Novel Regulation of Ski Protein Stability and Endosomal Sorting by Actin Cytoskeleton Dynamics in Hepatocytes*

Received for publication, May 7, 2014, and in revised form, December 21, 2014. Published, JBC Papers in Press, January 5, 2015, DOI 10.1074/jbc.M114.579532

Genaro Vázquez-Victoria†1, Cassandre Caligaris†, Eugenio Del Valle-Espínosa‡, Marcela Sosa-Garrocho‡, Nelly R. González-Arenas‡, Guadalupe Reyes-Cruz†, Marco A. Briones-Orta†‡, and Marina Macías-Silva‡3

From the †Departamento de Biología Celular y Desarrollo, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D. F. 04510, México and the ‡Departamento de Biología Celular, CINVESTAV-IPN, México, D. F. 07000, México

Cell proliferation is positively regulated by a myriad of signaling pathways downstream of growth factor receptors and G protein-coupled receptors (GPCRs), among others, and it is negatively regulated by homeostatic signals such as TGF-β. Liver development and regeneration are excellent models for studying cell proliferation in a physiological context that is distinct from cancer (1–3). The liver regenerates upon injury, and all hepatic cells coordinately go through the cell cycle to restore the original mass and function of the organ. Interestingly, the liver grows despite the presence of TGF-β, a potent antiproliferative cytokine for epithelial cells. The TGF-β pathway is a major modulator of hepatocyte proliferation and death. However, it is unclear how hepatocytes become insensitive to TGF-β antiproliferative effects under conditions such as liver regeneration or hepatocarcinoma development (4–8).

Ligands of the TGF-β family regulate gene expression through the activation of Ser/Thr kinase receptors and the phosphorylation of receptor-regulated Smads (R-Smads) (9–11). This canonical pathway is tightly regulated by negative feedback loops generated by the up-regulation of inhibitors such as Smad7 and SnoN, which are encoded by immediately early genes regulated by the TGF-β/Smad pathway (12). Any dysregulation of these negative feedback loops may contribute to the development of some diseases. TGF-β also regulates the levels of the Ski and SnoN corepressors by inducing their degradation via the ubiquitin-proteasome system. The phosphorylated R-Smads act as adaptors to recruit the E3 ubiquitin ligases that catalyze the polyubiquitination of the Ski and SnoN proteins (12, 13). This process facilitates TGF-β/Smad-induced transcriptional regulation of target genes. Therefore, any disruption of Ski and SnoN protein down-regulation might increase their protein levels and inhibit TGF-β signals.

The Ski corepressor is a negative regulator of TGF-β/Smad signaling that may also act as a transcriptional cofactor for some nuclear receptors and other transcriptional factors (14–17). Ski functions as a nuclear transcriptional corepressor for the Smad transcriptional factors by recruiting other corepressors, such as nuclear receptor co-repressor (NCoR) and Sin3A, and different histone deacetylases to repress TGF-β target genes such as smad7 and skil (12, 14, 18). However, the Ski protein is also localized in the cytoplasm of some cell types, where its function is less clear. It has been reported that overexpressed cytoplasmic Ski may escape from the down-regulation exerted by TGF-β and that it may sequester the Smad proteins to block

This is an Open Access article under the CC BY license.
TGF-β signals (17). To date, the TGF-β-independent functions of Ski are poorly studied, particularly the function of cytoplasmic Ski (19, 20).

A major cross-talk among diverse signaling pathways occurs during liver regeneration, where the antiproliferative actions of TGF-β may be under tight control exerted by different pathways, mainly those implicated in cell cycle promotion, such as the pathways downstream of growth factors receptors and GPCRs, among others (2). One of the mechanisms used by cells to prevent TGF-β-induced antimitotic actions, which have been observed in normal and some cancer cells, is the up-regulation of negative modulators of the canonical pathway, such as the Ski and SnoN corepressors (12). We previously reported that the inhibitory actions of Ski and SnoN against the TGF-β in proliferating hepatocytes, and we also suggested that the molecular mechanisms involved are influenced by the dynamics of the actin cytoskeleton. Furthermore, we show that Ski protein stability is increased during liver regeneration, where it may facilitate hepatocyte proliferation by controlling TGF-β signaling.

**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant hTGF-β1 (TGF-β) was obtained from PeproTech. Methyl-β-cyclodextrin (MβCD), CHAPS, sphingosine 1-phosphate (SIP1), lysophosphatidic acid (LPA), 3-isobutyl-1-methylxanthine (IBMX) and forskolin (F) were obtained from Sigma. Latrunculin B (LatB) and jasplakinolide (Jasp) compounds were obtained from Calbiochem. MG132 (a proteasome inhibitor), SB431542 (an AK5 receptor inhibitor), and Y27632 (a Rho-associated protein kinase (ROCK) inhibitor) were obtained from Tocris Bioscience. Culture reagents and media were obtained from Invitrogen. Anti-FLAG M2 and anti-β-tubulin mouse monoclonal antibodies were obtained from Sigma. The following antibodies were obtained from Santa Cruz Biotechnology: anti-SnoN (catalog no. H-317), anti-Ski (catalog no. H-329), and anti-hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (catalog no. V-20) rabbit polyclonal antibodies; anti-Smad2/3 (catalog no. N-19) and anti-Smad4 (catalog no. C-20) goat-polyclonal antibodies; and anti-Ski (catalog no. G8) and anti-flotillin-2 (catalog no. B-6) mouse monoclonal antibodies. Anti-Ski (catalog no. 07-060), anti-Smad4, and anti-phospho-Smad2 rabbit polyclonal antibodies were from Millipore. Anti-EEA1 and anti-GM130 mouse monoclonal antibodies were obtained from BD Transduction Laboratories. Anti-Smad2 and anti-Yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ) rabbit polyclonal antibodies were obtained from Cell Signaling Technology. Secondary anti-rabbit IgG and anti-rabbit IgG (light chain) HRP-coupled antibodies were from Zymed Laboratories Inc. and Jackson ImmunoResearch Laboratories, respectively. Secondary anti-mouse IgG HRP-coupled antibody was from Santa Cruz Biotechnology. Alexa Fluor 488 (anti-rabbit IgG) and Alexa Fluor 594 (anti-mouse IgG) secondary antibodies were from Molecular Probes. Alexa Fluor 594 anti-goat IgG secondary antibody was from Jackson ImmunoResearch Laboratories.

