Transforming growth factor β1 inhibits mitogen-activated protein kinase induced by basic fibroblast growth factor in smooth muscle cells

Eliane BERROU*, Michaëla FONTENAY-ROUPIE†, Rozenn QUARCK*, Fergus R. McKENZIE‡, Sylviane LÉVY-TOLEDANO*, Gérard TOBELEM* and Marijke BRYCKAERT*§

*Laboratoire de Physiopathologie Cellulaire et Moléculaire des Cellules du Sang et du Vaisseau, INSERM 348, IFR Circulation Lariboisière, Hôpital Lariboisière, 6 rue Guy Patin, 75475 Paris cedex 10, France, †Laboratoire d'Hématologie, Hôpital Cochin, 75679 Paris cedex 14, France, and ‡Centre de Biochimie-CNRS, Université de Nice, 06018 Nice, France

Stimulation of smooth muscle cells with basic fibroblast growth factor (bFGF) results in the activation of the mitogen-activated protein kinase (MAP kinase) cascade and leads to cell proliferation. We show that transforming growth factor β1 (TGF-β1), at concentrations that completely inhibited bFGF-induced mitogenic activity, decreased bFGF-induced MAP kinase activity. Under these conditions, tyrosine and threonine phosphorylations of MAP kinase were differentially affected depending on the time period of TGF-β1 pretreatment. After a short (30 min) TGF-β1 pretreatment, the bFGF-mediated increase in phosphorylation of $p42^{mapk}$ on threonine was inhibited, with no effect on the level of phosphotyrosine or decrease in the electrophoretic mobility of $p42^{mapk}$. This suggests that TGF-β1 inhibited MAP kinase activity through the action of a serine/threonine phosphatase. In contrast, a longer TGF-β1 pretreatment (4 h) partly inhibited the bFGF-induced MAP kinase mobility shift and correlated with the inhibition of phosphorylation on both threonine and tyrosine, suggesting that long-term TGF-β1 treatment prevented activation of the MAP kinase cascade or directly blocked MAP kinase. The ability of long-term (4 h) but not short-term (30 min) TGF-β1 pretreatment to inhibit MAP kinase activity was completely dependent on protein synthesis and suggests that TGF-β1 inhibits MAP kinase activity by two distinct mechanisms. These findings provide a molecular basis for the growth-inhibitory action of TGF-β1 on bFGF-induced mitogenic activity.

INTRODUCTION

Transforming growth factors β (TGF-β) are a family of disulphide-bonded 25 kDa dimeric proteins that modulate the growth of a wide variety of cell types. Three related proteins termed TGF-β1, TGF-β2 and TGF-β3 have been identified in various mammalian cells. TGF-β was originally identified by its ability to stimulate anchorage-independent growth of non-neoplastic fibroblasts [1]. TGF-β also has many other biological effects, including the regulation of cell differentiation, stimulation of extracellular matrix formation and modulation of the immune response [2]. TGF-β exerts its biological effects via binding to specific cell-surface receptors, which include the type I (53 kDa) and the type II (75 kDa) receptors respectively.

If the structural organization of type 1 and type II TGF-β receptors is well established [3–5], little is known about the TGF-β1-induced transduction mechanism [6–9]. TGF-β1 inhibits thrombin- and bFGF-induced DNA synthesis in fibroblasts without affecting phosphoinositide metabolism, activation of protein kinase C or intracellular Ca^{2+} mobilization [10]. In human bone marrow fibroblasts, TGF-β1-mediated inhibition of platelet-derived growth factor (PDGF)-induced proliferation correlates with inhibition of PDGF receptor autophosphorylation and tyrosine kinase activity [11,12]. This inhibitory effect was reversed by an inhibitor of type 1 and type 2A protein phosphatases (okadaic acid) and is apparently correlated with an activation of protein phosphatases [12]. A role for serine/threonine phosphatase activation by TGF-β1 was also suggested to arrest the growth of keratinocytes [13].

Recently the crucial role of mitogen-activated protein (MAP) kinase in signalling pathways involved in cell proliferation has been appreciated [14]. MAP kinase is a serine/threonine kinase that phosphorylates and activates various proteins including c-Myc [15], cytoplasmic phospholipase A_2 [16], p90rsk [17] and the epidermal growth factor (EGF) receptor [18]. Two highly related mammalian MAP kinases, $p44^{mapk}$ and $p42^{mapk}$, also called extracellular signal-regulated kinase 1 (ERK1) and ERK2, have been cloned and found to be ubiquitously expressed and activated by many growth factors and cytokines [19]. Basic fibroblast growth factor (bFGF)-induced mitogenic activity is mediated by the receptor-tyrosine kinase cascade and leads to the activation of MAP kinase. Phosphorylation of both tyrosine (Y185) and threonine (T183) residues [20] is required for full activity. This phosphorylation was mediated by a MAP kinase kinase (MAPKK or MEK). In fibroblasts, a biphasic activation of MAP kinase in response to bFGF [21] has been described. The second phase of activation correlates with the mitogenic activity induced by various growth factors [22], and the suppression of MAP kinase activation inhibits cell proliferation [23].

The objective of this study was to investigate the effect of TGF-β1 on MAP kinase activity induced by bFGF in smooth muscle cells from pig aorta. We show that the inhibition of bFGF mitogenicity by TGF-β1 involves the inhibition of bFGF-activated MAP kinase at the level of threonine and tyrosine

Abbreviations used: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor β1.

