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PI3K is required for the physical interaction and functional inhibition of NF-κB by β-catenin in colorectal cancer cells

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ABSTRACT

Activation of β-catenin and PI3K pathways are crucial for the oncogenesis of colorectal cancer (CRC). It remains controversial whether these two pathways function independently or cooperatively in the development and progression of CRC. We showed previously that β-catenin inhibited NF-κB activation by interacting with p65 and this inhibitory interaction involved an unidentified cellular protein. In this study, we found that the PI3K effect on NF-κB activity is dependent on the level of β-catenin in CRC cells. PI3K promoted NF-κB activity in the β-catenin-low RKO cells; whereas it inhibited NF-κB activity in the β-catenin-high HCT116, DLD-1, and SW480 cells. We showed that PI3K is required for the physical interaction and functional inhibition of NF-κB by β-catenin. Inhibition of PI3K released NF-κB suppression in β-catenin-high CRC cells, which conferred these cells with susceptibility to TNFα- and Fas-induced apoptosis. This is consistent with the observation showing that the level of β-catenin and activated Akt are both inversely correlated with the expression of Fas, a downstream target of NF-κB, in CRC specimens. Mechanistically, the PI3K subunit p85 formed a complex with β-catenin and NF-κB. Inhibition of PI3K disrupted the complex formation, leading to NF-κB activation. Our study not only provides new insight into the cross-talk among PI3K, β-catenin and NF-κB signaling pathways but also indicates that targeting PI3K may yield therapeutic efficacy in treating β-catenin-high CRC.

1. Introduction

Activation of Wnt/β-catenin signaling is an essential initiating event in the oncogenesis of CRC. The level of cytosolic β-catenin is regulated by ubiquitin proteosome degradation, which is dependent on phosphorylation of β-catenin by a multiprotein complex composed of tumor suppressor protein adenomatous polyposis coli (APC), Axin, and glycogen synthesis kinase-3β (GSK-3β) [1]. Defect in the complex, either inactive mutations on APC, Axin, or active mutations on the N-terminus of β-catenin leads to high level of cytosolic β-catenin [2,3]. Increased β-catenin activation results in the formation of benign tumors that eventually progress to carcinoma [1–3].

Phosphotidyl Inositol 3-Kinase (PI3K), a heterodimer composed of one regulatory (p85) and one catalytic (p110) subunits, is activated by receptor tyrosine kinases (RTK) and plays a central role in growth factor mediated oncogenesis [4]. The PI3K pathway is constitutively activated in many tumors, such as colon, breast, prostate, hematological, and lung cancers [5–7]. It remains unclear whether there is a cooperative oncogenic effect between PI3K and Wnt/β-catenin pathways in the development and progression of CRC.

NF-κB, an important signaling involved in inflammation, proliferation and apoptosis, plays a dual role in its biological functions [8]. On one hand, activated NF-κB increases resistance to apoptosis in many circumstances; on the other hand, activated NF-κB has been shown to promote apoptosis by inducing Fas expression [9] and is required for p53-mediated apoptosis [10].

We showed previously that β-catenin exhibits its oncogenic functions by interacting with p65 and inhibiting NF-κB activation in CRC cells. However, the interaction of β-catenin with p65 was indirect and required unidentified cellular proteins [11,12]. In this study, we discovered that the regulatory subunit p85 of PI3K is required for mediating the physical interaction between β-catenin and p65. Our study provides new insight into the cross-talk among PI3K, β-catenin and NF-κB pathways in CRC.

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2. Materials and methods

2.1. Cell culture and transfection assays

All cell lines were grown in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. HEK 293 cells, human CRC cell lines SW480, DLD-1, HCT116, and RKO [13] were used. 293 β-cateninS45Y stable transfectants (293-β-cat A and B) were described previously [14]. Transfections were performed with NF-κB-driven-luciferase plasmid (κB-luc) as reporter, and p65, β-catenin, or active mutant β-cateninS37A (β-catS37A), p85, p110, or mutant p85 as indicated. Plasmid pRL-tk (Promega) was used as internal control in all transfection assays. Apoptosis induction was performed by either anti-Fas antibody (CH11, Upstate; 0.5 μg/ml), or TNFα (10 ng/ml) for 18 h, with or without pretreatment with PI3K inhibitor LY294002 or Wortmannin (10 μM) for 3 h.

