plates and the cell number was counted 0 to 4 d after infection in uninfected cells and cells infected with Ad-N24-GFP or Ad-GFP. Mean ± SD of triplicate samples. B, N24 effects on DNA synthesis in FTC236 cells. Cells were infected with the adenovirus expressing N24-GFP or GFP as described in Materials and Methods. BrdUrd (10 μg/mL) was added 48 h after infection, and cells were cultured for an additional 3 h. Cells then were immunostained with anti-BrdUrd antibodies and FITC-conjugated secondary antibodies (green). The nuclei were counterstained with PI (red). Images then were taken with a laser scanning confocal microscope. Representative images of slides and percentage of BrdUrd-positive cells determined by counting 200 cells from triplicate samples (mean ± SD). C, Ad-N24-GFP effects on cell cycle progression. Cell cycle analyses were done on uninfected FTC236 cells, cells infected with Ad-N24-GFP or Ad-GFP, or cells treated with PI3K inhibitor LY294002 (final concentration: 20 μmol/L) as described in Materials and Methods. Representative histograms from an individual experiment. Similar results were obtained in three independent experiments. D, Rb protein expression in FTC236 cells. Lysates from FTC236, MCF-7, and T47D cells were prepared and then analyzed for the presence of Rb by Western blotting using a monoclonal antibody against Rb. Glyceraldehyde 3-phosphate dehydrogenase and proliferating cell nuclear antigen protein expression was also analyzed by Western blotting on the same membrane to show the equal loading of proteins and the integrity of the proteins in all lysates. E, Rb mRNA expression in FTC236 cells. Total RNA was isolated from FTC236, MCF-7, and T47D cells and subjected to reverse transcription-PCR analysis using primers and conditions described in Materials and Methods. F, effects of Ad-N24-GFP and LY294002 on the proteins involved in cell cycle progression and PI3K signaling in FTC236 cells. Cellular lysates were prepared and proteins were analyzed by Western blotting using antibodies against cyclin B1, mitotic protein monoclonal-2, proliferating cell nuclear antigen, and GFP as described in Materials and Methods. Glyceraldehyde 3-phosphate dehydrogenase expression also was analyzed with an appropriate antibody to show the equal loading of proteins in every well.
To further characterize the inhibition of cell proliferation by N24 in FTC236 cells, we examined the expression of several proteins involved in cell cycle progression by Western blotting (Fig. 2F). Cyclin B1 and phosphorylated proteins recognized by mitotic protein monoclonal-2 antibody are highly expressed during G2-M transition (15, 16). Significantly, both cyclin B1 and mitotic protein monoclonal-2 antibody-detected proteins were increased in FTC236 cells transformed with Ad-N24-GFP but were decreased in cells treated with LY294002. Furthermore, LY294002 markedly decreased the expression of proliferating cell nuclear antigen, which is a cofactor for DNA polymerase that is highly expressed during S phase (17), whereas N24 had little or no effect on proliferating cell nuclear antigen expression. Taken together, the foregoing divergent findings reflect the inhibition of cell cycle progression at different checkpoints of the cell cycle by N24 and LY294002 in FTC236 cells.

We determined the expression of Rb in the cell lines in which we found previously that N24 decreased cell proliferation and inhibited cell cycle progression at G0-G1, and all of them expressed Rb. We then analyzed several more lines and found another human thyroid cancer cell line, FTC238 (8), to be Rb deficient. As expected, N24 expression in FTC238 cells led to the cell cycle arrest at S and G2-M phases, further supporting the hypothesis that N24 inhibition on the cell cycle progression is mediated by Rb-dependent and Rb-independent mechanisms (data not shown).

