Increased Rac1b Expression Sustains Colorectal Tumor Cell Survival

Paulo Matos and Peter Jordan

Centre of Human Genetics, National Health Institute 'Dr. Ricardo Jorge', Lisbon, Portugal

Abstract

The small GTPase Rac1 can stimulate various signaling pathways that contribute to cell transformation. In particular, the activation of the NFκB transcription factor initiates an antiapoptotic response and promotes cell cycle progression through increased cyclin D1 expression. As a potential oncogenic mechanism to up-regulate this pathway, the overexpression of the Rac1b splicing variant was reported in some colorectal tumors. Rac1b exists predominantly in the active GTP-bound state and selectively promotes the pathway leading to NFκB activation. Here, we studied the role of endogenous Rac1b in colorectal cancer cells. We found that depletion of Rac1b by small interfering RNAs inhibited endogenous NFκB activation and reduced cell viability to 50% within 48 hours. This reduction was due to increased apoptosis, although a reduced G_1-S progression rate was also observed. These data show, for the first time, that colorectal cells expressing alternative spliced Rac1b also depend on Rac1b signaling to sustain their survival.
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Introduction

Rac1 is a member of the Rho family of small GTPases, which cycle between an inactive GDP-bound and an active GTP-bound state. Once in the active state, Rac1 can interact with downstream effector proteins and control signaling pathways that regulate the actin cytoskeleton and gene transcription (1, 2). One important pathway that links Rac1 signaling to gene expression is the reactive oxygen species–mediated activation of nuclear factor κB (NFκB; refs. 3, 4), which initiates an antiapoptotic transcriptional response and also promotes cell cycle progression by an increase in cyclin D1 expression (4-6). Rac signaling was shown to be essential for the transformation of cultured cells (7-9) and for tumor formation *in vivo* (10, 11), underlining that deregulated Rac1 activation is involved in tumor progression (12).

Rac1 activation *in vivo* occurs at the plasma membrane in response to appropriate external stimuli that control guanine nucleotide exchange factors, which stimulate the exchange of GDP for GTP, as well as GTPase-activating proteins, which accelerate the intrinsic GTPase activity, and guanine nucleotide dissociation inhibitors (Rho-GDI), which bind to Rac1, removing it from the plasma membrane and preventing its activation. In tumors, several mechanisms were reported to deregulate Rac1 activation, including changes in Rho GTPase expression (6, 13, 14), oncogenic activation of exchange factors (15, 16), or inactivation of Rho-GDI2 (17, 18). In addition, increased expression of alternative spliced Rac1b was previously reported in colorectal tumor samples (19). Rac1b differs in 57 nucleotides that correspond to the inclusion of exon 3b into the Rac1 mRNA (20). The resulting Rac1b protein fails to interact with Rho-GDI in cells and displays both an increased intrinsic nucleotide exchange activity and a decreased rate of GTP hydrolysis *in vitro*. Accordingly, Rac1b is isolated predominantly in the active GTP-bound state from cell lines (21-24). Unexpectedly, several classic Rac signaling pathways including lamellipodia formation or the activation of PAK1 or c-*Jun*-NH_2-kinase activities were not activated by Rac1b. However, Rac1b retains the ability to stimulate the NFκB pathway and was shown to induce IκBα phosphorylation, nuclear translocation of RelA, and transcriptional stimulation of a consensus NFκB promoter in a luciferase reporter construct (25, 26).

Previous studies found that Rac1b increased G_1-S progression, survival and transformation of NIH3T3 fibroblasts (23, 25, 26), promoted epithelial-mesenchymal transition of mouse mammary epithelial cells (24), and contributes to further activate Wnt signaling in HCT116 colorectal cells (27). However, the molecular details of how the overexpression of Rac1b can promote the progression of colorectal tumors (19) remains unclear. Here, we have used RNA interference to repress endogenous Rac1b in colorectal cancer cells and found a significant and specific decrease in cell viability. In particular, we show that the survival of colorectal cells requires Rac1b signaling.

