Mechanisms of Cytoplasmic β-Catenin Accumulation and Its Involvement in Tumorigenic Activities Mediated by Oncogenic Splicing Variant of the Receptor Originated from Nantes Tyrosine Kinase*

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The β-catenin pathway plays a critical role in the pathogenesis of certain types of cancers. To gain insight into mechanisms by which altered receptor tyrosine kinases regulate cytoplasmic β-catenin accumulation, the effect of an oncogenic receptor originated from Nantes (RON) variant on β-catenin accumulation and the role of β-catenin in RON-mediated tumorigenic activities were studied. In NIH3T3 cells harboring oncogenic variant RON160, increased β-catenin accumulation with tyrosine phosphorylation and nuclear translocation was observed. Overexpression of RON160 also resulted in increased expression of β-catenin target genes c-myc and cyclin D1. By analyzing cellular proteins that regulate β-catenin stabilities, it was found that RON160 activates the protein disheveled (DVL) and inactivates glycogen synthase kinase-3β by Ser-9 residue phosphorylation. These effects were channeled by RON160-activated PI 3-kinase-AKT pathways that are sensitive to specific inhibitors, such as wortmannin, but not to activated PI 3-kinase-AKT pathways that are sensitive to specific inhibitors, such as wortmannin, but not to chemical inhibitors. Silencing RON160 expression by small interfering RNA blocked not only β-catenin expression but also c-myc and cyclin D1 expression, suggesting that RON expression is required for the activation of the β-catenin signaling pathway. Moreover, it was found that knockdown of the β-catenin gene expression by small interfering RNA techniques reduces significantly the RON160-mediated NIH3T3 cell proliferation, focus-forming activities and anchorage-independent growth. Thus, the oncogenic RON variant regulates β-catenin stabilities through activation of DVL and inactivation of glycogen synthase kinase-3β. The activated β-catenin cascade is one of the pathways involved in tumorigenic activities mediated by the oncogenic RON variant.

The Wnt/β-catenin signaling pathway is involved in a variety of physiopathological processes, including body axis formation, central nervous system development, and tumor initiation and progression (1–3). The central part of Wnt signaling is the stabilization of cytoplasmic β-catenin followed by its nuclear translocation and association with T-cell transcription factors, which leads to the transcription of target genes (1–3). Because of its importance, the level of cytoplasmic β-catenin is tightly controlled by a regulatory multiprotein complex composed of axin, adenomatous polyposis coli, and glycogen synthase kinase (GSK)1–3β (4, 5). In unstimulated cells, β-catenin is associated with the regulatory multiprotein complex and phosphorylated by GSK-3β (4, 5). Phosphorylated β-catenin is then subjected to ubiquitination and degradation in proteasomes (6–8). Upon binding of the Wnt ligand to its Frizzled receptor, several intermediary components are activated through phosphorylation. One of these activated proteins is the cytoplasmic protein DVL (9). Phosphorylated DVL acts as a positive regulator that interacts with axin and prevents β-catenin phosphorylation by GSK-3β (10, 11). These events allow unphosphorylated β-catenin levels to accumulate and subsequently translocate into the nucleus to initiate the transcription of target genes, such as c-myc and cyclin D1 (12–14).

The oncogenic roles of the β-catenin pathway have been demonstrated in different types of human cancers, especially those from colon, liver, and ovarian cancer (15). Several mechanisms, including mutations in genes encoding β-catenin or adenomatous polyposis coli, are known to cause cytoplasmic β-catenin accumulation (16–19). Overexpression of receptor tyrosine kinases, such as oncogenic mutants of the MET proto-oncogene family, also contributes to abnormal accumulation of β-catenin, leading to tumorigenic phenotypes (20, 21).

The RON receptor tyrosine kinase is a 180-kDa heterodimeric protein composed of a 40-kDa extracellular α-chain and a 150-kDa transmembrane β-chain with intrinsic tyrosine kinase activity (22). The ligand for RON has been identified as MSP (23–25), also known as hepatocyte growth factor-like protein (26). RON expression is altered in certain human cancers, including those from breast and colon (27, 28). Altered RON expression is also accompanied by the generation of alternatively spliced RON variants with oncogenic potentials (27, 29). The generation of oncogenic variant RON160 is one such example (30). RON160 has an in-frame deletion of 109 amino acids in the RON β-chain extracellular domain (30). This deletion results in protein conformational changes leading to auto-

