TNFα induces sequential activation of Cdc42- and p38/p53-dependent pathways that antagonistically regulate filopodia formation

Gilles Gadea, Lauréline Roger, Christelle Anguille, Marion de Toledo, Véronique Gire and Pierre Roux*

Centre de Recherche en Biochimie Macromoléculaire, CNRS FRE 2593, IFR 24, 1919 route de Mende, 34293 Montpellier CEDEX 5, France

*Author for correspondence (e-mail: pierre.roux@crbm.cnrs.fr)

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Summary

Cell migration is an essential function in various physiological processes, including tissue repair and tumour invasion. Repair of tissue damage requires the recruitment of fibroblasts to sites of tissue injury, which is mediated in part by the cytokine tumour necrosis factor α (TNFα). As dynamic rearrangements of actin cytoskeleton control cell locomotion, this implicates that TNFα is a potent coordinator of cellular actin changes. We have investigated the role of TNFα in regulating the cortical actin-containing structures essential for cell locomotion called filopodia. Kinetic analysis of TNFα-treated mouse embryonic fibroblasts (MEFs) revealed a dual effect on filopodia formation: a rapid and transient induction mediated by Cdc42 GTPase that is then counteracted by a subsequent sustained inhibition requiring activation of the mitogen-activated protein kinase p38 but not Cdc42 activity. This inhibition also involves the tumour suppressor p53, given that it is activated in response to TNFα following the same time course as the decrease of filopodia formation. This functional activation of p53, measured by transcription induction of its target p21^{WAF1}(p21), is also associated with p38 kinase-dependent phosphorylation of p53 at serine 18. Furthermore, TNFα did not inhibit filopodia formation in MEFs treated with the transcription inhibitor actinomycin D, in p53-deficient MEFs, or MEFs expressing p53 mutants H273 or H175, which supports a role for the transcriptional activity of p53 in mediating TNFα-dependent filopodia inhibition. Our data delineate a novel inhibitory pathway in which TNFα prevents filopodia formation and cell migration through the activation of the mitogen-activated protein kinase (MAPK) p38, which in turn activates p53. This shows that TNFα on its own initiates antagonistic signals that modulate events linked to cell migration.

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Key words: p53, TNFα, Filopodia, p38 kinase, Cdc42, Phosphorylation

Introduction

The pro-inflammatory cytokine tumour necrosis factor α (TNFα) elicits pleiotropic biological responses, including lymphocyte and leukocyte activation, cell proliferation, differentiation and apoptosis. TNFα favours the immune response, in part by promoting lymphocyte and macrophage migration towards sites of infection. TNFα also induces cell migration of epithelial (Rosen et al., 1991) and fibroblastic cells (Postlethwaite and Seyer, 1990). While the mechanism by which TNFα regulates cell migration is still poorly understood, recent reports show that TNFα regulates F-actin-containing membrane protrusions known as filopodia in different cell types, including macrophages (Peppelenbosch et al., 1999; Puls et al., 1999) and fibroblasts (Gadea et al., 2002). Filopodia are linked to cell migration, where their role is presumably to sense the surrounding extracellular environment, providing spatial information to direct movement. Filopodia are usually generated during various physiological process, for example growth cone extension in neurons (Kater and Rehder, 1995) and migration of primary mesenchymal cells in sea urchin (McClay, 1999), as well as different cell types such as macrophages (Allen et al., 1997), primary embryo fibroblasts (Nobes and Hall, 1999) and astrocytes (Etienne-Manneville and Hall, 2001). Protrusion of filopodia appears to be tightly regulated by the Rho GTPase Cdc42 (Kozma et al., 1995; Nobes and Hall, 1995) (reviewed by Ridley et al., 1999), which probably stimulates actin polymerisation and thereby induces extension of filopodia.

TNFα participates in regulation of the actin cytoskeleton through specific targets, some of which have been identified. TNFα has been shown to stimulate filopodia formation through the activation of Cdc42 in fibroblasts (Puls et al., 1999) and in endothelial cells (Wojciak-Stothard et al., 1998). However, others reports have also clearly established that TNFα inhibits filopodia formation through a mechanism requiring the stress activated mitogen-activated protein kinase (MAPK) p38 in macrophages (Peppelenbosch et al., 1999) or the tumour suppressor p53 in fibroblasts (Gadea et al., 2002).

In an attempt to reconcile this apparent discrepancy and to clarify how TNFα affects filopodia formation, we analysed the signalling pathway connecting the membrane to p53-dependent filopodia inhibition in mouse embryonic fibroblasts

(MEFs). Our results delineate a novel inhibitory pathway in which TNFα blocks Cdc42-induced filopodia through the activation of p38 kinase that in turn activates transcriptional activity of p53, along with its phosphorylation at serine 18.

Materials and Methods

DNA constructs and reagents

pEGFPC1 p53-wt, pEGFPC1 p53-H273 and pEGFPC1 p53-H175 were obtained as previously described (Gadea et al., 2002). Constructs expressing myc epitope-tagged mutant Rac1, Cdc42 and RhoA proteins and their various mutants were kindly provided by P. Chavrier (Institute Curie, Paris, France) (Dutartre et al., 1996). The green fluorescent protein (GFP) fusion proteins were cloned in the pEGFP-C1 vector (Gauthier-Rouviere et al., 1998; Ory et al., 2000; Roux et al., 1997). The pCDNA3myc-NWASP containing the Cdc42-interacting domain of Wiskott-Aldrich syndrome protein (WASP) was previously described (Philips et al., 2000). The HA-tagged p38 kinase expressing vector (pECE-HA-p38) was a kind gift of A. Brunet (Institute for Biomedical Research, Boston, MA), and HA-MKK3 was from R. Davis (NCI, Frederick, MD). The pGL2B-mdm2 plasmid in which the reporter gene luciferase is controlled by the p53-responsive element of mdm2 (Barak et al., 1994) was a kind gift of E. Yonish-Rouach (CNRS/CEA, Fontenay-aux-Roses, France). pTKRL plasmid was from Promega. TNFα (Sigma) was used in all experiments at the concentration of 100 ng ml^{-1} on starved cells to avoid interference with serum. Actinomycin D (Sigma) was used at the concentration of 5 μg ml^{-1}.

Cell extracts, western blotting

Cells were lysed and analysed by immunoblotting as described previously (Zugasti et al., 2001). Each lane contained equal amounts of loaded proteins (40 μg). The primary antibodies used were anti-phospho-specific (serine 15) p53 (Ab-3; Oncogene research products), anti-p21 (C-19; Santa Cruz Biotechnology), anti-p53 (Pab 421; Ab-1; Oncogene Science), anti-GFP (Pab TP401, Clinisciences) and anti-p38 (Pab 9212, Cell Signaling). Gel electrophoresis, protein transfer and signal detection were performed as described previously (Gadea et al., 2002)

Cell culture and transfection

MEFs and $p53^{-/-}$MEFs were generated as previously described (Gadea et al., 2002) and were cultured at 37°C in the presence of 5% CO_2 in DMEM medium supplemented with 10% fetal calf serum (FCS). For immunofluorescence experiments, cells were plated on 18 mm diameter glass coverslips for 16-24 hours. Cells were transfected on coverslips at the confluence of approximately 30% by the lipofectamine method (0.1-1 μg of plasmid DNA per 35 mm diameter well containing three glass coverslips), as recommended by the supplier (Gibco-BRL). Four hours after the transfection, the medium was replaced by DMEM supplemented with 10% FCS. Expressing cells were observed under fluorescence microscope 6 to 10 hours after transfection.