Results and Discussion

We previously described that overexpression of wild-type Rac1b in serum-starved fibroblasts was sufficient to impair apoptosis and promote cell cycle progression through an increase in the endogenous levels of cyclin D1 (25). However, whether these findings were biologically relevant for the progression of colorectal tumors (19, 21), in which Rac1b

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overexpression was reported, remains unclear. We therefore studied colorectal cancer cells to determine the importance of Rac1b and inhibited its endogenous expression.

As shown in Fig. 1A some colorectal tumors overexpress Rac1b when compared with normal colon mucosa and the Caco-2 colorectal cancer cell line presented a level of Rac1b expression comparable to that found in Rac1b-positive colorectal tumors. In contrast, SW480 cells did not express Rac1b (Fig. 1A). We then used a CRIB domain–based pull-down assay to determine the level of active Rac1 and Rac1b GTPases in these cell lines (Fig. 1B). Consistent with previous results (21), Rac1b protein is expressed at low levels in Caco-2 cells, however, the amount of Rac1b present is sufficient to generate levels of active GTPase comparable to that of Rac1 found in SW480 cells. These cells were previously found to have a high level of Rac1 activation (21), probably due to the well-described fact that they carry an oncogenic mutation in K-ras, an upstream activator of Rac1 signaling.

Thus, we selected Caco-2 cells to analyze whether the repression of endogenous Rac1b affected cell viability and used SW480 cells, which in our hands do not express Rac1b, as control (it remains unclear why others have detected Rac1b expression in SW480 cells; see Materials and Methods for details; ref. 27). In order to repress endogenous Rac1b, we designed two small interfering RNA (siRNA) oligos targeting exon 3b–specific sequences within the 57-bp insertion that distinguishes Rac1b from Rac1 (20). The specificity of these siRNAs was first tested in cells overexpressing Myc-tagged versions of human Rac1 and Rac1b proteins. As shown in Fig. 1C, both oligos effectively repressed Rac1b expression without affecting Rac1 protein levels. Subsequently, the oligos were tested for their ability to repress endogenous levels of Rac1b. Treatment of Caco-2 cells with the Rac1b-specific oligos resulted in an >90% decrease in Rac1b expression after 48 hours, both at transcript and protein levels (Fig. 1D). Using these efficient and Rac1b-specific tools, their effect on cell viability was first quantified. Rac1b repression in Caco-2 cells with either siRac1b oligo A or B reduced cell viability by ~50% within 48 hours of treatment (Fig. 2A). However, neither of the oligos affected the viability of SW480 cells (that do not express Rac1b; Fig. 2B), demonstrating that the effect on Caco-2 cells depended on Rac1b repression and was not due to a nonspecific oligo-induced cytotoxic effect. A comparable effect on cell viability was also observed in other Rac1b-expressing colorectal cell lines such as HT29 or Co115 (data not shown).

We next investigated whether the decrease in cell viability following Rac1b repression reflected changes in cell cycle progression and/or apoptosis resistance because Rac signaling is known to be involved in both G_1-S progression (25, 28-30) and resistance to apoptosis (9, 25, 31, 32). The progression of Caco-2 cells into S phase was analyzed by bromodeoxyuridine (BrdUrd) incorporation 48 hours after transfection of the indicated siRNAs and revealed a consistent 1.6-fold decrease in G_1-S cell cycle progression [from 45% to 29% (oligo A) and 28% (oligo B); Fig. 3A]. This decrease was not observed in SW480 cells submitted to the same treatment (Fig. 3C).

Subsequently, apoptosis was assessed in Caco-2 and SW480 cells using the terminal nucleotidyl transferase–mediated nick

