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Identification of Genes, including the Gene Encoding p27Kip1, Regulated by Serine 276 Phosphorylation of the p65 Subunit of NF-κB

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Abstract

Phosphorylation of the p65 subunit of NF-κB is required for its transcriptional activity. Recent reports show that phosphorylation of p65 at serine 276 regulates only a subset of genes, such as those encoding IL-6, IL-8, Gro-β, and ICAM-1. In order to identify additional genes regulated by serine 276 phosphorylation, HepG2 hepatoma cells were infected with adenoviruses encoding either wild-type p65 or the S276A mutant of p65, followed by DNA microarray analysis. The results show that mutation of serine 276 affected the expression of several genes that encode proteins involved in cell cycle regulation, signal transduction, transcription, and metabolism. Notably, expression of S276A increased the mRNA and protein level of p27, a cell cycle inhibitory protein, which led to an increased association of p27 with cdk2, and inhibition of cdk2 activity. Furthermore, while wild-type NF-κB is known to increase cell proliferation in a number of different cancer cell lines, our data show that S276A inhibits cell proliferation. Evidence is mounting that NF-κB plays a pivotal role in oncogenesis. Therapeutic agents that regulate the phosphorylation of serine 276 and p27 gene expression, therefore, may be useful as anti-cancer agents in the future.

Keywords

NF-κB; p65; serine 276; p27; TNF-α

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Conflicts of Interesr Statement
None Declared

Introduction

Nuclear factor κB (NF-κB) is an inducible transcription factor critical for the expression of a variety of genes involved in inflammation, immunity, apoptosis and cell proliferation [1,2]. The most abundant form of this transcription factor is a heterodimer consisting of a p50 and p65 subunit. In most unstimulated cells, NF-κB is sequestered in the cytoplasm by the inhibitory protein, IκB. Upon stimulation of cells with various agents, including TNF-α, phorbol esters and lipopolysaccharide, IκB is phosphorylated and degraded, thus exposing the nuclear localization signal of NF-κB and allowing it to translocate to the nucleus and regulate gene expression [1,2].

An emerging area of interest is the role of p65 phosphorylation for the regulation of NF-κB activity. The phosphorylation of serine 276, which lies in the rel homology domain of p65, has been examined in some detail. Phosphorylation of this site is induced by lipopolysaccharide and TNF-α and is essential for the interaction of p65 with coregulators, including CBP [3–5]. Mutation of serine 276 to alanine affects the regulation of certain gene promoters [3,6]. For example, in $RelA^{-/-}$ MEFs, stable expression of wt p65 induces the transcription of several genes including intercellular adhesion molecule (ICAM-1), while stable expression of the S276A mutant of p65 does not induce ICAM-1 gene expression [6]. Both wt p65 and S276A, however, induce the expression of other genes, including the gene encoding manganese superoxide dismutase (MnSOD), to the same extent. Since ICAM-1 and MnSOD have different κB DNA cis-elements, it was concluded that phosphorylation of serine 276 modulates the transcriptional activity of NF-κB in a cis-acting element and promoter-specific context [6]. This suggests that the interaction of p65 with co-activators or other transcription components could be modulated in a DNA site-specific context. These observations also support the notion that the phosphorylation status of p65 is an important determinant of differential gene expression.

The phosphorylation of p65 on serine 276 both positively and negatively regulates gene expression. For instance, genes encoding chemokine CXC ligand 9, E-selectin, interleukins-6 and –8, and vascular cell adhesion molecule 1 are upregulated by an interaction of p65 with CBP/p300 [7–11]. The gene encoding phosphoenolpyruvate carboxykinase, on the other hand, is downregulated by p65 phosphorylation on serine 276, and the S276A mutant of p65 abolishes this effect [12].

The phosphorylation of p65 on serine 276 likely regulates the transcription of gene promoters through multiple mechanisms. Besides coactivators, p65 phosphorylated on serine 276 interacts with the positive transcription elongation factor b (P-TEFb), which is a complex containing cyclin-dependent kinase 9 and cyclin T1 subunits [13]. It was shown that the P-TEFb-phospho-Ser^{276} p65 complex binds primarily to a subset of gene promoters that are rapidly induced by TNF-α and that this complex is required for transcription of Gro-β and IL-8, but not IκBα [13].

It is important to identify genes that are regulated by the serine 276 phosphorylation of p65 because it may be possible to develop more specific NF-κB inhibitors that target the regulation of only certain subsets of genes. In order to further identify genes regulated by serine 276 phosphorylation, adenoviruses encoding either wild-type or the S276A mutant of p65 were constructed. RNA obtained from cells infected with these viruses was used in DNA microarray analysis to identify genes regulated by wild-type and mutant p65. The data reveal that p65 and S276A differentially regulate gene expression. Furthermore, S276A abrogates the expression of several genes regulated by TNF-α, thus acting in a dominant-negative fashion in some cases. Strikingly, S276A upregulates the expression of several genes that negatively regulate the cell cycle, including the gene encoding cyclin-dependent kinase (Cdk) inhibitors p27 and p18, and

two BTG family members, BTG2 and BTG3. While we were unable to detect p18 or BTG protein by immunoblot analysis in HepG2 cells, p27 protein levels increased in response to S276A expression. The increased p27 protein inhibited cdk2 kinase activity and cell proliferation. Furthermore, TNF-α, a physiological activator of NF-κB, downregulated p27 at both the gene and protein level. These results suggest that p65 phosphorylated on serine 276 may function to promote cell proliferation by repressing p27 expression.

Materials and Methods

DNA Constructs

The p27 promoter/luciferase promoter construct was a kind gift from Dr. Albert Nordin. Construction of expression vectors encoding p65 and S276A was described previously [12].

Adenovirus Construction

The p65-encoding adenovirus was a kind gift from Dr. Craig Logsdon. The cDNA encoding S276A was subcloned into the pShuttle-CMV vector and the cDNA encoding p27 was subcloned into the pAd-Track-CMV vector. Adenovirus was prepared using the pAd-Easy system [14]. Adenoviruses were propagated in HEK 293 cells and purified using the BD Adeno-X Virus Purification Kit (BD Biosciences, San Jose, CA). The number of virus particles per ml was calculated using the equation: virus particle/ml = OD_{260} × cuvette dilution × 10^{12}. The multiplicity of infection (MOI) was calculated as the number of virus particles per cell. HepG2 or MDA-MB-231 target cells were incubated for 48 h with either GFP-, p65- or S276A-encoding adenoviruses before experimentation to assure optimal expression of these proteins.

Cell Culture and Transient Transfection

HepG2 hepatoma cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum. Transient transfections were performed using FuGENE (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Cells were incubated for 48 h posttransfection, and cell extracts were prepared and subjected to luciferase assays. Background readings were subtracted from the luciferase assay values. Luciferase activity was normalized for the protein concentration in the cell lysate using the BioRad protein assay reagent.

DNA Microarray Analysis

Microarray Construction—Microarrays containing 11,520 cloned genes from the human sequence verified clone set from Research Genetics were generated by the Vanderbilt Microarray Shared Resource. Full gene lists and protocols are available at http://array.mc.vanderbilt.edu. Briefly, all cDNA clones were amplified and verified by gel electrophoresis. The dried PCR products were then resuspended in 40 μl VMSR buffer A (Vanderbilt Microarray Shared Resource, Nashville, TN) resulting in an average concentration of 400 – 700 μg/ml per product. The PCR products were then transposed to 384 well plates and robotically arrayed using a BioRobotics MicroGrid II microarray printing robot (Apogent Discoveries, Hudson, NH) onto polylysine-coated glass slides (Cel Associates, Pearland, TX). The microarrays were then crosslinked using 80 mjoules of ultraviolet energy (Stratgene Stratalinker, LaJolla, CA). Following crosslinking, the arrays were baked for two hours at 70° C in a standard oven and stored under low humidity conditions until use.

