**αE-catenin is not a significant regulator of β-catenin signaling in the developing mammalian brain**

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Accepted 31 January 2008
Journal of Cell Science 121, 1357-1362 Published by The Company of Biologists 2008
doi:10.1242/jcs.020537

Summary

β-catenin is a crucial mediator of the canonical Wnt-signaling pathway. α-catenin is a major β-catenin-binding protein, and overexpressed α-catenin can negatively regulate β-catenin activity. Thus, α-catenin may be an important modulator of the Wnt pathway. We show here that endogenous α-catenin has little impact on the transcriptional activity of β-catenin in developing mammalian organisms. We analyzed β-catenin signaling in mice with conditional deletion of αE-catenin (Ctnna1) in the developing central nervous system. This mutation results in brain hyperplasia and we investigated whether activation of β-catenin signaling may be at least partially responsible for this phenotype. To reveal potential quantitative or spatial changes in β-catenin signaling, we used mice carrying a β-catenin-signaling reporter transgene. In addition, we analyzed the expression of known endogenous targets of the β-catenin pathway and the amount and localization of β-catenin in mutant progenitor cells. We found that although loss of αE-catenin resulted in disruption of intercellular adhesion and hyperplasia in the developing brain, β-catenin signaling was not altered. We conclude that endogenous αE-catenin has no significant impact on β-catenin transcriptional activities in the developing mammalian brain.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/9/1357/DC1

Key words: Brain development, β-catenin signaling, α-catenin

Introduction

β-catenin is an adherens junction (AJ) protein involved in both intercellular adhesion and regulation of the canonical Wnt signaling pathway (Clevers, 2006). In adhesion, β-catenin links cadherins with α-catenin, and this interaction is crucial for the assembly and maintenance of AJs (Perez-Moreno and Fuchs, 2006). In Wnt signaling, β-catenin binds to the Lef/Tcf family of transcriptional factors and functions as a transcriptional co-activator (Clevers, 2006; Nelson and Nusse, 2004). Both the adhesion and signaling activities of β-catenin play a pivotal role in normal development and tissue homeostasis and it is often difficult to discern which of these functions is most critical at any given time of development or in the adult life of an organism. In fact, such a central position in both intercellular adhesion and signaling makes β-catenin a critical node that may be involved in orchestrating the behavior of individual cells assembled into a multicellular organism (Lien et al., 2006b).

In addition to its role in normal development and adult tissue homeostasis, the β-catenin-signaling pathway also plays a causal role in a variety of human malignancies (Clevers, 2006; Perez-Moreno and Fuchs, 2006; Polakís, 2007). Elucidation of the mechanisms responsible for regulation of β-catenin signaling is necessary for understanding of the Wnt pathway and developing efficient tools for anti-cancer therapy. The canonical Wnt signal transduction pathway is the principal regulator of β-catenin-mediated transcription (Clevers, 2006). Wnt interacts with its receptor Frizzled, which activates a signaling cascade that ultimately results in attenuation of the activity of the β-catenin destruction complex and accumulation of cytoplasmic and nuclear β-catenin. Stabilized β-catenin associates with Lef/Tcf transcription factors and activates the transcription of multiple genes including the classic targets of β-catenin pathway Myc, Ccdn1 (encoding cyclin D1) and Axin2 (He et al., 1998; Lustig et al., 2002; Tetsu and McCormick, 1999). Many β-catenin-binding proteins play a profound role in the regulation of its signaling activities. Interaction of β-catenin with Lef/Tcf and BCL9/BCL9-2 is required for signaling (Behrens et al., 1996; Brembeck et al., 2004; Kramps et al., 2002; Molenaar et al., 1996). By contrast, adenomatous polyposis coli (APC) and axin proteins are necessary for degradation of β-catenin and inactivating mutations in Apc and Axin2 result in abnormal activation of the Wnt pathway and predisposition to colorectal cancer (Behrens et al., 1998; Korinek et al., 1997; Morin et al., 1997; Polakís, 2007).

