The Phosphatidylinositol 3-Kinase/Akt Pathway Regulates Transforming Growth Factor-β Signaling by Destabilizing Ski and Inducing Smad7*§

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Ski is an oncoprotein that negatively regulates transforming growth factor (TGF-β) signaling. It acts as a transcriptional corepressor by binding to TGF-β signaling molecules, Smads. Efficient TGF-β signaling is facilitated by rapid proteasome-mediated degradation of Ski by TGF-β. Here we report that Ski is phosphorylated by Akt/PKB kinase. Akt phosphorylates Ski on a highly conserved Akt motif at threonine 458 both in vitro and in vivo. The phosphorylation of Ski at threonine 458 is induced by Akt pathway activators including insulin, insulin-like growth factor-1, and hepatocyte growth factor. The phosphorylation of Ski causes its destabilization and reduces Ski-mediated inhibition of expression of another negative regulator of TGF-β, Smad7. Induction of Smad7 levels leads to inactivation of TGF-β receptors and TGF-β signaling cascade, as indicated by reduced induction of TGF-β target p15. Therefore, Akt modulates TGF-β signaling by temporarily adjusting the levels of two TGF-β pathway negative regulators, Ski and Smad7. These novel findings demonstrate that Akt pathway activation directly impacts TGF-β signaling.

Transforming growth factor-β (TGF-β)† is a key regulator of several cellular processes including cell proliferation, differentiation, and apoptosis. TGF-β signaling is initiated by ligand-induced dimerization and phosphorylation of TGF-β type I and type II receptors. The activated type I receptor phosphorylates receptor-regulated Smads. This enhances the formation of heteromeric complexes between Smad4 and the receptor-regulated Smads, Smads 2 and 3. The Smad complex so formed is then translocated to the nucleus where it transcriptionally activates the expression of TGF-β target genes (1).

The TGF-β signaling cascade is well regulated and determined by post-translational modifications, protein localization, degradation, and inhibitory molecules (2, 3). An inhibitory Smad, Smad7, is a potent inhibitor of TGF-β receptor. It can stably associate with receptor type I and prevent the phosphorylation of the Smads (4, 5). By binding to the receptor, Smad7 may recruit ubiquitin ligases or phosphatase 1 to the site, leading to degradation or dephosphorylation of the type I receptor, respectively (6–8). In addition, Smad7 can disrupt the formation of the functional Smad-DNA complex at the Smad recognition elements and thus prevent the transcription (9). Ski and SnoN (Ski-related novel gene) are also negative regulators of TGF-β signaling. They block TGF-β signaling either at the transcriptional level by recruiting N-CoR-histone deacetylase complex to the promoter site or by binding and preventing the translocation of phosphorylated Smads to the nucleus (10–14). TGF-β induces rapid degradation of Ski and SnoN to allow unrestricted signaling (15–17). However, within a few hours, TGF-β reinstates a negative feedback loop by inducing the transcription and synthesis of SnoN and possibly the transcription and synthesis of Ski (15, 18). The ample means of control of the TGF-β pathway molecules and negative feedback loops ensure that TGF-β signaling is closely measured.

