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Down-regulation of the Tumor Suppressor PTEN by the Tumor Necrosis Factor- α /Nuclear Factor- κ B (NF- κ B)-inducing Kinase/NF- κ B Pathway Is Linked to a Default I κ B- α Autoregulatory Loop*

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The PTEN (phosphatase and tensin homolog deleted on chromosome ten) tumor suppressor gene affects multiple cellular processes including cell growth, proliferation, and cell migration by antagonizing phosphatidylinositol 3-kinase (PI3K). However, mechanisms by which PTEN expression is regulated have not been studied extensively. Similar to PTEN, tumor necrosis factor- α (TNF- α) affects a wide spectrum of diseases including inflammatory processes and cancer by acting as a mediator of apoptosis, inflammation, and immunity. In this study, we show that treatment of cancer cell lines with TNF- α decreases PTEN expression. In addition, overexpression of TNF- α downstream signaling targets, nuclear factor- κ B (NF- κ B)-inducing kinase (NIK) and p65 nuclear factor NF- κ B, lowers PTEN expression, suggesting that TNF- α -induced down-regulation of PTEN is mediated through a TNF- α /NIK/NF- κ B pathway. Down-regulation of PTEN by NIK/NF- κ B results in activation of the PI3K/Akt pathway and augmentation of TNF- α -induced PI3K/Akt stimulation. Importantly, we demonstrate that this effect is associated with a lack of an inhibitor of κ B (I κ B)- α autoregulatory loop. Moreover, these findings suggest the interaction between PI3K/Akt and NF- κ B via transcriptional regulation of PTEN and offer one possible explanation for increased tumorigenesis in systems in which NF- κ B is chronically activated. In such a tumor system, these findings suggest a positive feedback loop whereby Akt activation of NF- κ B further stimulates Akt via down-regulation of the PI3K inhibitor PTEN.

The tumor suppressor gene PTEN¹ (1, 2) (phosphatase and tensin homolog deleted on chromosome ten)/MMAC (mutated

in multiple advanced cancers), encodes a dual specificity phosphatase with lipid and protein phosphatase activity (3, 4). PTEN plays an important role in carcinogenesis of multiple human cancers (2, 5) and is the causative germ line mutation found in dominantly inherited Cowden disease and Bannayan-Zonana syndrome (6, 7). These syndromes are characterized by hamartoma formation in the gastrointestinal tract and other locations and a proclivity for cancer formation. PTEN modulates cell growth and survival by negatively regulating the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (4, 5, 8, 9), to decrease cell migration and invasion (10, 11), and to cause cell cycle inhibition (12, 13). Yet, despite the important role played by PTEN in cellular processes and the extensive characterization of mutations in human cancers, only a few reports have evaluated the regulation of PTEN expression. A p53-dependent regulation of the PTEN gene has been described (14); however, another study has suggested that p53 plays only a minor role in PTEN gene expression (15). The ligand-activated nuclear receptor peroxisome proliferator-activated receptor- γ has also been shown to up-regulate PTEN expression (16–18).

Tumor necrosis factor- α (TNF- α) is a secreted molecule that is involved in the control of tissue homeostasis and also in the progression of pathological conditions (20). It regulates many cellular processes including apoptosis, abnormal cell growth, inflammation, and angiogenesis (20). TNF- α binding to the subtype of receptors containing a death domain (TNFR1) recruits TRADD (TNFR1-associated death domain protein), FADD (Fas-associated-death domain protein) and FLICE (FADD-like ICE; also known as caspase 8) to induce apoptosis (19, 20). Alternatively, TNFR1 recruits RIP (receptor-interacting protein) to activate NF- κ B signaling (19), whereas TRADD can interact with TRAF2 (TNFR-associated factor 2) and activate the NF- κ B pathway through NIK (NF- κ B-inducing kinase) as well as AP-1 through c-Jun N-terminal kinase (21). On the other hand, TNF- α binding to the subtype of receptors without a death domain (TNFR2) recruits TRAF1 and TRAF2 and thus activates NF- κ B and AP-1 without influence on TRADD-dependent apoptosis (22). Finally, it is worth noting that TNF- α can also activate the PI3K/Akt pathway, which in turn facilitates NF- κ B activation by Akt (23). Importantly, the activation of NF- κ B inhibits the apoptosis induced by TNF- α through FADD and by other stimuli (24–26). In addition to its antiapoptotic effect, pivotal roles of NF- κ B have been described in immune and inflammatory responses through the regulation

kinase; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-PCR; TNF- α , tumor necrosis factor- α ; TNFR, tumor necrosis factor receptor; GSK, glycogen synthase kinase.

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¹ The abbreviations used are: PTEN, phosphatase and tensin homolog deleted on chromosome ten; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescence protein; I κ B, inhibitor of nuclear factor- κ B; NF- κ B, nuclear factor- κ B; NIK, nuclear factor- κ B-inducing

of pro- and anti-inflammatory genes (27) as well as in oncogenesis (28). NF- κ B is also implicated in growth control by regulating cyclin D1 (29) and c-Myc (30) expression. NF- κ B is a dimeric transcription factor formed by Rel family of proteins (RelA/p65, RelB, cRel, p50, p52) (for review, see Ref. 31). The predominant dimer in many cell types is the p65:p50 heterodimer. Its activity is regulated by I κ B (inhibitor of NF- κ B) isoforms, I κ B- α being the most prominent member, by complexing with NF- κ B in the cytoplasm and preventing nuclear translocation.

