Recent data investigating the role of the Smad anchor for receptor activation (SARA) in TGF-β signaling have suggested that it has a crucial function in both aiding the recruitment of Smad to the TGF-β receptor, and ensuring appropriate subcellular localization of the activated receptor-bound complex. The FYVE domain in SARA directs its localization to early endosomal compartments where it can interact with both the TGF-β receptors and Smads. However, the necessity of endocytosis in the TGF-β response remains controversial. We sought to examine the role of internalization in TGF-β Smad signaling in human kidney mesangial cells. Using co-immunoprecipitation studies, we show that endogenous Smad2 interacts with SARA after TGF-β1 stimulation. Inhibition of clathrin-mediated internalization only slightly affects TGF-β1-stimulated association between SARA and Smad2, Smad2 phosphorylation, or Smad2 interaction with Smad4. However, endocytosis inhibition decreases TGF-β1-induced Smad2 nuclear translocation and thus abrogates Smad2-dependent transcriptional responses. The TGF-β1-stimulated association between SARA and Smad2 peaks at 30 min followed by separation of the complex components. However, under conditions of inhibited endocytosis, Smad2 remains bound to SARA for at least 6 h without a significant decline in associated levels. This lack of complex dissociation correlates with a lack of Smad2 nuclear accumulation and reduction of Smad2-dependent ARE-Luc reporter activity. Our data therefore suggest that endocytosis plays a critical role in TGF-β signaling in mesangial cells, and that internalization enhances the dissociation of Smad2 from the TGF-β receptor-SARA complex, allowing Smad2 to accumulate in the nucleus and modulate target gene transcription.

Transforming growth factor (TGF)-β is a ubiquitously expressed cytokine that has varied roles, affecting cellular processes including proliferation, differentiation, apoptosis, fibrosis, and tumorigenesis (1). This diversity is likely caused by differential involvement of TGF-β downstream signaling mediators, and therefore understanding precise interactions among TGF-β signaling components is of critical importance.

TGF-β signaling is initiated when ligand-bound TGF-β type II receptor (TβRII) binds to, and phosphorylates, the TGF-β type I receptor (TβRI) (2–4). This phosphorylation, in the TβRI cytoplasmic GS region, leads to its activation and its ability to activate the receptor-regulated Smads (R-Smads), Smad2, and Smad3, by C-terminal serine phosphorylation. Once phosphorylated, the R-Smads form a heteromultimeric complex with the common mediator (Co)-Smad (Smad4) and accumulate in the nucleus to regulate transcriptional responses (2–4). However, the seemingly simple initial delineation of the Smad signaling pathway has gained considerable complexity from the recent discovery of a number of additional factors that interact with the Smads and can regulate the signaling outcome. Among these interacting factors are those with anchoring or chaperone activity that aid in recruitment of R-Smads to the TGF-β receptor complex. Thus far, the best characterized of this type of Smad cofactor is SARA (Smad anchor for receptor activation). SARA contains both a Smad-binding domain (SBD), which has been shown to interact with Smad2 and Smad3 (5), and a C-terminal, TGF-β receptor complex-interacting region (6). SARA is therefore proposed to play a role in presenting R-Smads to the receptor for phosphorylation.