§ To whom correspondence should be addressed.

phosphorylation. Also, the inhibition of MAP kinase activity after TGF-β1 pretreatment was prevented by cycloheximide, suggesting that the TGF-β1-induced inhibition requires protein synthesis to modify MAP kinase activity.

EXPERIMENTAL

Materials

Fetal calf serum, Dulbecco's modified Eagle's medium, L-glutamine, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, U.S.A.). Recombinant bFGF was obtained from Biotech Trade Service (St. Leon-Rot, Germany) and TGF-β1 from R&D Systems (U.K.). [*methyl*-^{3}H]Thymidine (74 GBq/mmol) and [γ-^{32}P]ATP (111 TBq/mmol) were from Amersham International (Buckinghamshire, U.K.). BSA, leupeptin, myelin basic protein, pepstatin A and protein A–Sepharose were obtained from Sigma (St. Louis, MO, U.S.A.). Antibody E1B is a rabbit polyclonal antibody raised against a C-terminal peptide of ERK1. Anti-(rat MAP kinase R2) (C-14), a rabbit polyclonal antibody raised against the C-terminal peptide of ERK1, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). MPM-2, a monoclonal antibody that specifically recognizes Thr-183 present in the regulatory region of p42^{mapk} only when Thr-183 is phosphorylated, and anti-phosphotyrosine antiserum were obtained from UBI (Lake Placid, NY, U.S.A.). Nitrocellulose sheets were purchased from Schleicher & Schuell (Dassel, Germany).

Cell culture

Smooth muscle cells were isolated from pig thoracic aorta and cultured as previously described [24]. The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 50 i.u./ml penicillin and 50 μg/ml streptomycin, at 37 °C in an air/CO_2 (19:1) atmosphere. Cultures were rendered quiescent by incubation in serum-free medium containing 0.2% BSA for 48 h. All experiments were performed with cell cultures having had fewer than eight passages.

DNA synthesis

Quiescent smooth muscle cells in six-well plates were pre-incubated with various concentrations of TGF-β1 (0–10 ng/ml) for either 30 min or 4 h before addition of bFGF (5 ng per well). After 30 h, [^{3}H]thymidine (1 μCi/ml) was added for 3 h. Cells were washed in PBS, pH 7.4, then treated with 10% ice-cold trichloroacetic acid for 10 min and lysed in 0.2 M NaOH. Radioactivity was quantified in a β-scintillation liquid counter (Beckman, Fullerton, CA, U.S.A.).

Immune complex MAP kinase assay

Quiescent smooth muscle cells in six-well plates were stimulated with 5 ng/ml bFGF in the presence or absence of various concentrations of TGF-β1 (0–10 ng/ml) for 15 min at 37 °C before lysis in Triton X-100 lysis buffer [50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μM pepstatin A and 1% (w/v) Triton X-100]. Equal amounts of cell lysate were first precleared for 1 h at 4 °C with 1 μl of rabbit serum and Protein A–Sepharose beads. Cell lysates were then immunoprecipitated overnight at 4 °C with 1 μg of polyclonal antibody against MAP kinase. Then protein A-Sepharose beads were added for 1 h. Immune complexes were collected by centrifugation at 12000 ***g*** (4 °C, 15 min) and washed four times in lysis buffer and once with kinase buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol and 10 mM *p*-nitrophenyl phosphate), before being resuspended in 40 μl of kinase buffer containing 10 μg of myelin basic protein, 50 μM unlabelled ATP and 3 μCi of [γ-^{32}P]ATP (5000 c.p.m./pmol) per sample. After 10 min at 30 °C the reaction was stopped by adding 40 μl of 2 × Laemmli's sample buffer, followed by boiling for 5 min. Samples were subjected to electrophoresis on an SDS/polyacrylamide gel [12% (w/v) gel]. The gel was then stained with Coomassie Blue, dried and autoradiographed.

Western blot analysis

Smooth muscle cells in 10 cm dishes were stimulated with bFGF (5 ng/ml) in the presence or absence of various concentrations of TGF-β1 (0–10 ng/ml) for 15 min at 37 °C. The cells were then washed twice with cold PBS and lysed in Triton X-100 lysis buffer. The cell lysates were immunoprecipitated as above with a polyclonal antibody against MAP kinase and were centrifuged at 12000 ***g*** for 15 min at 4 °C. Immune complexes were separated by SDS/PAGE (12% gel) and electroblotted onto nitrocellulose filters. Filters were blocked for 2 h in Tris-buffered saline containing 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 and 5% (w/v) non-fat dried milk and then incubated for 2 h at room temperature with the polyclonal antibody E1B-3 (1:1000), an anti-phosphotyrosine monoclonal antibody (1 μg/ml), or a monoclonal antibody MPM2 (1:2000). Filters were then incubated respectively with either horseradish peroxidase-conjugated goat anti-rabbit IgG (1:50000) or anti-mouse IgG (1:20000) for 1 h. The blots were made visible by using the Amersham enhanced chemiluminescence system, as recommended by the manufacturer.

Statistics

The figures represent the means ± S.E.M. for at least four independent experiments. Statistical significance was assessed with Student's *t*-test for paired comparisons. Where indicated, Figures represent the results from one experiment that was repeated at least three times.