RNA Labeling and MitoChip Hybridization—To produce targets for hybridization to the microarrays, 30μg of total RNA was labeled with fluorescent nucleotides by reverse transcription. The RNA was mixed with 2 μg of anchored oligo-dT (5′-

TTTTTTTTTTTTTTTTTT VN-3′) and incubated at 70°C for 5 minutes followed by 10 minutes at 25°C. The denatured and annealed RNA was then reverse transcribed in a 30 μl reaction mix containing reaction buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$ pH 8.3), 10 mM dithiothreitol, 500 μM dATP, 500 μM dGTP, 500 μM dTTP, 120 μM dCTP, 20 U SuperScript reverse transcriptase (Life Technologies, Gathiesburg, MD) and either 120 μM Cy5-dCTP (control samples) or 120 μM Cy3-dCTP (experimental samples). The reactions were incubated at 42°C for 2 h. Following incubation, 15 μl of 0.1M NaOH was added to degrade the remaining template RNA and the sample incubated at 70°C for 10 min. The reaction was neutralized by the addition of 15 μl of 0.1 M HCl followed by 440 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). The synthesized cDNA was purified using a silica-based DNA binding column (Qiagen). After purification, the target samples were desiccated and the final probe volume was adjusted to 55 μl hybridization buffer (25% formamide, 5x SSC, 0.1 % SDS, 10 μg yeast tRNA, 10 μg poly A RNA, 1 μg human COT-1 DNA). The target was denatured at 95° C for 2 min and added to the array. The probe and array were covered by a 24 mm × 60 mm coverslip (Lifterslip, Erie Scientific, Portsmouth, NH) and placed in a humidified hybridization chamber (Corning, Acton, MA) and incubated at 65°C for 12–16 h. Following hybridization, the arrays were washed with successive 5-minute washes in 2xSSC, 0.1%SDS; 1xSSC; and 0.1xSSC. After the final wash, the arrays were dried by centrifugation at 20xg and scanned using the Axon 4000B array scanner (Axon Instruments, Union City, CA).

Data Analysis—Microarrays were scanned using an Axon 4000B scanner and the raw data generated by the accompanying software, GenePix 4.0. Raw data were then analyzed using GeneTraffic 2.6 by Iobion Informatics, LLC. All 11,850 spots were first filtered and removed from analysis if the following criteria were not met:

1. Cy5 signal to background intensity ratio greater than 1.5
2. Cy3 signal to background intensity ratio greater than 1.5
3. Cy5 signal greater than 200
4. Cy3 signal greater than 200

The arrays were performed in triplicate. Data were normalized using the Lowess sub-grid method. Lists of features with a fold change of greater than two and a covariance of less than 30% (across the triplicate experiments) were generated for each treatment. These lists were then compared to each other to identify genes with interesting expression profiles across each category.

cDNA Synthesis and Quantitative Real-time PCR

Total RNA was isolated from HepG2 cells with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) using the instructions provided by the manufacturer. RNA was initially reverse transcribed to first-strand cDNA by PCR, using the conditions described below. PCR was performed using p27 and cyclophilin primers in a total volume of 50 μl in the presence of SYBR Green PCR Master Mix (Applied Biosystems). Primers used to amplify p27 are as follows: 5′-GCAGAGACATGGAAGAGGCG-3′ and 5′-CCTTCTCCACCTCTTGCCAC-3′. Primers used to amplify cyclophilin are as follows: 5′AAGGTGAAAGAAGGCATGAGCA-3′ and 5′-AGTTGTCCACAGTCGGAGATGG-3′. The reactions were performed under the following conditions: 48°C for 30 min (this step was included to synthesize cDNA), 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were performed using the ABI Prism 7700 sequence detection system, and the fluorescence was determined three times during each cycle. The data were analyzed via the Sequence Detection System version 1.7 software. Normalized fluorescence was plotted against cycle number (amplification plot), and the threshold suggested by the software was used to calculate the cycle at threshold (C_t). Results

of the real-time PCR were expressed as C_t, and the level of expression of p27 was indicated by the number of cycles required to achieve the threshold level of amplification.

Chromatin Immunoprecipitation (ChIP) Assays

HepG2 cells (1×10^8 cells per treatment) were kept in serum-free DMEM at 37 °C in 5% CO_2 for about 48 h. After a 1-h TNF-α treatment, protein-DNA cross-linking was performed with 1% formaldehyde in serum-free DMEM at room temperature for 10 min. The cells were then harvested into PBS buffer (ice-cold 1× PBS with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The cells were then pelleted and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, with protease inhibitors). After incubation on ice for 20 min, the lysates were mixed with 25-μm-diameter glass beads (MO-SCI Corp., Rolla, MO) and sonicated (VirSonic; 2.5-mm tip) at 4 °C 10 times with 15-s pulses at a setting of 6 (output of 6 watts) to shear chromatin to ~500-bp fragments. The supernatant from each sample was divided into aliquots for a subsequent 10-fold dilution in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, pH 8, 16.7 mM Tris-HCl, pH 8, with protease inhibitors). Each sample was pre-cleared to reduce nonspecific background using 40 μl of a 1:1 protein A/G-agarose slurry (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with 100 μg/ml salmon sperm DNA (Stratagene, La Jolla, CA) for 3 h at 4 °C on a rotating wheel. The beads were then pelleted, and the supernatant was transferred to a new tube. The precleared extracts were incubated overnight with 4 μg of the antibody or without antibody (as a negative control) at 4 °C on a rotating wheel. Immune complexes were collected using 40 μl of a 1:1 protein-A/G-agarose slurry plus 100 μg/ml salmon sperm DNA, with constant rotation for 3 h at 4 °C. The agarose beads were pelleted at 1000 × *g* for 2 min and washed for 10 min at room temperature with 1 ml of each of the following buffers in succession: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8, 20 mM Tris-HCl, pH 8, 500 mM NaCl, pH 8), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8), and two times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Chromatin complexes were eluted from the beads by two 1-h incubations with 160 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature. The DNA-protein cross-links were reversed by incubating the samples at 65 °C for ≥12 h. The RNA and protein in the samples were digested using an RNase mixture (Ambion, Austin, TX) and 50 μg/ml proteinase K, respectively. The DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and resuspended in 50 μl of nuclease-free water. Five microliter aliquots were used for each PCR or real time PCR utilizing primers specific for the p27 gene promoter. The forward and reverse primers used for the proximal κB element gene promoter were 5′-TCGCCAGTCCATTTGATCAG-3′ and 5′-GGAGCCAAAAGACACAGACC-3′, repectively. The forward and reverse primers used for the distal κB element were 5′-ACTCCCACACGCAGCCAATG-3′ and 5′-GCTGTCTCAGACACGTTTAG-3′, respectively. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The antibodies used for p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

[^{3}H]Thymidine Incorporation Assays

In all [^{3}H]thymidine incorporation experiments, cells were plated at 40,000 cells per well in 24-well plates, infected with adenoviruses, incubated for 48 h, and pulsed for the last 2 h of the treatment time with 40 μl of 0.1 mCi/ml [^{3}H]thymidine, as described previously [15].

