TGF-β inhibits p70 S6 kinase via protein phosphatase 2A to induce G_1 arrest

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On TGF-β binding, the TGF-β receptor directly phosphorylates and activates the transcription factors Smad2/3, leading to G_1 arrest. Here, we present evidence for a second, parallel, TGF-β-dependent pathway for cell cycle arrest, achieved via inhibition of $p70^{s6k}$. TGF-β induces association of its receptor with protein phosphatase-2A (PP2A)-Bα. Concomitantly, three PP2A-subunits, Bα, Aβ, and Cα, associate with $p70^{s6k}$, leading to its dephosphorylation and inactivation. Although either pathway is sufficient to induce G_1 arrest, abrogation of both, the inhibition of $p70^{s6k}$, and transcription through Smad proteins is required for release of epithelial cells from TGF-β-induced G_1 arrest. TGF-β thereby modulates the translational and posttranscriptional control of cell cycle progression.

[*Key Words*: TGF-β $p70^{s6k}$; PP2A; G_1 arrest; cell cycle]

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TGF-β inhibits G_1/S progression in a variety of eukaryotic cell types. Among these, untransformed epithelial cells are particularly sensitive to the growth inhibition by TGF-β. TGF-β binds the TGF-β receptor type I and type II (TβRI/TβRII). TβRI has been shown to transduce all known signals induced by TGF-β TβRI binds and phosphorylates the transcription factor Smad2 or, alternatively, its close homolog Smad3. Smad2 and 3 associate with Smad4 and modulate transcription of TGF-β responsive genes (Massague and Chen 2000; ten Dijke et al. 2000; Wrana and Attisano 2000).

It has been noted, however, that several other molecules interact with TβRI, indicating additional downstream effectors. FKBP12, the cellular target of the immunosuppressant Rapamycin, for example, binds the nonactive TβRI (Wang et al. 1994, 1996; Chen et al. 1997; Huse et al. 1999). In addition, the regulatory subunit Bα of protein phosphatase 2A (PP2A) has been shown to specifically interact with the activated TβRI (Griswold-Prenner et al. 1998). Among many other targets PP2A dephosphorylates and inactivates $p70^{s6k}$ (Ballou et al. 1988; Schonthal 1998; Goldberg 1999; Millward et al. 1999), a serine/threonine kinase that induces translation of mRNAs containing 5′TOP sequences (Jefferies et al. 1994; Pearson and Thomas 1995) and is essential for G_1/S progression (Lane et al. 1993).

In this study we first observe that inhibition of Smad signaling is not sufficient to release epithelial cells from TGF-β-induced G_1 arrest, indicating that there might be additional pathways for growth inhibition by TGF-β. We then show that PP2A and $p70^{s6k}$ are components of a TGF-β-induced signal transduction pathway to control protein translation and G_1/S progression. Dependent on PP2A-Bα, TGF-β inhibits $p70^{s6k}$ by establishing or stabilizing complex formation of PP2A with $p70^{s6k}$. Finally, we argue that repression of $p70^{s6k}$ functions as an alternative mechanism to Smad-mediated transcriptional control of the cell cycle. Once activated, either pathway is sufficient to induce TGF-β-dependent cell cycle arrest in G_1.

Results

To test whether the Smad signal transduction pathway is necessary and sufficient for growth control by TGF-β, we generated dominant-negative and constitutively active mutants of Smad2 and Smad4. The transcriptional activity of these mutant proteins was assessed by their potential to regulate TGF-β-induced transcription in polarized mammary epithelial cells, EpH4 (Reichmann et al. 1992). Multiclonal populations were analyzed for the retroviral-expressed mutant Smad proteins (Fig. 1A,B), the activity of TGF-β reporter constructs, endogenous TGF-β target genes (Fig. 1C), and cell cycle progression by Fluorescence Activated Cell Scanning (FACS; Fig. 1D,E). TβRI activates Smad2 by multiple phosphorylation of its C-terminal motif, SSMS. Mutation of those serines in Smad2 to acidic residues ($Smad2^{EDME}$) activated Smad2 to induce transcription (Fig. 1C; Macias-Silva et al. 1996; Liu et al. 1997; Souchelnytskyi et