Immunofluorescence and filopodia dynamic measurements

Six to ten hours after transfection, cells were fixed for 5 minutes in 3.7% formalin (in PBS); this was followed by a 5 minute permeabilisation with 0.1% Triton-X100 (in PBS) and incubation in PBS containing 0.1% BSA. Expression of GFP-tagged proteins was directly visualised, while expression of HA epitope-tagged proteins was visualised after 60 minutes incubation with the 12CA5 anti-HA monoclonal antibody (gift from D. Mathieu, CNRS, Montpellier, France) (1:2 dilution in PBS/BSA), followed by incubation with affinity-purified fluorescein-conjugated goat anti-mouse antibody (Cappel-ICN) (1:40 dilution). Cells were simultaneously stained for F-actin using rhodamine-conjugated phalloidin (0.5 U ml^{-1}) (Sigma). Cells were washed in PBS, and then mounted in Mowiol (Aldrich). To consider a cell having filopodia, we evaluated the number of filopodia on its surface: only F-actin stained cells displaying at least five filopodia were scored as being positive. Observations and analysis were performed as already described (Gadea et al., 2002)

Time-lapse imaging

Time-lapse differential interference contrast (DIC) light microscopy was performed on a Leica DL IRBE (Leica, Wetzlar, Germany) inverted microscope equipped with an automatic shutter and DIC filter sets, a 63× oil-immersion objective (NA1.3, Leica) sample heater (37°C) and a home-made CO_2 incubation chamber. Images were captured with a MicroMax 1300 CCD camera (RS-Princeton Instruments, Treuton, PA) imaging software, converted to TIFF files that were edited with NIH Image and compiled into QuickTime movies. The exposure time was set to 50 milliseconds.

Cdc42 activity assay

The Cdc42 activity assay was performed as described (Ory et al., 2000). Briefly, 3×10^5 cells, treated or not with TNFα, were lysed before incubation with GST-PAK fusion protein the Cdc42-binding domain (CRIB) from human PAK1B (amino acids 56-272) coupled to glutathione-Sepharose beads (Pharmacia Biotech). After precipitation, beads were washed four times with lysis buffer, eluted in SDS-PAGE sample buffer, immunoblotted with antibodies against Cdc42 (Transduction Laboratories). An aliquot of the total lysate used for precipitation was run alongside to quantify total Cdc42 GTPase present in cell lysates.

p53 transactivation assay

p53 transactivation was measured using the dual-luciferase assay system from Promega as already described (Gadea et al., 2002). Cells (5×10^4) were seeded onto 12-well plates and transfected 18 hours later in OptiMEM containing 0.440 μg of DNA (0.2 μg of appropriate plasmids, 0.2 μg of pGL2B-mdm2-luciferase plasmid and 0.04 μg of pTKRL plasmid) using 0.33 μl of Lipofectamine (Gibco-BRL) for 4 hours. Cells were then left for 10-14 hours in DMEM supplemented with 10% FCS, harvested in 250 μl of Passive Lysis Buffer (PLB, Promega), and luciferase activity was measured following the Dual-luciferase™ Reporter Assay Protocol as recommended by Promega, using a luminometer fitted with two injectors (Berthold).

p38 kinase assay

Cells, cultured in 60-mm dishes, were transfected or cotransfected with 2 μg of HA-p38 (provided by B. Derijard, Université Nice, France), Cdc42-N17 or N-Wasp, then serum-deprived for 10-12 hours before TNFα treatment for the indicated time point. Cells were washed in cold PBS, then lysed and analysed for p38 kinase activity as previously described (Philips et al., 2000). Cells were treated with SB 203580 at 10 μM.

Results

Cdc42 is required for rapid induction of TNFα-mediated filopodia formation but not for their subsequent decrease

To clarify the role of TNFα in filopodia formation, we first analysed the morphological changes associated with F-actin reorganisation during 8 hours of TNFα treatment in serum-arrested MEFs. As shown in Fig. 1A, TNFα rapidly induced the

