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Diverse effects of RacV12 on cell transformation by Raf: partial inhibition of morphological transformation versus deregulation of cell cycle control

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Abstract

Activated Raf kinases and Rac GTPases were shown to cooperate in the oncogenic transformation of fibroblasts, which is characterised by the disassembly of the cellular actin cytoskeleton, a nearly complete loss of focal adhesion complexes and deregulated cell proliferation. This is surprising since the Rac GTPase induces actin structures and the adhesion of suspended cells to extracellular matrix proteins. NIH 3T3 cells expressing a hydroxytamoxifen-inducible oncogenic c-Raf-1–oestrogen receptor fusion protein (c-Raf-1-BxB-ER™, N-BxB-ER™ cells) undergo morphological transformation upon stimulation of the Raf kinase. We show that treatment with the Rac, Rho and Cdc42 activating *Escherichia coli* toxin CNF1 or coexpression of an activated RacV12 mutant partially inhibits and reverses the disassembly of cellular actin structures and focal adhesion complexes by oncogenic Raf. Activation of the Rac GTPase restores actin structures and focal adhesion complexes at the cellular boundary, leading to spreading of the otherwise spindle-shaped Raf-transformed cells. Actin stress fibres, however, which are regulated by the function of the Rho GTPase, are disassembled by oncogenic Raf even in the presence of activated Rac and Rho. With respect to the RacV12-mediated spreading of Raf-transformed cells, we postulate an anti-oncogenic function of the activated Rac. Another feature of cell transformation is the deregulation of cell cycle control. NIH 3T3 cells expressing high levels of the c-Raf-1-BxB-ER™ protein undergo a cell cycle arrest upon stimulation of the oncogenic Raf kinase. Our results show that in N-BxB-ER™-RacV12 cells the expression of the activated RacV12 mediates cell proliferation in the presence of high-intensity Raf signals and high levels of the Cdk inhibitor p21[Cip1]. These results indicate a pro-oncogenic function of the Rac GTPase with respect to the deregulation of cell cycle control. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Raf; Rac; Morphological transformation; Actin cytoskeleton; Adhesion

1. Introduction

Oncogenic transformation of fibroblast cells is characterised by deregulated cell proliferation and profound morphological changes. The deregulation of cell proliferation enables the transformed cell to

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survive and proliferate in an environment lacking external factors necessary for cell cycle progression and cell survival. The morphological changes influence the attachment of the fibroblasts to the extracellular matrix such that the oncogenic cells have lost most of their adhesion contacts. Both properties together enable cells to migrate out of their natural tissues and form metastasis, a hallmark of malignant cancer. The c-Raf-1 kinase is a downstream effector of different mitogens and constitutive Raf activation is oncogenic [1]. Depending on the strength and duration of the Raf signal it exerts mitogenic or cell cycle inhibitory effects. High-intensity Raf signals inhibit DNA synthesis and mitosis by the induction of the cdk inhibitor $p21^{Cip1}$, whereas low-intensity Raf signals are sufficient to initiate DNA synthesis in arrested NIH 3T3 fibroblasts in the absence of external growth factors [2–6]. In the presence of high-intensity Raf signals it is proposed that a second cooperating oncogene is necessary to overcome the cell cycle block mediated by $p21^{Cip1}$. Proteins cooperating with Raf are the Myc transcriptional regulator and Rho and Rac GTPases [7–12]. The Ras GTPase is an upstream activator of Raf and constitutive activation of Ras is highly oncogenic [1,13]. Both the Rho and Rac GTPases cooperate with activated Raf proteins in cell transformation and are necessary for cell transformation by oncogenic Ras [8–12]. This is surprising since Rho and Rac are key regulators of the cellular actin filament and focal adhesion complexes [14], structures which are disassembled by the oncogenic action of Ras and Raf proteins. The formation of actin structures and the clustering of integrins to form focal adhesion complexes has been shown to be regulated by the members of the Rho family of small GTPases [14]. Actin stress fibres are induced by Rho activity, whereas the Rac and Cdc42 GTPases induce actin structures at the cellular boundary called lamellipodia and filopodia. The attachment of cells to the extracellular matrix, a process called cell spreading, has been shown to be strongly influenced by the action of the Rac and Cdc42 proteins on integrin clustering [15,16]. This paradox could be explained in the way that the action of the Rho and Rac proteins might contribute to the deregulation of cell proliferation, whereas their action in the regulation of actin structures and focal adhesion complexes is impaired by the oncogenic activation of the Ras and Raf proteins. Recent data demonstrate that the activated Rho protein inhibits the induction of $p21^{Cip1}$ by Ras [17]. Here we have addressed the question how constitutive Rac activity influences the morphological transformation and cell cycle progression of NIH 3T3 cells transformed by oncogenic c-Raf-1.