RESULTS

TGF-β1 inhibits bFGF-induced MAP kinase activity

To determine the effect of TGF-β1 on bFGF-induced MAP kinase activity, we initially tested the effect of TGF-β1 on bFGF-induced mitogenic activity at two distinct time points. Under conditions where 10 ng/ml TGF-β1 was added either 30 min or 4 h before 5 ng/ml bFGF, TGF-β1 significantly inhibited the bFGF-induced mitogenic activity by 88.5% and 79.5% respectively ($0.0005 < P \leqslant 0.005$) (Figure 1a). These conditions of treating smooth muscle cells with 10 ng/ml TGF-β1 and 5 ng/ml bFGF were used for subsequent studies.

We then explored the effect of TGF-β1 on bFGF-induced MAP kinase activity after a 15 min stimulation period corresponding to the first phase of activation of p42^{mapk} and p44^{mapk} (results not shown). Smooth muscle cells were pretreated for either 30 min or 4 h with TGF-β1 before the addition of bFGF. Stimulation with bFGF, which induced a significant increase in MAP kinase activity compared with the control without growth factor, was partly inhibited by both a 30 min and a 4 h TGF-β1 pretreatment (Figure 1b). Densitometric measurements confirmed a significant inhibition of bFGF-induced MAP kinase

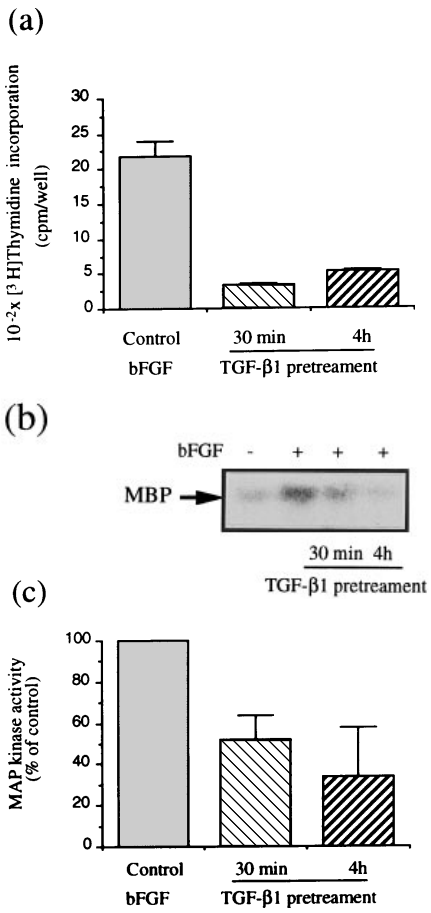

Figure 1 Effect of TGF-β1 on bFGF-induced MAP kinase activity

After 48 h of serum deprivation, smooth muscle cells were first pretreated for two different times (30 min and 4 h) with 10 ng/ml TGF-β1 and then incubated in the presence of 5 ng/ml bFGF. (**a**) After a 30 h stimulation period, 1 μCi/ml [^{3}H]thymidine was added for a 3 h pulse period. The radioactivity incorporated into trichloroacetic acid-insoluble material was determined. Results are expressed as c.p.m. per well and are the means $\pm$ S.E.M. for three separate experiments. (**b**) After a 15 min stimulation period, corresponding to the activation of the first phase of bFGF-induced MAP kinase activity, smooth muscle cells were lysed and cell lysates were immunoprecipitated with an anti-p42^{mapk} antibody, and MAP kinase activity was measured as described in the Experimental section. (**c**) Autoradiograms were scanned with a laser densitometer. For each experiment the control value obtained with bFGF alone was defined as 100%. The results after 30 min (solid bar) or 4 h (hatched bar) of TGF-β1 pretreatment are expressed relative to bFGF-induced MAP kinase activities and are the means $\pm$ S.E.M. for three separate experiments.

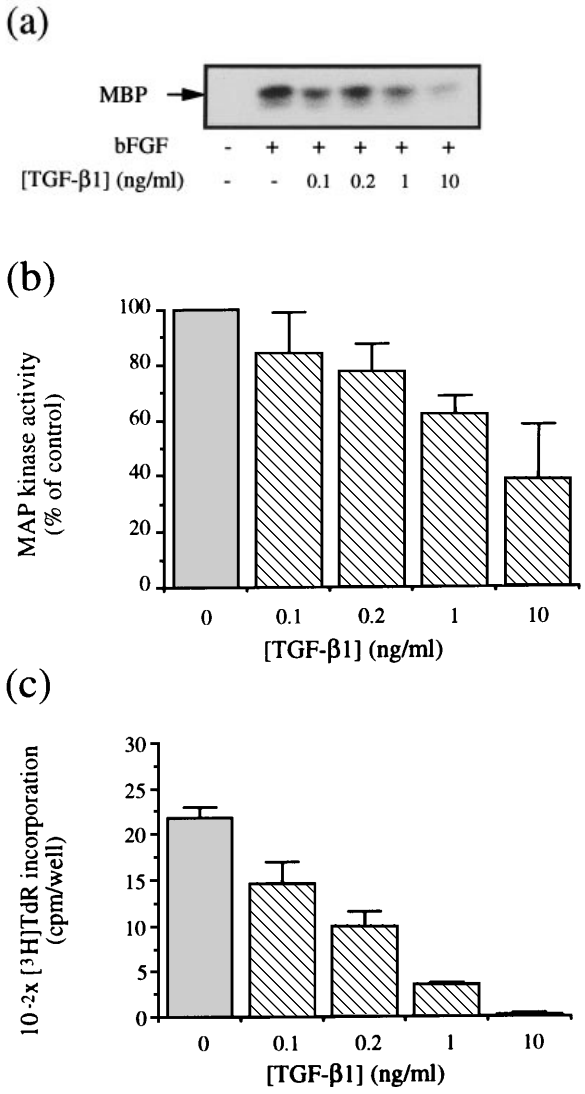

Figure 2 Dose-dependent effect of TGF-β1 inhibition on bFGF-induced MAP kinase activity after 30 min of TGF-β1 pretreatment

