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GADD34–PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor

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The cascade of phosphorylation is a pivotal event in transforming growth factor β (TGFβ) signaling. Reversible phosphorylation regulates fundamental aspects of cell activity. TGFβ-induced Smad7 binds to type I receptor (TGFβ type I receptor; TβRI) functioning as a receptor kinase antagonist. We found Smad7 interacts with growth arrest and DNA damage protein, GADD34, a regulatory subunit of the protein phosphatase 1 (PP1) holoenzyme, which subsequently recruits catalytic subunit of PP1 (PP1c) to dephosphorylate TβRI. Blocking Smad7 expression by RNA interference inhibits association of GADD34–PP1c complex with TβRI, indicating Smad7 acts as an adaptor protein in the formation of the PP1 holoenzyme that targets TβRI for dephosphorylation. SARA (Smad anchor for receptor activation) enhances the recruitment PP1c to the Smad7–GADD34 complex by controlling the specific subcellular localization of PP1c. Importantly, GADD34–PP1c recruited by Smad7 inhibits TGFβ-induced cell cycle arrest and mediates TGFβ resistance in responding to UV light irradiation. The dephosphorylation of TβRI mediated by Smad7 is an effective mechanism for governing negative feedback in TGFβ signaling.

Introduction

TGFβ superfamily members regulate cell fate by controlling proliferation, differentiation, and apoptosis and are therefore crucial for the development and maintenance of many different tissues (Derynck, 1994; Heldin et al., 1997; Kretzschmar and Massague, 1998; Miyazono, 2000; Attisano and Wrana, 2002). Deregulated TGFβ family signaling has been implicated in various human diseases, including autoimmune diseases, vascular disorders, and cancers (Derynck et al., 2001; Attisano and Wrana, 2002). TGFβ superfamily members elicit their cellular response through ligand-induced formation of heteromeric complexes of specific transmembrane types I and II kinase receptors. The type II receptor is a constitutively active kinase, which upon ligand-mediated heteromeric complex formation phosphorylates particular serine and threonine residues in the type I receptor juxtamembrane region (GS domain; Wieser et al., 1995), resulting in the activation of the type I receptor (Wrana et al., 1992, 1994a; Franzen et al., 1993). The activated type I receptor then transiently associates with and phosphorylates a subclass of a unique family of intracellular signaling molecules called Smad proteins. This subclass of Smads are receptor-regulated Smads (R-Smads; Heldin et al., 1997; Kretzschmar and Massague, 1998; Attisano and Wrana, 2002). Once phosphorylated by activated type I receptor at their COOH-terminal SSXS motif, R-Smads rapidly dissociate from the receptor to form complexes with common partner Smad, Smad4, and migrate into the nucleus where they regulate transcription of target genes (Derynck, 1994; Heldin et al., 1997; Attisano and Wrana, 2002). Thus, the activity of this pathway is tightly controlled by serine/threonine phosphorylation, which plays a key role in regulating protein–protein interactions that are critical in the elaboration of signaling responses (Derynck, 1994; Heldin et al., 1997; Miyazono, 2000; Attisano and Wrana, 2002).

With no exception, the phosphorylation state of cellular proteins is controlled by the opposing actions of protein kinases and phosphatases. There are two kinds of kinase/phosphatase in the mammalian system: protein tyrosine kinase/protein tyrosine phosphatase (PTP) and serine threonine kinase/protein phosphatase (PP). Receptor protein tyrosine kinases are all type I transmembrane proteins with a

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Abbreviations used in this paper: GADD, growth arrest and DNA damage; I-1, inhibitor 1; OA, okadaic acid; PP1, protein phosphatase 1; PP1c, catalytic subunit of protein phosphatase 1; PTP, protein tyrosine phosphatase; RNAi, RNA interference; R-Smads, receptor-regulated Smads; SARA, Smad anchor for receptor activation; siRNA, small interfering RNA; TβRI, transforming growth factor β type I receptor.

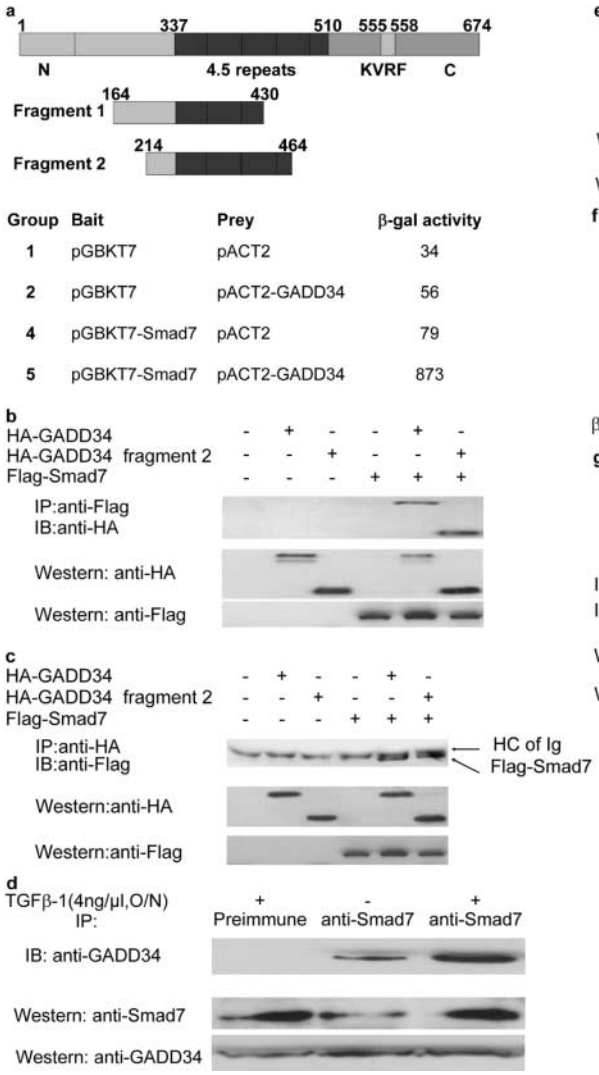

Figure 1. **GADD34 as a Smad7-interacting protein through its central repeats.** (a) GADD34 is shown schematically with conservative regions. Two fragments of GADD34 were isolated in a yeast two-hybrid screen using Smad7 as the bait. (b–e) Smad7 interacts with GADD34 in mammalian cells. (b and c) Immunoblots indicating the protein expression levels of the lysates of COS1 cells transfected with HA–GADD34 and Flag–Smad7 or HA–GADD34 fragment 2 are shown in the two lower panels. The upper panel shows anti-Flag immunoprecipitates probed with anti-HA antibody (b) or anti-HA immunoprecipitates probed with anti-Flag antibody (c). The bands at ~50 kD close to Smad7 in immunoprecipitate lanes were due to the presence of cross-reacting Ig heavy chain. (d and e) Endogenous GADD34 immunoprecipitated from Mv1Lu cells coprecipitated Smad7 as determined by immunoblotting. Conversely, immunoprecipiated endogenous Smad7 coprecipitated GADD34. (f and g) Similar experiments were performed in both yeast (f) and mammalian (g) systems to map Smad7 binding to GADD34.

cytoplasmic domain that has an intrinsic catalytic activity activated upon ligand binding. These phosphorylated substrates can hence be dephosphorylated by certain PTPs (Egloff et al., 1997; Bollen, 2001; Attisano and Wrana, 2002; Cohen, 2002). Mammalian members of the receptor serine threonine kinase family are receptors for ligands of TGFβ family. The counterpart of PTP here is protein phosphatase (PP), but no protein phosphatase was found directly involved in the dephosphorylation of major components in the TGFβ signaling pathway.