**Animals and Partial Hepatectomy Model—**Studies were performed on male Wistar rats ~200–250 g of weight. Animals were maintained on an ad libitum diet and used according to institutional guidelines (Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (UNAM)) for animal experimentation. Anesthetized rats were subjected to a ventral laparotomy, and the anterior two-thirds of the liver were removed. Animals were sacrificed 0, 2, 48, and 120 h after the 70% partial hepatectomy. The livers were harvested, and the nuclear extracts were obtained for protein analysis as described previously (21).

**Cell Lines, Hepatocyte Isolation, and Primary Culture—**The C9 (rat hepatocytes) and HepG2 (human hepatoma) cell lines were maintained in DMEM supplemented with 10% FBS plus antibiotics (penicillin/streptomycin). Rat hepatocytes were isolated using the collagenase perfusion method adapted from the protocol of Snorri Thorgeirsson as described previously (21). For primary culture, hepatocytes were seeded on plastic Petri dishes coated with 1% rat tail collagen type 1 (BD Biosciences), and cells were cultured for 4 h at 37°C in attachment medium with 10% FBS. Then the medium was changed to feeding medium (FBS-free), and hepatocytes were cultured for 24 h for further studies (22). Cells were serum-starved for 12 h before treatments. C9 cells were transiently transfected with the pcDNA3/mCherry-FLAG-Ski, pmCherry-C2/hAlix, or pcCR3.1/CD63-mCherry constructs using Lipofectamine 2000 (Invitrogen). The pcDNA3/FLAG-Ski WT construct was a gift from Dr. Céline Prunier (INSERM Bat Kourilsky, Hôpital Saint-Antoine, Paris, France). The pcCR3.1/CD63-mCherry construct was a gift from Dr. Paul D. Bieniasz (The Aaron Diamond AIDS Research Center, New York, NY). Addgene plasmid no. 21504 (pmCherry-C2/hAlix) was obtained from Dr. James Hurley (23).

**Immunoprecipitations and Western Blot Analyses—**To obtain whole cell protein extracts (total cell lysates), cells were lysed in SDS lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, and 1% SDS plus protease and phosphatase inhibitors) and then boiled for 10 min at 100°C, or cells were either lysed for 30 min with TNTE buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, and 1 mM EDTA plus protease and phosphatase inhibitors) or lysed for 1 h with modified RIP buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet-P40, 0.5% sodium deoxycholate, and 0.1% SDS plus protease and phosphatase inhibitors). From whole cell lysates, 50–75 μg of protein/ lane was separated by SDS-PAGE and assayed by immunoblotting using specific antibodies, whereas ~1.5–2 mg of protein was used for each immunoprecipitation (IP). Proteins were detected by either Immobilon Western (Millipore) or SuperSignal West Pico chemiluminescent substrates (Thermo). Densitometric analysis was carried out with ImageJ 1.47 software (National Institutes of Health).

**Subcellular Fractionation and Detergent-resistant Membrane (DRM) and non-DRM Fraction Preparation—**Nuclear and cytoplasmic fractions were prepared according to a modi-
SkI Protein Is Transiently Up-regulated during Liver Regeneration—The process of liver regeneration exhibits a spatio-temporal synchronization of many signaling pathways positively regulating cell proliferation. Paradoxically, some anti-mitotic signals, such as TGF-β, are increased during liver regeneration. We reported previously that the up-regulation of TGF-β pathway inhibitors such as SkI and SnoN may counteract TGF-β-induced antiproliferative actions during mouse liver regeneration, allowing for other TGF-β functions, such as extracellular matrix remodeling (21). At that time, we also proposed that the increase in SkI and SnoN protein levels and their association with the Smads might, in part, explain the resistance of hepatocytes to TGF-β signals (21). Since then, we have been interested in discovering the mechanisms whereby hepatocytes become refractory to TGF-β-induced antiproliferative signals, particularly in liver regeneration.

Initially, we observed that SkI and SnoN proteins were up-regulated in parallel with Smad2 protein phosphorylation in rat livers obtained from 2–120 h after partial heptectomy but not in sham-operated rats (Fig. 1A). These data suggest that, in this cell context, the TGF-β/Smad pathway seems to be unable to cause SkI and SnoN protein down-regulation. Interestingly, we found that a significant fraction of both proteins was associated with the Triton-insoluble fraction when primary cultured hepatocytes were lysed with TNTE buffer, whereas both proteins were better solubilized in RIPA buffer (data not shown).

