The ins and outs of APC and β -catenin nuclear transport

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Adenomatous polyposis coli (APC) and β -catenin, two key interacting proteins implicated in development and cancer, were recently found to traffic into and out of the nucleus in response to internal and external signals. The two proteins can enter and exit the nucleus independently, a discovery that has prompted debate about the previously proposed role of APC as a β -catenin chaperone. Here, we review the regulation of APC and β -catenin subcellular localization, in particular in cancer cells. We speculate that, in non-stimulated cells, APC actively exports β -catenin from the nucleus to the cytoplasm where its levels are regulated by degradation; and, conversely, that, in cancer cells or those stimulated by Wnt signaling, β -catenin degradation is inhibited and the accruing protein is capable of moving between the nucleus and cytoplasm independently of APC. Models that link APC and β -catenin transport to function are discussed.

Introduction

The Wnt/ β -catenin signal transduction pathway plays key roles in embryonic development and cancer (for a review, see Polakis, 2000). The Wnts are growth factors that bind Frizzled transmembrane receptors, producing a signal that is transduced to the nucleus via β -catenin (Figure 1; Cadigan and Nusse, 1997). Wnt activation inhibits the phosphorylation of β -catenin by glycogen synthase kinase 3 (GSK-3), thus also preventing its ubiquitylation and subsequent proteasomal degradation (Morin, 1999; Polakis, 2000). β -catenin is thereby stabilized and forms nuclear complexes with the lymphoid enhancer factor-1/T-cell specific transcription factor (LEF-1/TCF) family of transcription factors, inducing a genetic program that can lead to cell transformation (Polakis, 2000; Fodde *et al.*, 2001). Nuclear accumulation of β -catenin is also observed in cancers resulting from mutations in the β -catenin, adenomatous polyposis coli (APC) or Axin genes

(Polakis, 2000; Fodde *et al.*, 2001). The APC tumor suppressor binds to β -catenin and the scaffold protein Axin to form a complex promoting GSK-3 β phosphorylation of β -catenin. Inherited mutations in the APC gene cause the intestinal polyp disorder familial adenomatous polyposis (FAP), and >80% of colorectal cancers carry mutations that inactivate the APC protein (Miyoshi *et al.*, 1992; Powell *et al.*, 1992). Most of these mutations target the 'mutation cluster region' in the center of the APC gene, resulting in a truncated protein incapable of binding Axin and other regulatory proteins or associating with microtubules (the impact of mutations on APC functions are reviewed elsewhere: see Polakis, 2000; Dikovskaya *et al.*, 2001; Fodde *et al.*, 2001). Mutated APC can bind to β -catenin (albeit less efficiently), but cannot stimulate degradation (see Figure 2A) due to its failure to bind Axin (Polakis, 2000).

It was proposed that APC may regulate nuclear β -catenin by exporting it to the cytoplasm for degradation (Henderson, 2000; Neufeld *et al.*, 2000a; Rosin-Arbesfeld *et al.*, 2000). This 'chaperone' function of APC has recently been questioned, however, by the discovery that β -catenin can cycle between the nucleus and cytoplasm independently of APC (Eleftheriou *et al.*, 2001; Wiechens and Fagotto, 2001). In this review, we focus on the sequences and pathways that dictate nuclear transport/retention of APC and β -catenin and discuss how their altered localization may impact on their function.

APC subcellular localization

Immunolocalization studies have mapped endogenous APC to various sites throughout the cell, including the plasma membrane, where it colocalizes with the ends of microtubules (Nathke *et al.*, 1996; McCartney *et al.*, 2001) or cortical actin filaments (Nathke *et al.*, 1996; McCartney *et al.*, 2001; Rosin-Arbesfeld *et al.*, 2001), and the cytoplasm (Reinacher-Schick and Gumbiner,

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Fig. 1. Nuclear-cytoplasmic shuttling of β -catenin. In normal, non-stimulated cells, β -catenin (indicated here as ' β ') is bound to various interacting partners. Its distribution is therefore dictated by (a) retention in the nucleus, the cytoplasm and at the plasma membrane; (b) degradation in the cytoplasm; and (c) the movements of APC. In tumor cells (or Wnt-stimulated cells), β -catenin accrues to very high levels and is likely to shuttle independently of APC (wild-type or mutant). Some tumor-associated forms of β -catenin may show reduced anchorage by E-cadherin (Chan *et al.*, 2002). The functional implications of β -catenin shuttling are poorly understood.