Here we show that Smad7, an inhibitory Smad whose expression is induced by TGFβ (Hayashi et al., 1997; Nakao et al., 1997), interacts with growth arrest and DNA damage protein (GADD34; Hollander et al., 1997; Liebermann and Hoffman, 2002), a regulatory/targeting subunit of the protein phosphatase 1 (PP1) holoenzyme (Egloff et al., 1997; Aggen et al., 2000; Bollen, 2001; Cohen, 2002). The catalytic subunit of PP1, PP1c, is recruited to TFGβ type I receptor (TβRI)–Smad7–GADD34 complex through this regulatory subunit, GADD34, to dephosphorylate TβRI. Furthermore, GADD34 is induced by UV light irradiation along with Smad7 resulting UV light–induced TGFβ resistance in Mv1Lu cells. Blockage of GADD34 and Smad7 by RNA interference (RNAi) restores the resistance to TGFβ. Together, these results indicate that the formation of PP1 holoenzyme mediated by TGFβ-induced Smad7 functions as a negative feedback in TGFβ signaling pathway by dephosphorylating TβRI. This implies an important mechanism by which TGFβ regulates the development, maintenance, and tumorigenesis of different tissues.

Results

GADD34 functions as a Smad7-interacting protein through its central repeats

Although the phosphorylation cascade of TGFβ signaling is well characterized, little is known about the negative regulatory mechanism by phosphatases. To investigate the possible dephosphorylation mechanism of TGFβ signaling, we used the full length of Smad7 cDNA as a bait to screen a human chondrocyte cDNA library in a yeast two-hybrid system. Sequence analysis revealed that two of the positive clones were

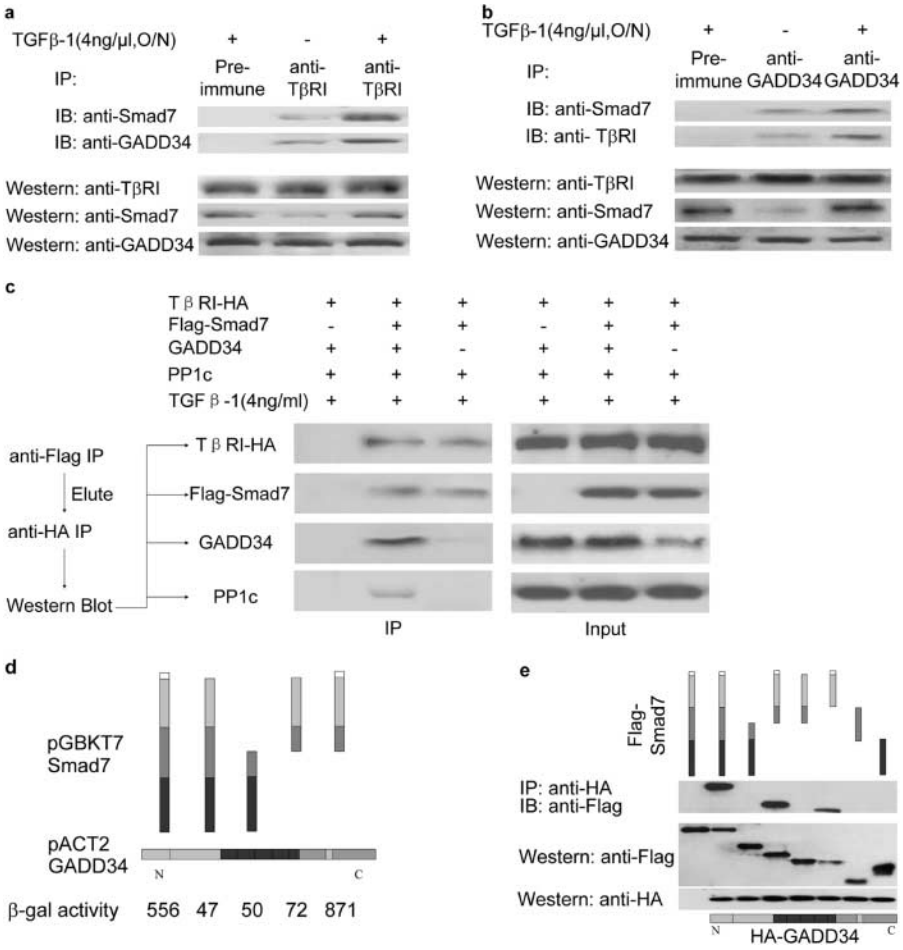

Figure 2. **TGFβ regulates the formation of TβRI–Smad7–GADD34 complexes via Smad7.** (a) Endogenous TβRI was immunoprecipitated from Mv1Lu cells with or without TGFβ-1 stimulation and precipitates were examined for the presence of GADD34 and Smad7 by immunoblotting. (b) Conversely, endogenous GADD34 was immunoprecipiated from Mv1Lu cells with or without TGFβ-1 stimulation and precipitates were examined for the presence of TβRI and Smad7. (c and d) Experiments were performed in both yeast (c) and mammalian (d) systems, as in Fig. 1, to map GADD34 binding to Smad7.

GADD34 and that both clones contain the GADD34 central repeat region (He et al., 1996; Hollander et al., 1997; Liebermann and Hoffman, 2002; and Fig. 1 a). β-Galactosidase liquid assays showed that Smad7 interacts with full-length GADD34, suggesting that the GADD34 central repeats mediate the interaction (Fig. 1 a).

Flag-tagged Smad7 was then cotransfected with HA-tagged GADD34 in COS1 cells to confirm the interaction in mammalian cells. The cell lysate was subjected to Smad7 immunoprecipitation and the associated GADD34 was immunoblotted. Conversely, GADD34 was immunoprecipitated and Smad7 was immunoblotted. The results showed that Smad7 coprecipitated with GADD34 (Fig. 1, b and c). The interaction was further confirmed with endogenous Smad7 and GADD34 and TGFβ significantly enhanced the interaction (Fig. 1, d and e). This is likely a result of TGFβ-induced Smad7 transcription (Hayashi et al., 1997; Nakao et al., 1997). To verify that GADD34 central repeats mediate the interaction, a series of truncated GADD34 plasmids were generated in both yeast two-hybrid and mammalian expression vectors. Consistent with library screen results, deletion of the GADD34 multiple 34–aa repeats abolished its interaction with Smad7 and a single repeat was not sufficient to mediate the interaction (Fig. 1 f). Immunoprecipitation assays with the same deletion constructs indicated that the GADD34 multiple 34–aa repeat mediates the interaction with Smad7 (Fig. 1, b and c, last lanes, and Fig. 1 g).

