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Oncogenic transformation by β -catenin: deletion analysis and characterization of selected target genes

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Genetic analysis of β -catenin-induced oncogenic transformation in chicken embryo fibroblasts (CEF) revealed the following prerequisites for oncogenicity: (1) removal of the N terminal phosphorylation sites targeted by glycogen synthase kinase 3 β (GSK3 β), (2) retention of the N terminal transactivation domain, and (3) retention of the armadillo repeats. The C terminal transactivation domain played an ancillary role in the transformation of CEF. There was a rough correlation between the transforming activity of various β -catenin constructs and their transactivation of the TOPFLASH reporter. Expression levels of the candidate target genes of β -catenin-LEF, *cyclin D1* and *myc* were not correlated with each other or with the transforming activity of β -catenin constructs. A new target gene, coding for inositol hexakisphosphate kinase 2 (IP6K2) was identified. Its expression showed concordance with the transforming activity of β -catenin constructs.

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Introduction

β -catenin is both a component of the cell adhesion system, interacting with E cadherin, and a member of the Wnt signaling cascade, affecting cell growth and differentiation (Polakis, 1999; Willert and Nusse, 1998). Cytoplasmic levels of β -catenin are regulated by a large protein complex that includes the adenomatous polyposis coli protein, axin, and glycogen synthase kinase 3 β (Bienz and Clevers, 2000; Eastman and Grosschedl, 1999; Miller *et al.*, 1999; Peifer and Polakis, 2000). In the absence of Wnt, the glycogen synthase kinase 3 β in the complex and casein kinase 1 α (CK1 α) phosphorylates β -catenin at amino terminal serine and threonine residues, marking it for ubiquitin-mediated degradation through the proteasome pathway (Liu *et al.*, 2002). Binding of Wnt protein to the cellular surface receptor Frizzled activates the cytoplasmic protein Dishevelled

that then recruits glycogen synthase kinase 3 β to its inhibitor Frat. Consequently the phosphorylation of β -catenin is decreased. Hypophosphorylated β -catenin escapes degradation and accumulates in the cytoplasm, eventually translocating to the nucleus. At this site, β -catenin combines with members of the T-cell factor/lymphoid-enhancer binding factor (TCF/LEF) family of high mobility group (HMG) proteins to form transcriptional regulator complexes that activate specific target genes.

Wnt signaling plays important roles in embryonic segment patterning in *Drosophila* and in the formation of the dorso-ventral axis in vertebrates (Nusse and Varmus, 1992). Mammalian Wnt was first identified as the product of an oncogene, activated by insertion of a retroviral genome in mouse mammary cancer (Nusse and Varmus, 1992). Aberrant regulation of the Wnt pathway is implicated in various human cancers (Bienz and Clevers, 2000; Morin, 1999; Peifer and Polakis, 2000; Polakis, 1999). Loss of function mutations in the adenomatous polyposis coli protein have been found in the familial adenomatous polyposis syndrome and in sporadic colon cancers. Mutated axins have been identified in hepatocellular carcinoma, colorectal cancer, and medulloblastoma (Dahmen *et al.*, 2001; Liu *et al.*, 2000; Satoh *et al.*, 2000). Gain of function mutations of β -catenin occurs in cancers of the colon, prostate, liver, endometrium, ovary, and in melanomas (Morin, 1999; Polakis, 2000). All these mutations result in nuclear accumulation of β -catenin and upregulation of LEF/TCF target genes.

We have shown previously that transactivating mutants of LEF-1 induce oncogenic transformation of chicken embryo fibroblasts (CEF) (Aoki *et al.*, 1999). LEF-1 fused to β -catenin or to transcriptional activation domains derived from the estrogen receptor or the herpes simplex virus protein VP16 was able to induce focus formation and anchorage-independent growth of CEF (Aoki *et al.*, 1999). These results suggested that the ability to induce activation of LEF/TCF target genes is important in the transforming activity of β -catenin. Here we present a structure/function analysis of β -catenin in oncogenic transformation of CEF, in transcriptional activation of reporter constructs, and activation of the known LEF/TCF targets c-Myc and cyclin D1. We also identify inositol hexakisphosphate kinase 2 (IK6P2) as a novel target of LEF-1.