Cdk2 Assays

Cdk2 kinase assays were performed as described previously [16]. Briefly, 1.5 to 3.0 mg of total cell extract was incubated with 4 μg of anti-Cdk2 antibody for 24 h at 4°C. The following day, 30 μl of protein A-Sepharose beads was added and mixed for an additional 2 h at 4°C. The beads were washed twice with detergent lysis buffer (described above) and twice with kinase assay buffer (50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1 mM NaF, and 0.1 mM Na_3VO_4). Kinase reactions were performed for 45 min at 37°C in kinase assay buffer and contained 20 μM [γ-^{32}P]ATP at a specific activity of 10 Ci/mmol and 5 μg of histone H1. Reactions were stopped with fourfold-concentrated Laemmli sample buffer, and reaction products were separated into several portions and resolved by SDS-PAGE on 12% gels. The gels were exposed to film to visualize phosphorylated histone H1. Radioactivity incorporated into histone H1 was quantitated using Packard Instant Imager electronic autoradiography.

Results

p65 and S276A regulate the expression of numerous hepatic genes

Experiments were designed to identify genes that are differentially regulated by p65 and the S276A mutant of p65 in HepG2 hepatoma cells. HepG2 cells were infected with an adenovirus expressing either wt p65 or the S276A p65 mutant. Another set of cells was infected with the same titer of a GFP-expressing adenovirus to control for the effect of viral infection on gene expression. Immunoblot analysis was performed to ensure that p65 and S276A were expressed at comparable levels in HepG2 cells (data not shown). Microarrays containing 11,520 cloned genes from the human sequence verified clone set from Research Genetics were generated by the Vanderbilt Microarray Shared Resource and utilized in this analysis. Microarrays were scanned using an Axon 4000B scanner and the raw data were generated by the accompanying software, GenePix 4.0 and analyzed using GeneTraffic 2.6, as described in Experimental Procedures. Data for the genes regulated by p65 and/or S276A by greater than two-fold are shown in Tables I-IV, with changes in gene expression of two-fold or greater in bold type. Genes are grouped according to the function of their gene product. Interestingly, many of these genes encode proteins involved in signal transduction, cell cycle regulation, electron transport and transcription. The regulation of the majority of these genes by NF-κB has not been described previously.

Table I summarizes data for genes both upregulated or downregulated by both p65 and S276A by greater than two-fold. Since p65 and S276A regulate these genes in the same manner, phosphorylation of S276 does not appear to be important for the regulation of this gene set. A different set of genes, however, is regulated by p65 but not significantly affected by S276A, suggesting that the phosphorylation of p65 on S276 is required for the regulation of these genes (Table II). Conversely, a somewhat unexpected result was that several genes are regulated by S276A, but not by p65 or are regulated in an opposite fashion by p65 (Table III). This result suggests that phosphorylation of p65 on S276 may be necessary to maintain the expression of these genes at a basal level or to prevent p65 from up- or downregulating these genes.

Experiments were also designed to determine whether S276A could also act as a dominant-negative regulator of TNF-α-activated gene transcription. GFP- or S276A-infected HepG2 cells were treated with TNF-α, an activator of p65. Despite its ability to regulate gene expression, S276A indeed acted in a dominant negative fashion and inhibited the expression of several genes regulated by TNF-α (Table IV). S276A alone had little effect on these genes. Since TNF-α causes phosphorylation of S276 [4,5], these data suggest that phosphorylation of S276 is important for the regulation of the genes listed in Table IV and further suggest that an interaction between p65 and CBP/p300 may be necessary for this regulation.

Regulation of the p27 promoter by p65

A striking result from the microarray analyses described above was the observation that S276A regulated several genes involved in cell cycle regulation in a manner opposite that of p65 (Table III). S276A induced the expression of genes encoding cyclin-dependent kinase inhibitor 1B (p27), cyclin-dependent kinase inhibitor 2C (p18), S100 calcium binding protein P and BTG family, member 2, and BTG family, member 3, while p65 had little effect or the opposite effect on these genes. During G1 to S phase progression of the cell cycle, several proteins, including p27 and p18, inhibit the activity of cdks to control the G1 to S phase cell cycle transition [17, 18]. Of the proteins encoded by these genes, p27 is the most highly characterized with regard to cell cycle control. To confirm the effect of S276A on p27 mRNA observed by microarray analysis, quantitative reverse transcriptase PCR was performed. PCR data confirmed the results observed with microarray analysis (Fig. 1A).

Since p27 is an important regulator of the cell cycle, the effect of S276A on p27 gene expression was further pursued. Reporter assays were performed using a p27-luciferase reporter construct, which consists of from −1609 to +178 of the p27 promoter upstream of the luciferase reporter gene, to determine if p65 and S276A have effects on p27 gene promoter activity. HepG2 cells were transiently transfected with this construct in the presence or absence of co-transfected expression vectors encoding p65 or S276A. In agreement with the microarray analysis, p65 repressed p27 gene expression, while S276A had a stimulatory effect (Fig. 1B).

As a further confirmation of p65-mediated regulation of the p27 gene promoter chromatin immunoprecipitation (ChIP) assays were performed to determine if p65 binds directly to the endogenous p27 gene promoter in response to TNF-α treatment (Fig. 1C). Two potential κB elements were identified between −1609 and +178 of the p27 promoter. The first potential element, positioned from −189 to −180, consists of the sequence GGGGCTTCCC. This element is referred to as the proximal κB element (pκB). The second potential element, positioned from −1330 to −1321, consists of the sequence GGGAAGATCC and is referred to as the distal κB element (dκB). PCR primers designed to amplify from −230 to −116 and from −1375 to −1270 of the human p27 promoter were used to determine if p65 binds to the region encompassing the two potential κB elements listed above. As shown in Fig. 1C, endogenous p65 binds to both of these sites after TNF-α treatment.

Regulation of p27 protein levels by NF-κB

Since the p27 gene is induced by S276A, p27 protein levels were also examined in HepG2 cells infected with increasing titers of the p65 or S276A adenovirus. Increased expression of S276A did indeed increase p27 protein levels, corresponding to the increase in mRNA observed by microarray analysis (Fig. 2A). The increase in p27 protein was not limited to HepG2 cells, as similar results were observed in the breast cancer cell line, MDA-MB-231 (Fig. 2A). These data and the data presented in Fig. 1, imply that TNF-α, a physiological activator of serine 276 phosphorylation, downregulates p27 protein levels. Furthermore, other activators of NF-κB, including phorbol esters and pervanadate, also decreased p27 protein levels (Fig. 2B).

Although S276A upregulates the genes encoding p18, BTG2, and BTG3, the proteins encoded by these genes were not detected in HepG2 cells (data not shown) and were not further studied.

S276A inhibits cell proliferation and cdk2

p27 is an inhibitor of cell cycle progression. Since S276A increased p27 levels, it was reasoned that S276A would inhibit cell proliferation. To test this hypothesis, [^{3}H]-thymidine incorporation assays were performed in HepG2 cells (Fig 3A). HepG2 cells were infected with increasing titers of GFP-, p65- or S276A-expressing adenoviruses for 48h, followed by the measurement of [^{3}H]-thymidine incorporation into DNA. As expected, S276A inhibited cell

proliferation in a dose-dependent manner. Similar results were observed in the breast cancer cell line, BT-549 (data not shown), suggesting that the effect of S276A on cell proliferation is not specific to hepatoma cells.

While it is possible that other genes regulated by S276A are responsible for the inhibition of DNA synthesis observed in HepG2 cells, the decrease in DNA synthesis by S276A strongly correlates with increased p27 protein levels. To further show that the level of p27 induced by S276A is sufficient to decrease DNA synthesis, HepG2 cells were infected with an adenovirus expressing p27 or S276A for 48 h followed by [^{3}H]-thymidine incorporation assays. As shown in Fig. 3B, the level of p27 necessary to inhibit DNA synthesis is similar to the level of p27 induced by S276A for inhibition of DNA synthesis, suggesting that the regulation of p27 by S276A is a major determinant of the effect of S276A on cell proliferation.