To understand better the role of PTEN in cellular processes and to formulate potential therapeutic management of diseases involving PTEN alteration, it is important to obtain insight into the regulation of PTEN expression. The role of TNF- α in activating NF- κ B has been well established. Although the PI3K/Akt pathway facilitates NF- κ B activation, whether NF- κ B, in turn, can affect the PI3K/Akt pathway antagonized by PTEN has not been examined. In this study, we show that treatment of cancer cell lines with TNF- α and subsequent activation of NIK and p65 NF- κ B decrease the expression of PTEN, thereby augmenting TNF- α activation of the PI3K/Akt pathway. This effect is associated with the absence of an I κ B- α autoregulatory loop.

EXPERIMENTAL PROCEDURES

Materials—TNF- α was purchased from Sigma. PI3K inhibitor, LY294002, was obtained from Cell Signaling (Beverly, MA) and dissolved in dimethyl sulfoxide. Akt inhibitor (1L-6-hydroxymethyl-chiroinositol 2-[(R)-2-O-methyl-3-o-octadecylcarbonate]) was purchased from Alexis Biochemicals (San Diego). Cell membrane-permeable, synthetic NF- κ B-binding peptide, SN50, and mutant control, SN50M, were obtained from Biomol (Plymouth Meeting, PA). Cell lysis buffer was purchased from Cell Signaling. NuPAGE 4–12% BisTris gel and Immunoblot polyvinylidene difluoride membranes were purchased from Invitrogen. The enhanced chemiluminescence (ECL) system was purchased from Amersham Biosciences, and SuperSignal West Dura Extended Duration Substrate was purchased from Pierce. The Bio-Rad Protein Assay was obtained from Bio-Rad Laboratories. Platinum *Taq* DNA polymerase and the PCRx enhancer system were purchased from Invitrogen. TriZol reagent was obtained from Invitrogen. X-ray film for autoradiography was from Eastman Kodak. Antibody against PTEN was obtained from Cell Signaling and BD Transduction Laboratories. Akt, phospho-Akt (Ser-473), and phospho-GSK-3 β were purchased from Cell Signaling. Anti-p65 NF- κ B, NIK, and normal rabbit, mouse, and rat horseradish peroxidase-conjugated antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- β -actin antibody was obtained from Sigma. The dual luciferase assay system, pRL-null plasmid, and pGL3-Basic reporter vector were obtained from Promega (Madison, WI). LipofectAMINE Plus reagent was purchased from Invitrogen. Cell culture reagents were from Cellgro (Herndon, VA). Adenovirus vector encoding p65 NF- κ B (Ad5p65), NIK (Ad5NIK), and hemagglutinin-tagged I κ B- α superrepressor (Ad5I κ B-AA) and its control vector (Ad5GFP) were gifts from Dr. Christian Jobin (University of North Carolina, Chapel Hill). The BD Adeno-X Virus Purification Kit and Adeno-X Rapid Titer Kit were purchased from BD Biosciences. Enzyme-linked immunosorbent assay (ELISA)-based transcription factor activity BD Mercury Transfactor kits for NF- κ B p65 and p50 were purchased from BD Biosciences. NE-PER Nuclear and Cytoplasmic Extraction Reagents were obtained from Pierce.

Cell Culture and Viral Infections—The human colon cancer cell lines HT29 and SW480, human cervical cancer cell line SiHa, and human embryonic kidney cell line HEK293 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). All cells were grown at 37 °C in a humidified incubator under 5% CO₂ and 95% air in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum. Cells were treated with TNF- α at the indicated doses in Dulbecco's modified Eagle's medium with fetal bovine serum. Adenoviruses were amplified in HEK293 cells, purified using the BD Adeno-X Virus Purification Kit, and titer determined using the Adeno-X Rapid Titer Kit. Cells were infected with adenovirus vectors at a 20 multiplicity of infection/cell, as described previously (32, 33).

Western Blot Analysis—Western blots were performed as described previously (33). Briefly, attached cells were collected and washed once

with phosphate-buffered saline. Whole-cell lysates were prepared using Cell Lysis Buffer containing 1 mM phenylmethylsulfonyl fluoride. Proteins (50–100 μ g/lane) were separated by NuPAGE 4–12% BisTris gel and transferred electrophoretically to Immuno-Blot polyvinylidene difluoride membrane. Membranes were blocked overnight at 4 °C or 1 h at room temperature with blocking solution (Tris-buffered saline solution containing 5% nonfat dry milk and 0.05% Tween 20). Blots were then incubated for 2–3 h at room temperature or at 4 °C overnight with primary antibodies in 1% buffer solution (Tris-buffered saline solution containing 1% nonfat dry milk and 0.05% Tween 20), washed twice with 1% buffer solution, and incubated with a horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. After two washes with Tris-buffered saline solution containing 0.05% Tween 20 followed by a final wash with Tris-buffered saline without Tween 20, the immune complexes were visualized using the ECL system or the SuperSignal West Dura system.