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Results

Deletion analysis of β -catenin induced oncogenic transformation

In order to identify the genetic determinants that are important in the oncogenic activity of β -catenin, we generated several β -catenin mutants as summarized in Figure 1. The mutants were expressed in CEF by the RCAS vector and tested for their ability to induce anchorage-independent growth in nutrient agar (Figure 2). Expression of the mutants was verified in Western blots, using two anti- β -catenin antibody (Figure 3). Wt β -catenin and b Δ NTAD showed moderate levels of expression. Morphology and size of the CEF agar colonies defined four levels of transforming potential: non-transforming, without significant growth in agar (0), weakly transforming, generating small colonies (grade 1), moderately transforming, characterized by large colonies of reticulate morphology (grade 2) and highly transforming with large, densely packed colonies (grade 3) (Figure 2). The efficiencies of agar colony formation expressed as numbers of colonies per 10^4 cells did not differ significantly between constructs of grades 1–3 transforming potential.

Wild-type β -catenin does not induce formation of cell colonies in nutrient agar (Table 1). Deletion of the GSK3 β and CK1 α phosphorylation sites at residues 33, 37, 41, and 45 in b Δ N or substitution of these phosphorylation sites with alanine residues (S33A) yields highly transforming β -catenin mutants. Deletion of the N-terminal transactivation domain (b Δ NTAD) leads to loss of transforming activity (b Δ NTAD), whereas deletion of the C-terminal transactivation domain in b Δ N/ Δ CTAD does not affect the efficiency of agar colony formation but changes the morphology of the colonies, inducing a less compact and more reticulate appearance. Deletion of the armadillo repeats causes loss of transforming activity (b Δ N/ Δ Arm). Deletion of both transactivation domains also yields an inactive construct (b Δ NTAD/ Δ CTAD). These observations support the following conclusions: stabilizing mutations in the N-terminal portion of β -catenin activate oncogenicity. The NTAD is essential for transformation, the CTAD is not required, but appears to enhance transforming activity. The armadillo repeats, needed for the binding of β -catenin to LEF, are also essential.

For a more detailed functional analysis of oncogenicity, we fused the N termini of β -catenin deletion mutants to a LEF construct modified by deletion of the context-dependent transactivation domains from its N terminus (Δ LN) (Figure 4). In these chimeras, the Δ LN component provides a DNA binding domain only; it lacks transactivating sequences. The fusion makes the armadillo domains that mediate the interaction of LEF and β -catenin redundant. The constructs then test primarily the roles of β -catenin transactivation domains in oncogenic transformation. Expression of these constructs was confirmed by Western blot, using

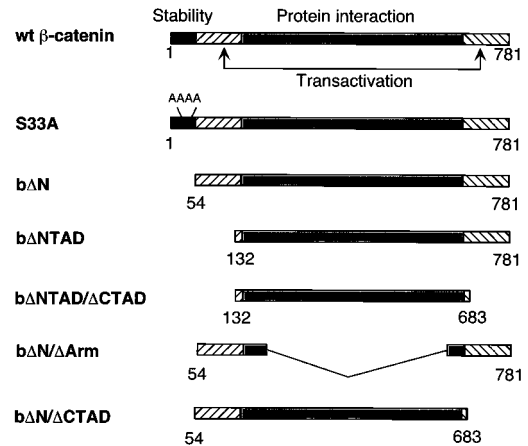


Figure 1 Schematic representation of β -catenin constructs. Approximate location of functional domains: black, protein instability; right-leaning hatch, N-terminal transactivation domain (NTAD); left-leaning hatch, C-terminal transactivation domain (CTAD); grey, armadillo repeats, protein–protein interaction. The S33A mutant carries alanine substitutions of the GSK3 β phosphorylation sites in the protein instability domain