These data also suggest that expression of S276A inhibits cdk2. To test this, cdk2 was immunoprecipitated from HepG2 cells infected with adenovirus expressing either GFP, p65, or S276A. As demonstrated in Fig. 3C, S276A expression caused inhibition of cdk2 activity, while p65 had little effect compared to the GFP control. Immunoblot analysis of the cdk2 immunoprecipitates showed that inhibition of cdk2 by S276A correlated with increased association of the cdk inhibitor p27 with cdk2 (Fig. 3C). p21, another cell cycle inhibitory protein, also coimmunoprecipitated with cdk2, however, cdk2-associated p21 levels did not change, showing the specific effect of S276A on p27 binding to cdk2.

Discussion

NF-κB is a dimeric transcription factor that plays an important role in a number of processes, including cell cycle control and cell survival. This transciption factor exists as a heterodimer of p50 and p65 subunits in most cell types and is sequestered in the cytoplasm by its inhibitor proteins, the IκBs [1,2]. NF-κB activity is controlled at several levels, including nuclear translocation, post-translational modifications, such as phosphorylation and acetylation, and DNA binding.

The role of NF-κB phosphorylation for the regulation of transcription is likely to be complex, as several serine residues of the p65 subunit are phosphorylated, and phosphorylation of these sites affects the activity of NF-κB and its interaction with co-regulators [3,4,6,19–26]. An even greater degree of complexity occurs at the level of NF-κB binding to DNA. The DNA microarray data presented in this study reflect the complex nature of p65-mediated gene regulation and the role of S276 phosphorylation. While S276A and p65 regulate several of the same genes similarly, p65 regulates genes that are not regulated by S276A (Table II). S276A is also capable of acting in a dominant-negative fashion by inhibiting the regulation of certain genes by TNF-α (Table IV). Intriguingly, S276A regulates a set of genes that are not highly regulated by p65 (Table III). One possible explanation for this observation is that S276A upregulates other transcription factors that regulate the expression of these genes. Alternatively, phosphorylation of wt p65 may function to repress or maintain the expression of these genes at a basal level.

Anrather, et. al. [6] recently demonstrated that mutation of serine 276 inhibits the ability of p65 to activate transcription through two different *cis*-acting elements. These consensus sequences consist of KGRAHWTYCC (R = purine, Y = pyrimidine, K = G or T, H = not G, W = A or T,), a site present in several genes, including IL-6 and ICAM-1, and GGRWWWYYYY, a site present in IL-2α and porcine ELAM-1. Mutation of serine 276 in p65, however, has no affect on the recognition of the sequence GGGRATTYCC, which is present in genes including MHC class I and human ELAM-1 [6]. It thus appears that site-specific phosphorylation of p65 directs NF-κB to a particular *cis*-acting element to regulate

specific subsets of genes. This point is underscored by our observation that S276A is capable of regulating a set of genes in a manner similar to wild-type p65 but does not regulate all of the genes affected by p65 (Table I). The p27 promoter has two κB elements. The downstream κB element has the sequence of GGGGCTTCCC, while the upstream sequence is GGGAAGATCC. Both of these DNA elements display characteristics consistent with the consensus sites regulated by serine 276 phosphorylation.

Most studies of p27 show that it is regulated at the protein level by several mechanisms, including proteasomal degradation. Our data using DNA microarray, real-time PCR and transient transfection all indicate that TNF-α and NF-κB regulate p27 at the gene level. Since S276A, which does not interact with CBP, upregulates p27 mRNA, it is possible that an interaction between p65 and CBP is necessary for downregulation of the p27 gene promoter by wild-type 65. Alternatively, it is possible that p65 interacts with a co-repressor through phosphorylation of S276 and this interaction is lost with the S276A mutant.

The data presented here suggest that the control of p27 at the gene level is important for the inhibition of cdk2 activity and cell proliferation in hepatoma cells. It is plausible that mechanisms used to increase p27 gene transcription could be used to inhibit the uncontrolled cell proliferation present in cancer cells. Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies, being the third largest cause of cancer deaths worldwide [27]. NF-κB likely contributes to the pathology of HCC since it is constitutively active in human HCC tumors [28,29]. Furthermore, inhibition of NF-κB in later stages of tumor development results in apoptosis of transformed hepatocytes and a failure to progress to HCC [30]. Further evidence for a role for NF-κB in HCC comes from animal studies. Administration of NF-κB inhibitors to rats with highly metastatic HCC significantly decreased lung metastasis [31]. NF-κB, therefore, is a potential target for the treatment and prevention of HCC.

Decreased p27 expression is associated with advanced tumor stages in HCC and a high expression of p27 is a favorable prognostic indicator for patients with HCC [27,32–35]. Given the constitutive activation of NF-κB in HCC and our preliminary data showing that inhibition of p65 phosphorylation on S276 leads to increased p27 protein levels, it would be beneficial to identify treatments that inhibit NF-κB phosphorylation of serine 276 *in vivo*. In the future, it may be possible to increase p27 gene transcription by disrupting the protein-protein interaction of p65 with coregulators using small molecule antagonists. A compound that specifically disrupts the interaction of phosphorylated CREB with CBP, but not with other co-activators such as transducer of regulated CREB, has already been identified, showing the feasibility of such an approach [36]. Alternatively, one may be able to inhibit the kinase that phosphorylates serine 276 to increase p27 expression and inhibit uncontrolled cell proliferation in cancer.

