Caveolin-1 Regulates Transforming Growth Factor (TGF)-β/SMAD Signaling through an Interaction with the TGF-β Type I Receptor*

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The transforming growth factor-β (TGF-β) signaling proceeds from the cell membrane to the nucleus through the cooperation of the type I and II serine/threonine kinase receptors and their downstream SMAD effectors. Although various regulatory proteins affecting TGF-β-mediated events have been described, relatively little is known about receptor interactions at the level of the plasma membrane. Caveolae are cholesterol-rich membrane microdomains that, along with their marker protein caveolin-1 (Cav-1), have been implicated in the compartmentalization and regulation of certain signaling events. Here, we demonstrate that specific components of the TGF-β cascade are associated with caveolin-1 in caveolae and that Cav-1 interacts with the Type I TGF-β receptor. Additionally, Cav-1 is able to suppress TGF-β-mediated phosphorylation of Smad-2 and subsequent downstream events. We localize the Type I TGF-β receptor interaction to the scaffolding domain of Cav-1 and show that it occurs in a physiologically relevant time frame, acting to rapidly dampen signaling initiated by the TGF-β receptor complex.

The transforming growth factor (TGF)β superfamily consists of a disparate group of polypeptide cytokines that regulate a plethora of biological processes. Members of this superfamily include the bone morphogenic proteins, the activins, and the TGF-βs, each with distinct roles in cellular differentiation, proliferation, apoptosis, and migration/motility, among others (see Refs. 1 and 2 for reviews). The prototype member of the group, TGF-β1, whose mechanism of action has been a focus of research for the past decade, classically initiates signaling at the plasma membrane by binding to a heterotetrameric complex consisting of two transmembrane serine/threonine kinases, known as Type I and Type II TGF-β receptors (TβR-I and TβR-II) (3, 4). The activation of this membrane complex occurs via the ligand-dependent phosphorylation of TβR-I by TβR-II (5, 6). In turn, TβR-I can act to phosphorylate its immediate downstream effectors, Smad-2 and Smad-3, members of the SMAD family of intracellular signaling molecules (7, 8). This phosphorylation induces a conformational change in Smad-2 and -3, thereby facilitating their heteromerization with another member of the family, Smad-4 (9). The SMAD complex then translocates to the nucleus, where it acts to regulate the transcription of various target genes (7, 9).

As in numerous other cellular processes, TGF-β-mediated signaling is subject to regulation and inhibition by a variety of mechanisms. The p42/44 MAP kinase pathway, interferon-γ STAT cascade, NF-κB, Smo/Ski oncoproteins, among others have been shown to act as negative regulators of TGF-β signaling primarily by interacting with, modifying, or regulating the SMAD proteins (10–15). Various groups have also observed regulation at the receptor level by the showing the interaction of certain intracellular proteins with the TβR-I. Of specific interest are the inhibitory proteins, Smad-6 and Smad-7, a functionally divergent subset of the SMAD protein family that can inhibit TGF-β signaling by directly interacting with the TβR-I (16–18). Other proteins such as FKBP12 have also been shown to interact with and negatively regulate TβR-I (19).

However, surprisingly little is known about the interactions of the TGF-β receptor complex with other membrane proteins. Although the compartmentalization of signaling molecules via scaffolding proteins is an emerging theme in signal transduction biology (20), the behavior and regulation of the TGF-β receptors at the membrane has thus far been a matter of speculation. Previous reports addressing the heterotetramerization of the TβR-I/II complex have shown a characteristic punctate membrane distribution (21, 22), and recently, the cloning of a SMAD anchor protein, SARA, also revealed a punctate distribution for both SARA and the TGF-β receptor complex (23).

Caveolae are ~50–100 nm vesicular invaginations of the plasma membrane and are thought to form as a result of a local accumulation of cholesterol, glycosphingolipids, and caveolin-1 (24–26). Caveolin-1, a 21–24-kDa integral membrane protein, is a principal component of caveolae membranes in vivo (27–29). Structurally, a hallmark of caveolin-1 and by extension, caveolae, is a characteristically punctate staining pattern at the plasma membrane (30). Although caveolae function in vesicular and cholesterol trafficking (31, 32), they have also been
implicated in signal transduction (33, 34). Biochemical and morphological experiments have shown that a variety of signaling molecules are concentrated within these plasma membrane microdomains, such as Src family tyrosine kinases, Ha-Ras, endothelial nitric oxide synthase, and heterotrimeric G proteins (35–40). Furthermore, several independent lines of evidence suggest that caveolin-1 plays a regulatory role in signaling, i.e., by functioning as a direct inhibitor of a variety of plasma-membrane-initiated signaling cascades (reviewed in Ref. 41).