To gain insight into this outcome, we focused on investigating mainly the regulation of SkI protein stability in hepatocytes and the signals involved. In this study, we observed that the amount of SkI protein immunoprecipitated from RIPA lysates was higher than the amount obtained from TNTE lysates. Moreover, the effect of TGF-β on SkI protein down-regulation was better observed in RIPA lysates (Fig. 1B). Therefore, most of the experiments were carried out with RIPA buffer unless indicated otherwise.

With this approach, we were able to detect a clear TGF-β-induced regulation of SkI and SnoN protein levels in freshly isolated hepatocytes from normal (PH 0 h) and regenerating livers (PH 48 h) (Fig. 1C). Interestingly, we observed that SkI
and also Smad2 proteins were associated with actin protein (co-IP) at the proliferation phase analyzed (i.e. 48 h post-hepatectomy) (Fig. 1C). Additionally, Ski has been described previously as a soluble nuclear protein in many cell lines, including the hepatoma cell line HepG2. Therefore, it was unexpected to observe that Ski protein was associated with a Triton-insoluble fraction in normal hepatocytes. When we analyzed subcellular localization of Ski in quiescent and regenerating livers (PH 48 h), the data showed that Ski exhibited mainly a nuclear localization. However, a significant fraction of Ski was cytoplasmic,

**FIGURE 1.** Ski protein levels are transiently up-regulated during liver regeneration after partial hepatectomy. A, nuclear protein extracts from regenerating rat livers after PH at the indicated times (or 2-h sham-operated rats) were used to evaluate Ski, SnoN, SnoN2, phospho-Smad2, and proliferating cell nuclear antigen (PCNA) protein levels by IP/WB (top panel). Bottom panel, densitometric analysis of Ski and SnoN protein levels as fold change induced by PH. Data are represented as mean ± S.D. of three independent experiments. PCNA protein was used as a cell proliferation marker. B, primary cultured hepatocytes were stimulated for 1 h without or with 0.2 μM TGF-β. Cells were lysed with either RIPA or TNTE buffer to obtain two fractions: SN (Triton-soluble fraction) and pellet (Triton-insoluble fraction resuspended in RIPA). Immunoblotting was performed for the indicated proteins from IP or cell lysates. C, hepatocytes were freshly isolated from livers obtained 0 or 48 h post-PH, stimulated for 1 h with 0.2 μM TGF-β, and lysed with RIPA buffer. Ski and Smad2/3 proteins were immunoprecipitated, and immunoblot analyses were performed to identify the indicated proteins from IP or cell lysates. D, nuclear (Nuc) and cytoplasmic (Cyto) protein extracts from regenerating rat livers after PH at the indicated times were used to evaluate the indicated proteins from IP or cell lysates (top panel). Bottom panel, densitometric analysis of the levels of cytoplasmic Ski-actin complexes expressed as fold-change induced by PH. Data are represented as mean ± S.D. of three independent experiments. E, cytoplasmic protein extracts were obtained from regenerating livers 0 or 48 h after PH, and then 35 μg of protein extracts was treated for 20 min with 0.5 μg/ml proteinase K in the absence or presence of 0.5% Triton X-100. Ski protein was immunoprecipitated, and immunoblot analyses were performed for the indicated proteins from IP or protein extracts. The densitometric analysis of Ski protein levels is shown as fold change over the control. GM130, tubulin, and flotillin-2 were used as controls for protein degradation (asterisk, GM130 fragment). F, mCherry-FLAG-Ski protein levels were detected by immunoblot analysis of RIPA lysates from transiently transfected C9 cells with the indicated amounts of plasmid (top panel). Transiently transfected C9 cells with 30 μg of plasmid were serum-starved for 24 h and then stimulated with 10% FBS for 24–48 h (bottom panel). Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (relative cell number). Data are represented as means ± S.D. from two independent experiments performed in triplicate.
and, intriguingly, the levels of cytoplasmic Ski protein coimmunoprecipitated with actin were higher 48 h post-PH (Fig. 1D, top panel). Fig. 1D, bottom panel, shows that the levels of cytoplasmic Ski-actin complexes increased ~2-fold after 48 h post-PH over the control (0 h).

In hepatocytes, Ski protein is partially localized to a Triton-insoluble fraction, which could be explained by a partial association with the cytoskeleton and/or with lipid raft-rich membranes. This hypothesis was proved to be correct by using a protease protection assay. We observed that proteinase K only partially degraded Ski protein from cytoplasmic fractions obtained from quiescent (PH 0 h) or regenerating hepatocytes (PH 48 h) in the absence or presence of 0.5% Triton X-100 (Fig. 1E). As controls, GM130, a cis-Golgi matrix protein, was degraded, whereas a protein associated with lipid raft domains such as flotillin-2 was resistant to degradation by proteinase K. The data suggest that a fraction of cytoplasmic Ski is protected from down-regulation in hepatocytes during regeneration, probably by recruitment to a lipid raft-rich compartment.

Next we analyzed whether the increase in Ski protein levels observed during liver regeneration may favor specific Ski functions, such as the promotion of cell proliferation. We observed that the proliferation rate of hepatic C9 cells in response to 10% FBS treatment for 24–48 h was increased (3- to 6-fold over the control) when Ski protein was transiently overexpressed (Fig. 1F). This result correlated well with the increase in Ski protein levels observed during the proliferative phase of liver regeneration (Fig. 1A) and in regenerating hepatocytes (Fig. 1C).