2.2. Immunoprecipitation and Western blotting

Immunoprecipitation was performed with 2 μg of antibodies against p65 or p50 (normal IgG as a negative control) in 1.0 mg lysate. Samples were first precleared with a nonspecific IgG antibody. Precleared lysates were then incubated with antibody against p65 or p50 for 1.5 h, then incubated with protein G agarose for overnight [13]. Antibodies to p65 (A), p50 (C-19), IκB-α (C-21), TRAF1 (G-20, H132), GFP (FL), and Fas (c-20) were purchased from Santa Cruz Biotechnology. β-catenin antibody was from BD Transduction Laboratories. Antibodies against p85 and p110 were from Upstate and Cell Signaling, respectively.

2.3. Electrophoresis mobility shift assay (EMSA)

EMSA was performed with an oligonucleotide probe containing the NF-κB binding site as described previously [11]. Eighty-fold of cold wild-type or mutant NF-κB oligonucleotides were used for competition in the nuclear extracts isolated from control or TNFα-treated (15 min) 293 cells; doses are as indicated. Antibodies used for supershifting the complex were as indicated. Antibodies to p65 (A) for supershift were from Santa Cruz Biotechnology.

2.4. Immunofluorescent (IF) staining

Following the transfection or treatment, cells cultured on glass coverslips were fixed with formaldehyde, permeabilized, and incubated with first antibody, followed by FITC-conjugated secondary antibody (Molecular Probes). Cells were analyzed using a fluorescence microscope, and digital images of FITC staining were captured.

2.5. Apoptosis assay

Apoptosis was measured by PI (propodium Iodide) staining as described [13]. The assays were performed and the mean ± SD were calculated from three independent experiments with triplicates.

2.6. Immunohistochemical staining

Tissue samples were deparaffinized and then subjected to a gradient of alcohol and washed five times. They were trypsinized in 0.05% trypsin in PBS and treated with 0.3% H_2O_2 in methanol. Then they were treated with 10% horse serum for 30 min. Sections were incubated with primary antibodies as indicated below for overnight; β-catenin antibody (Transduction Laboratories, 1:50 dilution), Fas antibody (Transduction Laboratories, 1:100), phospho-Akt (Ser473) antibody (Cell Signaling Technology; dilution 1:100). Sections were incubated with secondary antibodies Bio-Anti mouse or rabbit IgG (1:200 dilution) for 1 h and then incubated with avidin biotin-peroxidase complex diluted in PBS and visualized with amino-ethyl carbazole chromogen stock solution. The Fisher exact test was used for statistical analysis with $p < 0.05$ as statistical significance.

3. Results

3.1. The PI3K effect on NF-κB activity is dependent on the level of β-catenin in CRC cells

We showed previously that NF-κB activity inversely correlated with the level of cytosolic β-catenin in CRC cells, in which NF-κB activity is high in β-catenin-low RKO cells; whereas NF-κB activity is low in β-catenin-high HCT116, DLD-1, and SW480 [11,12]. To examine whether PI3K is also involved in the regulation of NF-κB activity, we ectopically expressed p85 or p110 with NF-κB-promoter luciferase construct in CRC cells. In RKO cells, expression of p85 or p110 increased NF-κB activity in a dose-dependent manner. However, expression of p85 or p110 resulted in repression of NF-κB activity in HCT116, DLD-1 and SW480 cells (Fig. 1). These results suggest that the effect of PI3K on NF-κB activity is dependent on the level of cytosolic β-catenin in CRC cells; PI3K activates NF-κB in β-catenin-low (RKO) cells, whereas it inhibits NF-κB in β-catenin-high (HCT116, DLD-1 and SW480) cells.

3.2. PI3K signaling is required for β-catenin-mediated NF-κB inhibition

To further examine the role of PI3K in β-catenin-mediated NF-κB suppression, we treated β-catenin-high HCT116 with TNFα and found that the expression of TRAF1, a major NF-κB downstream target gene, was inhibited (Fig. 2A). This data is consistent with the result of NF-κB luciferase activity measured above and indicates that the NF-κB pathway is suppressed in HCT116 cells. However, treatment with a PI3K inhibitor Wortmanin restored TNFα-induced TRAF1 expression in these cells. In addition, inhibition of PI3K by Wortmanin also increased TNFα-induced NF-κB DNA-binding activity in a gel-shift assay (Fig. 2B) and NF-κB luciferase activity in HCT116 cells (Fig. 2C). Similar results were obtained by using another PI3K inhibitor LY294002 (data not shown). Furthermore, we ectopically expressed GFP-p65 in HCT116 cells. We found that GFP-p65 was mainly localized in the cytoplasm, whereas a control GFP was evenly distributed in both the cytoplasm and the nucleus (Fig. 2D). However, treatment with LY294002 resulted in the nuclear translocation of GFP-p65. Taken together, these results suggest that PI3K is involved in regulation of NF-κB activity by β-catenin; inhibition of PI3K released the suppression of NF-κB activity by β-catenin in HCT116 cells.