Given the punctate plasmalemmal distribution of Cav-1 and its emerging role in various signaling cascades, we set out to investigate its relationship with the TGF-β receptor complex and substrate SMADs. Here, we demonstrate that Cav-1 and TβR-I are highly colocalized at the membrane, that TβR-I, TβR-II, and Smad-2 (but not Smad-4) cofractionate with caveolin-1 in caveolea enriched microdomains, and that caveolin-1 directly interacts with TβR-I in both heterologous and endogenous settings. We show that this interaction has functional consequences because Cav-1 is able to suppress TGF-β-mediated transcriptional activation. In addition, we demonstrate that Cav-1 diminishes the phosphorylation of Smad-2, disrupts its interaction with Smad-4, and prevents Smad-2 translocation to the nucleus in the ligand-activated state. This inhibition is mediated by an interaction between TβR-I and the scaffolding domain of Cav-1 and occurs in a physiologically relevant time frame. We show a rapid increase in the TβR-I/Cav-1 interaction upon ligand binding, and, by using an antisense strategy, we demonstrate that targeted down-regulation of caveolin-1 is sufficient to hyperactivate TGF-β signaling.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—The caveolin-1 mAb 2297 (used for immunoblotting) and mAb 2234 (used for immunofluorescence and immunoprecipitation) (30) were the gifts of Roberto Campos-Gonzalez (Transduction Laboratories, Inc.). The phospho-specific anti-SMAD2 rabbit pAb was a gift of Peter ten Dijke (Ludwig Institute for Cancer Research). The anti-FLAG tag mAb (Sigma), the anti-SMAD2 mAb (Transduction Laboratories, Inc.), and the anti-HA as well as the anti-TGF-β type I receptor rabbit pAb (Santa Cruz Biotechnology) were obtained commercially. Cell culture reagents were from Life Technologies, Inc. Recombinant human TGF-β1 was obtained commercially. NIH-3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% donor calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO₂.

Immunofluorescence—Unless specified, transient transfections were performed as previously described (44, 50). 293T cells plated on a 150-mm diameter plate were transfected with the appropriate plasmid(s). 36 h post-transfection, the cells were washed twice in PBS, scraped into 10 ml of PBS, spun down, and double-labeled with a 1:400 dilution of anti-caveolin-1 mAb 2234 and a 1:200 dilution of anti-HA rabbit pAb for 60 min. After rinsing three times with PBS, secondary antibodies (7.5 μg/ml) [lissamine-rhodamine-conjugated goat anti-rabbit and fluorescein isothiocyanate-conjugated goat anti-mouse antibodies] were added for a period of 60 min. Cells were washed twice with PBS and slides were mounted in antifade reagent (Molecular Probes). A Bio-Rad MR600 confocal fluorescence microscope was used for visualization of bound secondary antibodies. For assessment of SMAD2 translocation to the nucleus, a similar procedure was followed with the following differences: NIH-3T3 cells were transfected with caveolin-1, serum-starved for 20 h, and treated with TGF-β1 (4 ng/ml) for 45 min. A 1:400 dilution of anti-caveolin-1 mAb 2234 and a 1:200 dilution of anti-SMAD2 pAb were used for the caveolin-1 and endogenous SMAD2 staining, respectively.

Purification of Caveolae-enriched Membrane Fractions—Caveolea-enriched membrane fractions were purified essentially as previously described (35). For coimmunoprecipitation studies, NIH-3T3 cells (transfected with caveolin-1 and either TβR-I w.t. or TβR-I (T204D)) were fixed for 30 min in PBS containing 2% paraformaldehyde, rinsed with PBS, and quenched with 50 mM NH₄Cl for 10 min. The cells were then homogenized in prechilled buffer (PBS, 0.2% Nonidet P-40, 0.5% Tween-20, 0.5% Triton X-100) for 10 min on ice with a Dounce. After pelleting with a 1:400 dilution of anti-caveolin-1 mAb 2234 and a 1:200 dilution of anti-HA rabbit pAb for 60 min. After rinsing three times with PBS, secondary antibodies (7.5 μg/ml) [lissamine-rhodamine-conjugated goat anti-rabbit and fluorescein isothiocyanate-conjugated goat anti-mouse antibodies] were added for a period of 60 min. Cells were washed twice with PBS and slides were mounted in antifade reagent (Molecular Probes). A Bio-Rad MR600 confocal fluorescence microscope was used for visualization of bound secondary antibodies. For assessment of SMAD2 translocation to the nucleus, a similar procedure was followed with the following differences: NIH-3T3 cells were transfected with caveolin-1, serum-starved for 20 h, and treated with TGF-β1 (4 ng/ml) for 45 min. A 1:400 dilution of anti-caveolin-1 mAb 2234 and a 1:200 dilution of anti-SMAD2 pAb were used for the caveolin-1 and endogenous SMAD2 staining, respectively.

Immuno precipitation of Caveolin-1 with TGF-βRI—For coimmunoprecipitation of heterologously expressed proteins, 293T cells plated on 100-mm dishes were transfected with the appropriate plasmids. 36 h post-transfection, cells were lysed in lysis buffer (see “Immunoblotting”), clarified by centrifugation at 15,000 × g for 15 min, and precleared by incubation with protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. Supernatants were transferred to separate 1.5-ml microcentrifuge tubes containing anti-HA pAb or appropriate control antibodies (beads alone, preimmune serum) prebound to protein-A Sepharose. After incubation by rotation overnight at 4 °C, immunoprecipitates were washed three times with lysis buffer and subjected to immunoblot analysis with the anti-caveolin-1 2297 mAb probe. For coimmunoprecipitation of endogenous proteins, NIH-3T3 cells were plated on 100-mm dishes and lysed at confluency. The procedure was as described above with the anti-caveolin-1 2297 mAb, anti-TGF-β1 pAb, or preimmune rabbit pAbs as the preclearing materials.