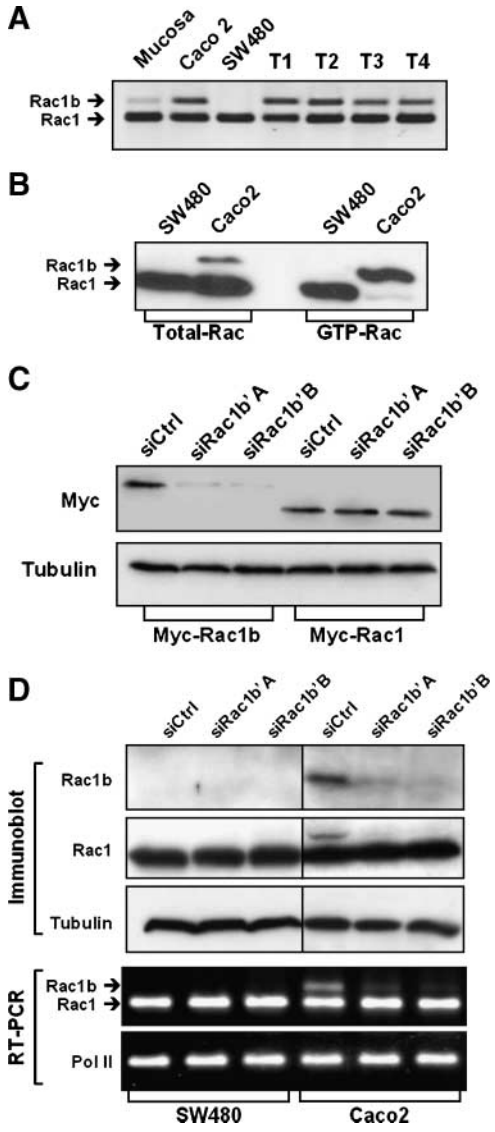

FIGURE 1. Expression and specific depletion of Rac1b in colorectal cell lines. **A.** The expression of endogenous Rac1 and Rac1b was amplified by reverse transcription-PCR in a representative normal colon mucosa, two colorectal cell lines and four primary colorectal tumors, which overexpress Rac1b. The ethidium bromide–stained agarose gel shows that Caco-2 cells expressed Rac1b at levels comparable with the tumor samples. **B.** Western blot analysis of Caco-2 and SW480 cells using an anti-Rac1 antibody demonstrating the amount of total Rac1 and Rac1b in these cell lines (*left*) versus the active GTP-bound Rac GTPase pools after isolation with a CRIB domain pull-down assay (*right*). Note that Caco-2 cells express little Rac1b protein compared with Rac1, but that the activation state of Rac1b largely exceeds that of Rac1. **C.** Specificity of the employed Rac1b siRNA oligonucleotides. NIH3T3 cells expressing either Myc-Rac1-wt or Myc-Rac1b-wt were transfected with a control (*siCtrl*) or one of two Rac1b-specific oligos (*siRac1b'A* or *siRac1b'B*), as indicated. Cells were lysed after 24 h and analyzed by Western blot with either anti-tubulin (loading control) or anti-Myc antibodies. Note that the two Rac1b siRNAs efficiently repressed Myc-Rac1b expression without affecting the Myc-Rac1 protein. **D.** Specific depletion of endogenous Rac1b in Caco-2 colorectal cells. Caco-2 and SW480 cells (controls, not expressing Rac1b) were transfected with the indicated siRNAs and analyzed after 24 h for the expression levels of Rac1b, both by Western blot with the indicated antibodies (*top*) and by reverse transcription-PCR (*bottom*). Tubulin protein levels and amplification of RNA polymerase II (*Pol II*) served as loading controls. Note that in Caco-2 cells, the endogenous Rac1b transcript and protein were depleted by ~90% whereas Rac1 levels remained unaffected in Caco-2 and SW480 cells.

end labeling (TUNEL) assay under the above experimental conditions. We observed a mean 3.6-fold increase in apoptosis (3.5-fold for oligo A and 3.8-fold for oligo B) 48 hours after repression of Rac1b expression in Caco-2 cells (Fig. 3B). In contrast, SW480 cells submitted to the same treatment showed no increase in apoptosis (Fig. 3D).

These results are further supported by Western blot analyses showing that in Caco-2 cells, the endogenous levels of cyclin D1 decreased and that cleaved PARP, a well known apoptosis marker, accumulated. Cyclin D1 and PARP protein levels remained unaffected in SW480 cells (Fig. 3E).