2001). When overexpressed, APC decorates the microtubule cytoskeleton (reviewed in Mimori-Kiyosue and Tsukita, 2001; Dikovskaya *et al.*, 2001), although such pronounced microtubule association has not been detected with endogenous APC. APC has been observed in the nuclei of human tumor cells (Neufeld and White, 1997), human colorectal epithelial cells (Deka *et al.*, 1999) and in rodent colon epithelial cells (Miyashiro *et al.*, 1995). It also localizes to the nucleus in colon cancer cells (Henderson, 2000) and in Cos7 cells (Neufeld *et al.*, 2000b) transfected with full-length APC cDNA. There are several indications that APC is actively involved in specific nuclear processes: it is occasionally detected in the nucleolus (Neufeld and White, 1997; Henderson, 2000), it can bind DNA *in vitro* (Deka *et al.*, 1999) and it colocalizes with the protein phosphatase 2A subunit B56 α in the nuclei of transfected cells (Galea *et al.*, 2001). In addition, the three potential DNA binding sites in APC are often deleted in cancer (Deka *et al.*, 1999). Despite these observations, it remains for APC to be directly linked to a specific nuclear process such as transcription or DNA repair/replication.

APC nuclear transport pathways

Nuclear import. APC can shuttle into and out of the nucleus (Henderson, 2000; Neufeld *et al.*, 2000b; Rosin-Arbesfeld *et al.*, 2000), and nuclear import of human APC is mediated by at least three sequences (Figure 2). Two are nuclear localization signals (NLSs) that function through the importin- α/β receptor pathway and are regulated by phosphorylation (Zhang *et al.*, 2000, 2001). These two NLSs are not essential for nuclear localization, however, since endogenous (Smits *et al.*, 1999; Galea *et al.*,

2001) and ectopically expressed (Galea *et al.*, 2001) APC mutants that lack them are still able to enter the nucleus. Additional import sequences have been mapped to the N-terminal 'arm' domain (Rosin-Arbesfeld *et al.*, 2000; Galea *et al.*, 2001), a sequence of seven Armadillo-like repeats resembling those found in β -catenin (Peifer *et al.*, 1994). While the mechanism of arm-dependent import is poorly defined, the APC arm-binding protein B56 α was reported to stimulate nuclear import, perhaps by acting as a nuclear chaperone (Galea *et al.*, 2001).

Nuclear export and cancer. Nuclear export of APC is mediated by the CRM1/Exportin receptor pathway (Henderson, 2000; Neufeld *et al.*, 2000b; Rosin-Arbesfeld *et al.*, 2000). APC contains at least five different nuclear export signals (NESs; Figure 2). Two classic Rev-type NESs (that closely resemble the one first identified in the HIV-1 Rev protein) are located at the N-terminus (Henderson, 2000; Neufeld *et al.*, 2000b), and three others are near the center within the β -catenin binding domain (Rosin-Arbesfeld *et al.*, 2000). The only published comparison of their individual activities revealed the N-terminal NES1 sequence to be the strongest signal (Galea *et al.*, 2001). NES1 is well conserved (Figure 2) and, unlike the central NESs, is not deleted in cancer. Different groups have observed endogenous mutant truncated APC predominantly in the cytoplasm (Smith *et al.*, 1993; Neufeld and White, 1997; Galea *et al.*, 2001; Tighe *et al.*, 2001) or, less frequently, in the nucleus (Rosin-Arbesfeld *et al.*, 2000). A transiently expressed form of APC(1–1309), by far the most common mutant APC in cancer (Polakis, 2000), is also primarily cytoplasmic but shifts to the nucleus following treatment with the CRM1 export inhibitor leptomycin B and by site-directed mutations in NES1 (Henderson, 2000; Galea *et al.*, 2001). Moreover, other disease-associated truncated forms of APC shuttle into and out of the nucleus (Galea *et al.*, 2001). While it is conceivable that the actual rate of nuclear import/export is reduced in cancer cells, the conservation of nuclear shuttling activity in cancer may be important for cell survival or growth. To date, the only known function associated with APC nuclear shuttling relates to the nuclear export and degradation of β -catenin (see below).