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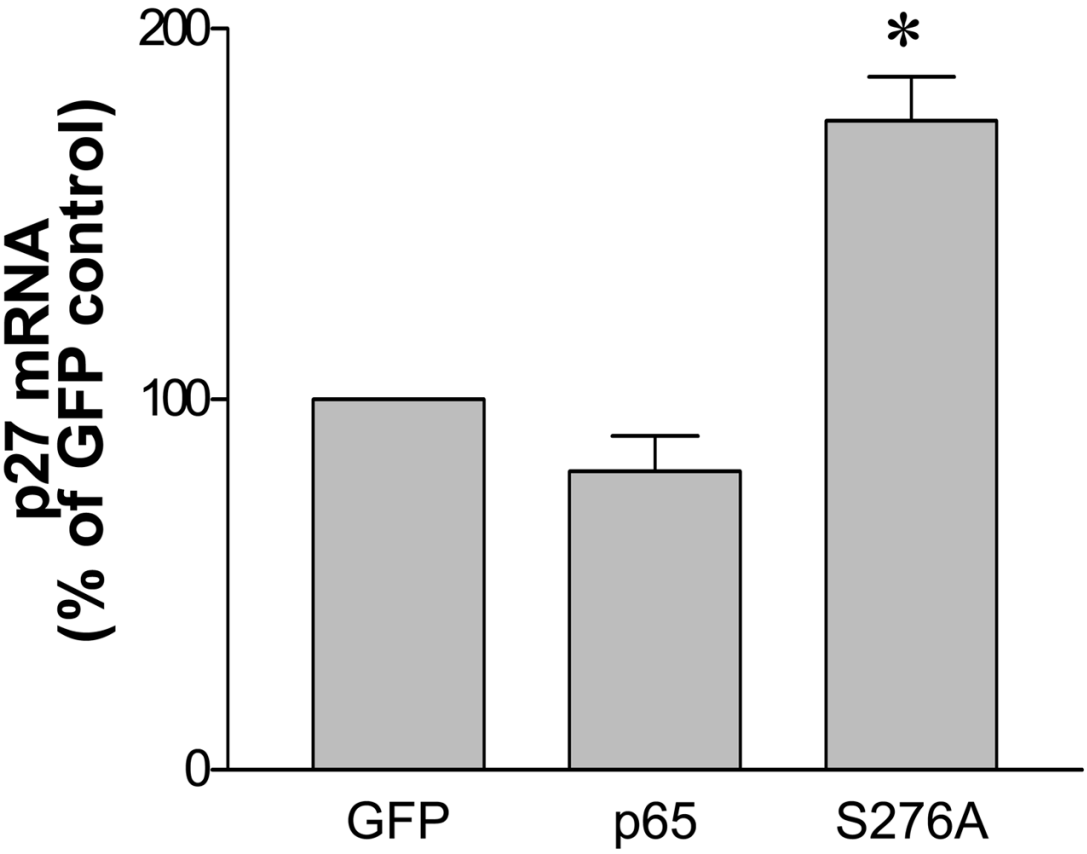
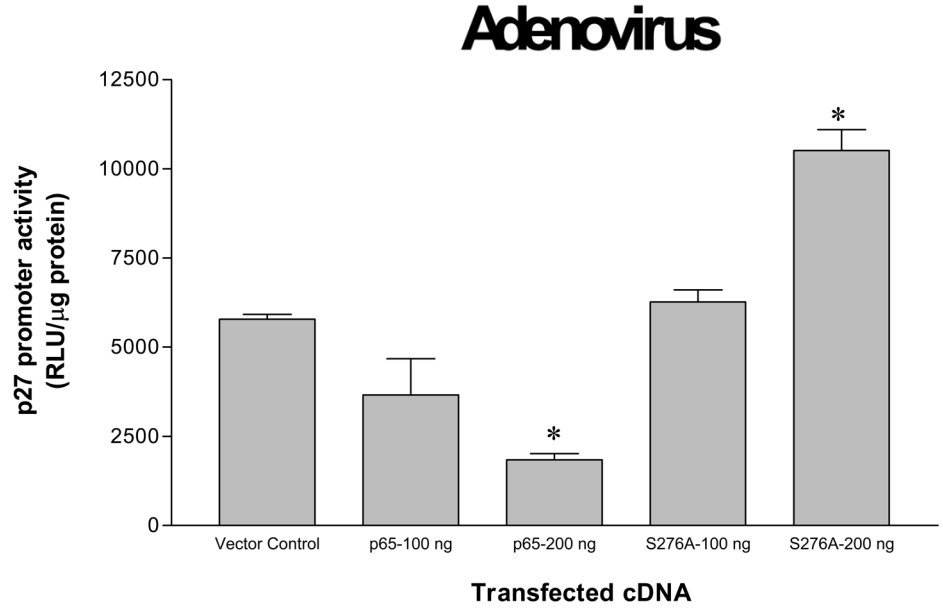




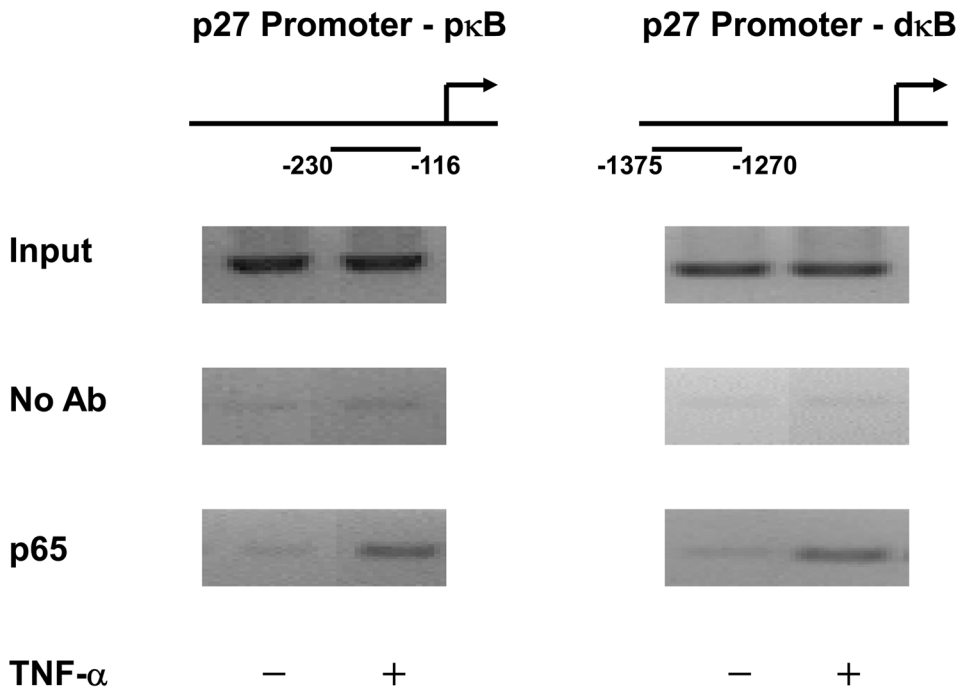


Fig. 1. p65 binds to the p27 promoter and represses p27 promoter activity

In panel A, HepG2 cells were infected with adenoviruses encoding GFP, p65, or S276A at an MOI of 50. Total RNA was isolated and p27 and cyclophilin mRNA were measured by real-time RT-PCR. p27 mRNA is normalized to that of cyclophilin and the amount of p27 mRNA detected in GFP-expressing cells is arbitrarily set at 100%. The graph represents three experiments ± S.E.M. In panel B, HepG2 cells were transiently transfected by a reporter construct consisting of −1609 to +178 of the p27 gene promoter upstream of the luciferase reporter gene. Cells were also co-transfected with either 100 or 200 ng of an expression vector encoding either p65 or S276A. DNA amounts were normalized using the plasmid pRc-RSV. Luciferase assays were performed and the results plotted as relative luminescence units per microgram of cell protein assayed. The data represent the average of triplicate determinations ± S.D. and is representative of three independent experiments. In panel C, the association of p65 with the endogenous p27 gene promoter was measured by ChIP assay in HepG2 cells. HepG2 cells were treated in the presence or absence of 1 ng/ml TNF-α for 24 h prior to the assay. The No Ab lane shows the results of immunoprecipitations performed in parallel without the application of primary antibodies. Input lanes show the results from samples not subjected to immunoprecipitation. Antibodies directed against endogenous p65 were used to measure the association of endogenous p65 with the p27 gene promoter. Primers designed to produce PCR products including the proximal κB (pκB) and distal κB (pκB) elements were used in these experiments.