**Ad-N24-GFP Induces Cell Cycle Arrest at S and G2-M Phases in Rb-Deficient HT29 Cells Generated by Short Hairpin RNA**

To provide further evidence for Rb-independent inhibition by N24, we used a plasmid expressing short hairpin RNA against Rb (12) to knock down Rb expression in HT29 cells. Stably transfected clones then were selected and their Rb expression was examined by Western blotting analysis. A cell line with significantly decreased Rb protein level (Fig. 3A) was further studied. Of note, Ad-N24-GFP inhibited cell proliferation and caused cell cycle arrest at S and G2-M phases in these modified HT29 cells (Fig. 3B and C). Our findings thus showed that N24 inhibited cell proliferation in both Rb+ and Rb− cancer cell lines.

**Ad-N24-GFP Inhibits the Xenograft Tumor Growth of HT29 and FTC236 Cells**

We next examined whether Ad-N24-GFP had activity in vivo against tumors derived from cancer cells that were Rb+ or Rb−. We first studied Ad-N24-GFP effects on the...
growth of HT29 tumor xenografts in athymic nude mice. In the first set of experiments, HT29 cells were infected with adenovirus and then injected into mice. The weights of resultant tumors were measured 3 weeks later at necropsy (Fig. 4A). Tumors derived from HT29 cells transformed with Ad-N24-GFP exhibited markedly decreased mean tumor weight when compared with tumors from HT29 cells transformed with Ad-GFP. Next, HT29 cancer cells were injected s.c. into nude mice, and after tumors became observable after 1 week, mice were injected intratumorally with 5 × 10^10 adenovirus particles (Ad-N24-GFP or Ad-GFP) or vehicle alone at weekly intervals for the next 3 weeks. Tumor size was measured twice a week for 4 weeks before the animals were sacrificed (Fig. 4B). Ad-N24-GFP injection significantly decreased tumor growth throughout the course of the experiment, and the final mean tumor weight of Ad-N24-GFP-treated mice decreased by 61% when compared with the mean tumor weight of Ad-GFP-treated mice (Fig. 4C). Immunohistochemical analysis of HT29 tumors showed that Ad-N24-GFP decreased the phosphorylation of Rb and the expression of Ki-67, a proliferation marker (Fig. 4D). No significant increase in apoptosis was observed in the tumors from mice treated with Ad-N24-GFP, consistent with the previous results that showed that N24 was cytostatic (data not shown).

Lastly, we examined the effects of Ad-N24-GFP on FTC236 tumor growth in vivo. FTC236 cells were injected into mice, and when tumors became observable (at ~10 days), 5 × 10^10 adenovirus particles were injected intratumorally weekly for 3 weeks. The weights of tumors were measured in mice sacrificed 1 week after final
injection (Fig. 5A). Tumors derived from FTC236 cells treated with Ad-N24-GFP exhibited markedly decreased mean tumor weight when compared with tumors treated with Ad-GFP. The expression of the proliferation marker, Ki-67, also was decreased consistently in tumors derived from FTC236 cells treated with Ad-N24-GFP (Fig. 5B).

Discussion

We showed previously that p55PIK, a regulatory subunit of PI3K, specifically interacts with a key regulator of cell cycle progression, Rb (6). Additionally, N24, a peptide inhibitor containing the NH₂ terminus of p55PIK, which binds to Rb, blocks this interaction and inhibits cell proliferation. We now report the generation of an adenovirus construct, Ad-N24-GFP, which expresses N24 fusion peptide. This adenovirus induces cell cycle arrest at G₀-G₁ in HT29, MCF-7, and T47D cells, which express Rb. Ad-N24-GFP also inhibited cell proliferation in FTC236 cells, which lack Rb. These data strongly suggest that p55PIK regulates cell proliferation by Rb-dependent and Rb-independent mechanisms, which can be inhibited by N24. In further support of this notion, we also observed N24 inhibition of cell proliferation in a HT29-derived cell line in which Rb expression was abrogated by short hairpin RNA. Additionally, we showed that Ad-N24-GFP markedly decreased the growth of tumor xenografts derived from both HT29 and FTC236 cells, suggesting that N24 has antitumoral effects against Rb⁺ and Rb⁻ cancers in vivo.