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The abbreviations used are: GSK, glycogen synthase kinase; DVL, disheveled; MSP, macrophage-stimulating protein; PI, phosphatidylinositol; RON, receptor originated from Nantes; si, small interfering; mAb, monoclonal antibody; BrdUrd, bromodeoxyuridine.
phosphorylation and increased kinase activities (27, 30). Expression of RON160 in NIH3T3 cells causes cellular transformation in vitro and tumor growth when inoculated into athymic nude mice (27, 30). Altered expression of RON or RON160 activates multiple signaling pathways that promote cell transformation and motile/invasive activities (31), indicating that RON-mediated signaling contributes to pathogenesis and progression of certain types of cancers. Consistent with these findings, a recent study has shown that silencing RON gene expression in colon cancer cells by small interfering (si)RNA techniques affects cytoplasmic β-catenin accumulation and reverses tumorigenic phenotypes of cancerous cells (32).

The present work was undertaken to study mechanisms by which oncogenic RON160 regulates cytoplasmic β-catenin accumulation. The role of β-catenin in RON160-mediated tumorigenic activities was also studied. Using NIH3T3 cells expressing RON160, we demonstrated that RON160 has the ability to cause cytoplasmic β-catenin accumulation, which leads to the increased expression of β-catenin target genes, such as c-myc and cyclin D1. These activities were manifested by the RON160-activated PI 3-kinase-AKT pathway with activation of DVL and inactivation of GSK-3β. By using siRNA techniques, we also demonstrated that the activated β-catenin pathway is required for the RON160-mediated tumorigenic activities.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**NIH3T3 cells and cells stably expressing wild-type RON (3T3-RON) or RON160 (3T3-RON160) were used as previously described (27). Human MSP was from Dr. E. J. Leonard (National Cancer Institute, Frederick, MD). Mouse mAb ID2 and rabbit IgG antibodies to human RON were used as described previously (27, 29). Mouse mAb to phospho-Tyr-100 to phosphotyrosine and cyclin D1 and rabbit polyclonal antibodies to c-myc, phospho-GSK-3βSer-9, and GSK-3β were from Cell Signaling Inc. (Beverly, MA). Mouse mAb to β-catenin was from Transduction Laboratories (San Diego, CA). Goat IgG antibodies to DVL and actin and mouse mAb to DVL-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PD98059, SB203580, and Wortmannin were from Calbiochem (San Diego, CA). ECL detection reagents were from Amersham Biosciences.

**Generation of RON and β-Catenin mRNA Silencing Vectors and Cell Lines—**The expression vector that produces RON-specific siRNA (psiR-RON) was used as previously described (32). To generate a vector that produces siRNA to mouse β-catenin, a 55-nucleotide that encodes two complementary sequences of 21 nt corresponding to the mouse β-catenin coding sequence (33, 34) separated by a hairpin structure, was inserted into the psiRNA-hH1neo vector (Invitrogen), yielding the vector psiR-βCTN. The mock vector (psiR-CTL) was used as the control.

**Immunoprecipitation and Western Blot Analysis—**Cells were homogenized in lysis buffer as described previously (27). Immunoprecipitation was performed by mixing cellular proteins (1 mg/sample) with antibodies specific to mouse β-catenin as detailed under “Experimental Procedures.” Results are from one of three experiments with similar outcome.

**Immunofluorescent Staining—**Cell fixation, permeabilization, and immunofluorescent staining was performed by using antibodies specific to mouse β-catenin as detailed under “Experimental Procedures.” Results are from one of three experiments with similar outcome.

**FIG. 1. Increased expression and phosphorylation of β-catenin in NIH3T3 cells expressing oncogenic variant RON160.** Generation of stable NIH3T3 cells expressing RON or RON160 has been described previously (27). Cellular proteins (50 μg/sample) from individual cell lines stimulated with (+) or without (−) MSP were immunoprecipitated by specific antibodies to RON or β-catenin coupled to protein G-Sepharose and separated in 10% SDS-PAGE under reduced conditions. Non-phosphorylated proteins were detected in Western blotting by antibodies to RON or β-catenin, respectively. Phosphorylated proteins were detected by mouse IgG antibodies to phosphotyrosine. Parental NIH3T3 cells were used as the control. The same membrane reprobed with goat IgG antibodies to actin served as a loading control.

