Cdc42 downregulates MMP-1 expression by inhibiting the ERK1/2 pathway

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Summary

The small GTPases of the Rho family are key intermediates in cellular signalling triggered by activated cell-adhesion receptors. In this study, we took advantage of RNA interference (RNAi) using small interfering RNAs (siRNAs) to define the roles of the best-characterized members of the RhoGTPase family, RhoA, Rac1 and Cdc42, in the control of MMP-1, MMP-2 and type-I-collagen expression in normal human skin fibroblasts (HSFs). A specific and long-lasting repression, up to 7 days after transfection, of the three GTPases was achieved by transient transfection of specific siRNA. The silencing of Cdc42, but not that of RhoA or Rac1, induced a 15-fold increase in MMP-1 secretion. This upregulation was confirmed at the mRNA level and observed with two different siRNAs targeting Cdc42. Such a regulation was also observed in various human cell lines and was rescued by re-expressing wild-type Cdc42 encoded by a construct bearing silent mutations impeding its recognition by the siRNA. By contrast, MMP-2 and type-I-collagen expression was not affected by the individual silencing of each Rho GTPase. Cytokine protein array, enzyme-linked immunosorbent assays and reverse-transcription PCR measurements revealed that ablation of Cdc42 induced an overexpression of interleukin 8 and MCP-1. Although these cytokines are known to induce the expression of MMP-1, we showed that they were not involved in the Cdc42-mediated upregulation of MMP-1. Silencing of Cdc42 also induced an increased phosphorylation of ERK1/2 and p38 MAP kinase. The use of chemical inhibitors on Cdc42-ablated cells revealed that the upregulation of MMP-1 is dependent on the ERK1/2 pathways, whereas the p38 MAP kinase pathway displayed an inhibitory role. Simultaneous knock-down of two or three Rho GTPases allowed us to demonstrate that the RhoA-ROCK pathway was not involved in this regulation but that the silencing of Rac1 reduced the effect of Cdc42 suppression. These data suggest that, in vivo, when cell/extracellular-matrix interactions via integrins induce cytoskeleton organization, MMP-1 expression is maintained at a low level by Cdc42 via a repression of the Rac1 and ERK1/2 pathways. Therefore, Cdc42 contributes to ECM homeostasis and connective tissue integrity.

Key words: RhoA, Rac1, Cdc42, MMP-1, siRNA, ERK1/2

Introduction

Interactions between cells and the extracellular matrix (ECM) components are primarily mediated through receptors of the integrins family (Hynes, 1992). Engagement of integrin triggers the assembly of focal adhesions and induces the reorganization of the cytoskeleton and the activation of various signalling cascades that culminate in the control of cell proliferation, survival, differentiation and gene expression (Danen et al., 1998). This includes the expression of genes coding for ECM components and for matrix metalloproteinases (MMPs), a family of zinc-dependent proteases that collectively degrade most components of the ECM and play a major role in physiological and pathological processes involving ECM remodelling. MMP-1, the founding member of this family of enzymes, is barely expressed in normal fibroblasts spread on a rigid support and is dramatically upregulated by inhibition of integrin functionality (Kheradmand et al., 1998) or by interfering with integrin-mediated cytoskeleton reorganization either with cytochalasin D (Lambert et al., 1998; Unemori and Werb, 1986) or by culturing fibroblasts in freely retracting collagen gels (Mauch et al., 1989; Lambert et al., 1992). Other MMPs, such as MMP-3, MMP-13 and the membrane bound MMP-14 (MT1-MMP; an MMP-2 activator), are similarly upregulated, whereas type-I- and type-III-collagen expression is repressed (Haas et al., 1998; Lambert et al., 2001a; Lambert et al., 2001b; Mauch et al., 1988).

The small GTPases of the Rho family are at the crossroads of signalling pathways initiated by receptors to diffusible biological mediators as well as by clustered integrins. These are key signalling molecules regulating the architecture of the cytoskeleton (Hall, 1998) and the assembly of proteins into focal adhesions (Hotchin and Hall, 1995). Some of them have been implicated in the control of MMP expression and activation. It has been reported that Rac1 activity is required for the induction of MMP-1 mediated by inactivation of the $\alpha5\beta1$ integrin (Kheradmand et al., 1998) as well as for type-I-collagen-dependent MMP-2 activation (Zhuge and Xu, 2001).

It has also been observed that constitutive activation of RhoA is sufficient to trigger MMP-1 expression in fibroblasts (Werner et al., 2001; Werner and Werb, 2002).

The function of the Rho GTPases is difficult to assess in cells that are refractory to transfection. Moreover, bacterial toxins and mutated Rho proteins are not fully specific for one Rho GTPase (Lerm et al., 2000; Ridley, 2001). In this report, we used RNAi by small interfering RNAs (siRNAs) (Elbashir et al., 2001) to analyse the roles of the best-characterized members of the Rho-GTPase family (RhoA, Rac1 and Cdc42) in the control of MMP-1, MMP-2 and type-I-collagen expression in normal human skin fibroblasts (HSFs) cultured on a rigid substrate. The silencing of Cdc42, but not of RhoA or Rac1, in HSFs induces a significant overexpression of MMP-1 at both the mRNA and the protein levels, whereas the expression of MMP-2 and type-I collagen is not affected. Multiple knockdown experiments and the use of chemical inhibitors demonstrate that the upregulation of MMP-1 is dependent on the Rac1 and ERK1/2 pathways, whereas the RhoA-ROCK pathway was not involved and the p38 mitogen-activated protein (MAP) kinase pathway displays an inhibitory role.

Materials and Methods

Reagents and cells

Y-27632 was kindly supplied by A. Yoshimura (Welfide, Osaka, Japan). PP2 and U0126 were from Calbiochem, Herbimycin A and LY294 from Sigma, and SB203580 from Alexis. Antibodies were purchased from the following manufacturers: mouse anti-RhoA (sc-418), mouse anti-Stat1α (sc-417), rabbit anti-myosin-regulatory-light-chain (sc-15370) and goat anti-phospho-myosin-regulatory-light-chain (sc-12896) from Santa Cruz Biotechnology; mouse anti-Rac1 (23A8) from Upstate Biotechnology; mouse anti-Cdc42 from BD Biosciences; rabbit anti-ERK1/2 (M-5670) and mouse monoclonal anti-phospho-ERK1/2 (M-8159) from Sigma; mouse monoclonal anti-human-p38 (AHO0782) and rabbit polyclonal anti-phospho-p38 (44-684Z) were from BioSource International; mouse anti-human-MMP-1 (Mab3307) from Chemicon; mouse blocking anti-human-MCP-1 (Mab279) and anti-human-interleukin-8 (Mab208) from R&D Systems. The blocking antibody concentrations used were ten times higher than those required to inhibit either the migration of monocytes induced by 20 ng ml^{-1} recombinant human MCP-1 (rhMCP-1) or the migration of neutrophils induced by 20 ng ml^{-1} recombinant human interleukin 8 (rhIL-8). HSFs were isolated by the explant procedure from normal human dermis and amplified in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% foetal calf serum (FCS) (Cambrex). Cells were trypsinized and seeded into three dishes every week and used between passages 8 and 13. Human breast adenocarcinoma cell line HS578T, human fibrosarcoma cell line HT1080 and human melanoma cell line A2058 were amplified in Dulbecco's modified Eagle's medium supplemented with 8% FCS and trypsinized and seeded into ten dishes every week. When indicated, cells were starved by serum deprivation for 16 hours.