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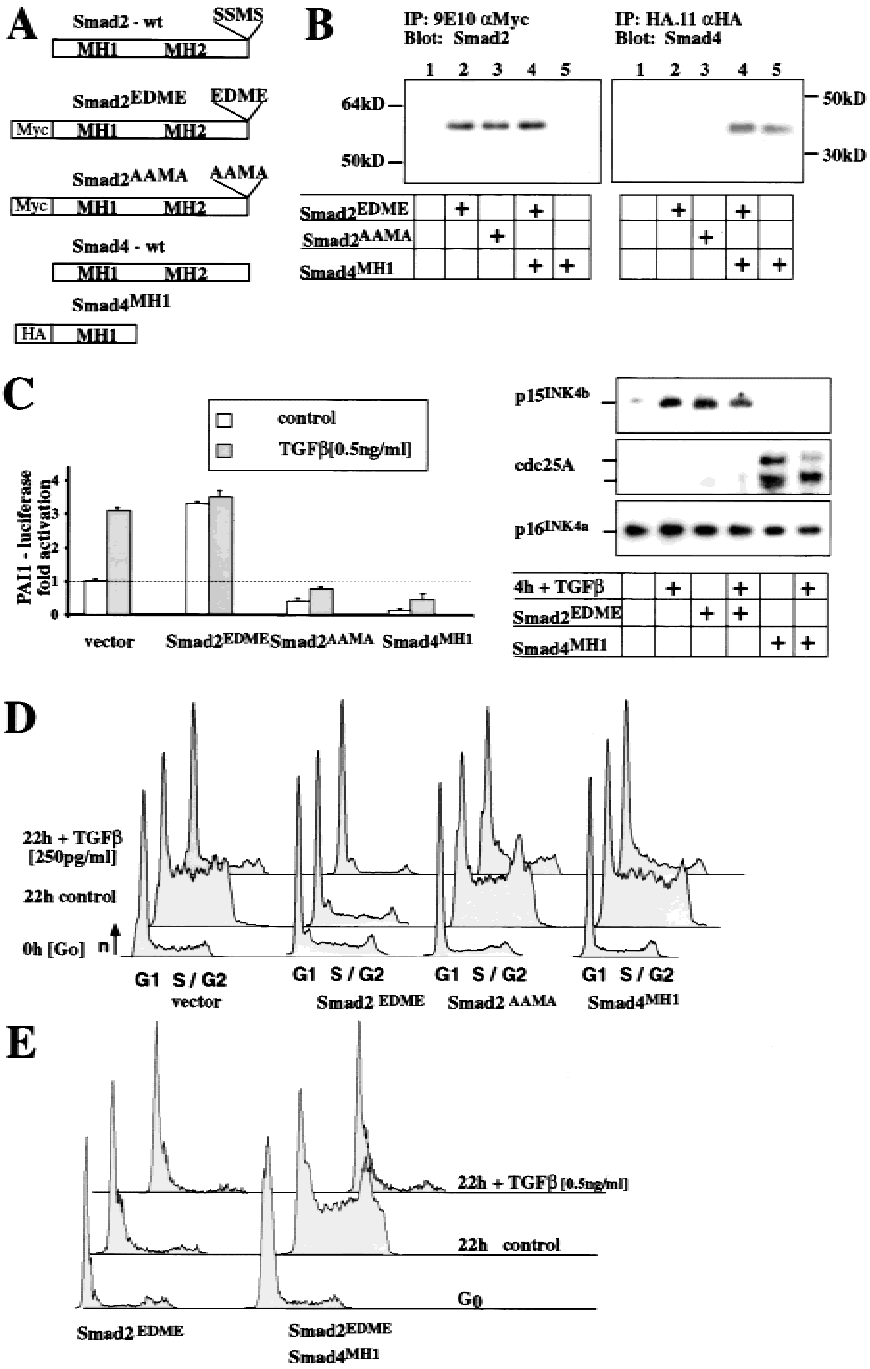

Figure 1. Smad proteins are sufficient and necessary for transcription but only sufficient and not necessary for G_1 arrest induced by TGF-β. (*A*,*B*) Smad-induced transcription was modified in EpH4 mammary epithelial cells by expressing activated Smad2 ($Smad2^{EDME}$) or dominant-negative Smad2 ($Smad2^{AAMA}$) or the Mad Homology domain 1 of Smad4 ($Smad4^{MH1}$) using high-titer retroviral vectors. (*C*) Transcription of transient TGF-β reporter normalized to β-actin control and Western blot for the endogenous p15 (TGF-β activated), cdc25A (TGF-β repressed), and p16 (control). (*D*) FACS-DNA profiles of EpH4 cells expressing the respective Smad protein. Cells were released from contact inhibition into cell cycle for 22 h, in the presence or absence of TGF-β. The activated $Smad2^{EDME}$ arrests cells in G_1 under all conditions and activates transcription; dominant-negative $Smad2^{AAMA}$ or $SMAD4^{MH1}$ were unable to prevent the TGF-β-induced G_1 arrest in EpH4 cells despite their ability tototo antagonize TGF-β-induced transcriptional effects. (*E*) The expression of $Smad4^{MH1}$ was sufficient to overcome $Smad2^{EDME}$-mediated cell cycle arrest but not TGF-β-mediated arrest, arguing for an additional, not transcription-mediated, mechanism for induction of G_1 arrest by TGF-β.

al. 1997). Mutation of the same serines to alanines ($Smad2^{AAMA}$) or expression of the DNA-binding domain of Smad4 ($Smad4^{MH1}$) inhibited TGF-β-induced transcription of a transient transfected PAI1 reporter or the endogenous $p15^{ink4b}$ gene (Fig. 1C). Both molecules thereby act as dominant-negative regulators of TGF-β-induced transcription.

TGF-β induces G_1 arrest (Fig. 1D) and $Smad2^{EDME}$ indeed mimicked this effect (Fig. 1D). Surprisingly, however, neither of the dominant-negative Smad proteins released from TGF-β-induced cell cycle arrest (Fig. 1D). Because receptor-activated Smads form a complex with Smad4, expression of $Smad4^{MH1}$ should inhibit the TGF-β activated Smad2 and the $Smad2^{EDME}$ mutant and relieve $Smad2^{EDME}$-mediated cell cycle arrest (Fig. 1D). Although expression of $Smad4^{MH1}$ indeed reversed the cell cycle arrest induced by the dominant active Smad2 ($Smad2^{EDME}$), it failed to release the same cells from TGF-β-induced G_1 arrest (Fig. 1E), indicating that TGF-β induces additional pathways to induce G_1 arrest, independent of Smad-mediated transcription.

Similar experiments performed previously in other

cell systems had identified Smad-induced transcription as the only mediator of TGF-β-induced G_1 arrest (Liu et al. 1997). In our experimental settings, Smad-induced transcription was likewise sufficient to induce G_1 arrest in epithelial cells, but was clearly not the only mechanism. We attempted therefore to identify the additional mechanism mediating TGF-β-induced G_1 arrest.