Additionally, SARA, by virtue of its FYVE domain binding to phosphatidylinositol 3-phosphate (PI3P), can interact with EEA1-positive, Rab 5-containing early endosomal compartments within the cell (6–10). Disruption of the SARA endocytic localization through either expression of a mutant SARA lacking the FYVE domain (SARA/D1-664) (6) or inhibition of PI3P generation by wortmannin treatment (7) causes a redistribution of SARA from punctate endocytic structures to the cytosol, and can prevent TGF-β-mediated transcriptional responses. However, while it has been clearly demonstrated that activated TβRII and TβRI are internalized into SARA-containing EEA1 endosomes, controversy remains as to the requirement for SARA binding and endocytosis in transducing the TGF-β signal. Although some reports indicate that TGF-β-mediated, Smad2-dependent transcription is reduced by inhibition of endocytosis (9–12), others find no effect of either inhibiting vesicle formation or interrupting SARA association on Smad2 (13) or Smad3 (14) responses at either the phosphorylation or transcriptional level. Additionally, a recent report investigating the role of endocytosis in the signaling mediated by the TGF-β family member, activin, through the Alk4 receptor, suggests that internalization is not required for Smad2 phosphorylation or transcriptional activity of the TGF-β1/activin-responsive p3TP-Lux transcriptional activation (15).
This inconsistency is likely due, at least in part, to the fact that almost every study of the Smad2-SARA interaction has been performed using transformed cell lines and overexpressed Smad, SARA, or TGF-β family receptors. Because high levels of SARA have been shown to lead to the formation of large non-vesicular aggregates (7) and to result in altered Smad localization (6), we sought to examine the specificity, localization and necessity of the interaction between endogenous SARA and Smad for TGF-β responsiveness.

We report here that endogenous SARA and Smad2 interact in response to TGF-β treatment. There is a requirement for endocytosis in Smad signaling in that inhibition of endocytic vesicle formation abrogates the TGF-β transcriptional response. Our data suggest that an internalization step, although dispensable for phosphorylation of Smad2 and its interaction with Smad4, may be required to allow dissociation of Smad2 from SARA followed by its nuclear accumulation and transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Active recombinant human TGF-β1 and PDGF-BB were purchased from R&D Systems (Minneapolis, MN). Goat anti-SARA (N-20), rabbit anti-SARA (H-300), goat anti-Smad2/3 (N-19), mouse anti-Smad2/3 (H-2), mouse anti-Smad4 (B-8), rabbit anti-Smad4 (H-552), goat anti-EEA1 (N-19), rabbit anti-HA (Y-11), rabbit anti-TgfRI (V-22), secondary anti-goat-IgG-horseradish peroxidase (HRP), anti-mouse IgG-HRP, chicken anti-goat rhodamine, -mouse FITC, and -rabbit FITC, were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit phospho-Smad2 (Ser465/467) was from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit secondary AlexaFluor594 and Alexa transferrin-594 were from Molecular Probes (Eugene, OR). Anti-rabbit IgG-HRP, luciferase, and -galactosidase activities.

**Potassium Depletion**—Cells were switched to 1% NBCS-containing media for 18–24 h prior to treatment. Media were then switched either to fresh 1% NBCS-containing media or to hypotonic media (50% Dulbecco’s modified Eagle’s medium/Ham’s F12 medium, supplemented with 20% heat-inactivated newborn calf serum (NBCS), glucose, penicillin/streptomycin, sodium pyruvate, Hepes buffer, and 8 μg/ml insulin (Invitrogen Life Technologies) as described previously (18), and were used between passages 5 and 8. Mouse mammary gland epithelial cells (NMuMG) were purchased from ATCC. They were passaged in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose, 10 μg/ml bovine serum albumin. PDGF stock is 20 μg/ml in water.

**Cell Culture**—Human mesangial cells were isolated by differential sieving of minced normal human renal cortex obtained from anonymous surgery or autopsy specimens. The cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium, supplemented with 20% heat-inactivated newborn calf serum (NBCS), glucose, penicillin/streptomycin, sodium pyruvate, Hepes buffer, and 8 μg/ml insulin (Invitrogen Life Technologies) as described previously (18), and were used between passages 5 and 8. Mouse mammary gland epithelial cells (NMuMG) were purchased from ATCC. They were passaged in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose, 10 μg/ml insulin, and 10% NBCS.

