Activation of Tumor-Specific Splice Variant Rac1b by Dishevelled Promotes Canonical Wnt Signaling and Decreased Adhesion of Colorectal Cancer Cells

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Abstract

Rac1b is a tumor-specific splice variant of the Rac1 GTPase that displays limited functional similarities to Rac1. We have shown previously a novel cross-talk between Rac1 and β-catenin, which induces canonical Wnt pathway activation in colorectal cancer cells. This prompted us to investigate if Rac1b, frequently overexpressed in colon tumors, contributes to Wnt pathway dysregulation. We show that Rac1b overexpression stimulates Tcf-mediated gene transcription, whereas depletion of Rac1b results in decreased expression of the Wnt target gene *cyclin D1*. Reconstitution experiments revealed an important difference between Rac1 and Rac1b such that Rac1b was capable of functionally interacting with Dishevelled-3 (Dvl-3) but not β-catenin to mediate synergistic induction of Wnt target genes. In agreement, Dvl-3 but not β-catenin caused increased activation of Rac1b levels, which may explain the functional cooperativity displayed in transcription assays. Furthermore, we show that Rac1b negatively regulates E-cadherin expression and results in decreased adhesion of colorectal cancer cells. RNA interference–mediated suppression of Rac1b resulted in reduced expression of *Slug*, a specific transcriptional repressor of E-cadherin, and a concomitant increase in E-cadherin transcript levels was observed. Intriguingly, mutation of the polybasic region of Rac1b resulted in complete loss of Rac1b stimulatory effects on transcription and suppressive effects on adhesion, indicating the importance of nuclear and membrane localization of Rac1b. Our results suggest that Rac1b overexpression may facilitate tumor progression by enhancing Dvl-3–mediated Wnt pathway signaling and induction of Wnt target genes specifically involved in decreasing the adhesive properties of colorectal cancer cells. [Cancer Res 2007;67(6):2469–79]

Introduction

Rac1 is one of the most extensively studied members of the Rho family of small GTP binding proteins. It regulates diverse cellular processes, which include actin cytoskeleton organization, membrane trafficking, proliferation, and gene expression (1–4). Similar to all members of the Ras superfamily proteins, the GTP binding/GTP hydrolysis state of Rac1 is tightly controlled by guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP), respectively. An additional level of regulation exists for Rho GTPases, whereby Rho-GDP dissociation inhibitors (Rho-GDI) sequester GDP-bound forms in the cytoplasm, preventing their activation. Exhaustive characterization of this GTPase has revealed the pivotal role it plays in the genesis of many cancers. A recently discovered splice variant of Rac1, designated as Rac1b, has also received much attention for its plausible role in tumorigenesis of colon and breast tissues. However, deciphering the functional consequences of its expression and contribution to tumorigenesis has been challenging because Rac1b shows limited similarities to known Rac1 functions.

Overexpression of Rac1, as well as altered function of Rac1-specific regulators (GEFs, GAPs, and GDIs) or downstream effectors, have been found in several cancers (5). Studies using activated point mutants of Rac1 (G12V or Q61L) indicate that aberrant activation of Rac1 can alter many cellular processes important for cancer progression. Rac1 is essential for cell cycle progression and activates several pathways important for cellular proliferation, such as serum response factor, cyclin D1, and E2F (6, 7). Rac1 also promotes cell survival by activating the nuclear factor-κB (NF-κB) pathway and by preventing anoikis and apoptosis (8–10). Rac1 can induce cellular transformation in rodent fibroblast models and is required for Ras-induced transformation (11–14). Rac1 also regulates cell-cell and cell-matrix adhesion as well as stimulates motility and invasion by modulating the actin cytoskeleton, activities important for tumor metastasis (15).

Rac1b is a naturally occurring splice variant, preferentially expressed in colon and breast tumors (16, 17). It is created by alternative splicing of an additional exon, resulting in a 19–amino acid insertion between codon 75 and 76 of Rac1, directly C-terminus to the switch II region. This structural modification enables it to behave as a constitutively activated GTPase, largely because of an accelerated GEF-independent guanine nucleotide exchange rate, a decreased intrinsic GTPase activity, and an inability to interact with Rho-GDI (18, 19). It has been postulated that the insertion may create a novel effector binding site, which may enable Rac1b to participate in signaling pathways related to neoplastic growth (19). Most studies to date indicate impaired effector signaling activity of Rac1b. Unlike Rac1, Rac1b does not activate the protein kinases c-Jun NH2-terminal kinase (JNK) and p21-activated kinase 1 and is not involved in lamellipodia formation. However, similar to Rac1, Rac1b can promote growth transformation of NIH3T3 cells as well as a loss of density-dependent and anchorage-dependent growth and stimulates AKT serine/threonine kinase, hence promoting cell survival (20). Furthermore, a constitutively activated Q61L Rac1b mutant, but

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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not wild-type (WT) Rac1b, can stimulate the transcription factor NF-κB and cyclin D1 expression (19, 20). Rac1b has been shown to induce epithelial-mesenchymal transition (EMT) in mouse mammary carcinoma cells via reactive oxygen species–dependent activation of the transcription factor Snail (21).

Although these studies implicate a role for Rac1b in cellular transformation, downstream signaling pathways by which this tumor-associated splice variant may contribute to colorectal tumorigenesis remain to be identified. The canonical β-catenin–mediated Wnt signaling pathway is aberrantly activated in the vast majority of colorectal cancers (22, 23). A hallmark feature of Wnt pathway activation is nuclear translocation of β-catenin, where it complexes with Tcf4 and inappropriately activates transcription of target genes important for tumorigenesis (24, 25). We have shown previously that Rac1 can cooperate with β-catenin to augment canonical Wnt pathway activation by promoting its nuclear accumulation and amplifying its transcription coactivator function (26). This prompted us to investigate if Rac1b, frequently overexpressed in colon tumors, contributes to Wnt pathway dysregulation, which is characteristically activated in these tumors. We describe that Rac1b is a downstream target of Dishevelled-3 (Dvl-3) and amplifies Dvl-3–initiated Wnt pathway activation leading to increased β-catenin/Tcf–dependent transcription. Furthermore, we show that Rac1b negatively regulates E-cadherin expression and results in decreased adhesion of colorectal cancer cells. Thus, our results contribute to the understanding of Rac1b as a putative accelerator of tumor progression by positively regulating the expression of proliferation-promoting genes and decreasing the adhesive properties of colorectal cancer cells.

Materials and Methods

Cell culture and transfection. HEK293, SW480, and MCF-7 cells were maintained in DMEM. HCT116 and HT29 cells were cultured in McCoy's 5A. SW48 and MDA-MB-231 cells were cultured in Leibovitz's L-15 medium. All media were supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA) and cultured at 37°C in a humidified atmosphere of 5% CO_2. Cells were transfected as described previously (26). For endogenous gene knockdown experiments, the following small interfering RNAs (siRNA) were used: Rac1 siRNA was a SMARTpool reagent (Dharmacon, Lafayette, CO), whereas Rac1b knockdown was achieved via an equal mixture of two siRNAs against the target sequences 5′-GAAACGUACGGUAAGGAUA-3′ and 5′-GGCAAAGACAAGCCGAUUG-3′. Gene expression data were normalized against transfections with a siCONTROL nontargeting siRNA (Dharmacon).

Plasmids. Dvl-3 construct was purchased from American Type Culture Collection (Manassas, VA). Activator protein (AP-1)–luc and serum response element (SRE)–luc luciferase reporter constructs were purchased from Stratagene (La Jolla, CA). To generate the Rac1b polybasic region (PBR) mutants, site-directed mutagenesis was done using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Using a WT FLAG-tagged Rac1b cDNA construct as template, the KKRKRK sequence at amino acids 202 to 207 of Rac1b was replaced sequentially by pairs of glutamines to create Q2, Q4, and Q6 mutants as depicted in Fig. 4*A*.

Reverse transcription-PCR. cDNA synthesis was done using 5 μg total RNA, random primer p(dN_6), and SuperScript II reverse transcriptase. Rac1 and Rac1b mRNA levels were analyzed using the following primers specific for both Rac1 and Rac1b: 5′-ATGCAGGCCATCAAGTGTGTG-3′(forward) and 5′-CAACAGCAGGCATTTTCTCTT-3′ (reverse). The PCR conditions used were as follows: 1 cycle at 94°C for 4 min; 35 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s; and 1 cycle at 72°C for 5 min. PCR products were separated on 1.5% agarose gel stained with ethidium bromide.