To identify signaling pathways essential for G_1/S progression in epithelial cells, we applied known cell cycle inhibitors. We found that Rapamycin, an immunosuppressant and Wortmannin or LY294002, two PI3-kinase inhibitors, each induced G_1 arrest in EpH4 cells (Fig. 2A). We asked whether Rapamycin or PI3-kinase inhibitors and TGF-β would synergize to induce G_1 arrest and tested each drug for synergism with TGF-β. To pursue this approach, dose response curves for cell cycle entry in the presence of drugs alone or with TGF-β were determined (Fig. 2A; data not shown). Rapamycin, Wortmannin, and LY294002 synergized with TGF-β to induce G_1 arrest when applied at nonsaturating conditions of either drug or factor alone (Fig. 2A), indicating that the drugs might modulate similar downstream targets as TGF-β to control cell cycle progression. The compounds, however, did not complement all aspects of TGF-β signaling, and neither drug significantly induced or synergized with Smad-mediated transcription (Fig. 2B).

All three compounds inhibit activation of p70^{s6k} (Cheatham et al. 1994; Chung et al. 1994; Petritsch et al. 1995). Overexpression of p70^{s6k} did not influence TGF-β-induced and Smad mediated transcription (data not shown). Next, we asked if regulation of p70^{s6k} activity is a rate limiting step in the control of G_1/S progression by TGF-β p70^{s6k} was overexpressed in EpH4 epithelial cells or derivatives coexpressing either dominant-negative Smad2 or Smad4 (Fig. 2C) and cells released into G_1/S in the absence or presence of TGF-β (Fig. 2D,E). In the absence of TGF-β cell cycle progression was not altered by expressing Smads and p70^{s6k} (Fig. 2D). Expression of both p70^{s6k} and the Smad2^{AAMA}, however, released a high proportion of epithelial cells from G_1 arrest into S-phase regardless of the presence of TGF-β (Fig. 2D). A kinase deficient p70^{s6k} (p70^{s6k} kin.def.) was not able to rescue from TGF-β-induced cell cycle arrest. Similar results were obtained when the dominant-negative Smad4 (Smad4^{MH1}) and p70^{s6k} were coexpressed (Fig. 2E; data not shown). Thus, p70^{s6k} confers resistance to TGF-β-induced, but Smad-independent, G_1 arrest.

To further test this model, we exposed cells expressing Smad4^{MH1} and p70^{s6k} to increasing concentrations of TGF-β. Whereas 100pg/mL TGF-β-induced complete G_1 arrest in EpH4 cells expressing either p70^{s6k} or Smad4^{MH1} (Fig. 2E), cells coexpressing p70^{s6k} and Smad4^{MH1} continued to undergo G_1/S progression efficiently even at a TGF-β concentration of 5ng/mL. Thus, the cooperation of p70^{s6k} and Smad4MH1 rendered the cells essentially insensitive to TGF-β-induced inhibition of cell cycle progression (Fig. 2E).

In mammalian cells, p70^{s6k} becomes activated on G_0/G_1 or M/G_1 transition, the activity peaks early in G_1 and declines on progression into S-phase (Edelmann et al. 1996). We therefore were interested if TGF-β would suppress p70^{s6k} activity in G_0 and early G_1 cells. EpH4 cells were cultured at confluency to induce early G_1 arrest and subsequently treated for various times with TGF-β TGF-β addition lead to a decrease of p70^{s6k} activity by 70% within 5 min compared with untreated lysates, and the inhibition was sustained for more than 1 h after factor addition (Fig. 3A,B). This rapid onset of inhibition indicates a direct posttranslational mechanism.

Having observed a TGF-β-induced inhibition of p70^{s6k} in arrested cells we examined its effect on p70^{s6k} activity in cells entering the cell cycle. Because overexpression of p70^{s6k} and inhibition of Smad signaling released epithelial cells from TGF-β-induced G_1 arrest (Fig. 2D,E), we anticipated that TGF-β would inhibit activation of p70^{s6k} during G_1/S progression. Indeed, the rapid increase of p70^{s6k} kinase activity observed in the untreated population was delayed and strongly attenuated in cells treated with TGF-β (Fig. 3B).

We next looked for additive effects of inhibition of growth factor pathways and TGF-β on p70^{s6k} activity. Activation of the epidermal growth factor receptor (EGFR) is essential for G_1/S progression of epithelial cells (data not shown). TGF-β and an inhibitor of the EGFR tyrosine kinase, PD 153.035, both strongly reduced the activity of p70^{s6k}. When TGF-β and PD 153.035 were added together, however, the inhibitory effect on p70^{s6k} was synergistic (Fig. 3C). To further examine the interplay of growth signals, we asked if inhibition of p70^{s6k} by TGF-β would antagonize activating signals from epithelial growth factors such as TGF-α that promote G_1/S progression. Unsynchronized proliferating EpH4 cells (50% in G_1 phase) were subjected to treatment with TGF-β TGF-α, or a combination of both. As expected, TGF-α stimulated p70^{s6k} and TGF-β reduced the kinase activity of p70^{s6k} with maximal inhibition after 60 min (Fig. 3D). When both factors were given at the same time, p70^{s6k} activation was intermediate in value, indicating, again, that growth inhibition by TGF-β antagonizes growth promoting signals at the level of p70^{s6k} activation.

We next analyzed if TGF-β-induced inhibition of p70^{s6k} is mediated by the ligand-activated TβRI and TβRII. Accordingly, we immunoprecipitated overexpressed p70^{s6k} from 293 cells, coexpressed with either wt TβRI and wt TβRII (Fig. 3E,F, lanes 6,7) or kinase-inactivated versions of TβRI and TβRII receptor (lanes 2,3) and stimulated the cells with TGF-β. The slower migrating form of p70^{s6k}, corresponding to a highly phosphorylated and enzymatically active form of p70^{s6k} (p70^{s6k} pp; Fig. 3E) and p70^{s6k} activity, was significantly reduced only in the presence of both wt TβR complex and TGF-β stimulation (Fig. 3E,F, lane 7). Thus, both the phosphorylation level and the kinase activity of p70^{s6k} are significantly decreased on TGF-β receptor stimulation.