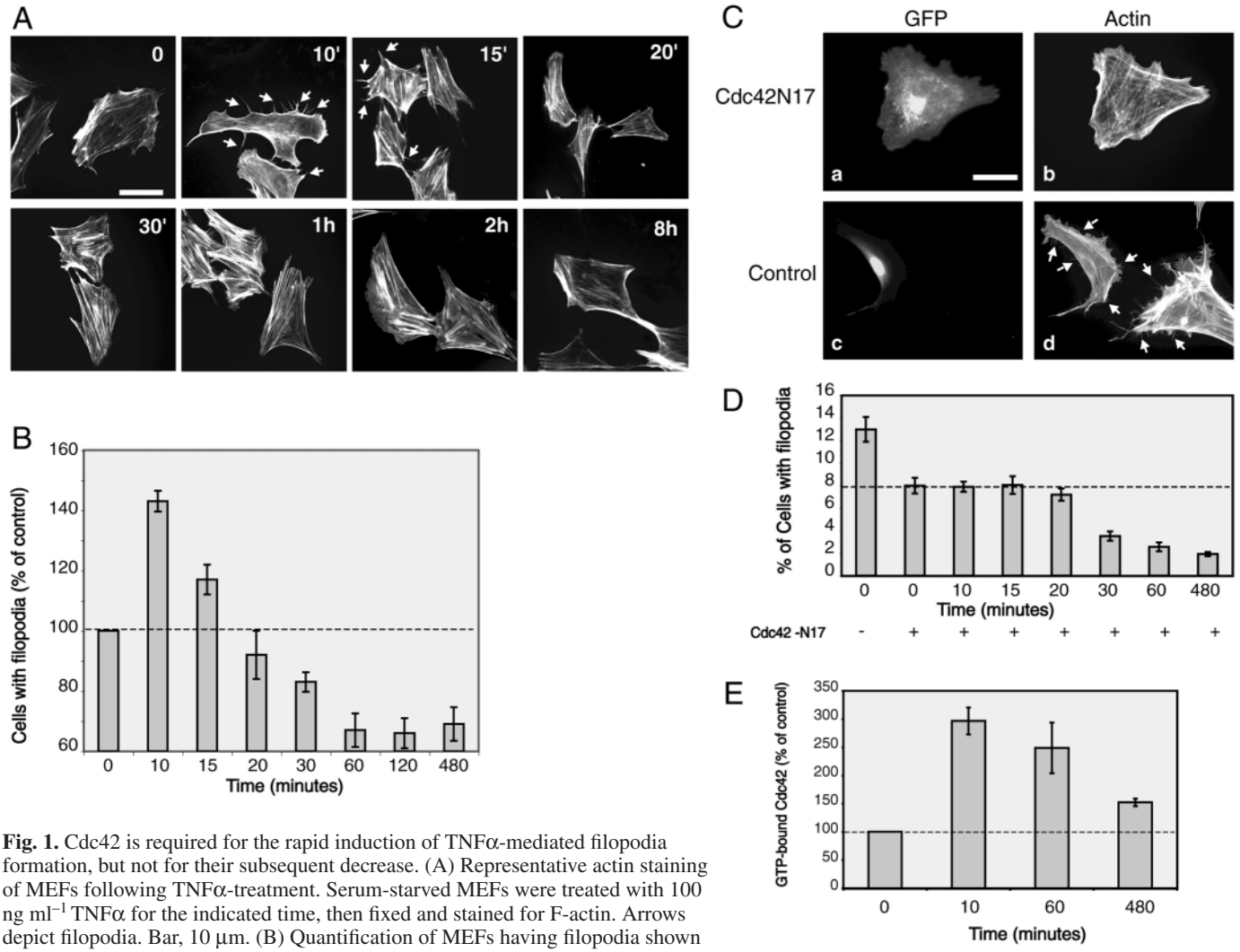


Fig. 1. Cdc42 is required for the rapid induction of TNFα-mediated filopodia formation, but not for their subsequent decrease. (A) Representative actin staining of MEFs following TNFα-treatment. Serum-starved MEFs were treated with 100 ng ml^{-1} TNFα for the indicated time, then fixed and stained for F-actin. Arrows depict filopodia. Bar, 10 μm. (B) Quantification of MEFs having filopodia shown in (A). The percentages of filopodia-positive cells following TNFα-treatment, relative to control cells, are shown. Values are means±s.d. of three independent experiments. Significance in induction of filopodia formation caused by TNFα compared with untreated: P=0.016 (Student's t-test). (C) Effect of inhibition of Cdc42 activity on TNFα-induced filopodia formation. MEFs were transfected with either GFP-tagged Cdc42-N17 (dominant negative form of Cdc42) (a and b) or with the control plasmid pEGFP (c and d). Serum-arrested transfected MEFs were treated with 100 ng ml^{-1} TNFα for 10 minutes, then fixed and analysed for F-actin organisation (b and d) and GFP staining (a and c). Arrows depict filopodia. Bar, 10 μm. (D) Quantification of Cdc42-N17 transfected MEFs having filopodia. MEFs transfected with GFP-tagged Cdc42-N17 were treated as in (A) with TNFα for the indicated times. The percentages of filopodia-positive cells following TNFα-treatment are shown. Values are means±s.d. of three independent experiments. (E) Cdc42 activity in TNFα-treated MEFs. Serum-arrested MEFs were treated with 100 ng ml^{-1} TNFα for various times as indicated, then lysed and the GTP-bound form of Cdc42 was assayed as described in Materials and Methods.

appearance of numerous long F-actin-rich hair-like filopodia after 10 minutes (arrows). Filopodia were no longer visible 8 hours after TNFα treatment. To quantify this result, MEFs bearing filopodia were counted at different times after induction. Filopodia formation was rapid and reached a maximum by 10 minutes, then started to decrease as soon as 5 minutes later (Fig. 1B). Interestingly, at later time points, the number of treated cells presenting filopodia at their surface strongly diminished relative to untreated cells. This suggests that the occurrence of filopodia was also affected by long-term exposure to TNFα. To ascertain that membrane structures on which TNFα exerts its regulatory activity are dynamic filopodia and not static retraction fibres, we performed time-lapse differential interference contrast light microscopy on TNFα-treated MEFs. As shown in the accompanying video (supplementary data, video 1), TNFα induced long and dynamic protrusions that rapidly extended and shortened. The morphology and dynamics of these structures were unambiguously characteristic of filopodia. Again, the number of filopodia at the cell surface rapidly increased and reached a maximum within 10 minutes, then decreased at later times, confirming our analysis of F-actin organisation.

It would appear that TNFα initially activates then inhibits filopodia formation in MEFs. Previous reports have identified the Rho GTPase Cdc42 as an essential mediator of TNFα-induced filopodia formation in serum-arrested fibroblasts (Puls et al., 1999; Sugihara et al., 2002) and in endothelial cells (Wojciak-Stothard et al., 1998). To verify the involvement of Cdc42 in the formation of TNFα-induced filopodia, MEFs

were transfected with green fluorescent protein-tagged dominant negative form of Cdc42 (GFP-Cdc42-N17), before TNFα-treatment for 10 minutes (Fig. 1C). Under these conditions, filopodia formation was no longer visible in cells expressing Cdc42-N17 (Fig. 1C, a and b), whereas filopodia still formed in cells expressing control pEGFP vector (Fig. 1C, c and d, arrows). Quantification (Fig. 1D) shows that filopodia formation that occurs without stimulation in 13% of MEFs was largely reduced by Cdc42-N17 expression. In addition, TNFα-dependent induction of filopodia formation was inhibited by Cdc42-N17 expression at early time points.

To determine whether Cdc42 activation correlates with the formation of TNFα-induced filopodia, we measured the level of active Cdc42 at different times in TNFα-treated MEFs. Cdc42 activity increased threefold in MEFs treated for 10 minutes with TNFα (Fig. 1E). However, the proportion of active Cdc42 hardly decreased 60 minutes post-induction and was still 1.5-fold above the basal level 8 hours after TNFα treatment. This indicated that a decrease in Cdc42 activity does not account for the drop of filopodia formation after the addition of TNFα.

Taken together, these results indicate that: first, TNFα first stimulates, then leads to inhibition of filopodia formation in serum-arrested MEFs; second, TNFα leads to a sustained activation of Cdc42; and third, Cdc42 activity correlated with increased levels of TNFα-stimulated filopodia formation but not with filopodia disappearance. This strongly suggests that TNFα negatively regulates filopodia formation independently of Cdc42.

TNFα induces p38 kinase activity independently of Cdc42 activity

In macrophages, TNFα inhibits filopodia formation through a mechanism requiring the MAPK p38 (Peppelenbosch et al., 1999). To check whether TNFα activates p38 in MEFs, we used an in vitro assay to measure the kinetics of p38 kinase activation by TNFα. p38 kinase activity was stimulated 2.3-fold 10 minutes after the addition of TNFα and then decreased (Fig. 2A).

We next stimulated cells with TNFα for 10 minutes, the time corresponding to maximal TNFα-dependent filopodia formation (as determined in Fig. 1B). Under these conditions, the activation of p38 was barely