siRNA transfection

21-nucleotide long siRNAs were chemically synthesized, desalted, deprotected and purified using polyacrylamide-gel electrophoresis (PAGE) (Eurogentec). The 5′-GAAGUCAAGCAUUUCUGUCTT-3′ and 5′-GACAGAAAUGCUUGACUUCTT-3′ oligoribonucleotides were used to inhibit RhoA synthesis; the 5′-CACCACUGUCCCAA-CACUCTT-3′ and 5′-GAGUGUUGGGACAGUGGUGTT-3′ oligori-bonucleotides were used to inhibit Rac1; and the 5′-GAUAACUCAC-CACUGUCCATT-3′ and 5′-UGGACAGUGGUGAGUUAUCTT-3′ oligoribonucleotides (1st siCdc42) or the 5′-GACUCCUUUCU-UGCUUGUUTT-3′ and 5′-AACAAGCAAGAAAGGAGUCTT-3′ oligoribonucleotides (2nd siCdc42) were used to inhibit Cdc42. As a control, we used a randomly mixed sequences of the 1st siCdc42 5′-AUACUUACGCACGCUCCAATT-3′ and 5′-UUGGAGCGUGC-GUAAGUAUTT-3′ oligoribonucleotides. Each pair of oligoribo-nucleotides was annealed at a concentration of 20 μM in 50 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. In some experiments, the two siRNAs targeting Cdc42 and the control siRNA were transcribed in vitro with the Silencer™ siRNA Construction Kit from Ambion (catalogue number 1620). Calcium-phosphate-mediated transfection was performed overnight (14-16 hours) on subconfluent cells with the final concentration of siRNA ranging from 0.6 nM to 60 nM. Cells were washed twice with PBS and once with complete medium; this last step was defined as time 0 after transfection. At 24 hours after transfection, each pool of transfected cells was trypsinized and seeded at subconfluence in six-well-plates. In each experiment, a series of wells was dedicated to the evaluation of the silencing of the Rho GTPases by western-blotting analysis.

Immunoblotting and zymography

Subconfluent HSFs were rinsed twice with and scraped in ice-cold PBS. One-third of the cell suspension was used to measure the DNA content by a fluorimetric technique and the remaining was lysed in SDS-PAGE lysis buffer. Samples equivalent to 0.5 μg DNA were separated on a 15% gel under reducing conditions. To analyse MMP-1 expression, serum-free medium was conditioned by subconfluent transfected fibroblasts at the indicated time after transfection. Medium conditioned by fibroblasts equivalent to 0.5 μg DNA were separated on a 10% gel under reducing conditions. Proteins were transferred to Immobilon-P PVDF membranes and immunodetected with the corresponding antibodies. The bands were visualized with the ECL system (Amersham). To determine MMP-2 secretion, aliquots of conditioned medium corresponding to 0.05 μg DNA were subjected to zymography analysis as previously described (Lambert et al., 2001b).

GTPase assays

The assay was carried out as previously described (Deroanne et al., 2003). Briefly, cells were chilled on ice and lysed in ice-cold buffer containing 1% Triton X-100, 25 mM HEPES, pH 7.3, 150 mM NaCl, 4% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 4 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin and 1 μg ml^{-1} pepstatin. Lysates were centrifuged for 8 minutes at 13,000 ***g***. Supernatants were immediately frozen in liquid nitrogen and stored at –80°C until used. An aliquot of each supernatant was denatured in SDS-PAGE lysis buffer before freezing to measure the total Rho GTPase content by western blotting. For pull-down assays, supernatants were incubated for 30 minutes with 30 μg GST-PBD fusion protein containing glutathione-*S*-transferase (GST) fused to the Cdc42- and Rac-binding region of PAK-1B or GST-RBD fusion protein containing GST fused to the Rho-binding region of rhotekin affinity, linked to glutathione-Sepharose beads (Ren et al., 1999; Sander et al., 1999). The beads were washed four times in lysis buffer and boiled in 60 μl SDS-PAGE lysis buffer.

RT-PCR analysis

The reverse-transcription polymerase chain reaction (RT-PCR) amplifications were performed in an automated thermocycler (GeneAmp PCR system 9600) using a GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin Elmer) with pairs of primers amplifying mRNAs encoding:
human MMP-1 (5′-GAGCAAACACATCTGAGGTACAGGA-3′ and 5′-TTGTCCCGATGATCTCCCCTGACA-3′)

human MMP-2 (5′-AGATCTTCTTCTTCAAGGACCGGTT-3′ and 5′-GGCTGGTCAGTGGCTTGGGGTA-3′)
human α_1I collagen (5′-CCCACCAATCACCTGCGTACAGA-3′ and 5′-TTCTTGGTCGGTGGGTGACTCTGA-3′)
human Stat-1 (5′-CGCACACAAAAGTGATGAACATGGA-3′ and 5′-GGCTGACGTTGGAGATCACCACA-3′)
human MCP-1 (5′-TAGCAGCCACCTTCATTCCCCAAG-3′ and 5′-AATGGTCTTGAAGATCACAGCTTC-3′)
human IL-8 (5′-GCCAAGGAGTGCTAAAGAACTTAG-3′ and 5′-GAATTCTCAGCCCTCTTCAAAAAC-3′)
human COX-2 (5′-AGAACTTGCATTGATGGTGACTGTTT-3′ and 5′-TTCTCCTTGAAAGGACTTATGGGTAA-3′)
human Cdc42 (5′-GCCCGTGACCTGAAGGCTGTCA-3′ and 5′-TGCTTTTAGTATGATGCCGACACCA-3′)
human mutated Cdc42 (mCdc42) (5′-GCCCGTGACCTGAAGGCT-GTCA-3′ and 5′-CAGTCGAGGCTGATCAGCGGTTTA-3′)
human 28S rRNA (5′-GTTCACCCACTAATAGGGAACGTGA-3′ and 5′-GGATTCTGACTTAGAGGCGTTCAGT-3′).

For MMP-1, MMP-2, α_1I and the 28S rRNA, the efficiency of RT-PCR was controlled by a synthetic RNA co-transcribed and co-amplified with the same primers as the endogenous RNA to yield an amplification product of larger size (Lambert et al., 2001a; Lambert et al., 2001b). For human Stat-1, MCP-1, IL-8, COX-2, Cdc42 and mCdc42, the lengths of the RT-PCR products were 234 bp, 171 bp, 222 bp, 282 bp, 195 bp and 205 bp, respectively. For quantitative RT-PCR measurements, 10 ng total RNA and a known copy number of the standard synthetic RNA for MMP-1, MMP-2, α_1I or 28S rRNA were used per 25 μl reaction mixture (final volume). The reverse transcription step (70°C for 15 minutes) was followed by a 2-minute incubation at 94°C to denature RNA-DNA heteroduplexes and then by amplification for 24 cycles (MMP-1), 25 cycles (MMP-2), 23 cycles (α_1I), 18 cycles (28S rRNA), 30 cycles (Stat1, MCP-1 and IL-8) or 23 cycles (Cdc42 and mCdc42) at 94°C for 15 seconds, 66°C for 20 seconds and 72°C for 10 seconds. The RT-PCR products were quantified after electrophoresis on a 10% polyacrylamide gel and staining (Gelstar, FMC BioProducts) using a Fluor-S™ MultiImager (Bio-Rad Laboratories, Life Science).

ELISA and cytokine proteoarray

The expression of cytokines was measured in serum-free medium conditioned between days 2 and 3 after transfection. A first screening of 79 cytokines was achieved with RayBio™ Human Cytokine Array Map V (RayBiotech; H0108005) following manufacturer's instructions. The expression levels of IL-8, MCP-1 and interferon-β were also analysed with enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (D8000C, DCP00 and 41400-1) following manufacturer's instructions.