After 48 h of serum deprivation, smooth muscle cells were pretreated with various concentrations of TGF-β1 (0–10 ng/ml) for 30 min; 5 ng/ml bFGF was then added and the samples were incubated for 15 min. (**a**) Cells were lysed and lysates were immunoprecipitated with an anti-p42^{mapk} antibody, and MAP kinase activity was measured as described in the Experimental section. MBP, myelin basic protein. (**b**) Autoradiograms were scanned with a laser densitometer. For each experiment the control value obtained with bFGF alone was defined as 100%. The results are expressed relative to bFGF-induced MAP kinase activities and are the means $\pm$ S.E.M. for three separate experiments. (**c**) After a 30 h stimulation period with 5 ng/ml bFGF, 1 μCi/ml [^{3}H]thymidine was added for a 3 h pulse period. The radioactivity incorporated into trichloroacetic acid-insoluble material was determined. Results are expressed as c.p.m. per well and are the means $\pm$ S.E.M. for three separate experiments.

activity (Figure 1c). The remaining activity represented only $52.3 \pm 17.3\%$ ($0.001 < P \leqslant 0.025$) and $34.6 \pm 24.0\%$ ($0.025 < P \leqslant 0.05$) of bFGF-induced MAP kinase activity. These results suggest that pretreatment of smooth muscle cells for either 30 min or 4 h in the presence of TGF-β1 affects bFGF-induced MAP kinase activity.

bFGF-induced MAP kinase activity is modulated by the concentration of TGF-β1 used for pretreatment

To confirm the effect of TGF-β1 pretreatment on MAP kinase activation we investigated the effect of various concentrations of TGF-β1 (0.1–10 ng/ml) on bFGF-induced MAP kinase activity. After a 30 min pretreatment with low concentrations of TGF-β1 (0.1 or 0.2 ng/ml), no detectable change in bFGF-induced MAP kinase activity was observed (Figures 2a and 2b), indicating that there was no significant effect of low concentrations of TGF-β1 on bFGF-induced MAP kinase activity. In contrast, increasing the concentration of TGF-β1 significantly inhibited bFGF-induced MAP kinase activity. The remaining activity represented $62.3 \pm 6.3\%$ with 1 ng/ml TGF-β1 ($0.0005 < P \leqslant 0.005$) and $38.7 \pm 20.4\%$ with 10 ng/ml TGF-β1 ($0.01 < P \leqslant 0.025$). To determine a possible relation between MAP kinase activity and mitogenic activity, we investigated the effect of different concentrations of TGF-β1 on the bFGF-induced increase in DNA

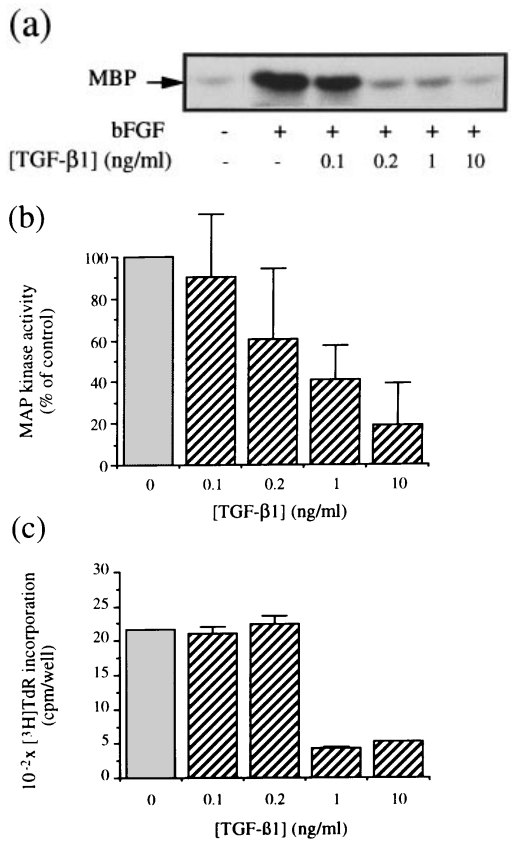

Figure 3 Dose-dependent effect of TGF-β1 inhibition on bFGF-induced MAP kinase activity after 4 h of TGF-β1 pretreatment

After 48 h of serum deprivation, smooth muscle cells were pretreated with various concentrations of TGF-β1 (0–10 ng/ml) for 4 h; 5 ng/ml bFGF was then added and the samples were incubated for 15 min. (**a**) Smooth muscle cells were lysed and cell lysates were immunoprecipitated with an anti-p42^{mapk} antibody, and MAP kinase activity was measured as described in the Experimental section. MBP, myelin basic protein. (**b**) Autoradiograms were scanned with a laser densitometer. For each experiment the control value obtained with bFGF alone was defined as 100%. The results are expressed relative to bFGF-induced MAP kinase activities and are the means $\pm$ S.E.M. for four separate experiments. (**c**) After a 30 h stimulation period with 5 ng/ml bFGF, 1 μCi/ml [^{3}H]thymidine was added for a 3 h pulse period. The radioactivity incorporated into trichloroacetic acid-soluble material was determined. Results are expressed as c.p.m. per well and are the means $\pm$ S.E.M. for three separate experiments.