Quantitative real-time reverse transcription-PCR. First-strand cDNA was synthesized from 5 μg RNA using SuperScript III reverse transcriptase (Invitrogen). A mixture of 2× SYBR Green (15 μL/well), 10 μmol/L of forward and reverse primer (0.6 μL each/well), 1 μL cDNA, and DNase-free water to bring the reaction to 30 μL was added to each well. Samples were analyzed in triplicate and carried out in an Applied Biosystems 7500 Real-time PCR System (Foster City, CA). Real-time results were collected and analyzed (standard curve method) using the ABI 7500 System software according to the manufacturer's protocol. Expression values were normalized with the *β-actin* gene expression values. Primer sequences used to amplify cDNA are described in Supplementary Table S1.

Luciferase reporter gene assays. Cells were plated at a seeding density of 2×10^5 per well of a 24-well dish 24 h before transfection. Luciferase reporter constructs (0.1 μg) were added to each well together with 0.03 μg pCMV β-galactosidase construct for normalization. For coexpression of additional proteins, FLAG-tagged WT or PBR-mutated Rac1b, WT β-catenin, or Dvl-3 constructs were added as indicated in figures. Cells were harvested after 24 h in 100 μL reporter lysis buffer (Promega, Madison, WI). Assays of luciferase and β-galactosidase activity were done as described previously (26).

Cell fractionation, coimmunoprecipitation, and Western blotting. Whole-cell lysates as well as cytoplasmic and nuclear extracts were prepared and detected as described (26). Coimmunoprecipitation experiments were done using both whole-cell lysates and fractionated cytoplasmic and nuclear lysates. The antibodies used in this study were horseradish peroxidase–conjugated anti-FLAG (Sigma, St. Louis, MO), monoclonal anti–β-catenin and monoclonal anti-paxillin (Transduction Laboratories, BD Biosciences, Franklin Lakes, NJ), monoclonal anti–topoisomerase II (Oncogene Research Products, San Diego, CA), monoclonal Dvl-3 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti–β-actin (Ambion, Austin, TX) as a loading control.

Active Rac pull-down experiments. Active GTP-bound Rac1 and Rac1b levels were determined using a nonradioactive Rac1 activation assay kit according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL). Following transfection, cells were washed in cold PBS and lysed on ice in 600 μL lysis buffer. Total lysates were cleared by centrifugation at 16,000 × *g* for 15 min, and 100 μL lysate was kept for protein quantitation of total Rac1/Rac1b protein. The remaining lysate was incubated for 30 min at 4°C with 20 μg PAK binding domain (PBD) agarose beads. Precipitated complexes were washed thrice with excess lysis buffer. After the final wash, the supernatant was discarded and 40 μL of 2× Laemmli sample buffer were added to the beads. Total lysates and precipitates were then analyzed by Western blot.

Fluorescence microscopy. HCT116 cells cultured on glass coverslips were transfected with indicated constructs and harvested after 24 h by fixing in formaldehyde (3.7%, 30 min, room temperature) and permeabilizing in Triton X-100 (0.2%, 10 min, room temperature). Cells were incubated in blocking buffer (1% goat serum, 1 h, room temperature) followed by incubation with appropriate primary and fluorescently labeled secondary antibodies. FLAG-tagged constructs were detected with rabbit polyclonal anti-FLAG antibody (Sigma) followed by Texas red–conjugated anti-rabbit polyclonal antibody (Molecular Probes, Eugene, OR). Endogenous E-cadherin was visualized using mouse monoclonal anti–E-cadherin (Transduction Laboratories) and antimouse FITC-conjugated secondary antibody (Molecular Probes). Actin was stained using an actin-phalloidin conjugate (Molecular Probes). The level of nonspecific background immunostaining was established by omitting primary antibodies. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined using the Olympus (Markham, Ontario, Canada) 1X-70 inverted deconvolution microscope (×100 lens magnification).

Adhesion assay. HCT116 colorectal cancer cells were seeded at a cell density of 2×10^6 per 60-mm tissue culture dish the day before transfection. Cells were transfected with indicated constructs, and DNA concentration was held constant at 8 μg per dish. Cells were harvested 24 h post-transfection by trypsinization, resuspended in serum-free McCoy's 5A medium at cell density of 100,000 and 400,000 cells/mL, and used in the Innocyte Adhesion Assay from Calbiochem (San Diego, CA). Briefly, 100 μL of prepared cell suspension were added, in triplicate, to wells of 96-well dish, coated with poly-L-lysine (general attachment) or bovine serum albumin (negative control), and incubated 2 h at 37°C. Wells were gently