TGF-β Induces a Transient Down-regulation of Ski Protein via the Proteasome—We first focused on studying the regulation of Ski protein stability and subcellular localization in normal hepatocytes. We observed that TGF-β induced a transient down-regulation of the Ski protein in a time-dependent manner in primary cultured hepatocytes (Fig. 2A). As an additional cell model, we decided to use the C9 cell line because it exhibits similar features as normal hepatocytes. In C9 cells, TGF-β also transiently decreased the levels of Ski protein and induced Smad2 phosphorylation (Fig. 2B). We observed that MG132
TGF-β and GPCR Signals Regulate Ski Protein Levels

Our data show that cytoplasmic Ski exhibits a punctate pattern of localization, which is typical of the endosomal compartment. To further characterize the subcellular localization of cytoplasmic Ski, we performed colocalization studies of Ski protein with different markers of multivesicular endosomes (MVE) and other organelles. Vesicles containing cholesterol-rich lipid rafts are commonly implicated in vesicular trafficking and also contribute to the formation of MVEs, which are relevant in different cellular processes such as protein degradation via lysosomes and protein export via exosomes. These MVEs may also serve as organelles able to temporally sequester or store signaling proteins, such as GSK3 (27). As shown in Fig. 3, endogenous Ski protein partially colocalized with the overexpressed MVE markers CD63-mCherry and mCherry-Alix and endogenous HRS. Additionally, Ski colocalized with flotillin-2, a lipid raft marker, and, to a lesser extent, with the early endosome marker EEA1 and with the Golgi marker GM130 (Fig. 3, Z stacks in merged images).

Furthermore, TGF-β treatment for 1 h caused a down-regulation of Ski protein in both the nucleus and cytoplasm, which correlated with Smad2 nuclear localization (Fig. 4A). Intriguingly, TGF-β also promoted a partial redistribution of cytoplasmic Ski protein to the perinuclear region after 3 h of treatment (Fig. 4A, magnified images). Additionally, TGF-β treatment enhanced Ski protein colocalization with CD63-mCherry, a late endocytic MVE and exosome marker (Fig. 4B, left panel). The index of colocalization of Ski-CD63 corresponds to 679 ± 136 counts (control) and 1818 ± 291 counts (TGF-β) (p < 0.004, n = 12), as shown in Fig. 4B, right panel. These data indicate that TGF-β treatment for 3 h promoted Ski-CD63 colocalization (2.7-fold over the control) in C9 cells. Together, our findings suggest that, in normal hepatocytes, cytoplasmic Ski protein exhibits different subcellular localizations, including its association with endosomes resembling MVEs, and it is also clear that Ski protein stability and its subcellular localization are regulated by TGF-β.

Ski Protein Is Partially Associated with Lipid Rafts—Our findings indicating that a fraction of the whole Ski protein is partially associated with a Triton-insoluble fraction suggest that Ski might be located in cholesterol-rich vesicles. To test this, C9 cells were pretreated with the cholesterol-chelating agents filipin and MβCD, followed by TGF-β treatment for 1 h, and lysed with TNTE or RIPA buffer. Ski protein was detected by IP/WB. As shown in Fig. 5A, MβCD treatment not only enriched Ski protein levels in the TNTE-soluble fraction but also disrupted the Ski-actin protein association in RIPA buffer. Despite the fact that neither agent altered the TGF-β effect on Ski protein stability (Fig. 5A), the ability of MβCD to extract cholesterol from the plasma membrane and intracellular compartments, promoting raft disruption and the solubilization of proteins from rafts (28), could explain the differential results obtained with MβCD and filipin treatments on the Ski-actin protein association. Moreover, MG132 pretreatment of C9 cells partially increased Ski protein levels in the Triton-insoluble fraction (pellet) (25% from total) compared with the soluble fraction (SN) (16% from total), and MβCD pretreatment blocked this effect (pellet) (13% from total) (Fig. 5B). These data suggest that Ski protein is partially associated with lipid rafts that are generally associated with the DRM (29, 30).

Next we decided to analyze DRM (cholesterol-rich) by separating DRM and non-DRM fractions from C9 cells. As shown...
in Fig. 5C, Ski protein was localized in both the DRM and non-DRM fractions from control cells. Notably, Ski protein at non-DRMs was more sensitive to TGF-β/H9252-induced down-regulation. It is possible that Ski protein becomes partially sequestered at MVEs to prevent total protein depletion after TGF-β stimulation. MG132 induced an accumulation of Ski protein in both the DRM and non-DRM fractions, whereas MβCD blocked Ski protein association with the DRM fraction (Fig. 5C). Therefore, our findings support the hypothesis that Ski protein is partially associated with lipid raft-rich vesicles in normal hepatocytes.

**Actin Cytoskeleton Dynamics Modulate Ski Protein Stability**—Our data suggesting that changes in actin cytoskeleton dynamics (actin polymerization or depolymerization) might control Ski protein stability led us to investigate different cellular contexts where the cytoskeleton rearrangement is relevant, such as cell-cell contact controlled by cellular confluence. Therefore, C9 cells were seeded at low (sparse) and high (confluent) density, and Ski protein stability was analyzed. As shown in Fig. 6A, Ski protein is highly stabilized in confluent C9 cells in both the nuclear and cytoplasmic compartments, suggesting that the Ski protein could be sensitive to actin cytoskeleton rearrangements that occur in cells when cell-cell junctions are decreased (sparse) or increased (confluent). Furthermore, TGF-β treatment efficiently down-regulated Ski protein levels in both sparse and confluent cells (Fig. 6B, top panel). Fig. 6B, bottom panel, shows that the levels of Ski protein were 6-fold higher in confluent than in sparse C9 cells.

