

Role of the Phosphatidylinositol 3'-Kinase/PTEN/Akt Kinase Pathway in the Overexpression of Fatty Acid Synthase in LNCaP Prostate Cancer Cells¹

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Abstract

One of the most common molecular changes in cancer cells is the overexpression of fatty acid synthase (FAS), a key metabolic enzyme catalyzing the terminal steps in the synthesis of long chain saturated fatty acids. As part of our efforts to elucidate the mechanisms responsible for FAS overexpression, we have addressed the question whether overexpression of FAS may be linked to the frequently observed inactivation of PTEN and subsequent activation of the phosphatidylinositol 3'-kinase (PI3k) pathway. Using LNCaP prostate cancer cells as an experimental paradigm of FAS-overexpressing PTEN-null cancer cells, we demonstrate that LY294002, an inhibitor of the PI3k pathway causes a dramatic decrease in FAS protein expression. Smaller but still substantial effects are seen at the FAS mRNA level and at the level of transcriptional activity of FAS promoter-reporter constructs. Consistent with these findings, reintroduction of PTEN results in decreased levels of FAS expression in a manner that is dependent on its lipid phosphatase activity. In support of a role for Akt/protein kinase B as a downstream effector, cotransfection of constitutively active Akt1/protein kinase B α abrogates the inhibitory effects of PTEN expression and restores FAS promoter activity. Taken together, these results demonstrate that inactivation of PTEN and subsequent activation of the PI3k/Akt kinase pathway may play an important role in the overexpression of the FAS protein in cancer cells.

Introduction

The majority of human cancers, including cancer of the breast, colon, ovary, lung, and prostate express elevated levels of FAS,³ a key metabolic enzyme catalyzing the terminal steps in the synthesis of long chain saturated fatty acids (1, 2). Increased FAS protein expression occurs very early in cancer development and is more pronounced in tumors with a poor prognosis. Inhibition of FAS activity inhibits tumor cell growth and induces apoptosis (3), rendering FAS an interesting target for anticancer drug development (4). Although very little is known about the mechanisms underlying the overexpression of the FAS protein in cancer cells, recent studies have revealed that FAS is up-regulated also at the mRNA level, together with several other enzymes of the same metabolic pathway (5, 6). This apparent coordinate regulation of lipogenic gene expression points to changes in the regulatory circuits controlling the program of lipogenic enzyme expression rather than in the FAS locus itself. Beside steroid hormones (7–11), factors that have been shown to play an important role in the

control of lipogenic enzyme expression in cancer cells are growth factors (12), of which the signaling pathways are frequently dysregulated and constitutively activated in cancer cells. One of the intracellular signaling pathways that frequently are activated in cancer cells is the PI3k/Akt kinase pathway (13). PI3k catalyzes the formation of the 3' phosphoinositides, phosphatidylinositol 3,4-diphosphate and phosphatidylinositol 3,4,5-triphosphate. Increases in 3' phosphoinositides lead to membrane translocation of downstream effectors such as the serine/threonine protein kinase Akt. On translocation, Akt is phosphorylated and activated, ultimately resulting in stimulation of cell growth and survival. PTEN, also known as MMAC1 or TEP1, opposes PI3k action by dephosphorylating 3' phosphoinositides (14, 15). In many human cancer tissues and cancer cell lines, PTEN is mutated or deleted, leading to constitutively high levels of 3' phosphoinositides and activation of Akt (14, 16–17).

As part of our studies to additionally elucidate the molecular mechanisms underlying the overexpression of lipogenic enzymes in cancer cells, we have used LNCaP prostate cancer cells as a paradigm of FAS-overexpressing PTEN-null cancer cells and have addressed the question to which extent inactivation of the PTEN tumor suppressor and subsequent activation of the PI3k/Akt pathway may contribute to the observed overexpression of FAS.

Materials and Methods

Culture of LNCaP Cells. The human prostatic adenocarcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA) and was maintained in a humidified atmosphere of 5% CO₂ in air in RPMI 1640 supplemented with 10% FCS, 3 mM L-glutamine, 100 μ g/ml streptomycin, and 100 units/ml penicillin (Invitrogen, Carlsbad, CA). LNCaP cells were used between passages 47 and 65. Where indicated the PI3k inhibitor LY294002, purchased from Biomol (Plymouth Meeting, PA), was added from a 1000-fold concentrated stock in DMSO. Control cultures received similar amounts of DMSO only. Final DMSO concentrations did not exceed 0.1%.

