Rac1, but Not Rac1B, Stimulates RelB-mediated Gene Transcription in Colorectal Cancer Cells

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Increased NF-κB-mediated transcription has been extensively linked to tumorigenesis and can be stimulated by deregulated Rac1 signaling. For example, the overexpression of Rac1b, a highly activated splicing variant of Rac1 with increased expression in colorectal tumors, stimulates NF-κB-mediated G1/S progression and cell survival, and was shown to promote cell transformation and epithelial-mesenchymal transition. Here we show evidence of further complexity between Rac1b and Rac1 signaling toward NF-κB in colorectal cells. Consistent with data from other cell types we demonstrate that both Rac1 and Rac1b stimulate transcriptional activation from reporter genes driven by NF-κB motifs or the cyclin D1 promoter in an IκBα- and reactive oxygen species-dependent manner. However, we found that in colorectal cells Rac1, but not Rac1b, induces nuclear translocation of RelB and p52, activates transcription from a RelB-specific reporter, and can be isolated in a complex with endogenous RelB and its inhibitor NF-κB2/p100. In addition, Rac1 colocalizes at the plasma membrane with RelB, p100, and c-Jun NH2-terminal kinase (JNK)-1, a core subunit of the E3 ubiquitin ligase that marks p100 for proteolytic processing to p52. Interestingly, this Rac1-specific pathway is not mediated via the production of reactive oxygen species. These data provide evidence that both Rac1 and Rac1b activate the canonical RelA-ΙκBα pathway, whereas Rac1 further stimulates NF-κB by inducing the RelB-NF-κB2/p100 pathway. The RelB pathway was reported to down-regulate canonical NF-κB activation during the inflammatory response, suggesting that increased levels of Rac1b in colorectal tumors may promote tumorigenesis by stimulating canonical NF-κB signaling while circumventing a negative feedback from the RelB pathway.

Rac1 belongs to the Rho family of small GTPases that regulate distinct signaling pathways involved in the control of many cellular processes (1, 2). Rac signaling activates, for example, the protein kinase PAK,3 the Jun NH2-terminal kinase (JNK)3 and p38 mitogen-activated protein kinase (MAPK) cascades, the phosphatidylinositol 3-kinase/AKT pathway and the production of reactive oxygen species (ROS). As a consequence, Rac1 signaling mediates changes in cell motility or adhesion and activates transcription factors such as NF-κB, AP1, and serum response factor, which can affect the expression of cell cycle regulatory and anti-apoptotic proteins. Through these effects aberrant Rac1 activity can also promote cellular transformation (1).

Recently, alternative splicing variant Rac1b was identified in colorectal and breast tumors (3, 4) and contains 19 additional amino acids between Rac codons 75 and 76. Rac1b differs in regulation and signaling properties from Rac1 (5–7). It was shown to exist predominantly in the active GTP-bound state in vivo and in vitro and found unable to interact with rho-GDI, thus lacking this mechanism of down-regulation. Expression of Rac1b caused growth transformation of NIH3T3 cells as shown by focus-formation assays and loss of density-dependent and anchorage-dependent growth regulation (7). In addition, expression of Rac1b under low-serum conditions was found sufficient to promote cyclin D1 accumulation and G1/S phase progression as well as increased resistance to apoptosis (8). An increase in Rac1b expression was reported during matrix metalloproteinase-3-induced epithelial-mesenchymal transition of mouse mammary epithelial cells (9). Interestingly, several of the classical Rac1 signaling pathways such as lamellipodia formation or activation of PAK or JNK are not stimulated by activated Rac1b. However, Rac1b was reported to stimulate AKT phosphorylation (7), ROS production (9), as well as phosphorylation of IκBα and nuclear translocation of RelA (5). Expression of the non-degradable super-repressor IκBα32A336 (IκBaa) (10) inhibited Rac1b downstream signaling toward G1/S progression and apoptosis resistance in NIH3T3 cells (8).