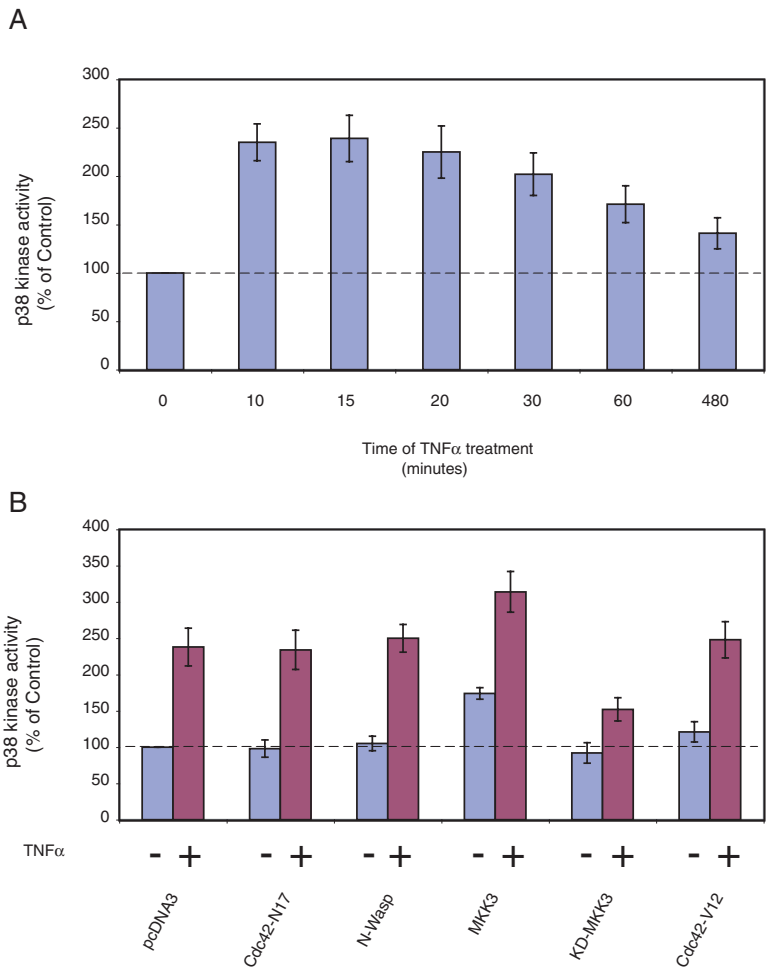
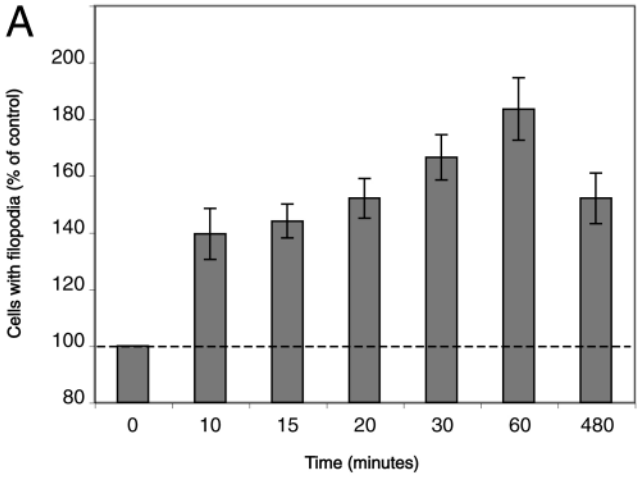
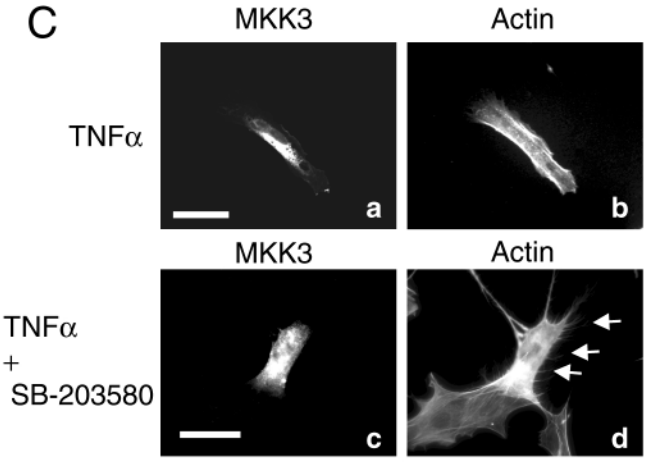


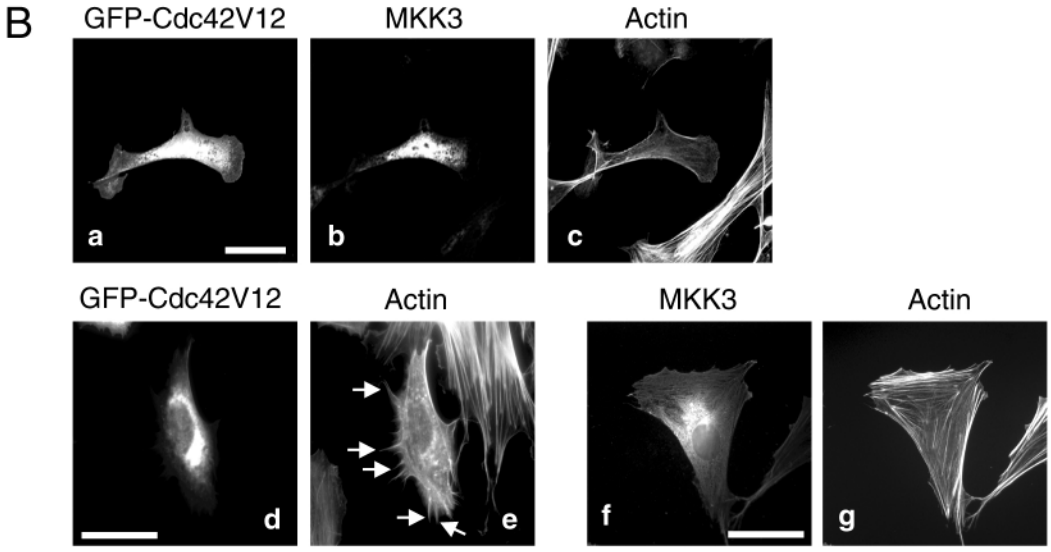
Fig. 2. p38 kinase is required for the inhibition of TNFα-induced filopodia formation. (A) p38 kinase assay in TNFα-treated MEFs. Serum-arrested cells were transfected with the HA-tagged p38 kinase, then treated with 100 ng ml^{-1} TNFα for the indicated times. Immunoprecipitated p38 kinase activity was determined as described in Materials and Methods. Values are the means±s.d. of three independent experiments. Statistical significance of p38 kinase activation caused by TNFα compared with untreated: P=0.061 (Student's t-test). (B) p38 kinase assay in transfected MEFs treated with TNFα. MEFs were transfected with the HA-tagged p38 kinase in the presence or not of vectors expressing either Cdc42-N17, N-Wasp, Cdc42-V12, MKK3, KD-MKK3 or pCDNA3. Serum-arrested cells were treated or not with 100 ng ml^{-1} TNFα for the indicated times. Immunoprecipitated p38 kinase activity was determined as described in Materials and Methods. The values are the means±s.d. of three independent experiments. Statistical significance of p38 kinase activation caused by TNFα compared with untreated: P=0.061 (Student's t-test).

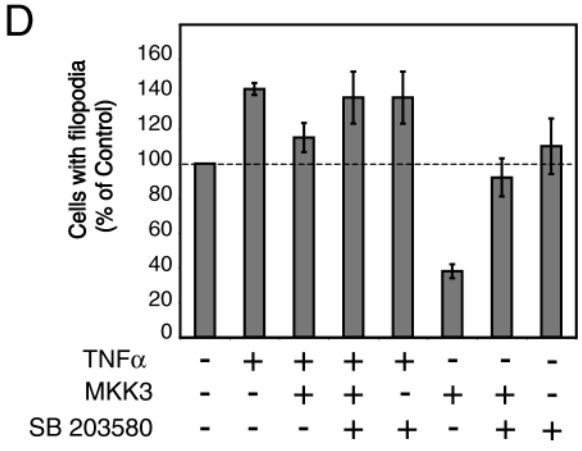
Fig. 3. Inhibition of TNFα-induced filopodia formation depends on p38 kinase activity. (A) Quantification of TNFα-treated MEFs having filopodia in the presence of SB 203580. The percentages of filopodia-positive cells treated with TNFα in the presence of the p38 kinase inhibitor SB 203580 relative to control cells are shown. Cells were fixed, stained for F-actin, then quantified for the presence of filopodia, as described in Materials and Methods. For each experiment, 100 cells were scored and results are expressed as the means±s.d. of three independent experiments. (B) Representative actin staining of transfected MEFs. MEFs were transiently transfected with GFP-labelled Cdc42-V12 (visualised in a and d) or with HA-tagged MKK3, or both. 20 hours later cells were observed for F-actin staining (c, e and g) and for MKK3 expression (b and f). Bar, 10 μm. (C) Representative actin staining of transfected MEFs following TNFα treatment. MEFs were transiently transfected with HA-tagged MKK3, then co-treated with TNFα and SB 203580. After fixation, cells were visualised and stained for MKK3 expression (a) and for F-actin (b). (D) Quantification of MEFs having filopodia shown in MEFs from B and C, transfected or not with MKK3, were pretreated or not with SB 203580 and/or with 100 ng ml^{-1} TNFα for the indicated time. Cells were fixed, stained for F-actin then quantified for the presence of filopodia. MEFs were scored positively when presenting at least five filopodia. For each experiment, 100 cells were scored and values are the means±s.d. of three independent experiments. (E) Quantification of MEFs having filopodia shown in MEFs from B, transfected or not with Cdc42-V12, were pretreated or not with SB 203580 and/or with TNFα for the indicated time. Scoring and analysis were performed as in D.