Immunoblot Analysis. LNCaP cells were plated in 60-mm dishes at a density of 10⁶ cells/dish and treated as indicated. For immunoblot analysis cells were washed and lysed in a reducing SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 0.715 M 2-mercaptoethanol, 8.7% glycerol). Protein concentrations were determined on diluted samples using a bicinchoninic acid procedure (Pierce Biochemical Company, Rockford, IL). Equal amounts of protein were separated on an SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane for FAS (Roche, Mannheim, Germany) or onto Hybond enhanced chemiluminescence membranes for all of the other antibodies (Amersham International, Buckinghamshire, United Kingdom). Membranes were blocked in a Tris-buffered saline solution with 5% nonfat dry milk and incubated with antibodies against Akt (Biosource International, Camarillo, CA), S⁴⁷³-phosphorylated Akt (Biosource), PTEN (Cell Signaling Technology, Beverly, MA), cytokeratin 18 (Dako, Glostrup, Denmark), or FAS. This latter antiserum was prepared in rabbits using purified FAS from SKBR-3 breast cancer cells as antigen and specifically recognizes the M_r 265,000 FAS protein. Immunoreactive signals were detected by incubation with horseradish peroxidase-conjugated secondary antibodies (Dako) followed by chemiluminescent detection of immunoreactive proteins (Renaissance; New England Nuclear, Dreiech, Germany).

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³ The abbreviations used are: FAS, fatty acid synthase; PI3k, phosphatidylinositol 3'-kinase; GFP, green fluorescent protein; Dox, doxycycline; myr-Akt, myristoylated form of Akt; HA hemagglutinin; SREBP, sterol regulatory element-binding protein.

RNA Preparation and Analysis. LNCaP cells were seeded in 60-mm dishes at a density of 10^6 cells/dish. The next day, medium was replaced and cells were treated as indicated in the legends to the figures. Plates were washed with Dulbecco's PBS (Invitrogen), snap-frozen in liquid nitrogen, and stored at -80°C . Total RNA was prepared using the guanidine/CsCl ultracentrifugation method (18). Northern blot analysis for FAS expression was carried out as described before (7). Equal loading of RNA was verified by hybridization with a probe for 18 S rRNA (7). This same procedure was followed to assess the expression of luciferase mRNA in transient transfections. A luciferase-specific probe was prepared from the pGL3b vector (Promega).

FAS Promoter-Reporter Studies. LNCaP cells were plated on day 0 in 60-mm dishes in RPMI 1640 containing 10% FCS at a density of 1×10^6 cells/dish. The next day transfection mixtures were prepared. For each plate, 2 ml of serum-free DMEM (Invitrogen) was supplemented with 2–3 μg of the FAS promoter-reporter construct described before (8). Where indicated, 0.5 μg of expression constructs encoding wild type PTEN (pSG5L-HA-PTENwt), mutant PTEN (pSG5L-HA-PTEN-G129E and pSGL5-HA-PTEN-G129R), Akt (pLNCX-HA-Akt), my-Akt (pLNCX-HA-myrAkt), or the corresponding empty vectors (pSG5L and pLNCX), all kindly provided by Dr. W. Sellers (Harvard Medical School, Boston, MA; Ref. 19), were added. After the addition of 9 μl of Transfast (Promega, Madison, WI) the transfection mixture was incubated for 15 min at room temperature. Cells were washed with serum-free medium, the transfection mixture was added, and cultures were incubated for 30 min in the CO_2 incubator. RPMI 1640 (3 ml) with 10% FCS was added. On day 3, medium was replaced. The next day, cells were washed with PBS and lysed in 500 μl of passive lysis buffer (Promega). Aliquots of 15 μl of cleared lysate were assayed for luciferase activity by using a luciferase reporter assay kit from Promega and a Berthold Microlumet LB 96P luminometer. Similar experiments were carried out in the presence of an expression vector encoding GFP (pEGFP; Clontech, Palo Alto, CA), to monitor potential changes in the number of transfected cells after transfection with PTEN. Expression of recombinant PTEN and Akt forms was assessed through immunoblot analysis with an antibody against the hemagglutinin (HA) epitope encoded for by the expression constructs (19).