NF-κB is a family of ubiquitously expressed transcription factors, which control the inflammatory response and the expression of genes involved in cell growth and survival. These properties are also intimately linked to the development of tumors (11–13). The NF-κB family is composed of five transcription factors that form homodimers or heterodimers with each other, namely RelA, RelB, c-Rel, p50, and p52. Unlike the three Rel proteins, p50 and p52 are produced from two inhibitor precursor proteins NF-κB1/p105 and NF-κB2/p100, respectively, through proteolytic processing. The NF-κB dimers remain transcriptionally inactive as long as they are associated with an ankyrin-repeat containing NF-κB inhibitor protein, such as IκBα, IκBβ, IκBε, or the NF-κB1/p105 or NF-κB2/p100 precursor proteins. Signaling pathways that activate NF-κB transcriptional activity involve phosphorylation of the NF-κB inhibitor proteins at specific serine residues by inhibitor of κB (IKK). The RING-H2-type E3 ubiquitin ligase complex SCF (Skp1/Cul-1-variable F-box protein complex) recognizes phosphorylated inhibitor proteins (14–17) and subsequent ubiquitination targets them for degradation by the 26 S proteasome (18). This enables NF-κB dimers to translocate into the nucleus and bind to the promoter regions of numerous target genes (19, 20).

Best understood is the tumor necrosis factor α-induced phosphorylation of IκBα by protein kinase IKKβ in a complex with kinase IKKa and a regulatory IKKγ/NEMO subunit. Phospho-IκBα is ubiquitinated by the SCF complex and then degraded, allowing the RelA/p50 dimer to translocate into the nucleus where it activates the tran-
scription of genes encoding cytokines, adhesion molecules, and inhibitors of apoptosis.

Besides this so-called classical pathway, a second conserved pathway of NF-κB activation has been characterized (21–23) that targets NF-κB/p100 rather than IκB for degradation, and involves the NF-κB-inducing kinase (NIK) upstream of IKKα but independent of IKKβ and IKKγ (22). Through this pathway IκKα controls B cell maturation, formation of secondary lymphoid organs, and induction of a specific set of NF-κB target genes (19, 22). The COOH-terminal phosphorylation of p100 is also recognized by the SCF complex resulting in ubiquitination of Lys-855, a site homologous to Lys-22 in IκBα and IκBγ (24). Subsequent degradation to p52. This second pathway specifically promotes the phosphorylation of IκBα at Ser-32 and Ser-36 (25).

An alternative approach to the production of ROS, and results in the phosphorylation of IκBα and inhibits both RelB nuclear localization and RelB-dependent transcriptional activation (26).

Signaling from GTP-Rac1 activates the IKK complex, probably via the production of ROS, and results in the phosphorylation of IκBα. In addition, the inactive RelA-p50-phospho-IκBα complex is recruited to sites at the plasma membrane where Rac1 is activated and brings it into proximity with the SCF ubiquitin ligase complex. Cullin-1 is a core component of the SCF complex and was shown to interact directly with active Rac1 (27), thus mediating membrane recruitment (28). This coreceptor is apparently shared by the variant Rac1b that was shown to induce phosphorylation of IκBα and to co-localize with phospho-IκBα at the plasma membrane (8). Here we show that in colorectal tumor cells Rac1 activates both the classical and the NF-κB/p100-RelB pathway, whereas Rac1b only activates the classical RelA-IκBα pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—DLD-1, SW480, and HT29 colorectal cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (Invitrogen) and regularly checked for absence of mycoplasm infection. For transfections, cells at 60–80% confluence were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions, and analyzed 16–20 h later. Total amounts of transfected DNA were 4 μg/60-mm dish for immunoprecipitation and pulldown assays and 2 μg of DNA per 35-mm dish for immunofluorescence and reporter assays. When required, the amount of DNA was adjusted with empty vector. Transfection efficiency in DLD-1 cells was 40–50% as judged by expression of 2 mg of pcDNA3-GFP-Rac1b.