TGFβ regulates the formation of TβRI–Smad7–GADD34 complex via Smad7

Because Smad7 acts as an antagonist in the TGFβ signaling pathway by binding to the TβRI (Wrana et al., 1994a,b; Wieser et al., 1995; Feng and Derynck, 1997; Attisano and Wrana, 2002), and GADD34 is a targeting subunit of PP1 (He et al., 1996; Hollander et al., 1997; Novoa et al., 2001; Liebermann and Hoffman, 2002), the interaction of Smad7 with GADD34 implicates a negative regulatory mechanism via the dephosphorylation of TβRI. Recent studies have revealed that PP1 negatively regulates decapentaplegic signaling in *Drosophila melanogaster* by affecting the phosphorylation state of TβRI (Bennett and Alphey, 2002). We therefore examined whether TGFβ mediates the binding of GADD34–PP1c serine/threonine phosphatase to its substrate, TβRI, because GADD34 is a target regulatory subunit of the PP1 holoenzyme. First, we examined whether GADD34 forms complexes with TβRI and Smad7 in mammalian cells. Immunoprecipitation assays were performed in TGFβ-responsive MvlLu cells treated with or without TGFβ-1. TGFβ-induced endogenous TβRI–Smad7–GADD34 complexes were immunoprecipitated with either anti-TβRI or anti-GADD34 antibody (Fig. 2, a and b). This complex is further confirmed by a sequential immunoprecipitation. COS1 cells were first cotransfected with TβRI–HA and PP1c with or without Flag–Smad7 and GADD34. After 2 h of stimulation with TGFβ-1, the cells were lysed, subjected to first immunoprecipitation with Flag

antibody, and the resultant precipitates were eluted from the protein G–Sepharose bead by Flag peptide competition and then subjected to second immunoprecipitation with HA antibody. The final precipitates were immunoblotted with antibodies against all these components (Fig. 2 c). The results indicated that the triple components complex, Smad7–TβRI–GADD34, were formed along with PP1c. To identify the region of Smad7 that binds GADD34, a series of truncated Smad7 truncation constructs were generated for a yeast two-hybrid assay (Fig. 2 d). The results indicate that the COOH terminus is responsible for the binding of Smad7 to GADD34. Immunoprecipitation experiments further corroborate the mapping results from yeast two-hybrid assays (Fig. 2 e). Together, the results demonstrate that TβRI forms complexes with GADD34 and that TGFβ enhances this interaction via Smad7, whose expression induced by TGFβ enhances the complex formation (Fig. 2, a and b).

Smad7 regulates recruitment of PP1c to Smad7–TβRI–GADD34 complex

As a catalytic subunit of PP1, PP1c is recruited to GADD34–Smad7–TβRI based on the sequential immunoprecipitation result (Fig. 2 c). We then investigated how this recruitment is regulated. MvlLu cells were treated with or without TGFβ-1. Cell lysates were immunoprecipitated with anti-TβRI antibody followed by immunoblotting with anti-PP1, anti-GADD34, and anti-Smad7 antibodies. Conversely, lysates were immunoprecipiated with anti-PP1c antibody and immunoblotted with anti-TβRI, anti-GADD34, and anti-Smad7 antibodies. The results demonstrate that PP1 coprecipitates with the triple complex and that TGFβ enhances the coprecipitation (Fig. 3, a and b). To determine whether TGFβ regulates the interaction through Smad7, we blocked Smad7 expression with Smad7 small interface RNA (siRNA; Hannon, 2002; McManus and Sharp, 2002). Fig. 3 c shows that blocking of Smad7 expression inhibits the recruitment of PP1c to the complex. Importantly, the amount of PP1c in the complex is proportional to the expression level of Smad7 (Hayashi et al., 1997; Imamura et al., 1997).

Dephosphorylation of TβRI by Smad7–GADD34–PP1c complex

Furthermore, we examined the potential dephosphorylation of TβRI by Smad7-recruited PP1 complex. Purified GST–TβRI–^{32}P was incubated with HA antibody immunoprecipitates from COS1 cells transfected with either HA–Smad7 or GADD34 or in combination with PP1c (Fig. 4 a). Transfection with Smad7 or GADD34 alone did not cause considerable dephosphorylation of TβRI. Dephosphorylation of TβRI was observed when both Smad7 and GADD34 were overexpressed (Fig. 4, a and b, lane 5). With co-overexpression of PP1c or addition of recombinant rabbit PP1c (rR-PP1c [0.05 μM]), TβRI was significantly dephosphorylated (Fig. 4, a and b, lanes 6 and 9), whereas addition of PP1 inhibitor 1 (I-1) inhibited TβRI dephosphorylation (Fig. 4, a and b, lane 7). To confirm the observation in cells, MvlLu cells were cotransfected with TβRI–HA, Smad7, GADD34, and PP1c and various other combinations of the genes and labeled with [^{32}P]orthophosphate (Fig. 4 c). The cell lysates were separated on a denatured gel and directly exposed to x-ray film. Results similar to those described above were obtained (Fig. 4, c and d, lanes 3–8). Okadaic acid (OA) was used as a phosphatase inhibitor. Importantly, deletion of COOH-terminal KVRF motif of GADD34 or the COOH terminus of Smad7-inhibited dephosphorylation activity (Fig. 4, c and d, lanes 9 and 10). PP1c alone did not cause significant dephosphorylation of TβRI either in vitro or in vivo (Fig. 4, a and b, lanes 4 and 8; Fig. 4, c and d, lane 5).

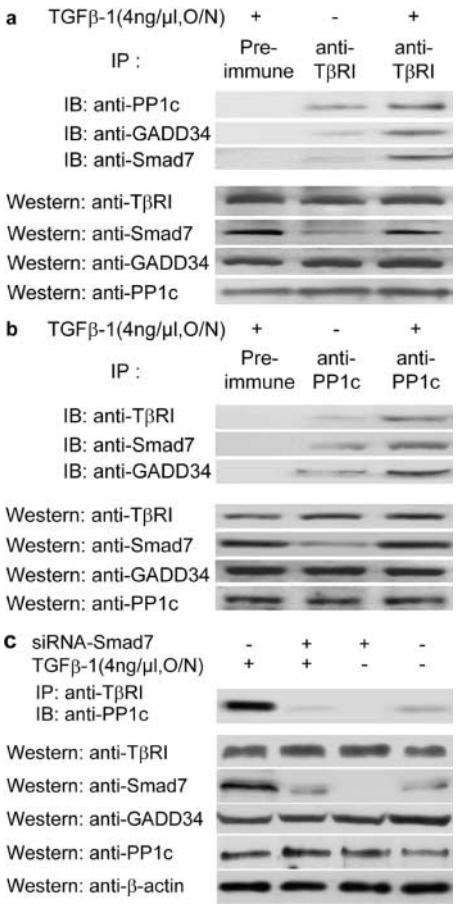

Figure 3. **Smad7 mediates recruitment of PP1c to TβRI.** (a and b) TGFβ regulates the interaction between TβRI and PP1c. Endogenous TβRI was immunoprecipitated from Mv1Lu cells with or without TGFβ-1 stimulation and precipitates were detected for the presence of PP1c, GADD34, and Smad7 by immunoblotting (a). Conversely, endogenous PP1 was immunoprecipitated and TβRI, Smad7, and GADD34 detected by immunoblotting (b). (c) Knockdown of Smad7 inhibits the formation of a GADD34–PP1c complex with TβRI. (c) Endogenous Smad7 in 293T cells was knocked down by using siRNA in the presence or absence of TGFβ-1. The cell lysates were immunoprecipitated with anti-TβRI and the presence of PP1c in the precipitate was detected by immunoblotting with anti-PP1.