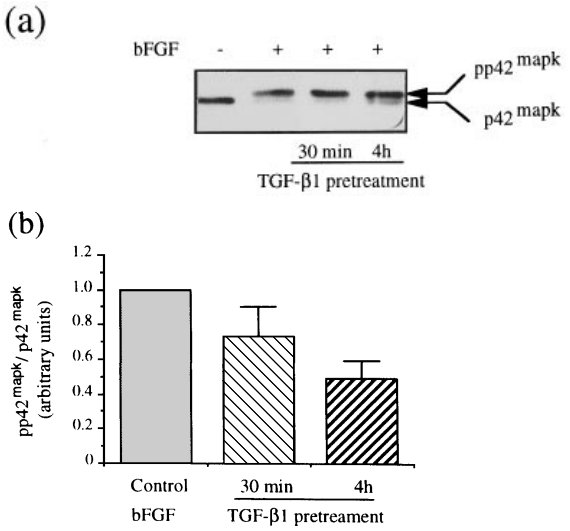

Figure 4 Effect of TGF-β1 pretreatment on the mobility shift of bFGF-induced MAP kinase

After 48 h of serum deprivation, smooth muscle cells were first pretreated for two different times (30 min and 4 h) with 10 ng/ml TGF-β1 and then incubated in the presence of 5 ng/ml bFGF. Smooth muscle cells were lysed and cell lysates were immunoprecipitated with an anti-p42^{mapk} antibody. (**a**) Immunoprecipitates were reduced and subjected to SDS/PAGE (12% gel) and Western blotting with an anti-p42^{mapk} and anti-p44^{mapk} antibody. (**b**) Autoradiograms were scanned with a laser densitometer. For each experiment the ratio of phosphorylated MAP kinase pp42^{mapk} to unphosphorylated MAP kinase p42^{mapk} was normalized to that of cells treated with bFGF alone and is expressed as the relative intensity. Results are the means $\pm$ S.E.M. for three separate experiments.

synthesis, as measured by [^{3}H]thymidine incorporation. Increased concentrations of TGF-β1 (0.1–10 ng/ml), added 30 min before bFGF, significantly inhibited the bFGF-induced mitogenic activity by 33–100% (Figure 2c), suggesting that TGF-β1 was a more potent inhibitor of bFGF-mediated [^{3}H]thymidine incorporation than of p42^{mapk} activation. The ability of TGF-β1 to inhibit bFGF-induced mitogenic activity was most pronounced at 1 and 10 ng/ml TGF-β.

To validate the hypothesis that 4 h of TGF-β1 pretreatment inhibited bFGF-induced MAP kinase activity, we analysed MAP kinase activity in the presence of various concentrations of TGF-β1 (0.1–10 ng/ml). No detectable change in bFGF-induced MAP kinase activity was observed in the presence of low concentrations of TGF-β1 (0.1 and 0.2 ng/ml) (Figures 3a and 3b). In contrast, increased concentrations of TGF-β1 significantly inhibited bFGF-induced MAP kinase activity. The remaining activity represented 41.3 $\pm$ 16.3% with 1 ng/ml TGF-β1 ($0.025 < P \leqslant 0.05$) and 19.1 $\pm$ 20.4% at 10 ng/ml ($0.01 < P \leqslant 0.025$) (Figures 3a and 3b). Under the same conditions that were used to test MAP kinase activity, we investigated the effect of different concentrations of TGF-β1 after 4 h of TGF-β1 pretreatment on bFGF-induced mitogenic activity (Figure 3c). No modification of bFGF-induced mitogenic activity was observed in the presence of low concentrations of TGF-β1 (0.1 and 0.2 ng/ml). In contrast, increased concentrations of TGF-β1 (1 and 10 ng/ml) significantly inhibited the bFGF-induced mitogenic activity by 78% and 76% ($0.0005 < P \leqslant 0.005$). Thus TGF-$\beta$1 at a concentration of both 1 and 10 ng/ml potently inhibits bFGF-induced mitogenic activity. In addition, the TGF-β1-mediated inhibition of MAP kinase activity was equivalent, irrespective of the time of TGF-β1 pretreatment.

Different effects of TGF-β1 pretreatment on the bFGF-induced mobility shift of p42^{mapk}

To identify a possible relationship between the TGF-β1-mediated inhibition of MAP kinase activity and a reduction in the level of MAP kinase phosphorylation, we investigated the state of phosphorylation of MAP kinase, which correlates with a reduction in the electrophoretic mobility of p42^{mapk} and p44^{mapk}. Smooth muscle cells were pretreated for either 30 min or 4 h with 10 ng/ml TGF-β1 before addition of 5 ng/ml bFGF. The lysate proteins were immunoprecipitated with anti-p42^{mapk} and then analysed by Western blotting by using an anti-(MAP kinase) antiserum that recognized p42^{mapk} and p44^{mapk}. After 15 min of bFGF stimulation there was a decrease in electrophoretic mobility of p42^{mapk} (Figure 4a); 30 min of TGF-β1 pretreatment was unable to modify the reduction in electrophoretic mobility of p42^{mapk} induced by bFGF alone, whereas 4 h of TGF-β1 pretreatment increased the proportion of unphosphorylated MAP kinase (Figure 4a). Densitometric measurements confirmed