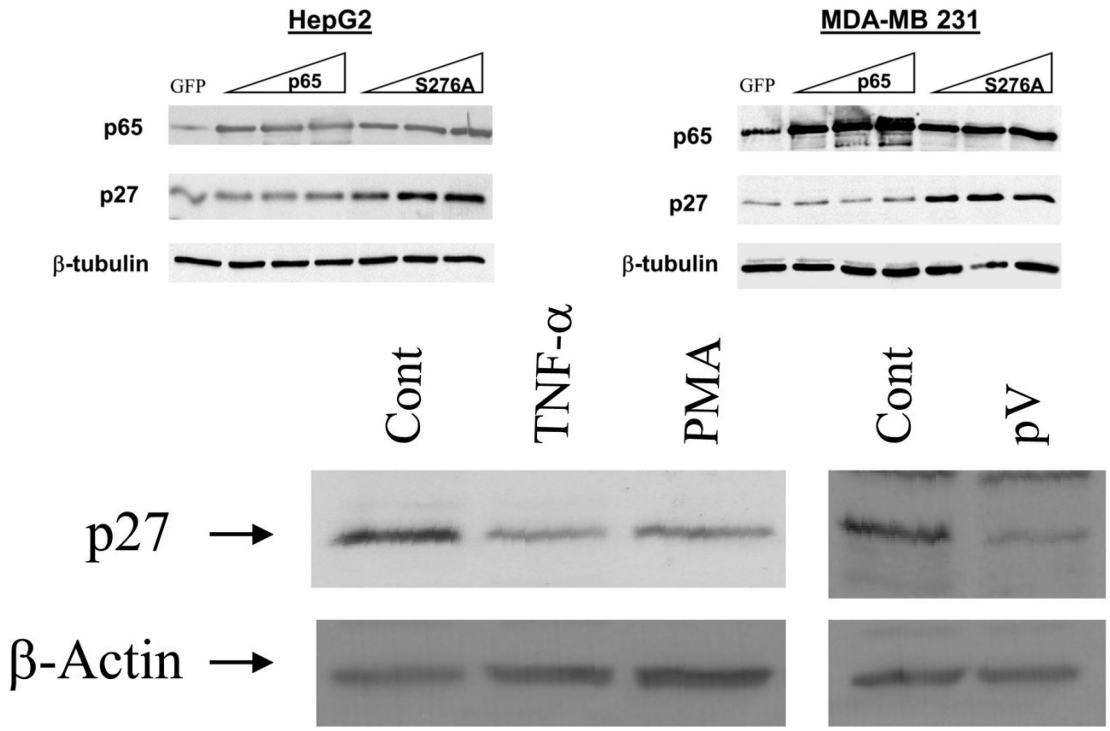
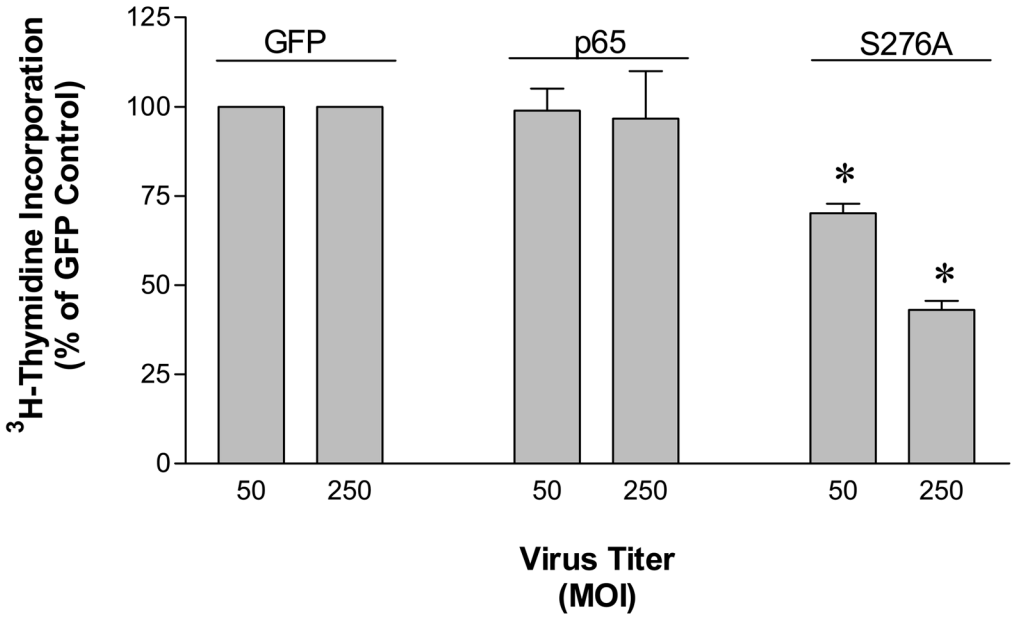
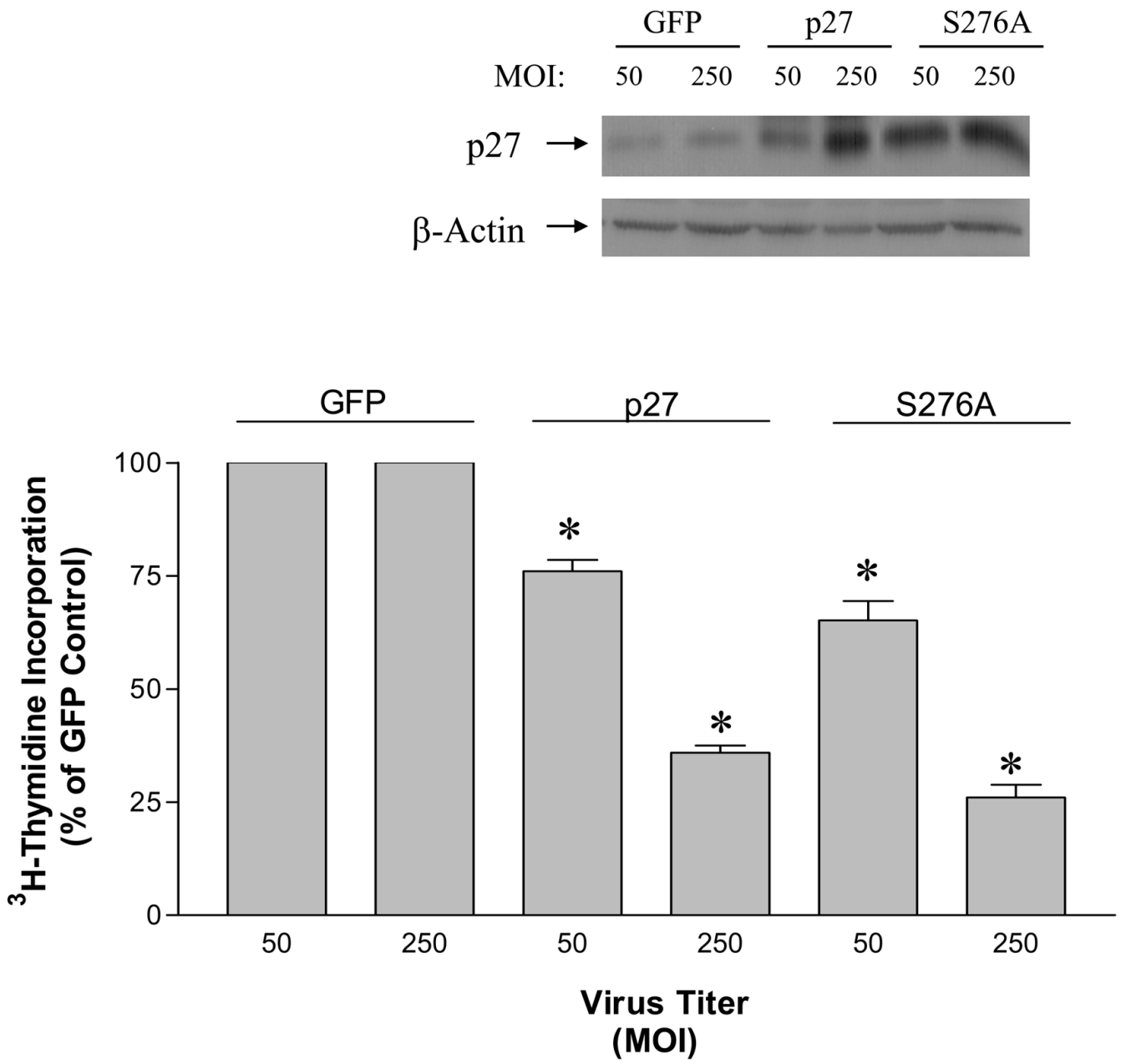


Fig. 2. S276A expression increased p27 protein levels

In panel A, HepG2 or MDA-MB-231 cells were infected with increasing titers of adenovirus (MOI = 25, 50 and 250) expressing p65, S276A, or GFP (MOI = 250). Cell lysates were prepared and immunoblot analysis was performed to examine expression of p65, p27, or β-tubulin (as a control for protein loading). In panel B, HepG2 cells were treated with 1 ng/ml TNF-α, 1 μM PMA, or 25 μM pervanadate (pV) for 24 h. Cell lysates were prepared and immunoblot analysis was performed to examine p27 and β-actin expression.