Next we tested whether pharmacological modulators of actin cytoskeleton dynamics might affect the stability of Ski protein and its association with actin. Strikingly, LatB, an inhibitor of actin polymerization, increased Ski protein stability (Fig. 6C), whereas Jasp, a potent inducer of actin polymerization, decreased Ski protein stability (Fig. 6C). Interestingly, actin cytoskeleton dynamics modulated Ski protein association to DRM and non-DRM fractions. Therefore, Jasp induced an increase in Ski protein levels associated with the DRM fraction, whereas LatB induced an increase in Ski protein levels associated with the non-DRM fraction and impaired the association of Ski protein with the DRM fraction (Fig. 6D). Notably, the Ski-actin interaction (co-IP) was increased in both the DRM and non-DRM fractions by Jasp treatment (Fig. 6D). Jasp treatment increased the levels of Ski-actin complexes 15-fold over the control, whereas LatB decreased such complexes 0.25-fold compared with the control (Fig. 6E). Neither LatB nor Jasp affected basal or TGF-β-induced Smad2 phosphorylation (data not shown). All of these data indicate that actin cytoskeleton dynamics control Ski protein stability and also strongly suggest that a fraction of cytoplasmic Ski is associated, possibly along with actin, to a subset of vesicles containing cholesterol-rich lipid rafts.

**GPCR Signaling Pathways Differentially Modulate Ski Protein Stability**—Knowing that GPCRs control diverse pathways that are highly active during liver regeneration and that it has been reported recently that these receptors can differentially...
regulate the dynamics of the actin cytoskeleton to control the Hippo pathway (2, 31, 32), we further analyzed whether Ski protein stability was regulated by either GPCRs controlling cAMP levels, such as the glucagon receptor, or GPCRs coupled to G12/13 and Rho-GTPase activation, such as the S1P and LPA receptors. When C9 cells were treated for different times with LPA or S1P, we observed that both stimuli induced a transient down-regulation of Ski protein (Fig. 7A). As a positive control, we observed that TAZ protein stability was increased (Fig. 7A).

Intriguingly, we did not see any significant modulation of Smad2 phosphorylation or its subcellular localization by agents controlling actin cytoskeleton dynamics or the Hippo pathway in hepatocytes (data not shown), even though it has been shown previously that the Hippo pathway controls Smad2 in embryonic cells (33). In addition, Ski protein down-regulation and Ski-actin association (co-IPs) induced by S1P were inhibited by pretreatment with MG132, LatB, and Y27632 (a ROCK inhibitor) (Fig. 7B). By contrast, we observed that stimulators of cAMP accumulation, such as glucagon or a mixture of F/IBMX, induced an increase in Ski protein stability (Fig. 7C) and that F/IBMX pretreatment inhibited the LPA- and S1P-mediated reduction of Ski stability (Fig. 7D, left panel). Fig. 7D, right panel, shows that S1P and LPA increased the levels of Ski-actin complexes 15-fold over basal in control cells. Intriguingly, M/H9252CD treatment prevented Ski down-regulation induced by S1P and also the Ski-actin interaction (Fig. 7E), but it did not change the Ski/Smad4 interaction (Fig. 7, E and F) or Ski subcellular localization (Fig. 7, F and G). Together, these data suggest considering the possibility that the association between Ski and actin may rely on the participation of an adaptor or scaffold protein rather than a direct interaction. However, this remains to be investigated.

On the basis of these results, we show a model depicting the proposed mechanism (Fig. 8). Accordingly, TGF-β/Smad pathway and/or the stabilization of actin filaments promoted by the GPCR/G13/Rho signaling axis can decrease Ski protein stability, whereas the GPCR/cAMP pathway disrupts actin filaments and, consequently, increases Ski protein stability in hepatocytes.
DISCUSSION

Ski protein is a major transcriptional corepressor for the TGF-β/Smad pathway. Therefore, its levels may determine the TGF-β signaling outcome (e.g. high Ski protein levels are associated with an inhibition of TGF-β-induced signals). The relevance of maintaining appropriate Ski protein levels for homeostasis has been clearly demonstrated. Recently, several in-frame mutations in exon 1 of Ski (the R-Smad binding region) have been identified as the main cause of the human Shprintzen-Goldberg syndrome, and increased TGF-β signaling has been associated with this pathogenesis (34). Furthermore, Ski knockout mice exhibit embryonic lethality, and heterozygous mice show a high sensitivity to developing cancer (35, 36). By contrast, transgenic mice overexpressing Ski protein exhibit increased muscle mass (17). Additionally, high levels of Ski protein have been involved in the switch from glycolysis to oxidative energy metabolism in cancer cells (37).