Regulation of PP1c intracellular localization close to Smad7–GADD34 complex by Smad anchor for receptor activation (SARA)

While pursuing the potential role of PP1c in TβRI signaling, Bennett and Alphey reported that PP1c binds to SARA and negatively regulates decapentaplegic signaling (Tsukazaki et al., 1998; Bennett and Alphey, 2002). Expression of dominant-negative SARA with a mutation in the PP1c-binding domain (F678A) resulted in hyperphosphorylation of the

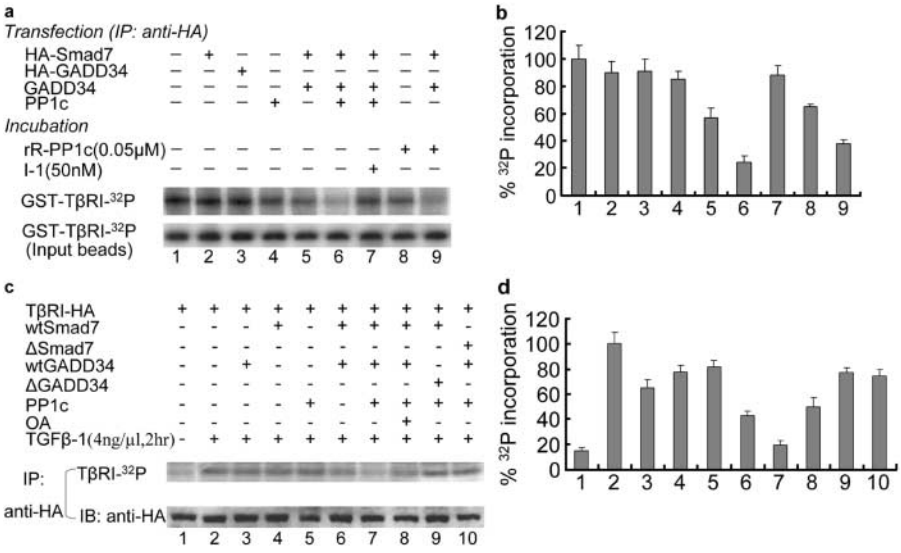

Figure 4. **Dephosphorylation of TβRI by Smad7-recruited PP1 complex.** (a and b) In vitro dephosphorylation assay. (a) GST–TβRI was phosphorylated by an in vitro phosphorylation reaction. GST–TβRI–^{32}P was incubated with different immunoprecipitates (anti-HA) from lysates of COS1 cells transfected with different combinations of genes, in the absence or presence of phosphatase inhibitor (I-1) or recombinant rabbit PP1c (rR-PP1c) as indicated. (b) The relative ^{32}P phosphorylation level of the type I receptor in a, normalized to input of GST–TβRI–^{32}P, is plotted as the mean ± SD from three experiments. (c and d) In vivo dephosphorylation assay. (c) Mv1Lu cells transfected with different combination of genes were labeled with [^{32}P]orthophosphate in the presence or absence of TGFβ-1 as indicated. TβRI–HA was immunoprecipitated from lysates of treated cells and separated by 8.5% SDS-PAGE. Gels were dried and exposed to Biomax M_r film (Eastman Kodak). ΔGADD34 is a mutant without the PP1c binding domain and ΔSmad7 is absent in its TβRI binding site. OA is an inhibitor for both PP1 and PP2. (d) The relative ^{32}P phosphorylation level of TβRI in c was plotted as the mean ± SD from three experiments.

type I receptor and stimulated expression of a TGFβ signaling target (Bennett and Alphey, 2002). SARA is known to recruit R-Smads to the TGFβ receptor by controlling the subcellular localization of R-Smads and by interacting with the TβRI complex (Tsukazaki et al., 1998). It also functions as an anchor for PP1c via its PP1c-binding motif (Bennett and Alphey, 2002). We first examined whether SARA–PP1c complex is able to dephosphorylate TβRI directly. Purified GST–TβRI–^{32}P was incubated with anti-Flag immunoprecipitates from cells transfected with Flag–GADD34, Flag–SARA, or Flag–GADD34 with mammalian dominant-negative SARA (F728A) in combination with PP1c and Smad7. SARA–PP1c complex did not show significant dephosphorylation of TβRI in comparison to control and Smad7–GADD34–PP1c complex (Fig. 5 a, lanes 1–3). These results suggest that SARA is not likely a targeting subunit for direct-

