FEBS Letters 584 (2010) 2305–2310

FEBS Letters

journal homepage: www.FEBSLetters.org

Rac1 modulates TGF-β1-mediated epithelial cell plasticity and MMP9 production in transformed keratinocytes

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ARTICLE INFO

Article history:
Received 9 October 2009
Revised 27 February 2010
Accepted 24 March 2010
Available online 28 March 2010

Edited by Lukas Huber

Keywords:
Keratinocyte
Transforming growth factor-β1
Metalloproteinase-9
Rac1
Snail1
Mitogen-activated protein kinase
Epithelial-mesenchymal transition
Migration

ABSTRACT

Transforming growth factor-β1 (TGF-β1) activates Rac1 GTPase in mouse transformed keratinocytes. Expression of a constitutively active Q61LRac1 mutant induced an epithelial to mesenchymal transition (EMT) linked to stimulation of cell migration and invasion. On the contrary, expression of a dominant-negative N17TRac1 abolished TGF-β1-induced cell scattering, migration and invasion. Moreover, Q61LRac1 enhanced metalloproteinase-9 (MMP9) production to levels comparable to those induced by TGF-β1, while N17TRac1 was inhibitory. TGF-β1-mediated EMT involves the expression of the E-cadherin repressor Snail1, regulated by the Rac1 and mitogen-activated protein kinase (MAPK) pathways. Furthermore, MMP9 production was MAPK-dependent, as the MEK inhibitor PD98059 decreased TGF-β1-induced MMP9 expression and secretion in Q61LRac1 expressing cells. We propose that regulation of TGF-β1-mediated plasticity of transformed keratinocytes requires the cooperation between the Rac1 and MAPK signalling pathways.

1. Introduction

Transforming growth factor-β1 (TGF-β1) is a potent inducer of epithelial-mesenchymal transition (EMT) [1], a phenotypic conversion by which epithelial cells lose their polarity and cohesiveness acquiring the morphology and migratory properties of fibroblasts. EMT is important in malignancy as it implies a profound rearrangement of the cytoskeleton that favours tumor cell invasion and metastasis [2]. Thus, it is not surprising that small GTPases of the Rho subfamily have been involved in TGF-β1-induced EMT [3]. Rho, Rac and Cdc42 members of small GTPases have been implicated in many cellular processes that contribute to tumor progression, including cytoskeletal remodelling, cell adhesion, transcriptional regulation and cell cycle progression [4].

In a previous work, we found that TGF-β1 promoted cell scattering and EMT of mouse transformed keratinocytes (PDV cells) [5]. This TGF-β1-mediated phenotypic conversion was associated with the development of highly aggressive spindle cell carcinomas [5–7] and expression/secretion of extracellular matrix proteinases, such as urokinase and metalloproteinase-9 (MMP9) gelatinase [8–10]. Increased synthesis and activation of gelatinases leads to degradation of collagen IV, a main component of the basement membrane, and favours vascularization since tumor angiogenesis depends on the activity of metalloproteinases, particularly that of MMP9 [11]. In addition, MMP9 proteolytically activates several members of the TGF-β family of growth factors [12], contributing to enhance the pool of active TGF-β in the tumor microenvironment.

In the current study, we show that Rac1 and mitogen-activated protein kinase (MAPK) signalling are important mediators of TGF-β1-induced EMT in transformed keratinocytes, as both pathways appear to regulate the expression of Snail1. Rac1 and MAPK signalling activity are also involved in TGF-β1-mediated production of MMP9.

Abbreviations: TGF-β1, transforming growth factor-β1; EMT, epithelial-mesenchymal transition; MMP9, metalloproteinase-9; GST, glutathione-S-transferase; PBS, phosphate buffered saline; F-actin, actin filaments; PAK, p21-activated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3-OH kinase

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.03.042

2. Materials and methods

2.1. Plasmids and antibodies

Dominant-negative (N17T) and constitutively active (Q61L) constructs of Rac1 [13] were kindly provided by J. Silvio Gutkind (National Institute of Health, Bethesda, USA). The reporter construct containing 1300 bp of the 5′-flanking region of the mouse MMP9 gene has been previously described [14]. Monoclonal antibodies (mAbs) for Rac1, E-cadherin (ECCD2) and β-actin (clone AC-15) were from Cytoskeleton (Denver CO, USA), Zymed laboratories (San Francisco CA, USA) and Sigma (Saint Louis MO, USA), respectively. Phospho-p44/42 ERK1,2 MAPK (Thr202/Tyr204) mAb was from Cell Signalling Technology Inc (Danvers, MA, USA). Antibodies for ERK1,2 (C-16), phospho-Akt (11E6), phospho-Smad2/3 (Ser 423/425) and Smad2/3 (FL-425) were from Santa Cruz Biotechnology (CA, USA). Appropriate secondary antibodies coupled to horseradish peroxidase or FITC were purchased from Sigma.