Previous work has shown that ectopic overexpression of Rac1b promotes proteolytic degradation of the inhibitory protein IκBα leading to the stimulation of NFκB transcriptional activity in colorectal cells as well as NFκB-dependent G_1-S progression and cell survival in NIH3T3 fibroblasts (25, 26). We therefore wanted to determine to what extent endogenous Rac1b signaling would contribute to the NFκB activity present in Caco-2 or SW480 colorectal cells and cotransfected them with the Rac1b-specific siRNAs and an NFκB-driven luciferase reporter vector system. As shown in Fig. 4A, 48 hours after the repression of Rac1b in Caco-2 cells, the NFκB-driven luciferase expression was reduced to a mean value of 64% (71% for oligo A and 57% for oligo B; $P < 0.001$). Consistently, the protein level of inhibitor IκBα was found stabilized in the corresponding cell lysates (Fig. 4B, *lane 2*). In order to provide direct evidence as to whether Rac1b acts upstream of the canonical NFκB activation cascade, the endogenous IκBα protein was depleted simultaneously with Rac1b. It should be noted that the down-regulation of IκBα by siRNAs was only moderately efficient (~40% reduction; see Fig. 4B, *lane 3*), probably because it results in the activation of NFκB, which in turn, leads to the transcriptional activation of the IκBα gene in a negative

