BASIC–ALIMENTARY TRACT

Oncogenic K-*ras* Stimulates Wnt Signaling in Colon Cancer Through Inhibition of GSK-3β

JINGNAN LI, YUSUKE MIZUKAMI, XIAOBO ZHANG, WON–SEOK JO, and DANIEL C. CHUNG
Gastrointestinal Unit, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

Background & Aims: **Two key genetic events underlying the development of colon cancer are activation of the K-*ras* and Wnt signaling pathways. We have previously shown that these 2 pathways can cooperate to regulate vascular endothelial growth factor (VEGF) gene expression. The goal of this study was to define the molecular basis for this interaction.** ***Methods:*** **The effects of K-*ras*^{Val12} on VEGF and T-cell factor 4 (TCF-4) promoter activity, nuclear levels of β-catenin and β-catenin/TCF-4 complexes, glycogen synthase kinase 3β (GSK-3β) phosphorylation, and GSK-3β kinase activity were measured. LY294002 and PD98059 were used to define the role of specific *ras* effector pathways.** ***Results:*** **Oncogenic K-*ras* up-regulated the activity of the VEGF promoter, and selective mutagenesis of TCF-4 binding sites significantly blocked this induction. K-*ras*^{Val12} also induced the activity of a heterologous TCF-4 reporter construct in Caco-2 and HeLa cells. LY294002 and dominant negative phosphatidylinositol 3-kinase nearly completely blocked this induction. K-*ras*^{Val12} increased the stability of β-catenin, the levels of nuclear β-catenin, and the formation of nuclear β-catenin/TCF-4 complexes, and these effects were also blocked by LY294002. Finally, K-*ras*^{Val12} inhibited the kinase activity of total cellular GSK-3β and GSK-3β complexed with Axin. This effect was not mediated through phosphorylation at serine 9 but did depend on phosphatidylinositol 3-kinase.** ***Conclusions:*** **Our results suggest a unique cooperative interaction between 2 critical oncogenic pathways in colorectal tumorigenesis and highlight the pivotal role of GSK-3β.**

Among all solid tumors, the molecular pathogenesis of colon cancer is one of the best understood.[1–4] Two key genetic events that underlie the development of precancerous colonic adenomas are activation of the K-*ras* and Wnt signaling pathways. Mutations of the K-*ras* oncogene are observed in up to 50% of colon cancers, and these mutations are detected at the stage of the adenomatous precursor.[5–7] K-*ras* can activate a variety of effector pathways, including RAF/MAPK, JNK, and phosphatidylinositol 3-kinase (PI3-K).[8–11] Some of the downstream gene targets of K-*ras* include vascular endothelial growth factor (VEGF), cyclin D1, and Cox-2, all of which contribute to the pathobiology of colon cancer.[12–16]

Mutations of *APC*, a key regulator of Wnt signaling, are identified in at least 70% of colon cancers and are characteristically observed in early colonic neoplasia.[17–19] One of the hallmarks of activated Wnt signaling is the accumulation of nuclear β-catenin. The cytoplasmic level of β-catenin is regulated by ubiquitin-mediated proteolysis after it is targeted for phosphorylation by glycogen synthase kinase 3β (GSK-3β). The phosphorylation of β-catenin by GSK-3β occurs in the context of a structural complex that includes Axin and APC.[20,21] When a Wnt signal is present, GSK-3β activity is inhibited.[22] The mechanisms underlying this regulation of GSK-3β by Wnt are poorly understood, but it has been recognized that the Dsh and Frat proteins play an important role.[23–25] Excess β-catenin is translocated to the nucleus, where it interacts with the T-cell factor 4 (TCF-4) transcription factor to induce the expression of specific target genes, including cyclin D1, VEGF, and c-*myc*, that promote cell growth and proliferation.[16,26–28]

Our previous studies have shown that the K-*ras* and Wnt pathways can cooperate to regulate the VEGF gene.[16] Investigators have shown that K-*ras* and Wnt signaling can also coordinately regulate other important target genes, including Cox-2 and gastrin.[29,30] The molecular mechanisms that link these 2 pathways are currently undefined. Studies in epidermal keratinocytes have shown that mutant H-*ras* can redistribute membrane-bound β-catenin to the cytoplasm in a PI3-K–dependent

Abbreviations used in this paper: GFP, green fluorescent protein; GSK-3β, glycogen synthase kinase 3β; PI3-K, phosphatidylinositol 3-kinase; TCF-4, T-cell factor 4; VEGF, vascular endothelial growth factor.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.02.067

manner.[31] These studies suggested that the p85α subunit may directly interact with β-catenin.

