Rac1b recruits Dishevelled and β-catenin to Wnt target gene promoters independent of Wnt3A stimulation

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Abstract. We previously reported a functional interaction between aberrant Wnt signaling and Rac1/Rac1b GTPases in tumorigenesis. In this study, we further investigated the mechanistic role of nuclear Rac1b. Using chromatin immunoprecipitation (ChIP) studies, we show that Rac1b resides at the promoters of Wnt target genes, c-Myc and Cyclin D1, in HCT116 cells with aberrant Wnt pathway. In HEK293T cells with intact Wnt signaling, Rac1b is tethered to these same gene promoters independent of Wnt3A stimulation and is further observed to recruit Dishevelled and β-catenin in the absence of Wnt3A stimulation. Our studies suggest a novel transcriptional co-activator role of Rac1b in β-catenin/TCF-mediated transcription.

Introduction

Rac1 GTPase is one of the members of RhoGTPase proteins that are essential regulators of various cellular processes such as maintenance of actin cytoskeleton, mitogenesis, activation of kinase cascade and transcription (1-3). Aberrant Rac1 activation is associated with tumorigenesis (4) Rac1b GTPase, an alternatively spliced variant of Rac1 gene, is preferentially over-expressed in a subset of breast and colon tumors (4-6). The Rac1b protein contains an in-frame insertion of 19 amino acids between Rac1 residues 75 and 76 immediately preceding the Switch II region (7). As a result of this inclusion within a key functional region, several differences exist between Rac1 and Rac1b. In vitro and in vivo studies have demonstrated that Rac1b possesses an impaired intrinsic GTPase activity, and consequently behaves as a constitutively active form of Rac1 (5,7-9). Over-expression of Rac1b was recently attributed to the enhanced expression of exon 3b inclusion splice factor ASF/SF2 (10). Unlike Rac1 GTPase, Rac1b does not lead to lamellipodia formation, or the activation of PAK1, AKT1, or c-Jun-NH2-kinase activities (11). However, Rac1b retains the ability to stimulate the NFκB pathway and was shown to induce transcriptional stimulation of a consensus NFκB promoter in a luciferase reporter construct (12,13). Rac1b is unable to bind to many regulators of Rho family GTPases, although it displays enhanced binding to SmgGDS, RACK1, and p120 catenin, proteins involved in cell-cell adhesion, motility, and transcriptional regulation (14). Rac1b signaling is essential for tumor cell viability and survival (11). Intriguingly, Rac1b also cooperates functionally with B-Raf V600E mutations to sustain colorectal cell viability suggesting that the two proteins constitute an alternative survival pathway to oncogenic K-ras in colon tumors (15).

Aberrant activation of the Wnt signaling pathway is one of the major causes of colorectal cancer (CRC) (16,17). Wnt signaling regulates expression of a wide range of target genes that direct cell proliferation, migration, establishment of cell polarity, development, specification of cell fate and adult stem cell proliferation (18-22). In the canonical Wnt signaling pathway, the core protein, β-catenin, plays a dual role in the cell, by participating in the maintenance of cadherin-mediated cell-cell contacts, and as a transcriptional co-activator in the nucleus. The list of potential co-activators present at the promoters of Wnt target genes is increasing, however, their specific roles are not well understood. Dishevelled plays a key role as a molecular branch point between the canonical and non-canonical Wnt signaling pathways. Dishevelled also exists in the nucleus and its nuclear localization is required for Dishevelled function in Wnt/β-catenin signaling (23,24). Gan et al (25) have shown the involvement of Dishevelled (Dvl-3) in the transcriptional regulation of a Wnt target gene, c-Myc, in SW480 colon cancer cells.

Our group previously reported cross-talk between canonical Wnt signaling and Rac1/Rac1b GTPases (26,27). We demonstrated that over-expression of the splice variant Rac1b facilitates tumour progression by enhancing Dvl-3-mediated Wnt pathway signaling and induction of Wnt target genes (26). We observed that Rac1b downregulated endogenous E-cadherin expression and decreased cell-cell adhesion of HCT116 colorectal cancer cells. 

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Abbreviations: Dvl-3, Dishevelled; TCF, T cell factor; ChIP chromatin immunoprecipitation

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cells and this effect was further augmented by combined action of Rac1b and Dvl-3. The classical mechanism of Rac1b function occurs through activation at the plasma membrane; however, it is becoming increasingly apparent that the oncogenic properties of Rac1b are also mediated by its entry into the nucleus. Mutation of the nuclear localization signal sequence in the C-terminal polybasic region (PBR) of Rac1b, however, resulted in a complete loss of Rac1b stimulatory effects on TCF-mediated Wnt target gene transcription and the suppressive effects seen on cell adhesion, indicating the importance of nuclear and membrane localization of Rac1b. In this study, we investigated the mechanistic role of nuclear Rac1b GTPase in Wnt signaling mediated tumorigenesis.