**DNA Plasmids and Constructs**—The following constructs were received as gifts: pcDNA3-IκBα and pcDNA3-HA-IκBα(A32A36) (10) from M. Karin (University of California, San Diego), pcDNA3-HA-RelA and -RelB from C. V. Paya (Mayo Clinic, Rochester, MN), pR-RK-NIK and pcDNA3-p100 from W. C. Greene (Gladstone Institute, University of California, San Francisco), the pCMV-964-CycD1-Luc vector from R. Pestell (Georgetown University, Washington, D.C.), and the 3x-kB-Luc vector from B. Baumann (University of Ulm, Ulm, Germany). Rac1 and Rac1b cDNAs and their Q61L mutants were cloned into pcDNA3-Myc or pcDNA3-Myc-Rho-GDI as described (5). pcDNA3-Myc-Rho-GDI was as described (5) and the GAP domain of BCR (BCR-GD) was cloned into pcDNA3-Myc as a BamHI/EcoRI fragment from the pGEX-BCR-GD vector (gift from V. Braga). p100 was cloned into pEGFPc2 (BD Biosciences) as a HindIII/EcoRI fragment from pcDNA3-p100. All constructs were confirmed by automated DNA sequencing. A triple RelB-specific binding motif from the Blc promoter (tGGGGAGATTGaa, Ref. 25) was in vitro synthesized with flanking Nhel/BglII sites and inserted in the pGL3-Luc vector (Promega).

**SDS-PAGE and Western Blotting**—Samples were prepared and detected as described (5). The antibodies used in this study were rabbit polyclonal anti-c-Myc A14 from Santa Cruz Biotechnology; rabbit anti-HA from Sigma; rabbit anti-GFP ab290 from Abcam, United Kingdom; anti-β-tubulin clone Tub2.1 from Sigma (as a loading control); anti-Rac1, anti-cullin-1, and anti-p100/p52 from Upstate Biotechnologies; and anti-phospho-IκBα and anti-RelB from Hypermartix. For densitometric analysis films from at least three independent experiments were digitalized and analyzed using ImageJ software (NIH).

**Active Rac Pulldown Assays and Immunoprecipitation**—Approximately 2 × 10⁶ DLD-1 cells were seeded in 60-mm dishes, transfected as indicated, and assayed 16–20 h later. Cells were washed in cold PBS, and lysed on ice in 250 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1% (v/v) Nonidet P-40, 100 mM NaCl, 10% (v/v) glycerol, 10 mM MgCl₂, and protease inhibitor mixture (Sigma)). Total lysates were cleared by centrifugation at 2,500 × g for 5 min and 0.1 volume was added to 2× Laemmli sample buffer. The remaining lysate was incubated for 1 h at 4°C with a biotinylated CRIB-domain peptide pre-coupled to streptavidin-agarose beads (Sigma) as previously described (5, 29). Precipitated complexes were washed three times with excess lysis buffer. After the final wash, the supernatant was discarded and 2 μl of 2× Laemmli sample buffer was added to the beads. Total lysates and precipitates were then analyzed by Western blot. The immunoprecipitation procedure was identical except that the lysates were incubated with mouse monoclonal anti-c-Myc (9E10, Sigma) or anti-HA (12CA5; Roche) antibodies at 2 μg ml⁻¹, pre-coupled to protein G-agarose beads (Roche). Precipitates were analyzed on Western blots as above. All results were confirmed in at least three independent experiments.

**Confocal Immunofluorescence Microscopy**—Cells were grown on 10×10-mm glass coverslips, transfected, and incubated as indicated above, then washed twice in PBS, immediately fixed with 3.7% (v/v) formaldehyde in PBS for 30 min at room temperature, and subsequently permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min at room temperature. Cells were then washed 3 times for 5 min in PBS containing 0.05% (v/v) Tween 20 (PBS-T), incubated for 1 h with the primary antibodies anti-RelB (1:50), anti-p100/p52 (1:100), or anti-cullin-1 (1:50), then washed 3 times in PBS-T and incubated for 30 min with conjugated goat anti-rabbit Texas Red (Jackson ImmunoResearch Laboratories). Coverslips were then washed 3 times in PBS-T, mounted in VectaShield (Vector Laboratories), and sealed with nail polish. Images were recorded with the 488- and 543-nm laser lines of a Zeiss LSM410 confocal microscope and processed with Adobe Photoshop software.