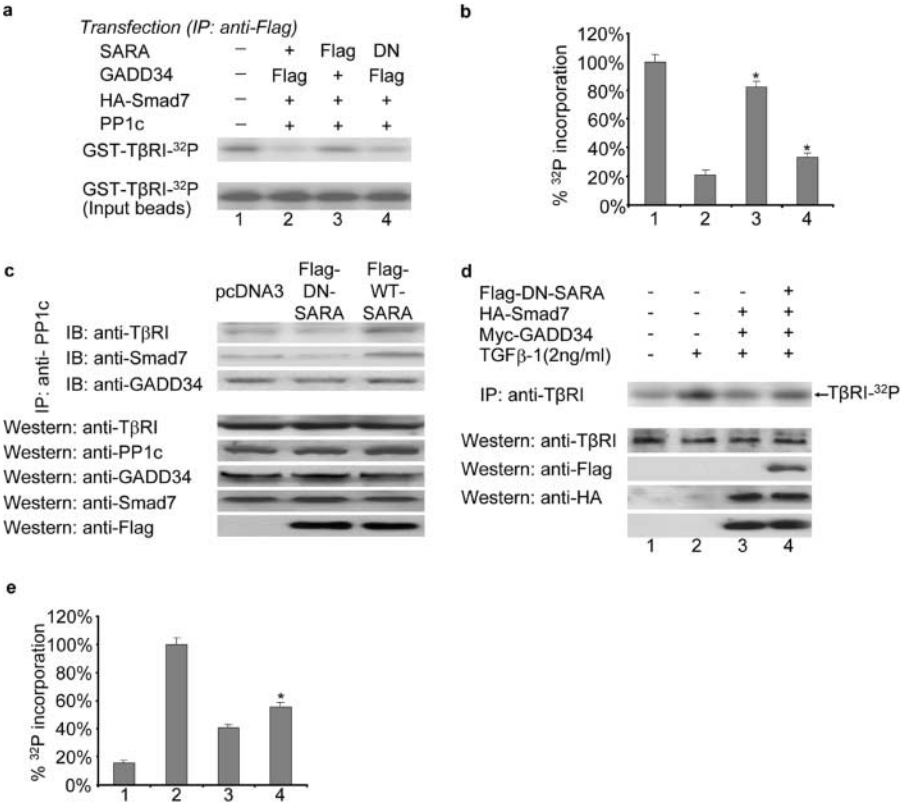

Figure 5. **The regulation of PP1 intracellular localization and facilitation of TβRI dephosphorylation by SARA.** (a and b) SARA–PP1c fails to dephosphorylate TβRI in vitro. (a) Similar in vitro dephosphorylation assay to that in Fig. 4 a was performed. GST–TβRI–^{32}P was incubated with different immunoprecipitates (anti-Flag) from lysates of COS1 cells transfected with different combinations of genes as indicated. In lanes 2 and 4 Flag was tagged to GADD34 and in lane 3 Flag was tagged to SARA. In lane 4 SARA was replaced with DN–SARA (F728A). (b) The relative ^{32}P phosphorylation level of the type I receptor in a, normalized to input of GST–TβRI–^{32}P, is plotted as the mean ± SD from three experiments. *$P < 0.05$, compared with lane 2. (c) The availability of PP1 to the complex is mediated by SARA. COS1 cells were transfected with either Flag–WT–SARA or Flag–DN–SARA (F728A). The cell lysates were immunoprecipitated with anti-PP1c, and the TβRI, Smad7, and GADD34 in the precipitate were probed by immunoblotting. (d and e) In in vivo dephosphorylation assay, dominant-negative SARA with a mutation in the PP1c-binding domain (F728A) inhibits the dephosphorylation of TβRI by Smad7-recruited PP1 complex. (d) Similar experiments to that in Fig. 4 c were performed with dominant-negative SARA (F678A). (e) The relative ^{32}P phosphorylation level of TβRI in panel d was plotted as the mean ± SD from three experiments. *$P < 0.05$, compared with lane 3.

ing the PP1 holoenzyme to dephosphorylate TβRI, as PP1c exhibits phosphatase activity only when it binds to its appropriate targeting subunit for specific substrate. Moreover, overexpression of dominant-negative SARA with mutation of PP1c-binding domain inhibits GADD34 complex–mediated dephosphorylation (Fig. 5, a and b, lane 4) indicating that SARA is likely a membrane anchor protein for PP1c. To investigate the potential role of SARA in recruitment of PP1c to the GADD34 complex, both wild-type and dominant-negative SARA were transfected into COS1 cells. As expected, the amount of PP1c in the complex was increased by addition of wild-type SARA and decreased by addition of dominant-negative SARA (Fig. 5 c). Together, these results suggest that PP1c is handed over to the targeting subunit GADD34 through SARA. In an in vivo phosphatase assay similar to experiment in Fig. 4 c, the effect of SARA on TβRI dephosphorylation was examined. Dominant-negative SARA suppressed the dephosphorylation of TβRI (Fig. 5, d and e). SARA appears to facilitate the dephosphorylation of TβRI in the Smad7-negative feedback loop by controlling the specific subcellular localization of PP1c.

GADD34–PP1c recruited by Smad7 inhibits TGFβ-induced cell cycle arrest and mediates TGFβ resistance

Finally, we attempted to characterize cellular function GADD34–PP1c in TGFβ signaling. First, we examined the effects of dephosphorylation of TβRI on TGFβ-induced gene transactivation. A TGFβ-responsive p3TP luciferase reporter construct was cotransfected into Mv1Lu cells with Smad7, GADD34, PP1, and/or dominant-negative SARA (Fig. 6 a). As expected, GADD34 or Smad7 alone inhibited TGFβ-induced transcription activation (Fig. 6 a, lanes 2 and 3). Furthermore, transactivation was almost completely blocked when Smad7 was coexpressed with either GADD34 or GADD34 and PP1c or GADD34 and SARA, whereas dominant-negative GADD34, Smad7, and SARA or OA reversed the Smad–GADD34-mediated inhibition to varying extents. As we know, TGFβ induces epithelial cell cycle arrest. GADD34–PP1c could inhibit such effect because the complex negatively regulates TGFβ-induced gene transcription. We then examined the effect of GADD34–PP1c complex on TGFβ-induced epithelium cell cycle arrest. Cells were cotransfected with Smad7, GADD34, PP1, and/or dominant-negative SARA as luciferase assay; GFP was also cotransfected for sorting the transfected cells. After treatment as indicated, cells were first sorted for cotransfected GFP and then DNA contents were quantified by FACS® (Fig. 6 b). These results indicate that GADD34–PP1c inhibits TGFβ-induced epithelium cell cycle arrest.

Considering GADD34 is a growth-arrested and DNA-damaged protein induced by different stresses, such as UV light and unfolded proteins, its involvement in here proposed complex implies that the regulation of TGFβ signaling by this PP1 complex may play an important role in stress-induced cell response. Interestingly, GADD34 and Smad7 expression can simultaneously be induced by UV light irradiation (Hollander et al., 1997; Quan et al., 2001). Furthermore, UV light irradiation–induced Smad7 is responsible for TGFβ resistance in the UV light–irradiated cells (Quan et al., 2001). We therefore examined whether the resistance to TGFβ is correlated with the induction of GADD34 and Smad7. We first transfected the cells with the constructs, including siRNA against Smad7 and GADD34, as indicated, and then exposed the cell to UV light to induce the GADD34 and Smad7 expression, which will cause TGFβ resistance in irradiated cells. Our luciferase assay indicates Smad7 and GADD34 are involved in TGFβ resistance in responding to UV light irradiation (Fig. 6 c) and that blockade of UV light–enhanced expression of GADD34 restores UV light–inhibited Smad2 nuclear translocation and downstream PAI-1 expression (Fig. 6 d).

Discussion

Precise control of cascade amplification of phosphorylation from receptor to R-Smad is a crucial component of the TGFβ signaling pathway (Wrana et al., 1992, 1994a; Wieser et al., 1995; Abdollah et al., 1997; Heldin et al., 1997; Attisano and Wrana, 2002). Here, we proposed a novel mechanism of Smad7-mediated dephosphorylation of TβRI. We first found Smad7 interacts with GADD34, a regulatory/targeting subunit of PP1 holoenzyme, and demonstrated the existence of an endogenous TβRI–Smad7–GADD34 triple complex, whose presence is regulated by TGFβ via its induction of Smad7. We then showed that this triple complex recruited a catalytic subunit of protein phosphatase1 (PP1c). Both in vitro and in vivo dephosphorylation assays demonstrated that the PP1 holoenzyme dephosphorylates TβRI, down-regulating the TGFβ signaling pathway. Smad7 acts as an adaptor protein for the formation of the complex and subsequent TβRI dephosphorylation (Fig. 7). Dephosphorylation of TβRI by Smad7-induced PP1c complex explains the central role that Smad7 plays in the negative feedback mechanism.