Materials and methods

Cell culture. HCT116 parental cells (ATCC, Manassas, VA) were cultured in McCoy's 5A media supplemented with 10% fetal bovine serum (FBS). HCT116 cells with stable-inducible expression of Rac1b were sub-cultured and maintained in McCoy's 5A media supplemented with 10% FBS, 10 µg/ml blasticidin and 150 µg/ml zeocine. 293T cells (ATCC) were cultured in ATCC-formulated DMEM medium supplemented in 10% FBS. L (L cells transfected only with pGKneo) and Wnt3A expressing cells (L cells transfected with pGK Wnt3A), a kind gift of Dr Liliana Attissano (University of Toronto, ON, Canada), were maintained in DMEM supplemented with 10% FBS. Wnt3A expressing cells were additionally supplemented with 400 µg/ml G418 (Innovigen, San Diego, CA).

Antibodies. Antibodies for co-immunoprecipitation and ChIP studies were as follows: Rac1b (Millipore, Billerica, MA), Monoclonal Anti-Flag M2 (Sigma, St. Louis, MO), β-catenin (H-102), Dvl-3 (4D3) and TCF 4 (H125) were all from Santa Cruz Biotechnology, Santa Cruz, CA.

Plasmids. Mammalian Rac1b expression construct (Rac1b-pcDNA3-Flag N) was a kind gift of Dr M.R. Ahmadian, Max-Planck Institute, Heidelberg, Germany. pcDNA™ 3.1 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, ~1x10⁵ HCT116 cells in 15-cm culture dishes were fixed for 10 min with 1% formaldehyde and then digested pCDNA 4/TO/myc-His (frame B) parent vector (Invitrogen). The expression of Rac1b in the newly generated construct was detected and confirmed after induction with doxycyclin at different time-points using Flag-HRP antibody.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed using the ChIP-IT™ Express Enzymatic kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, ~1x10⁶ HCT116 cells in 15-cm dishes were fixed for 10 min with 1% formaldehyde and then chromatin was prepared using ChIP-IT Express Enzymatic kit protocol. Chromatin was sheared with the Enzymatic Shearing Cocktail for 10 min at 37°C to generate optimally sheared chromatin fragments in the range of 200-1500 bp. Sheared and homogenized chromatin was immunoprecipitated using 3 µg of indicated antibodies. Chromatin samples were then eluted, subjected to cross-link reversal, treated with proteinase K. Recovered DNA was analyzed by PCR using primer pairs that flank Tcf-β-Catenin- or Cyclin D1 promoters. The sequences of the primers are as described previously (28). The negative control primers flank a region of genomic DNA of GADPH gene that is devoid of TBEs. The GADPH primers were: forward: 5'-ATGTTGTCGATCGG GATCT-3' and reverse: 5'-TGCCAAAAGCCTAGGGGA

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AGA-3'. The PCR products were run on 2.5% agarose gel and visualized with ethidium bromide.

**Results**

Subcellular distribution of Rac1b, Dvl-3 and β-catenin in HCT116 and HEK293T cells. HCT116 cells harbour constitutively active Wnt signaling due to β-catenin mutation and do not express endogenous Rac1b. To address the nuclear role of Rac1b in these cells, we first generated an HCT116 colorectal cancer cell line with stable and doxycyclin-inducible expression of flag-tagged Rac1b and verified its expression by Western blotting. We determined conditions for optimal expression of Rac1b by performing time point and doxycyclin induction dose curves. We observed robust expression of Rac1b as early as 7 h post-induction with doxycyclin and at a concentration as low as 0.01 µg/ml with no observable expression in cells treated with a drug vehicle control (Fig. 1).

To assess the subcellular distribution of Rac1b in stable inducible HCT116 colon cancer cells, we isolated cytoplasmic and nuclear extracts from cells with and without Rac1b induction. The purity of the nuclear and cytoplasmic fractions was verified by probing the Western blots with antibodies specific for nuclear and cytoplasmic marker proteins, topoisomerase II and paxillin, respectively. Robust Rac1b expression was found along with endogenous Dvl-3 and β-catenin in the cytoplasm as well as the nucleus (Fig. 2A). We observed that Rac1b does not affect endogenous Dvl-3 and β-catenin protein profiles since Rac1b induction did not alter the expression or cyto-nuclear distribution of these proteins.