TGF-β-activated TβRI has been reported to interact directly with the regulatory subunit Bα of PP2A (Griswold-Prenner et al. 1998). PP2A consist of three subunits, a structural regulatory subunit A, a variable regulatory subunit B, and catalytic subunit C. PP2A-C has

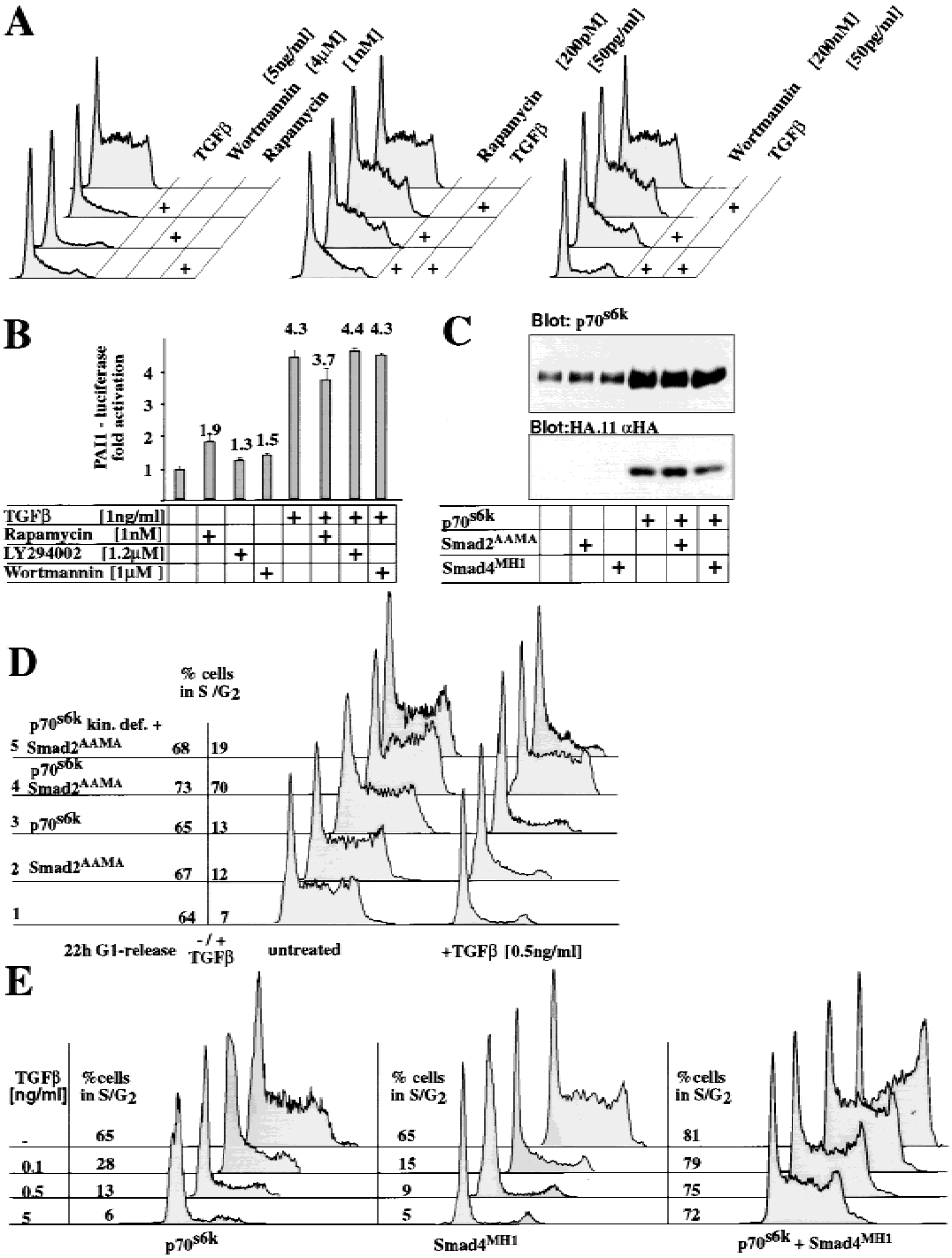

Figure 2. p70^{s6k} pathway is rate limiting for TGF-β-induced G_1 arrest. (*A*,*B*) Synergistic action of TGF-β with Wortmannin and Rapamycin on G_1 arrest, but not transcriptional activation of a TGF-β responsive promoter. (*A*) FACS analysis of EpH4 cells 22 h after cell cycle release. Cells were treated with various drugs as indicated during the entire release period. High concentrations of TGF-β Wortmannin or Rapamycin alone induce G_1 arrest in epithelial cells (left panel). Ineffective doses of Wortmannin and Rapamycin complement low, noninhibitory TGF-β concentrations to cause a complete G_1 arrest (central and right panels). (*B*) Wortmannin, LY 294002, and Rapamycin did not affect TGF-β-induced PAI-1 reporter gene transcription. (*C*,*D*) Overexpression of p70^{s6k} (*C*, Western blot) and FACS analysis determining proportion of cells in G_1 after TGF-β induction in dependence of p70^{s6k} and Smad2^{AAMA} expression (*D*). Only coexpression of p70^{s6k} and dominant-negative Smad2 protein release cells from TGF-β-induced G_1 arrest. Neither p70^{s6k} (*D*, lane *3*) nor Smad2^{AAMA} (*D*, lane *2*) alone are capable to relieve the TGF-β-mediated cell cycle arrest, whereas both proteins expressed together effectively do so (lane *4*). (*E*) FACS analysis of cells released from G_1 arrest 22 h after TGF-β induction in dependence of p70^{s6k} and Smad4^{MH1} expression. Activation of p70^{s6k} and inhibition of Smad-induced transcription are sufficient to completely protect cells from TGF-β-induced cell cycle arrest.