In addition to known downstream effectors of the classic Wnt pathway, AJ proteins can also influence β-catenin transcriptional activity. For example, cadherins negatively regulate β-catenin by competing for interaction with Lef/Tcf factors and perhaps, by tethering β-catenin to the cell plasma membrane away from the nucleus (Cox et al., 1996; Fagotto et al., 1996; Heasman et al., 1994; Orsulic et al., 1999; Sanson et al., 1996). It has been reported that another β-catenin-binding and AJ protein, α-catenin, is also a negative regulator of β-catenin-signaling pathway (Giannini et al., 2000a; Hwang et al., 2005; Merdek et al., 2004; Sehgal et al., 1997; Simcha et al., 1998). Overexpression of α-catenin antagonized the doryalisation effects of β-catenin in Xenopus embryos (Sehgal et al., 1997). In addition, overexpression of α-catenin in cell lines also resulted in attenuation of β-catenin transcriptional activity (Giannini et al., 2000b; Merdek et al., 2004; Simcha et al., 1998).
Finally, siRNA-mediated depletion of α-catenin in cultured chondrocytes resulted in a small, but statistically significant increase in β-catenin signaling (Hwang et al., 2005). Negative regulation of β-catenin transcriptional activity by α-catenin can be explained not only by sequestration of β-catenin away from the nucleus, but also by competition between α-catenin and DNA for binding to β-catenin/TCF complex (Giannini et al., 2000a). In contrast to these studies, abnormal activation of β-catenin was not detected in mouse keratinocytes lacking the major epithelial α-catenin, αE-catenin (Vasioukhin et al., 2001) and it is still unclear whether endogenous α-catenin can regulate the β-catenin-signaling pathway in vivo.

We have recently generated mice with conditional deletion of αE-catenin (Ctnna1) in the developing central nervous system and found that these animals display massive brain hyperplasia and dysplasia (Lien et al., 2006a). Although we revealed that abnormal activation of the Hedgehog-signaling pathway had an important role in hyperplasia in αE-catenin−/− brains, an abnormal increase in the total number of cells in the developing brain is also consistent with the potential activation of β-catenin signaling. As multiple studies demonstrated that α-catenin is a negative regulator of β-catenin transcriptional activity, we hypothesized that deletion of αE-catenin in the developing mouse brain activates β-catenin and that this activation may be at least partially responsible for hyperplasia in αE-catenin−/− animals. Surprisingly, our results revealed no significant changes in the β-catenin-signaling pathway in αE-catenin−/− mouse brains. These data suggest that endogenous α-catenin may have very little, if any, role in the regulation of β-catenin transcriptional activity in vivo.

Results and Discussion

αE-catenin is a prominent part of β-catenin protein complexes

Both αE-catenin and αN-catenin are expressed in the developing mammalian brain; however, these genes display striking cell-type specificity, with αE-catenin primarily expressed in the dividing neural progenitors and αN-catenin in differentiated neurons (Lien et al., 2006a; Stocker and Chenn, 2006) (supplementary material Fig. S1). We previously reported that conditional deletion of αE-catenin in the developing central nervous system using a Nestin-Cre driver results in the disruption of cell-cell junctions, dysplasia and hyperplasia of neural progenitors (Lien et al., 2006a). Nestin-Cre is activated in neural progenitors at embryonic day (E) 10.5 of development (Graus-Porta et al., 2001). Although no differences in cell numbers were detected between the wild-type and mutant brains in E12.5 embryos, E13.5 embryos displayed a 40% percent increase in the total number of cells in the mutant brains. The increase in cell numbers continued later in development; however, the most explosive hyperplasia in αE-catenin−/− brains took place early, between days E12.5 and E14.5 (Lien et al., 2006a). Since αE-catenin is a known binding partner of β-catenin and β-catenin plays an important role in regulating the proliferation of embryonic neural progenitor cells (Chenn and Walsh, 2002; Woodhead et al., 2006), we decided to analyze whether depletion of αE-catenin results in changes in transcriptional activity of β-catenin. Since the most drastic hyperplasia took place in αE-catenin−/− brains from E12.5 to E14.5, we concentrated on this developmental time point. We first analyzed potential changes in β-catenin protein complexes. For this purpose, β-catenin and its interacting proteins were immunoprecipitated from wild-type and αE-catenin−/− brains and analyzed by SDS-PAGE followed by western blotting with anti-β-catenin, anti-N-cadherin and anti-αE-catenin antibodies (Fig. 1A,B). Discrete protein bands corresponding to β-catenin, cadherins, α-catenin and few additional unidentified proteins were present in the immunoprecipitates from wild-type brains (Fig. 1A). As expected, levels of α-catenin were significantly decreased in β-catenin protein complexes from αE-catenin−/− brains. Surprisingly, this was the only major change that was detected by Coomassie Blue and Silver staining of the proteins immunoprecipitated with anti-β-catenin antibodies (Fig. 1A). We conclude that α-catenin is a prominent part of β-catenin protein complexes; however, depletion of α-catenin has little effect on interaction between β-catenin and other major β-catenin-binding proteins. Small amounts of α-catenin remaining in the complexes from mutant brains probably represent αN-catenin prominently expressed in the neurons of E14.5 brains (Fig. 1A), and the residual amounts of αE-catenin in the few brain cells (endothelial cells) that remained untargeted by the Nestin-Cre transgene (Fig. 1B).