Rescue of Cdc42 knockdown

The entire coding sequence of Cdc42 was amplified by RT-PCR. The amplification product was mutated by mean of a PCR-based approach with mutated primers. Six silent mutations were introduced in the sequence targeted by the 1st siCdc42 in order to make it resistant to this siRNA. The mutated Cdc42 cDNA (mCdc42) was cloned into pShuttle (Clontech Laboratories). Sequencing confirmed that the six expected mutations were introduced into the cDNA. Rescue experiments were carried out with HS578T cells cultured in six-well plates. The cells were seeded at a density of 1.5×10^5 cells per well. 8 hours after seeding, they were transfected with 20 nM siRNA against a scrambled part of *Cdc42*, as a control (siScr) or 20 nM siRNA against *Cdc42* (siCdc42) for 14-16 hours following the protocol described above. Immediately after the washing step, 1 μg empty pShuttle or 1 μg pShuttle-mCdc42 was transfected into cells for 20-24 hours with 3 μl Genejuice™ (Novagen) following the manufacturer's protocol. Each pool of transfected cells was trypsinized and seeded at subconfluence in six-well-plates. In each experiment, a series of wells was dedicated to the evaluation of the expression level of Cdc42 and MMP-1 by western-blotting analysis, and another series to evaluate the expression level of both endogenous and mutated Cdc42 mRNA by RT-PCR.

Results

Specific inhibition of RhoA, Rac1 and Cdc42 expression by siRNA

The RhoA-targeting siRNA designed in a previous work (Deroanne et al., 2003) served as a guideline to define the siRNA target sequences for Rac1 and Cdc42, which were chosen in the same region of the mRNA sequence. As a control, an irrelevant siRNA (siScr, a randomly mixed sequence of the 1st siRNA targeting Cdc42) was used. HSFs were transiently transfected with either 20 nM respective specific or control siRNA or treated with calcium phosphate alone. As shown in Fig. 1A, western-blot analysis of whole-cell lysates 3 days after transfection revealed a more than 90% reduction in the RhoA and Cdc42 protein levels, and an 80% reduction in the Rac1 protein level without any significant modulation of the amount of ERK1/2 used as control. The irrelevant siRNA had no effect on any of these proteins. The specific inactivation of the RhoA, Rac1 and Cdc42 pathways was confirmed by pull-down assays that revealed a similar repression of their GTP-bound forms (not shown). Interestingly, the silencing of one Rho GTPase altered neither the expression level nor the activation level of the others. Repression of Cdc42 by more than 80% of the

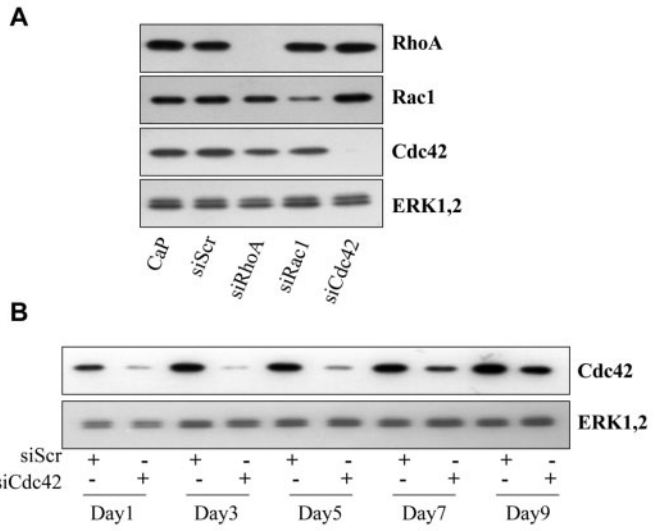

Fig. 1. Specific silencing of RhoA, Rac1 and Cdc42 mediated by transient transfection of siRNA. (A) Western-blot analysis of whole-cell lysates of HSFs transfected with calcium phosphate alone (CaP), with 20 nM siRNAs targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42), or with 20 nM irrelevant siRNA (siScr). 72 hours after transfection, the cells were lysed in SDS-PAGE loading buffer and analysed by immunoblotting with specific antibodies to RhoA, Rac1, Cdc42 and ERK1/2. (B) Western-blot analysis of whole-cell lysates of HSFs transfected with 20 nM siScr or with 20 nM siCdc42. Cells were lysed in SDS-PAGE loading buffer between day 1 and day 9 after transfection and analysed by immunoblotting with specific antibodies to Cdc42 and ERK1/2.

control was maintained for up to 7 days (Fig. 1B), whereas Rac1 and RhoA were inhibited for 5 and 9 days, respectively (not shown). As observed by phase-contrast microscopy, ablation of RhoA did not induce significant morphological alterations, whereas that of Rac1 decreased lamellipodium formation and ablation of Cdc42 induced a more 'dendritic' morphology (Fig. 2).

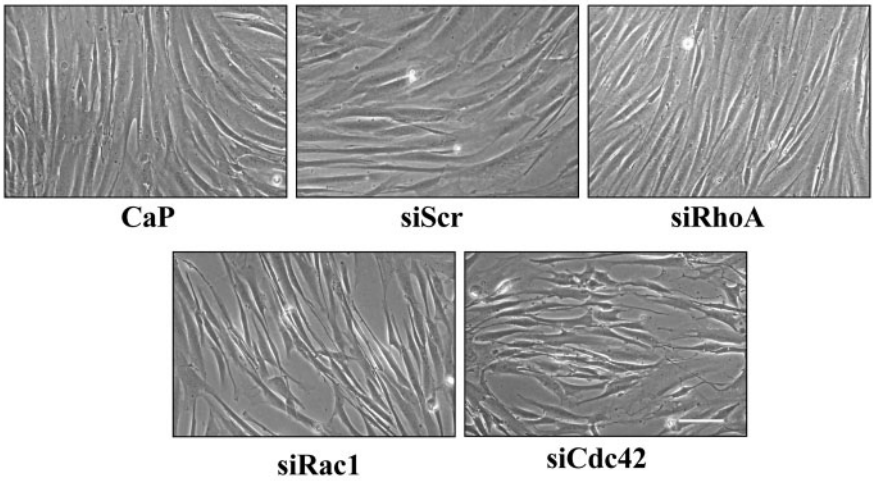

Fig. 2. Morphological effects of RhoA, Rac1 or Cdc42 silencing in HSFs. Representative phase-contrast microscopy of HSFs 72 hours after transfection with calcium phosphate alone (CaP), 20 nM irrelevant siRNA (siScr) or with 20 nM siRNA targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42). Bar, 20 μm.

Silencing of Cdc42 upregulates the expression of MMP-1

In HSFs spread on a rigid support, the expression of MMP-1 is low compared with conditions under which the actin cytoskeleton is disrupted by cytochalasin D (Lambert et al., 1998, Unemori and Werb, 1986) or by culturing cells in a freely retracting collagen gel (Lambert et al., 1992; Langholz et al., 1995; Mauch et al., 1989). In HSFs cultured in monolayer, the silencing of Cdc42 induced a 15-fold increase in MMP-1 secretion, as measured by western-blot analysis of conditioned medium. By contrast, the expression of MMP-1 was not affected by RhoA or Rac1 silencing (Fig. 3A,B). The induction of MMP-1 was already detectable at a concentration of 2 nM siRNA, reached a maximum at 6 nM and then levelled off up to 60 nM siRNA (Fig. 3C). The induction of MMP-1 expression by silencing Cdc42 was confirmed at the mRNA level by RT-PCR analysis (Fig. 4A,B). By contrast, the expression of α_1I collagen and MMP-2 was not affected by the silencing of the Rho GTPases (Fig. 4A,B). Analysis of serum-free medium conditioned by the transfected HSFs for gelatinase activity by zymography revealed that an equivalent level of the latent form of MMP-2 was present under all tested conditions, whereas the active forms were not detected (not shown). None of the siRNAs induced a significant overexpression of the signal transducer and activator of transcription 1 (Stat1) (Fig. 4C). On the contrary, the Rac1 siRNA significantly repressed the Stat1 steady-state level. Moreover, interferon-β expression was below the detection limit of our ELISA kit (not shown). This suggests that the phenotypic modulations induced by the siRNA are not related to the activation of the interferon system, as recently reported in other models (Sledz et al., 2003). A second siRNA targeting another sequence of Cdc42 repressed Cdc42 as efficiently as the previous one and induced a similar overexpression of MMP-1 at both the mRNA and protein levels (Fig. 5).