Coimmunoprecipitation of SMAD2/SMAD4 Complexes—Sets of 100-mm NIH-3T3 dishes were transfected with the appropriate plasmids. 36 h post-transfection, cells were lysed in RIPA/Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 0.1 μM okadi acid, and protease inhibitors), sonicated briefly to disrupt nuclei, and subjected to immunoblot analysis with anti-FLAG M2 mAb. For coimmunoprecipitation of endogenous proteins, NIH-3T3 cells were plated on 100-mm dishes and lysed at confluency. The procedure was as described above with the anti-caveolin-1 2297 mAb, anti-TGF-β1 pAb, or preimmune rabbit pAbs as the preclearing materials.

In Vivo Phosphorylation Experiments—NIH-3T3 cells plated on 100-mm dishes were transfected with the appropriate plasmids. 36 h post-transfection, cells were washed twice in Dulbeco’s modified Eagle’s medium lacking phosphate and incubated for 3 h in Dulbeco’s modified Eagle’s medium lacking phosphate supplemented with 1 μCi [³²P]orthophosphate/100-mm dish. Indicated plates were then addition ally treated with TGF-β1 (4 ng/ml) for 45 min. Cells were washed in ice-cold PBS and subjected to lysis in RIPA/Nonidet P-40 buffer and immunoprecipitation with anti-FLAG M2 antibody as outlined above. SDS-PAGE and subsequent autoradiography visualized the phosphorylated SMAD2 proteins.

Intra-caveolar SMAD2 Phosphorylation—293T cells plated on a 150-mm diameter plates were transfected with TβR-I (T204D), SMAD2, and either caveolin-1 or empty vector. Caveolea-enriched membrane fractions were purified as outlined above. Dilution with 1× MBS and subsequent centrifugation was used to concentrate the caveolar membranes into a 50-μl volume. Approximately 5 μg of these membranes...
was mixed 1:1 with 10 μl of 2× kinase reaction buffer (40 mM Hepes, pH 7.4, 10 mM MgCl₂, and 2 mM MnCl₂), and the reaction was initiated by the addition of 4 mM ATP (Sigma) for 15 min. Extent of phosphorylation was determined by immunoblot analysis with anti-phospho-SMAD2 pAb.

**Cytoplasmic/Nuclear Fractionation**—NIH-3T3 cells plated on 100-mm dishes were transfected with the appropriate plasmids. 36 h post-transfection, cells were washed once with PBS, scraped in 1 ml of Hypotonic lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors), passed 10 times through a loose fitting Dounce homogenizer, and centrifuged at 3000 rpm for 3 min. The supernatant (i.e. cytosolic fraction) was saved, whereas the pellet (nuclear fraction) was washed twice with hypotonic lysis buffer before resuspending in the same buffer and sonicating to disrupt nuclear membranes. Protein concentrations from both fractions were quantified using the BCA reagent, and equal amounts of protein were subjected to immunoblot analysis with the anti-FLAG mAb as the probe.

**Luciferase Reporter Assays for TGF-β Activity**—The A3-lux reporter construct was a gift of Malcolm Whitman (Harvard Medical School) (51). For assays involving TGF-β1 stimulation, each plate was transfected with the reporter of interest (A3-lux/FR-1 or A3-lux/TGF-RI/FR-1) at 36 h post-transfection. Results were expressed as a ratio of luciferase activity to β-galactosidase activity. Each experimental value represents the average of three separate transfections performed in parallel; error bars represent the observed S.D. All experiments were performed at least three times independently and yielded virtually identical results.

**TGF-β RI In Vitro Kinase Assay**—The kinase substrate used in this assay, the GST-SMAD2 fusion protein, was a gift of Mark de Caestecker (National Cancer Institute) (54). The purification of GST-SMAD2 was as described previously (30, 40, 55). Briefly, after expression in *Escherichia coli* (BL21 strain; Novagen, Inc.), GST-SMAD2 was affinity purified on glutathione-agarose beads, using the detergent sarcosyl for elution. In vitro kinase reactions were performed as described previously (55, with minor modifications. Briefly, immunoprecipitates and an appropriate fraction of the GST-SMAD2 eluate were equilibrated with kinase reaction buffer (20 mM Hepes, pH 7.4, 5 mM MgCl₂, and 1 mM MnCl₂), and the reaction was initiated by the addition of 4 mM ATP (Sigma). After 15 min of incubation at 25 °C, the reaction was terminated by addition of 2× SDS-PAGE sample buffer and boiling for 4 min. Phosphorylated GST-SMAD2 was detected by immunoblotting with the anti-phospho-SMAD2 rabbit pAb. In reactions involving the use of caveolin-derived peptides, prior to initiating the reaction, the immunoprecipitates were preincubated in kinase reaction buffer with the indicated peptide for 30 min at 4 °C. The caveolin-based peptides were synthesized using standard methodology and subjected to amino acid analysis and mass spectroscopy (Massachusetts Institute of Technology Biopolymers Laboratory and Research Genetics) to confirm their purity and composition, as we described previously (35, 48, 49, 57, 58). Peptides were dissolved in Me₂SO, and 100× stock solutions were prepared for use in experiments. In *in vitro* kinase assays assessing the autophosphorylation of TβRI were exactly as described above except for the addition of GST-SMAD2 and the use of 10 μCi of [γ³²P]ATP in lieu of ATP. Phosphorylated TβRI was visualized by autoradiography.