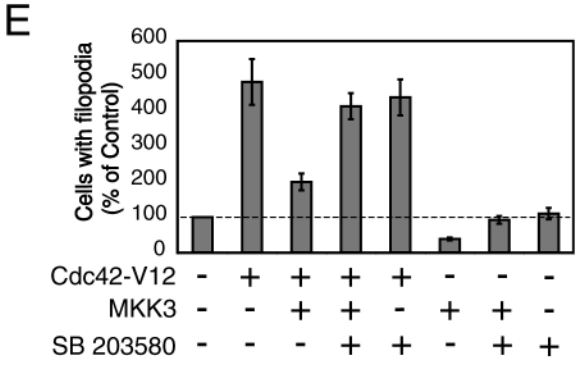
affected by the inhibition of endogenous Cdc42 with either Cdc42-N17, the dominant negative form of Cdc42, or the Cdc42-interacting domain of the Wiskott-Aldrich syndrome protein (WASP) that blocks endogenous Cdc42 activity through competition with its effector binding site (Aspenstrom et al., 1996) (Fig. 2B). TNFα-dependent activation of p38 at 10 minutes was even increased when MEFs were transfected with a specific activator of p38, the mitogen-activated protein kinase kinase MKK3. By contrast, expression of the kinase dead mutant of MKK3, KD-MKK3, strongly decreased, but did not completely abolish, p38 activation (Fig. 2B). This suggests that TNFα-dependent p38 activation is, at least in part, mediated by MKK3. In untreated MEFs, p38 activation was increased by MKK3 expression and to a lesser extent by Cdc42-V12 expression. As a control, we checked that p38 activity was unchanged by the expression of either Cdc42-N17 or N-Wasp.











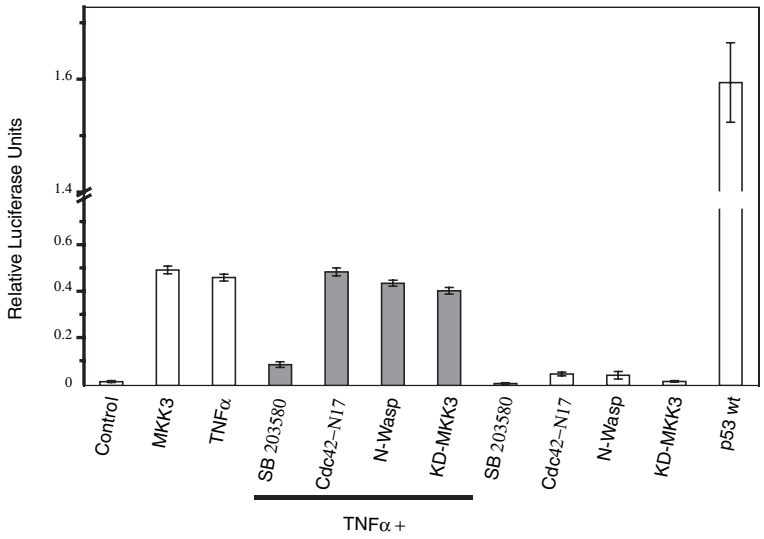


Fig. 4. p53-dependent transcriptional activity in TNFα-treated MEFs. MEFs were transfected with the firefly luciferase gene under the control of the p53 responsive element derived from the *mdm2* gene in combination with empty vector alone (Control), or MKK3, or Cdc42-N17, or N-Wasp, or KD-MKK3 or p53 wt as positive control. As indicated, cells were treated with either TNFα, or SB 203580, or both. The luciferase activity was assayed 24 hours later. The results are expressed as means±s.d. of four independent experiments.

These results indicate that TNFα activates p38 kinase activity independently of Cdc42.

p38 inhibits filopodia formation

To determine whether p38 mediates the inhibition of filopodia formation in MEFs treated by TNFα, we used the p38 pharmacological inhibitor SB 203580. Fig. 3A shows that, in the presence of the p38 inhibitor, the proportion of cells forming TNFα- induced filopodia did not decrease at 15 minutes (as was the case for TNFα-treated cells without SB 203580, compare to Fig. 1B), but rather persisted up to 8 hours. Therefore, loss of p38 function abolished the inhibition of filopodia formation induced by TNFα-treatment.

The above data suggest that p38 is an effector of TNFα signalling in MEFs. We next sought to examine whether its ectopic activation inhibits filopodia formation. To test this, we cotransfected MEFs with a constitutively activated mutant of Cdc42 fused to GFP (GFP-Cdc42-V12) and MKK3. We analysed their effect on cell morphology associated with F-actin organisation (Fig. 3B). Cells co-expressing Cdc42-V12 (Fig. 3B,a) and MKK3 (Fig. 3B,b) had no filopodia on their surface (Fig. 3B,c). As controls of filopodia formation, we have ascertained that, in MEFs, GFP-Cdc42-V12 expression (Fig. 3B,d) induced filopodia formation (e, arrows), whereas MKK3 alone (Fig. 3B,f) had no effect (Fig. 3B,g).

To eliminate the possibility that inhibition of filopodia was due to interference with overexpression of ectopic Cdc42, we stimulated endogenous Cdc42 activity with TNFα for 10 minutes (Fig. 3C). In this case, MEFs transfected with MKK3 (a) had no filopodia (b) except when cells were treated with the p38 inhibitor SB 203580 (c and d). Quantification of these results shows that both TNFα-mediated and Cdc42-V12-induced filopodia formation were prevented by the p38 activator MKK3 (respectively, in Fig. 3D and Fig. 3E). These inhibitions were largely reduced by the p38 inhibitor SB 203580. In addition, filopodia formation without TNFα-stimulation was also reduced by MKK3. When MEFs were treated

