Molecular Cell
Article

APC Is Essential for Targeting Phosphorylated β-Catenin to the SCF$^{β\text{-}TrCP}$ Ubiquitin Ligase

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DOI 10.1016/j.molcel.2008.10.023

SUMMARY

Ubiquitin-dependent proteolysis is an important mechanism that suppresses the β-catenin transcription factor in cells without Wnt stimulation. A critical step in this regulatory pathway is to create a SCF$^{β\text{-}TrCP}$ E3 ubiquitin ligase binding site for β-catenin. Here we show that the SCF$^{β\text{-}TrCP}$ binding site created by phosphorylation of β-catenin is highly vulnerable to protein phosphatase 2A (PP2A) and must be protected by the adenomatous polyposis coli (APC) tumor suppressor protein. Specifically, phosphorylated β-catenin associated with the wild-type APC protein is recruited to the SCF$^{β\text{-}TrCP}$ complex, ubiquitin conjugated, and degraded. A mutation in APC that deprives this protective function exposes the N-terminal phosphorylated serine/threonine residues of β-catenin to PP2A. Dephosphorylation at these residues by PP2A eliminates the SCF$^{β\text{-}TrCP}$ recognition site and blocks β-catenin ubiquitin conjugation. Thus, by acting to protect the E3 ligase binding site, APC ensures the ubiquitin conjugation of phosphorylated β-catenin.

INTRODUCTION

β-catenin is a transcription factor activated by Wnt signal stimulation during animal development and tissue homeostasis (reviewed by Logan and Nusse, 2004; Clevers, 2006). Although this activation is essential for stimulated cells, it is equally important for unstimulated cells to have a mechanism in place that can effectively prevent an abnormal activation of β-catenin. Ubiquitin-dependent proteolysis is an important mechanism that inactivates β-catenin in cells without Wnt stimulation. This regulatory pathway begins with the phosphorylation of β-catenin at its N-terminal conserved serine residues 33, 37, and 45, and threonine residue 41 (S33/S37/T41/S45). Phosphorylation of β-catenin occurs in a multiprotein complex consisting of adenomatous polyposis coli (APC) tumor suppressor protein, Axin, glycogen synthase kinase-3β (GSK-3β), and casein kinase 1 (CK1), among others (Behrens et al., 1998; Hart et al., 1998; Nakamura et al., 1998). Axin acts as a scaffold protein and contains distinctive binding domains for APC, β-catenin, GSK-3β, and CK-1. The role of APC in this complex is less clear, but it is generally believed that interactions among these proteins facilitate the phosphorylation of β-catenin by CK-1 and GSK-3β through a dual kinase mechanism (Liu et al., 2002). Phosphorylation of S33 and S37 creates a consensus β-TrCP recognition site at the N-terminal domain of β-catenin. After being released from the kinase complex, phosphorylated β-catenin is recognized by β-TrCP and recruited to the Skp1/Cul1/F-box$^{β\text{-}TrCP}$ (SCF$^{β\text{-}TrCP}$) E3 ubiquitin ligase (Marikawa and Elinson, 1998; Kitagawa et al., 1999; Hart et al., 1999). Ubiquitin-conjugated β-catenin is subsequently degraded by the 26S proteasome. Such a degradation pathway is essential for the normal development as well as for the tumor suppression (Clevers, 2006). Abnormal stabilization of β-catenin has been found to cause several types of human cancer.

Mutation in *APC* is perhaps the most common mechanism that stabilizes β-catenin during oncogenic transformation and the development of colorectal cancer (CRC) (reviewed by Polakis, 1997). *APC* mutation was originally identified as a genetic cause of familial adenomatous polyposis (FAP) (reviewed by Vogelstein and Kinzler, 2002). The first evidence linking the APC tumor suppressor to β-catenin came to the light when the two proteins were found to interact (Su et al., 1993; Rubinfeld et al., 1993). Subsequent studies reveal that one of the hallmarks of *APC* mutation is the stabilization and high levels accumulation of β-catenin in mutant cells (Clevers, 2006; Polakis, 2007). Stabilized β-catenin then enters into the nucleus and activates Wnt target genes. Introduction of full length, wild-type (WT) APC to the APC mutant cells reduces the high levels of β-catenin (Munemitsu et al., 1995) and suppresses the transcriptional activation of Wnt target genes, such as *MYC* (c-*myc*) and *CCND1* (cyclin D1; He et al., 1998; Tetsu and McCormick, 1999).

APC has been suggested to regulate β-catenin in a number of ways. First, it may promote the export of nuclear β-catenin upon the withdrawal of Wnt signaling (Henderson, 2000; Neufeld et al.,