2.2. Cell culture and transfection procedures

PDV cells were cultured in Ham's F-12 medium supplemented with amino acids and vitamins in the presence of 10% fetal bovine serum and antibiotics, as described [5]. For TGF-β treatments, human recombinant TGF-β1 (R&D Systems GmbH, Germany) was used at 10 ng/ml.

For stable transfections, $\sim 10^6$ cells seeded on 60 mm plates were transfected with 2 μg of the pcDNA3-N17TRac1, -Q61LRac1 plasmids or the empty pcDNA3 vector using Superfect (Qiagen, Hilden, Germany) following the manufacturer's instructions. Transfected cells were selected by growing in medium containing 10% fetal bovine serum and 400 μg/ml of G418 for two weeks. Individual clones were isolated by cloning rings.

Transient transfections to analyze MMP9 promoter activity were performed as previously described [10,14]. Firefly luciferase activity (Promega, Addison WI, USA) was standardized for β-galactosidase activity (Tropix, Bedford MA, USA).

The MEK inhibitor PD98059 (25 μM as final concentration) was added to the cells 30 min before stimulation for 48 h with TGF-β1.

2.3. RNA isolation and RT-PCR assays

Total RNA was obtained using Trizol and complementary DNA was generated by the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using oligo (dT) as a primer. Primer sequences for E-Cadherin, Snail1, Snail2 and E12/E47 have been previously described [15]. For murine MMP9 the following oligonucleotides were used: 5′-ACC-ACC-ACA-ACT-GAA-CCA-CA-3′ and 5′-ACC-AAC-CGT-CCT-TGA-AGA-AA-3′ (amplifies a fragment of 304 bp), and for murine GAPDH: 5′-ACC-ACA-GTC-CAT-GCC-ATC-AC-3′ and 5′-TCC-ACC-ACC-CTG-TTG-CTG-TA-3′ (amplifies a fragment of 450 bp). PCR products were obtained after 30–35 cycles of amplification with an annealing temperature of 60–65 °C.

2.4. Pull down, Western blot, zymographic and immunofluorescence assays

Western blots were performed as described elsewhere [10]. The level of active Rac1 (Rac1-GTP) in the cell lysates was measured using a glutathione-S-transferase (GST) fusion protein with the Rac1 binding domain of p21-activated kinase (PAK) (GST-PAK). Assays were performed as previously described [16].

Gelatinase activity was assayed in serum-free medium conditioned for 24 h in cell cultures treated or not with TGF-β1 subjected to SDS-PAGE zymography in gels containing 1 mg/ml of gelatine, as reported [9].

Detection of E-cadherin and actin filaments (F-actin) by immunofluorescence was performed in cells grown on glass coverslips fixed in cold methanol or with 3.7% formaldehyde in phosphate buffered saline and permeabilized with 0.1% Triton-X100 for 2 min at room temperature, respectively. For F-actin staining, phalloidin coupled to Alexa Fluor 594 (Molecular Probes, Eugene OR, USA) was used. Images were taken in a microscope equipped with epifluorescence using 400X magnification.

2.5. Migration and invasion assays

The motility properties of cell transfectants was analyzed in an in vitro wound healing assay [9]. Wounded cell cultures were allowed to grow for 24 h in the absence or presence of TGF-β1. The capacity of the cells to migrate through Matrigel-coated filters was assayed as described elsewhere [8].

2.6. Statistics

Data are given as means ± S.D. from at least three independent experiments. When necessary, statistical significance was evaluated using the Students' *t*-test. Differences were considered to be significant at a value of $P < 0.05$ (*&).