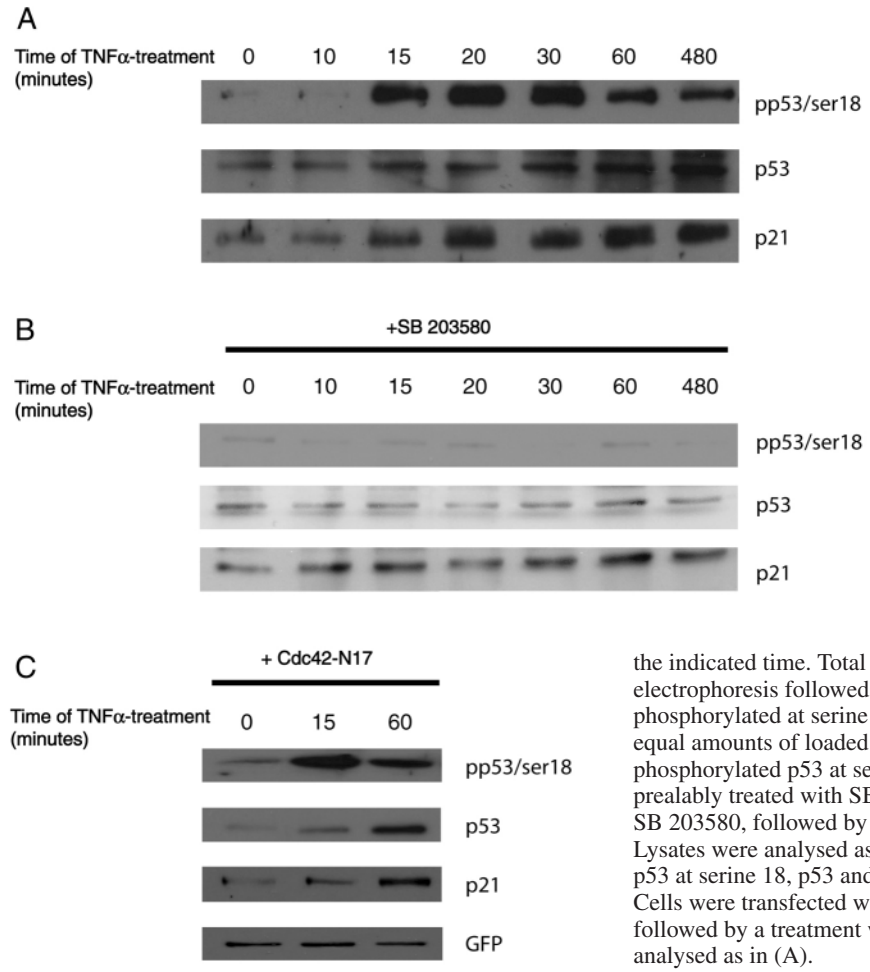


Fig. 5. TNFα activates p53 and induces its phosphorylation at serine 18 through p38 kinase. (A) Abundance and status of phosphorylated p53 at serine 18, p53 and p21 in TNFα-treated MEFs. Serum-arrested MEFs were treated with TNFα for the indicated time. Total protein lysates were prepared and analysed by gel electrophoresis followed by immuno-blotting analysis using antibodies to p53 phosphorylated at serine 18 (pp53/Ser18), p53 and p21. Each lane contains equal amounts of loaded proteins (40 μg). (B) Abundance and status of phosphorylated p53 at serine 18, p53 and p21 in TNFα-treated MEFs prealably treated with SB 203580. Serum-arrested cells were pre-treated with SB 203580, followed by a treatment with TNFα for the indicated time. Lysates were analysed as in (A). (C) Abundance and status of phosphorylated p53 at serine 18, p53 and p21 in TNFα-treated MEFs expressing Cdc42-N7. Cells were transfected with GFP-tagged Cdc42-N17 before serum-starvation, followed by a treatment with TNFα for the indicated time. Lysates were analysed as in (A).

with SB 203580 alone, the number of cells presenting filopodia was slightly increased. We conclude that activation of p38 counteracts Cdc42-induced filopodia formation in response to TNFα and that its activity is required for inhibition of filopodia formation in TNFα-treated MEFs.

TNFα-induced p53 activation depends on p38 kinase

Activation of the tumour suppressor p53 inhibits Cdc42-induced filopodia formation (Gadea et al., 2002), and TNFα induces p53 activation through a mechanism requiring p38 in the human cervical carcinoma cell line HeLa (Sayed et al., 2000). We