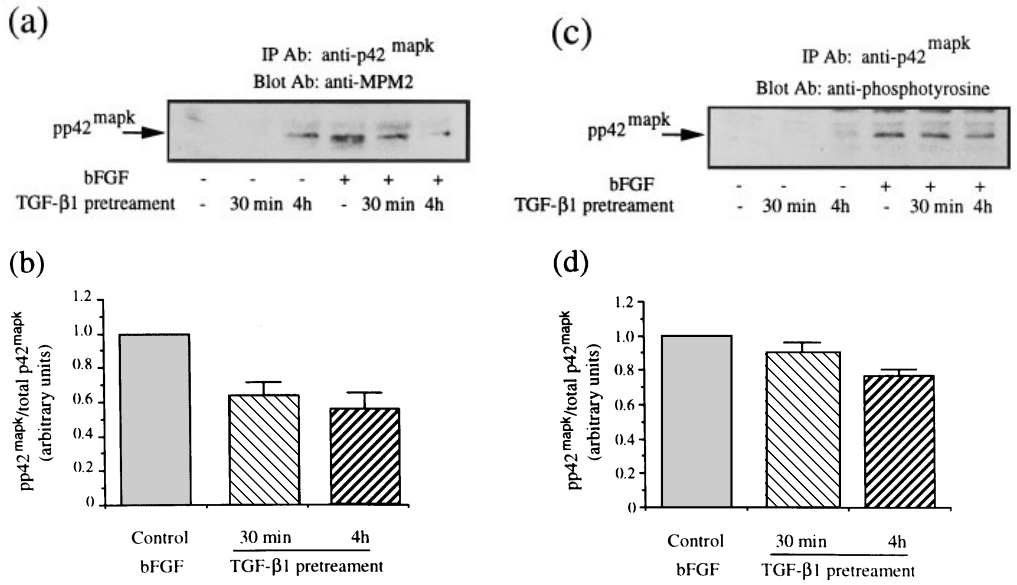

Figure 5 Effect of TGF-β1 pretreatment on bFGF-induced MAP phosphorylation

After 48 h of serum deprivation, smooth muscle cells were first pretreated for different times (30 min and 4 h) with 10 ng/ml TGF-β1 and then incubated in the presence of 5 ng/ml bFGF. Smooth muscle cells were lysed and cell lysates were immunoprecipitated with an anti-p42^{mapk} antibody. Immunoprecipitates were reduced and subjected to SDS/PAGE (12% gel) and Western blotting: with an anti-MPM2 antibody (**a**) and with an anti-phosphotyrosine antibody (**c**). Autoradiograms were scanned with a laser densitometer (**b** and **d**). For each experiment the ratio of phosphorylated MAP kinase (pp42^{mapk}) to total MAP kinase (p42^{mapk}) was normalized to that of cells treated with bFGF alone and is expressed as the relative intensity. The results are expressed relative to bFGF-induced MAP kinase phosphorylation and are the means $\pm$ S.E.M. for seven separate experiments.

these results: first, after 30 min of TGF-β1 pretreatment the ratio of phosphorylated MAP kinase to unphosphorylated MAP kinase was 0.86 ± 0.17 ($0.05 < P \leqslant 0.1$); secondly, after 4 h of TGF-β1 pretreatment this ratio decreased to 0.49 ± 0.10 ($0.01 < P \leqslant 0.025$) (Figure 4b). These results suggest that, although a 30 min TGF-β1 pretreatment did not modify the ability of bFGF to augment the level of phosphothreonine and phosphotyrosine on MAP kinase as determined by the electrophoretic mobility of MAP kinase, a 4 h TGF-β1 pretreatment did promote a decrease in the level of phosphothreonine and phosphotyrosine on MAP kinase.

Different effects of TGF-β1 pretreatment on tyrosine and threonine phosphorylations of p42^{mapk}

Phosphorylation of MAP kinase on either threonine or tyrosine is sufficient to induce a decrease in electrophoretic mobility [25]. Hence to test the hypothesis that one or two of the amino acids were affected, we investigated the state of phosphorylation of both threonine and tyrosine induced by bFGF after TGF-β1 pretreatment. Smooth muscle cells were pretreated for either 30 min or 4 h with 10 ng/ml TGF-β1 before addition of 5 ng/ml bFGF. The lysate proteins were immunoprecipitated with an anti-p42^{mapk} and then analysed by Western blotting by using either anti-MPM2 antibody, which recognizes the phosphothreonine epitope of MAP kinase, or an anti-phosphotyrosine antibody. After 15 min of bFGF stimulation there was an induction of the threonine phosphorylation of p42^{mapk}, whereas no phosphorylation was observed in quiescent unstimulated smooth muscle cells and smooth muscle cells pretreated for 30 min with TGF-β1 alone (Figure 5a). In the presence of TGF-β1, bFGF induces to a smaller extent the threonine phosphorylation of p42^{mapk}, which was confirmed by densitometric measurements (Figures 5a and 5b). After either 30 min or 4 h of TGF-β1 pretreatment, the ratios of phosphothreonine on MAP kinase to total MAP kinase were 0.64 ± 0.07 ($0.0005 < P \leqslant 0.005$) and 0.57 ± 0.10 ($0.0005 < P \leqslant 0.005$) respectively. Thus pretreatment of smooth muscle cells for both 30 min and 4 h with TGF-β1 decreased bFGF-induced threonine phosphorylation of p42^{mapk}. The fact that 4 h of TGF-β1 alone induced a threonine phosphorylation of p42^{mapk} suggested that different mechanisms could be implicated in the presence or absence of bFGF.