In many cancer cells, Ski overexpression occurs mainly in the nucleus, where it may recruit histone deacetylases to eventually repress TGF-β target genes. Some reports suggest that cytoplasmic Ski protein may act by sequestering Smad proteins and impairing their translocation to the nucleus. In some cancer cells, TGF-β induces Ski protein down-regulation via the proteasome, whereas, in other cancer cells, Ski protein seems to be resistant to TGF-β-induced degradation. Although the mechanisms underlying this cell-to-cell variability are unclear, it has been suggested that expression of specific E3 ubiquitin ligases may be important (38). Notably, there are very few studies of Ski and SnoN protein regulation and function in normal cells (39).

TGF-β-induced signaling is highly modulated during many physiological processes. One example is its regulation in cells with an elevated proliferation rate, such as those found in liver regeneration and cancer. Hepatic regeneration is an excellent model for studying the regulation of cell proliferation and how hepatic cells can proliferate in the presence of an antimitotic cytokine as TGF-β, which has been an enigma. We have proposed that the up-regulation of Ski and SnoN inhibitors might explain the transient resistance to TGF-β signals exhibited by...
hepatocytes during liver regeneration (21). Surprisingly, during this study, we found a novel mechanism for the regulation of Ski protein stability in normal hepatocytes involving a convergence of TGF-β and GPCR signals with actin cytoskeleton participation. Therefore, we focused on studying the molecular mechanisms involved in controlling Ski protein stability in normal hepatocytes.

In our model of normal hepatocytes, Ski protein appears to be distributed in both the nucleus and cytoplasm. Cytoplasmic Ski shows a punctate localization and partial association with the DRM fraction. We found that a fraction of cytoplasmic Ski is partially localized to a subset of lipid raft-rich vesicles, some of them containing MVE markers such as CD63 and Alix. We also found that nuclear and cytoplasmic Ski are quite sensitive to proteasome-dependent down-regulation in response to TGF-β or any stimulus that increases the polymerization of actin filaments, such as Jasp, S1P, or LPA. However, Ski protein stability is increased in response to any stimulus that decreases actin filament polymerization, such as LatB or glucagon-stimulated GPCR/cAMP signals (Fig. 7). To our knowledge, this is the first demonstration of Ski protein localization at the MVE in normal hepatocytes, and we also show that its stability is associated with the dynamics of the actin cytoskeleton. We suggest that this novel regulation of Ski protein stability is an event that possibly occurs during liver regeneration, when extracellular matrix remodeling modifies the architecture of the liver, caus-

FIGURE 7. GPCR signaling pathways differentially modulate Ski protein stability. A, C9 cells were stimulated with 1 μM LPA or 1 μM S1P for the indicated times. TAZ protein levels indicate LPA or S1P receptor activation. B, C9 cells were pretreated for 4 h without (Control) or with 25 μM MG132, 5 μM LatB, or 30 μM Y27632 and then stimulated for 1.5 h with 1 μM S1P and then stimulated for 1.5 h with 1 μM S1P or for 1 h with 1 μM LPA (left panel). Right panel, densitometric analysis of Ski-actin complex levels as fold change induced by S1P or LPA. Data are represented as mean ± S.D. of three independent experiments. C, C9 cells were pretreated with a mixture of 2 μM F/100 μM IBMX mixture or with 2 μM glucagon for the indicated times. D, C9 cells were preincubated for 1 h in the absence (Control) or presence of a mixture of 2 μM F/100 μM IBMX and then stimulated for 1.5 h with 1 μM S1P or for 1 h with 1 μM LPA (left panel). Right panel, densitometric analysis of Ski-actin complex levels as fold change induced by S1P or LPA. Data are represented as mean ± S.D. of three independent experiments. E, C9 cells were pretreated without (Control) or with MβCD for 4 h and stimulated with 1 μM S1P for 1.5 h. F, C9 cells were treated with MβCD for 3 h, and the nuclear (Nuc) and cytoplasmic (Cyto) fractions were obtained. In A–F, Ski protein was immunoprecipitated, and immunoblotting was performed for the indicated proteins from IP or cell lysates. G, C9 cells were treated with MβCD for 3 h, and endogenous Ski protein was detected by immunofluorescence. Nuclei were stained with DAPI. Scale bars = 20 μm.
ing hepatocytes to reorganize their cytoskeletons to migrate to new positions within the regenerating liver. Intriguingly, this novel regulation of Ski stability in normal hepatocytes seems to be lost in hepatoma cells.

Many signals are involved in regulating liver regeneration, and, possibly, many of them control the expression of the transcriptional coregulator Ski during this process. Therefore, our work is an initial approach to reveal the molecular mechanisms and the potential consequences of the regulation of Ski protein stability and sorting in hepatocytes. Although TGF-β signaling is present during regeneration, its inhibitory action on Ski protein levels may be counteracted by other signals that modulate hepatocyte polarity, a tightly regulated process that takes place during regeneration (40). However, Ski is a transcriptional coregulator for other signaling pathways distinct from TGF-β during regeneration (40). Nevertheless, the mechanisms involved have also not been fully elucidated.

In conclusion, our work demonstrates a novel mechanism that regulates Ski protein levels in normal hepatocytes through different stimuli (Fig. 8). In these cells, Ski protein is able to sense changes in actin cytoskeleton dynamics that control its own stability and endosomal sorting. Additionally, the regulation of Ski protein stability by GPCR pathways seems to be tightly associated with actin cytoskeleton dynamics.

**Acknowledgments**—We thank Drs. J. Vázquez-Prado and J. Chimal-Monroy for helpful discussions. We also thank Dr. J. A. García-Sáinz (IFC, UNAM) for the C9 and HepG2 cell lines and all members from Unidad de Imagenología, Bioterio, and Unidad de Cómputo at IFC, UNAM.