2. Materials and methods

2.1. Cell culture and transfections

NIH 3T3, N-EHneo, N-BxB-ER^{TM}-Cl-1 and N-BxB-ER^{TM}-RacV12 were grown in Dulbecco's modified Eagle medium (Gibco) supplemented with Glucose (Gibco), L-Glutamine (Gibco), 10% foetal calf serum (Hyclone), 100 units/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). The cells were incubated at 37°C, 5% CO_2 and 90% humidity. 6 µg/ml puromycin (Sigma) was added to the culture medium for puromycin selection and the maintenance of puromycin resistant cell lines. Cells were induced with hydroxytamoxifen by addition of a solution of 1 mM OHT in ethanol to the medium to a final concentration of 200 nM (5000× dilution). For CNF1 treatment GST-CNF1 was added to a final concentration of 300 ng/ml. NIH 3T3 cells were transfected with a high-efficiency liposome transfection method employing the Lipofectamine reagent supplied by Gibco (6 µl Lipofectamine/1.5 µg DNA). For the generation of the N-BxB-ER^{TM}-RacV12 cells 2×10^5 NIH 3T3 cells were seeded in a well of a six-well plate and transfected with 0.5 µg of the pBabe-puro-BxB-ER^{TM} vector [2] and 1.0 µg of pEXV-Val12-Rac1-myc tag vector [18,19]. Cell clones were isolated by puromycin selection in the absence of OHT. For the generation of N-EHneo cells 2×10^5 NIH 3T3 cells were seeded in a well of a six-well plate and transfected with 1.5 µg of the EHneo plasmid [20] directing the expression of a v-Raf protein. Foci of transformed cells were isolated by ring cloning and purified from untransformed cells by soft agar cloning.

2.2. Immunofluorescence staining

Cells were seeded on glass cover slides and fixed in

a 3.5% paraformaldehyde solution for 20 min at 4°C. After washing three times with PBS (Gibco) the cells were permeablised by treatment with PBS containing 0.1% Triton X-100 (Sigma) for 10 min at room temperature. The cells were washed three times with PBS and incubated in the presence of a monoclonal mouse anti-vinculin antibody (Sigma, hVIN-1, diluted 1:300) for 1 h. After washing three times with PBS the cells were incubated in the presence of polyclonal tetramethylrhodamine-isothiocyanate (TRITC)-conjugated goat anti-mouse antibodies (Dianova, 1:100 dilution) and fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes, Eugene, OR; 1:15 dilution) for 45 min.

2.3. Immunoblotting

Immunoblotting experiments were performed as described before [2]. The following antibodies were used: Myc 9E10 (mouse monoclonal, 2 μg/ml); mouse oestrogen receptor HL7 (rabbit polyclonal, 1:750); pRb G3-245 (mouse monoclonal, Pharmingen, 1 μg/ml), cyclin A C-19 (rabbit polyclonal, Santa Cruz, 1 μg/ml); cyclin D1 PRAD1 (rabbit polyclonal, Santa Cruz, 1 μg/ml); $p21^{Cip1}$ C-19 (rabbit polyclonal, Santa Cruz, 1 μg/ml); cdk4 C-22 (rabbit polyclonal, Santa Cruz, 1 μg/ml); phospho p44/p42 MAP kinase (Erk 1,2) Thr202/Tyr204 (rabbit polyclonal, New England Biolabs, 1:500).

3. Results

Analysis employing NIH 3T3 cells stably expressing a hydroxytamoxifen (OHT) inducible oncogenic c-Raf-1/oestrogen receptor fusion protein (c-Raf-1-BxB-ER^{TM}, N-BxB-ER^{TM} cells) has shown, that constitutive activation of the c-Raf-1 kinase is sufficient to transform NIH 3T3 mouse fibroblast cells [2,3]. Low-intensity Raf signals induce deregulated cell proliferation in the absence of external growth factors [2,3]. In contrast, a constitutive high-intensity Raf signal, as it is generated by OHT treatment of N-BxB-ER^{TM}-Cl-1 cells, blocks cell cycle progression [3]. Morphologically the activation of the oncogenic c-Raf-1-BxB-ER^{TM} kinase in NIH 3T3 cells leads to a disassembly of the actin filaments and a nearly complete loss of integrin-based focal adhesion complexes (Fig. 1B). With respect to the morphological transformation we found that cells exposed to high-intensity Raf signals, such as OHT treated N-BxB-ER^{TM}-Cl-1 cells, have a much more transformed phenotype, than cells which were exposed to a low-intensity Raf signal, such as N-EHneo cells (Fig. 1A). Adhesion of cells to extracellular matrices is largely mediated by members of the integrin family of transmembrane receptors [21]. In contrast to untransformed NIH 3T3 cells, which are flat and not refractile, the spindle-shaped transformed cells are refractile and only weakly attached to the tissue culture plate by a few integrin-based focal adhesion complexes at the end of the spindles (Fig. 1A,B).