Regulation of APC transport. APC localization is differentially regulated in normal tissues and cell lines: in normal human colorectal epithelium, APC is located in the nuclei of cells of the regenerating basal crypt more than in those nearer the apical region of the tubular glands (Deka *et al.*, 1999); in HT29 colon cancer cells, truncated APC shifts to the nucleus (and nucleolus) during early apoptosis (Efsthathiou *et al.*, 1998); and cellular APC accumulates in the nucleus of sub-confluent cells but is partly excluded in super-confluent cells (Zhang *et al.*, 2001). This latter finding was observed for mutant as well as wild-type APC (Brocardo *et al.*, 2001). Zhang *et al.* (2001) proposed that phosphorylation at putative casein kinase 2 and protein kinase A sites near the second NLS may regulate cell density-dependent APC nuclear import. Using a β -galactosidase fusion, they showed that mutation of these sites affects nuclear localization mediated by the second NLS. Further investigation will determine whether this also applies to full-length APC. Nuclear transport might also be regulated by other post-translational changes or by protein-protein interactions including the masking of APC transport signals.

Fig. 2. Map and key domains of human APC. (A) Nuclear import (NLS and 'arm' sequences) and export (NESs, R3, R4, R7) signals and protein binding sites are shown. ASEF (APC-stimulated guanine nucleotide exchange factor), the protein phosphatase 2A B56α subunit (PP2A-B56α) and kinesin superfamily-associated protein 3A (KAP3A) all bind to the ARM domain, whereas the end-binding protein 1 (EB1) and *Drosophila* discs large (DLG) proteins bind to the C-terminus (B) The NLS and NES sequences are shown in detail, including relative NES activities (Galea *et al.*, 2001). APC NES1 is conserved in human, *Drosophila* and mouse. MCR, mutation cluster region.

β-catenin subcellular localization

β-catenin was first identified as a component of cell–cell adhesion junctions (reviewed in Kemler, 1993). It binds directly to cadherins and, by associating with α-catenin, provides a link between the actin cytoskeleton and cell–cell junctions. β-catenin levels are often low in unstimulated cells, but both soluble and membrane-bound protein accumulates in the cytoplasm and nucleus in response to Wnt signaling (Polakis, 2000). The nuclear shift in β-catenin reflects an increase in total protein and enhanced nuclear targeting of the dephosphorylated form of the protein (Chan *et al.*, 2002; Staal *et al.*, 2002). The distribution of β-catenin varies depending on cell type or organism (Peifer and Wieschaus, 1990; Schneider *et al.*, 1996; Schohl and Fagotto, 2002) and is frequently altered in cancers due to mutations in the APC (Polakis, 2000), β-catenin (Morin, 1999) or Axin (Satoh *et al.*, 2000) genes. Nuclear β-catenin can also be increased experimentally by overexpressing stable β-catenin in transgenic

mice (Harada *et al.*, 1999) or in cell lines (Morin, 1999) or by drug-mediated blockage of β-catenin degradation (Simcha *et al.*, 1998; Henderson *et al.*, 2002), although this is not always the case (Staal *et al.*, 2002).

Nuclear import. β-catenin contains no recognizable NLS. Since its nuclear localization is stimulated by overexpression of LEF-1/TCF (Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.*, 1996), it has been proposed that β-catenin is imported by a piggy-back mechanism. However, LEF-1 is not essential for β-catenin import, as mutated β-catenin that does not bind LEF-1 can nevertheless enter the nucleus (Orsulic and Peifer, 1996; Prieve and Waterman, 1999). In digitonin-treated semi-permeabilized cells, where soluble factors are washed away but the nuclear envelope remains intact, β-catenin nuclear import occurred in the absence of transport factors such as the importins or the Ran GTPase (Fagotto *et al.*, 1998). Moreover, β-catenin was found to compete with importin-β for docking to components of the nuclear pore complex, suggesting that their passage through the nuclear pores involves an interaction with common sites (Fagotto *et al.*, 1998; Yokoya *et al.*, 1999). Indeed, the central arm repeats are required for β-catenin import (Funayama *et al.*, 1995), and these are structurally related to the importin-β HEAT repeats (Malik *et al.*, 1997) that bind the nuclear pore complex (Kutay *et al.*, 1997).