**Luciferase Reporter Assay**—Approximately 5 × 10⁵ DLD-1 cells were seeded in 35-mm dishes, co-transfected with 50 ng of the pRL-TK Reporter (a low-level, constitutively expressed Renilla luciferase as internal control; Promega) and 500 ng of cyclin D1, standard NF-κB, or RelB-p52 specific reporters (see above), together with 250 ng of the indicated constructs. In IκBα super-repressor titration experiments, cells were also transfected with 12.5–100 ng of pcDNA3-HA-IκBα(A32A36). Total DNA amount was adjusted to 2 μg with pcDNA3 empty vector. After 16–20 h post-transfection in the absence or presence of the NADPH-oxidase inhibitor diphenyleneiodonium chloride (DPI), cells were lysed, assayed with the Dual Luciferase Reporter Assay (Promega), following the manufacturer’s instructions, and measured in an Anthos Lcy-2 Luminometer. Lysates were assayed in duplicate and additional aliquots were analyzed by Western blot. All firefly luciferase values were first normalized to the corresponding values for Renilla luciferase and then plotted as fold increase over the value of empty vector. The background luciferase activity of the original pGL3 vector was determined in parallel transfections and used as a reference.
Rac1 but Not Rac1b Activates the RelB Pathway

RESULTS

Both Rac1 and Rac1b Stimulate κB Consensus- and Cyclin D1 Promoter-driven Gene Transcription—Previously, we reported that active Rac1b stimulates NF-κB-dependent responses in a mouse fibroblast model (8). To extend these studies to colorectal cell lines we first compared the amount and activation of Rac1 and Rac1b in three different colorectal cancer cell lines. As shown in Fig. 1A, SW480 cells expressed no detectable Rac1b and revealed only Rac1 in the fraction of active, GFP-loaded Rac isolated by the PAK CRIB(CD)-domain pulldown assay. In contrast, HT29 cells expressed Rac1b and the pulldown assay revealed predominant activation of Rac1b over Rac1. DLD-1 cells represented an intermediate phenotype with little Rac1b expressed and a predominant activation of Rac1 with some active Rac1b.

We therefore selected DLD-1 cells to study the effect of Rac1b overexpression on the activation of the transcription factor NF-κB. Cells were transfected with either Myc-empty vector, Myc-Rac1-L61 or Myc-Rac1b-L61, and a luciferase-based reporter vector. In one set of experiments the reporter was driven by the cyclin D1-promoter (a downstream target for NF-κB; Refs. 30–32) and co-transfected with a second Renilla luciferase reporter to normalize differences in transfection efficiency. In a second set of experiments a reporter regulated by a canonical NF-κB-consensus motif was co-transfected. As shown in Fig. 1B the cyclin D1 promoter was activated by expression of both active Rac1 and Rac1b to the same extent. Curiously, when the effect of both Rac variants on the canonical NF-κB reporter was measured, a difference was observed: whereas active Rac1 increased luciferase expression 11.3-fold, active Rac1b only induced a 5.2-fold increase (Fig. 1C). In six independent experiments this difference was highly reproducible: 2.2 ± 0.2-fold (p < 0.001).