3. Results and discussion

3.1. Rac1 controls cell morphology in transformed keratinocytes

TGF-β1 was able to increase the level of active Rac1-GTP in PDV cells without affecting Rac1 protein expression (Fig. 1A). Rac1 activation was visible at 5 min but raised a maximum (~3.5-fold) at 15–30 min of treatment which coincides with that of Smad2/Smad3 [17], suggesting that upregulation of Rac1 activity by TGF-β1 in PDV cells is Smad-independent.

In order to study the involvement of Rac1 in TGF-β1-induced cell migration, we transfected PDV cells with either a constitutively active (Q61L) or a dominant-negative (N17T) mutant form of Rac1 [13]. Cell clones expressing either Q61LRac1 or N17TRac1, designated as CAR and DNR, respectively, were selected as well as control cells transfected with the empty vector (designated as PDV/M). A total of three CAR and four DNR cell clones were isolated that exhibited each a similar morphology and behaviour. Expression of dominant-negative Rac1 blocked TGF-β1-mediated enhancement of Rac1-GTP levels in DNR cells (Fig. 1B). In contrast, CAR cells displayed increased basal Rac1-GTP levels with respect to control cells (Fig. 1B). Expression of the mutant forms of Rac1 had a profound effect on PDV cell morphology. PDV keratinocytes grow as cohesive islands forming stable cell–cell contacts [5]. These compact cell–cell junctions were disrupted in CAR cells that exhibited increased membrane extensions and ruffling activity compared to control cells (Fig. 1C). On the contrary, DNR cells were rounded and grew as tightly packed islands with strong cell–cell contacts (Fig. 1C).

Chronic exposure of PDV keratinocytes to TGF-β1 induces cell scattering during the first week of treatment followed by a complete EMT [5]. As shown in Fig. 2, a 48 h exposure of PDV/M cells to TGF-β1 induced the delocalization/downregulation of E-cadherin (Fig. 2A panels a and b) linked to the loss of cortical F-actin and induction of plasma membrane extensions (Fig. 2A, panels g and h). TGF-β1-mediated downregulation of E-cadherin was blocked in DNR cells (Fig. 2A panels e and f), while untreated

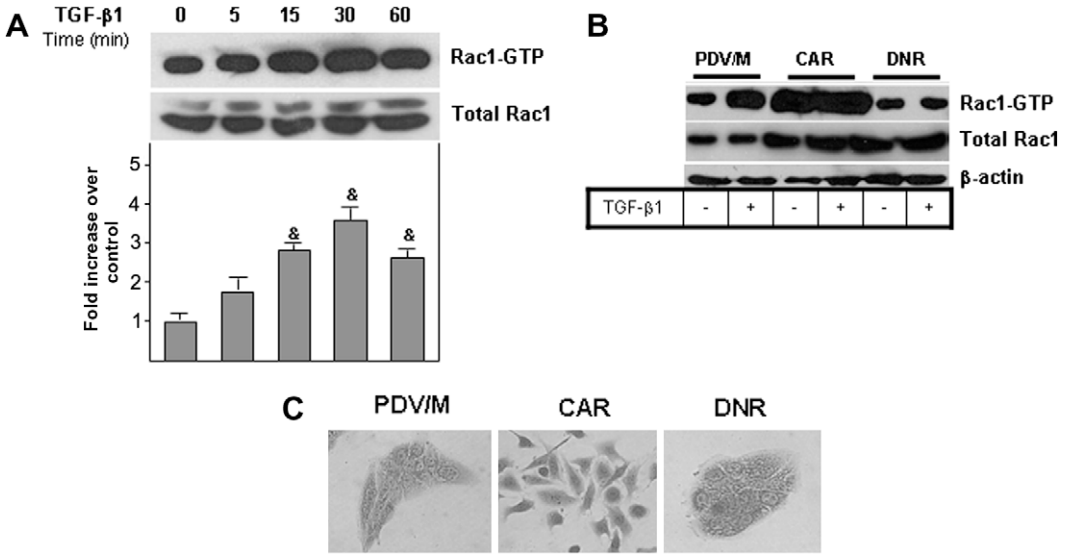

Fig. 1. TGF-β1 activates Rac1 in PDV cells and effects of Rac1 mutant forms on PDV cell morphology. (A) Active Rac1-GTP levels at different times of treatment with TGF-β1 were determined by a pull-down assay with GST-PAK beads. Quantification of Rac1-GTP levels relative to total Rac1 levels was performed by densitometric analysis. (B) Effects of dominant-negative (DNR) and constitutively active (CAR) Rac1 mutant forms in TGF-β1-induced upregulation of Rac1 activity. Levels of Rac1-GTP were determined in the cell transfectants as above. The total levels of Rac1 and β-actin (used as a control for protein loading) were determined by Western blotting. Note increased levels of Rac1 due to expression of exogenous mutant proteins in CAR and DNR cells with respect to control (PDV/M) cells. (C) Phase contrast micrographs of PDV/M, CAR and DNR cells.