To determine whether localization of β-catenin is changed in αE-catenin−/− brains, we performed immunofluorescent staining of cortical sections from E13.5 embryos with anti-N-cadherin and anti-β-catenin antibodies. Analysis of sections using confocal microscope revealed disruption of apical junctional complexes and disorganization of αE-catenin−/− neural progenitors (Fig. 1C–D’). Nevertheless, β-catenin in these cells was still present at the cell periphery and colocalized with N-cadherin. We conclude that despite the depletion of αE-catenin, the composition of other major proteins in β-catenin protein complexes remains unchanged and that β-catenin continues to colocalize with N-cadherin at the surface of αE-catenin−/− neural progenitor cells.

Depletion of αE-catenin has no impact on the total level of β-catenin and its nuclear localization

Wnt-mediated activation of β-catenin signaling usually results in inhibition of the β-catenin destruction complex and accumulation of β-catenin (Clevers, 2006). We analyzed potential changes in total levels of β-catenin in E12.5 and E13.5 wild-type and αE-catenin−/− mouse brains using western blot analysis (Fig. 2A). Although αE-catenin was depleted in the mutant brains, the total levels of β-catenin remained unchanged (Fig. 2A’).

Activated β-catenin localizes to the cell nucleus, which is indicative of activation of the β-catenin-signaling pathway. Nuclear β-catenin is difficult to detect using regular immunofluorescent staining approaches. However, it can be revealed using special antigen-retrieval protocols (Merrill et al., 2001). We used such a protocol to reveal potential changes in the nuclear localization of β-catenin in αE-catenin−/− mouse brains. No significant differences in the number or localization of cells with nuclear β-catenin were found between wild-type and αE-catenin−/− mouse brains (Fig. 2B–C”). We conclude that depletion of αE-catenin in the developing mouse brain does not alter the overall level or nuclear localization of β-catenin.

An in vivo reporter for Let/β-catenin transcriptional activity reveals no changes in the spatial distribution or level of β-catenin signaling in αE-catenin−/− brains

Although analysis of the total level of β-catenin or its nuclear localization can provide a general estimation of potential changes in the β-catenin signaling pathway, such measurements may not be sufficiently sensitive and specific. The endogenous transcriptional reporter system has proved to be a very useful tool for quantitative and spatial analysis of β-catenin transcriptional activity. Several β-catenin-signaling reporter mice have been developed and analyzed...
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Fig. 1. Depletion of αE-catenin has no major effect on interaction between β-catenin, N-cadherin and other β-catenin-binding proteins. (A,B) Total protein lysates from E14.5 wild-type (WT) and αE-catenin⁻/⁻ (knockout, KO) mouse brains were immunoprecipitated with control (IgG) or anti-β-catenin (β-cat) antibodies and the resulting protein complexes were separated by SDS-PAGE and stained with Colloidal Blue and Silver stain (A) or analyzed by western blot with anti-αE-catenin, N-cadherin or β-catenin antibodies (B). Note that although αE-catenin becomes depleted from β-catenin protein complexes, composition or relative abundance of other proteins does not change. Western blotting reveals no significant changes in the association between β-catenin and N-cadherin. (C-D'') Despite disruption of apical junctional complexes and loss of cell polarity, β-catenin continues to colocalize with N-cadherin at the periphery of αE-catenin⁻/⁻ neural progenitor cells. Cortical sections from E13.5 wild-type (WT) and αE-catenin⁻/⁻ (KO) embryos were stained with anti-N-cadherin (N-cad) and anti-β-catenin (β-cat) antibodies. Scale bar: 15.9 μm.