**REFERENCES**

1. Taub, R. (2004) Liver regeneration: from myth to mechanism. *Nat. Rev. Mol. Cell Biol.* 5, 836–847
2. Michalopoulos, G. K. (2007) Liver Regeneration. *J. Cell Physiol.* 213, 286–300
3. Godoy, P., Hewitt, N. J., Albrecht, U., Andersen, M. E., Ansari, N., Bhat-tacharya, S., Bode, J. G., Bolley, J., Borner, C., Böttger, J., Braeuning, A., Budinsky, R. A., Burkhardt, B., Cameron, N. R., Camussi, G., Cho, C. S., Choi, Y. J., Craig, Rowlands, J., Dahmen, U., Damm, G., Dirsch, O., Donato, M. T., Dong, J., Dooley, S., Drasdo, D., Eakins, R., Ferreira, K. S., Fonsato, V., Fraczek, J., Gebhardt, R., Gibson, A., Glanemann, M., Goldring, C. E., Gómez-Lechón, M. J., Groothuis, G. M., Gustavsson, L., Guyot, C., Hallif-dox, D., Hammad, S., Hayward, A., Häussinger, D., Hellerbrand, C., Hewitt, P., Hoehme, S., Holzhüter, H. G., Houston, J. B., Hrach, J., Ito, K., Jae-schke, H., Keitel, V., Kelm, J. M., Kevin Park, B., Kordes, C., Kullak-Ublick, G. A., LeCluyse, E. L., Lu, P., Luebe-Wheeler, J., Lutz, A., Maltman, D. I., Matz-Soja, M., McMullen, P., Merfort, I., Messner, S., Meyer, C., Mwinyi, J., Naisbitt, D. J., Nussler, A. K., Olinga, P., Pampaloni, F., Pi, J., Pluta, L., Przyborski, S. A., Ramachandran, A., Rogiers, V., Rowe, C., Schelcher, C., Schmich, K., Schwarz, M., Singh, B., Stelzer, E. H., Stieger, B., Stöber, R., Sugiyama, Y., Tetta, C., Thasler, W. E., Vanhaecke, T., Vinken, M., Weiss, T. S., Videra, A., Woods, C. G., Xu, J. J., Yarborough, K. M., and Hengstler, J. G. (2013) Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch. Toxicol.* 87, 1315–1530
4. Herrera, B., Alvarez, A. M., Beltrán, J., Valdés, F., Fabregat, I., and Fernández, M. (2004) Resistance to TGF-β-induced apoptosis in regeneration hepatocytes. J. Cell Physiol. 201, 385–392
5. Romero-Gallo, I., Sozmen, E. G., Chytli, A., Russell, W. E., Whitehead, R., Parks, W. T., Holdren, M. S., Her, M. F., Gautam, S., Magnuson, M., Moses, H. L., and Grady, W. M. (2005) Inactivation of TGF-β signaling in hepatocytes results in an increased proliferative response after partial hepatectomy. Oncogene 24, 3028–3041
6. Coulouarn, C., Factor, V. M., and Thorgeirsson, S. S. (2008) Transforming growth factor-β gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer. Hepatology 47, 2059–2067
7. Dooley, S., and ten Dijke, P. (2012) TGF-β in progression of liver disease. Cell Tissue Res. 347, 245–256
8. Mi, X., Lin, S., Yang, J., Chen, C., Chen, Y., Herzig, M. C., Washburn, K., Half, G. A., Walter, C. A., Sun, B., and Sun, L. Z. (2013) TGF-β signaling is often attenuated during hepatogenesis, but is retained for the malignancy of hepatocellular carcinoma cells. PLoS ONE 8, e63436
9. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994) Mechanism of activation of the TGF-β receptor. Nature 370, 341–347
10. Macías-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Tecalco-Cruz, A. C., Sosa-Garrocho, M., Vázquez-Victorio, G., Ortiz-dez, M. (2012) TGF-β signaling is negatively regulated by Ski in progression of liver disease. Cell 141, 290–303
11. Taelman, V. F., Dobrowolski, R., Plouhinec, J. L., Fuentealba, L. C., Vorwald, P. P., Gumper, I., Sabatini, D. D., and De Robertis E. M. (2010) Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. Cell 143, 1136–1148
12. Awasthi-Kalia, M., Schnetkamp, P. P., and Deans, J. P. (2001) Differential effects of filipin and methyl-β-cyclodextrin on B cell receptor signaling. Biochem. Biophys. Res. Commun. 287, 77–82
13. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. 1, 31–39
14. Delgado-Coello, B., Briones-Orta, M. A., Macías-Silva, M., and Mas-Oliva, J. (2010) Plasma membrane calcium ATPase isoform 3 expression in single cell types isolated from rat liver. Mol. Cell Biochem. 344, 117–124
15. Lee, H. H., Elia, N., Ghirlando, R., Lippincott-Schwartz, J., and Hurley, J. H. (2008) Midbody targeting of the ESCRT machinery by a noncanonical coiled coil in CEP55. Science 322, 576–580
16. Grewal, T., Heeren, J., Mewawala, D., Schnitgerhans, T., Wendt, D., Salomon, G., Enrich, C., Beisiegel, U., and Jäckle, S. (2000) Annexin V1 stimulates endocytosis and is involved in the trafficking of low density lipoprotein to the prelysosomal compartment. J. Biol. Chem. 275, 33806–33813
17. Slimane, T. A., Trugnan, G., Van Ijzendoorn, S. C., and Hoekstra, D. (2003) Raft-mediated trafficking of apical resident proteins occurs in both direct and transcytotic pathways in polarized hepatic cells: role of distinct lipid microdomains. Mol. Biol. Cell 14, 611–624