**Materials**—Active recombinant human TGF-β1 and PDGF-BB were purchased from R&D Systems (Minneapolis, MN). Goat anti-SARA (N-20), rabbit anti-SARA (H-300), goat anti-Smad2/3 (N-19), mouse anti-Smad2/3 (H-2), mouse anti-Smad4 (B-8), rabbit anti-Smad4 (H-552), goat anti-EEA1 (N-19), rabbit anti-HA (Y-11), rabbit anti-TgfRI (V-22), secondary anti-goat-IgG-horseradish peroxidase (HRP), anti-mouse IgG-HRP, chicken anti-goat rhodamine, -mouse FITC, and -rabbit FITC, were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit phospho-Smad2 (Ser465/467) was from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit secondary AlexaFluor594 and Alexa transferrin-594 were from Molecular Probes (Eugene, OR). Anti-rabbit IgG-HRP, luciferase, and -galactosidase activities were from Promega (Madison, WI). Anti-Smad2 and protein G-Sepharose were from Zymed Laboratories Inc. (South San Francisco, CA). Wild-type and K44A-mutant dynamin constructs were kindly provided by Dr. Sandra L. Schmid (16). ARE-Luc reporter construct and FAST-2, CMV-β-galactosidase, and either pcDNA3 empty vector control (EV), wild type dynamin (wt-Dyn), or K44A-mutant dynamin (K44A-Dyn). A, 3 h post-transfection, cells were treated with either vehicle (−) or 1 ng/ml TGF-β1 (+) for an additional 18–24 h prior to lysis and analysis for luciferase and β-galactosidase activities. B, 3 h post-transfection, cells were treated with either Me2SO (nystatin −) or 50 μg/ml nystatin (nystatin +) for 1 h prior to 18–24 h TGF-β1 treatment. Each luciferase measurement is corrected for β-galactosidase activities. Each bar represents triplicate measurements from a representative experiment. For each treatment set, the average TGF-β1 fold induction over control cells is indicated on top of each pair of bars.
Bradford protein assay (Bio-Rad) and prepared for immunoprecipitation or Western blotting. Alternatively, the cells were fractionated as described below.

**Cell Fractionation**—Cells were scraped into a detergent-free buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol) containing protease and phosphatase inhibitors. The cells were then disrupted by 15 strokes of a Dounce homogenizer, and centrifuged at 1,000 g for 5 min to remove nuclei. The supernatant was removed and further centrifuged at 100,000 g for 45 min. After centrifugation, the supernatant was saved as the cytosolic fraction. The pellet was resuspended in detergent-free buffer supplemented with 1% (final concentration) Triton X-100, sheared with a syringe, incubated for 30 min on ice, and centrifuged to remove insoluble material. This supernatant represents the soluble membrane fraction. The nuclei were resuspended in RIPA buffer, syringe-sheared, incubated for 30 min on ice, and centrifuged to remove insoluble material. This fraction represents the nuclear fraction. Protein concentration for each fraction was determined by Bradford protein assay.

**Immunoprecipitation and Western Blot**—300–600 μg of protein from total cell lysates were diluted in the same final volume of RIPA buffer containing protease and phosphatase inhibitors. Anti-SARA (N-20) or anti-Smad4 (H-552) antibody was added at a final concentration of 2 μg. Immunoprecipitations and Western blotting were performed as described previously (19).

**Transfection**—Cells were plated, in triplicate wells per condition, at 1.4 × 10⁵ cells per well in 6-well plates. The following day, medium was changed to 1 ml per well 1% NBCS-containing medium and transfected as described previously (20) using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). Cells were treated for 18–24 h with control vehicle or TGF-β1 then lysed with reporter lysis buffer.
and assayed for luciferase activity and also for β-galactosidase activity to correct for transfection efficiency.