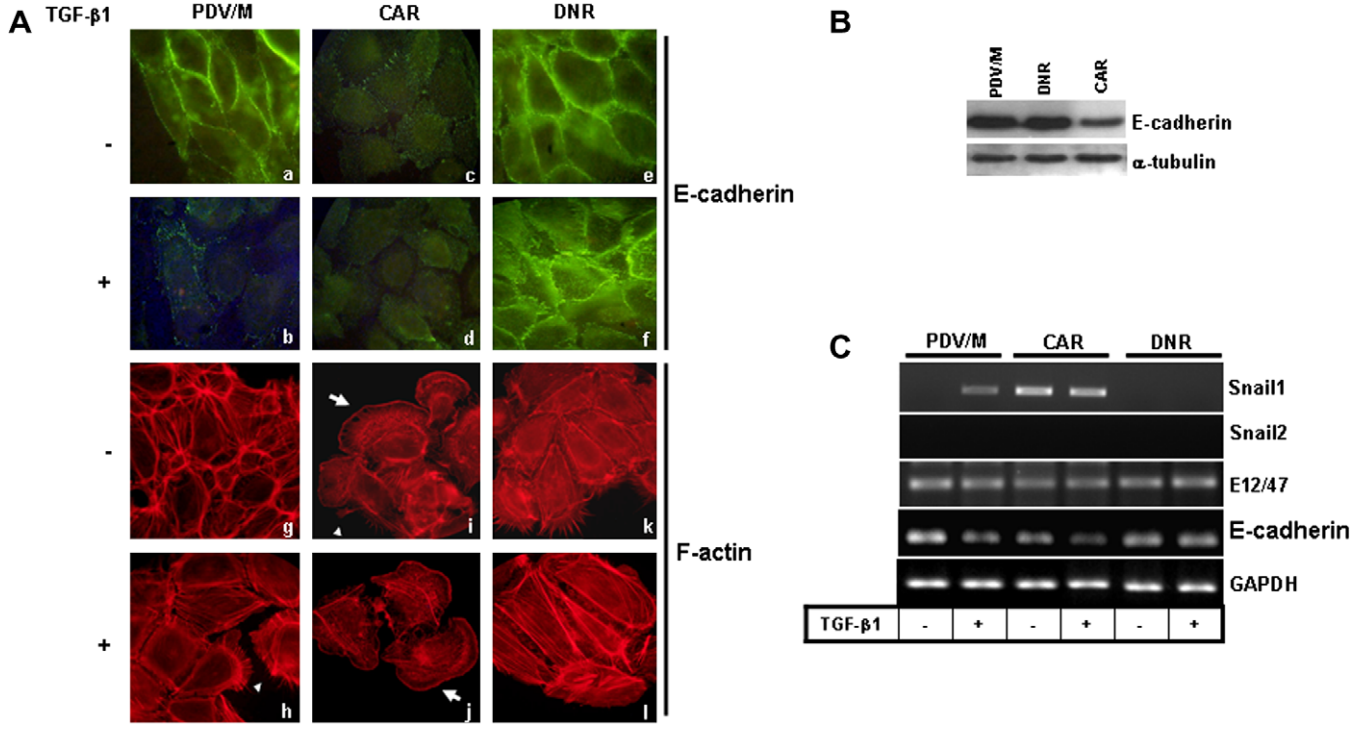

Fig. 2. Rac1-mediated EMT is associated with downregulation of E-cadherin and upregulation of Snail1 expression. (A) Immunofluorescence analysis of E-cadherin and F-actin in the cell transfectants before and after stimulation (48 h) with TGF-β1. Note that TGF-β1 promotes a reduction of E-cadherin staining at cell–cell contacts in control cells (a and b) that is blocked in DNR cells (e and f). Note also that CAR cells have wide lamellipodia (arrows) and that cell–cell junctions are severely disrupted (c and d). Increased number of cell-surface protrusions (arrowheads) can be seen in DNR cells (k) and TGF-β1-treated control cells (h) with respect to untreated control cells (g). (B) Western blot analysis of E-cadherin expression in the cell transfectants. α-tubulin was used as a control of protein loading. (C) Expression of E-cadherin, Snail1, Snail2 and E12/E47 mRNA transcripts by RT-PCR in the cell transfectants before and after stimulation (48 h) with TGF-β1. *GAPDH* was amplified as control for the amount of cDNA present in each sample.