Almost all adult and embryonic tissues express low levels of Ski. Despite its widespread expression, Ski regulates in particular muscle and neural lineage differentiation. High levels of Ski increase skeletal muscle mass in transgenic mouse and lead to muscle differentiation of quail embryo cells (19, 20). In contrast, Ski knock-out mice have defects in skeletal muscle development as well as in the central nervous system (21). The level of Ski is regulated by the ubiquitin-mediated proteasome pathway. Arkadia was identified as a Ski ubiquitin ligase that causes proteasomal degradation of Ski following TGF-β treatment in a Smad-dependent manner (22, 23). The level of Ski (and that of SnoN) is directly linked to its ability to repress TGF-β signaling. Overexpression of Ski (SnoN) leads to resistance to TGF-β-induced growth arrest (16, 24) and morphologically transforms chicken and quail embryo fibroblasts (25, 26). The levels of Ski correlate with tumor progression in several human cancers, including melanoma, esophageal squamous cell carcinoma, leukemia, and colorectal cancer (13, 27–29). However, Ski has been reported to act also as a tumor suppressor. Mouse embryonic fibroblasts lacking one copy of Ski have an increased susceptibility to chemical carcinogens (30). Cell proliferation is increased in Ski−/− mouse embryonic fibroblasts, and this capacity is suppressed by re-expression of Ski (30). Recently, it
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has been reported that silencing of Ski enhances the metastatic activity of TGF-β in breast and lung cancer cells (23). In addition, Ski represses oncogenic activity of c-Myc (31). In contrast, Ski mediates TGF-β-induced proliferative effects in Schwann cells by interacting with retinoblastoma protein, pRB (32, 33). Therefore the impact of Ski on cellular phenotype is cell type- and tissue type-dependent and is likely influenced by external signals regulating its levels and interactions with multiple cellular proteins (18).

The phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B (PKB))-dependent pathway supports cell growth and survival and intersects TGF-β-mediated signaling (reviewed in Refs. 34 and 35). The PI3K pathway suppresses several TGF-β-induced cell responses such as gene transcription, apoptosis, and phosphorylation of Smads (36–38). It has been proposed that Akt prevents the phosphorylation of Smad3 by direct interaction and that this leads to suppression of TGF-β-induced apoptosis (39, 40). Another model suggests that Akt prevents phosphorylation of Smads by activating mTOR mammalian target of rapamycin (mTOR) rather than by directly binding to Akt (38). PDK1 kinase, which activates Akt, has also been found to interact with Smads and to negatively regulate TGF-β-mediated signaling (41). On the other hand, TGF-β causes phosphorylation of Akt, an event that seems to be required for TGF-β-induced epithelial to mesenchymal transition (42).

In this study we show that the negative regulator of TGF-β signaling, Ski, is phosphorylated by Akt kinase on threonine 458. Phosphorylation of Ski causes its destabilization, causes up-regulation of Smad7, and reduces the activation of TGF-β signaling. The phosphorylation of Ski by Akt is a novel finding linking Akt to TGF-β signaling through TGF-β inhibitory molecules.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human osteosarcoma U-2 OS cells were cultured in Dulbecco's modified Eagle's medium containing 15% fetal calf serum (Autogen Bioclear, Calne, UK). Human kidney 293T epithelial cells and African green monkey kidney fibroblast-like Cos7 cells were grown in Dulbecco's modified Eagle's medium and 10% fetal calf serum. Human melanoma kidney fibroblast-like Cos7 cells were grown in Dulbecco's modified Eagle's medium containing 15% fetal calf serum (Autogen Bioclear, Calne, UK). Human melanoma 293T epithelial cells and African green monkey kidney fibroblast-like Cos7 cells were cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum. The cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C. All of the cell lines were obtained from the American Type Culture Collection (Manassas, VA).

LY294002, cycloheximide (CHX), and tricubine were purchased from Calbiochem. MG132 was from Affinity Research Products (Exeter, UK). Insulin was from Sigma-Aldrich. Insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), and epidermal growth factor were obtained from Calbiochem. TGF-β was from PeproTech (Rocky Hill, NJ).

Antibodies—Monoclonal antibody against HA tag was from Covance. Rabbit antibodies for Akt substrate, phospho-308 Akt (Akt phosphorylated on Thr-308), phospho-473 Akt (Akt phosphorylated on Ser-473), total Akt, and TGF-β receptor I were obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody against β-tubulin was purchased from BD Pharmingen (San Jose, CA). Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody was from Europa Bioproducts (Cambridge, UK). Rabbit anti-Ski antibody was from Upstate (Millipore). Goat anti-Ski (N-20), mouse monoclonal Sp-1 (LCG), rabbit p15, and Smad7 antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti-Smad2/3 was from Transduction Lab, and rabbit phosphospecific antibody for phospho-Smad2/3 was kindly provided by Dr. C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden).