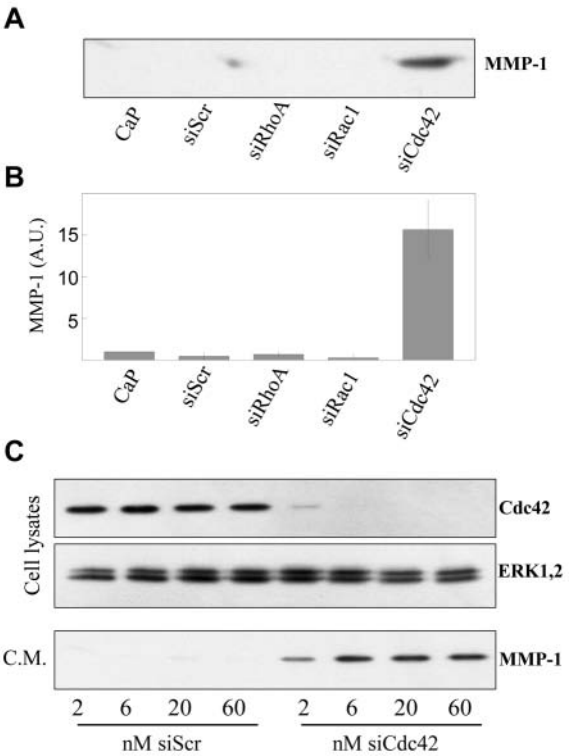

Fig. 3. Cdc42 silencing upregulates MMP-1 protein level. (A) Representative western-blot analysis of serum-free medium conditioned by HSFs for 16 hours between days 2 and 3 after transfection with calcium phosphate alone (CaP), with 20 nM siRNA targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42), or with 20 nM irrelevant siRNA (siScr). (B) Densitometric analysis of A. Results are the means±s.d. of three independent experiments. (C) Representative western-blot analysis of cell lysates of HSFs 72 hours after transfection (cell lysates) or serum-free medium conditioned by HSFs for 16 hours between days 2 and 3 after transfection (C.M.). Cells were transfected with the indicated concentrations of either an irrelevant siRNA (siScr) or the siRNA targeting Cdc42 (siCdc42).

MMP-1 overexpression induced by silencing Cdc42 requires Rac1

It has been reported in various models that a transient activation of either RhoA or Rac1 is a necessary step in the signal-transduction cascade leading to MMP-1 production (Kheradmand et al., 1998; Werner et al., 2001). To address their role in the induction of MMP-1 following Cdc42 silencing, we performed a simultaneous repression of two or the three Rho GTPases by co-transfection of two or three siRNAs. In these experiments, each Rho-GTPase-targeting siRNA was used at a

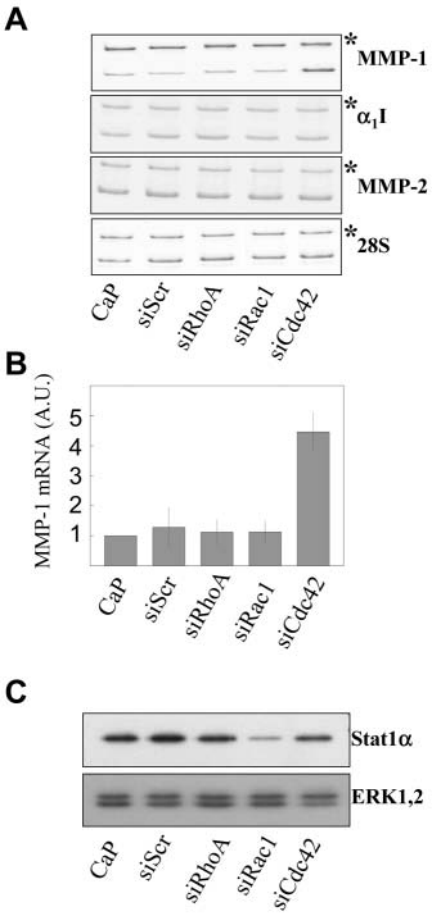

Fig. 4. Cdc42 silencing upregulates MMP-1 but not MMP-2 or α_1I mRNA steady-state levels. (A) Representative quantitative RT-PCR analysis of MMP-1, MMP-2 and α_1I mRNA levels, and of the 28S rRNA in total RNA extracted from HSFs cultured in DMEM containing 10% FCS 72 hours after transfection with calcium phosphate alone (CaP), an irrelevant siRNA (siScr) or an siRNA targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42). Sample-to-sample variations in RT-PCR efficiency are controlled by adding a known copy number of synthetic RNA co-transcribed and co-amplified with the same primers, to generate a product of larger size (*). (B) Densitometric analysis of A. Results are the means±s.d. of three independent experiments. (C) Western-blot analysis of whole-cell lysates of HSFs transfected with calcium phosphate alone (CaP), with 20 nM siRNA targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42), or with 20 nM irrelevant siRNA (siScr). 72 hours after transfection, the cells were lysed in SDS-PAGE loading buffer and analysed by immunoblotting with specific antibodies to Stat1α and ERK1/2.

final concentration of 10 nM. siScr was added to the transfection mix to reach a final concentration of 30 nM when only one or two specific siRNA were used. Western-blot analysis of whole-cell lysates collected 3 days after transfection confirmed the striking specificity of the repression of each Rho GTPase and revealed a lack of significant interference between the individual silencing after co-transfection with the siRNA targeting the other Rho GTPases (Fig. 6). Western-blot analysis of media conditioned by the transfected fibroblasts confirmed that only the silencing of

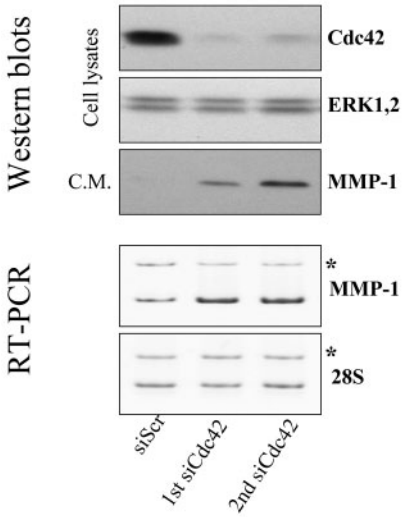

Fig. 5. A second siRNA targeting another region of the *Cdc42* mRNA also upregulates MMP-1. Representative western-blot analysis of whole-cell lysates and of serum-free medium conditioned by HSFs 72 hours after transfection with 20 nM irrelevant siRNA (siScr), the first siRNA targeting Cdc42 (1st siCdc42) or the second siRNA targeting Cdc42 (2nd siCdc42), using specific antibodies to Cdc42, ERK1/2 and MMP-1. RT-PCR analysis of *MMP-1* mRNA level and 28S rRNA was performed with total RNA extracted from HSFs cultured in DMEM containing 10% FCS 72 hours after transfection with 20 nM irrelevant siRNA (siScr), the first siRNA targeting Cdc42 (1st siCdc42) or the second siRNA targeting Cdc42 (2nd siCdc42). Sample-to-sample variations in RT-PCR efficiency are controlled using a known copy number of synthetic RNA co-transcribed and co-amplified with the same primers to generate a product of larger size (*).