To further confirm our contention, we generated a β-catenin stable transfectant in β-catenin-low RKO cells (RKO-β-cat). Treatment with TNFα in RKO cells induced a drastic TRAF1 expression, indicating that NF-κB is active in RKO cells (Fig. 2E). However, TNFα treatment did not induce TRAF1 expression in RKO-β-catenin cells. Strikingly, pretreatment with Wortmanin greatly restored TNFα-induced TRAF1 expression in RKO-β-catenin cells. These results indicate that β-catenin-mediated NF-κB suppression can be relieved by PI3K inhibition.

3.3. PI3K mediates the interaction of β-catenin with NF-κB (p65)

Our previous study indicated that β-catenin interacted p65 and this interaction required an unidentified cellular protein [11].

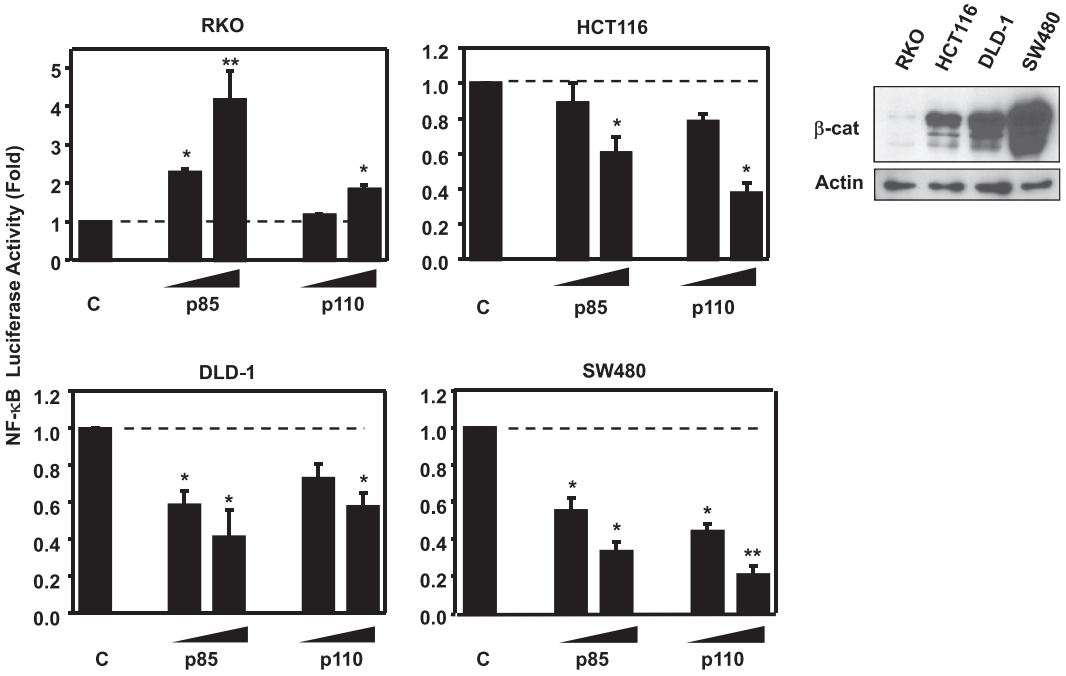

Fig. 1. The differential effects of PI3K on NF-κB activity are dependent on β-catenin level in CRC cells. Vector, or p85 and p110 subunit of PI3K were co-expressed with NF-κB-driven luciferase reporter in CRC cells. After 48 h, luciferase activities were determined and normalized (mean ± SD in three separate experiments). One and two asterisks indicate differences with $p < 0.05$ and $p < 0.01$, respectively. Expression of β-catenin in these cells was examined by Western blotting (right panel).