**RESULTS**

**TGF-β Type I Receptor Colocalizes, Cofractionates, and Interacts with Caveolin-1 in Caveolea-enriched Domains**—Given the punctate membrane immunostaining previously reported for the TβR-RII complex (21–23), a distribution resembling that of caveolin-1 and caveolea (30), we first investigated the possibility of colocalization between the two proteins in *vivo*. We utilized two different TβRI cDNA constructs (the HA-tagged wild-type form or a constitutively active T204D mutant). It is important to note that the TβR-RII (T204D) construct is a constitutively active mutant that can initiate TGF-β signaling independent of ligand binding or heteromerization with the TβR-RI (47). For most of the following studies we used NIH-3T3 cells readily responsive to TGF-β/SMAD signaling, or 293T, a Cav-1-negative cell line readily amenable to heterologous Cav-1 expression (50, 59). NIH-3T3 cells cotransfected with Cav-1 and either TβRI w.t. or TβRI (T204D) were immunostained and visualized by confocal microscopy. Confocal slices of areas delineating the plasma membrane revealed significant colocalization between the two proteins (Fig. 1). We utilized two different TβRI cDNA constructs (the HA-tagged wild-type form or a constitutively active T204D mutant). It is important to note that the TβR-RI (T204D) construct is a constitutively active mutant that can initiate TGF-β signaling independent of ligand binding or heteromerization with the TβR-RII (47). For most of the following studies we used NIH-3T3 cells readily responsive to TGF-β/SMAD signaling, or 293T, a Cav-1-negative cell line readily amenable to heterologous Cav-1 expression (50, 59). NIH-3T3 cells cotransfected with Cav-1 and either TβRI w.t. or TβRI (T204D) were immunostained and visualized by confocal microscopy. Confocal slices of areas delineating the plasma membrane revealed significant colocalization between the two proteins (Fig. 1). TβR-RI (T204D) was highly aligned with Cav-1, indicating that the two proteins can colocalize independent of ligand activation and TβR-RII. The alignment for cells transfected with TβR-RI w.t. was not complete, however, possibly indicating that the baseline distribution of TβR-RI is not solely restricted to areas of Cav-1 expression (Fig. 1). Biochemically, we have previously shown that caveolar microdomains can be separated from other cellular constituents using a sucrose gradient ultracentrifugation procedure. Via this

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**FIG. 1. Cav2.1 colocalizes with TβR-RI w.t. and TβR-RI (T204D).** NIH-3T3 cells were cotransfected with the cDNAs encoding caveolin-1 and either TβRI w.t. or TβRI (T204D). Cells were then doubly immunostained with antibodies that specifically recognize cav2.1 and HA (the hemagglutinin tag fused to each receptor). The bound primary antibodies were visualized with distinctly tagged secondary antibody probes (see "Experimental Procedures"). Both cell populations revealed significant membrane colocalization between cav2.1 and TβR-RI. Positive and negative controls omitting a given primary antibody and with cells singly transfected with a given cDNA were also performed and yielded the expected results (data not shown).
Caveolin-1 Inhibits TGF-β/SMAD Signaling by Blocking SMAD Activation.—Because TβR-I plays a pivotal role in the propagation of TGF-β signaling from the membrane to the nucleus, we were interested in the functional consequences of its interaction with Cav-1. First, we investigated the response of two commonly used TGF-β transcriptional reporter assays to heterologous Cav-1 expression. The A3-lux/Fast-1 and 3TP-lux systems utilize TGF-β-responsive promoter elements to drive the expression of a luciferase reporter gene (51, 52). NIH-3T3 cells were cotransfected with the appropriate luciferase reporters and a combination of TβR-I (T204D), Cav-1, or empty vector controls. Both reporters displayed robust activation in the presence of the constitutively active TβR-I, an effect that was dramatically reversed in cells coexpressing Cav-1 (Fig. 4A). In lieu of TβR-I (T204D), we used the same reporters to look at the activation of the endogenous TGF-β receptor complex by treating NIH-3T3 cells with TGF-β1 (4 ng/ml) for 8 h. Cav-1 again displayed inhibitory capacity in this respect, diminishing the ligand-activated state 3–4-fold (Fig. 4B). A K232R (kinase dead) mutant of the TβR-I had previously been shown to lack kinase activity and to act in dominant negative fashion by dimerizing with the wild-type TβR-I (46). A comparison of the effects displayed by Cav-1 with those of TβR-I (K232R) reveals a similar inhibitory capacity for both proteins (Fig. 4B). We have independently performed these assays using COS-7 cells yielding similar results,2 indicating that the Cav-1-mediated inhibition is not necessarily cell type-specific.