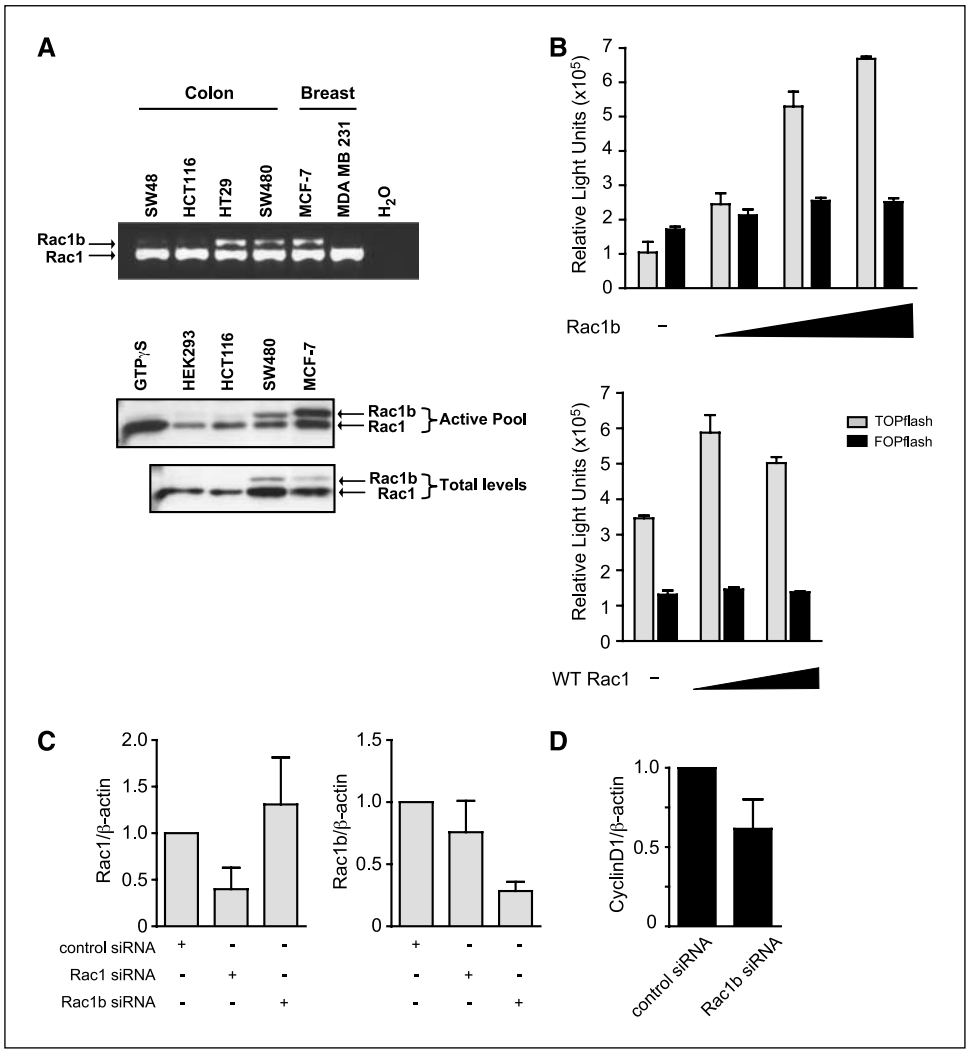

Figure 1. Rac1b expression stimulates transcription of Wnt-responsive promoter TOPflash in HCT116 colorectal cancer cells. *A, top,* endogenous Rac1b expression was analyzed by RT-PCR in a panel of human colorectal and breast cancer cell lines: breast (MDA-MB-231 and MCF-7) and colon (SW480, HT29, HCT116, and SW48); *bottom,* endogenous protein expression and activation status of Rac1b was analyzed by Western blotting of whole-cell lysates (10 μg) and PBD pull-down lysates, respectively, in human embryonic kidney HEK293 cells, HCT116 and SW480 colorectal cancer cells, and MCF-7 breast cancer cells. *B,* pTOPflash (□) or pFOPflash (■) reporter constructs were transiently transfected with increasing amount of Rac1b expression vector (0.2, 0.4, and 0.9 μg) or WT Rac1 (0.4 and 0.9 μg) for 24 h in HCT116 cells. Luciferase activity is expressed as total relative light units (*RLU*). *Columns,* average of experiments carried out in triplicate; *bars,* SE. Empty vector (−) was transfected to establish basal TOPflash/FOPflash activity. *C,* quantification of knockdown of endogenous Rac1b gene expression. HT29 cells were transfected with a scrambled control siRNA (negative control), Rac1 siRNA (targets both Rac1 and Rac1b), or siRNA selectively targeting Rac1b for 24 h and then subjected to real-time RT-PCR to quantitate Rac1/Rac1b gene expression. Data are triplicate values of at least two independent experiments. *D,* endogenous *cyclin D1* transcript expression was analyzed following specific Rac1b knockdown in HT29 cells under serum-starved conditions. Cells were transfected with Rac1b siRNA and then changed to low-serum conditions (0.1% fetal bovine serum) 4 h later. RNA was harvested for real-time RT-PCR after 20 h.

washed twice with PBS to remove nonadherent cells and then incubated with 100 μL alcein-AM green fluorescent dye solution for an additional 1 h at 37°C. Adherent cells were quantitated by measuring fluorescence of the samples using a fluorescent plate reader at excitation wavelength 485 nm and emission wavelength 520 nm.

Results

Rac1b induces transcriptional activation of Wnt-responsive promoters in colorectal cancer cells. The splice variant Rac1b exhibits selective expression, largely confined to colonic mucosa and crypt epithelial cells, and is overexpressed specifically in colon and breast tumors (16, 17). To assess the role of Rac1b in Wnt pathway regulation, we first determined its endogenous expression profile in a panel of colon cancer cell lines harboring aberrant Wnt pathway activation. Using reverse transcription-PCR (RT-PCR) analysis, we observed expression of the *Rac1b* transcript in SW480 and HT29 colorectal cancer cells as well as MCF-7 breast cancer cells and very low levels in HCT116 and SW48 colorectal cancer cells (Fig. 1*A, top*). Concordantly, cells expressing Rac1b transcript also showed Rac1b protein expression (Fig. 1*A, bottom*). The level of activation of Rac1b, assessed by PBD pull down-assay, was comparable with Rac1 in SW480 and MCF-7 cells, although examination of corresponding whole-cell lysates indicated far more Rac1 versus Rac1b total protein in these cells. This emphasizes that Rac1b is more efficiently activated than Rac1, which agrees with previously published reports (19, 20).

Because Rac1b expression was minimally detected in HCT116 cells, they were used to evaluate the role of Rac1b overexpression in Wnt pathway regulation. A hallmark feature of canonical Wnt pathway activation is β-catenin/Tcf–mediated transcription of Wnt target genes. β-catenin/Tcf–dependent gene expression was tested with the Wnt-responsive Tcf reporter TOPflash together with the mutant reporter FOPflash as a negative control. As shown in Fig. 1*B* (*top*), transient transfection of Rac1b led up to a 7-fold activation of β-catenin/Tcf–mediated transcription of TOPflash. Under similar conditions, WT Rac1 also stimulated TOPflash activity in HCT116 cells although much less robustly compared with Rac1b, reaching only a maximal 2-fold induction (Fig. 1*B, bottom*). To further elucidate the specific contribution of Rac1b in Wnt pathway activation, we down-regulated endogenous Rac1b in HT29 cells by transfection of small interfering RNAs (siRNA) targeting the splice insertion sequence within Rac1b. As shown in Fig. 1*C* (*right*), ~70% specific knockdown of Rac1b was achieved and did not affect WT Rac1 transcript levels. We examined the effects of Rac1b knockdown on the regulation of an endogenous Wnt target gene

cyclin D1 and observed ~40% reduction (Fig. 1*D*). These findings suggest that Rac1b expression contributes to inappropriate transcription of Wnt target genes in colorectal cancer cells.

Rac1b augments Dishevelled-mediated activation of Tcf/Lef–dependent transcription. We have shown previously that constitutively active V12Rac1 synergizes with stabilized β-catenin to activate transcription of Wnt target genes in HEK293 cells (26). To see if Rac1b acts analogously, Rac1b was cotransfected with the TOPflash reporter into HEK293 cells, which have an intact and therefore tightly regulated Wnt pathway. As shown in Fig. 2*A*, Rac1b failed to activate TOPflash activity on its own compared with 6-fold activation by β-catenin. Surprisingly, Rac1b did not show functional synergy with β-catenin as shown previously for V12Rac1. In agreement with our V12Rac1 studies, WT Rac1 showed a remarkable synergy with β-catenin, increasing TOPflash activity up to 30-fold (data not shown).

Failure to see a functional interaction between Rac1b and β-catenin in HEK293 cells that could account for the transcriptional activity observed in HCT116 cells prompted us to explore other Wnt signaling components that lay upstream of β-catenin along the Wnt signaling axis. Dishevelled is one such protein that is a well-established positive regulator of Wnt signaling, functioning very early in the cascade. It is believed that Dishevelled transmits Wnt signals from Frizzled receptors by inhibiting glycogen synthase kinase-3β activity through an unknown mechanism, enabling β-catenin to escape proteasomal degradation. Coexpression of Rac1b with Dvl-3 caused a striking transcriptional activation of up to 11-fold of TOPflash compared with 6-fold induction with Dvl-3 alone (Fig. 2*B*). Taken together, these data suggest that the functional cross-talk between Rac1b and Wnt pathway activation likely occurs downstream of Dvl-3 but upstream of β-catenin.

Having established a role for Rac1b as an upstream activator of transcription of Wnt target genes, we wished to compare its transcription regulatory function with respect to other well-established Rac1-specific transcriptional targets. Canonical Rac1 effector pathways that result in transcriptional regulation include NF-κB, mitogen-activated protein kinase (MAPK), and JNK pathways (8, 27, 28). As depicted in Fig. 2*C*, Rac1b very modestly activated NF-κB–dependent transcription in a dose-dependent manner and exerted no effect on AP-1– or SRE-dependent gene transcription, corresponding to MAPK and JNK pathway activation, respectively. Furthermore, Rac1b showed a dose-dependent activation of transcription of the cyclinD1 promoter (Fig. 2*D*), which agrees with our observations of Rac1b suppression causing reduced endogenous cyclin D1 expression (Fig. 1*D*). These