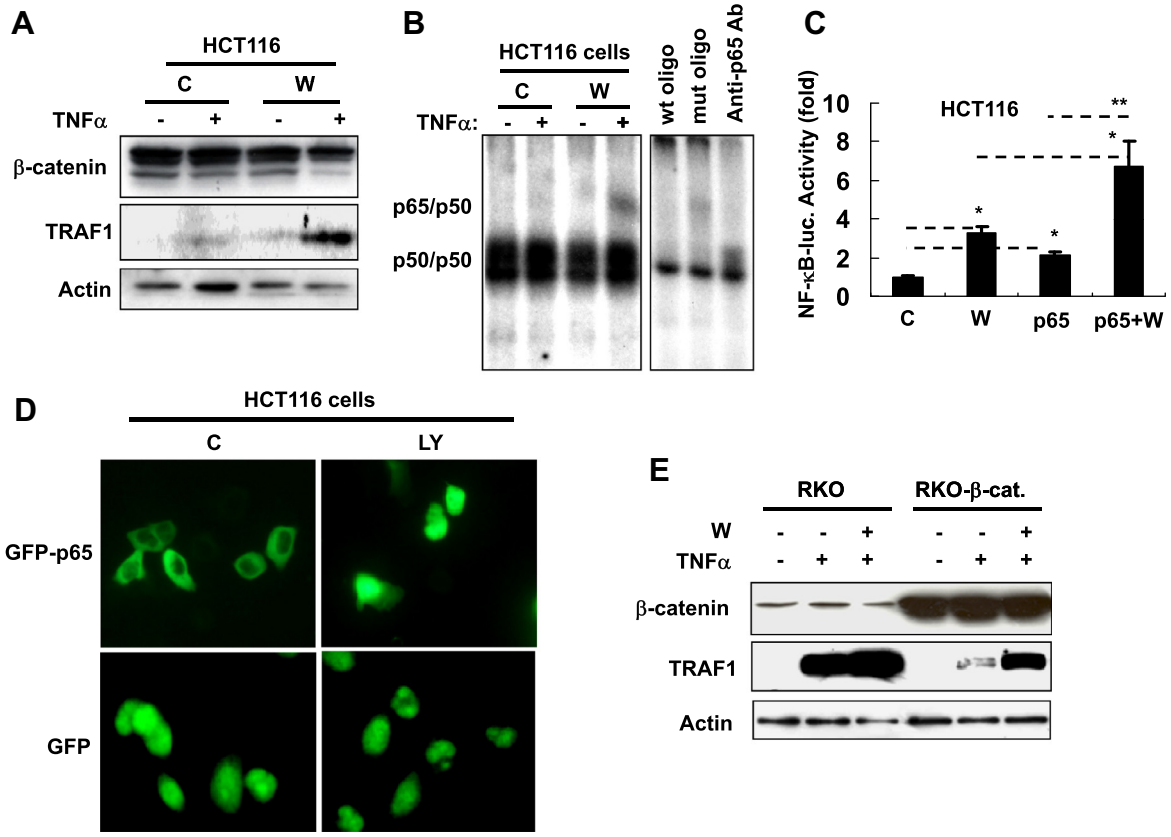

Fig. 2. PI3K signaling is required for β-catenin-mediated NF-κB suppression in CRC cells. (A) Expression of β-catenin and TRAF1 were analyzed by Western blotting in HCT116 cells treated with or without TNFα. (B) EMSA analysis for NF-κB-binding activity in HCT116 cells pretreated with or without PI3K inhibitor Wortmanin (W: 1 nM) for 3 h followed by TNFα stimulation (10 ng/ml for 30 min). (C) NF-kB-driven luciferase activity in HCT116 cells with treatments indicated above. (D) Cellular localization of GFP or GFP-p65 in HCT116 cells treated without (C) or with PI3K inhibitor LY294002 (LY) at 10 μM for 3 h was analyzed by immunofluorescence microscope. (E) Expression of β-catenin and TRAF1 were analyzed by Western blotting in cells after treatment indicated above.

Because PI3K signaling is required for the cross-regulation of NF-κB by β-catenin, we postulated that PI3K is involved in the interaction between β-catenin and p65. To test this idea, we co-expressed GFP-p65 and HA-p85 (or HA-mp85) in HEK293 cells stably expressed β-catenin (293-β-catenin cells) (Fig. 3A). Immunoprecipitation of p65 (using GFP antibody) identified the bound β-catenin (myc-tagged) and p85 (HA-tagged), indicating that these three molecules are in the same complex. However, expression of mutant p85 or pretreatment with Wortmanin significantly inhibited the interaction of p65 with β-catenin (Fig. 3A). These results suggest that: (1) p85 forms complex with p65 and β-catenin; and 2) PI3K (p85) is required for the physical interactions between p65 and β-catenin; inhibition of PI3K disrupts their interaction.