Cdc42 induces MMP-1 expression. The co-silencing of Rac1 and Cdc42, but not that of RhoA and Cdc42, significantly reduced the MMP-1 overexpression, suggesting that Rac1 participates in the upregulation of MMP-1 following Cdc42 silencing. The ROCK inhibitor Y27632 used at 10 μM (a concentration inhibiting more than 80% of myosin regulatory light chain phosphorylation), did not antagonize the MMP-1 overexpression induced by silencing Cdc42 (not shown), further suggesting that the RhoA-ROCK pathway is not involved in the process analysed here. It should be realized that Y27632 did not affect Cdc42 silencing (not shown).

Silencing of Cdc42 upregulates IL-8 and MCP-1 expression

To analyse the expression profile of cytokines by siCdc42-transfected HSFs, proteoarrays were used to evaluate the concentration of 79 different cytokines in the conditioned media. They revealed an overexpression of IL-8, IL-6 and MCP-1 by HSFs transfected with the Cdc42 siRNA (Fig. 7A). The upregulation of IL-8 and MCP-1, two cytokines known to induce MMP-1, was confirmed by RT-PCR and ELISA (Fig. 7B,C). It should be realized that IL-1α and IL-1β mRNAs were barely detectable in transfected HSFs and neither IL-1α nor IL-1β was detected by proteoarray analysis or by ELISA (not shown). The involvement of IL-8 and MCP-1 in the control of MMP-1 expression by Cdc42 was assessed by using blocking

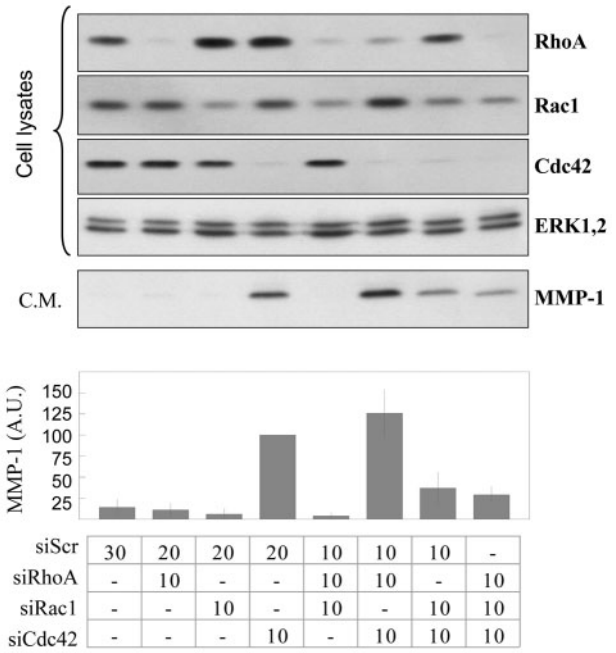

Fig. 6. Multiple knock down of Rho GTPases revealed a role for Rac1, but not RhoA, in MMP-1 overexpression following Cdc42 silencing. HSFs were transfected with the indicated siRNAs at a concentration of 10 nM. To reach the same final concentration of 30 nM siRNA in each condition, transfection mix were supplemented with the irrelevant siScr siRNA. Representative western-blot analysis of whole-cell lysates 72 hours after transfection (cell lysates) and of serum-free medium conditioned for 16 hours between days 2 and 3 after transfection. Cell lysates and conditioned medium (C.M.) were analysed by immunoblotting with specific antibodies to RhoA, Rac1, Cdc42, ERK1/2 and MMP-1. (bottom) Densitometric analysis of MMP-1 measurements by western-blot analysis of C.M. Results are expressed as the means±s.d. of three independent experiments. The concentrations of the siRNA in the table below the figures are in nM.

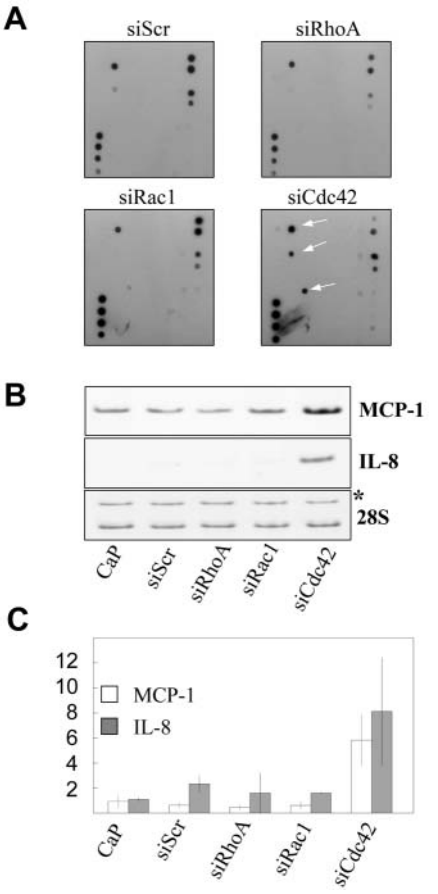

Fig. 7. MCP-1 and IL-8 overexpression following Cdc42 silencing. (A) Analysis of serum-free medium conditioned for 16 hours between days 2 and 3 after transfection by cytokine proteoarray. HSFs were transfected with 20 nM siRNA targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42), or with 20 nM irrelevant siRNA (siScr). White arrows indicate the positions of the IL-8, IL-6 and MCP-1 signals, respectively, from top to bottom (B) Representative semiquantitative RT-PCR analysis of *MCP-1* and *IL-8* mRNA level and of 28S rRNA in total RNA extracted from HSF 72 hours after transfection with calcium phosphate alone (CaP), an irrelevant siRNA (siScr) or an siRNA targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42). The efficiency of the 28S RT-PCR is controlled using a known copy number of synthetic RNA co-transcribed and co-amplified with the same primers to generate a product of larger size (*). (C) ELISA measurements of MCP-1 and IL-8 in serum-free medium conditioned by HSFs for 16 hours between days 2 and 3 after transfection.

antibodies. Treatment of transfected HSFs with up to 10 μg ml^{-1} blocking anti-IL-8 or anti-MCP-1 antibodies did not alter the induction of MMP-1 following silencing of Cdc42 (not shown). This suggests that MMP-1 overexpression occurs independently of that of IL-8 or MCP-1.

Induction of MMP-1 following Cdc42 silencing is mediated through the ERK1/2 pathway

We noted that, in starved HSFs, the electrophoretic pattern of ERK1/2 was modified following Cdc42 silencing, a shift like that observed when these proteins are phosphorylated. The analysis of the phosphorylation status of ERK1/2 and p38 with phosphorylation-specific antibodies revealed that ablation of Cdc42 induced an activation of both ERK1/2 and p38 compared with HSFs transfected with other siRNAs (Fig. 8A). The role of the ERK1/2, the p38 MAP kinase and phosphoinositide-3-kinase (PI3K) pathways in the intracellular signalling mediating the MMP-1 overexpression in Cdc42-ablated cells was evaluated by pharmacological inhibition. Transfected HSFs were treated with U0126 (a specific MEK1/2 inhibitor) and SB203580 (a specific p38-MAP-kinase inhibitor). The overexpression of MMP-1 following Cdc42 silencing was significantly decreased with a concentration of U0126 as low as 1 μM. Similar results were observed in HSFs transfected with the second siRNA targeting Cdc42 (Fig. 9). By contrast, inhibition of the p38 MAP kinase with SB203580 concentrations of 2-6 μM significantly increased the MMP-1 expression (Fig. 8) while barely affecting it at concentrations of 0.01-0.3 μM (Fig. 9). These observations confirm the involvement of the ERK1/2 pathway in the overexpression of MMP-1 following ablation of Cdc42. The contribution of the PI3K and Src signalling pathways was similarly investigated. The PI3K inhibitor LY294 did not affect MMP-1 expression in siCdc42-transfected HSFs (Fig. 8). The tyrosine-kinase