To analyse the influence of Rho GTPase activation on Raf-transformed NIH 3T3 mouse fibroblasts we employed the *Escherichia coli* toxin CNF1. As recently discovered, CNF1 activates the Rho, Rac and Cdc42 GTPases by deamidation of glutamine residues [22,23]. Treatment of OHT induced N-BxB-ER^{TM}-Cl-1 cells with CNF1 reverses the transformed morphology (Fig. 1A,B). The cells are flattened but have ruffled membrane structures around their nuclei (Fig. 1A). CNF1 also reverses the transformed morphology of v-Raf (N-EHneo) (Fig. 1A) and RasV12 transformed cells (data not shown). Analysis of the actin filament and integrin-based focal adhesion complexes shows that CNF1 restores actin structures and integrin-based focal adhesion complexes at the cellular boundary (Fig. 1B). However, the formation of Rho induced actin stress fibres is inhibited in the presence of constitutive Raf activity (Fig. 1B). These data indicate that constitutive activation of Rac by CNF1 overcomes the oncogenic activity of Raf on the disassembly of actin structures and integrin-based focal adhesion complexes at the cellular boundary. Oncogenic Raf, however, inhibits Rho-mediated formation of actin stress fibres.

To further characterise these findings we have analysed if a constitutively activated RacV12 mutant can rescue the morphological transformation of NIH 3T3 cells by oncogenic Raf. We established cell lines stably expressing the c-Raf-1-BxB-ER^{TM} kinase and the RacV12 protein (N-BxB-ER^{TM}-RacV12-1, 2). The expression of the transgenes was verified by immunoblot analysis (Fig. 3A). In contrast to N-BxB-ER^{TM}-Cl-1 cells [3], which express only the inducible oncogenic Raf kinase and become morpho-

logically transformed upon OHT induction, the N-BxB-ER^{TM}-RacV12 clones retain their flat morphology following OHT addition (Fig. 2A). The Raf kinase mediates the phosphorylation of the Erk kinases by activating the Mek kinase [1]. Compared to wild-type NIH 3T3 cells, all three cell lines exhibit high amounts of phosphorylated Erk kinases in the presence of OHT (Fig. 3A), indicating that the oncogenic c-Raf-1-BxB-ER^{TM} kinase has been activated by OHT treatment. Although N-BxB-ER^{TM}-RacV12 cells retain a flat morphology the cell structure changes in that these cells exhibit ruffled membrane structures around their nuclei (Fig. 2A). Analysis of the actin structures in N-BxB-ER^{TM}-Rac-V12-1 cells in the absence and presence of OHT revealed that the actin stress fibres were disassembled by the activity of the oncogenic c-Raf-1 kinase (Fig. 2B). Actin structures and integrin-based focal adhesion complexes at the cellular boundary, however, were not altered (Fig. 2B). Taken together these experiments show that oncogenic Raf is able to block the formation of Rho-mediated actin stress fibres, but is not able to overcome the activity of RacV12, mediating actin structures and integrin-based focal adhesion complexes at the cellular boundary.

Our results show that activated Rac proteins partially inhibit morphological Raf transformation by mediating cell spreading. This is a surprising result since Rac activity is required for Ras transformation,