CAR cells showed reduced E-cadherin staining (Fig. 2A panels c and d) and loss of cortical F-actin associated with the induction of extensive lamellipodia (Fig. 2A panels i and j). Intriguingly, untreated DNR cells showed a large number of long filopodia in the periphery of the colonies (Fig. 2A panel k), suggesting that mutant N17TRac1, which is thought to competitively inhibit the interaction of Rac1 with guanine-nucleotide exchange factors [13] induces membrane protrusions at the front of leading edge cells

which might involve the local activation of Cdc42 [18]. Both E-cadherin protein and mRNA expression were indeed slightly reduced, as shown by Western blotting (Fig. 2B) and RT-PCR assays (Fig. 2C). Since E-cadherin transcriptional repressors have also been found to be involved in EMT [19], we analyzed the expression of Snail1, Snail2 (also called Slug) and E12/E47 in our transfectants before and after TGF-β1 stimulation. Only Snail1 expression that was induced by TGF-β1 in PDV/M, but not in DNR cells, was constitutively upregulated in CAR cells (Fig. 2C). Snail2 was undetectable and the levels of E12/E47 did not change in the transfectants even after treatment with TGF-β1 (Fig. 2C). These results indicate that Rac1 mediates TGF-β1-induced expression of Snail1 in transformed keratinocytes.

3.2. Rac1 is required for TGF-β1-induced cell migration/invasion and MMP9 expression/secretion

We next tested whether Rac1 was necessary for TGF-β1-induced cell motility using a scratch wound assay. Untreated control cells were unable to close a wound made 24 h before, while untreated CAR cells and TGF-β1-stimulated control cells did it completely (Fig. 3A). In contrast, TGF-β1-stimulated cell motility was strongly inhibited (80–90%) in DNR cells (Fig. 3A). When the ability of the cell lines to migrate through Matrigel-coated filters was measured, similar results to those of the wound assay were obtained (Fig. 3B).

We have reported that dominant-negative inhibition of RhoA function in PDV cells induced a change towards a fibroblastic morphology while constitutive activation of RhoA promoted a more cohesive phenotype, the opposite effects to those seen with Rac1 mutant constructs [20]. Altogether, these results suggest that TGF-β1-mediated epithelial plasticity in transformed keratinocytes requires upregulation of Rac1 concomitantly to downregulation of RhoA activity. Nevertheless, a number of reports point to RhoA activation (sometimes in cooperation with Cdc42) as an essential requirement for TGF-β1-induced EMT [21–23]. These discrepancies might be cell type- or context-dependent and likely reflect a particular organization of the cytoskeleton and a different way for Rho GTPases to coordinately regulate cell migration [16,18]. Interestingly, Rac1 activation has been associated with a mesenchymal-type of movement of tumor cells characterized by focalized extracellular proteolysis at cell-surface protrusions in contrast to the amoeboid mode of tumor cell movement which is independent of proteinases and depends on RhoA activation [24].