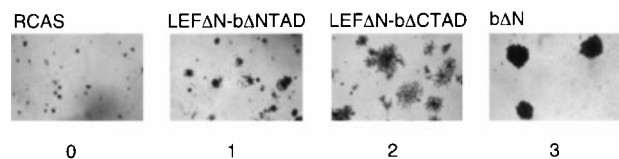


Figure 2 Transforming activity of β -catenin and LEF/ β -catenin fusion constructs. Transforming activity was scored by the size and morphology of the colonies. 0, no colonies, 1 indicates small colonies, 2 marks large colonies with reticulate morphology, and 3 refers to large, packed colonies. Typical examples of these colonies are shown

Table 1 Transforming potential of β -catenin constructs

Construct	Colony formation
wt β -catenin	0
S33A	3
b Δ N	3
b Δ NTAD	0
b Δ N/ Δ CTAD	2
b Δ N/ Δ Arm	0
b Δ NTAD/ Δ CTAD	0
Δ LN	0
Δ LN-bcat	2
Δ LN-b Δ N	3
Δ LN-b Δ NTAD	1
Δ LN-b Δ CTAD	2
Δ LN-b Δ N/ Δ Arm	2
Δ LN-bNTAD+	2
Δ LN-bCTAD	2
Δ LN Δ HMG-bCTAD	0
Δ LN-bNTAD	0
Δ LN-b Δ NTAD/ Δ CTAD	1
Δ LN-VP16	2

an anti-HA antibody and an anti-TCF antibody (Figure 3). The transforming activities of these constructs are summarized in Table 1. Δ LN does not induce anchorage independent growth by itself; fusion to wild-type β -catenin generates a moderately trans-

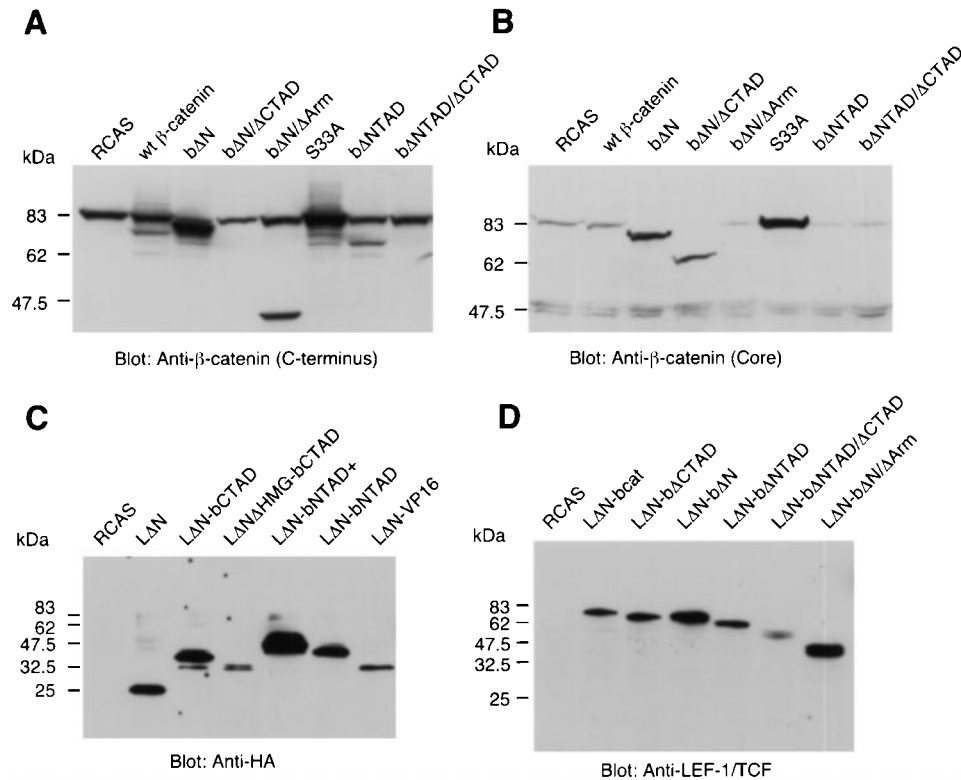


Figure 3 Expression of β -catenin and LEF/ β -catenin constructs in CEF. Cell lysates were analysed by Western blot with an anti- β -catenin antibody that recognizes the carboxyl terminus (a), the core region (b), an anti-HA epitope antibody (c) or with an anti-TCF antibody that recognizes the HMG domain of TCFs and LEF-1 (d)