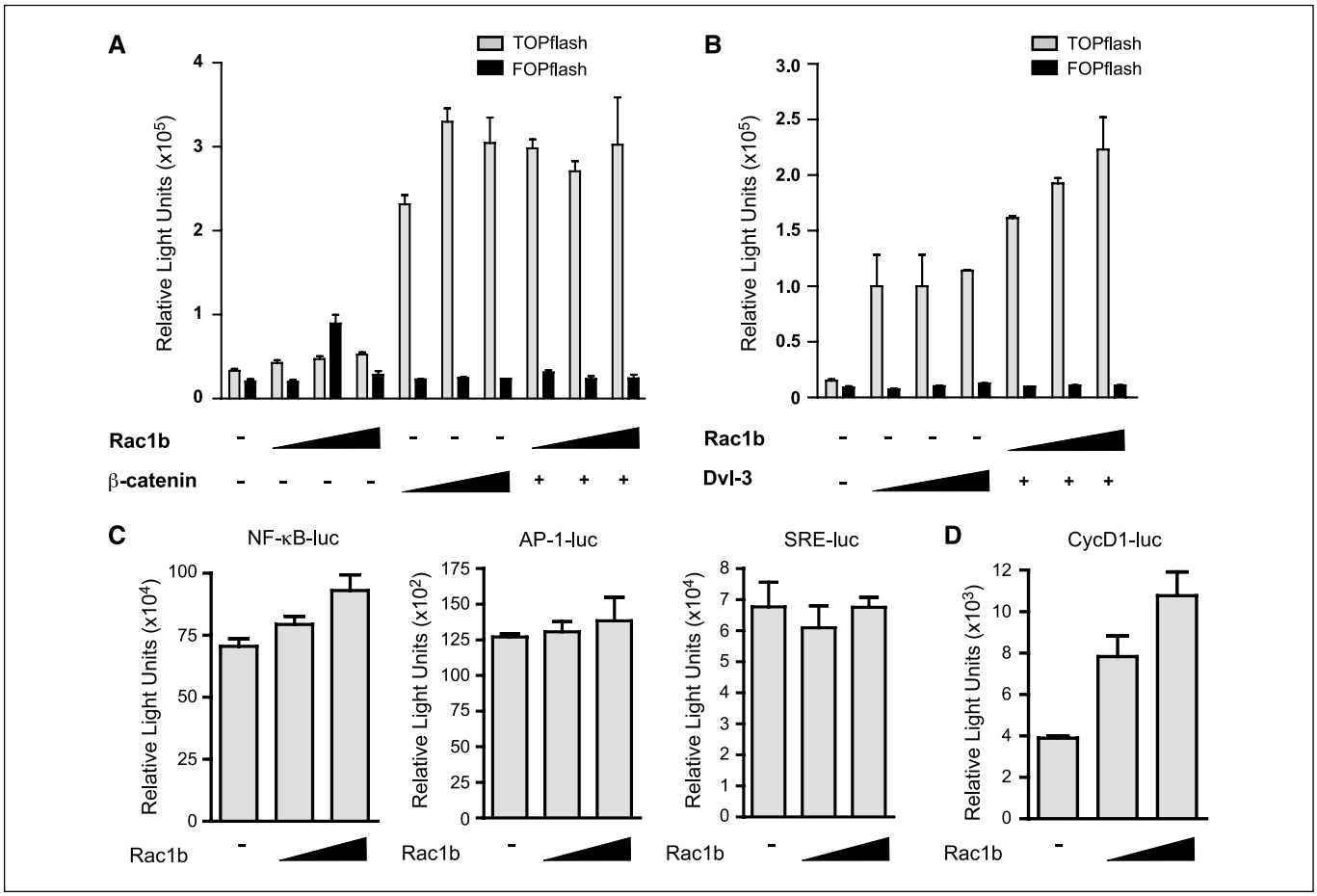

Figure 2. Rac1b cooperates with Dvl-3 to stimulate Wnt target gene transcription. *A* and *B,* pTOPflash (□) or pFOPflash (■) reporter constructs were transiently transfected with increasing amounts of Rac1b, β-catenin, or Dvl-3 expression vectors for 24 h in HEK293 cells. Amounts of expression constructs used are 0.2, 0.4, and 0.9 μg. The lowest amount of β-catenin or Dvl-3 (0.2 μg) was used in cotransfection experiments with Rac1b. *C* and *D,* HEK293 cells were cotransfected with 0.9 μg of empty vector (–) or 0.2 and 0.9 μg of Rac1b expression vector and either NF-κB–, AP-1–, or SRE-responsive promoter luciferase reporter constructs (*C*) or a full-length cyclin D1 promoter construct, CycD1-luc (*D*). Results are representative of at least three or more independent experiments. Luciferase activity is expressed as total relative light units. *Columns,* average; *bars,* SE (*A–D*).

differential transcriptional responses reiterate the selective signaling capability of Rac1b versus Rac1.

Dvl-3 activates Rac1b and together they synergistically stimulate cyclin D1 transcription. Because Rac1b was unable to activate TOPflash activity on its own but showed significant functional synergy in the presence of Dvl-3 in HEK293 cells, we speculated whether Rac1b activity was being modified by Dvl-3. Using the PBD assay, we found that expression of β-catenin did not change the activity of exogenously transfected Rac1b (Fig. 3*A, top*). In contrast, cotransfection with Dvl-3 resulted in a significant increase in Rac1b activation levels. These findings help to explain why we observed a differential response in our TOPflash assay (Fig. 2*B*). Rac1b is activated by Dvl-3 but not β-catenin and consequently proceeds to stimulate transcription from the Wnt-responsive promoter in a Dishevelled-dependent manner. *Cyclin D1* is a well-characterized target gene of both the Wnt and Rac1 signaling pathways (12, 29, 30). We observed cooperativity between Dvl-3 and Rac1b in regulation of the *cyclin D1* promoter (Fig. 3*B*). Combined expression of Dvl-3 with Rac1b yielded >5-fold activation of cyclin D1 promoter activity, which was greater than their effects individually, suggesting a synergistic interaction.

Expression of Dvl-3 and Rac1b has been shown in both the cytoplasm and the nucleus. A recent study has shown that nuclear translocation of Dvl is essential for its ability to activate Wnt/β-catenin signaling (31). In addition, we as well as others have reported previously the expression of Rac1 in the nucleus (26, 32, 33). For this reason, we examined complex formation of Dvl-3 and Rac1b in these specific cellular fractions. We observed complexes of Rac1b and Dvl-3 in the cytoplasm as well as the nucleus (Fig. 3*C*). Interestingly, Rac1b coimmunoprecipitated with Dvl-3 more efficiently in the nuclear fraction. Similarly, endogenous β-catenin also preferentially coimmunoprecipitated with Rac1b in the nuclear fraction. Protein expression levels of Rac1b, Dvl-3, and β-catenin in whole-cell lysates and cytoplasmic and nuclear fractions are shown. Blots were stripped and reprobed for marker proteins for cytoplasm and nucleus, paxillin and topoisomerase II, respectively. Considered together, our data suggest that Dvl-3 and β-catenin can both form complexes with Rac1b in the nucleus and cytoplasm.

PBR of Rac1b regulates its nuclear localization. The C-terminal PBR of Rac1 has been shown to function as a nuclear localization signal (NLS) and mutation of this region drastically reduces its nuclear entry (34). To examine if the PBR of Rac1b was involved in mediating its nuclear localization, we generated various FLAG-tagged Rac1b PBR mutant constructs, by substituting the basic amino acids of the PBR with the neutral amino acid glutamine, depicted in Fig. 4*A*. We examined cellular distribution of these mutants in fractionated lysates of HCT116 cells. As shown in Fig. 4*B*, progressive mutation of the PBR resulted in a significant progressive decrease in nuclear expression of Rac1b and was accompanied by a corresponding progressive increase in cytoplasmic expression of Rac1b. The Rac1b (Q6) mutant, with all six basic amino acids mutated, showed the greatest reduction in nuclear expression.

Mutation of the PBR did not compromise Rac1b activation levels. As shown in Fig. 4*C*, all PBR mutants (Q2, Q4, and Q6) showed similar levels of activation to WT PBR-intact Rac1b. We also examined the distribution of Rac1b and Rac1b (Q6) by fluorescence microscopy in HCT116 cells (Fig. 4*D*). Whereas Rac1b was prominently localized to the plasma membrane, and to a lesser extent in the cytoplasm and nucleus, Rac1b (Q6) transfectants showed complete loss of membrane staining, distinct exclusion