**FIG. 2. Nuclear localization of β-catenin in NIH3T3 cells expressing RON160.** NIH3T3, 3T3-RON, or 3T3-RON160 cells were serum-starved overnight, stimulated with MSP at 2 nM for 10 min, and then fixed. Immunofluorescent staining was performed by using antibodies specific to mouse β-catenin as detailed under “Experimental Procedures.” Results are from one of three experiments with similar outcome.
**RESULTS**

**Expression and Activation of RON or RONΔ160 in NIH3T3 Cells**

Results in Increased β-Catenin Expression and Phosphorylation—Previous studies have shown that RON mutants created experimentally cause cytoplasmic β-catenin accumulation in NIH3T3 or Madin-Darby canine kidney cells (20). To determine whether the oncogenic RON variant naturally occurring in colon cancer samples (27) exerts a similar effect, NIH3T3 cells stably expressing RONΔ160 were used. Results from Western blot analysis presented in Fig. 1, A and B, show the levels of RON or RONΔ160 and their phosphorylation status in the presence or absence of MSP. As shown in Fig. 1C, the levels of β-catenin in 3T3-RONΔ160 cells were significantly increased in comparison with those in parental NIH3T3 cells. The increased β-catenin expression was also evident in 3T3-RON cells. Phosphorylation assays showed that MSP-dependent RON activation causes β-catenin tyrosine phosphorylation in 3T3-RON cells (Fig. 1D). However, in 3T3-RONΔ160 cells, β-catenin was constitutively phosphorylated, even without MSP stimulation, and the phosphorylation was further increased upon MSP stimulation (Fig. 1D).

Increased β-catenin accumulation in 3T3-RONΔ160 cells was also accompanied by its nuclear translocation as determined by immunofluorescent staining (Fig. 2). Nuclear β-catenin was hardly detectable in parental NIH3T3 cells but clearly visible in 3T3-RONΔ160 cells, indicating that accumulation of β-catenin leads to its nuclear translocation. These results suggest that RONΔ160 has the ability to cause β-catenin accumulation, phosphorylation, and nuclear translocation.

**Expression of c-myc and cyclin D1**

Is Increased in 3T3-RONΔ160 Cells with Increased β-Catenin Accumulation—β-catenin nuclear translocation is often accompanied by increased expression of its target genes, such as c-myc and cyclin D1 (12–14). To determine whether this is the case for RONΔ160-mediated action, Western blot analysis of c-myc or cyclin D1 expression was performed using cell lysates prepared from 3T3-RON or 3T3-RONΔ160 cells. The results in Fig. 3 show that levels of c-myc were dramatically increased in 3T3-RONΔ160 cells but not in 3T3-RON cells. Similarly, expression of cyclin D1 was also significantly increased in 3T3-RONΔ160 cells. A marginal increase in cyclin D1 expression was seen in 3T3-RON cells. These results indicate that RONΔ160-mediated β-catenin accumulation is accompanied by increased expression of c-myc and cyclin D1.

**Increased DVL Expression and Phosphorylation Occur in 3T3-RONΔ160 Cells**

DVL activation is critical to maintaining the stability of cytoplasmic β-catenin (9). To determine whether RONΔ160-mediated β-catenin accumulation is related to DVL activation, the levels of DVL were first evaluated by Western blot analysis. As shown in Fig. 4A, expression of wild-type RON had no effect on DVL expression in NIH3T3 cells; however,
DVL expression was significantly increased in 3T3 cells expressing RONΔ160. Increased DVL was also accompanied by spontaneous phosphorylation, as evident in the shift in mobility of phosphorylated DVL proteins (Fig. 4A). MSP stimulation further increased DVL phosphorylation in a time-dependent manner, with maximal phosphorylation peaking at about 30 min post-stimulation (Fig. 4B). Using alkaline phosphatase, it was demonstrated that spontaneous or MSP-induced DVL phosphorylation is sensitive to the treatment of phosphatase, which completely eliminates the shift of the phosphorylated DVL bands (Fig. 4C). To determine whether DVL interacts with RONΔ160, the protein interaction assay was performed. By immunoprecipitation of DVL with a specific antibody, we found that DVL physically forms a protein complex with RONΔ160 and β-catenin, as evident by Western blotting using antibodies specific to RON or β-catenin, respectively (Fig. 4D).