The receptor-activated SMADs (Smad-2 and -3) are the first step in the propagation of TGF-β signals from the plasma membrane to the nucleus. The ligand-activated TβR-I directly phosphorylates Smad-2 and -3 at a C-terminal SSXS motif (7, 68), a modification that facilitates Smad-2/3 release and subsequent heteromerization with Smad-4 (9, 69). This interaction is followed by nuclear translocation of the complex with pleiotropic effects on the transcription of target genes (reviewed in Ref. 2). Because TβR-I activation is the initiating event in this cascade, the interaction of Cav-1 with the receptor should by extension disrupt Smad-2/3 phosphorylation, heteromerization with Smad-4, and translocation to the nucleus. We first investigated ligand-activated phosphorylation of SMADs by transfecting NIH-3T3 cells with FLAG-tagged Smad2 and either Cav-1 or empty vector, labeling with 32P-O4, and selectively treating with TGF-β1. As previously reported, the phosphorylation of Smad-2 was readily apparent in the TGF-β1-stimulated cells (54) (Fig. 5A). However, in cells coexpressing Cav-1, a dramatic reduction of this phosphorylation was observed. Note that although base-line levels are reduced, Cav-1 more dramatically attenuates Smad-2 phosphorylation in the TGF-β1-stimulated state.

The role of Cav-1 in SMAD phosphorylation was also investigated in a novel manner. Based on our sucrose density gradient experimentation above, we observed that TβR-I and Smad-2 are localized to caveolae-enriched microdomains, independent of Cav-1 coexpression. To test the functional significance of Cav-1, we cotransfected 293T cells with TβR-I (T204D), Smad-2, and either Cav-1 or empty vector and subjected the purified and concentrated caveolar fractions to an in vitro kinase reaction by adding 4 mATP. Fig. 5B shows that the constitutively active receptor can effectively phosphorylate cofractionated Smad-2 in these microdomains in the absence of Cav-1. In contrast, the fractions also containing Cav-1 significantly attenuate this process.

We next investigated the effects of Cav-1 on Smad-2/4 heteromerization. NIH-3T3 cells were transfected with FLAG-tagged Smad-2, HA-tagged Smad-4, and a combination of TβR-I (T204D), Cav-1, or empty vector controls. Immunoprecipitation of Smad-2 via anti-FLAG mAb revealed coprecipi-

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2 B. Razani, X. L. Zhang, M. Bitzer, G. von Gersdorff, E. P. Bottinger, and M. P. Lisanti, unpublished observations.
tated Smad-4. As predicted, cells expressing constitutively active 
TbR-I induced the formation of a Smad-2/Smad-4 complex,
whereas cotransfection with Cav-1 completely abrogated this
association (Fig. 6).

Finally, we tested the ability of Cav-1 to suppress TGF-
mediated nuclear translocation of Smad-2 both biochemically
and via microscopy. NIH-3T3 or 293T cells were cotransfected
with TbR-I (T204D), FLAG-tagged Smad-2, and either Cav-1 or
empty vector. Via hypotonic lysis, cells were fractionated into
fractions 4–6), whereas SMAD4 is entirely excluded from these
caveolae-enriched fractions. The distribution of total cellular
protein (as analyzed by Ponceau S staining) is shown in the top
panel, indicating that only a minute portion of total cellular
protein actually exists in caveolae.

**FIG. 2.** TGF-β Type I receptor and SMAD2 cofractionate with
caveolin-1 in caveolea-enriched microdomains. A and B, 293T
cells were individually transfected with the indicated cDNAs and sub-
jected to sucrose gradient centrifugation after homogenization in buffer
containing 1% Triton X-100 (see “Experimental Procedures”), a method
which separates Triton-resistant caveolea-rich domains (fractions 4–6)
from other cellular components (fractions 9–12). Immunoblot analysis
with anti-caveolin 2297 mAb, anti-HA pAb, and anti-FLAG mAb was
used to detect the Cav-1, TbR-I w.t./TbR-I (T204D)/TbR-II w.t./SMAD4,
and SMAD2 proteins, respectively. The Type I and Type II receptors
and SMAD2 proteins associate with Cav-1 (fractions 4–6), whereas SMAD4
is entirely excluded from these caveolea-enriched fractions. The distribu-
tion of total cellular protein (as analyzed by Ponceau 5 staining) is
shown in the top panel, indicating that only a minute portion of total
cellular protein actually exists in caveolea. C, analysis of two fractions
from selected gradients in A (fraction 5, caveolea origin; fraction 11,
noncaveolar origin) using equal protein quantities. Note that Cav-1 as
well as TbR-I w.t., TbR-I (T204D), and Smad-2 are concentrated in
caveolar fractions in contrast to Smad-4, which is entirely noncaveolar.

**FIG. 3.** Caveolin-1 interacts with TGF-β Type I receptor in both
heterologous and endogenous settings. A, 293T cells were trans-
fected with HA-tagged TbR-I (T204D) and Cav-1 either separately or
together, as indicated. Cell lysates were prepared and immunoprecipi-
tated (IP) with either anti-HA pAb or relevant negative controls (pre-
imune serum pAb and beads alone). Immunoprecipitates were re-
solved by SDS-PAGE and subjected to immunoblot analysis with anti-caveolin-1 2297 mAb. Note that Cav-1 is immunoprecipitated only in
cells coexpressing TbR-I (T204D) and Cav-1 (top panel). B, NIH-3T3
cells grown to confluence were subjected to immunoprecipitation with either anti-Cav-1 2234 mAb, anti-TbR-I pAb, or appropriate controls
(irrelevant mAb, preimmune serum pAb, and beads alone). Immunoblotting with anti-Cav-1 2297 mAb reveals an endogenous interaction between TbR-I and Cav-1. As compared with immunoprecipitated Cav-1 (first lane), the amount of Cav-1 associated with TbR-I can be estimated to be on the order of ~5–10%.
distinct cytoplasmic and nuclear fractions and the translocation of Smad-2 was analyzed. In both cell lines, Cav-1 reduced Smad-2 levels in the nuclear fraction (Fig. 7A). We used immunofluorescence confocal microscopy to corroborate these observations by transfecting NIH-3T3 cells with Cav-1 and comparing the TGF-β1-induced translocation of endogenous Smad-2 in transfected and nontransfected cells. Fig. 7B shows a mid-line confocal slice delineating the cytoplasms/nuclei of two closely juxtaposed cells. Although there is near complete nuclear translocation of Smad-2 in the untransfected cell, the neighboring Cav-1-expressing cell has a significant cytoplasmic pool of Smad-2.