Rac1, but Not Rac1b, Stimulates NF-κB Activity through an IkBα- and ROS-independent Pathway—Rac-induced stimulation of NF-κB-mediated transcription has been shown to involve the generation of ROS (33–36) and the subsequent phosphorylation and degradation of the IκBα inhibitor (5, 8, 28, 33). To test if this pathway was responsible for the stimulation of the NF-κB reporter in DLD-1 cells we inhibited both processes by using increasing amounts of either the non-phosphorylatable and non-degradable super-repressor IκBαA32A36, (IkBaa) (10) or DPI, a cell-permeable inhibitor of the NADPH oxidase widely used to block the generation of ROS (20, 33, 37, 38). Indeed, both co-expression of IkBaa and DPI treatment inhibited Rac1- and Rac1b-mediated stimulation of the NF-κB reporter (Fig. 2), placing Rac1/Rac1b signaling upstream of the proteolytic degradation of the inhibitor in a ROS-dependent pathway. Unexpectedly, however, only the Rac1b-mediated luciferase induction was suppressed to background levels. The stimulatory effect of Rac1, on the contrary, although significantly decreased, reached a stable threshold of around one-third the activation (Fig. 2).

These data suggested that there might be an additional pathway downstream of Rac1, but not of Rac1b, leading to NF-κB activation independently of ROS production and not efficiently repressed by IkBα.

Rac1 Stimulates RelB-p52-mediated NF-κB Activity—Because the consensus κB motifs present in the reporter vector used were previously described to be recognized by both the canonical (p50-p65RelA and p50-c-Rel) and the non-canonical (p52-RelB) NF-κB dimers, we determined the contribution of both Rac variants to these pathways in more detail. Whereas RelA and c-Rel require degradation of IκBα for activation, RelB is inhibited by p100, the proteolysis of which removes inhibition and simultaneously yields the RelB-dimer partner p52. We first determined the effect of activated Rac1 and Rac1b on the endogenous protein levels of IκBα and p52 in DLD-1 cells. The expression of both activated Rac1 and Rac1b was found to induce the degradation of IκBα to similar values. In contrast, only activated Rac1 was able to induce a significant increase in the transcriptionally competent p52 component of the non-canonical pathway (Fig. 3A). Although the absolute protein variations
were moderate due to a limited transfection efficiency of around 40%, they were, nevertheless, statistically significant and reproducible.

To confirm this effect of Rac1 we generated a luciferase reporter vector containing a RelB-p52-specific recognition sequence from the Bcl promoter that was recently reported (25). We found that, in contrast to the conventional kB reporter (see Fig. 2), this RelB-p52-specific reporter construct was not activated by expression of RelA but responded to the kinase inhibitor DPI (Fig. 3), was recently found involved in the NF-κB pathway (26, 27, 28) we wondered whether active Rac1 could also associate with the NF-κB reporter, Rac1 specifically stimulates the canonical IkBα-regulated pathway, Rac1 stimulates both the canonical and non-canonical RelB-mediated pathways, thus inducing a higher stimulation of the conventional NF-κB reporter.

Rac1, RelB, and p100/p52 Co-localize with Cullin-1 at the Plasma Membrane—We next asked whether the changes in intracellular localization that accompany activation of endogenous RelB could be demonstrated in vivo. DLD-1 cells were transfected with control GFP-empty vector, GFP-Rac1-L61, or GFP-Rac1b-L61, and then fixed and endogenous RelB and p100/p52 were localized by confocal immunofluorescence microscopy. In untransfected cells RelB and p100/p52 were found in a diffuse distribution throughout the cell and transfection with GFP or GFP-Rac1b-L61 had no effect on their localization (Fig. 4, A and B). Expression of activated Rac1, however, induced a clear nuclear translocation of endogenous RelB as well as of p52 (Fig. 4, C and D, white arrowheads), consistent with the observed stimulation of the luciferase reporter plasmids.

Interestingly, we also observed a plasma membrane-associated staining of endogenous RelB and p100/p52 that co-localized with active Rac1 in transfected cells (Fig. 4, C and D, white arrowheads). A Rac1-dependent membrane recruitment of the SCF E3 ubiquitin-protein ligase complex, that recognizes phosphorylated IkBα, was recently found involved in p50-p65 activation (28). The authors suggested that activated Rac1 mediates the association of the p50-RelA-phospho-IkBα complex with SCF at membrane ruffles, thus promoting the ubiquitination of phospho-IkBα and marking it for subsequent proteosomal degradation. Therefore we wondered whether, besides RelB and p100/p52, Rac1 also co-localized with the SCF complex at the plasma membrane of DLD-1 cells. Indeed, by labeling these cells for endogenous cullin-1, an essential subunit of the SCF complex, we found increased staining in Rac1-L61-labeled sites at the plasma membrane (Fig. 4D, white arrows). These data show that active Rac1 co-localizes with these three endogenous proteins and indicate that, as with the p50-RelA-phospho-IkBα complex, it locally promotes association of the RelB-p100 complex with the SCF E3 ubiquitin-protein ligase complex at the plasma membrane.