The association was also seen when RONΔ160 was first immunoprecipitated followed by probing with antibodies to DVL (data not shown). The protein complex formation was not observed in NIH3T3 cells expressing wild-type RON, even in the presence of MSP (data not shown). Thus, oncogenic RONΔ160 is capable of activating DVL by regulating its expression, phosphorylation, and association.

**Inactivation of GSK-3β through Ser-9 Phosphorylation Is Mediated by the RONΔ160-activated PI 3-Kinase-AKT Pathway**—Because degradation of β-catenin is triggered by GSK-3β, we sought to determine whether RONΔ160 expression elicits inhibitory effects on GSK-3β activities. As shown in Fig. 5A, RONΔ160 expression or activation by MSP had no effect on GSK-3β expression. The levels of GSK-3β were comparable among three cell lines. We then studied whether RONΔ160 expression regulates GSK-3β phosphorylation. GSK-3β phosphorylation at the Ser-9 residue was determined using antibodies specific to phospho-Ser-9 in GSK-3β. As shown in Fig. 5B, the GSK-3β Ser-9 residue was constitutively phosphorylated in 3T3-RONΔ160 cells. MSP stimulation further increased its phosphorylation, peaking at ~60 min after stimulation. Phosphorylated GSK-3β was not detected in parental NIH3T3 or 3T3-RON cells in the presence or absence of MSP (data not shown).

To determine potential signaling components involved in RONΔ160-mediated GSK-3β phosphorylation, 3T3-RONΔ160 cells were treated with optimal concentrations of individual chemical inhibitors specific to PI 3-kinase (wortmannin), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (PD98059), or p38 mitogen-activated protein kinase (SB203580), respectively. Phosphorylation of corresponding proteins was determined. As shown in Fig. 5C, AKT, the downstream component of the PI 3-kinase pathway, was constitutively phosphorylated in RONΔ160 cells. The levels of phosphorylation were further increased upon MSP stimulation. In the presence of the PI 3-kinase inhibitor wortmannin, spontaneous or MSP-dependent AKT phosphorylation was completely diminished. The inhibitory effect was also effective in GSK-3β phosphorylation. In the presence of wortmannin, the Ser-9 phosphorylation was significantly reduced. In contrast, no inhibitory effect of chemical inhibitors specific to mitogen-activated protein kinase/extracellular signal-regulated kinase kinase or p38 was observed (data not shown). These results suggest that the signaling protein AKT is probably involved in RONΔ160-mediated inactivation of GSK-3β.

**Silencing RONΔ160 Expression by siRNA Affects β-Catenin and Its Target Gene Expression or Protein Phosphorylation**—To determine whether increased β-catenin accumulation is indeed caused by RONΔ160, NIH3T3, 3T3-RON, and 3T3-RONΔ160 cells were transiently transfected with vectors expressing RON-specific siRNA followed by Western blot analysis of β-catenin expression. As shown in Fig. 6A, RON-specific siRNA completely knocked down RON and RONΔ160 expressions. This effect also diminished β-catenin expression. No silencing effect was seen when the control vector psiR-CTL was used. Interestingly, silencing RONΔ160 expression also affects the expression of the β-catenin target genes. As shown in Fig. 6B, levels of c-myc and cyclin D1 were diminished upon the introduction of the psiR-RON vector. In contrast, GSK-3β expression was not affected. Similarly, the control vector psiR-CTL had no effect on expression of c-myc or cyclin D1. It seems that knockdown of RON or RONΔ160 causes a chain reaction leading to diminished c-myc and cyclin D1 expression.

To study whether knockdown of RONΔ160 expression affects phosphorylation of β-catenin pathway components, we first studied total cellular phosphorylation patterns after 3T3-
RONΔ160 cells were transected with psiR-RON. Results in Fig. 6C show that expression of RON-specific siRNA dramatically alters cellular phosphorylation patterns in 3T3-RONΔ160 cells. Numerous phosphorylated bands disappeared upon silencing RONΔ160 expression, suggesting that knockdown of RONΔ160 has a significant impact on cellular phosphorylation status. To see changes at the single protein level, GSK-3β Ser-9 phosphorylation was measured. Results in Fig. 6D show that constitutively phosphorylated GSK-3β at the Ser-9 residue was dramatically reduced after RON-specific siRNA was introduced. This effect was not seen when cells were transfected with the psiR-CTL vector. Again, GSK-3β expression was not affected by psiR-RON. Taken together, these results suggest that silencing RONΔ160 expression not only causes diminished expression of β-catenin and its target genes but also alters the phosphorylation pattern of cellular proteins, including GSK-3β.