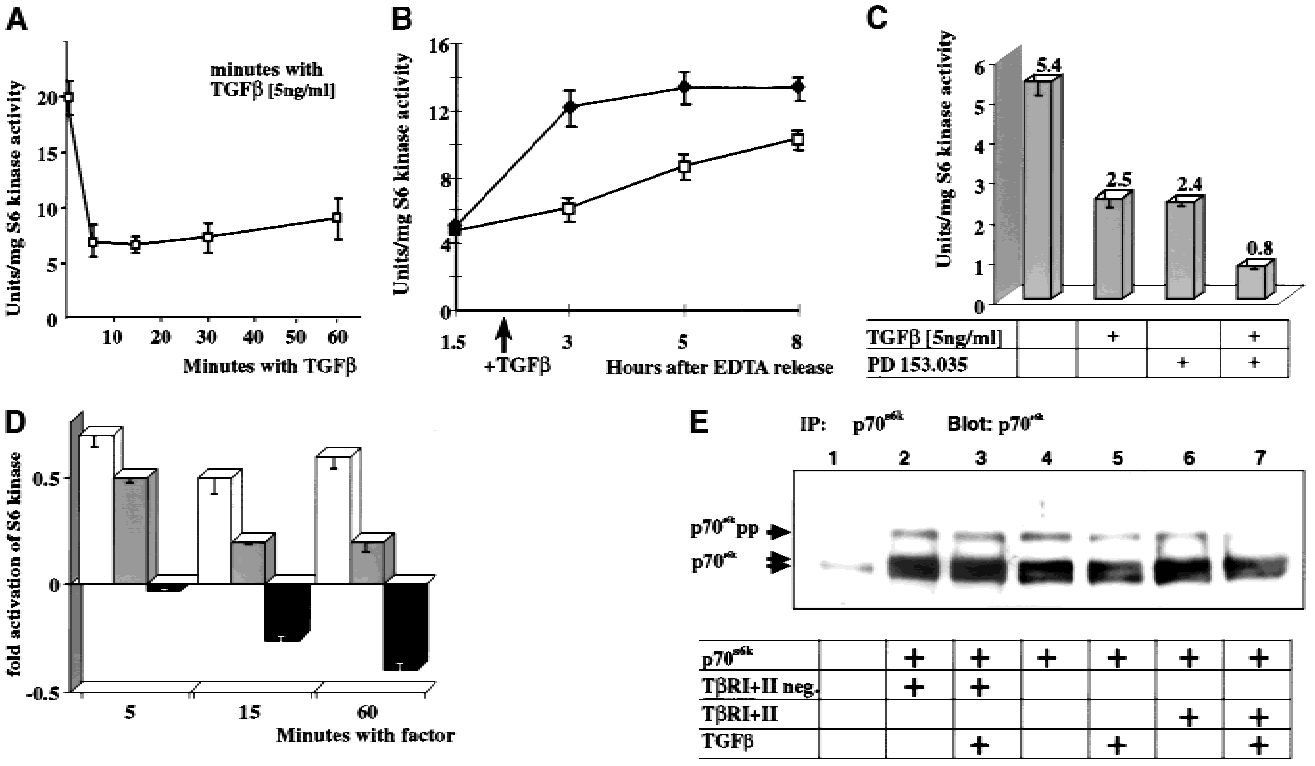

Figure 3. TGF-β inhibits p70^{s6k} and antagonizes EGFR signaling pathways at the level of p70^{s6k}. (*A*) p70^{s6k} kinase activity in EpH4 cells arrested in G_1 and exposed to TGF-β for various times. Sixty-five percent inhibition of p70^{s6k} activity by TGF-β was observed after 5 min, persisting for more than 1 h. (*B*) p70^{s6k} kinase activity was determined in epithelial cells, arrested in G_1 by contact inhibition, released into cell cycle, and treated with TGF-β or left untreated. TGF-β inhibits the increase of p70^{s6k} activity in early G_1. (♦) Control; (□) plus 5ng/mL TGF-β. (*C*) p70^{s6k} activity in response to TGF-β and an EGFR kinase inhibitor in epithelial EpH4 cells after 20 min. The inhibitory effects of a combination of TGF-β and PD 153.035 are synergistic. (*D*) p70^{s6k} activity in response to TGF-β and TGF-α in unsynchronized cycling cells. TGF-β inhibits the p70^{s6k}; TGF-α activates p70^{s6k} and partially neutralizes the inhibitory action of TGF-β on p70^{s6k} activity. (Open bars) 5 ng/mL of TGF-α; (gray bars) TGF-α + TGF-β; (black bars) 5ng/mL of TGF-β. (*E*,*F*) Immunoprecipitation and Western blot of p70^{s6k} transiently cotransfected in 293 cells with wild-type TβRI and II or inactive mutants (TβRI +II dn). (*E*) TGF-β inhibits the hyper-phosphorylation of p70^{s6k} in dependence on an activatable TGF-β–TβR complex. Serum-induced hyperphosphorylated forms of p70^{s6k} (p70^{s6k} pp) disappear only after TGF-β stimulation of the coexpressed wild-type TβRI and TβRII (lanes *6*,*7*), but not by stimulation of kinase inactivated TβRI/II (lane *2*,*3*). (*F*) Corresponding p70^{s6k} kinase activities determined in immunoprecipitates shown in *E*. The decrease of p70^{s6k} pp (Fig. 4a, lane 7) is accompanied by a reduction of p70^{s6k} kinase activity by more than 60%.