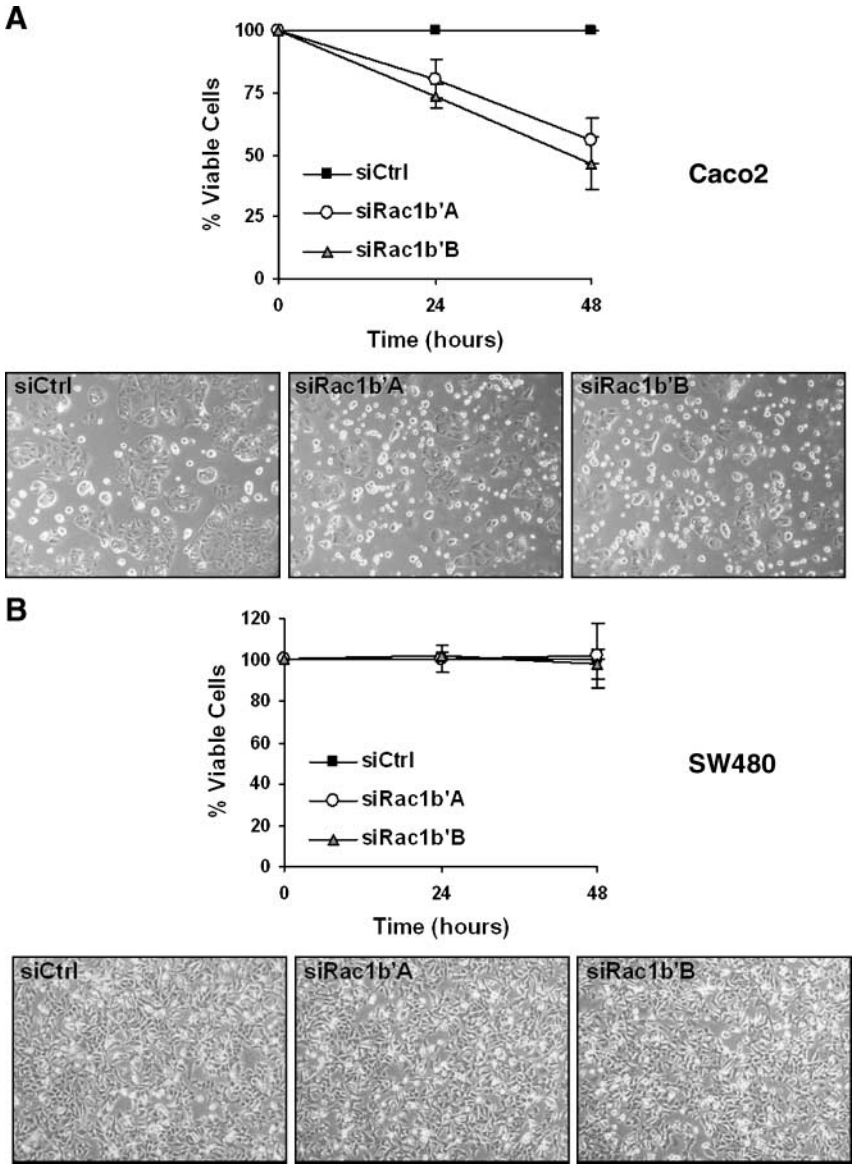

FIGURE 2. Depletion of Rac1b affects colorectal tumor cell viability. Caco-2 (**A**) or SW480 (**B**) cells were transfected with the indicated siRNAs and viable cells counted microscopically after 24 and 48 h. Points, mean values of three independent experiments, with at least 1,000 cells counted for each sample; bars, SD. Representative phase contrast images taken 48 h after treatment with the indicated siRNAs (*bottom*). Note that siRNA transfection did not affect the viability of SW480 cells, which do not express Rac1b.