**Knockdown of β-Catenin Gene Expression Impairs RONΔ160-mediated Cell-transforming Activities**—To address the role of β-catenin in RONΔ160-mediated oncogenic activities, we generated a vector expressing specific siRNA for mouse β-catenin (psiR-βCTN) according to the previously published results (33, 34). First, the effect of psiR-βCTN on cell proliferation was determined (Fig. 7A). Similar to the effect of the psiR-RON vector, expression of β-catenin-specific siRNA blocked the nuclear BrdUrd incorporation (25%), which is significantly lower than that in untransfected RONΔ160 cells (63%). No changes were seen in cells transfected with the control vector psiR-CTL. Second, the effect of psiR-βCTN on RONΔ160-mediated focus formation was evaluated (Fig. 8A). Transient expression of RONΔ160 in NIH3T3 cells results in the formation of numerous foci. As expected, RON-specific siRNA inhibited the focus formation. The control vector psiR-CTL had no effect. The inhibitory effect was also achieved when cells were transfected with psiR-βCTN. The percentages of psiR-βCTN-induced inhibition (61%) were comparable with those induced by psiR-RON (55%). Third, the effect of β-catenin siRNA on RONΔ160-mediated anchorage-independent growth was studied (Fig. 8B). NIH3T3 cells formed numerous colonies in soft agar after transfection with the RONΔ160 vector (70 colonies ±12/dish). The number of colonies was significantly reduced when cells were co-transfected with psiR-RON (27 colonies ±7) but not with control vectors (71 colonies ±9). When cells were co-transfected with psiR-βCTN, the number of the colonies was reduced (19 colonies ±7) to the level similar to the one caused by psiR-RON. These results suggest that knockdown of β-catenin expression reduces RONΔ160-mediated transforming activities.

**Introduction of β-Catenin-specific siRNA Reverses RONΔ160-mediated Morphological Changes**—To assess the role of β-catenin on RONΔ160-mediated cell morphological changes, RONΔ160 cells were transiently transfected with psiR-βCTN or control vectors, and cell morphological changes were monitored. Results presented in Fig. 9 show that expression of RONΔ160 alters NIH3T3 cell morphology, resulting in a more rounded-up phenotype, scattered appearance, and diminished cell-cell contacts. This transformed phenotype was largely reversed to fibroblast-like morphology when cells were transfected with psiR-βCTN. The reversed phenotypes were not seen in cells transfected with control vector psiR-CTL. As expected, expression of RON-specific siRNA reversed the transformed phenotype of RONΔ160 cells. Thus, β-catenin is im-
volved in RON160-mediated transforming phenotypes in NIH3T3 cells.

**DISCUSSION**

The purposes of this study were to investigate the cellular mechanisms by which the oncogenic RON160 variant regulates cytoplasmic β-catenin accumulation and the requirement of β-catenin in RON variant-mediated tumorigenic activities. Previous reports have shown that oncogenic mutants of the MET family, including METm1268t and RONm1254t, have the ability to activate the MET family, including METm1268t and RONm1254t, have the ability to activate the β-catenin pathway when expressed in NIH3T3 or Madin-Darby canine kidney cells (20). Activation of MET by hepatocyte growth factor/scatter factor in rat primary hepatocytes has also been shown to induce Wnt-independent β-catenin (36). Consistent with these reports, our recent studies have found that silencing the RON gene expression in colon cancer cell lines leads to diminished β-catenin expression (32). These results provide important clues, suggesting that the β-catenin pathway might be involved in RON160-mediated oncogenesis during progression of colorectal cancers. The data from this study extended our previous findings and demonstrated that expression of RON160, a naturally occurring oncogenic variant from colon cancer cells, in NIH3T3 cells causes accumulation of β-catenin in the cytoplasm. Increased β-catenin expression also leads to increases in its target gene expression, such as in c-myc and cyclin D1. These activities were initiated by RON160-mediated phosphorylation that activates DVL and inactivates GSK-3β, both of which are critical regulators for stability of cytoplasmic β-catenin (4, 5). To summarize these findings, a simple model as shown in Fig. 10 was used to depict the effect of RON160 in stabilizing β-catenin in cytoplasm. In addition, our data provide evidence showing that knockdown of β-catenin expression by siRNA techniques significantly impairs RON160-mediated NIH3T3 cell proliferation, focus-forming activities, and anchorage-dependent growth. Thus, RON160 is a potential regulator of β-catenin. The activated β-catenin pathway is one of the mechanisms involved in oncogenic RON variant-mediated pathogenesis of certain colon cancers.