Another link between PI3-K and GSK-3β has been shown through the study of insulin signaling. The insulin receptor can activate PI3-K and then PKB/Akt to inhibit GSK-3β in the regulation of glycogen metabolism.[32–34] Inhibition of GSK-3β activity is mediated through phosphorylation at serine 9. Although inhibition of GSK-3β is also central to the activation of Wnt signaling, phosphorylation at serine 9 does not appear to be a necessary event.[35,36] Nevertheless, some reports suggest that a link may exist between phosphorylation of GSK-3β at serine 9 and the downstream activation of Wnt target genes, indicating that this distinction may not be absolute.[37,38]

We have previously shown that VEGF is a novel target of the Wnt pathway in colon cancer and that K-*ras*^{Val12} functions synergistically to enhance Wnt signaling in the regulation of VEGF.[16] The goal of these studies was to gain further insight into the interaction of these pathways in colon cancer. Our results indicate that K-*ras*^{Val12} can up-regulate VEGF through TCF-4 binding elements in the VEGF promoter. Expression of K-*ras*^{Val12} increases the stability of β-catenin. This results in an increase in nuclear β-catenin levels as well as β-catenin/TCF-4 complexes that are bound to DNA. Furthermore, K-*ras*^{Val12} can inhibit the activity of GSK-3β, and this is not mediated through serine-9 phosphorylation. Inhibition of PI3-K blocks these effects of K-*ras*^{Val12}. Collectively, these findings show that K-*ras*^{Val12} can enhance Wnt signaling and that GSK-3β may serve as the link between these 2 pathways.

Materials and Methods

Cell Culture

HeLa, 293, and Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). Caco-2 cells were cultured in Eagle's minimum essential medium (American Type Culture Collection) supplemented with 20% fetal bovine serum (Cellgro, Herndon, VA) and 2% penicillin/streptomycin (BioWhittaker, East Rutherford, NJ). HeLa and 293 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. The cells were cultured at 37°C in 5% CO_2. The HeLa and Caco-2 cancer cell lines both carry a wild-type K-*ras* gene.[39,40] Caco-2 cells carry mutations in both the APC and β-catenin genes,[41] and the β-catenin gene is wild type in HeLa cells.[42] The 293 cell line is nontransformed and derived from human embryonic kidney.

Plasmids and Transfections

The human 1.9-kilobase VEGF-luciferase construct was previously described.[16] It contains VEGF promoter sequences from −850 base pairs to +1036 base pairs. Site-directed mutagenesis was performed to selectively alter 2 TCF-4 binding sites at −805 base pairs ("c" site: 5′-CTTTGAT) and −629 base pairs ("d" site: 5′-TTCCAAAG), designated 1.9-kilobase mTCF-4-c (5′-CTTTACT) and 1.9-kilobase mTCF-4-d (5′-TCACAAAG), respectively. The pGL3-OT and pGL3-OF luciferase reporter constructs contain 3 copies of wild-type or mutated consensus TCF-4 response elements, respectively. The K-*ras*^{Val12} expression vector (phr-K-*ras*) and empty vector (phr–green fluorescent protein [phr-GFP]) have been described.[43] The C-terminal HA-tagged GSK-3β wild-type and alanine-9 mutant vectors,[44] Flag-tagged Axin,[45] and dominant negative PI3-K vector (SRα-ΔP85) have been described.[46]

Transient transfections were performed using the cationic lipid Lipofectamine 2000 (Life Technologies, Inc, Gaithersburg, MD) according to the manufacturer's instructions. The cells were cultured in 24-well plates for reporter assay experiments. Transfections were performed when Caco-2 cells reached 50%–60% confluence and when HeLa and 293 cells reached 80% confluence.

A total of 0.4 μg of pGL3-OT/pGL3-OF or 0.6 μg of VEGF-luciferase reporter constructs was cotransfected with 0.2–0.3 μg of K-*ras* or empty vector. A total of 50 ng of the pRL-null vector was also transfected to normalize for transfection efficiency.[47] In selected studies, 0.2 μg of the SRα-ΔP85 vector or HA-tagged GSK-3β wild-type/alanine-9 mutant was also cotransfected. The total amount of plasmid DNA transfected was 0.8 μg. When cells reached 90% confluence after transfection (24 hours for HeLa cells and 48 hours for Caco-2 cells), the cells were harvested and firefly and Renilla luciferase activity were measured using a dual luciferase assay (Promega, Madison, WI). The firefly luciferase activity was normalized to Renilla luciferase activity. All experiments were performed in duplicate wells at least 3 times.

In selected studies, the cells were cultured in medium containing 20 μmol/L PD98059 or 50 μmol/L LY294002 (both from Cell Signaling Technology, Beverly, MA) for 12 hours.[48,49] To activate the Wnt pathway in HeLa cells, the GSK-3β inhibitor SB216763 (10 μmol/L) was added to the culture medium 12 hours before harvesting the cells.[50] PD98059 or LY294002 was added 4 hours after SB216763 in selected experiments. Control cells were treated with dimethyl sulfoxide.

Northern Blot Analysis

Total RNA was isolated using the TRIzol reagent (Life Technologies, Inc). Fifteen micrograms of total RNA was separated electrophoretically in 1% agarose-formaldehyde gels and then transferred to nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Northern blot hybridization was performed with a 2.4-kilobase human TCF-4 complemen-