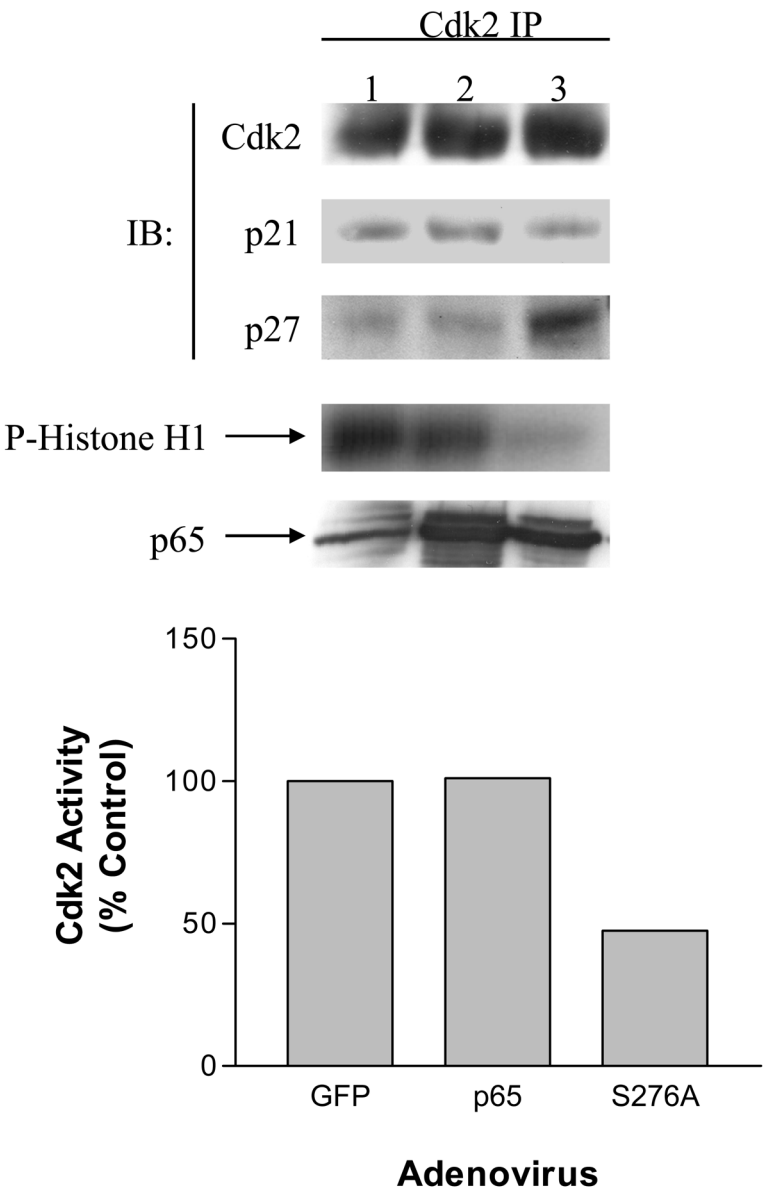


Fig. 3. S276A inhibited cell proliferation and cdk2 activity

To examine the effects of S276A on cell proliferation (panel A), HepG2 cells were subjected to [^{3}H]thymidine incorporation assays 48 h after viral infection with either the GFP-, p65-, or S276A-expressing adenovirus, as described in Experimental Procedures. The data presented are the result of three replicate determinations ± S.D. and are representative of three different experiments. In panel B, HepG2 cells were infected with an adenovirus expressing GFP, p27, or S276A (MOI = 50 or 250) and were subjected to [^{3}H]thymidine incorporation assays 48 h after viral infection. The data presented are the result of three replicate determinations ± S.D. and are representative of three different experiments. p27 expression was confirmed by immunoblot analysis of lysates prepared from cells infected with the GFP, p27, or S276A adenovirus. Cdk2 assays are shown in panel C. HepG2 were infected with adenoviruses expressing GFP (lane 1), p65 (lane 2), or S276A (lane 3) at an MOI of 50. To measure cdk2 activity, cdk2 was immunoprecipitated 48 h post-infection and the immunoprecipitates were incubated with [γ-^{32}P]ATP and histone H1, as a cdk2 substrate, as described in the

Experimental Procedures section. Phosphorylated histone is indicated by the arrow (panel C). Immunoblot analysis (IB) was performed to show equivalent immunoprecipitation of cdk2 from each sample. p21 and p27 immunoblot analysis was also performed to examine the association of these proteins with cdk2. Proteins from the same crude cell lysates used for cdk2 immunoprecipitation were probed with a p65-specific antibody in immunoblot analyses to confirm that p65 and S276A were expressed at comparable levels. The results shown are representative of three independent experiments.

Table I

Genes up- or down-regulated greater than two-fold by either p65 or S276A

GenBank accession no.	Gene name	Fold change	
		S276A	p65
Signal Transduction			
NM_000118	Endoglin	**3.21**	**2.85**
NM_003713	Phosphatidic acid phosphatase type 2B	**3.27**	**2.04**
Transcriptional Regulation			
NM_001328	C-terminal binding protein 1	**2.53**	**2.32**
Glucose/Lipid/Protein Metabolism			
NM_001701	Bile acid Coenzyme A: amino acid N-acyltransferase	**−2.39**	**−2.39**
Membrane Transporters			
NM_012449	Six transmembrane epithelial antigen of the prostate	**−2.56**	**−2.44**
Cell Adhesion/Motility			
NM_002026	Fibronectin 1	**2.07**	**3.34**
NM_002203	Integrin, alpha 2	**−3.10**	**−2.14**
Other			
NM_006383	DNA-dependent protein kinase catalytic subunit-interacting protein	**3.73**	**2.01**
NM_005620	S100 calcium binding protein A11	**−2.05**	**−3.61**
NM_007350	Pleckstrin homology-like domain, family A, member 1	**−2.60**	**−2.05**