The finding that RON160 activates DVL is interesting. DVL is a cytoplasmic protein that has no known enzymatic functions but does have several potential interaction motifs (9). Activation of DVL is characterized by phosphorylation upon the Wnt ligand binding to its Frizzled receptor, which positively regulates β-catenin accumulation (9). Although the underlying mechanisms are still largely unknown, it has been established that phosphorylated DVL associates with axin and...
\( \beta \)-Catenin in Oncogenic RON-mediated Activities

![Diagram](http://www.jbc.org/content/jbc/250/9/25093/fig9)

**FIG. 9.** Morphological changes in 3T3-RON160 cells transiently expressing RON- or \( \beta \)-catenin-specific siRNA. 3T3-RON160 cells \((1 \times 10^5 \text{ cells/dish})\) were transiently transfected with psiR-RON or psiR-\( \beta \)-CTN as described in the legend to Fig. 8. Parental NIH3T3 cells were used as the control. Morphological changes were observed 3 days after transfection under a microscope and photographed (magnification \( \times 20 \)). One of three experiments with similar results.

![Diagram](http://www.jbc.org/content/jbc/250/9/25093/fig10)

**FIG. 10.** A simple working model for RON160-mediated cytoplasmic \( \beta \)-catenin accumulation in NIH3T3 cells. Levels of cytoplasmic \( \beta \)-catenin are controlled by the regulatory multiprotein complex containing adenosomatous polyposis coli, axin, and GSK-3\( \beta \). In unstimulated cells, \( \beta \)-catenin is phosphorylated by GSK-3\( \beta \) and subjected to ubiquitination and degradation in proteasomes (off-state). Up-binding of the Wnt ligand to its Frizzled receptor, DVL, is phosphorylated and activated, which prevents \( \beta \)-catenin phosphorylation by GSK-3\( \beta \) and allows \( \beta \)-catenin levels to accumulate and subsequently translocate into the nucleus to initiate target gene transcription (on-state). Overexpression of RON160 results in increased DVL expression and phosphorylation. Activated RON160 also stimulates the PI 3-kinase-AKT pathway, which blocks GSK-3\( \beta \) activities. These events ultimately result in cytoplasmic \( \beta \)-catenin accumulation leading to its nuclear translocation and target gene transcription.

The enzymatic activities of GSK-3\( \beta \) are constitutively active in unstimulated cells (37, 38). Certain growth factors and signaling proteins are capable of inhibiting GSK-3\( \beta \) activities, which is characterized by phosphorylation of GSK-3\( \beta \) on residue Ser-9 (38–40). We can now add RON160 to the list of GSK-3\( \beta \) inhibitors. The effect of RON160 on GSK-3\( \beta \) differs from DVL. As shown in Fig. 5, RON160 does not regulate expression of the GSK-3\( \beta \) protein; instead, it causes phosphorylation of GSK-3\( \beta \) on Ser-9. The addition of MSP further increased Ser-9 phosphorylation in a time-dependent manner. Our results also show that AKT, the critical component in PI 3-kinase pathways, is involved in RON160-mediated GSK-3\( \beta \) inhibition. As demonstrated in Fig. 5C, expression of RON160 results in constitutive activation of AKT, as evident in the phosphorylation assay. Treatment of 3T3-RON160 cells with the PI 3-kinase inhibitor wortmannin blocks not only constitutive but also MSP-enhanced AKT phosphorylation. Wortmannin also exerted the inhibitory effect on GSK-3\( \beta \) activities. Both constitutive and induced Ser-9 phosphorylation was significantly impaired upon wortmannin treatment. Furthermore, we demonstrated that treatment of 3T3-RON160 cells with wortmannin results in progressive reduction of cytoplasmic \( \beta \)-catenin. These inhibitory activities were not observed in cells treated with other chemical inhibitors, such as PD98059 or SB203580, which target mitogen-activated protein kinase or p38 signaling pathways, respectively.