Plasmids and Transient Transfection—PCIneoSki-HA was kindly provided by Dr. S. Ishii (RIKEN Tsukuba Institute, Ibaraki, Japan). pCMV5-Akt1-HA, Akt1 kinase-dead (K179A), constitutively active myristylated (pCMV5-Akt1myr-HA), pCMV5Akt 2-HA, and pCMV5-Akt3-HA mammalian expression vectors were kindly provided by Dr. D. Alessi (University of Dundee, Dundee, UK). U2-OS and Cos7 cells were transiently transfected with FuGENE 6 or FuGENE HD (Roche Applied Science) reagent. 293T cells were transfected with JetPEI (PolyPlus Transfection, New York, NY).

Cell Lysates, Immunoblotting, and Immunoprecipitation—the cells were washed once with phosphate-buffered saline before lysis in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM orthovanadate, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, and 10 µg/ml aprotinin) on ice. The lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4 °C. Protein concentrations were determined using a Bio-Rad protein assay. 30–40 µg of total protein/lane was separated by SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose filters (Bio-Rad). The filters were blocked with 3% milk in TBST (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Following immunoblotting with the indicated antibodies and conjugates, the proteins were detected using chemiluminescence (Amersham Biosciences). For immunoprecipitation studies, the cell lysates were diluted with immunoprecipitation buffer (50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 150 mM NaCl) before adding the antibodies. Protein G-Sepharose beads (GE Healthcare, Uppsala, Sweden) were used to collect protein-antibody complexes followed by washes. Protein bands were quantified using FluorChem™ densitometry and AlphaEaseFC™ (Alpha Innotech Corporation, San Leandro, CA) software or ImageJ software of the exposed films as indicated in the figure legends.

Nuclear and Cytosolic Fractions—the cells were washed twice with phosphate-buffered saline and lysed in 200 µl of hypotonic buffer (20 mM Tris/HCl, pH 7.4, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol). The nuclei and cytosol were separated by centrifugation at 500 rpm for 5 min at 4 °C. 200 µl of hypotonic buffer containing 0.5 mM NaCl and protease inhibitors were added to the nuclear fraction and rotated for 30 min at 4 °C. Nuclear and cytosolic fractions were cleared by centrifugation at 13,000 rpm for 15 min at 4 °C.

Kinase Assay—PCIneo vector, PCIneoSkivt, and PCIneoSkiA were transfected into 293T cells and treated with 25 µM LY294002 overnight. Immunoprecipitation with anti-HA antibody was performed as indicated above, collected on Protein
G-Sepharose beads, and incubated for 30 min at 30 °C with human 100 ng of recombinant Akt (Cell Signaling Technology, Beverly, MA) in the presence of 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl₂, 0.05 mM dithiothreitol, 25 μM [γ-32P]ATP (PerkinElmer Life Science) in 5 mM MOPS pH 7.2 buffer. Incubation was stopped by adding Laemmli buffer and boiling.

**Stable Cell Lines**—U-2 OS cells were transfected with PCIneo vector, Ski wt, and Ski A expression vectors. After 24 h, the cells were replated and selected in the presence of 1 mg/ml G418 (Calbiochem, San Diego, CA).

**RT-PCR**—MDA-MB 435 cells were treated as indicated. RNA was isolated by using NucleoSpin RNAII Kit (Macherey Nagel, Düren, Germany), and semi-quantitative RT-PCR was performed according to the manufacturer's instructions (RobusT II RT-PCR kit; Finnzymes, Espoo, Finland). The primers for the amplification of the human Smad7 were as follows: forward, 5'-TCCAGATACCCGATGAGATTTC-3', and reverse, 5'-GATTAGCTCCGACCTTCT-3'.