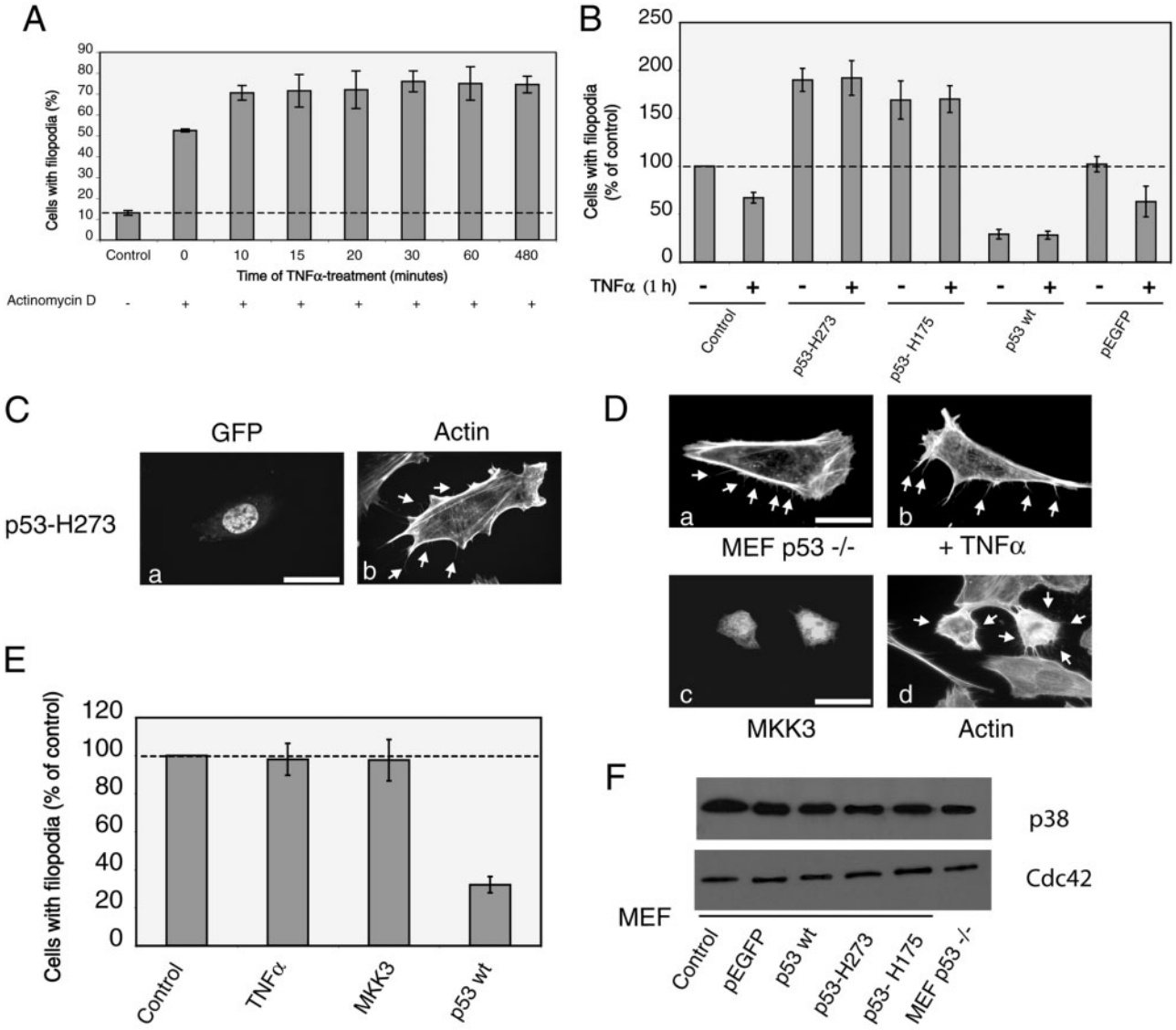


Fig. 6. Wild-type p53 activity is required to mediate TNFα-induced filopodia inhibition. (A) Effects of TNFα on filopodia formation in actinomycin D-treated MEFs. Serum-arrested cells were pretreated or not with the transcription inhibitor actinomycin D, followed by a treatment with 100 ng ml^{-1} TNFα for various times as indicated, fixed and stained for F-actin, then quantified for the presence of filopodia. For each experiment, 100 cells were scored and results are the mean±s.d. of three independent experiments. (B) Quantification of MEFs having filopodia. MEFs were transfected or not with plasmids encoding p53 wt, p53-H175, p53-H273 or pEGFP, then serum arrested before treatment or not with TNFα for 1 hour as indicated. After fixation and staining for F-actin, cells were analysed for the presence of filopodia as indicated in Fig. 3. (C) Representative actin staining of MEFs transfected MEFs with p53-H273. Serum arrested cells were treated (a and b) or not (c and d) with TNFα for 1 hour. Cells were fixed, visualised for p53 expression as revealed by GFP staining (a) and stained for F-actin (b). Arrows indicate filopodia. Bar, 10 μm. (D) Actin staining of $p53^{-/-}$MEFs. Cells were either untreated (a) or treated with TNFα for 1 hour (b), then fixed and stained for F-actin. In (c) and (d), $p53^{-/-}$MEFs were transfected with HA-tagged MKK3 before staining for MKK3 (c) or F-actin (d). Arrows indicate filopodia. Bar, 10 μm. (E) Quantification of $p53^{-/-}$MEFs having filopodia. The percentages of filopodia-positive cells relative to control cells are shown. $p53^{-/-}$MEFs were transfected or not with plasmids encoding pEGFP (Control), p53 wt, MKK3, or treated with TNFα for 1 hour, as indicated. Cells were fixed, stained for F-actin, then quantified for the presence of filopodia, as described in Materials and Methods. For each experiment, 100 cells were scored and results are expressed as the means±s.d. of three independent experiments. (F) Abundance of Cdc42 and p38 in MEFs expressing p53 mutants and in $p53^{-/-}$MEFs. MEFs were transfected with pEGFP, p53H273, p53 H175, p53 wt and $p53^{-/-}$MEFs before preparation of total protein lysates, and analysis by gel electrophores was followed by immunoblotting analysis using antibodies to Cdc42 and p38. Each lane contains equal amounts of loaded proteins (40 μg).

wondered whether p53 mediates p38 kinase-dependent inhibition of filopodia formation in TNFα-treated MEFs.

To test whether p38 mediates p53 activation by TNFα in MEFs (Fig. 4), we measured p53-driven transactivation of a luciferase reporter gene linked to the mdm2 promoter (Barak et al., 1994). As shown in Fig. 4, the p53-driven reporter gene was activated by TNFα treatment and by MKK3 expression. By contrast, the reporter gene was not activated by TNFα treatment when p38 was blocked by SB 203580 (Fig. 4). We conclude that p38 mediates TNFα-driven activation of p53 in MEFs.

As p38 has been shown to be stimulated by Cdc42 (Coso et al., 1995; Minden et al., 1995), we investigated whether the activation of p53 by TNFα is mediated by Cdc42 (Fig. 4). Neither short-term expression of Cdc42-N17 nor N-Wasp or KD-MKK3 affected reporter activation by TNFα in MEFs. In untreated MEFs, expression of Cdc42-N17 or N-Wasp led to a slight reporter activation, whereas KD-MKK3 and SB 203580 had no effect. By contrast, p53 wt used here as positive control strongly stimulated the mdm2-luciferase reporter gene (Fig. 4). Therefore, we conclude that Cdc42 does not mediated TNFα-induced p53 activation.

p38 kinase mediates phosphorylation of p53 at serine 18 in response to TNFα

Phosphorylation of p53 protein at multiple sites is a critical event in the stabilisation and activation of p53 (Ashcroft et al., 1999; Oren, 1999; Shieh et al., 1997). Among these phosphorylation sites, serine 18 in mouse, which corresponds to serine 15 in human p53, is considered as a crucial site that greatly contributes to p53 stabilisation following various genotoxic stresses (Lakin and Jackson, 1999; Tibbetts et al., 1999).

To examine whether p53 was phosphorylated at serine 18 in cells treated with TNFα, we performed western blot analysis using a phosphospecific antibody against serine 18 of p53 during 8 hours of TNFα treatment in serum-arrested MEFs. As shown in Fig. 5, TNFα rapidly induced the phosphorylation of p53 at serine 18 within 15 minutes. The level of phosphorylation reached a maximum at 20 minutes following exposure of cells to TNFα (Fig. 5, panel A). Analysis of the same extract for total p53 protein levels showed that p53 progressively accumulated, beginning at 15 minutes and continuing up to 8 hours. To ascertain whether TNFα-induced phosphorylation and stabilisation of p53 reflects its transcriptional activity, we followed the expression of a p53 target gene, the cyclin-dependent kinase-inhibitor p21. As shown in Fig. 5A, levels of p21 were rapidly increased after 15 minutes of treatment and reached a maximum at 20 minutes. The kinetic of p21 accumulation was similar to those of serine 18 phosphorylation of p53.

To further determine whether TNFα-induced p53 phosphorylation at serine 18 requires p38 kinase, serum-arrested MEFs were pre-treated with SB 203580 before treatment with TNFα. This significantly inhibited phosphorylation and accumulation of p53 and p21. Therefore, the intrinsic kinase activity of p38 is required for TNFα-induced serine 18 phosphorylation and functional activation of p53.

We conclude that TNFα-mediated phosphorylation at serine 18 of p53, its activation, and the subsequent expression of p21, coincide with the onset of filopodia inhibition (see Fig. 1A) and the maximum level of TNFα-dependent p38 kinase activation (see Fig. 2A).

To confirm that the induction of p53 by TNFα is not Cdc42-dependent, Cdc42-N17 was expressed in MEFs before serum starvation and treatment with TNFα. Fig. 5C shows that inhibition of endogenous Cdc42 had no effect on TNFα-induced phosphorylation of p53 at serine 18, stabilisation of p53 and induction of p21. This strengthens the point that the TNFα-dependent activation of the p53 pathway occurs independently of Cdc42.

Transcriptional activity of p53 is required for TNFα/p38-induced filopodia inhibition

We then sought to examine whether p53 transcriptional activity is required to inhibit filopodia formation in response to TNFα. We first analysed filopodia formation at different times in TNFα-treated MEFs using the transcription inhibitor actinomycin D. Fig. 6A clearly indicates that, in TNFα-untreated cells, filopodia formation was dramatically increased (fourfold) when transcription was blocked. In response to TNFα, the proportion of cells forming filopodia was enhanced at 10 minutes, but did not decrease for up to 8 hours. These data argue that filopodia inhibition induced by TNFα is largely dependent on transcriptional activity.

To further examine whether p53 transcriptional activity is necessary for TNFα-induced inhibition of filopodia formation, we used two dominant negative mutants of p53 – namely, p53-H175 and p53-H273 – that have lost DNA binding activity and their subsequent transcriptional activities. We tested their effect on filopodia formation of MEFs treated or not with TNFα for 1 hour, the time required to obtain filopodia inhibition as determined in Fig. 1. Quantitative analysis of F-actin organisation in transfected cells showed that both mutants, but not wild-type p53, increased filopodia formation of untreated cells and also prevented the inhibition of filopodia formation induced by TNFα observed at 1 hour (Fig. 6B). An example of this can be seen in Fig. 6C, which shows that expression of p53-H273 (a) led to persistence of filopodia (b) (arrows) at the surface of TNFα-treated MEFs for 1 hour.