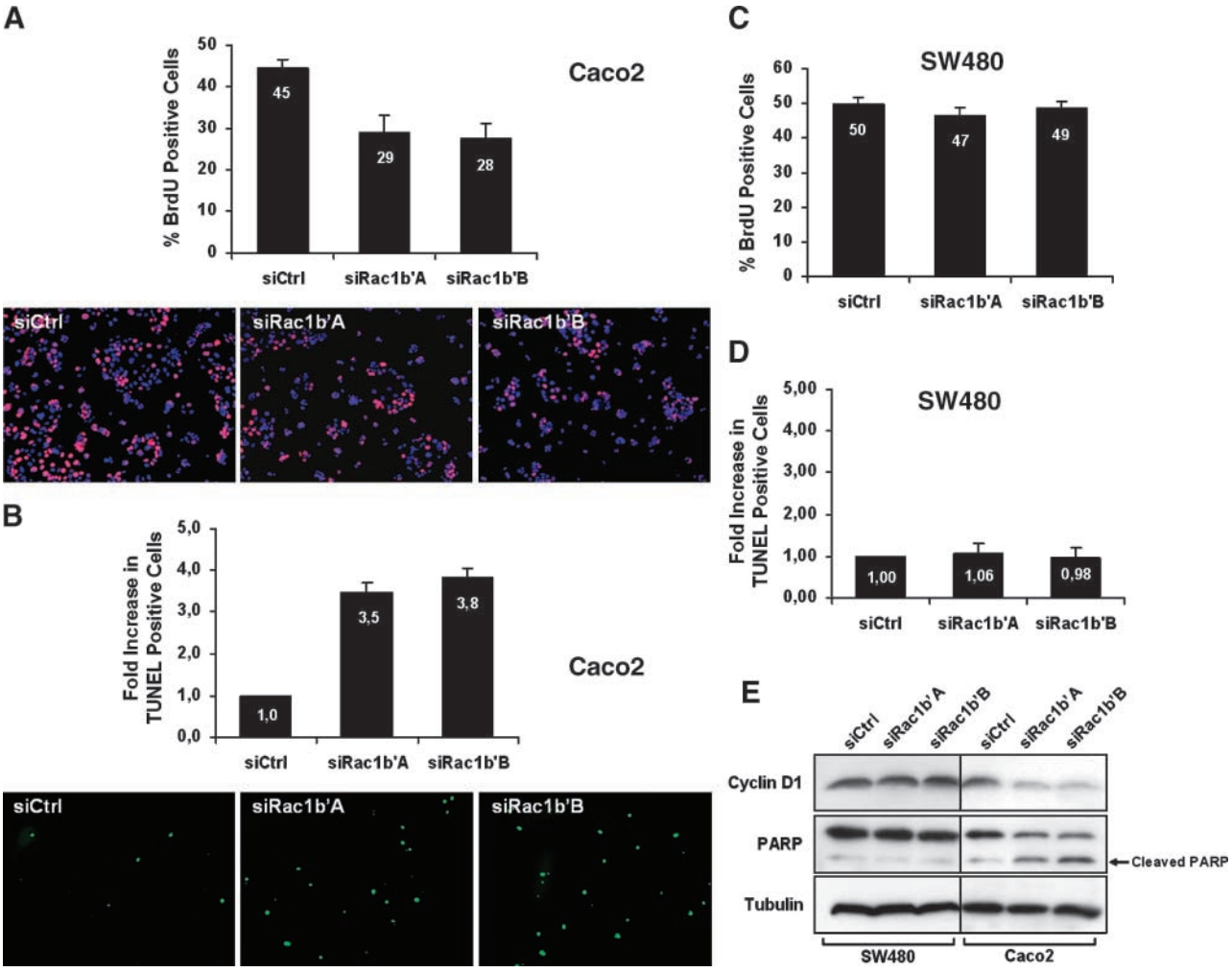

FIGURE 3. Depletion of Rac1b impairs cell cycle progression and promotes apoptosis in colorectal tumor cells. Caco-2 (**A**) or SW480 (**C**) cells were transfected with the indicated siRNAs, pulse-labeled with BrdUrd after 48 h, and S phase cells counted microscopically. Depletion of Rac1b in Caco-2 cells produced a 1.6-fold decrease (45% to 28%) in BrdUrd incorporation but had no effect on SW480 cells. Representative fluorescence images of Caco-2 cells show 4′,6-diamidino-2-phenylindole–stained nuclei (*blue*) and incorporated BrdUrd (*red*). Caco-2 (**B**) or SW480 (**D**) cells were transfected with the indicated siRNAs and the number of apoptotic cells determined after 48 h with the TUNEL assay. Depletion of Rac1b increased cell death 3.6-fold in Caco-2 cells and representative fluorescence images of TUNEL-stained cells (**B,** *bottom*) are shown. Columns, mean values of three independent experiments, with at least 1,000 cells counted for each sample; bars, SD. **E.** In a parallel Western blot analysis, the cyclin D1 and caspase-cleaved PARP protein levels were determined in Caco-2 and SW480 cells following transfection with the indicated siRNAs (β-tubulin levels served as loading controls).

feedback loop required for postinduction inactivation of NFκB (33-35). Despite these limitations, the level of IκBα suppression achieved resulted in a significant stimulation of NFκB activity and, importantly, was sufficient to revert the inhibitory effect of Rac1b suppression on this pathway (Fig. 4A, *lane 4*). Moreover, the codepletion of Rac1b and IκBα protein levels significantly rescued the induction of apoptosis observed in cells depleted of Rac1b alone (Fig. 4C). These observations provide strong evidence that the mechanism of apoptotic induction in Rac1b-depleted colorectal cells is mainly mediated by the canonical IκBα-regulated NFκB activation cascade. Comparable results were obtained in the Rac1b-expressing HT29 cell line. In contrast, the Rac1b-specific siRNAs had no effect on NFκB activity in SW480 cells, although NFκB could be inhibited in these cells upon treatment with the Rac1-inhibiting drug NSC23766 (data not shown). These data indicate that in proliferating Caco-2 cells, Rac1b signaling significantly contributes to cell survival through stimulation of the NFκB pathway.

Together, our results indicate a direct role for Rac1b signaling in maintaining the viability of colorectal cancer cells that overexpress this variant, particularly regarding the prevention of apoptosis. The antiapoptotic function of Rac1 signaling has already previously been found essential for experimental cell transformation (8, 9, 32) or mouse tumor models (10, 11). On the other hand, Rac1 signaling may also promote apoptosis in certain cell types and this can involve the activation of the downstream c-*Jun*-NH_2-kinase cascade in intestinal epithelial cells (36) or of the p38 mitogen-activated protein kinase (37, 38). In this respect, it is important to underline that Rac1b, although highly activated, differs from Rac1 signaling by favoring the NFκB pathway without

stimulating other classic Rac1 signaling targets including c-*Jun*-NH_2-kinase (21, 23) and p38 mitogen-activated protein kinase (data not shown). We therefore postulate that the increase of Rac1b observed in some colorectal tumors can contribute to tumor progression by selectively boosting the antiapoptotic and proproliferative branches of Rac1 signaling whereas circumventing the antagonistic effects generated by other Rac1 effectors. Thus, our results identify alternative spliced Rac1b as a novel antiproliferative target for small-molecule inhibitors or RNA interference–based therapeutics in a group of colorectal tumors.