**RESULTS**

**Ski Interacts with Akt Kinase**—Akt has been shown to interfere with the TGF-β signaling pathway by negatively regulating Smad3-mediated signaling (39, 40). However, its possible impact on other TGF-β signaling mediators has not been addressed. In the course of investigating possible new mediators we noticed that Ski contains a highly conserved sequence of Akt motif, RXRXX(S/T) (43). We therefore first determined whether Ski interacts with Akt. Cos7 cells were co-transfected with Akt1-HA and Ski-HA. We performed reciprocal immunoprecipitation of cell lysates with Ski and Akt antibodies followed by Western blotting. Both proteins were found to co-immunoprecipitate with each other (Fig. 1A).

In mammals Akt exists in three isoforms, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. Akt1 and Akt2 are ubiquitously expressed, whereas Akt3 is mainly expressed in brain, heart, and kidney (44 – 47). To assess whether there is a preference of a particular isoform to interact with Ski, we expressed the Akt isoforms in combination with Ski and performed immunoprecipitation with anti-Ski antibody. We found that Akt1 and Akt2 but not Akt3 co-immunoprecipitate with Ski (Fig. 1B). Next we investigated whether Akt activity is required for Ski interaction.

We expressed Ski with wt Akt, a kinase-defective mutant, or a constitutively active mutant (Akt-Myr). Additionally, we treated cells expressing Ski and Akt1 wt with LY294002, a synthetic inhibitor of the p110 catalytic subunit of PI3K (47) to inactivate Akt. Cell lysates were then immunoprecipitated with an anti-Ski antibody. These immunoprecipitation studies indicated that both active and inactive forms of Akt bind Ski (Fig. 1C). However, we were unable to find any interaction between endogenous Ski and Akt (not shown). This suggests that the interaction by the kinase and the target is only a brief enzyme substrate interaction similar to numerous other interactions of Akt substrates (48 – 51).

**Ski is Phosphorylated by Akt at Threonine 458**—A putative Akt phosphorylation site, threonine 458 in human Ski, is highly conserved across species (Fig. 2A). To explore whether Akt phosphorylates Ski on this site, we mutated threonine 458 to alanine (Ski A). We also mutated threonine 458 to aspartic acid (Ski D) to create a phosphorylation mimicking mutant. We then expressed Ski wt, Ski A, and Ski D in Cos7 cells. The cell lysates were precipitated with anti-Ski antibody, and the immunocomplexes were blotted with an Akt phosphorylation site antibody (52). This antibody recognizes a phosphorylated serine or threonine at the conserved Akt phosphorylation site. The Akt substrate antibody recognized Ski wt and Ski D mutant but not Ski A mutant (Fig. 2B). To confirm that Akt directly phosphorylates Ski at this site, we performed an in vitro Akt kinase assay. We expressed Ski wt or Ski A mutant and treated the cells with LY294002 to inactivate Akt and to reduce basal phosphorylation of Ski. Immunoprecipitates were incubated with recombinant Akt and blotted with anti-Akt substrate antibody. The addition of recombinant Akt markedly increased the phosphorylation of Ski wt but not the Ski A mutant (Fig. 2C). In vitro Akt assay was also repeated by incubating Ski wt and Ski A with recombinant Akt in the presence of [γ-32P]ATP. Only Ski wt but not Ski A was phosphorylated by Akt, confirming direct phosphorylation on the threonine 458 by Akt (Fig. 2D). To further confirm the involvement of Akt in phosphorylation of Ski in vivo, we expressed Ski wt in U2-OS cells, which have a very low basal level of endogenous Ski. Following transfection, we treated the cells with LY294002 or another Akt inhibitor, tricibine. The endogenous Akt constitutively phosphorylated Ski in U2-OS cells, the effect of which was attenuated by blocking the activity of Akt by chemical inhibitors (Fig. 3A).