**Immunocytochemistry—** Cells were grown to 70% confluence on gelatin-coated glass coverslips in 6-well dishes. For determination of Smad nuclear translocation, cells were transfected with wild type- or K44A-dynamin using FuGENE 6 reagent (as described above). To confirm dynamin effectiveness cells were co-transfected with HA-tagged TβRI along with either wild type or K44A-dynamin. To determine SARA subcellular localization and to confirm inhibition of endocytosis, cells were serum deprived and potassium depleted as described above. Cells were then either treated with TGF-β1 for various time points, or labeled for 30 min with Alexa transferrin 594 (50 µg/ml). Coverslips were prepared for immunocytochemistry by fixing in 3.7% formaldehyde for 15 min, followed by permeabilization with ice-cold 0.5% Triton X-100 for 5 min, and blocking in 3% bovine serum albumin-phosphate-buffered saline for 20 min. Antibody solutions were layered onto coverslips at a final concentration of 2 µg/ml for Smad (H-2) or 4 µg/ml for HA (Y-11), SARA (H-300), EEA1 (N-19), or TβRI (V-22) and incubated in a humidified chamber for 2 h at 37 °C, followed by three 5-min washes in phosphate-buffered saline. The coverslips were then incubated in secondary antibody for 30–60 min with 2.5 µg/ml either chicken anti-goat-rhodamine, chicken anti-mouse-FITC, chicken anti-rabbit-FITC, or AlexaFluor594 goat anti-rabbit secondary antibodies. To rule out background fluorescence, negative controls were incubated with secondary antibodies only. After three 5-min washes, cover slips were mounted onto glass slides, and images were scanned using a Zeiss LSM510 laser scanning confocal microscope.

**RESULTS**

**TGF-β1 Stimulates Smad2 and Smad3 Association with SARA—** There are relatively few examples of SARA interaction with R-Smads (5, 6, 9, 10, 21, 22), only one of which showed interaction of endogenous SARA with R-Smads (6), and none in human mesangial cells. We therefore first sought to examine the Smad2-SARA interaction in serum-deprived mesangial cells treated with 1 ng/ml (40 pM) TGF-β1 for various time periods. After immunoprecipitation with anti-SARA antibody, the immunoprecipitated complexes were analyzed by Western blot with an antibody that recognizes both Smad2 and Smad3. As shown in Fig. 1A, there is little Smad2 or Smad3 detected in association with SARA in the absence of treatment. However, with 30 min of TGF-β1 stimulation, there is a marked increase in co-immunoprecipitated Smad2. This interaction is apparent within 5 min (data not shown), peaks at 30 min, and continues to be somewhat increased over basal levels 24 h after treatment. Increased association is not caused by changes in total cellular SARA (1A) or Smad2 (1B) expression levels, which do not vary throughout 24 h of treatment with TGF-β1. Interestingly, while Smad3 is strongly expressed in mesangial cells (Fig. 1B, lower panel), there is only a slight band revealed for Smad3-associated SARA (Fig. 1A).

TGF-β1 activates Smad2 through receptor-mediated C-terminal phosphorylation, and the pattern of TGF-β1-induced Smad2-SARA association parallels that of Smad2 phosphorylation as detected using an antibody specific for phosphoryserine 465/467 (Fig. 1B). PDGF, which does not induce the phosphorylation of Smad2 (Fig. 1B and Ref. 20), does not stimulate its association with SARA (Fig. 1A). The TGF-β1-induced association between SARA and Smad2 does not appear to be restricted to mesangial cells, as we detect an increased association after 30 min TGF-β1 treatment of an unrelated mouse mammary gland epithelial cell line (NMuMg) (Fig. 1C).