The findings that RON160-mediated AKT activation results in \( \beta \)-catenin accumulation are different from those reported by others (38, 39). Previous reports have shown that GSK-3\( \beta \) inhibition by growth factor-mediated PI 3-kinase pathways does not lead to increased \( \beta \)-catenin accumulation (38, 39). However, our data suggest that RON160-mediated AKT activation is critical in inhibiting GSK-3\( \beta \) activities leading to increased \( \beta \)-catenin accumulation. Consistent with this, a recent study (38) has shown that overexpression of DVL results in AKT activation. Activated AKT bound to the axin-GSK-3\( \beta \) complex in the presence of DVL caused GSK-3\( \beta \) phosphorylation at the Ser-9 residue and increased cytoplasmic \( \beta \)-catenin accumulation. Considering the effect of RON160 on DVL expression and activation, we believe that constitutive activation of AKT in RON160 cells could be mediated by DVL characterized by increased protein expression and phosphorylation in 3T3-RON160 cells, which ultimately leads to \( \beta \)-catenin accumulation. Definitely, the direct effect of RON160 on activation of PI 3-kinase and AKT may also be involved. Thus, more studies are needed in the future to determine the relationship between RON160-mediated DVL activation and AKT phosphorylation.

Prevents GSK-3\( \beta \) from phosphorylation of \( \beta \)-catenin (9). The data in Fig. 4 implies that oncogenic RON160 has the ability to regulate DVL. First, RON160 forms a protein complex with DVL, which also includes \( \beta \)-catenin. This effect was not observed in cells expressing wild-type RON protein, even when stimulated by MSP. The recruitment of DVL to RON160 provides a close proximity in which to relay the signals to the \( \beta \)-catenin-regulatory complex composed of adenosomatous polyposis coli, axin, and GSK-3\( \beta \). Second, the levels of the DVL protein are significantly increased in cells expressing RON160 (but not wild-type RON) suggesting that RON160 is capable of regulating DVL at the protein level. Third, MSP-dependent or -independent activation of RON160 causes DVL phosphorylation, with a shift in mobility of the DVL bands relative to that observed in control cells. This mobility shift was similar to that described by others (10) as a result of phosphorylation of DVL in response to Wnt signals that stabilize \( \beta \)-catenin. Finally, the increased DVL expression and phosphorylation are associated with inactivation of GSK-3\( \beta \), leading to increased \( \beta \)-catenin accumulation, which suggests that activated DVL could exert the inhibitory effect on GSK-3\( \beta \) and stabilize \( \beta \)-catenin in the cytoplasm. Thus, RON160 is an activator of DVL. Currently, we do not now how RON160 regulates DVL expression and phosphorylation. It will be of great interest in the future to determine the underlying mechanisms by which RON160 regulates DVL protein expression and activation.

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Oncogenic RON variant-mediated tumorigenic activities are manifested through activated signaling pathways, such as RAS, PI 3-kinase, mitogen-activated protein kinase, signal transducers and activators of transcription, and others (31). The importance of these signaling proteins in the oncogenic activities of RON is still being investigated. The results presented in this study show that the β-catenin pathway contributes to RONΔ160-mediated tumorigenic activities. This conclusion was obtained using siRNA techniques in which β-catenin-specific siRNA was generated to block endogenous β-catenin expression. We showed that RONΔ160-mediated replication of NIH3T3 cells is diminished when the β-catenin gene expression is silenced by siRNA. Moreover, RONΔ160-mediated focus-forming activities and anchorage-independent growth of NIH3T3 cells were reduced upon knockdown of β-catenin. These inhibitory effects are comparable with those induced by silencing RONΔ160 expression. As demonstrated in Fig. 9, the inhibition of β-catenin significantly reverses the transformed cellular phenotypes caused by expression of RONΔ160. Thus, the activated β-catenin pathway participates in RONΔ160-mediated tumorigenic activities. It is very likely that, in RONΔ160-expressing cells, the β-catenin pathways collaborate with other signaling cascades that drive cells toward transformation and malignancy. Thus, studying the cellular mechanisms by which RONΔ160 activates the β-catenin pathway could provide insight into the roles of altered receptor tyrosine kinases in the pathogenesis of colorectal cancers.

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Mechanisms of Cytoplasmic β-Catenin Accumulation and Its Involvement in Tumorigenic Activities Mediated by Oncogenic Splicing Variant of the Receptor Originated from Nantes Tyrosine Kinase

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