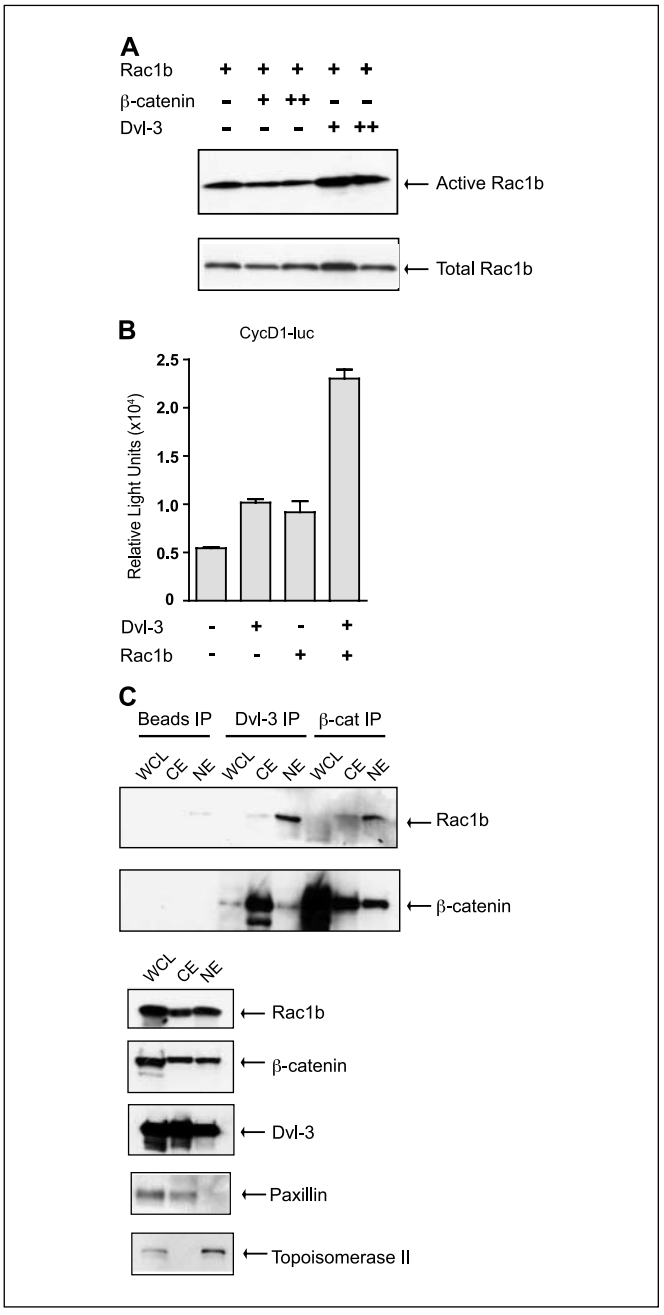

Figure 3. Dvl-3 stimulates activation of Rac1b and cooperatively induces transcription of cyclin D1. *A,* FLAG-tagged Rac1b was transiently cotransfected with increasing concentrations of either β-catenin or Dvl-3 (0, 1, and 4 μg) to determine activation status in transiently transfected HEK293 cells. *Top,* active GTP-bound Rac1b was determined by glutathione *S*-transferase (GST) pull-down assays using the PBD followed by immunoblot analysis using anti-FLAG antibody to detect exogenous Rac1b; *bottom,* total expression levels of exogenously transfected FLAG-tagged Rac1b were determined by immunoblot analysis of whole-cell lysates (10 μg). *B,* HEK293 cells were transfected with cyclin D1 promoter-luciferase construct (*CycD1-luc*) and Dvl-3 or Rac1b (0.4 μg) alone or in combination. Luciferase activity is expressed as total relative light units. *Columns,* average of experiment carried out in triplicate; *bars,* SE. *C, top,* complex formation of Rac1b with Dvl-3 and β-catenin was examined in HEK293 cells cotransfected with FLAG-tagged Rac1b and Dvl-3 for 24 h. Whole-cell lysates (*WCL*), cytoplasmic extracts (*CE*), or nuclear extracts (*NE*) were immunoprecipitated (*IP*) with anti–Dvl-3 or anti–β-catenin antibody or agarose beads alone (negative control). Immunoblotting was done with indicated antibodies. FLAG-specific antibody was used to detect FLAG-tagged Rac1b. *Bottom,* 10 μg WCL, CE, and NE were immunoblotted with β-catenin, Dvl, or FLAG antibodies to confirm expression of endogenous β-catenin and exogenous Rac1b and Dvl-3 in different cellular fractions.

from the nucleus, and diffuse cytoplasmic staining. The cells were costained with actin to distinguish individual cells. Taken together, the PBR of Rac1b controls both membrane localization and nuclear accumulation of Rac1b. We used the Rac1b (Q6) mutant in subsequent experiments because it exhibited the greatest reduction in plasma membrane and nuclear localization.

Mutation of PBR compromises the transcription activator function of Rac1b and the association of Dishevelled and β-catenin with Rac1b. To gain insight into the role of subcellular localization on Rac1b functions, we examined the transcription activator potential of PBR-mutated Rac1b. As shown in Fig. 5*A*, Rac1b (Q6) was unable to activate transcription of the TOPflash promoter in HCT116 cells compared with WT Rac1b (Fig. 1*B*). Consistent with these findings, the Rac1b (Q6) mutant also failed to stimulate transcription from the NF-κB promoter (Fig. 5*B*) and cyclin D1 promoter (Fig. 5*C*). These data show that the PBR of Rac1b is important for mediating transcriptional activation. Furthermore, disrupting plasma membrane and/or nuclear localization of Rac1b interferes with this activity.

Because PBR-mutated Rac1b (Q6) was unable to stimulate TOPflash activity, we speculated that activation by Dvl-3 may also be compromised. Comparison of the activation levels of WT Rac1b and Rac1b (Q6) in the presence of Dvl-3 indicate that Dvl-3 is unable to further stimulate the activation of Rac1b (Q6) (Fig. 5*D, left*). Because we have shown that Rac1b can complex with Dvl-3 and β-catenin (Fig. 3*C*), we examined whether they were associated with active Rac1b by reprobing the PBD blot with Dvl-3 and β-catenin antibodies. As shown in Fig. 5*D* (*left*), Dvl-3 and β-catenin were associated with active GTP-bound Rac1b. Interestingly, the Rac1b (Q6) pull-down fractions contained considerably reduced levels of Dvl-3 and β-catenin. The reduced binding to Rac1b (Q6) was not due to lack of expression of Dvl-3 and β-catenin, which was confirmed by Western blotting of whole-cell lysates (Fig. 5*D, right*). Because Rac1b (Q6) was refractory to further activation by