Materials and Methods

Cell Lines

Caco-2 cells were maintained in RPMI and SW480 in DMEM, both supplemented with 10% (v/v) fetal bovine serum whereas NIH3T3 cells were grown in DMEM containing 10% (v/v) newborn calf serum (all reagents were from Life Technologies Invitrogen Corporation). Cells were regularly checked for the absence of mycoplasm infection. Because a previous article reported that SW480 cells express Rac1b (27), we have grown our SW480 cells in different media (DMEM, MEM, RPMI), used different serum batches (Life Technologies, Sigma), varying passage numbers, and a series of cell densities but were unable to detect Rac1b under any condition. We further obtained SW480 extracts from two other labs (Dr. Raquel Seruca, IPATIMUP, University of Porto; Dr. Eric Chastre, INSERM U773, Paris) and found that these cells also showed no significant Rac1b expression.

Plasmid Constructs and siRNA Oligos

Rac1 and Rac1b cDNAs were cloned into pcDNA3-Myc as previously described (21). SiRNA oligos were ordered from MWG Biotech with the following sequences: siRac1b′A 5′-CAG UUG GAG AAA CGU ACG GTT; siRac1b′B 5′-CGU ACG GUA AGG AUA UAA CTT; siCtrl was a mixture of standard control siRNAs against green fluorescent protein 5′-GGC UAC GUC CAG GAG CGC ACC TT and Luciferase GL2 5′-CGU ACG CGG AAU ACU UCG ATT.

siRNA Transfection

For RNA interference experiments, cells at 30% to 40% confluence were transfected using LipofectAMINE 2000 (Invitrogen) and 200 pmol of the indicated siRNAs, according to the manufacturer's instructions, and analyzed 24 and 48 h later. For the control of oligo specificity, NIH3T3 cells were first transfected with the indicated siRNA for 24 h, followed by a second transfection with 500 ng of the indicated Rac1 or Rac1b constructs, and analyzed following 16 to 20 h.

Reverse Transcription-PCR Expression Analysis

Total RNA was extracted from cell lines with the RNAeasy kit (Qiagen) and 1 μg was reverse-transcribed using random primers (Invitrogen) and Ready-to-Go You-Prime beads (GE Healthcare). Primers for specific amplification of Rac1 and Rac1b were RacATG (5′-CAG GCC ATC AAG TGT GTG GTG G) and Rac281-r (5′-CGG ACA TTT TCA AAT GAT GCA GG) in 28 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. Tumor and mucosa RNA samples were as previously described (19).