In our current study, we observed that Ad-N24-GFP inhibited the cell proliferation of FTC236 cells by inducing cell cycle arrest at S and G₂-M phases. In conjunction with previous (6) and current findings using Ad-N24-GFP in cells, which express Rb, these observations suggest that p55PIK may be involved in S and G₂-M as well as G₀-G₁ phase transitions, although the latter may predominate when Rb is present. In this connection, PI3K activation is most commonly associated with G₁-S progression (4, 18); however, several studies also have implicated the PI3K signaling in S-phase entry and G₂-M transition (19, 20). In addition, NIH3T3 cells exhibit a strong PI3K activity peak at G₀-G₁ phase and two smaller peaks at mid-G₁- and M-phase entry (21). Taken together with our current findings, it suggests that PI3K signaling has multiple effects on cell cycle progression and highlights the important need to determine the precise roles of p55PIK and other PI3K regulatory subunits, particularly in the regulation of the S and G₂-M phases of the cell cycle. With respect to p55PIK, it would appear that the pathway(s), which regulates S- and G₂-M-phase transitions, is mediated by p55PIK interaction with a protein(s) other than Rb. The identification of such a protein(s) and the elucidation of its signaling pathways downstream from its interactions with p55PIK will be important for future studies, particularly because the loss or inactivation of Rb is observed in many human cancers (22, 23). Based on the foregoing data, we

Figure 5. Effects of Ad-N24-GFP on the growth of FTC236 tumor xenografts in athymic nude mice. **, P < 0.01, Ad-N24-GFP group compared with Ad-GFP group.

Figure 6. Model showing mechanisms of Rb-dependent and Rb-independent cell cycle arrest by N24 peptide. See text for description.
propose a model showing the potential mechanisms for N24 peptide inhibition of cell proliferation at different checkpoints during the cell cycle (Fig. 6). In G0-G1, p55PIK interacts with Rb-containing complexes, which include E2F/DP. Mitogens or other cell proliferation signals activate p55PIK-P13K associated with Rb. This is followed by phosphorylation of Rb and dissociation of E2F/DP leading to cell cycle progression and cell proliferation. In the presence of N24 peptide, interaction of p55PIK with Rb is disrupted, and phosphorylation of Rb is inhibited, leading to cell cycle arrest at G0-G1. In a similar manner, N24 peptide disrupts p55PIK-P13K interaction with other proteins (X) that regulate different checkpoints of the cell cycle (G2-M and S phases), leading to cell cycle arrest in a Rb-independent manner.

P13K catalytic subunits bind to the regulatory subunits (p85, p55, and p50) with similar affinity, so signaling specificity may be determined by the regulatory subunits rather than the p110 catalytic subunits (24). Activation of Akt and its downstream signaling pathways are thought to be mediated mainly by P13Ks; however, the precise contributions to Akt activation by the various p110/ regulatory subunit heterodimers are not known. LY294009, which targets all P13Ks including those containing p55PIK, inhibited the phosphorylation of Akt and blocked cell proliferation and G0-G1 transition in all the cancer cell lines studied. N24 inhibited cell proliferation but did not change phosphorylation of Akt significantly (data not shown). Several reasons may account for this: we showed previously that p55PIK is almost entirely localized in nucleus, so nuclear compartmentalization likely enables p55PIK to modulate nuclear processes such as cell cycle progression (6). This nuclear compartmentalization also may limit the role of p55PIK on Akt phosphorylation because only a small fraction of total Akt is in the nucleus, and only this pool of Akt could potentially be activated by p55PIK. Thus, inhibition of p55PIK signaling by N24 would be expected to have little effect on total phosphorylation of Akt. Secondly, the expression of p55PIK is much lower than p85 regulatory subunits in most cells, so p55PIK may only play a minor role in Akt phosphorylation relative to p85. In any event, our data show that P13Ks are involved in multiple signaling pathways that regulate cell cycle progression; moreover, p55PIK plays essential roles in cell cycle progression. In further support of the importance of these p55PIK pathways in cell cycle progression, recent studies showed that p55PIK small interfering RNA decreased cell proliferation in several ovarian cancer cell lines (7). We also have observed similar findings on cell proliferation using p55PIK small interfering RNA in MCF-7 cells.6