**Clathrin-mediated Endocytosis Is Required for Smad2-dependent Transcriptional Activity—** Because SARA has been shown to associate with EEA1-positive early endosomal compartments (7), and its mislocalization has been suggested to disrupt Smad2 signaling, we sought to determine whether human mesangial cells require endocytosis to signal through Smad2 after TGF-β1 stimulation. We performed transient transfection of mesangial cells with the Smad2-specific ARE-Luc reporter construct along with a FAST-2 expression construct and either wild-type or K44A mutant dynamin. This K44A mutant of dynamin does not
allow exocytosis of vesicles from the cell membrane and thereby blocks clathrin-mediated endocytosis (16). Whereas wild-type dynamin does not affect the TGF-β1 induction of ARE-Luc, this activation was significantly inhibited in the presence of the dynamin mutant (Fig. 2A), confirming a role for internalization in Smad2 transcriptional activity. Because blocking the function of dynamin would also block caveolar excision from the membrane (23), and because lipid raft/caveolar internalization may mediate the ubiquitin-dependent degradation of the TβR (24), we sought to rule out the possibility of caveolar compartmentalization by using nystatin to disrupt cellular cholesterol and thereby interfere with caveolar formation (25). Nystatin treatment has only a minimal inhibitory effect in comparison to that of the K44A-dynamin mutant (Fig. 2B). Therefore the internalization requirement in TGF-β1-mediated Smad2 signaling is likely specific to a non-caveolar, clathrin-mediated endocytic event.

Inhibition of Endocytosis Abrogates TGF-β1-stimulated Nuclear Translocation of Smad2—Having established that endocytosis is required for Smad2 signaling, we sought to further investigate the specific role of internalization after TGF-β receptor stimulation. To determine whether endocytosis is required for nuclear Smad2 accumulation, we performed cell fractionation experiments under conditions of cellular potassium depletion to inhibit clathrin-coated pit formation (26). In agreement with other reports (9, 11), under conditions of inhibited endocytosis, the level of nuclear Smad2 accumulation in response to TGF-β1 is decreased compared with that of potassium control conditions (Fig. 3A). This is confirmed by immunocytochemical analysis for intracellular localization of Smad2. Whereas there is a striking increase in detection of Smad in the nucleus of TGF-β1 treated cells under potassium depletion conditions (Fig. 3B compare panels b to a), potassium depletion completely abrogates this response (Fig. 3B, panels c and d). To ensure that the depletion of intracellular potassium had, in fact, reduced vesicle formation and thus endosomal association of SARA, we performed immunocytochemical analysis to detect SARA localization under potassium-depleted versus potassium-containing conditions. In control conditions, SARA shows the distinct punctuate staining pattern associated with endosomal localization (Fig. 4A, left panel). As expected (6–9), these punctuate structures also contain the early endosomal marker EEA1 (27). However, in potassium-depleted cells SARA immunostaining becomes faint and diffuse throughout the cell and no longer shows extensive co-localization with EEA1 (Fig. 4A, right panel). To confirm that the levels of SARA expression were not altered by potassium depletion, we performed Western blot analysis of SARA from cells treated with TGF-β1 for various duration in either potassium-control or potassium-depleted conditions. As shown in Fig. 4B, throughout 6 h of treatment, there are no significant changes in the levels of SARA total protein expression. To further confirm that potassium depletion is inhibiting endosomal internalization, we treated the cells with fluorescently tagged transferrin (AF-tf594). Transferrin is known to internalize via clathrin-mediated endocytosis (28). As shown in Fig. 4C, top panel, cells incubated with AF-tf594 for 30 min show a punctuate fluorescence. In contrast, in potassium-depleted conditions (4C, bottom panel) there is a lack of internalized AF-tf594. These results suggest that our potassium depletion conditions are effectively blocking endocytic internalization. However, there remains the possibility that the relatively nonspecific conditions of potassium depletion may be introducing alternate effects on the cells that disrupt the ability of Smad2 to translocate to the nucleus. Therefore, we used the K44A-dynamin mutant as an alternative means to block endocytic internalization. Cells were transfected with either HA-tagged wt-dynamin or K44A-dynamin and stained for Smad and HA. As shown in Fig. 5A, K44A-dynamin decreases TGF-β1-induced nuclear accumulation of Smad2, whereas wild-type dynamin-expressing cells respond with nuclear accumulation similar to untransfected neighboring cells. This inhibitory effect of K44A-dynamin on translocation is seen in every transfected cell at both an early time point (Fig. 5A, panels a–d), corresponding to peak Smad2 phosphorylation (Fig. 1B) as well as at a longer duration of treatment of 6 h (Fig. 5A, e–h). This confirms the effect seen with potassium depletion in that Smad nuclear translo-
Endocytosis Requirement in TGF-β1-mediated Signaling