**Insulin, IGF-I, and HGF Induce Phosphorylation of Ski by Akt**—Akt is activated by survival signals, such as growth factor signaling. To investigate the involvement of growth factors in the phosphorylation of Ski, we treated serum-starved cells with
insulin. For this purpose, we used U2-OS cells ectopically expressing Ski. Insulin induced time-dependent phosphorylation of Ski concomitant with the activation of Akt as evidenced by Akt phosphorylation on residues 308 and 473 (Fig. 3B). To assess the effect of Akt on endogenous Ski, we serum-starved MDA-MB 435 melanoma cells, treated the cells with LY294002 as indicated, then treated the cells with insulin, and analyzed cell lysates for phosphorylation of endogenous Ski (Fig. 3C). Insulin induced the phosphorylation of endogenous Ski, and this phosphorylation was attenuated with pretreatment with LY294002. Furthermore, we found that other Akt pathway-activating growth factors, IGF-I and HGF, but not epidermal growth factor, increase the phosphorylation of Ski (Fig. 3D).

**Localization of Phosphorylated Ski**—Ski is localized both in the cytoplasm and in the nucleus, and on the other hand, Akt-mediated phosphorylation is known to cause relocalization of many of its target proteins, like p27, Skp2, and FoxO (53–56). To investigate whether Akt-mediated phosphorylation of Ski affects its localization, we performed nuclear and cytoplasmic fractionation of Ski wt but did not affect the degradation of the Ski A mutant. Accordingly, the levels of both Ski wt and Ski A were increased following proteasomal block. To verify whether Ski is destabilized by phosphorylation, we wanted to determine the half-life of Ski wt and Ski A in the absence and presence of insulin (Fig. 6). Insulin treatment for 30 min prior to the addition of CHX markedly lowered the basal level of Ski wt but did not affect the degradation of the Ski A mutant.

Next we studied the degradation of endogenous Ski by TGF-β in the presence or absence of insulin (Fig. 7A). The cells were first pretreated with insulin (30 min) as indicated, and TGF-β was then added for the indicated times. Following the 30-min insulin pretreatment, the level of Ski was reduced by half, in concordance with the results in cells with ectopic Ski expression (Fig. 6). Similarly, in concordance with previous publications, TGF-β rapidly reduced the level of Ski in MDA-MB 435 cells with an estimated half-life of 35 min (Fig. 7B). Interestingly, following the initial rapid degradation of Ski phosphorylation and Smad3-dependent activation of TGF-β-induced genes (39, 40). Because Ski binds Smad3, we wanted to investigate whether Smad3 is also present in the Akt and Ski complex. We therefore expressed Ski and Akt with increasing amounts of Smad3. As shown in Fig. 5, Akt and Ski interact in the absence of Smad3 overexpression, and the expression of Smad3 reduces the complex formation between Akt and Ski. This suggests that Smad3 is not required for the complex formation between Akt and Ski but that it competes with Akt in binding to Ski. We also tested whether phosphorylation of Ski affects its binding to either Smad3 and Smad4. Ski wt as well as Ski A and D mutants were equally capable of associating with Smad3 and Smad4 (supplemental Fig. S1). These findings indicate that Akt, Smad3, and Ski can form a multiprotein complex independent of Ski phosphorylation status but that in the presence of excess of Smad3, an interaction between Ski and Smad3 is favored.