RNA Isolation and Real Time RT-PCR—Total RNA was isolated using TriZol reagent. Applied Biosystem Assays-by-Design (P/N 4331348) 20 \times assay mixture (Foster City, CA) of primers and TaqMan[®] MGB probes (FAM[™] dye-labeled) for the target gene, human PTEN (NCBI accession no. NM_000314), and predeveloped 18 S rRNA (VIC[™]-dye-labeled probe) TaqMan[®] assay reagent (P/N 4319413E) for internal control were utilized. Human PTEN primers were designed to span the exon 6-exon 7 junction so as not to detect genomic DNA. Primer and probe sequences were searched against the Celera Genomic data base (www.celera.com) to confirm specificity. The probe and primer sequences of human PTEN were as follows: probe, TGAGGATTGCAAGTTC; forward, CAAGATGATGTTTGAACCTAT-TCCAATG; reverse, CCTTTAGCTGGCAGACCACAA. A validation experiment was performed to test the efficiency of the target amplification and the efficiency of the reference amplification. The absolute value of the slope of log input amount versus ΔC_T was <0.1. Singleplex one-step RT-PCR was performed with 20 ng of RNA for both target gene (PTEN) and endogenous control. The reagent used was TaqMan[®] one-step RT-PCR master mix reagent kit (P/N 4309169). The cycling parameters for one-step RT-PCR were as follows: RT, 48 °C for 30 min; AmpliTaq activation, 95 °C for 10 min; denaturation, 95 °C for 15 s; and annealing/extension, 60 °C for 1 min (repeat 40 times). Triplicate C_T values were analyzed using Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained after normalization with an endogenous reference (18 S).

ELISA-based Transcription Factor Activity Assay—Nuclear protein from HT29 cells was extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents and quantified using the protein assay dye reagent. ELISA-based transcription factor activity assay was performed following the manufacturer's protocol. Briefly, 10 μ g of nuclear extracts were incubated within 96-well format chamber coated with oligonucleotides containing consensus binding sequences for either p50 or p65 NF- κ B. Bound transcription factors were then detected by a specific anti-p50 or -p65 antibodies followed by a horseradish peroxidase-conjugated secondary antibody, which was used to detect the bound primary antibody. For competition assays, excess competitor oligonucleotides were coincubated with nuclear protein. For p50 and p65 transcription factor activity assays, 500-ng and 1,000-ng competitor oligonucleotides were used, respectively. Samples were run in triplicates, and all experiments were performed on at least two separate occasions.

Transient Transfection, Luciferase Assays—A PCR fragment encompassing the human PTEN promoter was amplified with the primers PTENa 5'-GGTAACCTCAGACTCGAGTCAGTGA-3' and PTENb 5'-TGGCTGCCATGGCTGGGAGCCTGTG-3' using Platinum *Taq* DNA polymerase and the PCRx enhancer system following the instructions of the supplier. The PCR fragment was cut with the restriction enzymes XhoI and NcoI and inserted in the corresponding sites of the pGL3-Basic reporter vector. Cell transfections were conducted as described previously (33). Briefly, HT29 cells were seeded at 1×10^5 cells into 24-well plates in triplicate 24 h prior to transfection. Cells were then transiently transfected with 0.4 μ g of the PTEN-promoter firefly luciferase reporter plasmid and 0.05 μ g of the *Renilla* reporter pRL-null to normalize for variation in transfection efficiency, using LipofectAMINE Plus transfection agent following the manufacturer's recommended protocol. Cells were harvested for measurement of firefly and *Renilla* luciferase activities using the dual luciferase assay system. Firefly luciferase activity was determined by subtracting background signal and normalized to the *Renilla* activity. Fold inhibition was calculated by dividing treatment values by control values.