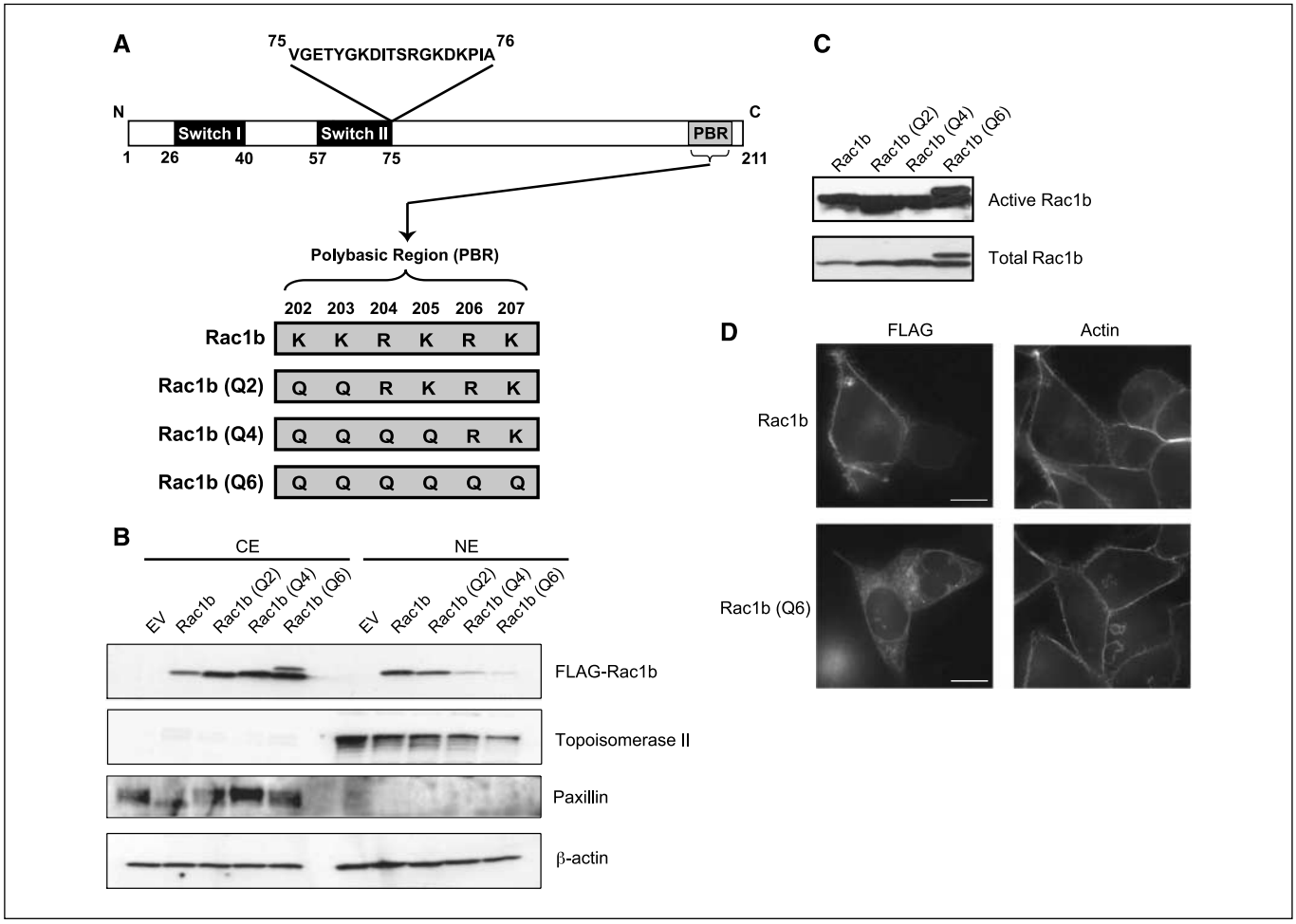

Figure 4. The PBR of Rac1b regulates its nuclear and plasma membrane localization but not activity. *A,* schematic representation of the Rac1b protein sequence and an enlarged view of the C-terminal PBR sequence of WT Rac1b and the substitutions that were made to alter PBR function (Q2, Q4, and Q6). *B,* the expression of FLAG-tagged Rac1b and the various FLAG-tagged PBR mutants (Q2, Q4, and Q6) were determined by Western blotting of fractionated cytoplasmic and nuclear lysates of transiently transfected HEK293 cells. Blots were stripped and reprobed with topoisomerase II, paxillin, or β-actin antibodies to assess purity of nuclear and cytosolic fractions and to normalize for protein loading, respectively. Interestingly, a doublet was observed in the Rac1b (Q6)–transfected cells. The doublet could also be detected by an anti-Rac1 antibody on Western blots (data not shown). In addition, RT-PCR analysis of Rac1b (Q6) plasmid DNA did not show an additional band. We conclude that the doublet is specific for Rac1b and may result due to a posttranslational modification. *C,* the activation status of Rac1b and PBR-mutated forms of Rac1b were assessed by PBD assay. Indicated FLAG-tagged constructs were transiently transfected into HEK293 cells for 24 h. *Top,* active GTP-bound Rac1b expression levels determined by PBD-GST pull-down assays followed by immunoblot analysis using anti-FLAG antibody; *bottom,* total cellular expression of transfected Rac1b constructs determined by immunoblot analysis of whole-cell lysates (10 μg) with anti-FLAG antibody. *D,* the distributions of FLAG-tagged Rac1b and Rac1b (Q6) in HCT116 cells were determined by fluorescence microscopy. Cells were stained with actin-phalloidin to demarcate individual cells. Approximately 50 to 60 transfected cells were examined. *Bar,* 10 μm.

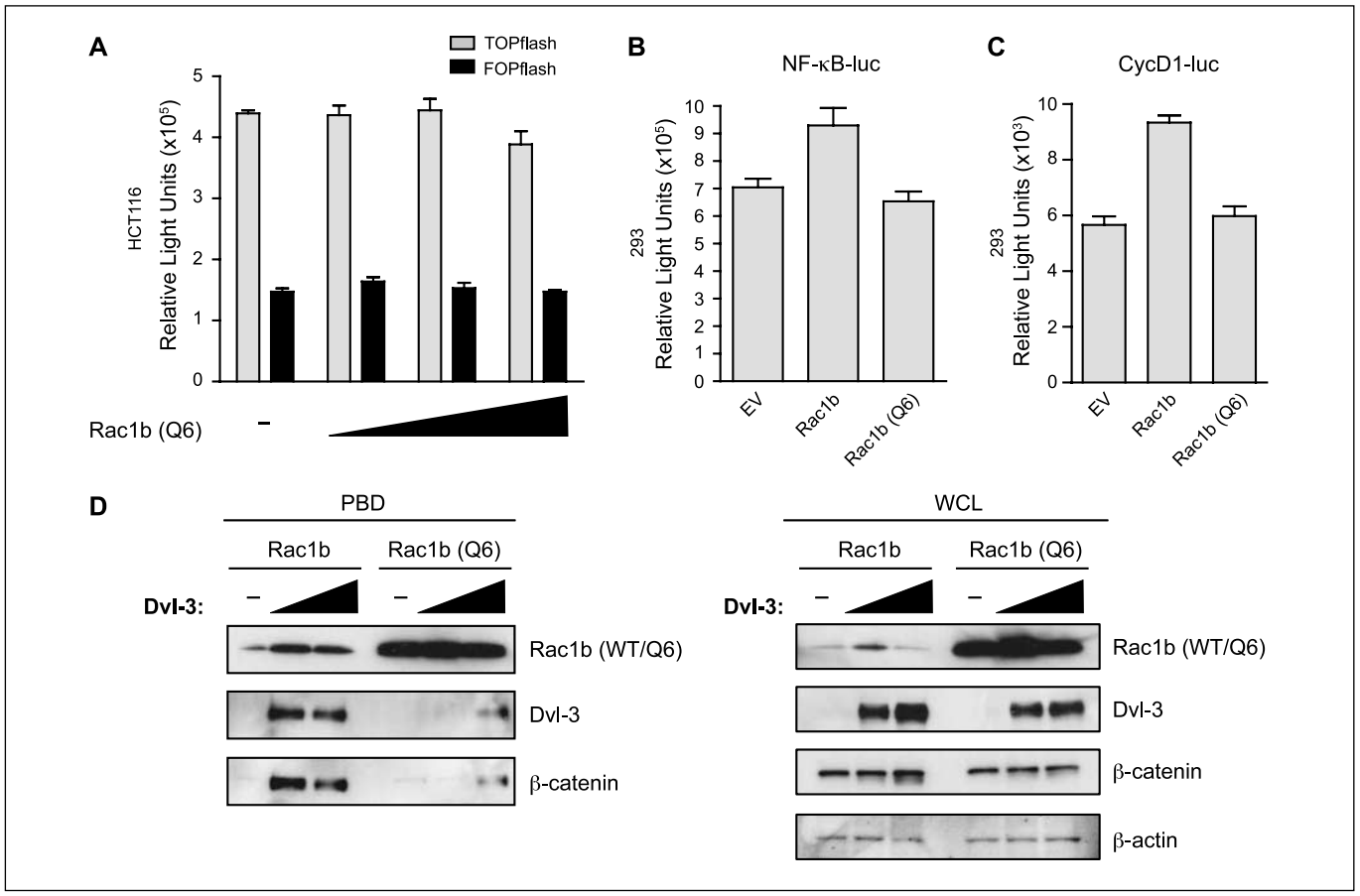

Figure 5. Mutation of Rac1b PBR disrupts Rac1b-mediated transcriptional activation and activation by Dvl-3. *A,* HCT116 cells were transfected with pTOPflash/pFOPflash promoter luciferase constructs and increasing doses of PBR-mutated Rac1b (Q6; 0.2, 0.4, and 0.9 μg). *B* and *C,* HEK293 cells were transfected with indicated promoter-luciferase constructs and either WT Rac1b or Rac1b (Q6; 0.4 μg). *D,* activation status of FLAG-tagged WT Rac1b or Rac1b (Q6) in the absence (−) or presence of Dvl-3 (1 or 4 μg). Indicated constructs were transiently transfected into HEK293 cells for 24 h. *Left,* active GTP-bound Rac1b/Q6 expression levels determined by PBD-GST pull-down assays followed by immunoblot analysis using anti-FLAG antibody. Blots were stripped and reprobed with Dvl-3 and β-catenin antibodies. *Right,* total cellular expression of transfected Rac1b/Q6 constructs determined by immunoblot analysis of whole-cell lysates (10 μg) with anti-FLAG antibody. Blots were stripped and reprobed for Dvl-3, β-catenin, and β-actin. Luciferase activity is expressed as total relative light units. *Columns,* average of experiments carried out in triplicate; *bars,* SE (*A–C*). Data are representative of three independent experiments (*A–D*).

Dvl-3 and we failed to see association of Dvl-3 or β-catenin with Rac1b (Q6), these observations strongly support the role of the PBR in mediating binding and activation of Rac1b by Dvl-3.