APC-dependent export of β-catenin in normal cells

The first evidence that β-catenin could exit from the nucleus came from Yokoya *et al.* (1999) and Prieve and Waterman (1999). Yokoya and colleagues observed the nuclear export of recombinant β-catenin in microinjected cells, whereas Prieve and Waterman reported a cytoplasmic shift of *Xenopus* β-catenin following actinomycin D treatment of human peripheral blood lymphocytes. More recently, it was proposed that APC transports β-catenin from the nucleus to the cytoplasm for degradation, based on steady-state analysis of shifts in APC/β-catenin localization in transfected cells. When cells expressing truncated APC were also transfected with an APC construct containing active or inactive NESs, cellular β-catenin localization shifted in parallel to that of APC (Henderson, 2000; Neufeld *et al.*, 2000a; Rosin-Arbesfeld *et al.*, 2000). We now further extend this hypothesis, proposing that APC exerts this influence on β-catenin localization most effectively in cells where β-catenin is efficiently regulated by degradation (i.e. in unstimulated or non-cancerous cells; Figure 1). In such cells, there is little uncomplexed β-catenin and its distribution is determined by its interaction with APC or by its anchorage to LEF-1/TCF in the nucleus or cadherins at junctions (see below). There is evidence that ectopic and endogenous APC can associate with β-catenin in the nucleus (Henderson, 2000; Neufeld *et al.*, 2000a), and, given that β-catenin also forms part of an *in vivo* complex with APC and kinesin motor proteins (Jimbo *et al.*, 2002), it is possible that β-catenin–APC complexes are carried along microtubules from the nucleus to the plasma membrane or to sites of degradation. We emphasize that APC-mediated translocation of β-catenin through the nuclear pore has not been formally demonstrated and that evidence to this effect will help to refine current transport models.

Nuclear accumulation of β -catenin in cancer is independent of APC

β -catenin has been observed to accumulate in the nuclei of colon cancer cells, which led to the hypothesis that mutant APC 'traps' β -catenin in the nucleus (Rosin-Arbesfeld *et al.*, 2000). However, overexpression of APC(1–1309), the most frequently occurring APC cancer mutant, shifts β -catenin from the nucleus to the cytoplasm (Henderson, 2000). This mutant therefore retains the ability to bind and regulate localization but lacks the Axin binding sites required for β -catenin degradation (Fodde *et al.*, 2001). Therefore, it seems more likely that it is the inability of APC to promote β -catenin degradation, rather than a lack of export function, that causes the nuclear accumulation of β -catenin in APC-mutant tumor cells. Indeed, the evidence for APC-independent nuclear accumulation of β -catenin is overwhelming (Behrens *et al.*, 1996; Huber *et al.*, 1996; Simcha *et al.*, 1998; Harada *et al.*, 1999; Kongkanunt *et al.*, 1999; Satoh *et al.*, 2000; Henderson *et al.*, 2002). In cancer cells (or following Wnt signaling), β -catenin is stabilized and simply accrues to levels well in excess of the threshold for APC regulation (Munemitsu *et al.*, 1995) and enters the nucleus, where it is then subject to nuclear retention by proteins such as LEF-1 (Figure 1).

Evidence for APC- and CRM1-independent β -catenin nuclear export

The first indication that β -catenin can exit the nucleus independently of APC came from the analysis of a *Xenopus* β -catenin mutant ($\Delta 19$), which has a small deletion within its arm domain that disrupts its binding to APC, LEF-1 and E-cadherin (Prieve and Waterman, 1999). This β -catenin mutant was primarily nuclear but capable of nuclear export (Prieve and Waterman, 1999). In more recent microinjection assays in *Xenopus* oocytes, the export of β -catenin was shown to be independent of the CRM1 exporter and of RanGTP (Wiechens and Fagotto, 2001). In this system, the N- and C-terminal sequences promoted β -catenin nuclear export (and import), although they share no homology with known transport signals (Wiechens and Fagotto, 2001). It was further shown that recombinant β -catenin shuttled into and out of the nucleus of human cells in which soluble cytoplasmic factors had been washed away by digitonin permeabilization of the plasma membrane. Confirming the physiological relevance of the above studies, Eleftheriou *et al.* (2001) used *in vitro* assays with digitonin-permeabilized SW480 colon cancer cells, which express high levels of nuclear β -catenin, and showed that the endogenous protein exited the nucleus independently of APC. These latter two studies demonstrate that β -catenin nuclear export can occur even when the CRM1 export pathway is blocked by the CRM1 inhibitor leptomycin B. However, a small proportion of nuclear β -catenin was refractory to export in the presence of leptomycin B and may reflect the fraction associated with other components such as APC (Eleftheriou *et al.*, 2001). These findings suggest that, in human cancer cells, β -catenin nuclear export occurs primarily independently of APC and the CRM1 export pathway (Figure 1).