P13Ks have been considered promising targets for the treatment of cancer as well as cardiovascular, inflammatory, and autoimmune diseases. Although inhibitors of the P13K catalytic subunits have been shown to be effective for various cancers (5), the lack of specificity and isooform selectivity of these inhibitors has limited their clinical application. For example, general inhibitors such as LY294002 and its derivatives target the catalytic subunits of P13Ks; hence, they inhibit multiple downstream signaling pathways mediated by all P13Ks. It is likely that the toxicity of broad-spectrum P13K inhibitors is due in part to the inhibition of specific P13Ks subserving critical functions in normal cells. In particular, inhibitors of P13K catalytic activity perturb glucose homeostasis in vivo because P13K plays a key role in insulin signaling by linking the insulin receptor and insulin receptor substrate proteins to downstream effectors (25, 26). Recently, isosomal-specific catalytic subunit inhibitors have been developed and employed successfully in various conditions. For example, specific inhibitors against p110α, a catalytic subunit isoform of p110α mainly expressed in leukocytes, have been effective against autoimmune diseases (27, 28). Similarly, isoform-specific catalytic subunit inhibitors against p110α, an isoform that plays a prominent role in the growth of some tumors, were efficacious against some tumors and exhibited improved therapeutic indices compared with LY294002 (27, 28).

In contradistinction to catalytic subunit inhibition, our studies show a new strategy for P13K inhibition directed against regulatory subunit signaling as a p55PIK regulatory subunit inhibitor such as N24 blocks cellular proliferation via disruption of a key protein-protein interaction(s). Our studies also show the therapeutic potential of this strategy, as N24 is effective in blocking the growth of xenograft tumors from colon and thyroid cancers. Moreover, N24 did not cause changes in phosphorylation of Akt and other proteins involved in P13K signaling pathways. These findings thus raise the possibility that N24 or its peptidomimetics may be effective therapies for cancer, which have less side effects than p110 catalytic subunit inhibitors by virtue of their specific effects on cell signaling pathways mediated by p55PIK. It also is possible N24 and its peptidomimetics could be used in combination with p110 inhibitors (perhaps at a lower dose) to create more effective and safer regimens for inhibiting P13K signaling pathways. Because they are directed toward downstream target, such as Rb, N24 and its peptidomimetics also may be effective in treating tumors that are activated by oncogenes and growth factors. In this connection, we have observed that N24 can block cell proliferation stimulated by epidermal growth factor and insulin-like growth factor in several cell lines (6). The foregoing data also show that Ad-N24-GFP itself may be a potentially useful agent for treatment of human cancers, particularly for local antitumor therapy. Of note, intratumoral injection of adenovirus expressing p53 tumor suppressor has been used clinically to treat head and neck cancers (29).

In summary, our studies with Ad-N24-GFP show that a specific P13K regulatory subunit isoform, p55PIK, has differential and essential roles of in cell cycle progression by Rb-dependent and Rb-independent mechanisms. These findings show the utility of targeting p55PIK signaling to inhibit cell proliferation and tumor growth. They also

6 Oetting and Yen, unpublished results.
provide the rationale for development of N24 or peptidomimetics as a new class of anticancer drugs that may have advantages over present PI3K inhibitors that target the catalytic subunits. Last, we show that preventing the delivery of an enzyme to its proper intracellular address by blocking the protein/protein interactions of a regulatory subunit can be as effective as blocking the catalytic activity of the enzyme itself. It is expected that the application of strategy will lead to new molecular targets and more specific strategies for modulating enzyme activities in the cell.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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