forming construct, inducing colonies of reticulate morphology (Δ AN-bcat). The transforming activity of this construct suggests a stabilizing effect of Δ AN on the β -catenin component. Removal of the GSK3 β -phosphorylation sites enhanced transforming activity (Δ AN-b Δ N), and deletion of the NTAD of β -catenin greatly reduces, but does not entirely abolish transforming activity (Δ AN-b Δ NTAD). Removal of the CTAD has a less drastic effect, and again results in reticulate colonies (Δ AN-b Δ CTAD). Deletion of the armadillo repeats still permits transformation; the NTAD or CTAD by themselves have oncogenic activity if linked to Δ AN (Δ AN-bNTAD+ and Δ AN-bCTAD). Removal of the HMG DNA binding domain from Δ AN causes loss of transforming activity (Δ AN Δ HMG-bCTAD). Δ AN-VP16 served as a positive control. These data, obtained with the fusion constructs, are in accord with the results from the β -catenin deletion mutants, confirming the critical role of NTAD and the enhancing role of CTAD in transformation and demonstrating that the function of the armadillo repeats in transformation can be replaced by covalent linkage. DNA binding is essential for transformation. Two observations with the fusion constructs are unexpected: the inactivity of Δ AN-bNTAD and the low but significant oncogenicity of the armadillo repeats by themselves when linked to Δ AN (Δ AN-b Δ NTAD/ Δ CTAD). Possible explanations of these findings will be considered in the Discussion.

Transcriptional activation induced by the β -catenin constructs

Oncogenic transformation by β -catenin is believed to result from translocation of the protein in the nucleus where it activates transcription in association with LEF. Therefore, we determined transactivation induced by β -catenin mutants and the Δ AN- β -catenin fusion constructs. Reporter assays were performed in HEK293 cells using the TOPFLASH construct with four copies of the LEF/TCF binding site upstream of the minimal promoter and the firefly luciferase cDNA. We chose HEK293 cells because they showed low background activity using the TOPFLASH reporter as compared to CEF. We observed similar transactivation potentials of the β -catenin constructs in CEF, but detailed comparisons were difficult because of decreased sensitivity. Luciferase activities were calibrated by determining Renilla luciferase generated by co-transfected pRL-CMV reporter constructs. Wild-type β -catenin, b Δ N, b Δ NTAD produced similar intermediate values of transactivation. The Δ AN fusions with b Δ N, bCTAD and β -catenin were more active, and the S33A mutant was the best transactivator in this test, about three times more active than either wild-type β -catenin, b Δ N or b Δ NTAD (Figure 5).

The results show a very rough correlation between transactivation and transformation with some notable exceptions. The transforming activities of Δ AN-