The Caveolin-1 Scaffolding Domain Mediates the Functional Interaction with TGF-β Type I Receptor—The demonstration of an association between caveolin-1 and TβR-I and its inhibitory effects on downstream SMAD signaling led us to determine the Cav-1 domains possibly mediating this functional interaction via in vitro kinase assays. We affinity purified a GST-Smad-2 fusion protein and used it as a physiologically relevant substrate for immunoprecipitated TβR-I (T204D). We have previously described a series of caveolin-derived peptides spanning various regions of the caveolin molecule (Fig. 8A) (35, 70). Using these peptides in combination with TβR-I (T204D) and GST-Smad-2, we were able to localize an inhibitory region in the Cav-1 molecule. Of the several peptides derived from the N- or C-terminal regions, only two displayed a potent suppression of GST-Smad-2 phosphorylation, namely residues 61–101 (the

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**Fig. 4.** Caveolin-1 functionally regulates TGF-β/SMAD signaling at the transcriptional level. A, NIH-3T3 cells were transfected with either A3-lux/Fast-1 (left panel) or 3TP-lux (right panel) (the TGF-β-responsive luciferase reporters) and a combination of TβR-I (T204D), Cav-1, or empty vector controls. Note that Cav-1 inhibits signaling mediated by the constitutively active TβR-I in both reporter systems. B, as in A, the A3-lux/Fast-1 and 3TP-lux reporter systems were utilized. However, instead of the constitutively active receptor, TGF-β1 ligand (4 ng/ml) was used to stimulate endogenous TβR-I in NIH-3T3 cells for 8 h, as indicated. In addition, the ability of Cav-1 to diminish signaling was compared with that of TβR-I (K232R), a kinase-dead receptor mutant. Note that Cav-1 significantly diminishes the transcriptional response, an effect on the same order of potency as the dominant negative K232R mutant receptor. In both panels, luciferase activities are expressed as ratios normalized to β-galactosidase activity, and each experimental value represented graphically is the average of three separate transfections performed in parallel. Error bars represent the observed S.D.}

**Fig. 5.** Caveolin-1 inhibits base-line and TGF-β1-stimulated phosphorylation of the receptor-linked SMAD (SMAD2). A, NIH-3T3 cells were cotransfected with FLAG-tagged SMAD2 and either Cav-1 or empty vector, incubated in phosphate/serum-free medium supplemented with 32PO₄, and selectively treated with TGF-β1 (4 ng/ml) for 45 min. Immunoprecipitation of SMAD2 via the anti-FLAG mAb revealed the phosphorylated protein. Note that although Cav-1 reduces base-line levels, it dramatically affects SMAD2 phosphorylation in the TGF-β1-stimulated state (top panel). Total SMAD2 levels are indicated in the bottom panel. B, 293T cells were transfected with TβR-I (T204D), SMAD2, and either caveolin-1 or empty vector. Caveolae-enriched membrane fractions were purified, concentrated, and subjected to in vitro kinase assays by the addition of 4 mM ATP (see “Experimental Procedures”). The presence of Cav-1 in these microdomains is sufficient to inhibit SMAD2 phosphorylation by the constitutively active TβR-I.
Caveolin-1 oligomerization domain and its 82–101 truncation (the Cav-1 scaffolding domain). Importantly, when the Cav-1 scaffolding domain is divided into two halves (residues 84–92 and 93–101), this inhibition is completely abrogated (Fig. 8B). The TβRI (T204D) has been shown to retain autophosphorylation activity in vitro (46, 47). Therefore, we conducted kinase assays as above using only the immunoprecipitated TβRI (T204D). The same peptides that displayed inhibition of Smad-2 phosphorylation also abrogated the autophosphorylation of TβRI (Fig. 8C), indicating that the Cav-1 scaffolding domain acts to block TβRI kinase activity.

We next investigated the relevance of the Cav-1 scaffolding domain to TGF-β signaling in vivo. We have previously described the construction of a Cav-1 mutant containing a deletion of residues 61–100 (Cav-1 Δ61–100), schematically shown in Fig. 9A (71). 293T cells transfected with HA-tagged TβRI w.t. and either c-Myc-tagged Cav-1 FL or Cav-1 Δ61–100, were subjected to immunoprecipitation using either anti-HA pAb or control preimmune serum pAb. Note that Cav-1 FL specifically interacts with immunoprecipitated TβRI w.t., whereas the Cav-1 Δ61–100 mutant does not (Fig. 9B).