been shown to dephosphorylate and inactivate p70^{s6k} (Ballou et al. 1988; Peterson et al. 1999). This indicated that TGF-β-induced inhibition of p70^{s6k} might be mediated by PP2A. We therefore tested whether TGF-β might regulate the interaction of PP2A with p70^{s6k}. HA-tagged p70^{s6k} was subjected to anti-HA-mediated immunoprecipitation. The endogenous catalytic unit of PP2A (PP2A-C) formed a complex with p70^{s6k} only in the presence of the active TGF-β receptor type I and II and only on addition of TGF-β (Fig. 4A, lane 6). No interaction was observed when kinase-dead versions of TβRI and TβRII were coexpressed with p70^{s6k} and stimulated with TGF-β. Furthermore, inhibition of PP2A catalytic activity by addition of the phosphatase inhibitor okadaic acid at concentrations specific for PP2A disrupted formation of the TGF-β-induced PP2A-C/p70^{s6k} complex. Taken together, these findings support the hypothesis that TGF-β inhibits p70^{s6k} by inducing or stabilizing the interaction of PP2A-C with its substrate p70^{s6k}. A similar mechanism has recently been postulated for the inhibition of p70^{s6k} by Rapamycin (Peterson et al. 1999).

In certain cell lines such as mink lung epithelial cells (Mv1Lu), TGF-β does not inhibit p70^{s6k}, and Smad signaling is both sufficient and necessary for TGF-β-mediated G_1 arrest (Like and Massague 1986; Liu et al. 1997). Moreover, in contrast to wild-type murine embryonic fibroblasts (MEFs), MEFs lacking Smad3 do not respond to growth inhibition by TGF-β (Zhu et al. 1998; Datto et al. 1999; Yang et al. 1999). To understand this fundamental difference between primary embryonic fibroblasts