Rac1b reduces E-cadherin expression and cellular adhesion of colorectal cancer cells. Transcriptional activation of Wnt target genes requires β-catenin accumulation and translocation to the nucleus. Rac1b does not likely modulate proteasomal degradation of β-catenin because our observations were made in HCT116 cells, which already contain stabilized β-catenin (35). Recently, it was shown that loss of E-cadherin can lead to Wnt-dependent transcription in colorectal cancer cells (36). Therefore, we next explored whether Rac1b may mediate its positive effects on transcription by altering E-cadherin expression in HCT116 cells. We observed that the total amount of endogenous E-cadherin protein was decreased in the presence of Rac1b (Fig. 6*A*). Densitometry indicated a 40% reduction of endogenous E-cadherin levels in the presence of Rac1b. In agreement, specific suppression of Rac1b using Rac1b siRNA resulted in a modest increase in the expression of E-cadherin (Supplementary Fig. S1). Interestingly, E-cadherin levels gradually returned to basal (untransfected) levels with progressive mutation of the PBR, such that Rac1b (Q6) showed minimal effects on E-cadherin expression (Fig. 6*A*). This shows that the PBR is required for Rac1b-mediated regulation of E-cadherin. Because the PBR is important in regulating the targeting of Rac1b, it is possible that the loss of E-cadherin regulation is related to change in its cellular localization.

Next, we wished to see if the membrane pool of E-cadherin reflected the same changes as total E-cadherin. Overexpression of Rac1b changed the distribution of endogenous E-cadherin by causing a marked reduction of E-cadherin staining at the plasma membrane (Fig. 6*B*) compared with untransfected cells. In contrast, PBR-mutated Rac1b (Q6) did not alter E-cadherin staining at the plasma membrane, which remained intact at cell-cell junctions.

Changes in E-cadherin expression often translate to changes in cell-cell adhesion. We wanted to assess whether expression of Rac1b changed the adhesive properties of HCT116 cells. Expression of Rac1b decreased the adhesion of HCT116 cells, whereas Rac1b (Q6)–transfected cells showed no change (Fig. 6*C, left*). Interestingly, V12Rac1 caused an even more robust reduction in cell adhesion compared with Rac1b. Because V12Rac1 is a constitutively active mutant, we predicted that the greater reduction in adhesion was attributed to its greater level of activity. Because we

have shown that Dvl-3 can increase the activation level of Rac1b, we speculated whether Rac1b would decrease adhesion to a greater extent in the presence of Dvl-3. Indeed, cotransfection of Dvl-3 with Rac1b caused ~80% reduction in adhesion of HCT116 cells compared with Rac1b or Dvl-3 alone, which individually caused a 50% reduction in adhesion (Fig. 6*C, right*). A primary mechanism of E-cadherin down-regulation in tumors is a result of the action of transcriptional repressors (e.g., proteins of the Snail/Slug family), which bind to E-box elements within the promoter region of E-cadherin (37). This prompted us to investigate whether such underlying molecular mechanisms were involved in mediating the Rac1b-associated decrease in E-cadherin expression. Suppression of endogenous Rac1b in HT29 cells resulted in increased E-cadherin transcript expression and a concomitant decrease in Slug

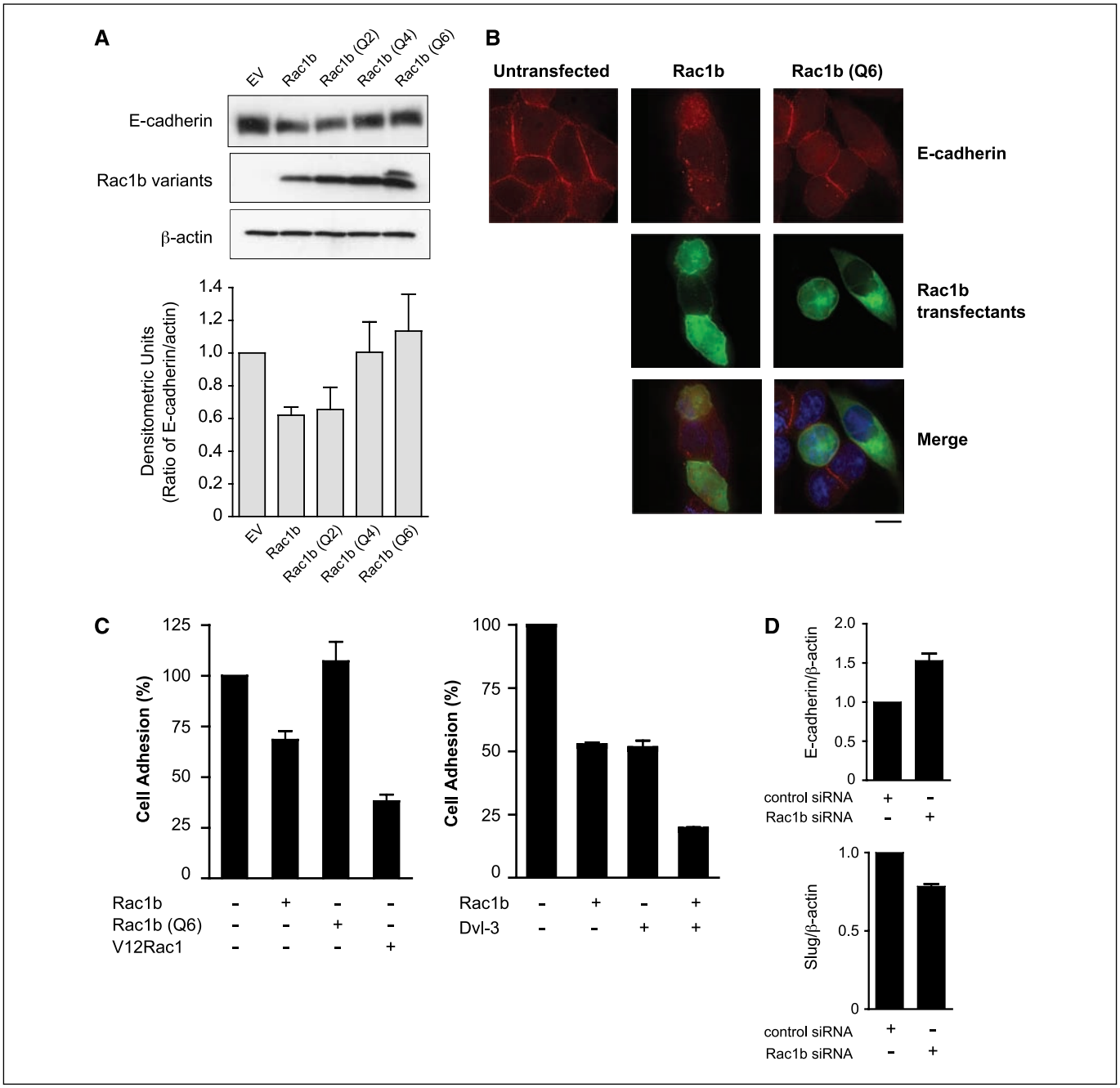

Figure 6. Rac1b decreases the expression of E-cadherin and cellular adhesion in HCT116 cells. *A, top,* Western blot analysis of the total amount of E-cadherin after 24 h of transfection with Rac1b or PBR mutants of Rac1b. FLAG-tagged Rac1b and PBR-mutant expression was detected by probing blots with FLAG antibody. Expression of actin was used as a loading control. *Bottom,* the band of densitometry was normalized with actin to allow quantification. Data show a significant decrease in E-cadherin expression mediated by Rac1b, which is abolished on mutation of the PBR region. Data are representative of three independent experiments. *B,* distribution of endogenous E-cadherin (red fluorescence) was compared in HCT116 cells transfected with FLAG-tagged Rac1b versus FLAG-tagged Rac1b (Q6), or untransfected cells by fluorescence microscopy. Rac1b and Rac1b (Q6) transfectants were identified by green fluorescence. *Bar,* 10 μm. *C,* HCT116 cells were transfected for 24 h with indicated constructs followed by assessment of adhesion on poly-L-lysine–coated surface. Results are the percentage of adhesion compared with empty vector–transfected cells. Representative data obtained from triplicate values. Data are representative of at least two independent experiments. *D, E-cadherin* (*top*) and *Slug* (*bottom*) transcript levels in response to RNA interference–mediated inhibition of Rac1b expression in HT29 cells.

(Snai2) transcript levels (Fig. 6*D*). We did not observe any change in Snail transcript levels under similar conditions (data not shown). These data agree of a repressive role for Rac1b in cellular adhesion by inhibition of E-cadherin transcription.

Discussion

Because its discovery as a splice variant specifically overexpressed in colon and breast tumors, there has been considerable interest in characterizing the functions of Rac1b to understand how it may contribute to tumorigenesis in these tissues (16, 17). Our data show functionality of naturally occurring Rac1b in modulating Wnt signaling by augmenting Dishevelled-mediated effects on Tcf-dependent target gene transcription. In addition, we show that Rac1b decreases membrane E-cadherin expression and reduces adhesion of colorectal cancer cells and this is likely via upregulation of Slug, the E-cadherin-specific transcriptional repressor.