**FIG. 6. Smad2 associates with SARA under conditions of inhibited endocytosis.** Cells were either serum-starved only (C) or serum-starved and then incubated in potassium containing (K(+)) or potassium-depleted (K(−)) conditions, followed by 30 min of treatment with either vehicle (−) or 1 ng/ml TGF-β1 (+). Cells were immunoprecipitated with SARA and then blotted first for Smad2/3 (top) followed by blotting for SARA (bottom). Representative blots from one of three separate experiments are shown.

**FIG. 7. Smad2 can be phosphorylated when endocytosis is inhibited.** Cells were treated as described in Fig. 4 and either immunoprecipitated with Smad2/3 (N-19) and developed for phosphoserine (top) followed by re-probing for Smad2/3 (middle), or whole cell lysates were blotted for C-terminal Smad2 phosphorylation (bottom).

**FIG. 8. Phosphorylated Smad2 can co-precipitate with SARA.** Smad2 was immunoprecipitated from cells treated with TGF-β1 for 30 min under control or potassium-deprived conditions. The immunoprecipitate was probed first for C-terminal phospho-Smad2 (top panel), followed by stripping and re-probing for Smad2 (middle), and then for SARA (bottom).

Phosphorylation of Smad2 Does Not Require Internalization and Can Occur While Smad2 Is in Association with SARA—Interfering with endocytosis via potassium depletion could inhibit Smad2 signaling because mislocalization of SARA may not allow the SARA-Smad2 complex to be efficiently formed. To examine this possibility, we performed co-immunoprecipitations to determine the effect of potassium depletion on the levels of TGF-β1-stimulated SARA-Smad2 association. As demonstrated in Fig. 6, the level of association is only slightly reduced by inhibition of internalization. Therefore the complex forms in response to TGF-β1 even though SARA localization is altered (Fig. 4A).

As one of the potential functions of the endosome may be to act as an intracellular compartment to enhance the duration and level of membrane-associated signaling complexes, the requirement for endocytosis may be to enhance Smad2 receptor-mediated phosphorylation (9, 11). To investigate this possibility, we analyzed Smad2 phosphorylation under control or potassium-depleted conditions. Total Smad2 phosphorylation was determined by immunoprecipitation of Smad2 followed by phosphoserine Western blotting. Additionally we examined the TGF-β receptor-mediated C-terminal phosphorylation using an antibody specific for Smad2-phosphoserine 465/467. Although there may be a slight reduction in overall phosphorylated Smad2 levels, we found that neither Smad2 total serine phosphorylation nor Smad2 C-terminal phosphorylation were considerably reduced when endocytosis was inhibited (Fig. 7). If both the TGF-β1-induced Smad2-SARA complex formation and Smad2 phosphorylation are only minimally affected by potassium depletion, it would imply that the inhibition of endocytosis still allows for a membrane-associated complex of SARA and phosphorylated Smad2. To examine this possibility, SARA was immunoprecipitated from cells under control or potassium-depleted conditions, and the complexes were analyzed by immunoblotting with anti-phospho-Smad2. Phosphorylated Smad2 co-immunoprecipitates with SARA after treatment with TGF-β1 (Fig. 8). Moreover, phosphorylation of Smad2 in association with SARA is not significantly affected by inhibition of endocytosis. Therefore, our data suggest that endocytosis is not critical for Smad2/SARA complex formation or for Smad2 phosphorylation in response to TGF-β1.
Smad2 supernatant does not show association with SARA (Fig. 9C, middle panels). Therefore, it appears that the Smad4 that is not associated with Smad2 is also not associated with SARA. Further, SARA immunoprecipitated from the lysates after Smad2 was removed no longer shows association with Smad4 (Fig. 9C, right panels). Thus it appears that endogenous Smad2 forms a complex with both SARA and Smad4, and that these complexes are not mutually exclusive.

