TGF- β inhibits p70 S6 kinase via protein phosphatase 2A to induce G₁ arrest

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On TGF- β binding, the TGF- β receptor directly phosphorylates and activates the transcription factors Smad2/3, leading to G₁ 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)-B α . Concomitantly, three PP2A-subunits, B α , A β , and C α , associate with p70^{S6k}, leading to its dephosphorylation and inactivation. Although either pathway is sufficient to induce G₁ 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 G₁ arrest. TGF- β thereby modulates the translational and posttranscriptional control of cell cycle progression.

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TGF- β inhibits G₁/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 (T β RI/T β RII). T β RI has been shown to transduce all known signals induced by TGF- β . T β RI 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 T β RI, indicating additional downstream effectors. FKBP12, the cellular target of the immunosuppressant Rapamycin, for example, binds the nonactive T β RI (Wang et al. 1994, 1996; Chen et al. 1997; Huse et al. 1999). In addition, the regulatory subunit B α of protein phosphatase 2A (PP2A) has been shown to specifically interact with the activated T β RI (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 G₁/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 G₁ 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 G₁/S progression. Dependent on PP2A-B α , 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 G₁.

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). T β RI 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 to 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 PAIL 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 G₁ arrest (Liu et al. 1997). In our experimental settings, Smad-induced transcription was likewise sufficient to induce G₁ arrest in epithelial cells, but was clearly not the only mechanism. We attempted therefore to identify the additional mechanism mediating TGF- β -induced G₁ arrest.

To identify signaling pathways essential for G₁/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 G₁ arrest in EpH4 cells (Fig. 2A). We asked whether Rapamycin or PI3-kinase inhibitors and TGF- β would synergize to induce G₁ 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 G₁ 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 G₁/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 G₁/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 G₁ 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, G₁ 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 G₁ arrest in EpH4 cells expressing either p70^{s6k} or Smad4^{MH1} (Fig. 2E), cells coexpressing p70^{s6k} and Smad4^{MH1} continued to undergo G₁/S progression efficiently even at a TGF- β concentration of 5ng/mL. Thus, the cooperation of p70^{s6k} and Smad4^{MH1} rendered the cells essentially insensitive to TGF- β -induced inhibition of cell cycle progression (Fig. 2E).

In mammalian cells, p70^{s6k} becomes activated on G₀/G₁ or M/G₁ transition, the activity peaks early in G₁ and declines on progression into S-phase (Edelmann et al.

1996). We therefore were interested if TGF- β would suppress p70^{s6k} activity in G₀ and early G₁ cells. EpH4 cells were cultured at confluency to induce early G₁ 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 G₁ arrest (Fig. 2D,E), we anticipated that TGF- β would inhibit activation of p70^{s6k} during G₁/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 G₁/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 G₁/S progression. Unsynchronized proliferating EpH4 cells (50% in G₁ 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 T β RI and T β RII. Accordingly, we immunoprecipitated overexpressed p70^{s6k} from 293 cells, coexpressed with either wt T β RI and wt T β RII (Fig. 3E,F, lanes 6,7) or kinase-inactivated versions of T β RI and T β RII 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 T β R 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 T β RI has been reported to interact directly with the regulatory subunit B α 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 G₁ arrest. (A,B) Synergistic action of TGF-β with Wortmannin and Rapamycin on G₁ 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 G₁ arrest in epithelial cells (left panel). Ineffective doses of Wortmannin and Rapamycin complement low, noninhibitory TGF-β concentrations to cause a complete G₁ 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 G₁ 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 G₁ 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 G₁ arrest 22 h after TGF-β induction in dependence of p70^{s6k} and Smad4^{MHI} expression. Activation of p70^{s6k} and inhibition of Smad-induced transcription are sufficient to completely protect cells from TGF-β-induced cell cycle arrest.

TGF- β inhibits p70^{s6k} by activation of PP2A

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 G₁ 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 G₁ 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 G₁. (♦) Control; (□) plus 5 ng/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) 5 ng/mL of TGF- β . (E,F) Immunoprecipitation and Western blot of p70^{s6k} transiently cotransfected in 293 cells with wild-type T β RI and II or inactive mutants (T β RI +II dn). (E) TGF- β inhibits the hyper-phosphorylation of p70^{s6k} in dependence on an activatable TGF- β -T β R complex. Serum-induced hyperphosphorylated forms of p70^{s6k} (p70^{s6k} pp) disappear only after TGF- β stimulation of the coexpressed wild-type T β RI and T β RII (lanes 6,7), but not by stimulation of kinase inactivated T β RI/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 T β RI and T β RII 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 G₁ 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

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 T β RI and T β RII were expressed at comparable levels in all three cell types (data not shown). We found, however, that the regulatory subunit B α 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-B α subunit was detected with endogenous T β RI/T β RII 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 G₁ phase of the cell cycle with either Rapamycin, TGF- β , or Wortmannin. On p70^{s6k} immunoprecipitation, complex formation with PP2A-B α 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-B α was immunoprecipitated from lysates of EpH4 cells in early G₁, we were able to detect significantly increased amounts of p70^{s6k} associated with PP2A-B α 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 G₁, 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-B α -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 A β (PR65) regulatory subunit has recently been genetically linked to human lung and colon cancer (Wang et al. 1998). A β was expressed at comparable levels in both cell types, EpH4 and Mv1Lu (Fig. 4F). Interestingly, we detected A β 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 G₁ 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 G₁-arrest. Both, inhibition of p70^{s6k} and activation of Smad-induced transcription are independently sufficient to induce G₁ arrest.

subunits PP2A-A β and PP2A-B α . PP2A-B α interacts with T β RI and thereby physically links TGF- β to the control of PP2A and to p70^{s6k}. On receptor activation, PP2A-B α specifically binds the activated T β RI and is catalytically activated by TGF- β (Griswold-Prenner et al. 1998; data not shown). PP2A-B α then recruits PP2A-A β and PP2A-C to bind and dephosphorylate p70^{s6k}. We were, however, not able to detect complexes containing at the same time PP2A, T β RI 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 up-regulation of proteins important for G₁/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 G₁ 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-B α itself appears to be a prerequisite for the PP2A-mediated inhibition of p70^{s6k} by TGF- β . Cells with nondetectable PP2A-B α 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-B α has not been investigated; PP2A-A β , 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

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

EpH4 cells were infected with supernatants of BOS23 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 G₁/S progression studies, the cells were seeded at a confluent density, arrested in G₀/G₁ 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 G₁/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 MgCl₂, 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 MgCl₂, 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

T β RI, T β RII 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 T β RI and T β RII 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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