RelB and p100/p52 Co-precipitate with Activated Rac1—Because SCF recruitment is mediated by direct interaction of Rac1 with Cullin-1 (27, 28) we wondered whether active RelB could also associate with the RelB-p100 complex. This was first tested in co-immunoprecipitation experiments. When DLD-1 cells were co-transfected with either Myc-empty vector, Myc-Rac1-L61 or Myc-Rac1b-L61, and HA-RelB, the anti-Myc antibody precipitated RelB together with Rac1-L61 but not Rac1b-L61 or empty vector (Fig. 5A). No co-precipitation was observed between Myc-Rac1-L61 and HA-RelA (Fig. 5A). Interestingly, overexpressed GFP-tagged p100 failed to co-precipitate with Myc-Rac1-L61, although it was clearly pulled down when HA-RelB was precipitated (Fig. 5B). This suggested that an interaction between activated Rac1 and the RelB-p100 complex would occur mainly through RelB.

If membrane recruitment involves the formation of a complex in vivo then one would expect that both RelB and p100 could be isolated from cells together with the pool of endogenous GTP-bound Rac1. To test this, DLD-1 cells were lysed and endogenous active Rac1 isolated...
through PAK-CD pulldown assays. We found that endogenous RelB, p100, and p52 were clearly present in the active Rac1 fraction (Fig. 6). As a control, endogenous Rac1 activation was down-regulated by expression of either Rho-GDI or the Bcr-GAP domain. The corresponding pulldown fractions revealed the absence of active Rac1 and showed that RelB and p100/p52 were no longer isolated under these conditions. Furthermore, RelB or p100/p52 were not pulled down from cells in which Rac1b is the predominantly activated variant (see Fig. 6), such as HT29 cells, or DLD-1 cells in the presence of Rho-GDI (which down-regulates Rac1 but not Rac1b; see Refs. 5 and 7). The observation that endogenous RelB and p100 were pulled down together with endogenous levels of active Rac1 from DLD-1 cells strongly support the role of Rac1 in promoting RelB-p52 activation.

**DISCUSSION**

The activation of the transcription factor NF-κB by Rac1 signaling has been well documented in the past and some knowledge on the involved pathways was gained. Several studies have implicated Rac1 stimulation of the NADPH oxidase, and the consequent production of ROS, as a major mechanism behind Rac1-mediated induction of NF-κB transcriptional activity (20, 33, 34, 37, 39). In addition, it was also reported that the activation of NF-κB downstream of Rac1 can involve a MEKK1-dependent cascade (40–42) and other studies have found a role for the kinases PAK and NIK (43, 44). Rac1-induced NF-κB activation can promote tumorigenesis through increased transcription of matrix metalloproteinases (45), anti-apoptotic genes (11, 38), and cyclin D1 (30–32).
Increased cyclin D1 expression occurs in cells that enter the cell cycle, and characterizes a variety of tumors (46–47). Through the ROS pathway, Rac-mediated activation of NF-κB/H9260 promotes increased cyclin D1 expression and subsequent cell cycle progression (30, 48). This activity is important to protect Ras-transformed cells from apoptosis (38). The same pathway is apparently also activated by the alternative splicing variant of Rac. We found that both active Rac1 and Rac1b stimulate transcription of luciferase reporters driven by either NF-κB/H9260 consensus motifs, or by the cyclin D1 promoter (Fig. 1). These data are in agreement with our previous findings in starved NIH3T3 fibroblasts that expression of Rac1b increased cyclin D1 protein levels, promoted G1/S progression, and increased cell survival of serum-starved fibroblasts (8). These effects involved phosphorylation of IkBα and nuclear translocation of RelA, and were inhibited by expression of the non-degradable super-repressor IkBaa (5). Rac1b has been shown to increase production of mitochondrial ROS in mouse mammary carcinoma cells (9) and we show that activation of NF-κB by Rac1b is also dependent on ROS production (Fig. 2). The Rac-induced generation of ROS was found to depend on a COOH-terminal effector-binding site called the insert region (residues 124–135) (34, 49), which apparently is unaffected in Rac1b.