**Smad2 Does Not Separate from SARA under Conditions of Inhibited Endocytosis**—The fact that Smad2 can associate with SARA, can be phosphorylated, and can at least be in proximity with Smad4 without requiring internalization suggests that the requirement for endocytosis lies somewhere in between these initial events and Smad2 translocation to the nucleus. This led us to speculate that the potential role for internalization in the Smad2 signaling pathway is to allow dissociation of the complex components, thereby allowing separation of the Smad2-Smad4 complex from the TβR-associated SARA as required for Smad2 nuclear accumulation. As shown in Fig. 10A, co-immunoprecipitation to examine the Smad2-SARA complex over longer durations of TGF-β1 treatment, indicate that, in control conditions, Smad2 dissociates from SARA between 0.5 and 4 h of treatment (left panel). In contrast, when endocytosis is inhibited, Smad2 is induced by TGF-β1 to associate with SARA, and remains bound for at least 6 h (right panel). This trend, illustrated in Fig. 10B, demonstrates that without endocytosis Smad2 remains bound to SARA, and suggests that the separation of this complex occurs subsequent to internalization. Because potassium depletion for 6 h may be a relatively harsh condition, it is possible that the lack of SARA-Smad dissociation is because of a generally unhealthy state of the cells. Therefore to rule out a nonspecific toxicity effect, we transfected the cells with a CMV-β-galactosidase reporter construct and analyzed the β-galactosidase activity of cells under potassium control or depletion conditions. As shown in Fig. 10C, cells that are incubated under potassium-depleted conditions do not vary in β-galactosidase activity compared with control cells, nor does TGF-β1 treatment affect the transcriptional response. Additionally, if cells are incubated under potassium-depleted and control conditions for 1 h and then switched back to 1% NBCS-containing media for another 18 h, they have β-galactosidase activities identical to cells that have not undergone any media change (Fig. 10C). This suggests that depletion of intracellular potassium is not causing the cells to become apoptotic, and that the lack of Smad2 responsiveness is due specifically to the inhibition of endocytosis. Therefore our data suggest that the inhibition of internalization alters the dissociation of the Smad2-SARA complex, which is required for nuclear accumulation of Smad2 (Figs. 3 and 5) and thus for Smad2-mediated signaling (Fig. 2).

**DISCUSSION**

In this report we show that endogenous SARA and Smad2 associate in response to TGF-β1 stimulation in human mesangial cell. Interestingly, under conditions of inhibited endocytic vesicle formation, we see both phosphorylation of Smad2 and its association with Smad4, but not Smad2 nuclear translocation or transcriptional activity. This suggests that neither phosphorylation of endogenous Smad2 nor its hemezimerization with Smad4 is sufficient to fully propagate TGF-β1-stimulated Smad signaling. Our data confirm a critical role for endocytosis in TGF-β signaling through Smad2.

Based on our data and the data of others, SARA and Smad would associate with the TβR at the plasma membrane where Smad2 is phosphorylated which allows for its interaction with Smad4. In our proposed model, endocytic internalization would
allow the Smad2-Smad4 complexes to separate from SARA and enter the nucleus to modulate Smad2-dependent transcription. The robust TGF-β1-stimulated interaction between SARA and Smad2 in our studies appears to differ from those of the initial report characterizing SARA (6) in which it was suggested that SARA preferentially interacts with unphosphorylated Smads. In that report, Tsukazaki et al. (6) proposed a TGF-β-induced dissociation of SARA and Smad due to Smad phosphorylation. More recent work (22) supports this model, showing that phosphorylated Smad2 has a reduced affinity for SARA. While we detect the phosphorylated form of Smad2 in association with endogenous SARA, we also demonstrate a separation of Smad2 from SARA after the peak time of Smad2 phosphorylation. However, one novel finding of the present study is that this separation only occurs under conditions, which allow endocytosis. Therefore, our results do not contradict the idea that Smad2 phosphorylation induces dissociation from SARA, but rather suggest that this dissociation is not immediate upon stimulation of the TGF-β receptor and requires an intermediate internalization step.