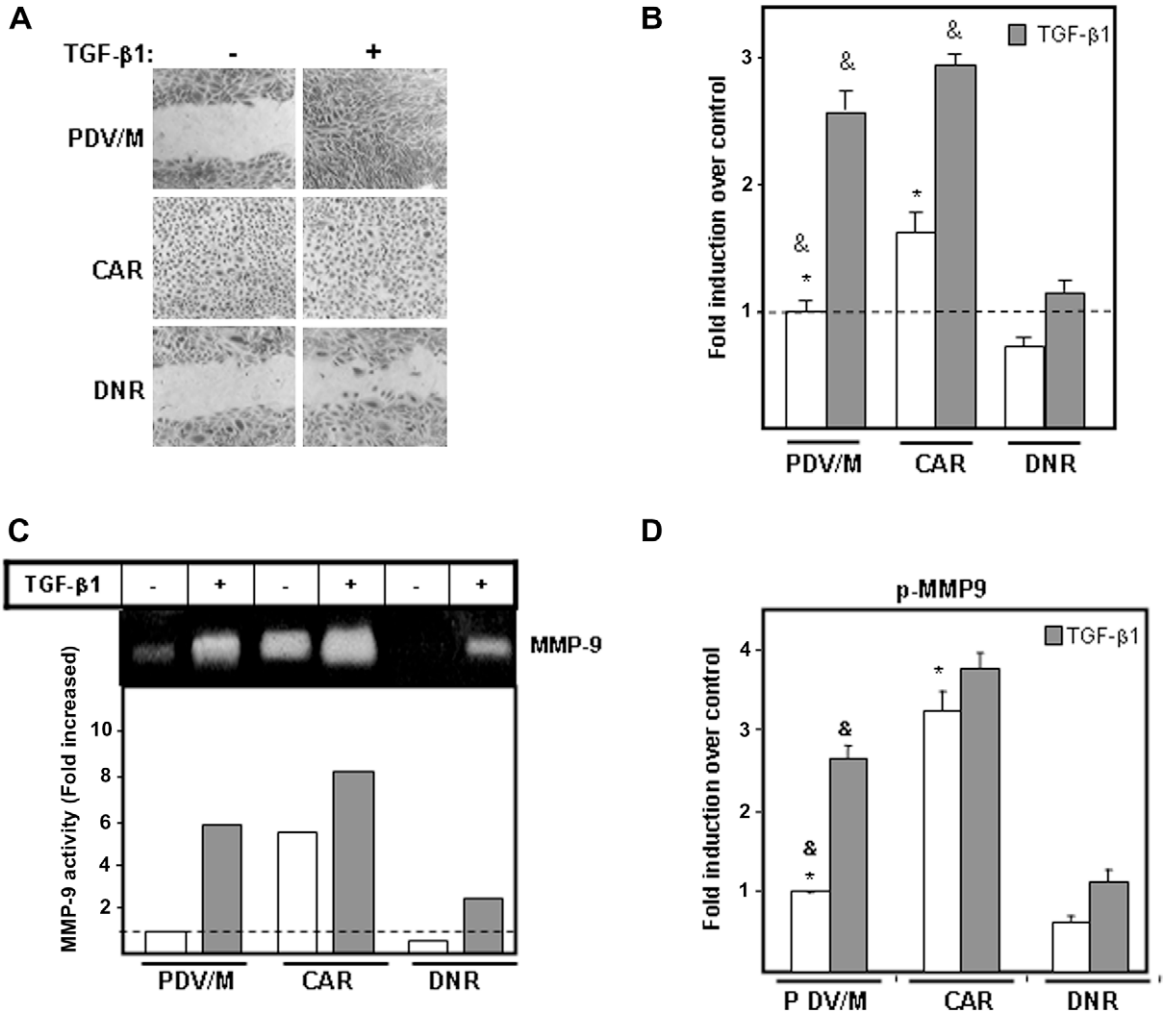

Fig. 3. Effect of TGF-β1 on the migratory and invasive abilities of the cell transfectants and on MMP9 production. (A) Scratch wound assay. Areas free of cells were examined 24 h after wounding in the absence or presence of TGF-β1. Cells were fixed and stained with crystal violet. (B) Invasion assay through Matrigel-coated filters. The percentage of migrated cells was calculated with respect to the total viable cells seeded on the upper chamber at the end of the incubation period (72 h) in the absence or presence of TGF-β1. (C) MMP9 gelatinase activity was determined by zymography in the conditioned media of cell transfectants untreated or treated with TGF-β1 for 48 h. Quantification of MMP9 activity levels was performed by densitometric analysis. (D) MMP9 promoter activity was assayed in cells unstimulated and stimulated with TGF-β1 for 48 h. *,&Statistically significant differences ($P < 0.05$).