Active Rac1b is unable to induce lamellipodia formation or to activate the protein kinases PAK and JNK (5–8). Previous studies have shown that lamellipodia induction, PAK binding, and JNK activation were dispensable for Rac1 transforming activity (50), whereas

FIGURE 4. Active Rac1 but not Rac1b co-localizes with RelB, p100, and cullin-1 at the plasma membrane. DLD-1 cells were transfected with the indicated constructs, incubated for 16–20 h, then fixed and processed for fluorescence confocal microscopy using anti-RelB (panels A–C), anti-cullin-1 (panel D), or anti-p100/p52 (panel E) antibodies (see “Experimental Procedures”). Note that only activated Rac1 induced the nuclear translocation of endogenous RelB (arrowhead in panel C) and co-localized with RelB at the plasma membrane (white arrow in panel C). In addition, active Rac1 co-localizes at the plasma membrane with endogenous cullin-1 (white arrows in panel D) and with p100/p52 (white arrow in panel E), and induced nuclear translocation of p52 (arrowhead in panel E).

FIGURE 5. Activated Rac1 co-immunoprecipitates (IP) with RelB but not p100. A, DLD-1 cells expressing pcDNA3-HA-RelB were co-transfected with empty vector, pcDNA3-Myc-Rac1-L61, or pcDNA3-Myc-Rac1b-L61. Note that after co-immunoprecipitation with anti-Myc antibody (9E10) RelB co-precipitated with activated Rac1 but not with activated Rac1b. As a control, Myc-Rac1-L61 was co-transfected with either pcDNA3-HA-RelA or pcDNA3-HA-RelB and shown to co-precipitate only with RelB. B, DLD-1 cells expressing pEGFP-p100 were co-transfected with empty vector, pcDNA3-Myc-Rac1-L61, pcDNA3-Myc-Rac1b-L61, or pcDNA3-HA-RelB. Note that after immunoprecipitation with anti-Myc (9E10) or anti-HA antibodies, p100 was only co-precipitated together with HA-RelB.

FIGURE 6. Endogenous GTP-Rac1 but not Rac1b can be isolated in a complex with endogenous RelB, p100, and p52. DLD-1 cells transfected with empty vector, pcDNA3-Myc-BCR-GAP domain (BCR-GD), or pcDNA3-Myc-RhoGDI were lysed and the GTP-bound Rac fractions were isolated by a PAK CRIB-domain pulldown assay. Note that the fraction of endogenous GTP-Rac1 contains endogenous RelB, p100, and p52 and that this co-isolation was prevented by Rac down-regulation in the presence of the BCR-GAP domain or of RhoGDI. Although RhoGDI expression substantially increased the amount of activated Rac1b, probably by relaxing its competition with Rac1 for guanine nucleotide exchange factors, it did not result in the pull-down of RelB-p100 complexes. Moreover, a pull-down from HT29 cells (lane 1), in which the level of GTP-Rac1b is very high compared with DLD-1 cells, also revealed no detectable levels of RelB or p100.
NF-κB-mediated stimulation of cyclin D1 expression best correlated with Rac1-transforming activity (30). Therefore, the selective signaling properties of Rac1b appear sufficient to play a role in cell transformation. Indeed, Rac1b-expressing fibroblasts were reported to lose density-dependent and anchorage-dependent growth (8), and expression in mouse mammary carcinoma cells induced epithelial-mesenchymal transition via a ROS-dependent activation of the transcription factor snail (9).