SARA was originally known as a membrane-bound anchor protein for the recruitment of R-Smads and PP1c (Tsukazaki et al., 1998; Bennett and Alphey, 2002). Mutation in SARA's PP1c binding site (F728A) inhibited the recruitment of PP1c to the triple complex. Overexpression of wild-type SARA, however, enhanced the interaction of PP1c with the triple complex, consequently enhancing the dephosphorylation of TβRI. Importantly, we also found that the SARA–PP1c complex is not able to dephosphorylate TβRI directly. SARA is not a targeting subunit of PP1 to dephosphorylate TβRI. GADD34 is the essential targeting subunit of the PP1 holoenzyme, directing PP1c-mediated TβRI dephosphorylation. SARA appears to serve only as an anchor protein to enhance the availability of PP1c to GADD34 (Fig. 7). A previous study has shown that R-Smads interact with and are recruited by SARA, but once phosphorylated by TβRI, they dissociate from SARA to form a complex with Smad4. So, phosphorylated R-Smad is not a component of this PP1 holoenzyme complex.

It seems that there are three mechanisms by which Smad7 negatively regulates TGFβ signaling: (1) mechanical blockage of R-Smad's phosphorylation, (2) proteasomal degradation, and (3) dephosphorylation of TβRI. Smad7 was initially found involved in the regulation of a variety of physiological and pathological processes such as shear stress

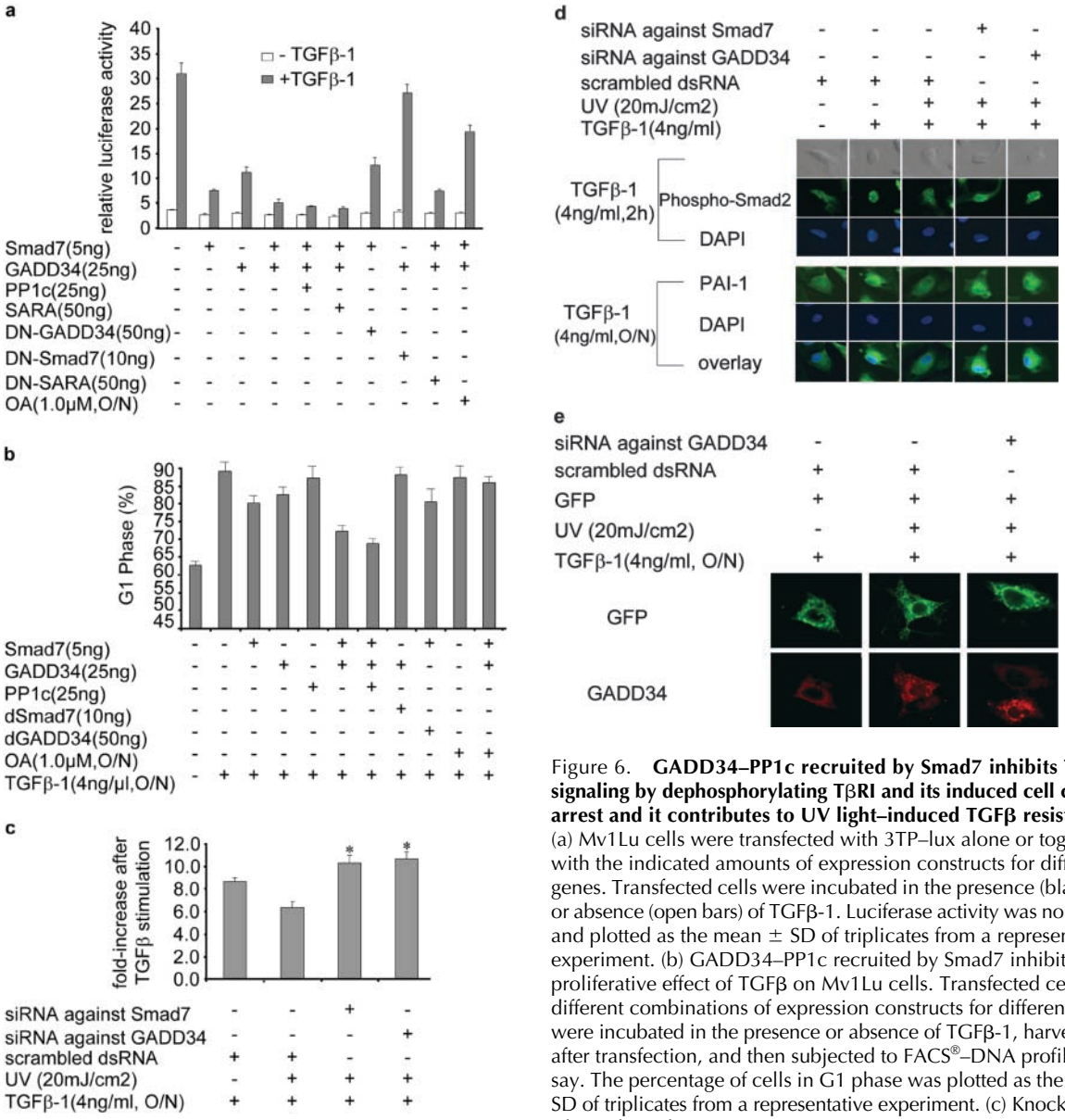

Figure 6. **GADD34–PP1c recruited by Smad7 inhibits TGFβ signaling by dephosphorylating TβRI and its induced cell cycle arrest and it contributes to UV light–induced TGFβ resistance.** (a) Mv1Lu cells were transfected with 3TP–lux alone or together with the indicated amounts of expression constructs for different genes. Transfected cells were incubated in the presence (black bars) or absence (open bars) of TGFβ-1. Luciferase activity was normalized and plotted as the mean ± SD of triplicates from a representative experiment. (b) GADD34–PP1c recruited by Smad7 inhibits antiproliferative effect of TGFβ on Mv1Lu cells. Transfected cells with different combinations of expression constructs for different genes were incubated in the presence or absence of TGFβ-1, harvested 2 d after transfection, and then subjected to FACS®–DNA profiling assay. The percentage of cells in G1 phase was plotted as the mean ± SD of triplicates from a representative experiment. (c) Knockdown of Smad7 and GADD34 expression resensitizes TGFβ signaling in UV light–irradiated cells. The day before UV light irradiation, HepG2 cells were transfected with 3TP–lux together with the indicated siRNAs or scrambled dsRNA. 24 h after UV light irradiation, luciferase activity was assayed and normalized and plotted as the mean ± SD of triplicates from a representative experiment. *$P < 0.05$, compared with lane 2. (d) UV light irradiation inhibits Smad2 nuclear translocation and downstream gene expression, while blocking UV light–enhanced expression of Smad7 or GADD34 rescues it. HepG2 Cells were transfected with siRNA against Smad7 or GADD34 the day before UV light irradiation. The cells were stimulated by TGFβ for 2 h (Smad2 translocation) or 24 h (PAI-1 induction) after UV light irradiation. Immunostaining were then performed to visualize the intracellular translocation of Smad2 and expression of PAI-1. (e) GADD34 induced by UV light irradiation is knocked down by RNAi. GFP expression vector (one tenth the amount of siRNA) was cotransfected into HepG2 cells with siRNA against GADD34. The siRN-transfected cell (last column, showing GFP positive) showed less expression of GADD34, whereas the siRNA-untransfected cell (last column, showing GFP negative, at bottom of the panel) with GADD34 intact compared with siRNA-untransfected UV light–irradiated cell in middle column.