We have previously shown that TGF-β1 promotes the expression of MMP9 associated with the stimulation of invasive properties in PDV cells [10]. In order to analyze whether Rac1 is involved in TGF-β1-induced MMP9 production, we studied the secretion of MMP9 gelatinase activity in the conditioned media of the cell lines. As shown in Fig. 3C, stimulation of MMP9 production by TGF-β1 was strongly inhibited in DNR cells, while substantial MMP9 levels were secreted by CAR cells in basal conditions that were further enhanced by TGF-β1. On the other hand, stimulation of MMP9 production by TGF-β1 was strongly inhibited in DNR cells. The ability of TGF-β1 to transcriptionally stimulate MMP9 expression was also evaluated in the cell lines expressing a reporter construct containing the luciferase gene under the control of the MMP9 promoter [14]. The results obtained paralleled those of the zymography (Fig. 3D).

3.3. Rac1-mediated cell plasticity and MMP9 production require MAPK signalling activity

MMP9 has been found to play an important role in cell migration and tumor progression [11]. Expression of MMP9 in tissues is generally low but it is transcriptionally activated by different cytokines and growth factors, including TGF-β. TGF-β1 has been reported to induce MMP9 expression in keratinocytes [10,25] and other cell types [26–28] by a variety of mechanisms. Thus, for example, the Smad pathway has been involved in TGF-β1-induced MMP9 expression in human breast and head and neck carcinoma cell lines [26,27], while in oral carcinoma cells MMP9 induction appears to depend on SNAI1 and Ets-1 transcription factors [28]. We also have shown that the Ras/mitogen-activated protein kinase (MAPK) pathway mediates MMP9 induction by TGF-β1 in PDV transformed keratinocytes [10]. Therefore, we studied the activation of Smad, MAPK and phosphatidylinositol-3-OH kinase (PI3 K) pathways in our cell transfectants by analyzing the levels of phosphorylated Smad2,3, ERK1,2 and AKT. All these pathways were stimulated by TGF-β1 in transformed keratinocytes (Fig. 4A). However, whereas basal levels of p-AKT and p-Smad2,3 were low in CAR and DNR cells and increased after TGF-β1 treatment, p-ERK1,2 levels were already constitutively upregulated in untreated CAR cells. In addition, TGF-β1-induced ERK1,2 activation was substantially inhibited in DNR cells, suggesting that besides Ras [17] Rac1 mediates TGF-β1 activation of ERK1,2 in transformed keratinocytes. This is not surprising since Ras/Raf and Rac/Raf show synergism in both ERK1,2 activation and cell transformation [29].

Finally, we analyzed the effect of inhibiting the MAPK pathway on the expression of Snail1, E-cadherin and MMP9 in PDV/M and CAR cells. The MEK inhibitor PD98059 impaired induction of Snail1 by TGF-β1 in PDV/M cells (Fig. 4B), which is in accordance with our