The described effect of Rac1b on transcriptional activity appears to differ from data reported by Singh et al. (7) that could not induce a significant increase in reporter activity by expressing Rac1b in serum-starved fibroblasts. Whereas we used constitutively active Rac1 and Rac1b mutants in our study (5), Singh et al. (7) compared wild type Rac1b with an active Rac1 mutant, assuming that Rac1b is a constitutively active Rac variant. Our previous data have suggested that Rac1b is highly activated but still subject to guanine nucleotide exchange factor-mediated activation and GAP-triggered inactivation (5). As a consequence, we found that prolonged serum starvation of fibroblasts may lead to a progressive loss of wild type Rac1b activation (8). In addition we found that Rac1b, in comparison with Rac1, induces a lower stimulation of the consensus NF-κB reporter. These differences together may explain the discrepancies with regard to the transcriptional reporter activation in both studies.

The major novelty in this work is that Rac1 signaling also activates the RelB-p52 pathway, whereas Rac1b only stimulates the IκBα-dependent NF-κB pathway. The expression of active Rac1 promoted accumulation of endogenous p52 protein (see Fig. 3). In DLD-1 cells only a small amount of p52 was usually detected relative to its precursor p100, indicating that the regulated processing of p100 is the rate-limiting step in these cells. This processing requires first of all phosphorylation of p100 at two COOH-terminal serine residues (Ser-866 and Ser-870), for example, by IKKα, which can be activated by the kinase NIK (21, 22). How exactly Rac signaling activates p100 phosphorylation is unclear, however, we show that, in contrast to IκBα phosphorylation, it does not require ROS production (Fig. 2). p52 can form a transcriptionally competent dimer with RelB in the nucleus (19), and we further observed that Rac1-induced generation of p52 was accompanied by nuclear translocation of RelB and p52 (Fig. 4). Finally, using a RelB-motif driven luciferase reporter we found a specific transcriptional activation upon expression of Rac1 in DLD-1 cells (Fig. 3). The RelB reporter revealed a significant response to Rac1 or NIK but not to Rac1b or RelA expression. From these data we can conclude that active Rac1 stimulates gene transcription through both the canonical RelA-IκBα-dependent and RelB-p100-mediated NF-κB pathways. Stimulation of the canonical pathway by Rac1 and Rac1b requires ROS production, whereas activation of the RelB pathway is ROS-independent (see Fig. 7).

A second novel finding in this work concerns co-localization at the plasma membrane of active Rac1 and proteins involved in the activation of the RelB pathway, namely RelB, NF-κB2/p100/p52, and cullin-1, a core component of E3 ubiquitin ligase complexes (17, 51). Direct interaction of p100-Rac1 by specific F-box protein 

NF-κB2/p100/p52, and cullin-1, a core component of E3 ubiquitin ligase complexes (17, 51). Direct interaction of p100-Rac1 by specific F-box protein 

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NF-κB2/p100/p52, and cullin-1, a core component of E3 ubiquitin ligase complexes (17, 51). Direct interaction of p100-Rac1 by specific F-box protein.
transcription by a ROS-independent mechanism, the data suggested to us that Rac1 might play a specific role in RelB/p100 recruitment that Rac1b cannot exert. We provide evidence indicating that active Rac1 may directly assemble a protein complex containing the inactive RelB-p100 complex. First, the active GTP-bound fraction of endogenous Rac1 was isolated from DLD-1 cells by PAK-CRIB domain pulldown assays and the presence of endogenous RelB, p100, and some processed p52 could be detected. Their presence was strictly dependent on Rac1 activation because down-regulation of Rac1 by expression of Rho-GDI that down-regulates Rac1b but not Rac1 promotes G1/S progression, cell survival (8), and transformation of fibroblasts (7). A detailed study on the transcriptional responses induced by RelA and RelB, namely in colorectal cells, may validate this hypothesis in the future.

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