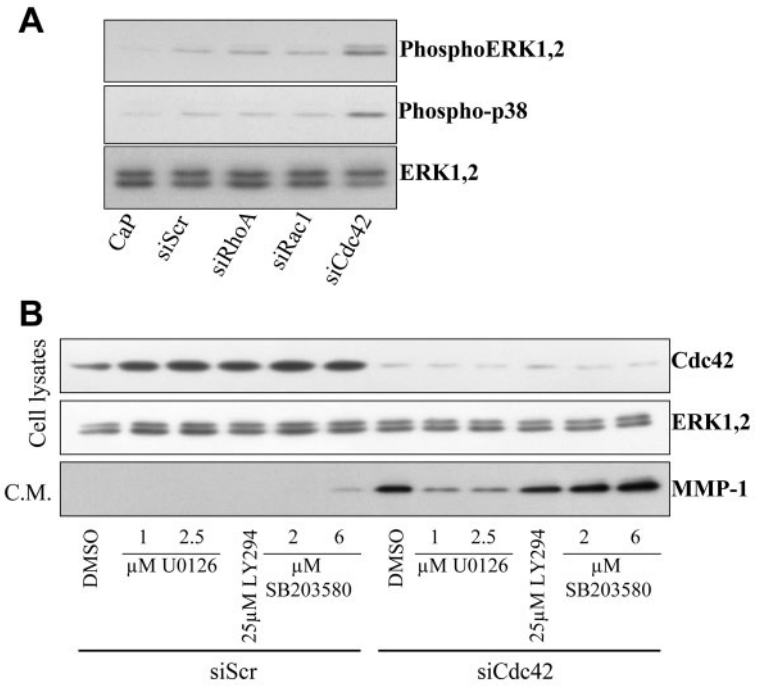

Fig. 8. Overexpression of MMP-1 following Cdc42 silencing is mediated through the ERK1/2 pathway. (A) Western-blot analysis of whole-cell lysates from starved HSFs 72 hours after transfection with calcium phosphate alone (CaP), 20 nM irrelevant siRNA (siScr) or 20 nM siRNA targeting RhoA (siRhoA), Rac1 (siRac1) or Cdc42 (siCdc42). Cell lysates were analysed by immunoblotting with specific antibodies to phospho-ERK1/2, phospho-p38 and total ERK1/2 (ERK1,2). (B) Representative western-blot analysis of whole-cell lysates 72 hours after transfection (cell lysates) and of serum-free medium conditioned for 16 hours between days 2 and 3 after transfection (C.M.). HSFs were transfected with 20 nM irrelevant siRNA (siScr) or with 20 nM siRNA targeting Cdc42 (siCdc42) and cultured between days 1 and 3 after transfection with the indicated concentrations of the MEK-kinase inhibitor U0126, of the PI3K inhibitor LY294 and of the p38-MAP-kinase inhibitor SB203580. Cell lysates and C.M. were analysed by immunoblotting with specific antibodies to Cdc42, ERK1/2 and MMP-1.

inhibitor Herbimycin A, used at 260 nM, completely suppressed the induction of MMP-1, while a more specific Src-kinase inhibitor PP2 (1 µM) was inactive (not shown). It should be realized that these inhibitors did not modify the silencing of Cdc42 (illustrated for U0126, SB203580 and LY294 in Figs 8, 9).

Induction of MMP-1 following Cdc42 is observed in human cell lines from various origins

To extend the significance of our results, the control of MMP-1 expression by Cdc42 was also tested in human cell lines from various origins. Human breast adenocarcinoma cell HS578T, human fibrosarcoma cells HT1080 and human melanoma cells A2058 were transfected with either the irrelevant siRNA (siScr) or the first siRNA targeting Cdc42 (siCdc42). Western-blot analysis of whole cell lysates 72 hours after transfection revealed a significant silencing of Cdc42 by siCdc42. Western-blot analysis of the corresponding conditioned medium demonstrated a significant overexpression of MMP-1 following Cdc42 in the three cell lines (Fig. 10), suggesting that this mechanism of regulation is not limited to HSFs.

Rescue of Cdc42 silencing repressed MMP-1 overexpression

To confirm the role of Cdc42 in this control of MMP-1, the silencing of Cdc42 was rescued by expressing a *Cdc42* mRNA made resistant to the siRNA by six silent mutations and coding for a wild-type Cdc42 protein (mCdc42). Although each cell line was easily transfected with the siRNA, preliminary analysis revealed that HS578T cells are by far the most efficiently transfected cells with an expression vector for the enhanced green fluorescent protein (not shown). Rescue experiments were therefore carried out with this cell type. HS578T seeded in six-well plates were first transfected overnight with 20 nM either siScr or siCdc42. Then, the cells were washed and immediately transfected with 1 µg empty pShuttle or 1 µg pShuttle-mCdc42 for 20-24 hours. The specific measurement of the mCdc42 mRNA was achieved by RT-PCR using a primer complementary to a nucleotide sequence of pShuttle located between the multiple cloning site and the polyadenylation site (i.e. in the specific 3′ untranslated region of the mCdc42 mRNA). The lack of amplification product when the reverse-transcription step was omitted demonstrated that only mCdc42 mRNA was amplified (Fig. 11, top left). RT-PCR analysis of total RNA extracted from HS578T 72 hours after transfection demonstrated that: (i) the silencing of the endogenous Cdc42 mRNA by the siCdc42 was as efficient after transfection of either the empty pShuttle or the pShuttle-mCdc42; (ii) the mutated *Cdc42* mRNA was not ablated by the siCdc42 (Fig. 11), and western-blot analysis of whole-cell lysates and of medium conditioned by HS578T cells demonstrated that the re-expression of Cdc42 by transfecting pShuttle-mCdc42 after transfection of siCdc42 significantly decreased the expression of MMP-1 (Fig. 11). These results definitively confirm the specificity of the negative regulation operated by Cdc42 on MMP-1 expression.

Discussion

Interactions of cells with their support are primarily mediated by the heterodimeric receptors of the integrin family (Hynes, 1992). These receptors are thought to play a key role in the control of cell behaviour by the ECM, in part through their ability to drive cytoskeleton organization, which influences the pattern of gene expression (Danen et al., 1998). There is extensive evidence that integrins control actin polymerization via members of the Rho family of small GTPases (Juliano et al., 2002). Among them, RhoA, Rac1 and Cdc42 are the best characterized and elicit distinct effects on actin structures. RhoA regulates the formation of the actin stress fibres through bundling of pre-existing filaments, whereas Rac1 and Cdc42 are required for the formation of lamellipodia and filopodia, respectively, via de novo actin polymerization (Hall, 1998). In fibroblasts, the expression of MMP-1 is tightly controlled by