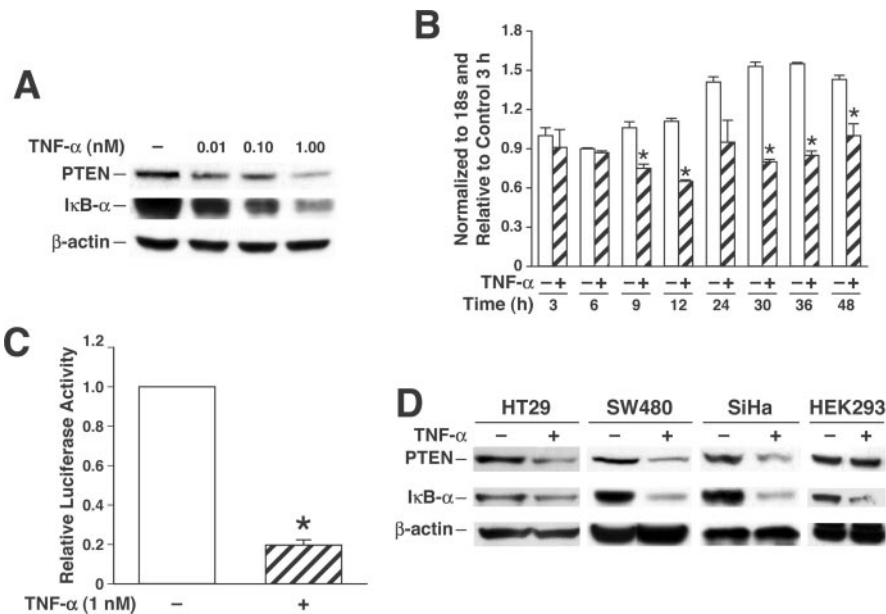


FIG. 1. TNF- α decreases PTEN expression. *A*, HT29 cells were treated with 0.01–1 nM TNF- α for 24 h. Attached cells were collected, lysed, and proteins extracted for Western blot analysis. Blotting for PTEN and I κ B- α shows a TNF- α dose-dependent decrease in PTEN and I κ B- α protein expression. β -Actin levels show equal loading of protein. *B*, HT29 cells were treated with 1 nM TNF- α over a 3–48-h time course. For each time point, cells were collected and total RNA extracted for PTEN real time RT-PCR analysis. Analysis shows suppression of PTEN mRNA starting at 9 h for TNF- α -treated cells. Untreated cells displayed an increasing level of PTEN expression up to 48 h. The experiments were repeated on three separate occasions (mean \pm S.E. for double determinations; * = $p < 0.05$ compared with control). *C*, to evaluate whether the decrease in PTEN mRNA expression is caused by transcription suppression, a PTEN promoter luciferase construct was transfected into HT29 cells. After 24 h post-transfection, cells were treated with 1 nM TNF- α for 24 h. Luciferase assay shows significant suppression of PTEN promoter activity (mean \pm S.E. for triple determinations; * = $p < 0.05$ compared with control). *D*, other human cell lines were treated with 1 nM TNF- α for 24 h to determine whether TNF- α had an effect on PTEN expression similar to that in HT29. Similar results were obtained for SW480 and SiHa, but in HEK293 cells, PTEN levels remained unchanged despite reduction in I κ B- α levels.

Statistical Analysis—All data presented with statistical analysis were analyzed using a two-sample *t* test. All tests were assessed at the 0.05 level of significance.

RESULTS

TNF- α Treatment Decreases PTEN Expression—To examine the effect of TNF- α on PTEN expression, we used the human colon cancer cell line HT29, a cell line without mutation in the endogenous PTEN gene sequence.² HT29 is used extensively as a model of intestinal epithelial cell proliferation/differentiation and colorectal cancer (34). Cells growing in serum-containing medium were treated with increasing concentrations of TNF- α (0.01–1.0 nM) for 24 h and extracted for protein expression. Western blotting demonstrated a TNF- α -mediated dose-dependent decrease in PTEN (Fig. 1A). Concomitant with PTEN decrease, a similar reduction in I κ B- α was also observed, demonstrating TNF- α -induced I κ B- α degradation. To examine whether the reduction of PTEN protein was linked to a modification in gene expression, HT29 cells were treated with 1 nM TNF- α over a time course, and total cellular mRNA was extracted for analysis of PTEN expression by real time RT-PCR. As shown in Fig. 1B, TNF- α caused a significant reduction in PTEN mRNA, which was apparent after 9 h of treatment and continued over 24 h. In addition, we constructed a reporter plasmid containing 1,365 bp of the human PTEN promoter (NCBI accession no. NM_000314) inserted upstream of the luciferase reporter gene. This plasmid was transfected into HT29 cells, and luciferase assay was performed 24 h after 1 nM TNF- α treatment; luciferase activity was reduced in TNF- α -treated cells as compared with untreated cells (Fig. 1C). Together, these data indicate that TNF- α down-regulates PTEN expression at the level of gene transcription.

To assess whether the TNF- α effect on PTEN is limited to certain cell types, we examined other human-derived cell lines. Similar to results obtained in HT29 cells, decreased PTEN protein expression was noted in the colon cancer cell line SW480 and in the cervical cancer cell line SiHa (Fig. 1D). However, a reduction in PTEN expression was not observed in the human embryonic kidney cell line HEK293, suggesting that the TNF- α -mediated reduction in PTEN expression is cell line-dependent.

NF- κ B Activation Is Sustained in HT29 Cells Treated with TNF- α —Because the NF- κ B pathway is an important target of TNF- α signaling, we analyzed key elements of this pathway after TNF- α treatment in HT29 cells. For this purpose, cells were treated with a single dose of TNF- α (1 nM) and analyzed over a 72-h time course. As expected from the above results, PTEN protein expression was decreased 24, 48, and 72 h after TNF- α treatment (Fig. 2A). Concomitant with PTEN reduction, this treatment produced a sustained reduction in I κ B- α throughout the 72-h time course.

To confirm that NF- κ B activation accompanied the TNF- α -induced reduction of I κ B- α , nuclear protein was extracted from treated and untreated HT29 cells and analyzed by ELISA-based DNA binding activity of NF- κ B using a consensus double-stranded oligonucleotide (35). Analysis for p50 NF- κ B showed maximal p50 binding activity at 8 h of TNF- α treatment (Fig. 2B), whereas maximal activity for p65 NF- κ B was observed within 1 h of TNF- α treatment (Fig. 2C). The specificity of DNA binding was confirmed by competition assays, where free floating excess oligonucleotide blocked both p50 and p65 NF- κ B binding activities. These data corroborate the finding that TNF- α treatment increases NF- κ B binding activity and correlates with reduction in I κ B- α expression. In addition, these data show that NF- κ B activity is sustained without signs of inhibition as might be expected because of an I κ B autoregulatory inhibition.