We also examined these endogenous interactions in HCT116 cells. Immunoprecipitation of p85 pulled down p110, confirming that these two subunits forms heterodimer. However, this heterodimeric interaction was disrupted when cells were treated with Wortmanin (top left, Fig. 3B). As expected, immunoprecipitation of p65 also pulled down β-catenin, and this endogenous interaction was abrogated when the cells were pretreated with Wortmanin (bottom left, Fig. 3B). Similar result was obtained with LY294002 (data not shown). These results are consistent with the data in Fig. 3A, in which PI3K is required for the physical interaction of β-catenin with p65. Because the interaction between β-catenin and NF-κB (p65) is indirect based on our previous study, this data suggest that the p85 subunit of PI3K is the potential mediator for bridging the interaction of β-catenin with p65.

We also examined the subcellular localization of β-catenin and NF-κB in HCT116 cells by immunofluorescent analysis. We found that GFP-p65 (green) was mainly co-localized with β-catenin (red) in the cytoplasm (Fig. 3C). However, treatment with LY294002 resulted in the nuclear translocation of GFP-p65 (Green) (an active form of NF-κB) and the membrane retention of β-catenin (red). This observation is consistent with data above and supports that PI3K is required for the physical interaction and functional inhibition of NF-κB by β-catenin.

3.4. PI3K suppression leads NF-κB activation and enhances apoptosis in β-catenin-high CRC cells

To characterize the biological effect of PI3K in this cross-signaling regulation, we examined the apoptotic susceptibility mediated by anti-Fas antibody and TNFα in HCT116 cells. As expected, both anti-Fas antibody and TNFα induced apoptosis in HCT116 cells; however, treatment with LY294002 (LY) substantially increased apoptosis by these two agents (Fig. 4A). Since inhibition of PI3 K leads to nuclear translocation of NF-κB (Fig. 3C), we then examined the effect of NF-κB activation in these cells. As expected, ectopic expression of GFP-p65 induced more apoptosis than GFP (Fig. 4B). Notably, treatment with LY2940002 greatly induced apoptosis in GFP-p65 group than in GFP group (Fig. 4B). These results indicate that NF-κB activation promotes apoptosis in β-catenin-high HCT116 cells, and inhibition of PI3K greatly enhances this effect. In line with this idea, inhibition of PI3K also