**Phosphorylation of Ski at Threonine 458 Causes Its Destabilization**—TGF-β controls Ski proteasomal degradation and its rapid turnover to allow transmission of Smad-signaling (22, 57). To assess whether Akt-mediated phosphorylation of Ski impacts Ski half-life, we first produced stable U2-OS cell lines expressing Ski wt or Ski threonine 458 alanine mutant. The cells were treated with CHX to block protein synthesis, MG132 to block proteasomal degradation, insulin to activate Akt, and LY294002 to inactivate Akt, and Ski wt and A mutant levels were assessed (Fig. 6A). We noticed that insulin reduced the level of wild type Ski but not Ski A mutant. Accordingly, the inactivation of Akt with LY294002 increased the level of Ski wt but did not affect the level of Ski A. The levels of both Ski wt and Ski A were increased following proteasomal block. To verify whether Ski is destabilized by phosphorylation, we wanted to determine the half-life of Ski wt and Ski A in the presence and absence of insulin (Fig. 6B). Insulin treatment for 30 min prior to the addition of CHX markedly lowered the basal level of Ski wt but did not affect the degradation of the Ski A mutant.
following insulin treatment, the degradation rate of Ski by TGF-β decreased \(T_{1/2} = 60\) min in the insulin-exposed cells. Therefore, although insulin destabilizes Ski, it does not augment the TGF-β mediated Ski degradation.

**DISCUSSION**

In this study we show that the negative regulator of TGF-β pathway, Ski, is phosphorylated by Akt and that this phosphorylation is enhanced by activation of PI3K/Akt pathway with growth factors like insulin, IGF-1, and HGF. Phosphorylation of Ski is the trigger that causes the following chain of events: first the destabilization of Ski and the reduction of its ability to inhibit the expression of Smad7, thus increasing Smad7 levels, and finally the quenching of TGF-β signaling. Based on these

**Destabilization of Ski by Insulin Induces the Expression of Smad7**—It has been previously reported that Ski constitutively blocks the transcription of Smad7 mRNA \((58, 59)\). Because insulin treatment did not increase the rate of degradation of Ski by TGF-β, we decided to investigate whether insulin-induced phosphorylation and destabilization of Ski affects Smad7 protein levels. We found that, in fact, concomitant with reduced Ski levels, insulin treatment increased Smad7 levels after 3 h of treatment (Fig. 8A). Smad7 mRNA levels peaked after 2–3 h of stimulation with insulin and returned to the basal level after 4 h (Fig. 8B). This finding is in accordance with the observation that although insulin rapidly reduced the level of endogenous Ski, its level was recovered after 4–5 h (Fig. 8, A and B). Because Smad7 causes inactivation of TGF-β receptors, we also investigated the phosphorylation of Smad2 and Smad3 following insulin stimulation. The cells were first incubated with insulin for 1.5–4.5 h, followed by the addition of TGF-β for the last 30 min. As shown in Fig. 8C, pretreatment of the cells with insulin for more than 2.5 h clearly attenuated Smad2/3 phosphorylation but did not reduce either total Smad2/3 or TGF-β receptor I levels. We wanted to further assess the effect of insulin on the regulation of TGF-β downstream targets. p15 is a well characterized cell cycle inhibitor that contributes to TGF-β-mediated G1 phase arrest \((1)\). As shown in Fig. 8D, TGF-β induces p15 \((lane 2)\), and this effect is quenched with extended treatments with insulin \((5–7\ h, \ lanes\ 6–8)\). This indicates that TGF-β is unable to induce p15 expression when the cells have been pretreated with insulin for 3 h or more. Kinetically, this parallels the elevation of Smad7 and the decreased phosphorylation of Smads (Fig. 8, A–C). This finding is consistent with insulin-mediated inhibition of TGF-β signaling through temporal regulation of Ski and Smad7.