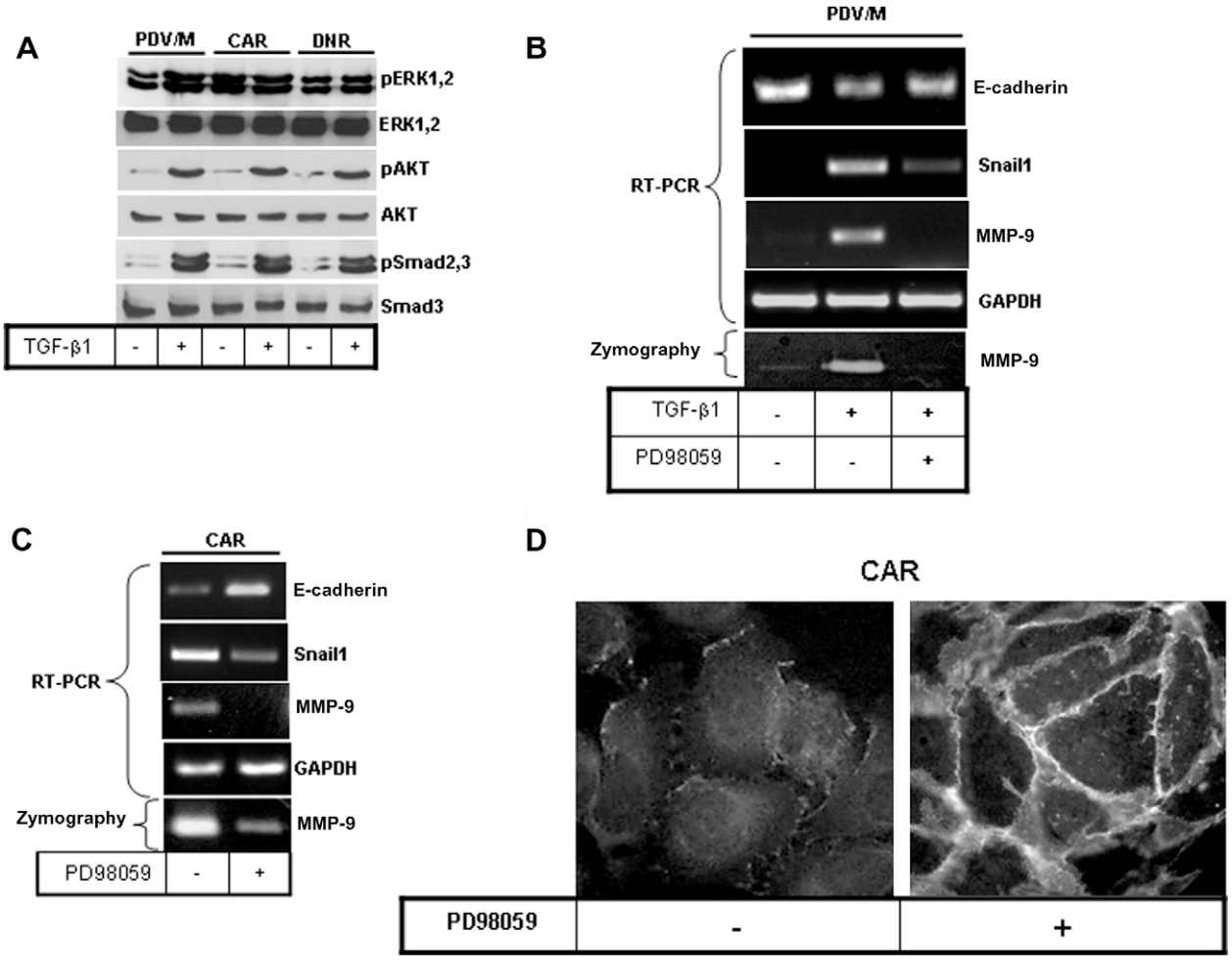

Fig. 4. Rac1-mediated epithelial cell plasticity requires MAPK signalling activity. (A) Western blot analysis of phospho-Erk1,2, phospho-Akt and phospho-Smad2,3 levels relative to the total Erk1,2. Akt and Smad3 expression protein levels in the cell transfectants before and after stimulation for 30 min with TGF-β1. (B and C) Effect of PD98059 on E-cadherin, Snail1 and MMP9 expression in PDV/M control cells unstimulated and stimulated (48 h) with TGF-β1 (B) and CAR cells (C). Expression of E-cadherin, Snail1 and MMP9 mRNA transcripts was determined by RT-PCR. MMP9 gelatinase activity was determined by zymography in the conditioned media. (D) Immunofluorescence analysis of E-cadherin in CAR cells untreated and treated with PD98059.

previous finding that TGF-β1-mediated Snail1 expression in epithelial cells is Smad-independent and involves MAPK signalling [30]. PD98059 also blocked TGF-β1 induction of MMP9 in PDV/M cells (Fig. 4B), as previously reported [10]. The MEK inhibitor reduced MMP9 expression/secretion and Snail1 levels in CAR cells (Fig. 4C), the latter allowing the restoration of E-cadherin expression at cell–cell contacts (Fig. 4C and D). Therefore, TGF-β1-mediated cell plasticity and MMP9 production requires the cooperation between MAPK and Rac1 signalling activity. As Rac1 can be activated by Ras [18], and TGF-β1 activates Ras much earlier than Rac1 in PDV cells: maximal Ras activation occurred at 2 min with a rapid decay at 10 min [17], it remains to be determined whether activation of Rac1 by TGF-β1 in PDV cells involves Ras.

Acknowledgments

The financial support of the Fund for Science and Technology of Chile (grants FONDECYT #1050476 and # 3000045 to J.F.S.), the University of Chile (DID # I003-99/2 to J.F.S.), the Ministry of Science and Technological Development, Republic of Serbia (Grant #145048) and the Spanish Ministry of Science and Innovation (SAF2007-63821 to M.Q.) is gratefully acknowledged. We thank Silvio Gutkind for his useful gift of Rac1 mutant constructs.

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