² S. Kim, C. Domon-Dell, J. Kang, D. H. Chung, J.-N. Freund, and B. M. Evers, unpublished data.

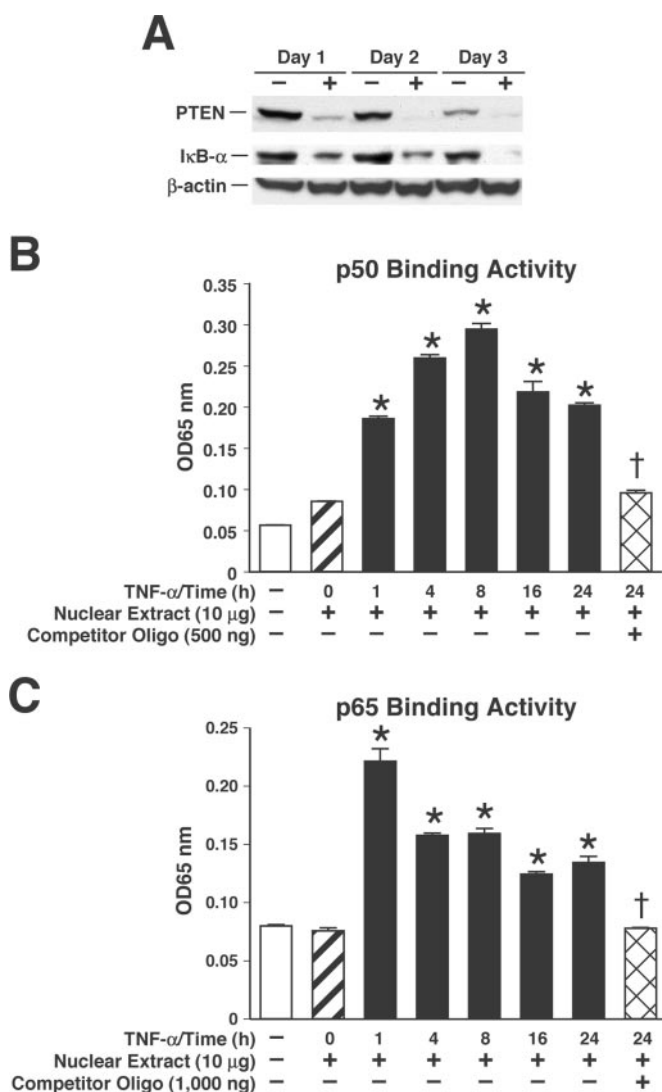


FIG. 2. NF- κ B activation is sustained in HT29 cells treated with TNF- α . *A*, HT29 cells were treated with 1 nM TNF- α and incubated for 24–72 h. Western blot analysis shows persistent reduction in PTEN and I κ B- α expression for each time point. *B* and *C*, HT29 cells were treated with 1 nM TNF- α over a 24-h time course. Treated cells were collected and nuclear proteins extracted for ELISA-based NF- κ B activity assay. Nuclear extracts (10 μ g) were incubated in 96-well plates lined with oligonucleotide specific for either p50 or p65 NF- κ B. Specific binding was detected by either anti-p50 or -p65 antibodies. Analysis of ELISA results shows maximal p50 binding activity at 8 h post-TNF- α treatment compared with maximal binding activity at 1 h for p65 binding activity. At all time points up to 24 h, NF- κ B binding activities were higher than base line. Competition assay using excess free floating p50 binding oligonucleotide (500 ng/well) or p65 binding oligonucleotide (1,000 ng) shows complete blockage of binding activity. Experiments were repeated twice (mean \pm S.E. for triplicate determinations; * = $p < 0.05$ compared with control; † = $p < 0.05$ compared with 24-h treatment time point without competition oligonucleotide).

NIK and Activation of p65 NF- κ B Decrease PTEN Expression—TNF- α binding to TNFR1 activates NF- κ B through recruitment of the TRAF2 adaptor protein and the serine/threonine kinase NIK (21). Subsequently, NIK phosphorylates I κ B kinase, which targets I κ B- α for ubiquitination and proteasome-mediated degradation, thus allowing NF- κ B to translocate into the cell nucleus (36, 37). To determine whether TNF- α -induced PTEN reduction is mediated through NIK, an adenovirus containing the NIK gene was used to infect HT29 cells. Consistent with the effect observed previously by TNF- α treatment, NIK overexpression decreased PTEN expression as well as the level of I κ B- α , suggesting NF- κ B activation (Fig. 3A). Conversely, we