**FIGURE 3. Insulin and other growth factors induce phosphorylation of Ski.** A, basal phosphorylation of Ski is abrogated by a PI3K inhibitor, LY294002, and an Akt inhibitor, tricibine. U-2 OS cells were transiently transfected with Ski for 24 h and were treated with LY294002 \((25\ \mu M)\) or tricibine \((5\ \mu M)\) overnight. The lysates were immunoprecipitated \((IP)\) and then Western blotted \((WB)\) with either Akt substrate or anti-HA antibodies as shown. The cellular lysates were analyzed as control. B, time course of the phosphorylation of Ski after insulin treatment. U-2 OS cells were transiently transfected with Ski for 24 h, serum-starved overnight, and then stimulated with insulin \((100\ nM)\) for the times indicated. The lysates were immunoprecipitated with anti-Ski antibody and probed with anti-Akt substrate, anti-HA, and phospho-Akt antibodies \((P308Akt\ and\ P473Akt)\) as indicated. C, insulin increases phosphorylation of endogenous Ski. MDA-MB 435 cells were serum-starved overnight and treated with LY294002 \((25\ \mu M)\) for 6 h prior to adding insulin \((100\ nM)\) for 1 h. Ski was immunoprecipitated and probed with anti-Akt substrate antibody, anti-Ski antibody, and anti-phospho-Akt antibody. D, U-2 OS cells were transfected with Ski, starved and treated with insulin \((100\ nM),\ IGF-1\ (10\ ng/ml),\ HGF\ (50\ ng/ml),\ and\ epidermal\ growth\ factor\ (100\ ng/ml)\) for 1 h. Ski, Akt, and their phospho-forms were analyzed as in A. The cellular lysates were analyzed as control. The data in C and D are representative of two similar experiments.
findings we propose a novel model of how insulin and other growth factors activating Akt pathway can modulate TGF-β signaling cascade (Fig. 8E).

Both Ski and Smad7 are negative regulators of TGF-β signaling. Ski inhibits TGF-β signaling mainly transcriptionally by recruiting the nuclear co-repressor N-CoR complex to the Smad complex at the promoter sites blunting TGF-β-induced transcriptional activation. Therefore Ski must be degraded to allow the signaling to proceed. In fact, within few hours TGF-β induces the production of Ski (Fig. 8B), turning down its own transcription (18). Smad7 has a similar role as a feedback loop inhibitor. TGF-β stimulation in some cases transports Smad7 from the nucleus to the plasma membrane where it binds to the receptor I and prevents phosphorylation of Smads (60). Alternatively, it is degraded together with the TGF-β receptors by the ubiquitin-proteasome pathway (6, 7). However, all of these events are transient. At a later time point TGF-β induces the production of Smad7 to a steady state level. It has already been shown that Ski binds to Smad-binding elements, which antagonizes the induction of Smad7 mRNA and, conversely, that the silencing of Ski increases Smad7 mRNA levels (58, 59). By causing the degradation of Ski, TGF-β induces Smad7 production, which shuts down TGF-β receptors. However, upon resumption of Ski levels the Smad7-mediated negative repression is switched off (Fig. 8E).