We next performed a similar series of experiments with an anti-phosphotyrosine antibody (Figures 5c and 5d). Under these conditions, bFGF induced a significant increase in the level of tyrosine phosphorylation of p42^{mapk} compared with unstimulated smooth muscle cells or with 30 min and 4 h of TGF-β1 pretreatment alone. After 30 min of pretreatment, TGF-β1 did not significantly modify the tyrosine phosphorylation of p42^{mapk}; the ratio of phosphotyrosine MAP kinase to total MAP kinase was 0.91 ± 0.06 after 30 min of TGF-β1 pretreatment, whereas after 4 h a small but significant inhibition (0.78 ± 0.04; $0.0005 < P \leqslant 0.005$) was observed. These results indicate that (1) treatment of smooth muscle cells for 30 min with TGF-β1 diminished the bFGF-induced phosphorylation of p42^{mapk} on threonine, and (2) treatment of smooth muscle cells for 4 h inhibited both threonine and tyrosine phosphorylations, suggesting the presence of an additional mechanism at later time points.

TGF-β1 pretreatment induces protein synthesis that could inhibit MAP kinase activity

To explore whether TGF-β1 mediated inhibition of MAP kinase requires protein synthesis, MAP kinase activity was examined after TGF-β1 pretreatment in the presence of 100 μg of cycloheximide per well (Figures 6a and 6b). Under these conditions, 30 min of TGF-β1 pretreatment inhibited bFGF-stimulated MAP kinase activity ($51.5 \pm 17.5\%$; $0.025 < P \leqslant 0.05$). In con-

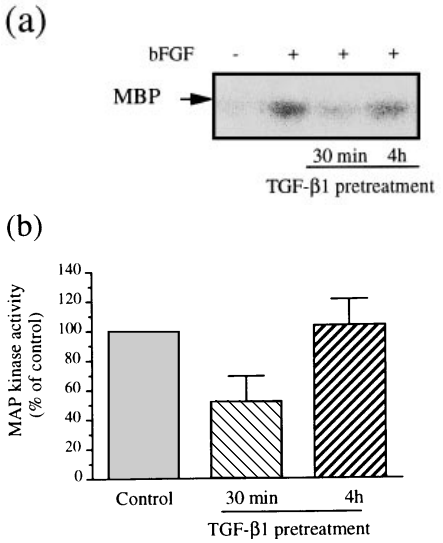

Figure 6 Effect of TGF-β1 pretreatment on bFGF-induced MAP kinase activity in the presence of cycloheximide

After 48 h of serum deprivation, smooth muscle cells were pretreated with 10 ng/ml TGF-β1 for 30 min or 4 h in the presence of 100 μg/ml cycloheximide. bFGF was then added and samples were incubated for 15 min. Smooth muscle cells were lysed and cell lysates were immunoprecipitated with an anti-p42^{mapk} antibody and MAP kinase activity was measured as described in the Experimental section (**a**). MBP, myelin basic protein. (**b**) Autoradiograms were scanned with a laser densitometer. For each experiment the control value obtained with bFGF alone was defined as 100%. The results after 30 min or 4 h of TGF-β1 pretreatment are expressed as percentages of the control bFGF and are the means $\pm$ S.E.M. for three separate experiments.

trast, after 4 h of TGF-β1 pretreatment in the presence of cycloheximide, complete restoration of MAP kinase activity was observed ($102.7 \pm 17.6\%$) compared with the control bFGF without TGF-β1. This suggests that two different mechanisms are involved in the inhibition of MAP kinase activity. Thus, after 30 min of TGF-β1 pretreatment, inhibition of MAP kinase activity occurred without protein synthesis, whereas after 4 h of TGF-β1 treatment this inhibition was fully dependent on protein synthesis.

DISCUSSION

We have investigated the effect of TGF-β1 on the bFGF-induced signal transduction mechanism directly implicated in cell proliferation. The transduction mechanism induced by TGF-β1 is as yet poorly defined, but here we present evidence to support the proposal that TGF-β1 inhibits bFGF-induced MAP kinase activity. Our results demonstrate a significant inhibition of the first phase of MAP kinase activity, which could participate in the inhibition of mitogenic activity. Surprisingly, depending on the period of TGF-β1 pretreatment, phosphorylations of MAP kinase on tyrosine and threonine were differently affected. In addition, after 4 h of TGF-β1 pretreatment, this MAP kinase activity was completely restored in the presence of cycloheximide, suggesting a role for TGF-β1-induced protein synthesis in the inhibition of MAP kinase activity. However, after 30 min of TGF-β1 pretreatment, the addition of cycloheximide did not abolish the inhibition of MAP kinase. Thus after 30 min and 4 h of TGF-β1 pretreatment, different phosphatases and different mechanisms of inhibition were implicated.

Previous reports have shown that MAP kinase activation is required for growth factor-stimulated cells to progress and enter S-phase [21–23]. For this reason we first examined the effect of TGF-β1 on bFGF-induced sustained activation of MAP kinase [26]. After either 30 min or 4 h of incubation, TGF-β1 inhibited MAP kinase activity induced by bFGF. This inhibition of MAP kinase activity was observed for TGF-β1 concentrations that were able to inhibit bFGF-induced mitogenic activity completely. These results suggest three possible mechanisms of inhibition: (1) an upstream inhibition of the MAP kinase cascade resulting from an inhibition of MAP kinase activity, (2) an inhibition of the phosphorylation of threonine and/or tyrosine required for full MAP kinase activity or (3) the presence of an inhibitor that blocks MAP kinase activity.