If the scaffolding domain is indeed the region of caveolin that binds to TβRI, it would be predicted that coinoculation of NIH-3T3 cell lysates with Cav-1 peptides containing this domain (as also utilized in Fig. 8) would competitively disrupt the Cav-1/TβRI complex. Fig. 9C shows that indeed only peptides containing the scaffolding domain (i.e. 61–101 and 82–101) are capable of abrogating the interaction of Cav-1 with immunoprecipitated TβRI.

To demonstrate that the scaffolding domain is also functionally important in vivo, we utilized the previously tested A3-lux/Fast-1 luciferase reporter system. NIH-3T3 cells were cotransfected with the A3-lux/Fast-1 reporter and a combination of TβRI (T204D), Cav-1 FL, Cav-1 Δ61–100, or empty vector controls. Cav-1 FL inhibits the signaling mediated by the constitutively active TβRI (also see Fig. 4B), whereas the Cav-1 Δ61–100 mutant has no effect (Fig. 9D).

The Caveolin-1/TGF-β Type I Receptor Interaction Occurs in a Physiologically Relevant Time Frame and Is Important for Dampening TGF-β Signaling—Various investigators have reported the phosphorylation kinetics of Smad-2 to occur gradually with a $t_{1/2}$ of ~5–10 min, peaking at 20–30 min (2, 54). The demonstration of an endogenous interaction between Cav-1 and TβRI (Fig. 3B) led us to investigate whether this association occurs with altered kinetics in the ligand-stimulated state and whether it occurs within the time frame of SMAD phosphorylation. Serum-starved NIH-3T3 cells (grown to confluence) were treated with TGF-β1 (4 ng/ml) over an 80-min period and subjected to immunoprecipitation with anti-TβRI. The Cav-1/TβRI interaction gradually increases from base line, peaking at 40 min post-stimulation (Fig. 10, top panel). The total expression of Cav-1 is unaffected upon TGF-β1 treatment (Fig. 10, middle panel), indicating that the observed interaction is independent of transcriptional regulation. Furthermore, note that the base-line Cav-1/TβRI interaction in this serum-starved and ligand-unstimulated setting is minimal in contrast to the ~5–10% serum-stimulated interaction observed in Fig. 3B.
We also determined the phosphorylation state of Smad-2 and observed an increase in phosphorylation with expected kinetics (i.e., maximal at 20–30 min and plateauing thereafter; Fig. 10, lower panel). Note that the peak of Cav-1/TβR-I interaction (30–40 min) occurs slightly after the peak of phosphorylation level of Smad-2 (20–30 min). Given this time frame, it is plausible that Cav-1 can act to dampen TGF-β signaling by gradually sequestering more of the available TβR-I pool.

To test this hypothesis, we studied the kinetics of TGF-β signaling in cells with perturbed caveolin levels. We have previously described the use of an antisense construct in down-modulating caveolin-1 levels in NIH-3T3 cells (72). Cells harboring antisense caveolin-1 display significantly reduced Cav-1 protein levels and a concomitant loss of morphological caveolae (72). Confluent plates of serum-starved parental NIH-3T3 cells and their antisense-Cav-1 counterparts were treated with TGF-β1 (4 ng/ml) over a 60-min period and subjected to immunoblot analysis. As expected, baseline phosphorylation of Smad-2 was negligible in both cell types and gradually increased in the TGF-β-stimulated state (Fig. 11A). However, starting at the 15-min time point, cells harboring antisense Cav-1 displayed significantly higher Smad2 phosphorylation than the parental cells. Note that the total Smad2 levels remain equal and unaltered in both cell types. In addition, the antisense-expressing cells still produce caveolin-1 but at dramatically reduced levels (Fig. 11A). Quantitation of the Smad-2 phosphorylation levels in both cell types was also conducted by densitometry of the above results (Fig. 11B). Given the time frame in which Smad2 is activated in the antisense cells (i.e. unaltered at baseline but hyperactivated thereafter), caveolin-1 can act to physiologically dampen TGF-β signaling.

DISCUSSION

By using several independent and complementary approaches, we have examined the role of Cav-1 in TGF-β/SMAD signaling. We demonstrated significant colocalization between the punctate distributions previously reported for both Cav-1 and TβR-I and observed that TβR-I, TβR-II, and Smad-2 (but not Smad-4) cofractionate with Cav-1 in caveolae-enriched domains. Support for a direct interaction between Cav-1 and TβR-I was provided by coimmunoprecipitation studies in both heterologous and endogenous settings. This interaction has functional consequences because Cav-1 was able to suppress TGF-β-mediated transcriptional activation. In addition, we showed that Cav-1 diminishes the phosphorylation of Smad-2, disrupts its interaction with Smad-4 and prevents the nuclear translocation of Smad-2 in the ligand-activated state. This inhibition was mediated via an interaction between TβR-I and the scaffolding domain of Cav-1, because only peptides derived from this region displayed potent inhibition of TβR-I kinase activity.
activity in vitro and were able to disrupt the Cav-1/TβR-I interaction in vivo. Furthermore, a Cav-1 mutant harboring a deletion of this domain was unable to either interact with the receptor or functionally suppress TGF-β signaling. We also demonstrated that the endogenous Cav-1/TβR-I association occurs rapidly after ligand-activation and showed that antisense-mediated down-regulation of caveolin-1 in NIH-3T3 cells was sufficient to hyperactivate TGF-β-stimulated Smad-2 phosphorylation. Taken together, our results support a novel role for caveolin-1 as an important negative regulator of TGF-β signaling.