in the vascular epithelium (Topper et al., 1997; Ishisaki et al., 1998; Kleeff et al., 1999; Nakao et al., 1999). It functions as an intracellular receptor antagonists by binding stably to activated TβRI to prevent phosphorylation of R-Smads. The physical blocking of TβRI requires the interaction between Smad7 and TβRI, which is also the initial step of the PP1c complex–mediated dephosphorylation. This mechanism may explain why Smad7 binds to phosphorylated TβRI with a much higher affinity than to dephosphorylated TβRI. TGFβ ignites the phosphorylation TβRI with subsequent signaling and induction of Smad7, which further initiates the formation of GADD34–PP1 complex for TβRI dephosphorylation. Therefore, it is likely that the phosphorylation state of TβRI regulates the interaction between Smad7 and TβRI. So, dominant-negative GADD34 inhibits the dephosphorylation of TβRI, which leads to a longer stable association of Smad7 with phosphorylated TβRI, inhibiting TGFβ signaling. This could be the reason

that dominant-negative GADD34 with an absent PP1c binding site appears not efficiently rescue the inhibition of TGFβ signaling in transcriptional response and cell cycle assays. This observation is further confirmed by the fact that the blocking of GADD34 expression by RNAi eliminates the effect of dnGADD34 in the UV light irradiation experiment. Physical blockage is only one step of Smad7 inhibition, and each Smad7 molecule can only inhibit one TβRI receptor in the physical blockage model. Whereas, the involvement of PP1c dephosphorylation, Smad7 could inhibit TβRI much more efficiently through enzymatic activity. In the third proteasome degradation mechanism, Smad7 was found to act as an adaptor protein to bind to Smurf2 to form an E3 ubiquitin ligase that targets TβRI for its degradation (Kavsak et al., 2000). This is an irreversible and terminal destruction of TβRI, a different level of regulation.

Because cytokines such as interferon γ and TNFα also induce Smad7 expression, and TβRI is occasionally phosphorylated by constitutively active type II receptor (TβRII; Ventura et al., 1994; Chen et al., 1995) or other kinases in the absence of ligands (Topper et al., 1997; Ulloa et al., 1999; Zhang and Derynck, 1999; Bitzer et al., 2000), there is a basal level of induced Smad7 expression. Importantly, the interaction between ubiquitin ligase Smurf2 and Smad7 is induced by IFNγ. Therefore, the mechanism by which Smad7 targets TβRI for degradation is important for the turnover of TβRI and IFNγ-dependent inhibition of TGFβ signaling (Kavsak et al., 2000). Our data show this basal level Smad7 still mediates the recruitment of PP1 holoenzyme to minimize the background signaling initiated by random, promiscuous phosphorylation of TβRI, which may in turn be important for maintenance of cell function. Taken together, these data imply that Smad7, in different cellular contexts, differentially regulates cellular activity by a preferential mechanism, although all three mechanisms may act simultaneously to contribute to the final response of the cell.

GADD34 was initially reported to be induced by various types of cellular stress and DNA damage, such as UV light irradiation and unfolded proteins, and its function in overcoming a protein synthesis checkpoint is supported by the fact that the γ(1)34.5 domain necessary for averting the total shutoff of protein synthesis in herpes simplex virus–infected cells maps to the COOH-terminal domain of the γ(1)34.5 protein. This region is highly homologous to the corresponding domains in MyD116 and GADD34 (He et al., 1998). Moreover, GADD34 has been implicated in the dephosphorylation of eIF2α in a negative feedback loop that inhibits stress-induced gene expression and that might promote recovery from translational inhibition in the unfolded protein response (Novoa et al., 2001). The involvement of GADD34 in our proposed complex implies that the regulation of TGFβ signaling by this PP1 complex may play an important role in stress-induced cell response. Interestingly, GADD34 and Smad7 expression can simultaneously be induced by UV light irradiation (Hollander et al., 1997; Quan et al., 2001). Furthermore, cellular stress caused by UV light irradiation has been known to confer TGFβ resistance in Mv1Lu cells (Quan et al., 2001). UV light–induced TGFβ resistance in Mv1Lu cells is likely attributable to up-regulated expressions of Smad7 and GADD34 and that disruption of this up-regulation will resensitize the cell to TGFβ signaling. Blocking expression of GADD34 and Smad7 with RNAi not only restored TGFβ signaling in UV light–irradiated cells, but rescued the suppressed expression of downstream gene, PAI-1. Dephosphorylation of TβRI by Smad7-mediated PP1 complex is a quick reversible mechanism and it plays a very important role in regulating TGFβ signaling in certain cellular context, such as cellular stress, DNA damage, and induced growth arrest, which further indicates the diversity of cell growth regulation under different cellular context. It will be of substantial interest to investigate the role of our proposed complex in the tumorigenesis of some TGFβ-resistant tumors, developmental events, and other TGFβ-mediated disorders.

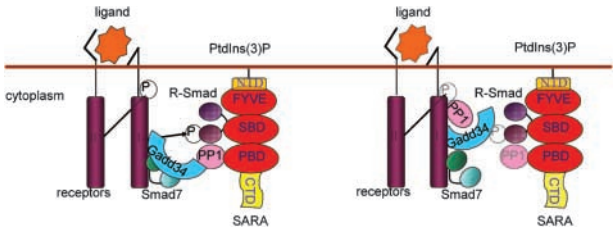

Figure 7. **A model for dephosphorylation of TβRI by Smad7-mediated PP1 holoenzyme in TGFβ signaling.** See Discussion for details. SBD, Smad-binding domain; PBD, phosphatase-binding domain; CTD, COOH-terminal domain; NTD, NH_2-terminal domain.

Materials and methods

Yeast two-hybrid assay

A full-length wild-type Smad7 coding sequence was cloned into pGBKT7 (CLONTECH Laboratories, Inc.) to generate the bait plasmid with which a human chondrocyte cDNA library was screened according to the manufacturer's instructions (CLONTECH Laboratories, Inc.). The interaction between Smad7 and GADD34 were further confirmed with a β-gal filter lift assay and quantified by a liquid β-gal assay according to the manufacturer's instructions (CLONTECH Laboratories, Inc.). Liquid β-gal assay was also performed for Smad7 and GADD34 interaction domain analysis by using different truncation mutations of Smad7 and GADD34.

Immunoprecipitation and immunoblotting

Cells transfected by LipofectAMINE (GIBCO BRL) were lysed with radioimmune precipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (as described above for cell homogenization) and phosphatase inhibitors (10 mM sodium orthovanadate, 1 μM OA, and 50 mM sodium β-glycerophosphate). Lysates were immunoprecipiated by incubation with the appropriate antibodies, followed by adsorption to protein G Sepharose. Immunoprecipitates were separated by SDS-PAGE, blotted onto a PVDF (Bio-Rad Laboratories) membrane, and visualized by enhanced chemiluminescence (ECL Kit; Amersham Biosciences). For mapping the interaction domains between Smad7 and GADD34 in mammalian cells, a series of different deletion constructs were epitope tagged with Flag or HA and subcloned into pcDNA3. All immunoprecipitation and blotting antibodies were obtained from commercial sources: monoclonal anti–Flag M2 and anti–β-actin (Sigma-Aldrich), anti-HA (Babco), polyclonal anti-TβRI (Genex Bioscience, Inc.), polyclonal goat anti-Smad7, polyclonal anti-GADD34, and monoclonal anti-PP1 (Santa Cruz Biotechnology, Inc.).