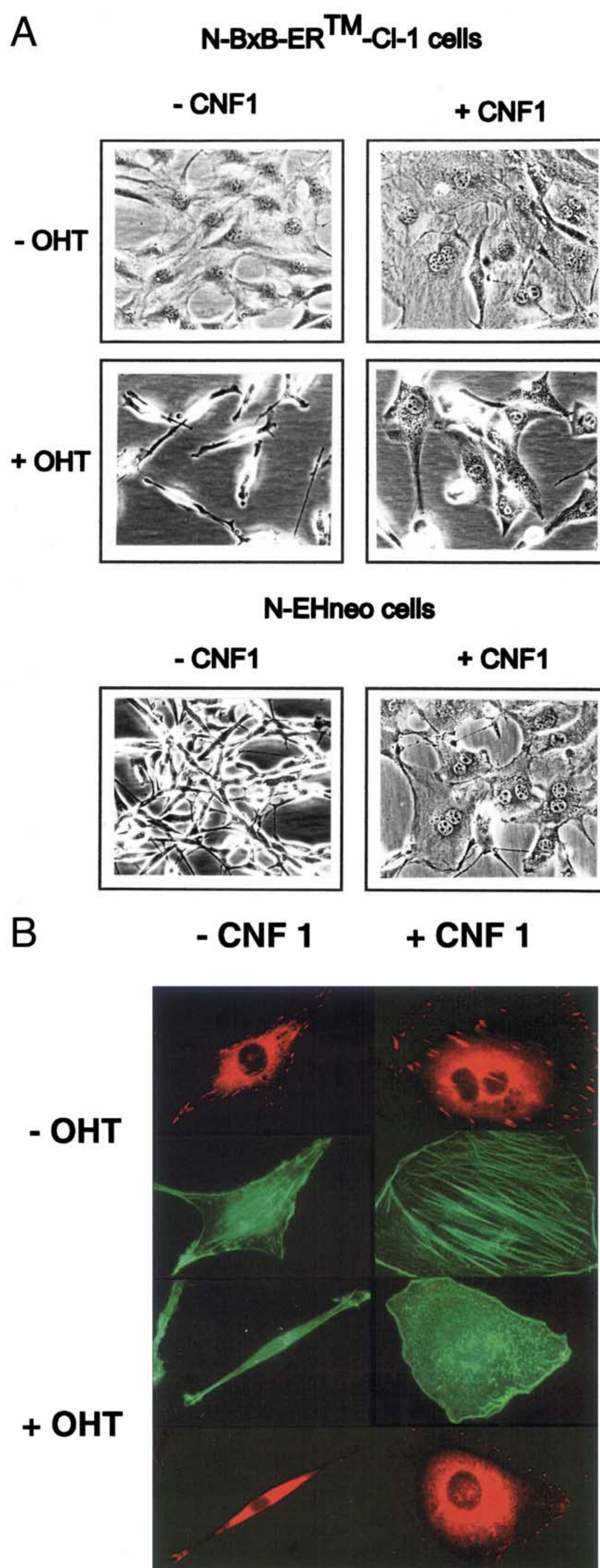

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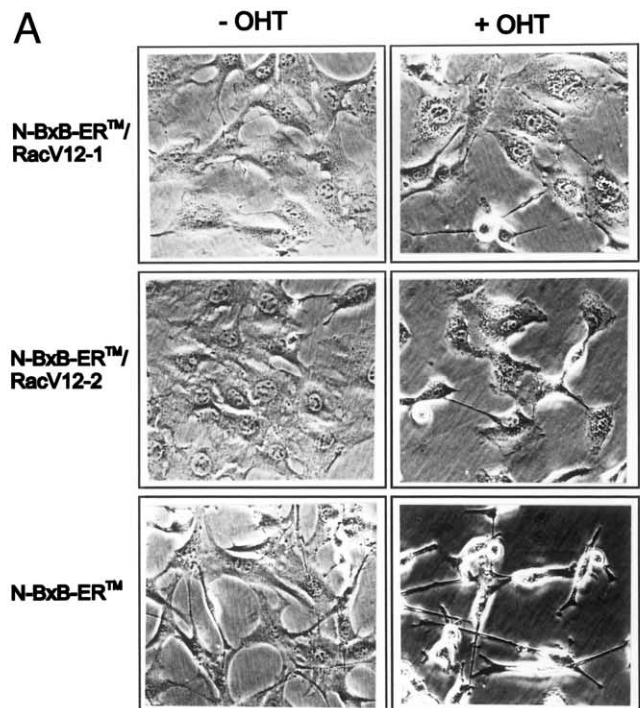
Fig. 1. *Escherichia coli* toxin CNF1 partially reverses the morphology of Raf-transformed cells. (A) NIH 3T3 cells stably expressing the c-Raf-1-BxB-ER^{TM} fusion protein (N-BxB-ER^{TM}-Cl-1) exhibit a transformed morphology upon induction of the oncogenic Raf kinase by hydroxytamoxifen. Addition of CNF1 to N-BxB-ER^{TM}-Cl-1 cells in the presence of OHT reverses the transformed spindle-shaped rounded morphology towards flat and adherent cells. CNF1 also reverses the transformed morphology of NIH 3T3 cells stably expressing an oncogenic v-Raf protein (N-EHneo). (B) Immunofluorescence staining of the actin structure and focal adhesion complexes of N-BxB-ER^{TM} cells in the presence and absence of OHT and CNF1. Proliferating N-BxB-ER^{TM}-Cl-1 cells, untreated, and treated with OHT, CNF1 and OHT/CNF1 were fixed with paraformaldehyde and stained with FITC–phalloidin (green) for their actin structures and with mouse anti-vinculin/TRITC–goat anti-mouse antibodies (red) for their integrin-based focal adhesion complexes. Vinculin is present in integrin-based focal adhesion complexes [14]. The stained cells were analysed with the help of a fluorescence microscope.