Regulation of β -catenin shuttling: implications for signaling

The above discussion centers on how β -catenin can enter and exit the cell nucleus, but the functional implications of β -catenin shuttling are not yet apparent. At present, we can speculate that β -catenin may act as a messenger, mediating cross-talk between the plasma membrane and the nucleus where it associates with cell–cell junctions and transcription complexes, respectively. Such communication might involve post-translational modifications of β -catenin, including glycosylation, acetylation, ubiquitylation or phosphorylation. In this regard, we note that the dephosphorylated form of β -catenin (induced by mutations or Wnt signaling) is more prominent in the nucleus and more transcriptionally active than its phosphorylated counterpart (Staal *et al.*, 2002). The increased nuclear localization of dephosphorylated ($\Delta N45$) β -catenin partly reflects reduced membrane anchorage by E-cadherin (Chan *et al.*, 2002).

Consistent with a role in cell signaling, β -catenin shuttling is regulated by retention and release at the plasma membrane and in the nucleus. Nuclear retention is mediated by the LEF-1/TCF transcription factors (Behrens *et al.*, 1996; Huber *et al.*, 1996; Simcha *et al.*, 1998). Interaction with TCF-3 blocks nuclear export of β -catenin in *Xenopus* oocytes (Wiechens and Fagotto, 2001), and LEF-1 represses β -catenin export and degradation in SW480 cells (Henderson *et al.*, 2002). Other nuclear partners may anchor β -catenin or indirectly affect retention/export by modulating the β -catenin-LEF-1/TCF complex, as has been shown recently for histone deacetylase 1 (Henderson *et al.*, 2002). Moreover, casein kinase 1 ϵ -mediated phosphorylation of β -catenin (Gao *et al.*, 2002) or TCF-3 (Lee *et al.*, 2001) enhances the β -catenin-LEF-1/TCF interaction, stabilizing β -catenin in part by nuclear retention (Lee *et al.*, 2001; Henderson *et al.*, 2002).

Plasma membrane retention of β -catenin is mediated by the cadherins (Fagotto *et al.*, 1996; Orsulic *et al.*, 1999), whereas Axin appears to be able to trap β -catenin in the cytoplasm (Tolwinski and Wieschaus, 2001). In primary colon tumors, β -catenin is predominantly in the nuclei of dedifferentiated mesenchyme-like tumor cells at the invasive front but cytoplasmic/membrane-localized in central areas of the tumor (Brabletz *et al.*, 2001). These shifts in β -catenin localization may reflect changes in cadherins and the local cell microenvironment (Brabletz *et al.*, 2001), although it is not known whether they involve changes in nuclear transport or anchorage. Signaling pathways that modulate the membrane anchorage of β -catenin often impact on cell adhesion. For example, the influx of extracellular calcium disrupts cell–cell adhesion by enhancing E-cadherin cleavage and degradation, causing β -catenin to move into the cytoplasm and nucleus (Ito *et al.*, 1999). Membrane release is also triggered by the heterotrimeric G proteins G $\alpha 12$ and G $\alpha 13$ (Meigs *et al.*, 2001) and insulin-like growth factor-induced tyrosine phosphorylation of β -catenin (Playford *et al.*, 2000). The newly synthesized soluble pool of β -catenin is not necessarily competent for nuclear signaling, however, as N-terminal phosphorylation appears to tag β -catenin not only for ubiquitylation/degradation but also reduces its nuclear activity (Staal *et al.*, 2002). The impact of protein modifications and cancer mutations on β -catenin shuttling, retention and function will provide clues for unraveling its involvement in nucleus–membrane communication.

Conclusions and perspectives

APC and β -catenin display dynamic subcellular distributions. Their localization is influenced by nuclear-cytoplasmic transport, nuclear and membrane retention and cytoskeletal associations, although the extent of these influences is not yet known. Wnt causes β -catenin to enter the nucleus, but it is not clear whether other changes at the cell surface (e.g. changes in the adhesion complex) also induce nuclear translocation of β -catenin. In this regard, the physiological conditions under which membrane-associated β -catenin is released are poorly understood. Key research questions include the following: (i) how is β -catenin nuclear transport regulated; (ii) does nuclear retention/stabilization of β -catenin promote feedback regulation of β -catenin signaling; and (iii) is it possible that β -catenin transfers signals from the nucleus to the plasma membrane, and how might this occur? With regard to the role of APC, it is important to determine when APC regulates β -catenin localization: is it only in unstimulated normal cells, as predicted here? Also, given that wild-type and mutated forms of APC shuttle into and out of the nucleus, what does APC do inside the nucleus, which proteins does it associate with and can APC also transmit signals between the nucleus and the plasma membrane? The answers to these questions will continue to surprise and provide fresh insights into developmental signaling and cancer.

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