Adenoviral Expression of PTEN. The Adeno-X Tet-Off expression system was obtained from Clontech. A PCR fragment encompassing the coding region of the *PTEN* gene was generated using a forward primer 5' GAAGGATCCAC-CATGACAGCCATCATCAAAGAG adapted with a *Bam*HI site (italicized) and the reverse primer 5' GAACTAGATCAGACTTTTGTAAATTGTGTATGCTG containing an *Xba*I site (italicized). After digestion with *Bam*HI and *Xho*I the fragment was ligated into the pTRE-Shuttle vector. Integrity of the construct was verified by restriction digestion and DNA sequence analysis. To create the phosphatase-death PTEN-G129R construct, codon 129 was mutated using the Quikchange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The Tet-responsive expression cassette from the recombinant pTRE-Shuttle plasmids was excised using I-CeuI and PI-SceI, and ligated into the predigested Adeno-X viral DNA. Recombinant Adeno-X viral DNA was propagated in *Escherichia coli*, linearized by digestion with *Pac*I, and transfected into low passage HEK 293 cells. To produce high-titer adenovirus stocks, HEK 293 cells were infected with recombinant virus, and growth medium was collected when most of the cells had detached. LNCaP cells were plated in 60-mm dishes at a density of 10^6 cells/dish. Two days later, cells were coinfecting with the Tet-responsive recombinant virus and with the tetracyclin-controlled transactivator virus, Adeno-X Tet-Off, at a multiplicity of infection of ~ 5 plaque-forming units of each virus strain/cell. After incubation for 2 h in the CO_2 incubator at 37°C , the virus-containing medium was removed and fresh growth medium (MEM α + 10% Tet-approved FCS from Clontech) was added. Where indicated, Dox (Clontech) was added to a final concentration of 1 $\mu\text{g}/\text{ml}$. Later (42 h), cells were harvested and protein extracts were made for immunoblot analysis as described above.

Results

In a first series of experiments to test whether high levels of FAS expression in LNCaP cells are linked to the activation of the PI3K/Akt kinase pathway, we have treated LNCaP cells with different concentrations of the PI3K inhibitor LY294002. Efficacy of the inhibitor was monitored by its ability to block phosphorylation and activation of one of the main downstream effectors, Akt. Akt phosphorylation was

assessed by Western blot analysis using a commercially available antibody specifically recognizing the Akt protein only when it is phosphorylated (at the S⁴⁷³ position). Potential effects on FAS protein levels were assessed by Western blot analysis using a polyclonal anti-FAS antiserum that was developed in our laboratory using purified FAS from SKBR-3 breast tumor cells. As shown in Fig. 1, increasing concentrations of LY294002 caused a gradual decrease in FAS protein levels. At concentrations that fully blocked activation of Akt (16 μM), the FAS protein completely disappeared. At these concentrations of LY294002, no overt signs of cellular toxicity or apoptosis were observed, and levels of other proteins such as total (both phosphorylated and unphosphorylated) Akt and cytokeratin 18 remained unaffected or even slightly increased.

To examine whether FAS expression is also affected at the mRNA level, we treated LNCaP cells with a maximally effective concentration of LY294002 (16 μM) and prepared RNA 36 h after addition of the inhibitor. Hybridization with a FAS-specific probe revealed that treatment with LY294002 resulted in a 2–3-fold reduction in steady state FAS mRNA levels (Fig. 2A). These changes were not because of differences in the amount of total RNA loaded on the blot as verified by hybridization with an 18 S rRNA probe.

To test whether LY294002 also affects FAS transcription, LNCaP cells were transiently transfected with a 178-bp FAS promoter-reporter construct (FAS-luc) that harbors all of the elements necessary for high level expression in LNCaP cells (8). Consistent with the effects at the mRNA level, treatment of transfected cells with LY294002 caused a 2–4-fold decrease in reporter activity (Fig. 2B). This decrease in reporter activity was not because of changes in the number of transfected cells nor in their viability, as verified by cotransfection with an expression construct encoding GFP.

To confirm that inactivation of the PI3K is responsible for the decrease in FAS expression, we cotransfected LNCaP cells with an expression construct encoding PTEN. PTEN is a dual action phosphatase dephosphorylating both 3'phosphoinositides (thus catalyzing the reverse reaction of PI3k) and phosphoproteins (20). To distinguish between these two actions we used constructs encoding mutant forms of PTEN, which lack only the lipid phosphatase function (PTEN-G129E) or lack both lipid and protein phosphatase functions (PTEN-G129R). As shown in Fig. 3A, reintroduction of wild-type PTEN resulted in a 2.5–3-fold reduction in FAS promoter-reporter activity. In contrast, none of the mutated PTEN proteins showed any inhibition of FAS promoter-reporter activity, despite equally efficient expression of the different PTEN forms (data not shown). To exclude that the inhibitory effects on luciferase activity are caused by PTEN-induced