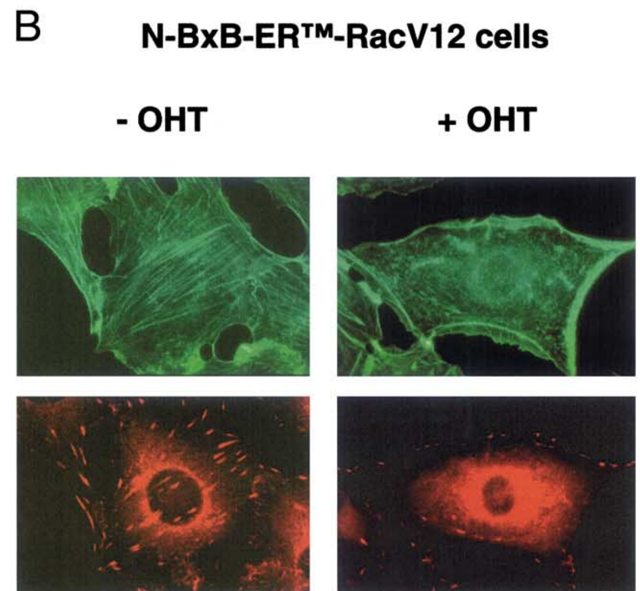


Fig. 2. Activated RacV12 inhibits morphological transformation by oncogenic c-Raf-1. (A) The morphology of N-BxB-ER^{TM}-Cl-1 (N-BxB-ER^{TM}) and N-BxB-ER^{TM}-RacV12-1, 2 cells was analysed by phase-contrast microscopy in the presence of the c-Raf-1-BxB-ER^{TM} activating hydroxoytamoxifen. (B) Actin structure of N-BxB-ER^{TM}-RacV12-1 cells in the presence and absence of OHT. N-BxB-ER^{TM}-RacV12-1 cells were cultured in the presence and absence of OHT, fixed with paraformaldehyde and stained with FITC-conjugated phalloidin (green) for their actin structures and with mouse anti-vinculin/TRITC–goat anti-mouse antibodies (red) for their integrin-based focal adhesion complexes. Vinculin is present in integrin-based focal adhesion complexes [14]. The cells were analysed by fluorescence microscopy.

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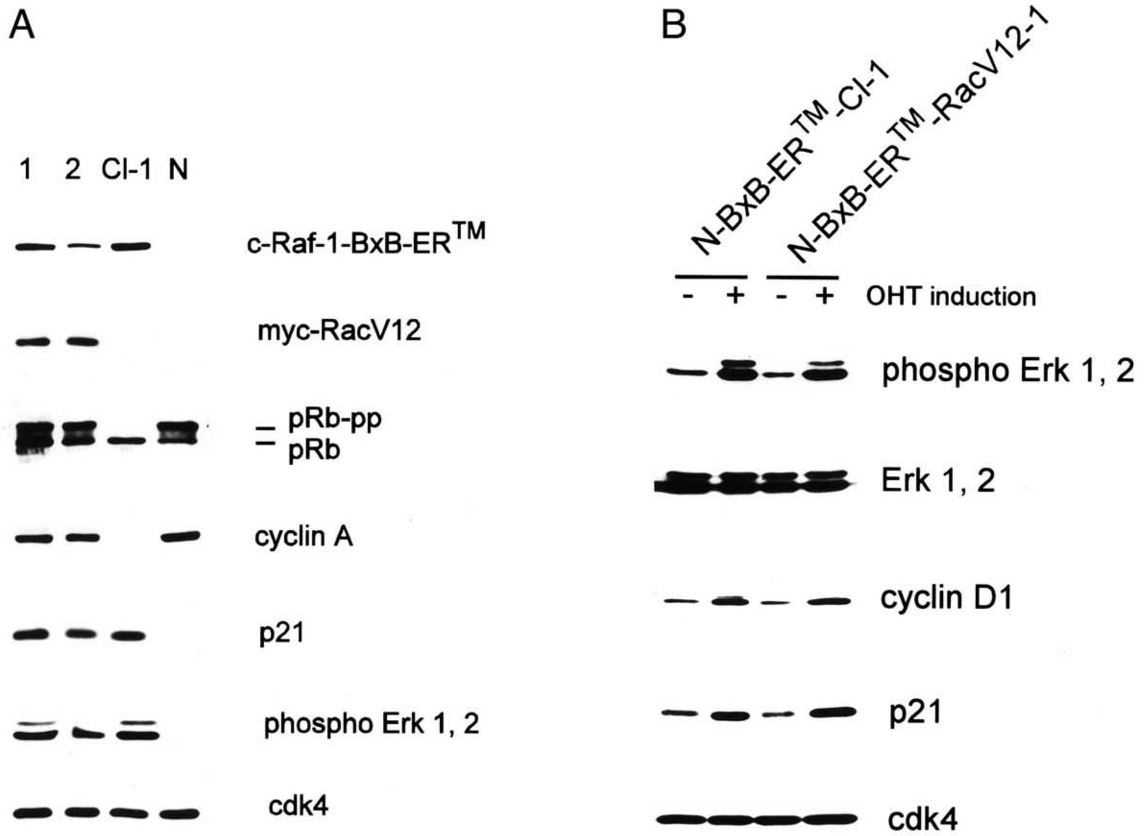
and activated Rac and Raf mutants cooperate in the oncogenic transformation of fibroblasts as analysed by focus formation assays [8–12]. We have therefore asked the question whether constitutive Rac signals might contribute to the deregulation of cell cycle control mechanisms, an other hallmark of oncogenic transformation. Low-intensity Raf signals have been shown to induce cell proliferation, whereas high constitutive Raf signals, as those generated in OHT treated N-BxB-ER^{TM}-Cl-1 cells, block cell cycle progression by the induction of the expression of the p21^{Cip1} cdk inhibitor [2,3]. A read-out system for Raf signal intensities is the degree of Erk phosphorylation. N-BxB-ER^{TM}-Cl-1 cells exhibit high levels of phosphorylated Erk kinases and p21^{Cip1}, when induced with OHT (Fig. 3A,B). The high Raf signals in N-BxB-ER^{TM}-Cl-1 cells cause a down-regulation of pRb hyper-phosphorylation as documented by the loss of the slower migrating pRb-pp proteins (Fig. 3A). Flow cytometry analysis revealed that the cells arrest in G1 and G2/M phase of the cell cycle (Fig. 3C). By analysing the phosphorylation of the Erk1, 2 proteins we found that OHT induced N-BxB-ER^{TM}-RacV12-1 cells exhibit Raf signal intensities comparable to the signals in N-BxB-ER^{TM}-Cl-1 cells (Fig. 3A,B). The high-intensity signals lead in the case of N-BxB-ER^{TM}-Cl-1 and N-BxB-ER^{TM}-RacV12 cells to the expression of high levels of the cdk inhibitor p21^{Cip1} (Fig. 3A,B). In contrast to N-BxB-ER^{TM}-Cl-1 cells, however, N-BxB-ER^{TM}-RacV12 cells are able to proliferate in the presence of the high-intensity Raf signals and elevated levels of the p21^{Cip1} cdk inhibitor (Fig. 3A,B,C). The pRb protein is a cellular phosphorylation target for cdk activity [24,25]. Cdk-mediated pRb phosphorylation is an essential step in the regulation of the G1/S transition. Despite the high levels of p21^{Cip1} expression the pRb hyperphosphorylation is not affected (Fig. 3A), indicating that p21^{Cip1} does not inhibit the cdk activity in OHT treated N-BxB-ER^{TM}-RacV12 cells. We have confirmed these data by analysing the activity of the