In this study we addressed the intersection of the TGF-β and insulin-activated Akt kinase pathway. Our findings indicate a
Akt is anti-apoptotic and supports cell growth and division, whereas TGF-β signaling, at least in vitro, inhibits proliferation and promotes apoptosis. Earlier studies have proposed that Akt counteracts TGF-β signaling by direct binding and retention of Smad3 in the cytoplasm (39, 40). Additionally, mammalian target of rapamycin has been shown to mediate suppression of Smad3 by Akt (38). We provide here an alternative model. We show that Akt interacts and phosphorylates Ski directly, thereby causing its destabilization. The phosphorylation of Ski on threonine 458 is observed by physiological Akt activators and on the endogenous protein. Therefore it seems likely that the regulation and interaction observed here are physiologically meaningful. Moreover, we find that Smad3 competes with the Akt-Ski complex, indicating that Smad3 is not required for Akt-Ski interaction and in fact displaces Akt from the Ski-Smad3 complex. However, because this study and the previous studies addressing Smad3-Akt interactions rely on ectopically expressed proteins, the stoichiometry and physiological relevance of the trimeric Akt-Smad3-Ski complex are not clear and will require further study. Indeed, we find no evidence for Akt-Ski interaction at physiological levels of the proteins. Hence, the Akt kinase activity toward its substrate Ski is consistent with Akt phosphorylation has been shown to lead to the cytoplasmic translocation of several nuclear proteins, and the threonine 458 phosphorylation site found here is adjacent to the Ski nuclear localization signal (residues 452–458), we did not observe a definite relocalization of Ski following Akt overexpression or changes in the localization of Ski A or D mutants (not shown). However, we clearly detected phosphorylated Ski both in nuclear and cytoplasmic fractions. Interestingly, it has been previously reported that Ski protein levels vary during the cell cycle, being lowest in the G2/M phase (18, 64). The stabilization of Ski in G2/M was believed to be due to phosphorylation by CDK1/cyclin B, although no putative phosphorylation site was identified. Redistribution of Ski to centrosomes and the mitotic spindle was also reported (64). Although not directly addressed in our rapid cascade of events initiated by Ski phosphorylation on threonine 458 (15 min) following insulin treatment, degradation of Ski (30 min), up-regulation of Smad7 (2 h) followed by reconstitution of Ski levels (3–4 h), and a consequent decrease in Smad7. TGF-β transiently reduces Ski levels, which is likely potentiated by the action of Akt to destabilize Ski, thus increasing the TGF-β-mediated signaling. However, we find no evidence of additive degradation of Ski by insulin and TGF-β. On the other hand, Ski complex at the Smad7 promoter site is unstable (59), indicating that relatively minor changes in Ski levels affect Smad7 transcription. As demonstrated in Fig. 8, insulin is able to reduce Ski levels sufficiently to augment the production of Smad7 and inactivate Ski phosphorylation. Moreover, the Akt-Ski interaction is temporally blocked. We suggest that the fine tuning of Ski and Smad7 levels is a part of normal regulation of TGF-β signaling cascade. Based on the attenuation of Smad2/3 phosphorylation and decreased p15 induction, we propose that the TGF-β and Akt pathways converge in a temporal manner, leading to repression of the TGF-β signals. It is unlikely that these temporal events, lasting only for a few hours, would be captured in static reporter-based assays measuring more long term outcomes.

**FIGURE 8.** Insulin-mediated destabilization of Ski induces expression of Smad7 and reduces TGF-β signaling. **A**, insulin destabilizes Ski and increases Smad7 expression. MDA-MB 435 cells were treated with insulin (100 nM) as indicated, and cell lysates were immunoblotted (WB) with anti-Ski, anti-Smad7, anti-P473Akt, anti-Smad2/3 or anti-TGF-βRI antibodies. **B**, insulin increases Smad7 expression temporarily. MDA-MB 435 cells were serum-starved overnight, stimulated with insulin followed by the addition of TGF-β (3 ng/ml) for the last 2 h or TGF-β alone (lane 2). **C**, model of parallel regulation of TGF-β and Akt pathways of TGF-β downstream regulators. The data shown are representative of at least two similar experiments.
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study, it is possible that some of the fluctuation observed in Ski levels is due to growth factor-mediated Akt signaling during the G1 phase.

Controlled TGF-β signaling is essential for cell and tissue homeostasis. However, during tumor evolution, the TGF-β pathway evolves to support epithelial-mesenchymal transition, cell invasion, and metastasis (65). Therefore the actions of TGF-β pathway inhibitors, like Ski, are likely to possess opposite functions during various stages of tumorigenesis. This is also reflected by the contrasting reports of both tumorigenic and anti-tumorigenic activities of Ski. Therefore, the exact role of Ski in carcinogenesis has not been established. Highly metastatic breast and melanoma cell lines contain the high Ski levels associated with poor prognosis (13, 23). In contrast, silencing of Ski tumor suppressive properties may reflect its repression of TGF-β tumor promoting signals. PI3K/Akt pathway is often activated in cancer and may cause aberrant regulation of Ski and, in consequence, Smad7 and the TGF-β-signaling pathway. Further studies are clearly needed to elucidate the connections between Ski, Akt, and TGF-β-signaling components in carcinogenesis.

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