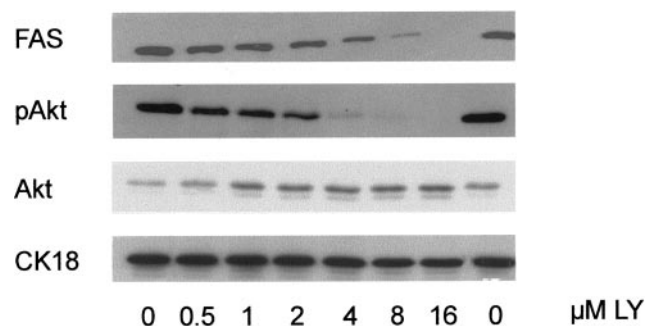


Fig. 1. Effects of LY294002 on FAS protein expression in LNCaP cells. LNCaP cells were treated with different concentrations of LY294002 (LY) or with DMSO vehicle. After treatment (48 h, 1 h for pAkt), total cellular extracts were made. Equal amounts of protein were subjected to Western blot analysis using antibodies against FAS, S⁴⁷³-phosphorylated Akt (pAkt), total Akt (Akt), or cytokeratin 18 (CK18). Immunoreactive bands were visualized using a peroxidase-coupled secondary antibody and a chemiluminescent Western blot analysis system. The data shown are representative of two independent experiments.

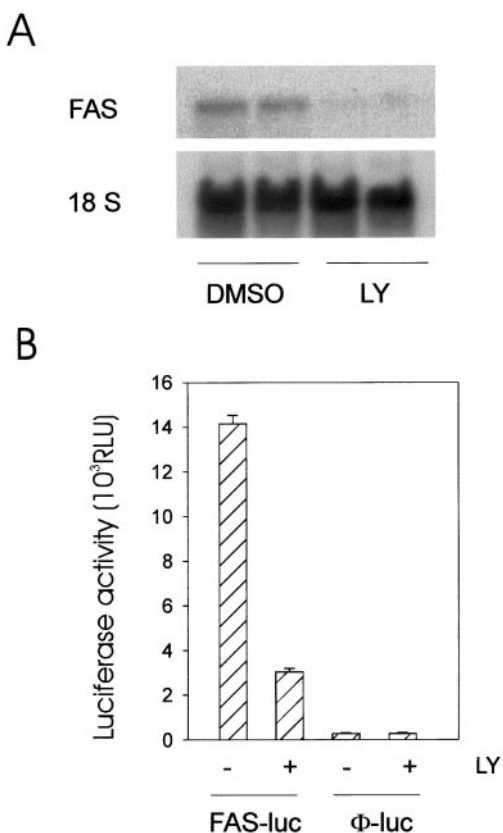


Fig. 2. Effects of LY294002 on steady state FAS mRNA expression (A) and on the activity of a FAS promoter-reporter construct (B) in LNCaP cells. A, LNCaP cells were treated (in duplicate) with LY294002 ($16 \mu\text{M}$) or with DMSO vehicle. Later (36 h), cells were harvested and examined for FAS mRNA expression through Northern blot analysis with a radiolabeled FAS cDNA probe. Equal loading of RNA was verified using an 18 S rRNA probe. B, LNCaP cells were transiently transfected with a plasmid containing a luciferase reporter gene driven by a 178-bp FAS promoter fragment (FAS-luc) or with the corresponding empty vector lacking the FAS promoter (Φ -luc). The next day cells were treated with LY294002 (LY; $16 \mu\text{M}$) or with DMSO vehicle. After 24 h of incubation, cells were lysed, and luciferase activity was measured. Columns, means of incubations performed in triplicate; bars, \pm SE.

apoptosis and subsequent cell loss (21), similar transfection experiments were carried out in the presence of an expression vector encoding GFP. Although limited changes in the number of GFP-positive cells were noticed (data not shown), these effects could not fully account for the observed reduction of FAS promoter-reporter activity induced by expression of PTEN.