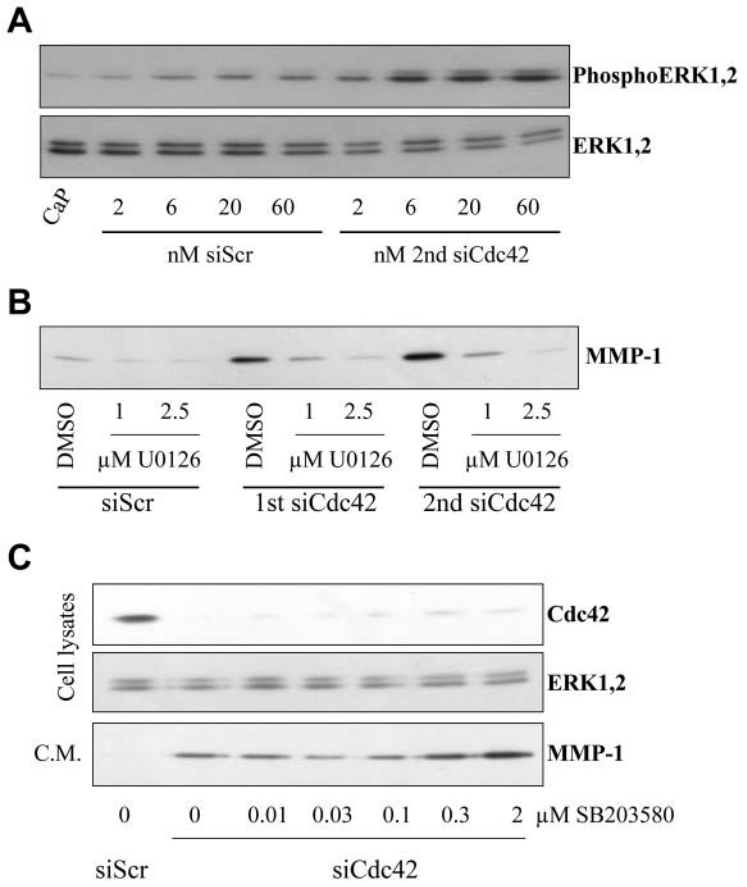

Fig. 9. The ERK1/2 pathway is also required for MMP-1 overexpression following Cdc42 silencing with a second siRNA. (A) Representative western-blot analysis of whole-cell lysates of starved HSFs 72 hours after transfection with calcium phosphate alone (CaP) or with the indicated concentrations of an irrelevant siRNA (siScr) or the second siRNA targeting Cdc42 (2nd siCdc42). Cell lysates were analysed by immunoblotting with specific antibodies to phospho-ERK1/2 and ERK1/2. (B,C) Representative western-blot analysis of serum-free medium conditioned by HSFs for 16 hours between days 2 and 3 after transfection with 20 nM irrelevant siRNA (siScr), with 20 nM first siRNA targeting Cdc42 (1st siCdc42) or with 20 nM second siRNA targeting Cdc42 (2nd siCdc42) and cultured between days 1 and 3 after transfection with the indicated concentrations of the MEK-kinase inhibitor U0126 (B) or the p38-MAP-kinase inhibitor SB203580 (C).

integrin-mediated interactions with ECM components. Fibroblasts spread on a rigid substrate express low levels of MMP-1. Inhibiting integrin function by mean of blocking antibodies (Kheradmand et al., 1998) or interfering with integrin-mediated cytoskeleton organisation with cytochalasin D or by culturing cells in floating collagen gels dramatically increases MMP expression (Mauch et al., 1989; Lambert et al., 1992; Lambert et al., 2001b). We hypothesized that the low expression level of MMP-1 in fibroblasts spread on a rigid substrate involves small GTPases of the Rho family. The conventional methods to target the Rho GTPase pathways include the mutated forms of the Rho proteins (Feig, 1999), the p21-binding domain of their effectors (Nur-E-Kamal et al., 1999) and a class of bacterial toxins that modulate their activity (Lerm et al., 2000). Although widely used, each of these approaches has its own drawbacks. Dominant negative forms could be less specific than expected because some guanine-nucleotide exchange factors are shared by several Rho GTPases. Moreover, overexpression of mutated proteins is precluded in cells that are refractory to transfection and might not be adequate to many experimental situations. Similarly, the limited specificity of bacterial toxins did not allow differential analysis of highly homologous Rho proteins.

In this report, we took advantage of RNA interference to investigate the roles of RhoA, Rac1 and Cdc42 in the control of the expression of MMP-1, MMP-2 and type-I collagen. This experimental approach proved to be especially suitable for these investigations in the hard-to-transfect primary HSFs. It was very efficient and specific at silencing each GTPase without significant alteration of the overall protein synthesis, as suggested by the constant steady-state level of ERK1/2 used as control. It is well-established that activated GTPases translocated to the cell membrane, where they bind and activate their downstream effectors (del Pozo et al., 2000). In this cell compartment, the GTPases could have a half-life different from that of the cytoplasmic inactive GDP-bound form. The repression of both active and inactive forms was confirmed with a pull-down assay and definitively validated the siRNA approach. This targeting was very specific and, furthermore, we did not observe any compensatory modulation of the steady-state levels or activation levels of one GTPase caused by the silencing of another GTPase. These results are in agreement with recent observations in Rac1-deficient macrophage (Wells et al., 2004).

Recently, Sledz et al. (Sledz et al., 2003) reported the activation of the interferon system and observed a strong upregulation of Stat1 upon siRNA transfection in T98G cells. In our system, this side effect did not occur because the Stat1 protein level was not increased upon transfection with 20 nM siRNAs. Moreover, the expression level of interferon-β was below the detection limit of the ELISA kit used here (not shown). These results suggest that the interferon system is not activated in HSFs following siRNA transfection. This discrepancy could be related to the type of cell used and/or to the transfection method [calcium-phosphate precipitation in our study rather than Oligofectamine™ in the study of Sledz et al. (Sledz et al., 2003)]. The repression of Stat1 by the Rac1 siRNA as we observed here would be worth investigating further in another context.

Titration of the siRNA targeting Cdc42 revealed that the induction of MMP-1 is observed at concentrations as low as 2 nM, reached a plateau around 6 nM and did not increase further up to 60 nM, demonstrating that this effect is saturable. The use of a second siRNA targeting another sequence of Cdc42 confirmed the role of Cdc42 in the control of MMP-1 expression. Silencing of Cdc42 induced a significant increase of MMP-1 expression in human cell lines of various origins, suggesting that the negative regulation of MMP-1 by Cdc42 is a widespread mechanism. In HS578T cells, the effect of Cdc42 silencing was rescued by re-expressing wild-type Cdc42. The residual expression of MMP-1 upon rescue of Cdc42 silencing is probably caused by the 10-30% of cells that have not been

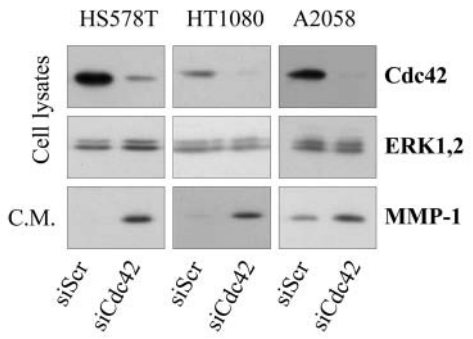

Fig. 10. MMP-1 overexpression following Cdc42 silencing is observed in various human cell lines. Representative western-blot analysis of cell lysates of human breast adenocarcinoma cell line HS578T, fibrosarcoma cell line HT1080 and melanoma cell line A2058 72 hours after transfection, and of serum-free medium conditioned by the same cells for 16 hours between days 2 and 3 after transfection (C.M.). Cells were transfected with 20 nM irrelevant siRNA (siScr) or 20 nM first siRNA targeting Cdc42 (siCdc42). Cell lysates and C.M. were analysed by immunoblotting with specific antibodies to Cdc42, ERK1/2 and MMP-1.