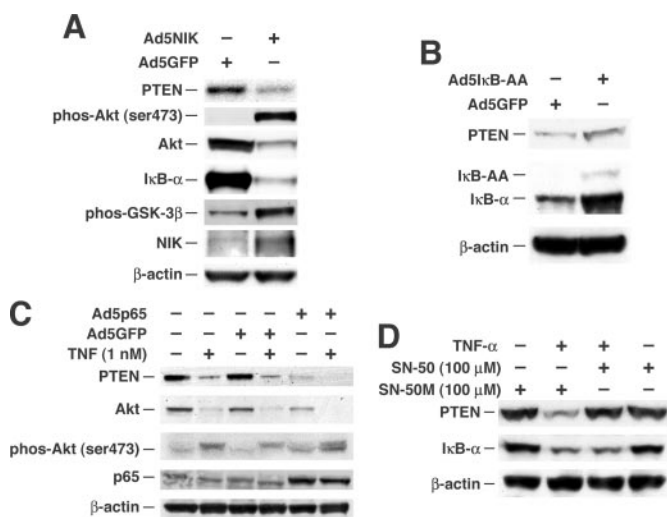


FIG. 3. NIK and activation of p65 NF- κ B decrease PTEN expression. *A*, adenoviruses (Ad5NIK) carrying the NIK gene were infected into HT29 cells at a multiplicity of infection of 20. Ad5NIK-infected cells were compared with cells infected with control adenovirus (Ad5GFP) carrying green fluorescence protein. After a 48-h incubation, attached cells were collected and proteins extracted for Western blot analysis. PTEN expression was found to be lower for Ad5NIK-infected cells. In addition, a decrease in total Akt and I κ B- α expression but an increase in phospho-Akt (Ser-473) and phospho-GSK-3 β levels were observed for Ad5NIK-infected cells. NIK overexpression was confirmed for Ad5NIK-infected cells. *B*, HT29 cells were infected with Ad5I κ B-AA, which contains the superrepressor I κ B- α gene, at a multiplicity of infection of 20. Western blot analysis shows expression of I κ B-AA, which has a higher molecular weight than endogenous I κ B- α because of hemagglutinin protein attachment. Ad5I κ B-AA infection increased PTEN and I κ B- α expression consistent with results obtained as in *A*. *C*, adenovirus Ad5p65 carrying the p65 subunit of NF- κ B was infected into HT29 cells at a multiplicity of infection of 20. Ad5GFP was used as control infection. After 48 h postinfection, attached cells were collected for Western blot analysis. Ad5p65 infection decreased PTEN and Akt expression but increased phospho-Akt expression similar to the result with Ad5NIK infection. Anti-p65 blotting shows p65 overexpression for Ad5p65-infected cells. *D*, HT29 cells were pretreated with cell membrane-permeable SN50 peptide that binds to unbound NF- κ B 30 min prior to TNF- α treatment. SN50M, a mutant variant of SN50 which does not bind to NF- κ B, was used as control peptide. After a 24-h incubation with TNF- α and SN50, attached cells were collected and proteins extracted for Western blot analysis. SN50 completely blocked TNF- α -induced reduction in PTEN expression, whereas SN50M had no effect. The I κ B- α level was decreased for TNF- α - and SN50-treated cells as expected because SN50 does not prevent TNF- α -mediated I κ B- α degradation. Equal β -actin shows even loading of proteins.

analyzed the effect of the expression of the superrepressor of I κ B- α after cell infection with the recombinant adenovirus Ad5I κ B-AA. The superrepressor of I κ B- α does not undergo degradation upon TNF- α stimulation (38), hence it sequesters NF- κ B in the cytoplasm. We found that the infection of I κ B- α superrepressor actually increased PTEN expression and the endogenous level of I κ B- α (Fig. 3B). Next, we examined whether NF- κ B can directly suppress PTEN expression by overexpressing the p65 subunit of NF- κ B in HT29 cells. Overexpression of p65 protein decreased PTEN expression and, in combination with TNF- α treatment, an additive suppression was observed (Fig. 3C).

To provide additional evidence that the TNF- α -induced PTEN decrease is functionally dependent on the activation of the NF- κ B pathway, HT29 cells were treated with 1 nM TNF- α together with SN50, a cell-permeable peptide that binds to uncomplexed NF- κ B and prevents nuclear translocation of NF- κ B (39). As shown in Fig. 3D, SN50 peptide prevented the decrease in PTEN expression in TNF- α -treated cells, without affecting the decrease in I κ B- α , confirming that this inhibitor acts downstream of I κ B on the translocation of NF- κ B. More-

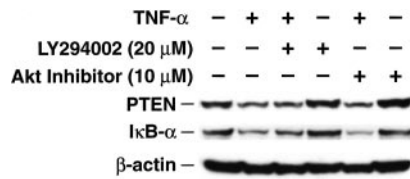


FIG. 4. **TNF- α -induced down-regulation of PTEN is not dependent on PI3K.** HT29 cells were pretreated with 20 μ M specific PI3K inhibitor LY294002 or 10 μ M Akt inhibitor (for the full name of the compound, see "Experimental Procedures") 30 min prior to 1 nM TNF- α treatment. After a 24-h coincubation, attached cells were collected and protein extracted for Western blot analysis. Neither LY294002 nor Akt inhibitor blocked TNF- α -induced down-regulation of PTEN or I κ B- α degradation. Equal β -actin levels show even protein loading.

over, the mutant inactive peptide SN50M was unable to prevent the TNF- α -induced PTEN decrease. Together, these data indicate that TNF- α down-regulates PTEN expression by activating the NIK/I κ B kinase/I κ B/NF- κ B pathway.