To confirm these results also at the level of the endogenous FAS protein, we turned to an inducible adenoviral expression system, because it allows a higher efficiency of gene delivery than our standard transfection technique (90% versus 20%, as visualized using control constructs encoding β -galactosidase or GFP). Moreover, the use of an inducible system eliminates the need to compare cultures infected by different viral preparations (e.g., one with insert and one without insert). As shown in Fig. 3B, the expression system we used (Adeno X Tet-Off) is turned on in the absence of tetracycline or its analogue, Dox, and can be turned off in its presence. Functionality of the PTEN protein was confirmed by monitoring its inhibitory effect on the phosphorylation of Akt. Consistent with all of our other data, PTEN expression caused a marked decline in FAS protein expression concurrent with inactivation of Akt. Under the conditions used (5 plaque-forming units/cell; 42 h induction) no significant changes in total protein content of the cultures were noticed (data not shown) nor were there overt signs of cell toxicity or induction of cell death. Consistent with this notion, levels of control proteins such as cytokeratin 18 were unaffected by PTEN expression.

Because Akt is one of the main downstream effectors of PI3k and because inhibition of PI3k or reintroduction of wild-type PTEN blocks activation of Akt in LNCaP cells (see Figs. 1 and 3B), we were interested to see whether Akt functions as the downstream effector of PI3k action on FAS expression. To answer this question we examined whether Akt could reverse the inhibitory effect of PTEN. To introduce active Akt into LNCaP cells, we used an expression vector encoding a myr-Akt. This form is constitutively active irrespective of the phosphatidylinositol 3,4,5-triphosphate levels. As shown in Fig. 4, expression of constitutively active Akt completely reversed the inhibitory effects of PTEN on FAS promoter-reporter activity. As expected, wild-type Akt, which is supposed to be poorly active in the presence of functional PTEN, had only slight effects. To exclude that these effects on luciferase activity are because of general changes in protein translation and/or stability of the luciferase protein, luciferase expression was examined also at the mRNA level. Consistent with an earlier report (17), transfection with PTEN and/or Akt resulted in similar effects at the mRNA level as at the level of luciferase activity (data not shown).

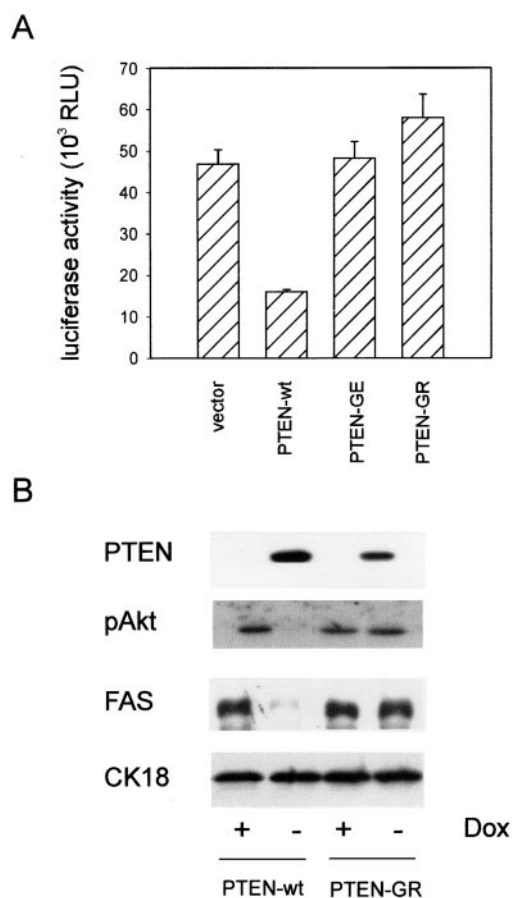


Fig. 3. Ectopic expression of PTEN in PTEN-null cells represses FAS expression in LNCaP cells. A, LNCaP cells were transiently transfected with the FAS promoter-reporter construct (FAS-luc) together with the empty pSGL5 expression vector (vector) or with the same vector encoding wild-type PTEN (PTEN-wt), or mutated forms of PTEN lacking only lipid phosphatase activity (PTEN-GE) or lacking both lipid and protein phosphatase activity (pPTEN-GR). After transfection (36 h), cells were lysed, and luciferase activity was measured. The data shown are representative of three independent experiments. Columns, means of incubations performed in triplicate; bars, \pm SE. B, to examine the impact of reconstitution of PTEN on endogenous FAS protein levels, LNCaP cells were infected with adenoviruses encoding wild-type or mutant PTEN (PTEN-wt and PTEN-GR) in a Dox-controlled manner. Dox was added as indicated at a final concentration of $1 \mu\text{g/ml}$. Two days later, protein extracts were made and subjected to Western blot analysis with antibodies against PTEN, S⁴⁷³-phosphorylated Akt, FAS, and cytokeratin 18. Immunoreactive bands were visualized using a peroxidase-coupled secondary antibody and a chemiluminescent Western blot analysis system.