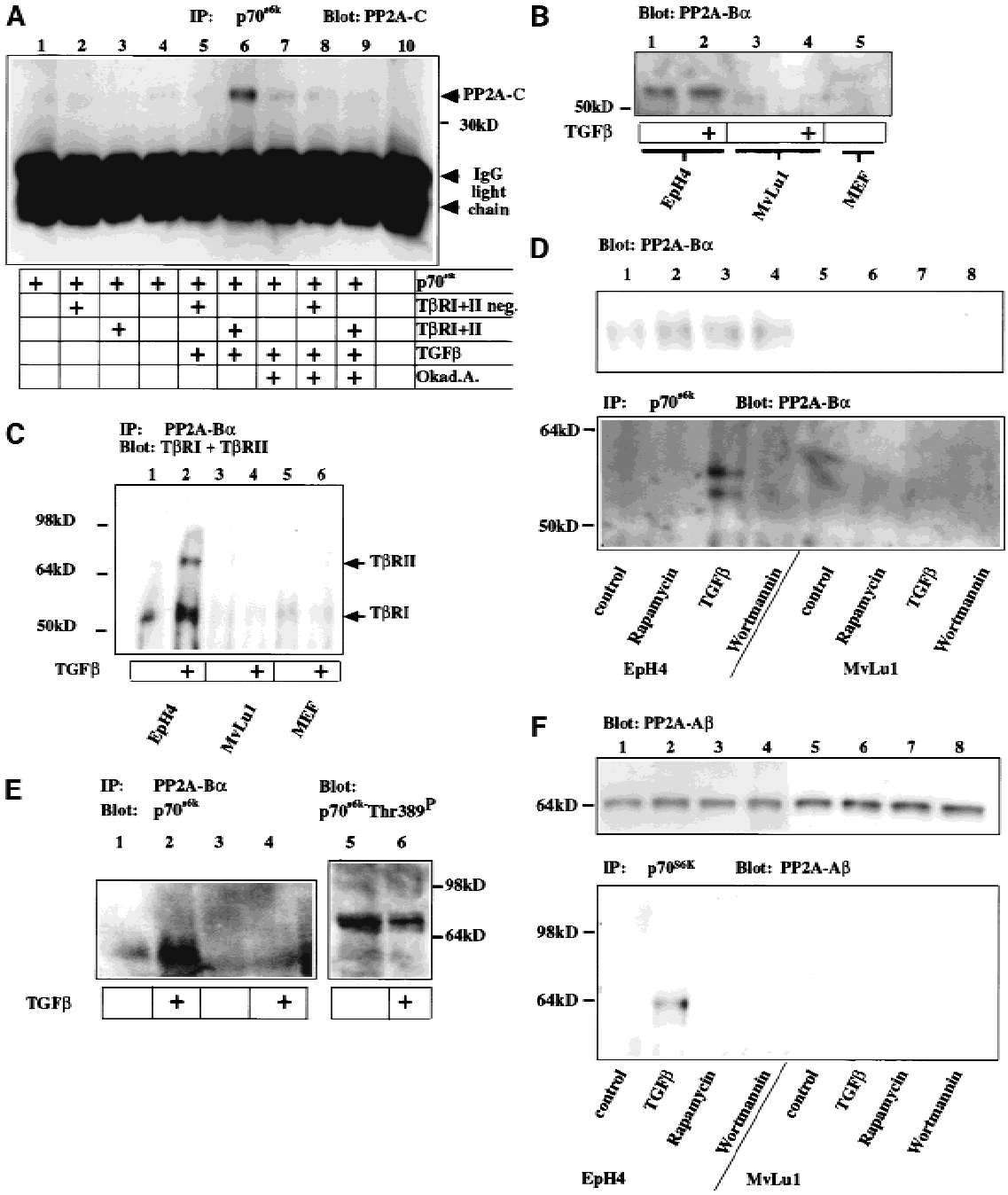

Figure 4. TGF-β induces de-phosphorylation of $p70^{s6k}$ by stimulating association of PP2A subunits C, Aβ and Bα and $p70^{s6k}$ and association of PP2A-Bα and TβRI. (*A*) Western blot for PP2A-C after immunoprecipitation of $p70^{s6k}$ TβR expressing cells were treated with TGF-β and/or the PP2A inhibitor okadaic acid or left untreated. Interaction of PP2A with $p70^{s6k}$ is stimulated in cells expressing the TβRI/TβRII receptor complex on addition of TGF-β (lane *6*), but inhibited by the presence of okadaic acid (Okad.A., lane *9*). Coexpression of inactive TβRI/TβRII neg. fails to stimulate interaction (lanes *2*,*5*,*8*) above basal binding activity (lanes *1*,*4*,*7*). (*B*) Western blot for the expression of the endogenous regulatory subunit PP2A-Bα EpH4 epithelial cells express high levels of PP2A-Bα, and its expression is not altered by 1 h of TGF-β induction. Endogenous levels of PP2A-Bα in Mv1Lu cells and primary embryonic fibroblasts are below detection limits. (*C*) Immunoprecipitation for PP2A-Bα and Western blot for TβRI and TβRII. PP2A-Bα interacts with TβRI and TβRII on TGF-β stimulation in EpH4, but similar complexes are not detectable in Mv1Lu cells or primary embryonic mouse fibroblasts (MEF). (*D*) Western blot for PP2A-Bα after immunoprecipitation of $p70^{s6k}$ in EpH4 or Mv1Lu cells. PP2A-Bα interaction with $p70^{s6k}$ is observed specifically in response to TGF-β in EpH4 cells and not in response to other inhibitors of $p70^{s6k}$ or in Mv1Lu cells. (*E*) Western blot for $p70^{s6k}$ after immunoprecipitation of PP2A-Bα (lanes *1*,*2*) or using a control antibody (lanes *3*,*4*) and direct Western blot for Thr389 of $p70^{s6k}$ from cells stimulated with TGF-β in early G_1. In response to TGF-β, PP2A-Bα is found in complexes with $p70^{s6k}$ and Thr 389 phosphorylation of $p70^{s6k}$ decreases. (*F*) Western blot for PP2A-Aβ before (top panel) and after (bottom panel) immunoprecipitation with $p70^{s6k}$. In EpH4 cells, Aβ interacts with $p70^{s6k}$ specifically in response to TGF-β.

and Mv1Lu cells on the one hand and epithelial cells on the other, we analysed the expression of the PP2A subunits and TGF-β receptors in these cell types. Both TβRI and TβRII were expressed at comparable levels in all three cell types (data not shown). We found, however, that the regulatory subunit Bα of PP2A is expressed in EpH4 cells (Fig. 4B), in primary small airway epithelial cells, and in a variety of epithelial tumor cell lines (data not shown), but not in Mv1Lu cells or primary MEFs (Fig. 4B). Therefore, it was not surprising that interaction of the endogenous PP2A-Bα subunit was detected with endogenous TβRI/TβRII in response to TGF-β in EpH4 cells, but not in Mv1Lu cells or primary MEFs (Fig. 4C). To examine the endogenous complexes formed by PP2A and p70^{s6k}, EpH4 and Mv1Lu cells were treated in early G_1 phase of the cell cycle with either Rapamycin, TGF-β, or Wortmannin. On p70^{s6k} immunoprecipitation, complex formation with PP2A-Bα was only detected in EpH4 cells in response to TGF-β, indicating a TGF-β specific mechanism of p70^{s6k} inhibition presumably absent in Mv1Lu cells (Fig. 4D). Moreover, if PP2A-Bα was immunoprecipitated from lysates of EpH4 cells in early G_1, we were able to detect significantly increased amounts of p70^{s6k} associated with PP2A-Bα in TGF-β treated cells (Fig. 4E). To better characterize p70^{s6k} regulation, we analyzed the phosphorylation status of the Thr 389 in the linker domain of p70^{s6k} (Fig. 4E). Phosphorylation of Thr 389 correlates with the kinase activity of p70^{s6k} (Pullen et al. 1998; Weng et al. 1998). Thr 389 of p70^{s6k} was found to be phosphorylated in EpH4 cells in early G_1, but significantly reduced on TGF-β stimulation (Fig. 4E). These data further support our model that the inhibition of p70^{s6k} by TGF-β is dependent on PP2A-Bα-mediated association of PP2A-C with p70^{s6k}.

We then analyzed which A subunit of PP2A would interact with p70^{s6k} in response to TGF-β The Aβ (PR65) regulatory subunit has recently been genetically linked to human lung and colon cancer (Wang et al. 1998). Aβ was expressed at comparable levels in both cells types, EpH4 and Mv1Lu (Fig. 4F). Interestingly, we detected Aβ associated with p70^{s6k} only in EpH4 cells, but not in Mv1Lu cells, and only in response to TGF-β, but not in response to Rapamycin or Wortmannin (Fig. 4F).

Discussion

Taken together, our results show that TGF-β interferes with two independent pathways to induce G_1 arrest in epithelial cells. The first is the pathway of transcriptional control mediated by Smad proteins (Eppert et al. 1996; Lagna et al. 1996; Macias-Silva et al. 1996; Zhang et al. 1996; Nakao et al. 1997). The second leads to the inhibition of p70^{s6k} via PP2A (Fig. 5). This represents a novel observation with potential implication for our understanding of cell cycle regulation, cancer biology, and cancer development.