Table II

Genes up- or down-regulated by p65

GenBank accession no.	Gene name	Fold change	
		S276A	p65
Signal Transduction			
NM_002996	Chemokine (C-X3-C motif) ligand 1	−1.05	**2.80**
NM_003810	Tumor necrosis factor (ligand) superfamily, member 10	−1.18	**3.07**
NM_002648	Pim-1 oncogene	−1.28	**2.06**
NM_012425	Ras suppressor protein 1	−1.09	**−2.00**
Cell Cycle Regulation			
NM_004748	Cell cycle progression 8 protein	1.18	**−2.15**
Glucose/Lipid/Protein Metabolism			
NM_001902	Cystathionase O-acyltransferase 3	1.09	**−2.14**
Membrane Transporters			
NM_000166	Gap junction protein, beta 1	−1.22	**2.13**
XM_010822	Putative L-type neutral amino acid transporter	1.43	**−2.00**
NM_007107	Signal sequence receptor, gamma	1.24	**−2.17**
NM_005827	Solute carrier family 35, member B1	1.10	**−2.45**
NM_006516	Solute carrier family 2 (GLUT1)	−1.05	**−2.45**
Serine/Cysteine Protease Inhibitors			
NM_000185	Serine proteinase inhibitor, clade D, member 1	−1.47	**2.12**
NM_000354	Serine proteinase inhibitor, clade A, member 7	1.04	**2.07**
NM_000488	Serine proteinase inhibitor, clade C, member 1	−1.16	**−2.11**
Electron Transport/Redox Regulation			
NM_002064	Glutaredoxin	1.05	**−2.02**
NM_018947	Cytochrome c	1.04	**−2.40**
Other			
NM_017660	p66 alpha	−1.25	**4.01**
NM_007021	Decidual protein induced	−1.51	**3.66**
NM_016305	Synovial sarcoma translocation gene	1.09	**3.20**
NM_153186	Kidney ankyrin repeat-containing protein	1.06	**2.50**
NM_002960	S100 calcium binding protein A3	1.17	**2.29**
NM_021175	Hepcidin antimicrobial peptide	−1.24	**2.08**
NM_005065	Sel-1 suppressor of lin-12-like	1.02	**−2.00**
NM_016009	SH3-domain GRB2-like endophilin B1	−1.17	**−2.02**
NM_006947	Signal recognition particle 72kDa	1.19	**−2.03**
NM_000270	Nucleoside phosphorylase	−1.07	**−2.07**
NM_003758	Eukaryotic translation initiation factor 3, subunit 1 alpha	−1.09	**−2.11**
NM_006010	Arginine-rich, mutated in early stage tumors	1.49	**−2.14**
NM_002970	Spermidine/spermine N1-acetyltransferase	1.19	**−2.14**
NM_000214	Jagged 1	−1.22	**−2.19**
NM_003299	Tumor rejection antigen (gp96) 1	1.00	**−2.28**
NM_021237	Selenoprotein K	1.65	**−2.30**

GenBank accession no.	Gene name	Fold change	
		S276A	p65
NM_002539	Ornithine decarboxylase 1	1.07	**−2.34**
NM_004184	Tryptophanyl-tRNA synthetase	1.19	**−2.70**
NM_012328	DnaJ (Hsp40) homolog, subfamily B, member 9	1.01	**−2.64**
NM_004911	Protein disulfide isomerase related protein	1.15	**−2.78**

Table III

Genes up- or down-regulated by S276A

GenBank accession no.	Gene name	Fold change S276A	Fold change p65
Membrane Receptor			
NM_003641	Interferon induced transmembrane protein 1 (9–27)	**2.00**	1.16
Signal Transduction			
NM_004419	Dual specificity phosphatase 5	**−2.32**	1.25
Transcriptional Regulation			
NM_006290	Tumor necrosis factor, alpha-induced protein 3	**3.31**	−1.12
NM_004906	Wilms tumor 1 associated protein	**3.20**	−1.65
NM_003403	YY1 transcription factor	**2.47**	−1.09
NM_006015	SWI/SNF related, subfamily f, member 1	**2.46**	1.00
NM_001430	Endothelial PAS domain protein 1	**2.36**	1.15
NM_003998	Nuclear factor of kappa light polypeptide (p105)	**2.29**	−1.21
NM_004343	Calreticulin	**2.25**	−1.23
NM_002166	Inhibitor of DNA binding 2	**2.15**	1.21
NM_015869	Peroxisome proliferative activated receptor, gamma (PPAR-γ)	**−2.20**	−1.21
NM_021969	Nuclear receptor subfamily 0, group B, member 2	**−2.22**	1.48
Cell Cycle Regulation			
NM_004064	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	**2.39**	−1.39
NM_001262	Cyclin-dependent kinase inhibitor 2C (p18)	**2.07**	-
NM_005980	S100 calcium binding protein P	**2.19**	−1.25
NM_006806	BTG family, member 3	**2.03**	−1.26
NM_006763	BTG family, member 2	**2.01**	1.39
Glucose/Lipid/Protein Metabolism			
NM_021122	Fatty-acid-Coenzyme A ligase, long-chain 2	**2.36**	−1.27
NM_005566	Lactate dehydrogenase A	**2.09**	−1.21
Tissue inhibitors of matrix metalloproteinases/Matrix metalloproteinases			
NM_003255	Tissue inhibitor of metalloproteinase 2	**4.19**	1.23
Membrane Transporters			
NM_017510	gp25L2 protein	**2.52**	1.03
Other			
NM_006041	Heparan sulfate 3-O-sulfotransferase 3B1	**3.39**	1.20
NM_000917	Procollagen-proline, 2-oxoglutarate 4-dioxygenase	**2.90**	1.05
NM_014447	Arfaptin 1	**2.44**	1.09
NM_006058	TNFAIP3 interacting protein 1	**2.41**	1.05
NM_005067	Seven in absentia homolog 2	**2.32**	1.10
NM_025076	UDP-glucuronate decarboxylase 1	**2.29**	−1.08
NM_016069	Mitochondria-associated protein involved in GMCSF signaling	**2.26**	−1.21
NM_004766	Coatomer protein complex, subunit beta	**2.11**	1.15
NM_000161	GTP cyclohydrolase 1	**2.06**	−1.34
NM_005803	Flotillin 1	**2.02**	−1.30

GenBank accession no.	Gene name	Fold change	
		S276A	p65
NM_001145	Angiogenin, RNase A family, 5	**−2.09**	1.08
NM_021139	UDP glycosyltransferase 2 family, polypeptide B4	**−2.14**	−1.02

Table IV

Dominant-negative effect of S276A on genes regulated by TNF-α

GenBank accession no.	Gene name	Fold change		
		S276A	TNF-α + GFP	TNF-α + S276A
Membrane Receptor				
NM_001470	GABA B receptor, 1	1.20	**7.44**	−1.69
Signal Transduction				
NM_002996	Chemokine (C-X3-C motif) ligand 1	−1.05	**3.44**	−1.60
NM_003246	Thrombospondin 1	0.85	**3.12**	−1.20
NM_004414	Down syndrome critical region gene 1	0.91	**2.03**	−1.67
Transcriptional Regulation				
NM_021008	Deformed epidermal autoregulatory factor 1	1.04	**2.14**	−1.49
NM_000346	SRY (sex determining region Y)-box 9	0.74	**2.11**	−1.67
NM_015885	PCF11p homolog	1.42	**2.00**	1.02
NM_005384	Nuclear factor, interleukin 3 regulated	1.06	**−2.00**	1.24
XM_167633	Thyroid hormone receptor interactor 8	−1.23	**−2.04**	1.02
NM_001675	Activating transcription factor 4	−1.10	**−2.12**	1.06
NM_170695	TGFβ-induced factor (TALE family homeobox)	−1.33	**−2.95**	1.23
Glucose/Lipid/Protein Metabolism				
NM_000596	Insulin-like growth factor binding protein 1	−1.07	**−2.49**	1.12
Cytoskeleton Formation				
U35637	Nebulin	0.96	**2.57**	**−2.56**
Electron Transport/Redox Regulation				
NM_002083	Glutathione peroxidase 2	−1.14	**2.05**	1.20
Other				
NM_0059501	Metallothionein 1G	−1.17	**2.28**	−1.03
NM_005952	Metallothionein 1X	−1.07	**2.10**	−1.05
NM_005947	Metallothionein 1B	−1.24	**2.02**	−1.11