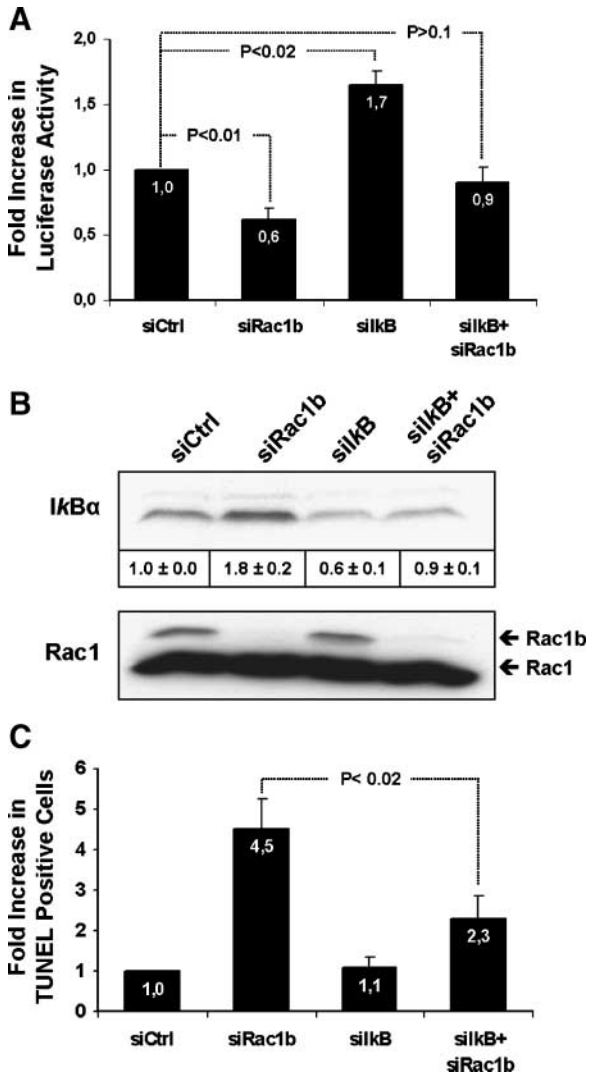

FIGURE 4. Depletion of Rac1b reduces endogenous NFκB activity in colorectal tumor cells and acts via IκBα. **A.** Caco-2 cells were transfected with the indicated siRNAs, and after 24 h, transfected again with a mixture of an NFκB-driven luciferase reporter plus a constitutively transcribed control vector. Cells were lysed 24 h later and analyzed using the Dual Luciferase Reporter Assay (Promega). Depletion of Rac1b in Caco-2 cells decreased luciferase activity to a mean value of 64%, whereas simultaneous depletion of Rac1b and IκBα abolished the effect on NFκB. **B.** Western blot analysis of the cell lysates analyzed in **A** using an anti-IκBα antibody (*top*) to detect protein levels of the inhibitor (numbers indicate densitometric quantification). The bottom of the blotting membrane was cut and further analyzed with anti-Rac1 antibodies to document the specific depletion of Rac1b whereas Rac1 protein levels remained unaffected and served as loading controls. **C.** Apoptotic Caco-2 cells were counted using the TUNEL assay after 24 h of transfection with the indicated siRNAs. Note that the simultaneous knockdown of IκBα significantly rescued the induction of apoptosis induced by depletion of Rac1b alone. Columns, mean values of three independent experiments; bars, SD (*P* values were statistically significant).

SDS-PAGE, Western Blotting, and CRIB-Peptide Pull-Down Assays

Samples were prepared and detected as previously described (21). The antibodies used in this study were rabbit anti–c-Myc A14 from Santa Cruz Biotechnology, mouse anti–β-tubulin clone Tub2.1 from Sigma-Aldrich (as a loading control), mouse anti–cyclin D1 clone DCS6 from BD Biosciences PharMingen (Erembodegem), rabbit anti-cleaved PARP from New England Biolabs, monoclonal anti-Rac1 from Upstate Biotechnologies, rabbit anti-Rac1b polyclonal serum as previously described (21). For densitometric analysis, film exposures from at least three independent experiments were digitalized and analyzed using ImageJ software (NIH). Active Rac CRIB-peptide pull-down assays were done as previously described (26).

Viability, Cell Death, and G_1-S Progression Assays

Cells were seeded in 35 mm dishes containing 10 mm coverslips, transfected as described above and analyzed at 0, 24, and 48 h. Prior to each time point, cells were incubated with 60 μmol/L of 5-bromo-2-deoxyuridine (BrdUrd; Sigma Aldrich) for 60 min, after which, coverslips were retrieved and cells were either stained with trypan blue (Sigma-Aldrich Quimica) or fixed. Apoptosis was detected by the fluorescence TUNEL assay (In-situ cell death kit, Roche Applied Science) and BrdUrd incorporation (progression into S phase) detected with mouse anti-BrdUrd (Roche Applied Science) and goat anti-mouse Texas red (Jackson ImmunoResearch Laboratories). For each time point, phase contrast or fluorescence images were digitally recorded in 10 randomly chosen fields (200× magnification) and both positive and negative cells were scored. A minimum of 1,000 cells were counted and all results confirmed in at least three independent experiments.

NFκB Reporter Assay

Reporter assay experiments have been described previously (26). Briefly, cells were transfected with siRNAs as described above, and 24 h later, cotransfected with an NFκB-driven firefly luciferase reporter and the control Renilla luciferase reporter construct. Cells were lysed 24 h later, analyzed using the Dual Luciferase Reporter Assay (Promega) according to the manufacturer's instructions, and measured in an Anthos Lucy-2 Luminometer. Lysates were assayed in duplicate and an aliquot preserved for Western blot analysis. All experiments were repeated in at least three independent transfection assays.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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