We show here that repression of p70^{s6k} coincides with its dephosphorylation and association of p70^{s6k} with three subunits of PP2A: PP2A-C and the two regulatory

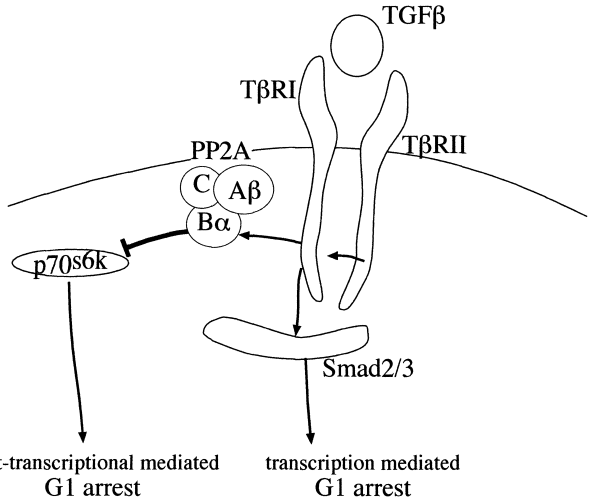

Figure 5. Model for the two independent pathways initiated by TGF-β to induce G_1-arrest. Both, inhibition of p70^{s6k} and activation of Smad-induced transcription are independently sufficient to induce G_1 arrest.

subunits PP2A-Aβ and PP2A-Bα. PP2A-Bα interacts with TβRI and thereby physically links TGF-β to the control of PP2A and to p70^{s6k}. On receptor activation, PP2A-Bα specifically binds the activated TβRI and is catalytically activated by TGF-β (Griswold-Prenner et al. 1998; data not shown). PP2A-Bα then recruits PP2A-Aβ and PP2A-C to bind and dephosphorylate p70^{s6k}. We were, however, not able to detect complexes containing at the same time PP2A, TβRI and p70^{s6k}, indicating that on activation the phosphatase is released from the receptor to bind to the target molecule. Immunolocalization of the endogenous proteins supports this model (M. Oft, unpubl.). p70^{s6k} activity controls the translational upregulation of proteins important for G_1/S progression and is itself essential for cell cycle progression (Lane et al. 1993; Pearson and Thomas 1995). Most of the transcripts isolated to date represent ribosomal proteins and elongation factors of protein synthesis (Jefferies et al. 1994). TGF-β-induced inactivation of p70^{s6k} leads to the translational regulation of a group of cell cycle regulators for G_1 progression (M. Oft, unpubl.). It remains unclear, however, if the repression of those cell cycle regulators result from global repression of protein translation or represent a class of specifically translationally repressed mRNAs. It is conceivable that the regulation of crucial components of the cell cycle machinery is mediated at the transcriptional, translational, and posttranslational levels.

Expression of the regulatory subunit PP2A-Bα itself appears to be a prerequisite for the PP2A-mediated inhibition of p70^{s6k} by TGF-β. Cells with nondetectable PP2A-Bα expression remain solely responsive to TGF-β-mediated transcriptional responses (Liu et al. 1997; Zhu et al. 1998; Datto et al. 1999; Yang et al. 1999). p70^{s6k} is not inhibited by TGF-β in these cells (Like and Massague 1986; data not shown), which reflects the differential sensitivity of epithelial cells and mesenchymal cells to

growth inhibitory effects of TGF-β. The chromosomal localization of PP2A-Bα has not been investigated; PP2A-Aβ, however, has been mapped to a human tumor suppressor locus on 11q22–24 and appears to be mutated in a subset of human lung tumors (Wang et al. 1998). It is tempting to speculate that mutations of the regulatory subunits of PP2A in human tumors abolish the regulation of $p70^{s6k}$ to TGF-β and confer a selective advantage to growing tumors.

Material and methods

Cell culture and cell cycle analysis

PAI1-promoter transcription assays were normalized to a internal β-actin–β-galactosidase control, and numbers represent the average of three independent experiments.

EpH4 cells were infected with supernatants of BOSC23 virus-producing cells (Pear et al. 1993), drug selected, tested for the transgene expression, and immediately analyzed as a mass culture to exclude clonal variations. For G_1/S progression studies, the cells were seeded at a confluent density, arrested in G_0/G_1 by confluency for two days, EDTA released and replated as single cells. The majority of cells enter S-phase after 18 h. Samples for all G_1/S profiles shown were collected 22 h after EDTA release. The results were, however, verified extensively throughout a 16-h to 30-h time window.

$p70^{s6k}$ kinase assays

Cells were washed twice with 10 mL cold Extraction Buffer (20 mM Tris at pH 7.5, 20 mM EDTA, 15 mM $MgCl_2$, 40 mM 4-nitrophenyl phosphate [pNPP], 1 mM dithiothreitol [DTT], and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). For s6 kinase assays $p70^{s6k}$ was immunoprecipitated using polyclonal antibodies against $p70^{s6k}$ (Edelmann et al. 1996) or the HA-epitope and G protein–coupled sepharose beads. Precipitated beads were resuspended in S6 kinase assay buffer (50 mM Tris at pH 7.5, 0.1 mM EGTA, 5% ethylene glycol, 5 mM DTT, 10 mM $MgCl_2$, 0.1% Triton X-100, and 0.25 mg/mL BSA) and assayed for S6 kinase activity using the ribosomal 40S subunit as a substrate as described previously (Petritsch et al. 1995). Immunoprecipitation of $p90^{rsk}$ and subsequent kinase assays revealed that s6 phosphorylation by $p90^{rsk}$ is negligible in EpH4 cells. All kinase assays were performed in duplicates and repeated twice.

Immunoprecipitations

TβRI, TβRII or their derivatives with inactivated kinase domains and HA-tagged $p70^{s6k}$ were transiently transfected into 293 cells. Cell lysates were collected 24 h after transfection and 20 min after stimulation with TGF-β or okadaic acid and subjected to anti-HA- or anti-$p70^{s6k}$ immunoprecipitation.

To precipitate endogenous TβRI and TβRII kinase-phosphatase complexes, lysates for immunoprecipitation were taken after 20 min of TGF-β treatment, normalized for protein content, and subjected to immunoprecipitation using the respective antibodies and Protein G sepharose beads.

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