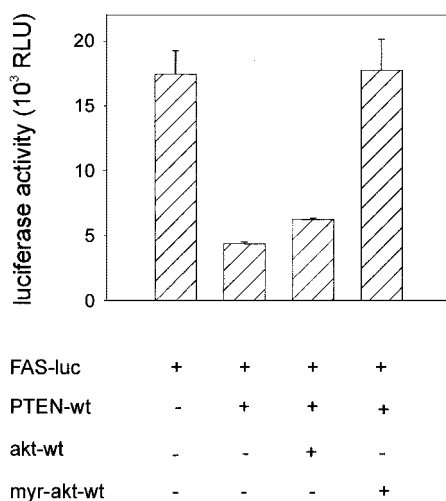


Fig. 4. Akt is able to reverse the inhibitory effect of PTEN on FAS promoter-reporter activity. LNCaP cells were transiently transfected with a FAS promoter-reporter construct (*FAS-luc*) together with expression constructs encoding wild-type PTEN (*PTEN-wt*), wild type Akt (*akt-wt*), or myr-Akt (*myr-akt-wt*). After transfection (36 h), cells were lysed and luciferase activity was measured. The data shown are representative of two independent experiments. Columns, means of incubations performed in triplicate; bars, \pm SE.

Discussion

Although FAS is overexpressed in most human cancers and holds significant promise as a cancer marker and as a target for antineoplastic therapy, very little is known about the molecular events that lead to overexpression of this key metabolic enzyme in cancer cells. In this study we demonstrate that the frequently observed loss of PTEN function and consequent activation of the PI3k/Akt kinase pathway may play an important role in the overexpression of FAS in cancer cells. Treatment of LNCaP prostate cancer cells, lacking endogenous PTEN, with the PI3k inhibitor LY294002 results in a dramatic drop in FAS protein expression, and in decreased levels of steady-state FAS mRNA and transcriptional activity. Reconstitution of PTEN into these cells results in a similar drop in FAS expression. PTEN-induced down-regulation of FAS is dependent on the lipid phosphatase function of PTEN and can be overcome by activation of Akt signaling.

Our finding that LY294002 completely abolishes FAS protein expression while causing a more limited (2–4-fold) decrease in FAS promoter-reporter activity and in steady state mRNA levels (2–3-fold), suggests that PI3k/Akt acts at the transcriptional level with additional effects at the protein level. Transcriptional effects may in part be because of activation of specific lipogenic transcription factors such as sterol-regulatory element-binding protein-1 (SREBP-1). This transcription factor has been shown previously to be activated by PI3k and Akt in hepatocytes, and is involved in the activation of tumor-associated FAS transcription by androgens and by growth factors (7–12, 22–24). However, preliminary studies aimed at the additional elucidation of this mechanism revealed that deletion of the SREBP-binding site in the FAS promoter only partially abolishes the effects of LY294002 and of PTEN (data not shown). This suggests that additional and perhaps more general effects on transcription may be involved. This latter hypothesis is supported by earlier findings that many apparently unrelated promoters are affected by PTEN and by the PI3k/Akt kinase pathway in LNCaP cells (14, 17). In regard to the additional effects of PI3k/Akt at the protein level, it is worth mentioning that regulators of protein translation are among the downstream targets of the PI3k/Akt pathway (13, 25). Modulation of these regulators may affect FAS protein levels through a more general effect on protein translation. However, FAS seems to be affected more

severely than several other proteins (*e.g.*, Akt, CK18; see Figs. 1 and 3B). This suggests that FAS is more sensitive to these effects than other proteins or that also other mechanisms, more selectively affecting FAS protein levels (*e.g.*, phosphorylation-induced protein stabilization), are involved.

Whatever the exact mechanism by which the PI3k/Akt pathway activates FAS expression in LNCaP cells, to the best of our knowledge, this is the first direct demonstration of a link between the activation of a specific signal transduction pathway and overexpression of FAS expression in cancer cells. To which extent this pathway ultimately contributes to the overexpression of FAS in various cancers *in vivo* awaits additional investigation. One possible strategy to pursue this question is to look for correlations between activation of the PI3k/Akt pathway and increased FAS protein expression in clinical cancer samples by immunohistochemistry.

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