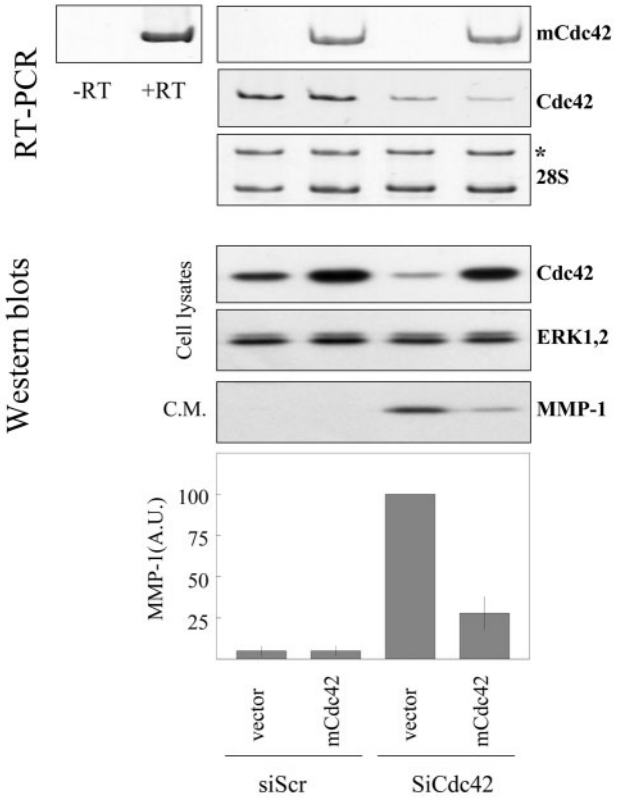

Fig. 11. The rescue of Cdc42 knock down represses MMP-1 overexpression. RT-PCR analysis of Cdc42 and mutated Cdc42 (mCdc42) mRNA, and 28S rRNA levels was performed with total RNA extracted from HS578T cells transfected with 20 nM irrelevant siRNA (siScr) or 20 nM first siRNA targeting Cdc42 (siCdc42) and 1 μg either empty pShuttle (vector) or pShuttle-mCdc42 (mCdc42). Total RNA was extracted 72 hours after transfection with the siRNA. (top left) The amplification of mCdc42 mRNA performed with total RNA extracted from HS578T cells 48 hours after transfection with pShuttle-mCdc42, with (+RT) or without (–RT) the reverse transcription step. Representative western-blot analysis with specific antibodies to Cdc42, ERK1/2 and MMP-1 of whole-cell lysates and of serum-free medium conditioned (C.M.) by HS578T cells transfected with 20 nM irrelevant siRNA (siScr) or 20 nM first siRNA targeting Cdc42 (siCdc42) and 1 μg empty pShuttle (vector) or pShuttle-mCdc42 (mCdc42). (bottom) Densitometric analysis of MMP-1 measurements by western-blot analysis of C.M. Results are the means±s.d. of three independent experiments.

transfected by pShuttle-mCdc42. These data definitively confirmed the involvement of Cdc42 in the control of MMP-1 expression.

The exquisite flexibility of siRNA technology allowed us to knock down the three Rho GTPases simultaneously with a specificity that cannot be reached by any other method currently used. By mean of multiple knock-down experiments, a role was demonstrated for Rac1 but not for RhoA in MMP-1 overexpression following Cdc42 silencing. These results highlight an antagonistic effect of Cdc42 and Rac1 on MMP-1 expression. Because Rac1 activity does not seem to be affected by the silencing of Cdc42, we hypothesize that one or more pathways downstream of Rac1 are targeted by Cdc42. WASP and WAVE family proteins are potential candidates because they are common intracellular signalling effectors of the Cdc42 and Rac1 pathways involved in the regulation of the actin dynamics (Takenawa and Miki, 2001). The participation of Rac1 in the overexpression of MMP-1 is in agreement with a previous report demonstrating that the requirement for Rac1 activity in MMP-1 overexpression is mediated by anti-integrin blocking antibodies (Kheradmand et al., 1998). Rac1 is the most 'resistant' GTPase to siRNA ablation. It could not be repressed lower than 20-30% of its original steady-state level in our HSFs. This might explain why co-silencing of Cdc42 and Rac1 did not result in a complete suppression of MMP-1 overexpression. Finally, the lack of inhibition of MMP-1 expression by the ROCK inhibitor Y-27632 definitively ruled out the involvement of the RhoA-ROCK pathway in the MMP-1 upregulation following Cdc42 silencing (not shown).

In fibroblasts, it has been reported that IL-1α and IL-1β are signalling intermediates acting through autocrine loops to mediate MMP-1 upregulation triggered by cytoskeleton disorganization (West-Mays et al., 1995), phagocytosis (Werner et al., 2001), integrin inactivation (Kheradmand et al., 1998) or interaction with specific ECM components (Utani et al., 2003). However, these cytokines were undetectable at the protein level and barely detectable at the mRNA level in transfected HSFs, excluding their involvement in the process studied in this report. The expression profile of cytokines in transfected HSFs was further analysed by using proteoarrays. Among the 79 cytokines tested, only MCP-1 and IL-8 were modulated by the silencing of Cdc42. Although these cytokines are potential inducers of MMP-1 expression in HSFs (Yamamoto et al., 2000), they were not involved in our experimental conditions, as suggested by the lack of effect of anti-MCP-1 and anti-IL-8 blocking antibodies. Because MMP and prostaglandin levels often parallel each other, the *COX-2* mRNA level was also analysed by RT-PCR (not shown). However, the lack of induction of *COX-2* mRNA following Cdc42 silencing suggests that prostaglandin synthesis was not affected.

Our results point to an involvement of ERK1/2 in the overexpression of MMP-1 following Cdc42 silencing, whereas

the p38-MAP-kinase pathway plays an inhibitory role, as suggested by the increased expression of MMP-1 in fibroblasts treated with the p38-MAP-kinase inhibitor (Fig. 8), probably by inhibiting the activation of ERK1/2 (data not shown). SB203580 has been shown to inhibit MMP-1 expression at low concentrations (Xu et al., 2001). However, in our model, SB203580 concentrations of 0.01-0.30 μM barely affected the MMP-1 expression suggesting that the p38 pathway did not play a key role in the regulation studied here. Several reports point to the divergent regulation of MMPs by the ERK1/2 and p38 pathways (Ridley et al., 1997; Brauchle et al., 2000; Reunanen et al., 2002). Interestingly, Rac1 and Cdc42 were reported to control p38-MAP-kinase activation (Minden et al., 1995), a process responsible for the inhibition of both ERK1/2 activation and MMP-1 expression (Singh et al., 1999; Westermarck et al., 2001), whereas a specific inhibitor of the p38-MAP-kinase pathway is known to activate the ERK1/2 pathway (Aguirre-Ghiso et al., 2003). Moreover, the inhibition of the Rac1 and Cdc42 pathways resulted in ERK1/2 activation (Zugasti et al., 2001). The PI3K and Src-kinase pathways are also potentially involved in the regulation of MMPs in fibroblasts (Vincenti et al., 1996; Liao et al., 2003). However, their specific inhibition did not affect MMP-1 expression in our model.

In fibroblasts, an antagonistic regulation of MMP-1 and type-I collagen is usually observed by modulating cell-ECM interactions or integrin-mediated cytoskeleton organization (Lambert et al., 1992; Lambert et al., 1998). However, in three-dimensional collagen gel, the signalling pathways modulating the expression of these genes are not coupled (Langholz et al., 1995). This is also the case in our model, in which the type-I-collagen expression was not regulated following Cdc42 silencing, suggesting that Cdc42 does not exert a general control on the integrin-regulated genes.

In summary, our study is an additional evidence of the power of RNAi to analyse Rho GTPase functions. This approach enabled us to underscore the key role played by Cdc42 in the control of the expression of MMP-1, one main enzyme involved in the degradation of the most abundant ECM protein, type-I collagen. Our data suggest that, in vivo, when integrin-mediated interactions between cells and ECM induce cytoskeleton organization, MMP-1 expression is maintained at a low level by Cdc42 via a repression of the Rac1 and ERK1/2 pathways. The different regulation of MMP-1 and type-I collagen could play a key role in ECM homeostasis and connective-tissue remodelling, as well as in the control of the mechanochemical information delivered to the cells by the ECM, which governs the differentiation state of various cell types (Deroanne et al., 2001; Ingber, 2002). A deregulation of this function of Cdc42 could be at the origin of pathologies associated with excessive matrix degradation such as arthritis, ulcers, atherosclerosis, aneurysms and even tumour progression, because it was recently reported that MMP-1 overexpression in invasive melanoma cells is mediated by the ERK1/2 pathway (Huntington et al., 2004).

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