TNF- α -induced Down-regulation of PTEN Is Not Dependent on PI3K—TNF- α activates the PI3K pathway through TNFR/insulin receptor substrate 1-mediated tyrosine phosphorylation of the p85 regulatory subunit of PI3K (40). It was reported previously that TNF- α activation of NF- κ B requires both NIK and PI3K/Akt (23); however, in different cell types, TNF- α activation of NF- κ B is not dependent on PI3K/Akt, whereas platelet-derived growth factor-mediated activation of NF- κ B involves PI3K/Akt activation of I κ B kinase (41). In the case of HT29 cells infected with the NIK-encoding adenovirus, we found a decrease in the total amount of Akt protein but a strong activation of this kinase assessed by its phosphorylation at residue Ser-473 (Fig. 3A). Confirming the functional activation of Akt, increased phosphorylation of a downstream substrate of Akt, GSK-3 β (42), was also observed in NIK-overexpressing cells (Fig. 3A). These results suggest that Akt is activated directly by TNF- α signaling through insulin receptor substrate-1 and PI3K to enhance to TNF- α /NIK-dependent down-regulation of PTEN and that activation of Akt is augmented by the decrease in PTEN, which antagonizes Akt activation by PI3K inhibition.

To examine whether TNF- α -mediated down-regulation of PTEN is dependent on the PI3K/Akt pathway in HT29 cells, the PI3K inhibitor LY294002 (43) and the Akt inhibitor (44) were used to pretreat the cells prior to TNF- α incubation. Both LY294002 and the selective Akt inhibitor failed to prevent the decrease in I κ B- α and PTEN in TNF- α -treated HT29 cells (Fig. 4). These data suggest that the down-regulation of PTEN by TNF- α is mediated by NIK, independent of the PI3K/Akt pathway, and that the activation of Akt in TNF- α -treated cells is augmented resulting from the decay of the antagonizing phosphatase PTEN.

I κ B- α Autoregulatory Loop Is Absent in HT29—NF- κ B activity is controlled by I κ B isoforms (I κ B- α , - β , and - ϵ), which sequester NF- κ B to the cytoplasm, whereas TNF- α stimulation and subsequent I κ B degradation allow NF- κ B nuclear translocation and DNA binding (31). NF- κ B signaling is complex in that NF- κ B activity is modulated by rapid transcriptional autoregulatory loop involving NF- κ B mediated I κ B- α synthesis, which occurs within hours of TNF- α stimulation (45, 46). In addition, it was shown recently that NF- κ B-mediated I κ B- α synthesis provides a strong negative feedback to inhibit NF- κ B activation (47).

To obtain insight into why PTEN expression is down-regulated in HT29 cells by TNF- α but such an effect failed to occur in HEK293 cells, we examined the changes in expression level of I κ B- α and PTEN, when cells are treated with 1 nM TNF- α over a time course. As shown in Fig. 5A, TNF- α treatment

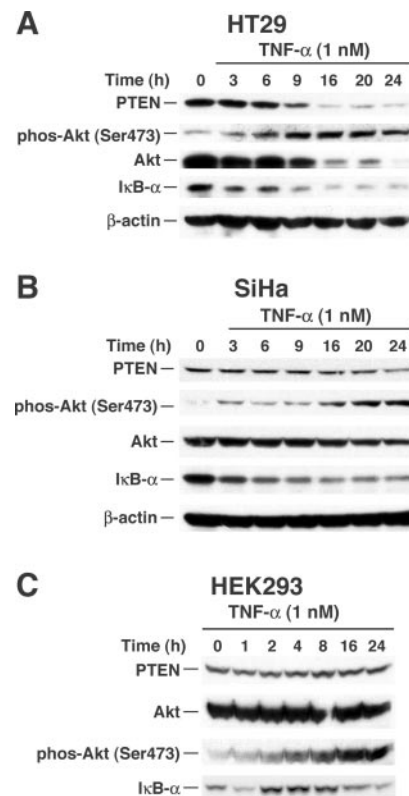


FIG. 5. **I κ B- α autoregulatory loop is absent in HT29.** A, HT29 cells were treated with 1 nM TNF- α over a 24-h time course. Attached cells were collected at each time point and proteins extracted for Western blot analysis. A gradual decrease in PTEN and Akt but an increase in phospho-Akt (*phos-Akt*) expressions were observed with prolonged TNF- α treatment. A gradual decrease in I κ B- α expression was noted without I κ B- α resynthesis. B, SiHa cells were treated with TNF- α as in A and analyzed. Although not as prominent as HT29 cells, there is a decrease in PTEN Akt and I κ B- α levels associated with an increase in phospho-Akt (*phos-Akt*) expression. C, HEK293 cells were treated with TNF- α as in A and analyzed similarly. Unlike HT29 results, rapid reduction in I κ B- α expression was noted at 1 h TNF- α treatment. This was followed by rapid recovery in I κ B- α expression starting at 2 h, which was sustained up to 16 h.