RNAi

To silence endogenous Smad7 and GADD34 expression, single-stranded 21-nt RNAs directed against Smad7 and GADD34 were chemically synthesized and purified (Ambion). The target sequences were 5′-AGGUCACCACCAUCCCCACUU-3′ and 5′-GUCAAUUUGCAGAUGGCCAUU-3′, respectively. siRNA duplexes were generated and transfected into cells using the Silencer™ siRNA transfection kit (Ambion) according to the manufacturer's instructions. The amount of transfected siRNA was kept constant by addition of scrambled dsRNA provided by the manufacturer.

In vitro phosphorylation and dephosphorylation

GST-TβRI was purified from bacterial lysates by absorption to glutathione–agarose beads as described elsewhere (He et al., 1996). GST–TβRI beads were washed with phosphorylation buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.4 mM EDTA, 1 mM dithiothreitol, 2 mM orthovanadate, 10 mM NaF, 5 mM β-glycerophosphate, and 10 μM ATP) containing a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride and 10 μg/ml antipain, chymostatin, leupeptin, and pepstatin A). 50 μCi of [γ^{32}P]ATP was then added to the mixture and incubated for 20 min at 30°C with anti-HA precipitates of TβRII–HA-transfected cells and protein G–Sepharose beads. The protein G–Sepharose beads and particulate material were pelleted at 14,000 *g* for 20 min, washed again with dephosphorylation buffer (20 mM Tris-HCl, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.8 mM ATP), and incubated in the same buffer with different precipitates from cells transfected with the indicated genes in the presence or absence of 0.05 μM recombinant PP1 catalytic subunit (γ isoform from rabbit; Sigma-Aldrich) or its inhibitors (1.0 μM OA and 50 nM I-1; Sigma-Aldrich). Phosphorylation status was analyzed on an 8.5% SDS-PAGE gel and autoradiography. Phosphatase activity of the precipitates was determined by phosphorylation status of the supposed substrate, TβRI–HA.

Metabolic ^{32}P labeling and in vivo phosphorylation state detection

36 h after transfection with different combination of genes, cells were washed twice with phosphate-free DME containing 2% dialyzed fetal calf serum, incubated in the same medium for 4 h, and then labeled with 1 mCi/ml [^{32}P]orthophosphate (PerkinElmer) for an additional 2 h at 37°C in the absence or presence of TGFβ-1. The cells were washed again with the same medium and incubated with regular DME/ 2% FBS for another 2 h with or without PP1 inhibitor treatment. The ^{32}P-labeled cells were then washed with ice-cold PBS and lysed with radioimmunoprecipitation assay buffer. TβRI–HA was immunoprecipitated with anti-HA as described above. The resultant precipitates were separated by 8.5% SDS-PAGE. Gels were dried and exposed to Biomax M_r or MS film (Eastman Kodak Co.). After autoradiographic analysis, dried gels were rehydrated with transfer buffer, and transferred onto PVDF membranes. For equal loading confirmation, the transfected TβRI–HA was visualized by the ECL^{Plus} Western blotting detection system (Amersham Biosciences).

Transcriptional response assay

Mv1Lu cells were transiently transfected either with 3TP-Lux alone or together with indicated constructs using LipofectAMINE (GIBCO BRL) transfection. Total DNA was kept constant by the addition of pcDNA3 plasmid. 24 h after transfection, cells were incubated overnight with or without 4 ng/ml TGFβ-1. Luciferase activity was measured using the Dual Luciferase assay kit (Promega) according to the manufacturer's instructions.

Cell cycle analysis

Transfected cells were harvested in PBS containing 0.1% BSA and then washed once in PBS containing 1% FBS, centrifuged, resuspended in 0.5 ml of PBS, and fixed by adding 5 ml of cold absolute ethanol. Fixed cells were stored at 4°C until the time of analysis. Immediately before analysis on the flow cytometer, the fixed cells were centrifuged at 1,600 rpm for 5 min, washed once with PBS/1% FBS, and then incubated at 37°C for 2 h in propidium iodide/RNase A solution (10 μg/ml propidium iodide in 0.76 mM sodium citrate at pH 7.0; 100 ng/ml RNase A in 10 mM Tris-HCl, 15 mM NaCl at pH 7.5) diluted into PBS/1% FBS. Cells were first sorted for cotransfected GFP and then DNA contents were quantified. FACS® sorting was performed on a FACStar® machine and analyzed with CellQuest program.

UV light irradiation

Subconfluent cells were incubated in serum-free medium overnight. The next morning, the media were removed and cells were covered with a thin layer of PBS and irradiated with UV light (20 mJ/cm^2) using four FS24T12UVB-HO bulbs. A Kodacel filter was used to eliminate wavelengths below 290 nm (UVC). The irradiation intensity was monitored with an IL400A radiometer and a SED240/UVB/W photodetector (International Light). After irradiation, the PBS was replaced with the original media. Cellular viability 24 h after UV light irradiation was near 100% based on cell morphology and number. For luciferase assay, the day before UV light irradiation, cells were transfected with 3-TP luciferase reporter construct and TGFβ-1 (4 ng/ml) was added after UV light irradiation overnight before luciferase activity assay. siRNA was transfected the day before UV light irradiation.

Immunolocalization

After UV light irradiation and ligand stimulation, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 on ice in blocking buffer (10%BSA in PBS), and labeled with antibodies in PBS with 2% BSA. GADD34 was visualized by immunostaining with rabbit antibody against GADD34 (Santa Cruz) and goat anti–rabbit Texas red–conjugated IgG (Amersham Biosciences). For Smad2 translocation observation, TGFβ-1 (4 ng/ml) was added for 2 h after UV light irradiation, and phosphorylated Smad2 was visualized by immunostaining with rabbit antibody against phosphorylated Smad2 at 465 and 467 residues (Biosource International) and goat anti–rabbit fluorescein-conjugated IgG. For PAI-1 induction observation, immunostaining performed with antibodies (rabbit anti–PAI-1 from Santa Cruz; goat anti–rabbit fluorescein-conjugated IgG from Amersham Biosciences) 24 h after UV light irradiation. Digital pictures were taken with an Olympus, IX TRINOC camera under Olympus, IX70 Inverted Research Microscope (Olympus) with objective lenses of Hoffman Modulation Contrast ®, HMC 10 LWD PL FL, 0.3NA ∝/1, OPTICS INC at room temperature, and proceeded with MagnaFire® SP imaging software (Optronics).

Online supplemental material

Cultured cells were homogenized and fractionated as described previously (Chan and Leder, 1996). Fractions were collected and analyzed by Western blotting. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200307151/DC1.

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