We have previously established that mouse embryonic fibroblasts lacking p53 ($p53^{-/-}$MEFs) exhibit constitutive membrane filopodia (Gadea et al., 2002). To confirm that inhibition of filopodia formation by TNFα-p38 signalling was due to p53 activity, we analysed F-actin organisation of $p53^{-/-}$MEFs treated with TNFα for 1 hour (Fig. 6D). Under these conditions, the enhanced number of filopodia observed in untreated $p53^{-/-}$MEFs (a, arrows) was not affected (b, arrows). MKK3 expression in $p53^{-/-}$MEFs (Fig. 6D,c) did not alter the formation of filopodia at the cell surface (Fig. 6D,d). Quantification of these results showed that the number of cells bearing filopodia was no longer affected by TNFα treatment or MKK3 expression, contrary to p53 wt expression, which strongly reduced it (Fig. 6E). Finally, we checked that expression of p53 wt and p53 mutants in MEFs or abrogation of p53 function in $p53^{-/-}$MEFs had no effect on the levels of p38 and Cdc42 (Fig. 6F).

Taken together, these results strongly suggest that p53 wild-type activity is required for TNFα- and p38 kinase-induced inhibition of filopodia formation.

Discussion

An essential mechanism by which TNFα implements its pro-inflammatory properties is to favour the migration of specialised cells towards site of infection. The data presented here show that TNFα regulates the appearance of actin-based structures that are important for cell locomotion, called filopodia. TNFα activates two independent signalling pathways, the effects of which are antagonistic. Filopodia formation is promoted by the stimulation of the Rho GTPase Cdc42, and then prevented by the activation of the tumour suppressor p53 through the MAP kinase p38.

TNFα has already been implicated in actin reorganisation; in Swiss 3T3 fibroblasts, TNFα has been shown to activate Cdc42, leading to filopodia formation and subsequently to Rac and Rho activation (Puls et al., 1999). This induction is transient, since Puls et al. (Puls et al., 1999) detected Cdc42-activation and subsequent filopodia formation after 10 minutes of TNFα treatment, while 30 minutes later, filopodia and lamellipodia had largely disappeared. The transient induction of filopodia is also characteristic of constitutive activated Cdc42 (Kozma et al., 1995; Nobes and Hall, 1995). However, treatment with the p38 kinase inhibitor SB 203580 leads to a prolonged presence of filopodia at the surface of cells expressing constitutively activated Cdc42 (data not shown). Similarly, our data have established that TNFα stimulates Cdc42 activity and filopodia formation within 10 minutes. Longer exposure to TNFα inhibits Cdc42-mediated filopodia extension through a mechanism requiring p38 and p53 activities in MEFs. Hence, TNFα has a biphasic effect: at early times it triggers Cdc42-induced filopodia formation, whereas at later times it leads to a p38/p53-dependent inhibition of Cdc42-induced filopodia. The inhibition of filopodia formation takes place 15-30 minutes after the beginning of TNFα treatment, corresponding to the time necessary to detect an increase in the transactivation activity of p53 (Fig. 5).

Our data indicate that inhibition of endogenous Cdc42 either by the dominant negative form of Cdc42, or the Cdc42-interacting domain of the Wiskott-Aldrich syndrome protein (WASP), has no effect on the ability of TNFα to induce p38 kinase, suggesting that TNFα activates p38 kinase independently of Cdc42. A similar conclusion has been reached for the stimulation of another stress MAP kinase, the c-Jun N-terminal kinase (JNK), whose activation by TNFα is also independent of Cdc42 (Puls et al., 1999). The above observations suggest the co-existence of two independent pathways generated by TNFα: one leading to actin polymerisation through Cdc42 and another promoting the activation of the MAP kinases p38 and JNK.

In our experiments, TNFα-treatment and MKK3 expression have a synergistic effect in stimulating p38. In addition, TNFα-dependent p38 activation was strongly decreased, but not completely inhibited, by a dominant negative mutant of MKK3 (Fig. 2A). These data indicate that MKK3 partly mediates the effect of TNFα on p38 activation, and suggest the existence of a MKK3-independent pathway. One potential candidate could be the mitogen-activated protein kinase kinase MKK6, a kinase closely related to MKK3. In support of this, it is already known that TNFα activates two MAP kinase kinases, MKK3 and MKK6, which in turn phosphorylate and activate p38 kinase (McDermott and O'Neill, 2002; Winston et al., 1997). Furthermore, MKK3 and MKK6 are also known to mediate the activation of p38 by the pro-inflammatory cytokine, the interleukin 1 (IL-1) in macrophages (McDermott and O'Neill, 2002).

p38 MAP kinase has been shown to stimulate p53 in response to various stress agents, including UV radiation (Bulavin et al., 1999; Huang et al., 1999; Keller et al., 1999), chemotherapeutic agents (Sanchez-Prieto et al., 2000), osmotic shock (Kishi et al., 2001), nitric oxide (Kim et al., 2002), anisomycin, arsenite and TNFα (Sayed et al., 2000). p38 has also been described to be physically associated with p53 in vivo (Sanchez-Prieto et al., 2000) and the p38-p53 signalling pathway undergoes a negative feedback mediated by the protein phosphatase Wip1, induced by p53 itself (Takekawa et al., 2000). p53 is phosphorylated at multiple sites in vivo (Prives and Hall, 1999). Many p53 phosphorylation sites are clustered in the N-terminal activation domain (amino-acids 1-42), whereas the others are more dispersed in the C-terminal region of the molecule. Several studies have shown that phosphorylation of the mouse p53 protein at serine 18 (corresponding to serine 15 in human) may play a crucial role in the stabilisation and functional activation of p53 by compromising interactions with its negative regulator MDM2 (Shieh et al., 1997) and by increasing binding to the p300 co-activator protein (Lambert et al., 1998). The importance of p53 phosphorylation at serine 18 by p38 kinase was shown by the observation that increased accumulation of serine 18-phosphorylated p53 was reduced by the p38 kinase inhibitor SB 203580. In human cells, serine 15 phosphorylation of p53 has been shown to be induced by a variety of DNA-damaging agents (Levine, 1997; She et al., 2000; Siliciano et al., 1997). Our data indicate that serine 18 phosphorylation of p53 is also induced by TNFα through a mechanism requiring p38 kinase.

The predominant biochemical activity of p53 relies on both transactivation and transcriptional repression of specific target genes. In this manner, p53 implements its antiproliferative activities: the cell-cycle arrest in G_1/S and G_2/M phases or induction of apoptotic cell death. Here, we show that the inhibition of TNFα-mediated filopodia formation correlates with the activation of p53 as a transcriptional transactivator (Fig. 5A). Furthermore, TNFα does not inhibit filopodia formation either in MEFs treated with the transcription inhibitor actinomycin D, p53-deficient MEFs, or MEFs expressing DNA-binding deficient mutants p53-H273 or p53-H175 (Fig. 6). Taken together, these data support a role for the transcriptional activity of p53 in mediating filopodia inhibition in response to TNFα.

To date, p38-dependent activation of p53 has been shown to trigger either cell cycle arrest or apoptosis. Here, we have shown that a TNFα-p38-p53 signalling module also serves as regulator of cellular morphology in inhibiting filopodia, thereby implicating this signalling module in the control of cell migration. For example, during skin wounding, fibroblasts would migrate to the wound site and then remain in place under the influence of TNFα (Postlethwaite and Seyer, 1990).

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