18. Sangi, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., and Sabin, D. M. (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141, 341–351
19. Soeta, C., Suzuki, M., Suzuki, S., Naito, K., Tachi, C., and Tojo, H. (2001) Peroxisome proliferators-activated receptor (PPARγ) mediates a Ski oncoprotein-induced shift from glycolysis to oxidative energy metabolism. J. Biol. Chem. 286, 40013–40024
20. Briones-Orta, M. A., Levy, L., Madsen, C. D., Das, D., Erker, Y., Sahai, E., and Hill, C. S. (2013) Arkadia regulates tumor metastasis by modulation of the TGF-β signaling pathway. Cancer Res. 73, 1800–1810
21. Krakowski, A. R., Laboureur, J., Mauriel, A., Bissell, M. J., and Loo, K. (2005) Cytoplasmic SnO2 in normal tissues and nonmalignant cells antagonizes TGF-β signaling by sequestering of the Smad proteins. Proc. Natl. Acad. Sci. 102, 12437–12442
22. Wang, L., and Boyer, J. L. (2004) The maintenance and generation of stem cells: regulating the division and differentiation of stem cells through the hippo pathway. J. Biol. Chem. 279, 28483–28490
23. Lee, H. H., Elia, N., Ghirlando, R., Lippincott-Schwartz, J., and Hurley, J. H. (2008) Midbody targeting of the ESCRT machinery by a noncanonical coiled coil in CEP55. Science 322, 576–580
24. Grewal, T., Heeren, J., Mewawala, D., Schnitgerhans, T., Wendt, D., Salomon, G., Enrich, C., Beisiegel, U., and Jäckle, S. (2000) Annexin V1 stimulates endocytosis and is involved in the trafficking of low density lipoprotein to the prelysosomal compartment. J. Biol. Chem. 275, 33806–33813
25. Slimane, T. A., Trugnan, G., Van Ijzendoorn, S. C., and Hoekstra, D. (2003) Raft-mediated trafficking of apical resident proteins occurs in both direct and transcytotic pathways in polarized hepatic cells: role of distinct lipid microdomains. Mol. Biol. Cell 14, 611–624
26. Sangi, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., and Sabin, D. M. (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141, 341–351
27. Soeta, C., Suzuki, M., Suzuki, S., Naito, K., Tachi, C., and Tojo, H. (2001) Peroxisome proliferators-activated receptor (PPARγ) mediates a Ski oncoprotein-induced shift from glycolysis to oxidative energy metabolism. J. Biol. Chem. 286, 40013–40024
28. Briones-Orta, M. A., Levy, L., Madsen, C. D., Das, D., Erker, Y., Sahai, E., and Hill, C. S. (2013) Arkadia regulates tumor metastasis by modulation of the TGF-β signaling pathway. Cancer Res. 73, 1800–1810
29. Krakowski, A. R., Laboureur, J., Mauriel, A., Bissell, M. J., and Loo, K. (2005) Cytoplasmic SnO2 in normal tissues and nonmalignant cells antagonizes TGF-β signaling by sequestering of the Smad proteins. Proc. Natl. Acad. Sci. 102, 12437–12442
30. Wang, L., and Boyer, J. L. (2004) The maintenance and generation of stem cells: regulating the division and differentiation of stem cells through the hippo pathway. J. Biol. Chem. 279, 28483–28490
43. Liu, X., Li, P., Liu, P., Xiong, R., Zhang, E., Chen, X., Gu, D., Zhao, Y., Wang, Z., and Zhou, Y. (2008) The essential role for c-Ski in mediating TGF-β1-induced bi-directional effects on skin fibroblast proliferation through a feedback loop. *Biochem. J.* **409**, 289–297

44. Kokura, K., Kim, H., Shinagawa, T., Khan, M. M., Nomura, T., and Ishii, S. (2003) The Ski-binding protein C184M negatively regulates tumor growth factor-β signaling by sequestering the smad proteins in the cytoplasm. *J. Biol. Chem.* **278**, 20133–20139

45. Prunier, C., Pessah, M., Ferrand, N., Seo, S. R., Howe, P., and Atfi, A. (2003) The oncoprotein Ski acts as an antagonist if transforming growth factor-β signaling by suppressing Smad2 phosphorylation. *J. Biol. Chem.* **278**, 26249–26257

46. Ferrand, N., Atfi, A., and Prunier, C. (2010) The oncoprotein c-ski functions as a direct antagonist of the transforming growth factor-β type 1 receptor. *Cancer Res.* **70**, 8457–8466

47. Jacob, C., Grabner, H., Atanasoski, S., and Suter, U. (2008) Expression and localization of Ski determine cell type-specific TGF-β signaling effects on the cell cycle. *J. Cell Biol.* **182**, 519–530

48. Marcelain, K., and Hayman, M. J. (2005) The Ski oncoprotein is up-regulated and localized at the centrosomes and mitotic spindle during mitosis. *Oncogene* **24**, 4321–4329