The mechanism by which subsequent internalization of the membrane-localized receptor complex propagates the TGF-β signal remains unclear. While receptor-mediated endocytosis can be a means to blunt signals through the direction of receptors away from surface ligand interactions, endocytic vesicles may perform other functions such as the creation of specialized signaling compartments to enhance signal intensity (11). A further role for endocytosis was recently described by Di Guglielmo et al. (24) who showed that internalization of the TGF-β receptor into EEA1 endosomal vesicles led to SARA-associated enhanced signal activation, but that internalization to caveolar compartments led to Smurf2- and Smad7-associated receptor degradation. If either route of internalization was blocked, there was an increase in distribution to the other vesicular compartment, which suggests that one function of endocytosis may be to enhance signal propagation by sequestering the receptor from its degradation-associated caveolar pathway. However, a recent report by Mitchell et al. (27) suggests that clathrin-mediated endocytosis is the crucial event in both TgfR, rab11-dependent, recycling, and degradation, with raft/caveolae-dependent endocytosis being of minor importance.

Evidence suggests that SARA functions as a cytosolic retention factor (29). SARA binds to the “hydrophobic corridor” in the MH2 domain of Smad2. Smad2 also contains a nucleoporin-interacting site that allows Smad2 to interact directly with nucleoporins CAN/Nup214 and Nup153, and this site overlaps the Smad2 SARA binding site. Thus the nuclear transport of Smad2 can be regulated through competitive binding, where TGF-β-induced Smad2 phosphorylation reduces its affinity for SARA and allows interaction with the nuclear pore complex. The proposed competitive interaction between Smad2-SARA and Smad2-CAN/Nup214 involves di-
rect interaction of Smad2 with the nucleoporins. Because we do not observe Smad2 separation from SARA without internalization, a further possible role of the endocytic vesicle in TGF-β1 signaling might be to aid in the transport of Smad2 to the nucleus. This type of requirement for endocytic vesicular transport in nucleocytoplasmic flux has been described previously for the transcription factor Stat3 in response to EGF stimulation (30).

Another unique finding of our studies is the presence of Smad4 in the SARA immunoprecipitates upon TGF-β1 treatment. The difference in our data from results described previously showing that Smad2 and Smad3 generate different downstream signals upon TGF-β1 stimulation, it would be interesting to examine potential differences in the requirement for SARA association or endocytosis in Smad3 signaling. One report of SARA-Smad3 association shows SARA preferentially binds monomeric Smad3 with phosphorylation-induced dissociation and activity followed by Smad3 trimerization leading to Ski-mediated down-regulation of Smad3 (31). Additionally, a recent article by Lin et al. (10) examined the role of the promyelocytic leukemia protein (pml) as a potential bringing protein between Smad2/3 and SARA. This report showed that add-back of wild-type pml, but not a Smad3-binding deficient mutant pml, restored TGF-β1 responsiveness in pml-null fibroblasts. In contrast, another group has suggested that TGF-β-induced Smad3 signaling may not require its association with SARA (14).

In summary, we have shown that endogenous Smad2 associates with SARA following TGF-β1 treatment in human mesangial cells. Endocytosis, while not critical for Smad2 phosphorylation or for Smad4 association with the receptor-bound Smad2-SARA complex, is required downstream in order to facilitate the separation of the Smad complex from SARA and allow its nuclear accumulation and transcriptional activity.

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