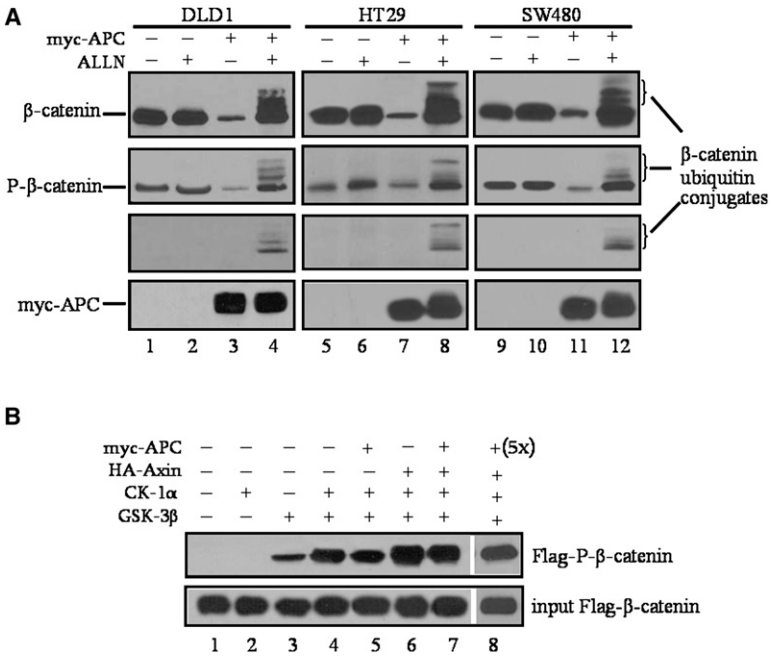

Figure 1. APC Restores β-Catenin Ubiquitination in CRC Tumor Cells

(A) Cell extracts from DLD1, HT29, and SW480 were incubated with (+) or without (−) myc-tagged wild-type APC (myc-APC) in the presence (+) or absence (−) of proteasome inhibitor ALLN. Following incubation for 4 hr at room temperature, β-catenin was immunoprecipitated (IP) by a rabbit anti-β-catenin antibody. Levels of ubiquitin-conjugated and non-conjugated β-catenin in the IP products were determined by immunoblotting (IB) with a mouse anti-β-catenin (upper panel), a rabbit anti-phospho-S33/S37/T41 β-catenin-specific antibody (second panel), and a mouse anti-ubiquitin antibody (third panel), respectively. The bottom panel shows that equal amount of myc-APC was used in relevant reactions. P-β-catenin designates phosphorylated β-catenin.

(B) Phosphorylation of β-catenin was reconstituted in vitro as described in the Experimental Procedures. Components included in the reaction were designated on the top of each lane. In all reactions, β-catenin was used as a GST-fusion protein. Following the incubation, levels of Flag-tagged phospho-β-catenin (Flag-P-β-catenin) were determined by IB with a rabbit anti-phospho-S33/S37/T41 β-catenin specific antibody (upper panel). The lower panel shows that equal amount of input Flag-β-catenin was used in each reaction.

2000). Second, it may sequester β-catenin in the cytoplasm, thereby preventing its association with Tcf4 in the nucleus (Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). The last and perhaps the most important function of APC is linked to β-catenin degradation (Polakis, 2007; Clevers, 2006). Paradoxically, this later function can be substituted by overexpression of Axin or β-TrCP in APC mutant CRC cells (Nakamura et al., 1998; Behrens et al., 1998; Hart et al., 1998, 1999). APC is a large protein with an estimated molecular weight of 330 kDa and contains two separated domains capable of interacting with β-catenin. The first domain consists of three 15 amino acid (aa) repeats, whereas the second domain contains seven 20 aa repeats (Polakis, 1997). Mutational analyses of human CRC indicate that mutant APC proteins often retain the first domain but have truncations wherein parts of or the entire second domain are missing (Vogelstein and Kinzler, 2002). This second domain also contains three 25 aa SAMP (Ser-Ala-Met-Pro) repeats. The SAMP repeats are dispersed among the 20 aa repeats and interact with Axin. Biochemical and genetic studies have shown that both 20 aa and SAMP repeats are required for the downregulation of β-catenin. It is thus suggested that APC may act to promote β-catenin to interact with Axin in the phosphorylation complex like a scaffold protein (Bienz and Clevers, 2000). Yet it is not clear why two scaffold proteins should be required in the same phosphorylation complex. In addition, Axin already contains specific interaction domains for the kinases and substrates in the complex. Unlike the classical scaffold protein Axin, APC does not contain a kinase docking site and is incapable of interacting with GSK-3β and CK-1. Accordingly, phosphorylation of β-catenin has been shown to occur in APC mutant tumor cell lines and in a kinase reaction without the presence of the WT APC (Liu et al., 2002; Xing et al., 2004; Ha et al., 2004; Yang et al., 2006). Hence, a second model has recently been proposed (Xing et al., 2003, 2004). This model suggests that phosphorylated β-catenin is turned over to APC from Axin and then released from the kinase complex. However, it is not clear why phosphorylated β-catenin has to be turned over to APC before being released from the kinase complex. Thus, the precise function of APC in association with β-catenin degradation remains to be resolved.

In this report, we present evidence demonstrating that APC acts to ensure the ubiquitin conjugation of phosphorylated β-catenin. We show that phosphorylated β-catenin associated with the WT APC protein is recruited to the $SCF^{\beta\text{-TrCP}}$ E3 ubiquitin ligase, conjugated with ubiquitin, and degraded. In the absence of the WT APC protein, phosphorylated β-catenin is rapidly dephosphorylated by serine/threonine protein phosphatase 2A (PP2A). Dephosphorylation by PP2A instantly eliminates the β-TrCP binding site and consequently prevents β-catenin entering into the downstream ubiquitination machinery. These findings provide insight into the functional mechanism of APC and may offer a basis for developing treatment of the disease through the prevention of β-catenin dephosphorylation.

RESULTS

APC Restores β-Catenin Ubiquitination in APC Mutant Cells

To gain a better understanding of APC, we used a reconstituted cell-free system to investigate how WT APC restores the β-catenin degradation in APC mutant cells. A similar system was previously used to explore the biochemical process of β-catenin degradation in *Xenopus* egg extracts (Salic et al., 2000). We examined a number of cell lines and decided to use extracts prepared from CRC cell lines DLD1, HT29, and SW480. These cell lines are well characterized and known to have mutant APC incapable of promoting β-catenin degradation. Accordingly, they contain very high levels of β-catenin (Morin et al., 1997). We confirmed the existence of high levels of β-catenin in these extracts by immunoblotting (IB) (Figure 1A,