To investigate whether activation of the Wnt signaling could alter the subcellular expression and/or distribution of Rac1b in a cell system with intact canonical Wnt pathway, we treated human embryonic kidney 293T (HEK293T) cells with Wnt3A conditioned medium (WCM) in order to stimulate the canonical Wnt signaling pathway. Control L-cell conditioned medium (LCM) is devoid of secreted Wnt3A protein and was used as a negative control. We first confirmed the ability of Wnt3A conditioned medium to stimulate the canonical Wnt signaling pathway in HEK293T cells by performing the classical β-catenin stability assay as described previously (28). HEK293T cells do not express endogenous Rac1b (Fig. 2B). Following
ectopic expression of Rac1b in HEK293T cells treated with LCM- and WCM, Rac1b was found in both cytoplasmic and nuclear compartments. We observed that activation of Wnt signaling following WCM treatment of HEK293T cells did not modulate the expression levels of Rac1b (as detected by Rac1b-specific antibody) nor did it affect the cytonuclear distribution of Rac1b, indicating that Wnt signaling activation does not regulate Rac1b in these cells.

**Rac1b interacts with endogenous β-catenin, Dvl-3 and TCF-4.**

Our observation of co-localization of Rac1b, β-catenin, and Dishevelled (Dvl3) in HCT116 stable inducible cells prompted us to investigate if these proteins also interact with each other. Whole cell lysates from HCT116 cells with and without Rac1b induction were immunoprecipitated with antibodies to Dvl-3, β-catenin and TCF-4. As shown in Fig. 3, Rac1b was found to co-precipitate with Dvl-3, β-catenin and TCF-4 suggesting that Rac1b is involved in complex formation with these proteins. These observations are similar to our previous findings where we and others have previously shown Dvl-3, β-catenin and TCF-4 to bind c-Myc and Cyclin D1. We observed that Rac1b is indeed at the promoters of Wnt target genes in colon cancer cells with constitutively active Wnt signaling. We and others have previously shown Dvl-3, β-catenin and TCF-4 to bind c-Myc and Cyclin D1 promoters in vivo in colon cancer cells (25,28). However, to our knowledge, this is the first report demonstrating the presence of Rac1b at the promoter of endogenous Wnt targets in HCT116 cells.

Next, we examined if the binding of Rac1b to these promoters is dependent on Wnt stimulation of HEK293T cells by performing chromatin immunoprecipitation (ChIP) studies. We had already established that WCM treatment of HEK293T cells did not modulate the expression levels of ectopic Rac1b (as detected by Rac1b-specific antibody) nor did it have any effect on cyto-nuclear distribution of Rac1b (Fig. 2B). As expected, Wnt3A stimulation of HEK293T cells leads to an increase in the amount of both Dvl-3 and β-catenin proteins.

**Figure 4.** (A) Rac1b binds to the promoter of Wnt target genes in HCT116: HCT116 cells with stable inducible expression of Flag-tagged Rac1b were treated with (I) and without (U) 0.01 µg/ml Doxycyclin for 24 h. Chromatin was isolated as described in Materials and methods and subjected to immunoprecipitation with Flag, Dvl-3, β-catenin and TCF-4 antibodies. IgG was used as a negative control. Enriched chromatin was PCR amplified using the primers that flank TCF binding sites (TBEs) in C-Myc or Cyclin D1 promoters. GAPDH primers as described in Materials and methods were used as negative controls. Input represents 10% of chromatin used for immunoprecipitation. (B) Rac1b binds to the promoter of Wnt target genes in Wnt-independent manner and recruits β-catenin and Dvl-3 to the c-Myc and Cyclin D1 promoters in the absence of Wnt3A stimulation of HEK293T cells: HEK293 T cells were plated and transfected with empty vector (pCDN 3.1) and Rac1b expressing plasmid constructs (Rac1b-pcDNA3-Flag N). Next day the medium was removed and cells were treated with L-CM (LCM) and Wnt3A-CM (WCM) for 24 h. For ChIP assay, chromatin was subjected to immunoprecipitation using Dvl-3, β-catenin and Flag antibodies. Enriched chromatin was analyzed by PCR using primers that flank the TBEs in Wnt target genes, c-Myc and Cyclin D1. GAPDH primers as described in Materials and methods were used as negative controls. Input represents 10% of chromatin used for immunoprecipitation.
present at the \( c\)-myc and \( Cyclin\ D1 \) promoters (Fig. 4B) in the absence of Rac1b. Intriguingly, we observed that when Rac1b is present, it binds to the promoters of endogenous Wnt target genes, \( c\)-myc and \( Cyclin\ D1 \), independent of Wnt3A stimulation. Furthermore, Rac1b recruits Dvl-3 and \( \beta\)-catenin to these same promoters independent of Wnt3A stimulation. Taken together, these observations lend further support for a novel role of Rac1b in the nucleus.