Depletion of αE-catenin has no impact on the levels of endogenous transcriptional targets of β-catenin signaling

The synthetic TOPGAL promoter may not be able to faithfully reproduce the complexity of transcriptional regulation at the endogenous promoter sequences, which are controlled by β-catenin transcriptional activity. Although β-catenin can control transcription of many genes in a tissue- and time-specific manner, Myc, CcnD1 and Axin2, are considered to be the classic endogenous transcriptional targets of β-catenin signaling (He et al., 1998; Lustig et al., 2002; Tetsu and McCormick, 1999). To examine whether depletion of αE-catenin results in changes in the transcriptional levels of these genes, we performed real-time PCR analysis using total RNA extracted from E12.5 and E13.5 wild-type and αE-catenin⁻/⁻ mouse brains. Although transition from E12.5 to E13.5 is associated with significant hyperplasia in αE-catenin⁻/⁻ mice, we found no statistically significant changes in the levels of Myc, CcnD1 or Axin2 mRNA in wild-type and αE-catenin⁻/⁻ mouse brains (Fig. 4). Thus, levels of the known endogenous transcriptional targets of β-catenin signaling are unchanged at the time of the most drastic increase in total cell number in αE-catenin⁻/⁻ mouse brains.

In summary, we used a loss-of-function approach to determine the potential role of endogenous αE-catenin in the regulation of β-catenin transcriptional activity in vivo, we used TOPGAL mice (DasGupta and Fuchs, 1999). These animals carry the transgene containing three Lef/Tcf binding sites in front of a c-fos minimal promoter and LacZ reporter gene (DasGupta and Fuchs, 1999). We crossed our αE-cateninlox/lox/Nestin-Cre mice (Lien et al., 2006a) with TOPGAL mice. Staining the brains of TOPGAL αE-catenin⁺/⁺ E12.5 and E13.5 embryos for β-galactosidase activity revealed a pattern of reporter expression that was similar to the pattern observed previously with other reporters of Lef/Tcf signaling (Maretto et al., 2003; Moriyama et al., 2007). β-catenin signaling in the developing mouse telencephalon was highly compartmentalized, with the reporter displaying high levels of activity in the E12.5 dorsal cortex, especially in the cingulate cortex area (Fig. 3B). In the E13.5 cortex, the area displaying active β-catenin signaling expands to encompass the developing hippocampus (Fig. 3C). Remarkably, an almost identical pattern of staining was observed in both E12.5 and E13.5 TOPGAL and αE-cateninlox/lox/Nestin-Cre/TOPGAL mice (Fig. 3B’-C’). Therefore, we conclude that depletion of αE-catenin does not change the spatial pattern of active β-catenin signaling in the developing mouse telencephalon.

Although staining of tissue sections for LacZ is useful for spatial localization of β-catenin signaling, quantification of this enzymatic staining is challenging. To determine whether the overall levels of the reporter were different in αE-catenin⁻/⁻ brains, we performed western blot analyses of the total protein extracts from TOPGAL wild-type and αE-catenin⁻/⁻ mouse brains with anti-β-galactosidase antibodies. We found no significant differences in the level of the reporter between wild-type and mutant mouse brains (Fig. 3D). Overall, we conclude that neither spatial distribution nor the level of expression of the Lef/Tcf reporter construct were significantly altered in the αE-catenin⁻/⁻ brain.
catenin signaling in the developing brain. For this purpose, we analyzed the level and localization of β-catenin, activity of β-catenin signaling via a TOPGAL reporter construct and the levels of known endogenous transcriptional targets of the β-catenin-signaling pathway. We did not find significant changes in β-catenin-mediated transcriptional activity in αE-catenin−/− neural progenitor cells. Although it is possible that our analysis was not sensitive enough to detect potential minor changes in β-catenin signaling, our results indicate that α-catenin has very little impact on β-catenin signaling in vivo. This is different from the results obtained using overexpression or knockdown of α-catenin in cultured cell lines (Giannini et al., 2000a; Hwang et al., 2005; Merdek et al., 2004; Sehgal et al., 1997; Simcha et al., 1998). It is possible that small amounts of αN-catenin in neural progenitors compensate for the loss of αE-catenin; however, this is unlikely, because αE-catenin−/− progenitors display prominent cell-cell adhesion defects. Both αE-catenin and αN-catenin are completely competent in AJ formation (Hirano et al., 1992). Therefore, disruption of AJs in αE-catenin−/− progenitors indicates the absence of any compensation by αN-catenin. The most likely reason for differences between our results and previously published studies are the different model systems that were utilized. We believe that our in vivo approach may be more relevant for the analysis of α-catenin, because the absence of a three-dimensional tissue organization in cells in culture may introduce major changes, which are simply not pertinent to the situation in the live organism. This is especially critical for studies on catenins, because these proteins are directly involved in tissue organization via their role in AJ formation.
Materials and Methods

Mice
Mice with Nestin-Cre-mediated conditional deletion of αE-catenin in the developing central nervous system were generated as described (Lien et al., 2006a). TOPGAL Le/Tcf reporter mice were obtained from Elaine Fuchs (DasGupta and Fuchs, 1999). To obtain TOPGAL/Nestin-Cre/αE-catenin<sup>lox/lox</sup> mice, we crossed TOPGAL females with Nestin-Cre/αE-catenin<sup>lox/lox</sup> males, and the resulting TOPGAL/Nestin-Cre/αE-catenin<sup>lox/lox</sup> males were crossed with αE-catenin<sup>fl/fl</sup> females. All mice were on C57BL/6J genetic background.