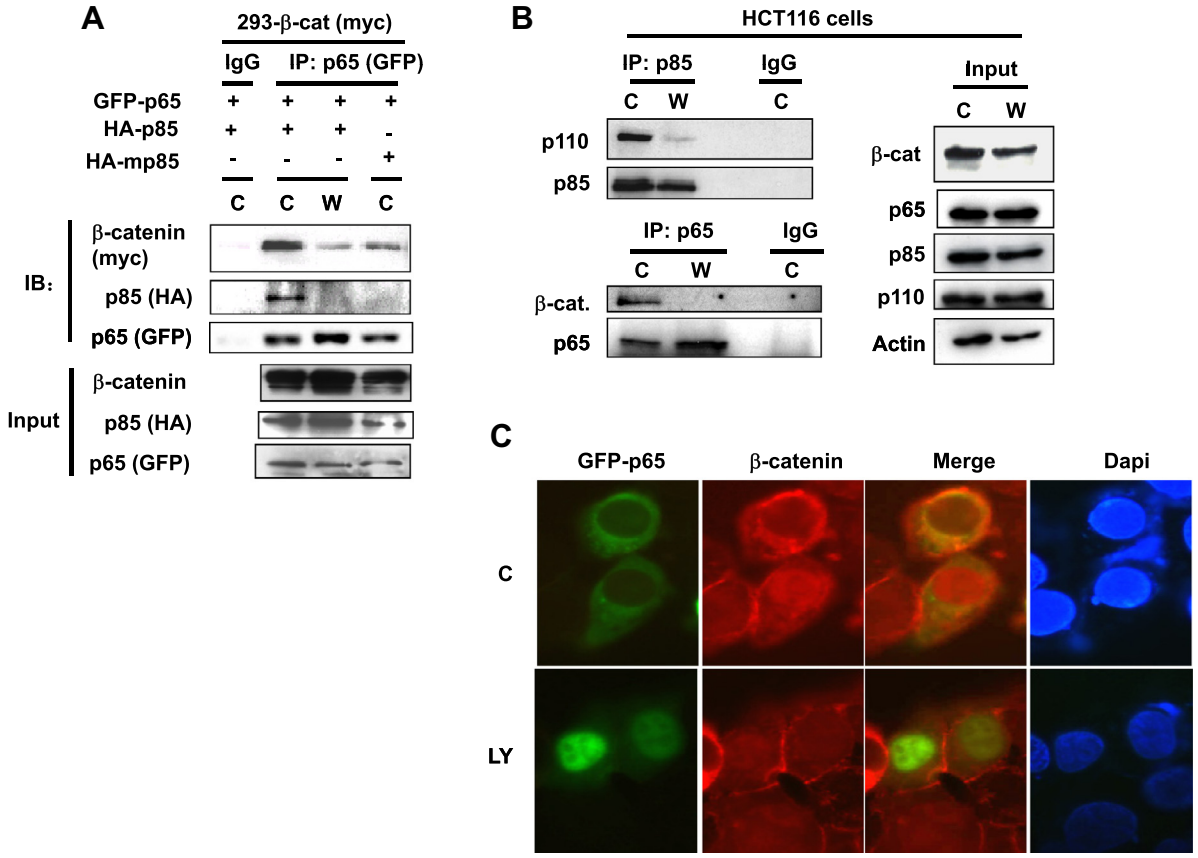

Fig. 3. PI3K is required for the physical interaction of β-catenin with NF-κB. (A) GFP-p65, HA-p85 (wild-type or mutant) were co-expressed in 293-β-catenin cells. Cells were treated without (C) or with PI3K inhibitor wortmannin (W) at 1 nM for 3 h. GFP-p65 was immunoprecipitated, and the associated β-catenin and p85 were examined by Western blotting. (B) HCT116 cells were treated without (C) or with wortmanin (W). Endogenous p85 and p65 were immunoprecipitated, the bound p110 and β-catenin were detected by Western blotting, respectively. (C) GFP or GFP-p65 was expressed in HCT116 cells treated without or with PI3K inhibitor LY294002 (LY). The cellular localization of p65 (green) and β-catenin (red) were analyzed by immunofluorescent staining. Nuclei are stained with Dapi (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