To date, a variety of physiological regulators of TGF-β have been identified. Many of these molecules (e.g., the p42/44 MAP kinases, SmoN/Ski oncoproteins, STAT proteins, etc) act to alter the function of the SMAD proteins by either modifying their phosphorylation state, disrupting their interaction with downstream partners, or preventing their capacity to affect transcription (11). The known repertoire of molecules affecting TGF-β signaling at the level of receptor is also expanding.

The regulation of signaling mediated by Cav-1 and its kinetics of interaction with TβR-I imply a distinct mechanism of TGF-β inhibition. In contrast to Smad-7, which is a TGF-β-inducible gene (17), Cav-1 expression remains unaffected in the first 80 min of TGF-β1 stimulation. Because the transcriptional response of Smad-7 occurs maximally at 60 min (14, 17) and presumably longer for robust protein expression, the negative regulation mediated by Smad-7 is clearly different than that of Cav-1. The inhibitory effect of FKBP12 on TβR-I is released...
upon ligand binding, indicating that its cellular function might be to control aberrant TGF-β signaling in the ligand-independent state (19). This is in contrast to Cav-1, where its interaction with TβR-I actually increases upon ligand activation and plateaus at 40 min. Various investigators have reported the phosphorylation kinetics of Smad-2 to occur gradually with a t1/2 of ~5–10 min, peaking at 20–30 min (2, 54). This response rate is inversely correlated with the observed gradual increase in Cav-1/TβR-I interaction, leading credence to the possibility of a Cav-1-mediated dampening mechanism. In support of these observations, we showed that NIH-3T3 cells harboring an antisense Cav-1 construct behave similar to parental cells under serum-starved conditions, but display a 2–2.5-fold hyperactivation of Smad2 phosphorylation upon TGF-β-stimulation.

At this time, we cannot rule out the presence of intervening proteins which mediate the Cav-1/TβR-I interaction. Given the ability of peptides derived from the Cav-1 scaffolding domain to potently inhibit TβR-I enzymatic activity in vitro and disrupt the Cav-1/TβR-I complex in vivo, a direct interaction between the two proteins is likely. By using phage display libraries, we have previously identified ligands for the caveolin scaffolding domain. These peptide ligands or “caveolin-binding motifs” are as follows: ΦXΦXXXΦΦ, ΦXXXΦXXXΦ, and ΦXΦXXXΦΦXXΦ, where Φ indicates an aromatic residue, Trp, Phe, or Tyr (75). More recent analysis indicates that motifs with a mixture of appropriately spaced aromatic and hydrophobic residues (i.e., Leu, Ile, and Val) could also serve to bind caveolin (76). Because functional caveolin-binding motifs have been deduced in tyrosine kinases, serine/threonine kinases, and endothelial nitric oxide synthase (reviewed in Ref. 41), the Cav-1/TβR-I interaction could presumably occur in this manner. Indeed, there are several candidate caveolin-binding motifs in TβR-I (42; QYLPYYDLV, 381IMKHFESF, and 386FESFKRADY) (3). Most of these motifs are present in the intracellular kinase domain of the receptor (more specifically, subdomains IX and X as delineated in Ref. 77). Therefore, the inhibitory effects of Cav-1 on TβR-I kinase activity and downstream signaling could be mediated by a direct interaction of the Cav-1 scaffolding domain with the TβR-I kinase domain.

What physiological roles might the inhibitory interaction of Cav-1 with TβR-I serve? Although we addressed only a subset of TGF-β growth factors (i.e., TGF-β1), their pleiotropic effects on cellular physiology includes some intriguing highlights. In contrast to its role as an anti-mitogen in the early stages of tumor growth, TGF-β1 appears to act as a promoter of metastasis and tumor cell migration in the later stages (78). By cooperating with matrix metalloproteinases on the deposition and remodeling of the extracellular matrix, TGF-β signals appear to promote tumor invasion and angiogenesis (79). These responses are in direct contrast to ones elicited by Cav-1. We have recently shown that Cav-1 inhibits lamellipod extension and cellular migration in a metastatic mammary adenocarcinoma cell line (80). In addition, VEGF, a potent angiogenesis...
factor, is capable of down-regulating Cav-1 expression in an endothelial-derived cell line (81). Consequently, a loss of Cav-1 regulation on TGF-β signaling might be an important step in the progression to cellular migration and metastasis.

Furthermore, TGF-β signaling plays an extremely important role in cellular differentiation. The progression of Schwann cell, myocyte, adipocyte, endothelial cell, and other lineages are regulated by TGF-β, and in many cases the attainment of a terminal phenotype depends on a cessation of TGF-β signaling (reviewed in Ref. 82). Cav-1 and other members of the caveolin family are up-regulated during such processes and expressed at high levels in terminally differentiated cells, including many of the TGF-β-responsive lineages (83). For example, in the adipogenesis model system 3T3-L1, Cav-1 expression is up-regulated 25-fold in the transition from 3T3-L1 fibroblasts to adipocytes (84). In contrast, TGF-β signaling has been shown to potently inhibit this adipocyte conversion (85). Therefore, the attenuation of TGF-β signals by Cav-1 could be an important mechanism for the controlled progression of developmental events.

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