As phosphorylation of both tyrosine and threonine is required for full MAP kinase activity, we initially investigated the decrease in electrophoretic mobility of MAP kinase, which is directly correlated with the state of phosphorylation of MAP kinase [25]. The fact that TGF-β1, at concentrations that inhibit MAP kinase activity and after 30 min of pretreatment, had no inhibitory effect on the reduction of electrophoretic mobility suggests that TGF-β1 did not interfere with upstream signalling from MAP kinase but acted directly on MAP kinase. This suggests an apparently novel mechanism of MAP kinase inhibition. It has previously been noted that the MAP kinase cascade might be inhibited at the level of the upstream activator, Raf-1, resulting in a decrease in MAP kinase phosphorylation and hence MAP kinase activity [17–20]. Inhibition of PDGF-BB-induced MAP kinase signalling by protein kinase A occurs between the receptor and MAP kinase kinase [27]. Elevation of intracellular cAMP levels abrogated the first phase of EGF-induced MAP kinase activity, which was correlated with growth arrest [28,29]. In addition, increased concentrations of cAMP block EGF-induced activation of Raf-1 and consequently MAP kinase activation in Rat1hER fibroblasts [30].

The observation that 30 min of TGF-β1 pretreatment did not affect the decrease in electrophoretic mobility of MAP kinase did not exclude the possibility that (1) phosphorylation of one of the two regulatory amino acids (tyrosine or threonine) was affected and/or (2) TGF-β1 could act directly on MAP kinase activity. To test the hypothesis that TGF-β1 could induce an activation of a phosphatase, we investigated tyrosine and threonine phosphorylations of MAP kinases after p42^{mapk} immunoprecipitation. As TGF-β1 inhibited the bFGF-mediated increase in threonine phosphorylation of MAP kinase without any effect on tyrosine, an upstream inhibition of the MAP kinase cascade is not involved, which suggests that the induction of serine/threonine phosphatase activity might have occurred. Several reports have described the induction of phosphatase activity by TGF-β1 [12,13]. TGF-β1 has been shown to up-regulate serine/threonine phosphatase activity in keratinocytes [13] and modulate PDGF-induced mitogenic activity and phosphorylation of the PDGF receptor via activation of serine/threonine phosphatases [12]. In our study, activation of serine/threonine phosphatases was perhaps involved in the inhibition of MAP kinase activity. These results were in agreement with those of Gruppuso et al. [13], who showed that incubation of keratinocytes with TGF-β1 rapidly activated (within 30 min) protein serine/threonine phosphatases, whereas tyrosine phosphatases were activated only after 48 h [13]. In addition, different types of phosphatase specific for MAP kinases have been described in the literature. *In vitro*, 3CH134, CL100 and HVH1 seemed to be dual-specific tyrosine/threonine phosphatases that were highly specific for MAP kinases [30–34]. The 3CH134 gene was originally identified as an immediate-early gene induced by serum in mouse fibroblasts [30]. Recently this phosphatase was

described to regulate MAP kinase in smooth muscle cells [31]. In our experiments, after 30 min of TGF-β1 pretreatment and where only the level of phosphothreonine present on MAP kinase was affected, this phosphatase was not implicated. The inactivation of MAP kinase could have involved a dual-specificity phosphatase. Protein phosphatase 2A can dephosphorylate Thr-183 in p42^{mapk} *in vitro*, and treatment of cells with okadaic acid, a potent inhibitor of serine/threonine phosphatases PP2A and PP1, leads to a transient activation of MAP kinase [35]. A recent report described a rapid inactivation of p42^{mapk} within 30 min of stimulation with growth factors, which was catalysed by a protein phosphatase 2A [36]. In addition, during interphase of the first mitotic cycle in *Xenopus*, when MAP kinase is dephosphorylated and inactive, a MAP kinase phosphatase with the properties of the catalytic subunit of PP2A was purified from extracts [37].

After 4 h of TGF-β1 pretreatment, TGF-β1 partly inhibited the mobility shift of MAP kinase, an effect correlated with the inhibition of tyrosine and threonine phosphorylation. This suggests that additional phosphatases are involved in the TGF-β1 mechanism of inhibition of MAP kinase activity. Moreover, the fact that 4 h of TGF-β1 was by itself sufficient to induce a threonine phosphorylation of MAP kinase suggested that these phosphatases could be activated in the presence of bFGF. These results do not exclude the possibility that TGF-β1 induces inhibition of the MAP kinase cascade upstream of MAP kinase or a direct inhibition of the phosphorylation of threonine and tyrosine of MAP kinase. The hypothesis that another mechanism was involved after 4 h of TGF-β1 pretreatment was boosted by the complete restoration of MAP kinase activity after 4 h in the presence of cycloheximide. This suggested that early protein synthesis was involved. Because 30 min in the presence of cycloheximide did not restore MAP kinase activity, this mechanism of inhibition probably did not require protein synthesis. However, we have to take into account that such cycloheximide treatment could prevent the production of dual-specificity MAP kinase phosphatases, thus leading to a potentiation of MAP kinase activity [30].

Although this study has not established the nature of the phosphatases acting on MAP kinase activity that are induced by TGF-β1, the results show that TGF-β1 can attenuate the bFGF-induced MAP kinase activation pathway by two different mechanisms involving phosphatases. These phosphatases remain to be identified. Moreover these findings suggest that the effect of TGF-β1 on MAP kinase activity can be regarded as a general inhibitory mechanism on growth factor-induced mitogenic activity.

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