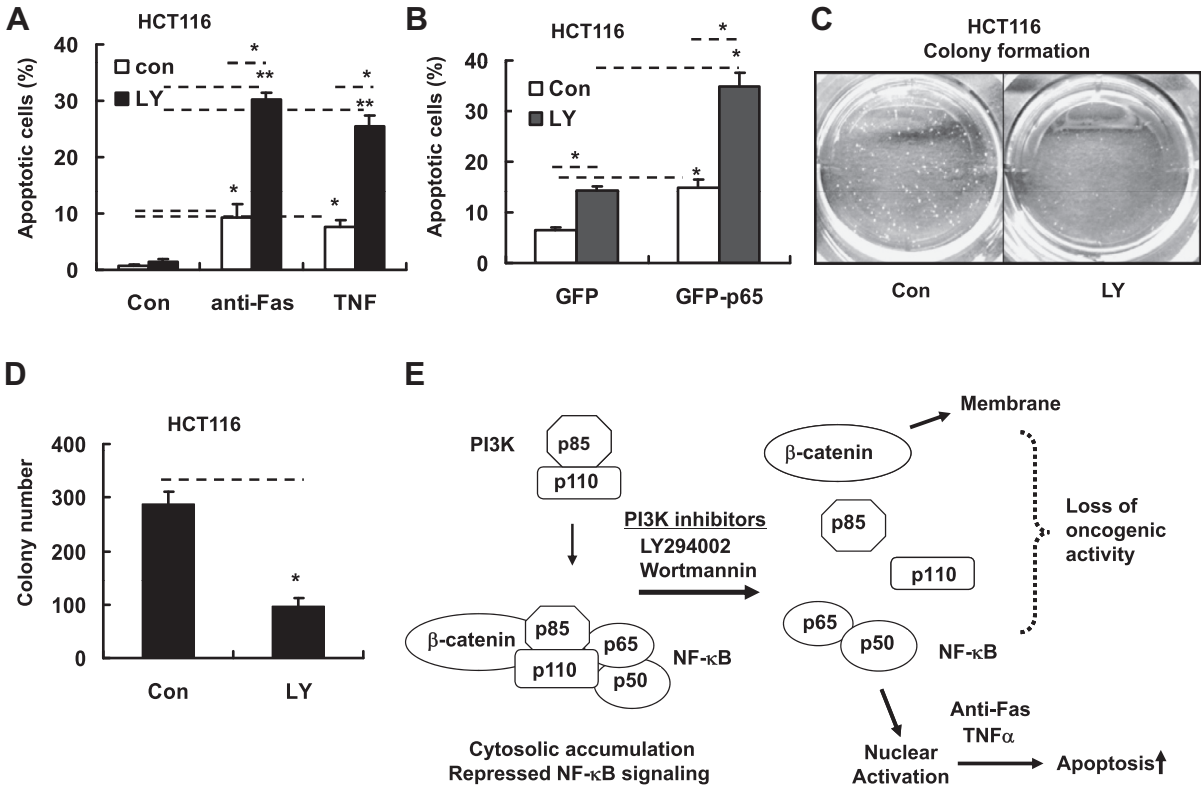

Fig. 4. Inhibition of PI3K signaling promoted apoptosis in CRC cells. (A) HCT116 cells were pretreated with or without PI3K inhibitor LY294002 (LY) followed by anti-Fas antibody (1 μg/ml) or TNFα (10 ng/ml) treatment, apoptosis was measured by FACS analysis. Results are expressed as mean ± SD) ($n = 3$). One and two asterisks indicate $p < 0.05$ and $p < 0.01$, respectively. (B) GFP or GFP-p65 was expressed in HCT116 cells followed with or without (C) LY294002 (LY) treatment, apoptosis was measured as above. (C) Anchorage-independent growth (colony-formation) was analyzed in HCT116 cells treated with or without LY294002. Representative image is shown. (D) Statistical analysis of results from C. (E) A proposed model to illustrate the signaling cross-talk among PI3K, β-catenin and NF-κB pathways.

suppressed colony formation and growth in HCT116 cells (Fig. 4C and D) and DLD-1 cells (data not shown), presumably mediated by NF-κB activation. Thus, the oncogenic effect of PI3K in β-catenin-high colorectal cancer cells is associated with NF-κB repression [11,12]. Taken together, our results suggest that in β-catenin-high CRC cells, p85 subunit of PI3K interacts with β-catenin and NF-κB. Inhibition of PI3K by PI3K inhibitors disrupts the heterodimeric formation of PI3K and the interaction of β-catenin with p65, leading to the dissociated p85 and p110, as well as membrane retention of β-catenin and nuclear translocation of NF-κB (p65), which promotes apoptosis in CRC cells (Fig. 4E).

3.5. Phosphorylated Akt and β-catenin inversely correlate with Fas expression in CRC samples

Fas (CD95), a NF-κB downstream target, is expressed at high level in normal colonic epithelial cells, and its expression is frequently lost in human CRC. We showed previously that Fas expression and Fas-mediated apoptotic pathway were suppressed by cytosolic β-catenin via NF-κB inhibition [11]. To investigate whether there is a correlation of PI3K, β-catenin and NF-κB pathways in tumor samples, we examined phosphorylated Akt (Ser473; Akt-P), cytosolic β-catenin and Fas expression in human CRC specimens by immunohistochemical (IHC) staining (Supplementary Fig. 1). Consistent with the previous report, we found that cytosolic β-catenin is inversely correlated with Fas expression ($p = 0.002 < 0.01$, Supplementary Table 1A), suggesting that cytosolic β-catenin plays a critical role in suppressing Fas expression. However, activated Akt-P is not inversely correlated with Fas expression ($p = 0.704 > 0.05$, Supplementary Table 1B), implying that activated PI3K/Akt signaling alone does not directly repress Fas expression. Interestingly, in Akt-P negative group, β-catenin is not inversely correlated with Fas expression ($p = 0.095 > 0.05$, Supplementary Table 1C), suggesting that in absence of PI3K/Akt activation, cytosolic β-catenin alone is not sufficient to repress Fas expression. Intriguingly, in Akt-P positive group, β-catenin is inversely correlated with Fas expression ($p = 0.021 < 0.05$, Supplementary Table 1D), supporting our contention that PI3K/Akt activation is required for Fas repression, presumably through β-catenin-mediated NF-κB inhibition. In addition, we found that Akt-P does not correlate with cytosolic β-catenin ($p = 0.128 > 0.05$) (Supplementary Table 1E), suggesting that activation of these two pathways occurs through two independent events. Taken together, these clinical sample analyses support that PI3K activation is critical for suppression of Fas expression through β-catenin-mediated NF-κB inhibition.