**Discussion**

In the present study, we explored the nuclear role of Rac1b in a colon cancer cell line with aberrant Wnt signaling due to constitutive \( \beta\)-catenin expression (HCT116), as well as in human embryonic kidney cells (HEK293T) with intact Wnt signaling as a model base system. While it is well known that the classical mechanism of Rac1b function occurs through activation in the cytoplasm and/or at the plasma membrane, emerging evidence indicates that the oncogenic properties of Rac1b are also mediated by its entry into the nucleus. After establishing cross talk between Rac1GTPase and Wnt signaling, we reported for the first time that ectopically expressed constitutively active mutant of Rac1, V12 Rac1GTPase is associated with a subset of Wnt-responsive gene promoters in vivo. In addition, endogenous Tiam 1, a Rac1-specific activator, associated with nuclear TCF-binding sites to form a complex of Rac-Tiam-\( \beta\)-catenin-TCF to enhance Wnt target gene transcription. In extension of work previously published by our group and after establishing the nuclear localization of Rac1b, we were interested in exploring if Rac1b is recruited to these promoters. We show that Rac1b indeed associates with the \( \beta\)-catenin-TCF complex in vivo in colon cells with inherently dysregulated Wnt signaling. This makes us believe that, aside from the nuclear \( \beta\)-catenin-TCF complex, which is a prerequisite for transcription, a myriad of proteins may act as transcriptional co-activators consequently leading to enhanced dysregulated expression of endogenous oncogenic targets. Besides its over-expression in a subset of colon and breast tumors, our work demonstrates an alternate mechanistic role of nuclear Rac1b that may further enhance tumor progression.

Wnt proteins constitute a family of secreted signaling molecules that regulate pathways essential for development, and its aberrant activation drives oncogenesis in a multitude of human cancers. The total expression levels of Dvl-3 and \( \beta\)-catenin are inherently elevated in HCT116 cells due to a \( \beta\)-catenin mutation that results in its stabilization, and consequently, the constitutive activation of the canonical Wnt pathway. It is well known that \( \beta\)-catenin is an essential nuclear effector of canonical Wnt signaling. Extracellular Wnt ligands activate Dishevelled, which is believed to function as a scaffold protein bridging the receptors and downstream components of Wnt signaling pathway leading to activation of \( \beta\)-catenin target gene. Nuclear localization of Dishevelled is required for its function in the Wnt/\( \beta\)-catenin pathway. A subsequent study further demonstrated that, in addition to its cytoplasmic role in regulating the stability of \( \beta\)-catenin, Dishevelled could independently interact with c-Jun and \( \beta\)-catenin in the nucleus to mediate the formation of a transcription complex comprising...
Dishevelled, c-Jun, β-catenin, and TCF. Recent studies have shown a novel role of Dishevelled in the canonical Wnt signaling pathway by mediating the transcriptional activity of the β-catenin/TCF complex. Gan et al (25) demonstrated that Dvl-3 is recruited to the promoter of Wnt target gene, c-myc, in a Wnt3A-dependent manner, whereas binding of TCF-4 to the promoter was independent of Wnt3A stimulation. Similarly, our previous work showed that Rac1 and the Rac1-specific activator Tiam1 are also components of transcriptionally active β-catenin/TCF-4 complexes at c-Myc and Cyclin D1 promoters; and that the presence of Rac1 and Tiam1 within these complexes serves to enhance target gene transcription (28). We found that while Rac1b is also present at the gene target promoters in vivo, this binding is not dependent on Wnt signaling. Further, Rac1b recruits Dvl-3 and β-catenin to these promoters independent of Wnt3A stimulation.

Based on our current data as well as previously published studies, we propose a model as illustrated in Fig. 5 to describe the novel role of Rac1b in the nucleus. Despite functional differences between Rac1 and Rac1b, both proteins are found to be present in complexes with TCF/β-catenin in colon cancer cells, and also bind the promoters of Wnt target genes. Dvl-3 and β-catenin are known to bind these Wnt target promoters only upon Wnt3A stimulation of HEK293T cells. We now demonstrate, for the first time, that Rac1b is associated with the promoters of Wnt target genes, c-Myc and Cyclin D1, independent of Wnt activation. Further, similar to Wnt3A stimulation, Rac1b also leads to the recruitment of Dvl-3 and β-catenin to these promoters, independent of Wnt activation. Using a reporter assay, we have previously shown that, in HEK293T cells, Rac1b augments Dvl-3-mediated activation of TCF/LEF-dependent transcription. Co-expression of Rac1b and Dvl-3 in HEK293T cells caused a striking transcriptional activation of TOPFlash and Cyclin D1-luc constructs compared to expression of individual proteins. Taken together, we speculate that it is this novel nuclear role for Rac1b in co-operation with Wnt components that leads to augmented transcription of Wnt target genes, and subsequently tumorigenesis. The data presented here suggests the existence of an additional alternative mechanism of Rac1b mediated activation of Wnt signaling, and underscores the molecular complexity associated in the signaling networks leading to cancer. We believe that this new mechanism further extends our understanding of functional role of nuclear Rac1b in cell signaling.

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References