A
1
2
Cl-1
N
c-Raf-1-BxB-ER^TM
myc-RacV12
pRb-pp
pRb
cyclin A
p21
phospho Erk 1, 2
cdk4
B
N-BxB-ER^TM-Cl-1
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phospho Erk 1, 2
Erk 1, 2
cyclin D1
p21
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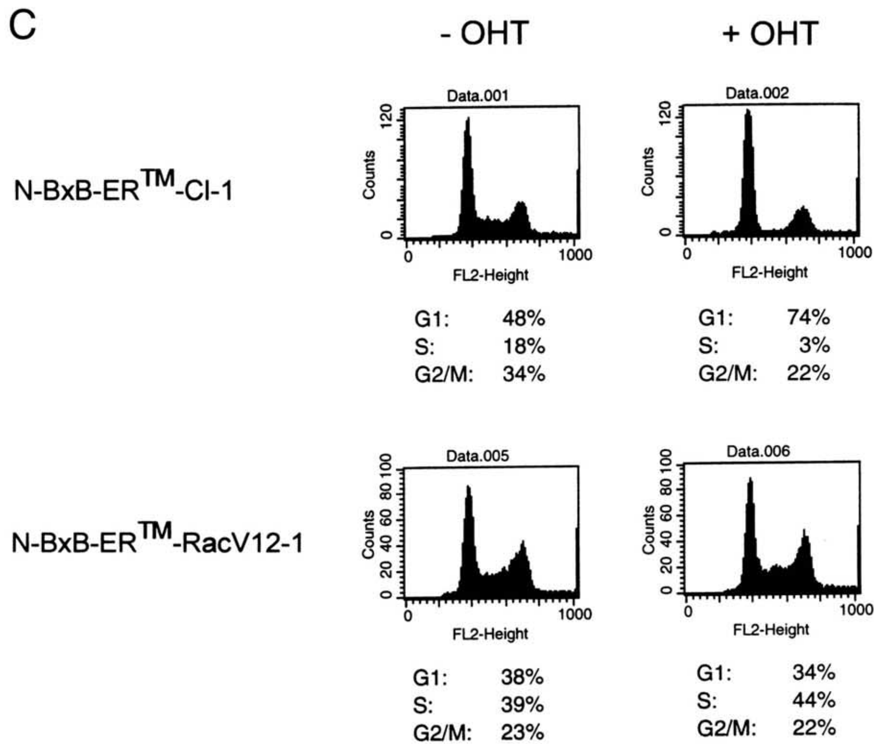
C
- OHT
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N-BxB-ER^TM-Cl-1
N-BxB-ER^TM-RacV12-1
Data.001
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Data.006
Counts
FL2-Height
G1: 48%
S: 18%
G2/M: 34%
G1: 74%
S: 3%
G2/M: 22%
G1: 38%
S: 39%
G2/M: 23%
G1: 34%
S: 44%
G2/M: 22%