4. Discussion

β-catenin is known to have two major functions, as a protein associated with E-cadherin in cell–cell adhesion, and as a transcription factor in the nucleus. However, cytosolic β-catenin may not be limited as a transcriptional factor. Cytosolic β-catenin has been shown to interact with retinoic acid receptor (RAR) and modulate the RAR pathway [15]. We showed previously that cytosolic β-catenin also interacts with and inhibits NF-κB activation. However, the interaction of β-catenin with p65 was found to be indirect and required an unidentified molecule to bridge this interaction [11,12]. Here we showed that PI3K represents a long-sought cellular mediator for bridging the interaction between β-catenin and NF-κB. Our study provides new insight into the intricate signaling cross-talk among PI3K, β-catenin and NF-κB pathways in CRC.

Sustained β-catenin activation is the critical initiation step for CRC. Constitutive activation of PI3K signaling pathway has also been found CRC [5–7], suggesting that the crosstalk between these two pathways plays important role in the development and progression of CRC. It had been widely assumed that this cross-talk occurs at the level of GSK-3β [16], a common target that is regulated by Wnt/β-catenin and PI3K/Akt pathways. However, a recent study indicated that GSK-3β is not the merging point of this cross-talk due to different compartmentalization of Axin and PI3K [17]. We showed that PI3K (p85) and β-catenin can cross-talk through protein–protein interaction. Our data also indicated that PI3K is critical in modulating β-catenin-mediating NF-κB suppression. The detailed interactions between p85 of PI3K with β-catenin and p65 remain unclear; however, β-catenin is tyrosine phosphorylated at residues 142, 489, and 654 [18,19], it is likely that the SH2 domain of p85 can interact with tyrosine phosphorylated β-catenin. Further systematic and thorough investigation will resolve this mystery.

Aberrant NF-κB activation has been detected in many cancers and correlates with tumor progression, disease relapse and poor prognosis. However, NF-κB activation is frequently associated with reduced oncogenic activity or decreased malignant phenotype in CRC. For example, Aspirin activates NF-κB pathway and induces apoptosis in intestinal neoplasia in CRC animal model [20,21]. In addition, non-steroidal anti-inflammatory drug (NSAID) diclofenac inhibits Wnt/β-catenin signaling by activating NF-κB; and expression of p65 suppresses β-catenin signaling in HCT116 cells [22]. Furthermore, expression of p65 enhances curcumin-mediated apoptosis in HCT116 cells [23]. Lastly, expression of wild-type APC in SW480 cells that have truncated APC gene leads to decreased β-catenin and increased NF-κB activity [24]. These observations are consistent with our results and support our contention that NF-κB activation, at least in CRC cells with high level of β-catenin, promotes apoptosis. Despite these important observations, the underlying molecular mechanism is unclear at that time. Our current study indicates that PI3K is required for the physical interaction and functional inhibition of NF-κB by β-catenin. Our study provides a plausible mechanism for these important observations.

Normal colonic epithelial cells express Fas and are relatively sensitive to Fas ligand-mediated apoptosis. By contrast, colon tumor cells are relatively resistant to Fas ligand-mediated apoptosis, which is attributed by downregulation of Fas expression [25,26], inactivating mutations of Fas [27] and defective translocation of Fas to the cell surface [28] in CRC. Indeed, deficiency of Fas/CD95 increased intestinal tumor burden in $APC^{min/+}$ mice [29], demonstrating that defect in Fas ligand-mediated apoptotic pathway promotes tumorigenesis in CRC. Fas expression is reduced in 75% of CRC whereas strong expression is observed in the adjacent normal colorectal epithelium [30]. Fas gene promoter contains two NF-κB binding sites, suggesting that NF-κB activation contributes to the increased Fas expression in CRC. In line with our idea, cytosolic β-catenin in the activated Akt-P group, but not in the inactivated Akt-P group, was found to inversely correlate with Fas expression. This result supports the conclusion that activation of PI3K signaling is required for β-catenin-mediated NF-κB inhibition. Both cytosolic β-catenin and PI3K/Akt activation are critical for repressing Fas expression, leading to survival and proliferation of CRC cells.

In summary, PI3K is required for the physical interaction and functional inhibition of NF-κB by β-catenin in CRC. Inhibition of PI3K signaling disrupts this inhibitory effect, resulting in NF-κB activation, which confers CRC cells with sensitivity to Fas- and TNFα-mediated apoptosis. Our study not only identifies a new signaling cross-talk among PI3K, β-catenin and NF-KB pathways but also implicates that targeting the PI3K pathway may represent a novel therapeutic approach for treating β-catenin-high CRC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.135.

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