gradually decreased PTEN in HT29 cells; this was because of the progressive and sustained decrease in I κ B- α levels. Thus, no I κ B- α rebound synthesis was observed in HT29 cells beyond 3 h of TNF- α treatment. Similar, but not as dramatic, results were noted after TNF- α treatment of SiHa cells (Fig. 5B). On the contrary, TNF- α treatment did not result in decreased PTEN expression in HEK293 cells (Fig. 5B). Noteworthy, TNF- α produced a rapid initial decrease in I κ B- α at 1 h, but this decline was followed by rebound synthesis with resultant increase in I κ B- α levels which exceeded basal expression up to 16 h (Fig. 5B). Therefore, the decay in PTEN protein in TNF- α -treated HT29 cells is associated with an abnormal deficiency in the I κ B- α autoregulatory loop. These data suggest that sustained NF- κ B activation because of prolonged I κ B- α suppression may be responsible for the differential effect noted in PTEN expression in these two cell lines.

DISCUSSION

In our present study, we have shown that TNF- α /NF- κ B down-regulates PTEN expression and facilitates transduction of the TNF- α signal to activate Akt. Down-regulation of PTEN by NF- κ B implicates its role in modulation of PI3K/Akt because TNF- α /TNFR also transduce signals through activation of the PI3K/Akt pathway (40). A high basal level of Akt phosphorylation is observed in multiple PTEN-deficient cell lines and tumors from PTEN-deficient mice (5, 8). Furthermore, in the

absence of growth factor stimulation, the level of Akt phosphorylation is normally low. These and other findings have suggested that regulation of PI3K/Akt signal transduction is modulated strongly by PTEN expression. In other words, inhibition of PI3K by PTEN is mainly dependent on its level of expression within the cell. Our finding that TNF- α /NF- κ B activation can affect PTEN and Akt levels reveals another level of complexity which can occur between the PI3K/Akt and NF- κ B pathways.

Other important pathways, such as Ras, which may converge on the PI3K/Akt and NF- κ B pathways add further complexity. Oncogenic Ras, the most widely mutated human proto-oncogene (48), activates PI3K/Akt and Raf/mitogen-activated protein kinase pathways (49). It also affects NF- κ B through transcriptional up-regulation of RelA/p65 subunit (50, 51). In turn, NF- κ B is known to regulate p53 expression positively (52, 53). Furthermore, it has been shown that NF- κ B is required to suppress p53-independent apoptosis induced by oncogenic Ras (28). These findings are suggestive and support the hypothesis that NF- κ B-mediated down-regulation of PTEN expression may augment the effect of oncogenic Ras in tumor cells.

In addition, we have shown that overexpression of NIK or the p65 subunit of NF- κ B increases Akt activity as evidenced by increased phosphorylated form of Akt and its downstream target GSK-3 β . The data presented suggest that the increased Akt activity is facilitated by reduction in PTEN expression. NF- κ B-induced activation of Akt also suggests an interesting positive feedback loop, where activated Akt further facilitates NF- κ B activation. Such a feedback loop may augment Akt activity in tumors and increase tumor growth and invasion.

The NF- κ B inhibitor I κ B- α is degraded rapidly upon TNF- α stimulation and then resynthesized after NF- κ B stimulation. This effect was also reported in HT29 cells by Place *et al.* (54). The rapid decrease in I κ B- α levels was noted within 15 min post-TNF- α treatment with rebound synthesis noted at 30 min. However, the data presented were limited to a 1-h treatment. We have shown in our study that in HT29 cells, the expected I κ B- α oscillation with prolonged TNF- α treatment does not occur. Contrary to another cell line examined, HEK293, I κ B- α degradation is prolonged in HT29 cells with TNF- α treatment. One implication of the role of I κ B- α as a temporal regulatory switch to turn off NF- κ B by resynthesis of I κ B- α is the hypothesis that some NF- κ B-responsive genes are activated with a short pulse of NF- κ B, whereas other genes need longer exposure to activate transcription. The bimodal temporal signal activation of NF- κ B/I κ B- α to up-regulate the two classes of NF- κ B target genes (short pulse *versus* persistent NF- κ B activation) was demonstrated for NF- κ B-induced activation of the chemokine interleukin-10 and RANTES (regulated on activation normal T cell expressed and secreted) genes (47). The mechanism for the bimodal effect is, however, unknown. Furthermore, bimodal temporal regulation of NF- κ B/I κ B- α to down-regulate (rather than up-regulate) a target gene has not been demonstrated. Our data suggest that PTEN down-regulation requires a persistent NF- κ B activation; hence, PTEN belongs to the second class of NF- κ B targeted genes.

In summary, we analyzed the effect of TNF- α on PTEN expression and found that TNF- α -induced NF- κ B activation suppresses PTEN expression. Specifically, overexpression of the p65 subunit of NF- κ B decreases PTEN expression and results in increased Akt activation. These findings elucidate the interaction between PTEN/PI3K/Akt and NF- κ B at the level of transcription and offer one possible explanation for increased tumorigenesis and inflammation in systems where NF- κ B is chronically activated.

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