The contribution of Rac1b to Wnt pathway activation may represent an important mechanism by which it promotes colon tumorigenesis. We have shown previously that V12Rac1 requires the presence of stabilized β-catenin to mediate its stimulatory effects on transcription. In agreement with these data, coexpression of WT Rac1 with β-catenin also resulted in synergistic induction of TOPflash promoter activity in HEK293 cells (data not shown). Unlike Rac1, Rac1b was unable to stimulate Tcf-dependent transcription in HEK293 cells, despite coexpression of β-catenin. However, when exogenous Dvl-3 was coexpressed with Rac1b in these cells, we observed striking synergistic activation of TOPflash. Interestingly, Dvl proteins have been noted to form cytoplasmic puncta when overexpressed in cells, and their formation has been correlated with their ability to induce Wnt pathway activation (38, 39). In this regard, we observed a striking increase in the size and number of Dvl-3 punctae in HCT116 cells cotransfected with Dvl-3 and either Rac1b or L61Rac1 compared with Dvl-3 alone (Supplementary Fig. S2). Interestingly, we also observed Rac1b and L61Rac1 colocalize with Dvl-3 within these punctae. Understanding how these vesicles form and correlate with Wnt signaling activity may uncover new roles for Dvl and Rac1b/Rac1 proteins in regulating the canonical arm of Wnt signaling.

It is tempting to speculate that Rac1b and Rac1 may act at different stages of tumor progression to further exacerbate Wnt signaling in colonic tissues. Our data show that Rac1b and Rac1 likely require different cellular cues, at which they can participate in Wnt signaling. In contrast to Rac1, which can synergize with β-catenin to augment Wnt signaling, Rac1b requires a signal further upstream, likely stemming from Dishevelled proteins. This implies that expression of Rac1b in colorectal tumor cells that already harbor inherent Dishevelled-dependent activation of Wnt pathway may increase the intensity of Wnt signaling within those cells. This may be a critical step in tumor progression, assisting tumor cells to metastasize to distal sites. Clinical evidence suggests that Wnt signaling acts differently at different stages of tumor progression (40). This is best exemplified by nuclear staining for β-catenin, a hallmark of Wnt pathway activation, in colorectal carcinomas, which often show a heterogeneous pattern, with strongest nuclear enrichment at the invasion front. The molecular basis for this differential distribution of β-catenin is not known, but it has been speculated that signals from the mesenchymal tissue, which surrounds the invasive tumor cells, might superactivate the pathway by unknown ways. Matrix metalloproteinases (MMP) are important microenvironmental factors present in mesenchymal tissue, implicated in tumor initiation and progression. Interestingly, some MMPs are Wnt target genes (41). Recently, Rac1b was shown to be the key mediator of MMP-3–induced malignant transformation of mouse mammary epithelial cells (21). Specifically, MMP-3 treatment induced Rac1b expression, which was responsible for the EMT of the cells. It is tempting to speculate that a positive feedback loop exists, in which initial Wnt pathway activation induces expression of a subset of gene products, such as MMPs, which feedback in an autocrine or paracrine manner, on Wnt-dysregulated cells to induce Rac1b expression. In turn, as our data suggest, Rac1b expression would further augment nuclear Wnt signaling in the cancer-initiated cells, promoting tumor progression by aiding tumor cells in invasion and metastasis. Whether such a feedback loop exists warrants future investigation.

Another important point worth noting is that overexpression of the naturally occurring WT form of Rac1b was sufficient to induce changes in Wnt signaling. Unlike the widely used 'artificial' constitutively active mutants (G12V or Q61L), WT forms of Rho proteins are amenable to regulation and so their use can facilitate identification of upstream or downstream signals that may be critical in modulating expression and activity. In this context, our findings are significant because overexpression of WT Rac1b had a profound stimulatory effect, cooperating with Dvl-3 to increase nuclear Wnt signaling events. Furthermore, we show that expression of Dvl-3, but not β-catenin, leads to increased activation of Rac1b. This is likely the reason why Rac1b augmented Tcf-mediated transcription in the presence of Dvl-3 but not β-catenin. Perhaps association of Dvl-3 with Rac1b not only further activates Rac1b by modulating GEF/GAP activity but also may recruit other proteins, such as β-catenin, to form functional signaling complexes. Our findings that Dvl-3 and β-catenin were predominantly present in the active GTP-bound fractions of Rac1b, in Dvl-dependent manner, support this idea. Mechanisms of how Dvl-3 may increase activity of Rac1b are currently under investigation in our laboratory. Aberrant expression of Dishevelled has been observed in multiple cancers and has been linked to activation of Wnt/β-catenin signaling and cell growth in these cancers. It would be interesting to examine Rac1b expression and activity and turnover in these tumors (42–44).

Because we observed nuclear expression of Rac1b, we wondered if the putative NLS in the PBR was responsible. Substitution of all six basic amino acids of the Rac1b PBR with neutral glutamines resulted in almost complete exclusion of Rac1b from the nucleus, as well as the plasma membrane. PBR-mutated Rac1b was unable to activate transcription from TOPflash, cyclin D1, and NF-κB promoters. It is tempting to speculate that the nuclear pool of Rac1b is likely critical for transcription of Wnt target genes. Interestingly, Dvl-3 was unable to augment activity of PBR-mutated Rac1b, suggesting that it may mediate these effects within the nucleus. In this regard, nuclear expression of many Rac1-specific proteins, including GEFs and downstream effectors, further supports the notion of Rac1 and Rac1b participating in nuclear signaling pathways (33, 45). In agreement with this hypothesis, we observed Dvl-3 coimmunoprecipitating with Rac1b mostly in the nuclear fraction of cells, despite these proteins being present in the cytoplasm, which lends further support for these effects occurring in the nucleus.

In the colon, E-cadherin–mediated cell adhesion is critical in the transition from adenoma to carcinoma, and reexpression of WT E-cadherin in cancer cell lines reduces their invasiveness (46, 47). Here, we show that expression of Rac1b in HCT116 colorectal

cancer cells reduces endogenous E-cadherin expression and cellular adhesion. Intriguingly, coexpression of Dvl-3 with Rac1b resulted in a striking reduction of adhesion compared with effects seen by either protein alone. Furthermore, we show that both of these effects disappear on mutation of the PBR of Rac1b. Because nuclear localization of Rac1b is disrupted by mutation of the PBR, it is possible that Rac1b functions in the nucleus to alter E-cadherin expression. We observed decreased Slug expression and increased E-cadherin transcript expression on suppression of Rac1b in HT29 cells. These data support an important nuclear role for Rac1b, whereby it positively regulates Slug transcription, which consequently represses E-cadherin expression leading to diminished cellular adhesion. Interestingly, *Slug* has been reported to be a Wnt target gene and shown to have Tcf-binding elements within its promoter region (48). We propose that Rac1b expression contributes to tumor progression by synergizing with Wnt components to modulate Slug expression and therefore the adhesive properties of cells. Future studies aimed at elucidating how Rac1b regulates Slug and the discovery of other Rac1b/Wnt target genes will further our understanding of the role of Rac1b in tumor progression.

It is tempting to speculate that because Rac1b, being a tumor-specific protein, seems to share only a selective repertoire of Rac1 functions, it is likely that those pathways retained by Rac1b are critical for tumorigenesis, by perhaps imparting a growth advantage and/or increased capacity for invasion and metastasis. In this regard, further activation of Wnt signaling in colon cancer cells by Rac1b may accelerate tumor progression in this tissue. Tumorigenesis is an evolutionary process, where successive genetic changes transform a normal cell into a self-sufficient, resilient "super" cancer cell capable of metastasis. Mounting evidence suggests that deregulation of Rac1 signaling, through overexpression of itself or its regulators, has dire consequences on cell physiology, changing signaling circuitry and contributing to cellular transformation. Changes in Rac1 splicing to create Rac1b, a much more efficient and active GTPase, likely represents a further evolutionary advantage for cancer development. Future studies aimed at identifying the mechanisms involved in activation of Rac1b expression as well as clinical studies correlating Rac1b expression with extent of colorectal cancer will increase our understanding of Rac1b in tumorigenesis and may also be of prognostic value or provide novel therapeutic targets.

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