Fig. 3. Activated RacV12 allows cell proliferation and retinoblastoma protein (pRb) hyperphosphorylation in the presence of high levels of the cdk inhibitor p21^{Cip1}. (A) Immunoblot analysis of the expression of the transgene products c-Raf-1-BxB-ER^{TM} and RacV12, the phosphorylation status of the Erk 1, 2 kinases and the retinoblastoma protein pRb (the hyperphosphorylated form is designated as pRb-pp), and the cell cycle machinery components cyclin A and p21^{Cip1}. Equal amounts of total lysates of N-BxB-ER^{TM}-RacV12-1 (1), N-BxB-ER^{TM}-RacV12-2 (2), N-BxB-ER^{TM}-Cl-1 (Cl-1) and NIH 3T3 cells (N) were separated by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose or PVDF membranes. The expression of the indicated proteins was analysed by immunodetection. To document equal protein loading the expression of the cdk4 protein has been analysed. The different cell lines were subconfluent and cultured under high serum conditions (10%). In order to analyse the activation of oncogenic c-Raf-1 protein, c-Raf-1-BxB-ER^{TM}-expressing cell lines were cultured for 36 h in the presence of OHT. (B) Expression of p21^{Cip1} and cyclin D1 in N-BxB-ER^{TM}-Cl-1 and N-BxB-ER^{TM}-RacV12-1 cells in the presence and absence of 4-hydroxytamoxifen. The cells were cultured subconfluent under high serum (10%) conditions. The OHT-induced cells were incubated for 36 h in the presence of the inducer. Total protein lysates were separated by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose and PVDF membranes. The expression of the indicated proteins was analysed by immunodetection. In order to verify equal protein loading the expression of the cdk4 and Erk1, 2 kinases was analysed. In order to control the induction of the c-Raf-1-BxB-ER^{TM} kinase by OHT the phosphorylation status of the Erk1, 2 kinase was analysed with the help of phospho Erk-specific antibodies. (C) Subconfluent N-BxB-ER^{TM}-Cl-1 and N-BxB-ER^{TM}-RacV12-1 cells were cultured under high serum conditions in the absence and presence of OHT. The ethanol-fixed cells were stained with propidium iodide and analysed for their DNA content by flow cytometry as desired earlier [2]. The distribution of cells in the different phases of the cell cycle were quantified with the computer program ModFit (Verity Software).
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cdk2 kinase, which is not affected in the presence of the high levels of p21^{Cip1} in N-BxB-ER^{TM}-RacV12-1 cells (data not shown). Consistent with the results on the pRb phosphorylation status, the cells do not cease the cell cycle as shown by the expression cyclin A protein, whose expression correlates with the proliferative status of cells (Fig. 3A), and the cell cycle distribution of N-BxB-ER^{TM}-RacV12-1 cells (Fig. 3C). Even in the presence of OHT N-BxB-ER^{TM}-RacV12-1 cells exhibit a significant proportion of cells in S phase, compared to the complete loss of the S-phase population in OHT-treated N-BxB-ER^{TM}-Cl-1 cells (Fig. 3C).

4. Discussion

We show that the mutational activation of the Rac GTPase influences the transformation of Raf in different ways. RacV12 partially inhibits the morphological transformation and favours deregulated cell proliferation in the presence of high p21^{Cip1} cdk inhibitor levels. The mechanisms by which the oncogenic Raf kinase mediates morphological transformation are unknown. Our results demonstrate distinct sensitivities of individual cellular structures towards the constitutive activation of Raf signals. Actin stress fibre formation is regulated by the Rho GTPase [14,26]. Even under conditions where the CNF1 toxin has activated the Rho protein, the on-

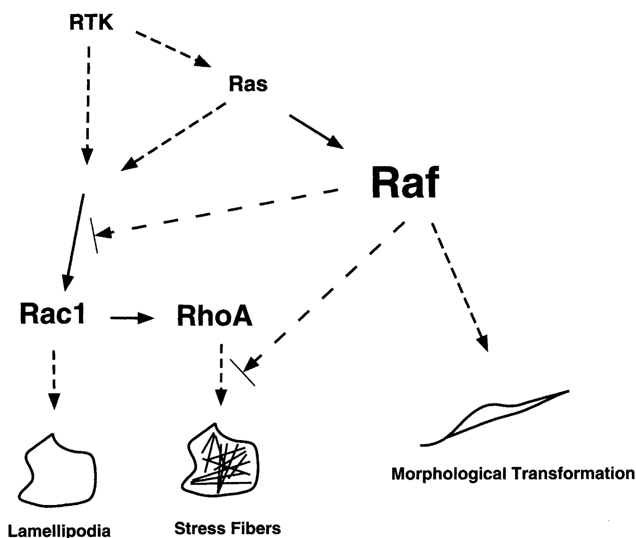

Fig. 4. Co-expression of activated Rac and Raf proteins impairs the oncogenic activity of Raf to disassemble focal adhesion complexes and actin structures at the cellular boundary. The finding that mutational activation of Rac partially reverses the morphological oncogenic activity of Raf indicates that during Raf-induced oncogenic transformation upstream activators of Rac are inhibited, which then cause the severe morphological changes found in transformed fibroblasts. We found actin stress fibres destroyed by oncogenic Raf even in the presence of CNF1-activated Rho. This could be explained by the fact that oncogenic Raf inhibits downstream events of Rho signalling, regulating the formation of actin stress fibres.