Immunoprecipitation and western blotting
Total proteins were extracted from embryonic brains with immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Brij, 10% glycerol, 0.1 mM EDTA, 0.5 mM MgCl<sub>2</sub>, 20 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate and a cocktail of protease inhibitors). Extracts were precleared by centrifugation at 25,000 g for 15 minutes at 4°C and supernatants were incubated with 30 µl of 50% slurry of Protein-A-Sepharose (Amersham) for 30 minutes. Resulting extracts were rotated at 4°C for 1 hour with anti-β-catenin antibody (Sigma) and then for 1 hour with 50 µl of 50% slurry of Protein-A-Sepharose conjugated to rabbit anti-mouse antibody. Sepharose beads were washed four times with IP buffer and bound proteins were released by addition of LDS loading buffer and heating at 100°C for 5 minutes. Immunoprecipitated proteins or total protein extracts were resolved by NuPAGE electrophoresis (Invitrogen) and transferred to Immobilon P membrane (Millipore) or stained with Colloidal Blue or Silver stain (Invitrogen). The membranes were incubated with anti-αE-catenin (1:500, gift from Shoichino Tsukita, Kyoto University, Japan), anti-αE-catenin (NCAT2; 1:500, University of Iowa Hydridoma Bank) anti-β-catenin (1:2000, Sigma), anti-β-actin (1:10,000, Sigma), anti-N-cadherin (1:2000, Zymed), or anti-β-galactosidase (1:2000, Rockland) antibodies at 4°C overnight. The membranes were washed and incubated with anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase (Jackson Laboratories) at a dilution of 1:10,000. The blots were developed using ECL chemiluminescence detection reagent (Pierce).

Immunofluorescence and immunohistochemistry
Immunofluorescence staining was performed as described (Klezovitch et al., 2004). Stained sections were analyzed using the Zeiss LSM510 confocal two photon microscope. For nuclear β-catenin staining, brain tissues were first fixed in 4% paraformaldehyde for 15 minutes on ice, washed in PBS, processed, embedded in paraffin and sectioned. The resulting sections were deparaffinized, hydrated, subjected to antigen retrieval by autoclaving for 15 minutes in the antigen unmasking solution (Vector Laboratories, H3300) and incubated with primary anti-β-catenin antibodies (1:2000, Sigma, C-7082) overnight at 4°C, as described above. The ABC (mouse on mouse) MOM kit (Vector Laboratories) was used for immunohistochemical detection of primary antibodies (Jackson Laboratories). Secondary antibodies were detected with DAB peroxidase substrate kit (Vector Laboratories).

X-gal staining
E12.5 to E13.5 mouse embryos were pre-fixed for 20 minutes in 4% paraformaldehyde in PBS on ice, washed four times in cold PBS and incubated in 30% sucrose in PBS at 4°C overnight. Subsequently, mouse heads were embedded in OCT (Tissue-Tek) and cryo-sectioned at 7 µm. Sections were post-fixed in 0.5% glutaraldehyde in PBS for 2 minutes at room temperature, rinsed seven times with PBS and then stained overnight in the dark, at room temperature with X-gal solution (100 mM Na phosphate pH 7.3, 1.3 mM MgCl<sub>2</sub>, 3 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 3 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1 mg/ml X-gal). Sections were counterstained with Nuclear Fast Red (Vector Labs).

Total brain cell number counting
To determine the total brain cell numbers, brains were dissected, incubated in DMEM media containing 0.6 mg/ml papain (Worthington) and 20 µg/ml DNase (Sigma) for 20 minutes at room temperature, and dissociated to a single cell suspension by trituration. Cells were counted using Z1 Coulter particle counter (Beckman Coulter).

We thank all members of V. Vasioukhin’s laboratory for help and the critical reading of the manuscript; Akira Nagafuchi, Shoichino Tsukita and the University of Iowa Developmental Studies Hydridoma Bank for generous gift of antibodies; Elaine Fuchs for the gift of TOPGAL mice; and Vi Nguyen for help with mouse genotyping. This work was supported by NCI grant 1R01CA098161 to V.V.

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