cogenic Raf kinase causes a disassembly of the stress fibre structures (Fig. 1B). This indicates an inhibitory mechanism downstream of the Rho signal transducer (Fig. 4). This conclusion was recently confirmed by a study showing that sustained Raf-MAP kinase signalling downregulates ROCK and Rho-kinase, two Rho effectors required for actin stress fibre formation [27]. In the presence of activated Rac, however, the oncogenic Raf protein is not able to disassemble actin structures and focal adhesion complexes at the cellular boundary (Fig. 2B). These data suggest that oncogenic Raf inhibits upstream activators of Rac in order to impair the Rac-mediated adhesion of cell to the extracellular matrix (Fig. 4). Similar results were found in epithelial cells where sustained Raf/MAP kinase signalling causes transcriptional down-regulation of Tiam1, an activator of Rac [28]. Expression of RacV12 in mesenchymal RasV12 or RafCAAX-transformed epithelial cells restores the epithelial phenotype [28]. A down-regulation of Tiam1 expression could also be detected in N-BxB-ER^{TM} and N-BxB-ER^{TM}-RacV12 cells following OHT stimulation (data not shown).

Previous studies convincingly demonstrate that activated Rac GTPases enhance oncogenic RafCAAX and Ras-triggered morphological transformation [8,10,11]. These studies are in contrast to the data presented in this study and to the data by others described above [28]. The signal strength turned out to be a critical parameter in the decision of a cell to proliferate or to cease the cell cycle [29]. Therefore, a possible explanation for the discrepancy could be a different Rho and Rac signal intensity in the studies. It remains to be analysed whether a modulation of the Rho or Rac signal strength could in one case enhance morphological transformation and in the other case partially inhibit it. Our experiments are the only studies which have employed the activated Raf–oestrogen receptor fusion protein. Since the treatment of the viral Raf-transformed N-EHneo cells with CNF-1 partially reverted the transformed morphology (Fig. 1A) and RacV12 was shown to revert the transformed morphology of RafCAAX transformed cells [28], we exclude the use of the c-Raf-1-BxB-ER^{TM} protein as an explanation for the differences in morphological cell transformation.

High-intensity Raf signals mediate a cell cycle arrest by the upregulation of the cdk inhibitor p21^{Cip1}. In the presence of RacV12 expression, high-intensity Raf signals were unable to mediate a proliferative arrest (Fig. 3). In contrast to the Rac related Rho GTPase, which was shown to inhibit the upregulation of p21^{Cip1} expression [17], the activated Rac protein does not influence the expression of the cdk inhibitor (Fig. 3A,B). Despite the high levels of the cdk inhibitor, the activity of cdk kinases is not influenced since a cellular phosphorylation target, the pRB protein, is highly phosphorylated (Fig. 3A) and we detect cdk2 activity in in vitro complex kinase assays (data not shown). These data indicate that Rac activity blocks the inhibitory action of p21^{Cip1} on cdk kinase activity.

The overall relation between cdks, cyclins, and cdk inhibitors determines if p21^{Cip1} will act as an inhibitor. Since both RacV12 and Raf induce the expression of cyclin D1 [2,30], we have especially analysed the expression of this cyclin. A synergistic effect of both activated oncogenes on cyclin D1 expression would have been able to super-induce the cyclin D1 expression and shift the complex equilibrium of the cell cycle components in the direction of cell cycle progression. Comparison of Raf-induced with Raf/Rac-induced cells did not reveal any differences in the induction levels of cyclin D1 (Fig. 3B). Therefore, a synergistic activation and a role of cyclin D1 in the rescue mechanism can be excluded. A detailed analysis of the expression of all components of the cell cycle machinery in N-BxB-ER^{TM}-Cl-1 and N-BxB-ER^{TM}-RacV12-1 cells may shed light on the mechanism of how cell proliferation is mediated in OHT induced N-BxB-ER^{TM}-RacV12 cells.

Here we show that RacV12 has anti- and pro-oncogenic influences on the transformation by Raf. It will be interesting to analyse in detail which of the different pathways mediates the divers functions of RacV12 on cell transformation. Separate effector pathways for cell spreading and the deregulation of cell cycle control might exist. Delineation of these pathways should help to find new strategies for the development of anti-cancer drugs, targeting the morphological transformation of cells.

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