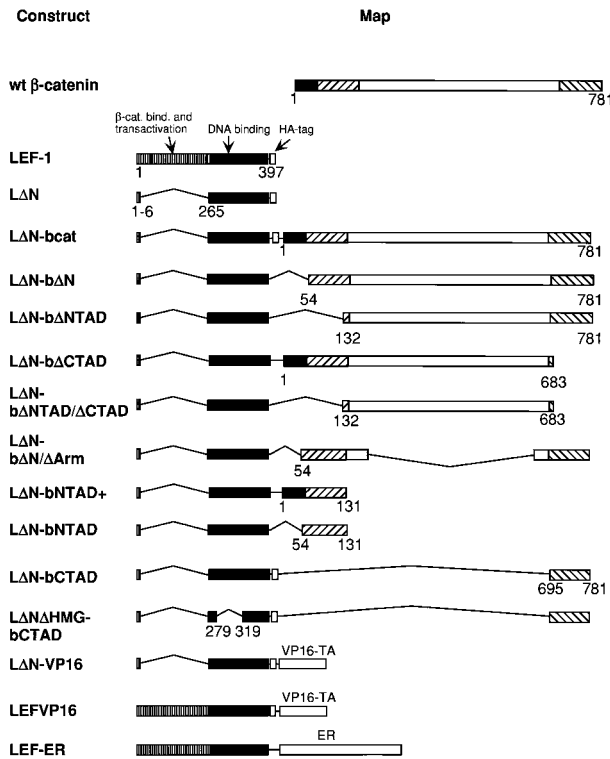


Figure 4 Schematic representation of LEF Δ N fusion constructs. Approximate location of functional domains: vertical hatch, β -catenin binding and context dependent transactivation domains; dark grey, HMG DNA binding domain; white, HA tag. VP16-TA: VP16 transactivation domain of herpes simplex virus. ER: the hormone-binding domain of the human estrogen receptor

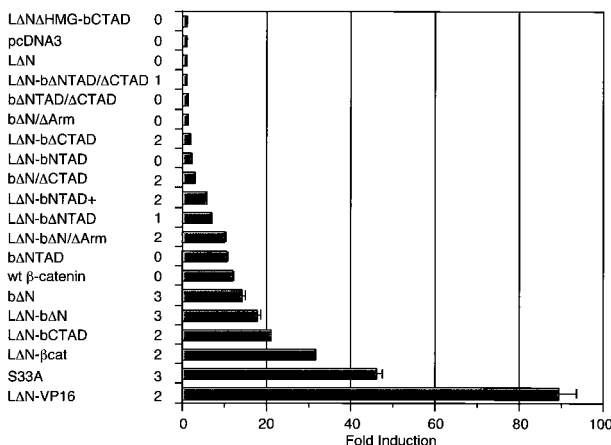


Figure 5 Activation of the TOPFLASH reporter by β -catenin constructs and LEF Δ N fusion constructs. Human HEK293 cells were transfected with the TOPFLASH reporter plasmid and pcDNA3 expression plasmids for various β -catenin and LEF Δ N fusion constructs. Luciferase activities were determined as described in Materials and methods and are expressed as fold induction relative to the reporter gene activity in control cells transfected with the reporter and empty pcDNA3 vector. The transforming activity is shown with the designations of the constructs

b Δ CTAD and b Δ N/ Δ CTAD are not backed up by corresponding levels of transactivation, and the lack of transforming activity seen with wild-type β -catenin and with b Δ NTAD contrasts with their ability to activate transcription. A possible explanation for some of these deviations could be provided by a peculiarity of the TOPFLASH reporter assay. It appears to measure primarily the activity of the β -catenin CTAD and less so that of the NTAD. For instance, Δ N-bCTAD shows threefold higher transactivation than Δ N-bNTAD+. Deletion of the CTAD leads to a greater reduction in transactivation in the TOPFLASH test than loss of the NTAD (compare Δ N-b Δ CTAD to Δ N-b Δ NTAD). Yet our deletion analysis has shown that it is the NTAD that is of principal importance for transformation, not the CTAD. It is also possible that the response of the reporter construct to the β -catenin mutants differs significantly from that of essential transformation-related target genes.

Expression of LEF/TCF target genes in β -catenin transformed CEF

Because of the imperfect correlation between transactivation as measured in reporter assays and transformation, we investigated the expression of LEF/TCF target genes, seeking targets whose differential transcription correlates with the transforming activity of the mutant β -catenin constructs.

We began by determining mRNA levels of two previously reported targets of LEF/TCF, cyclin D1 and Myc (Tetsu and McCormick, 1999; He *et al.*, 1998) in CEF expressing various transforming and non-transformation β -catenin constructs. Northern blots using 15 μ g total RNA from CEF stably transfected with the constructs were prepared and were hybridized with cyclin D1 and *c-myc* probes (Figure 6). GAPDH and cyclin D2 served as controls. Cyclin D1 was significantly upregulated by most transforming constructs with the notable exception of Δ N-b Δ CTAD, Δ N-bCTAD and Δ N-bNTAD+. Transcription of Myc was also stimulated by transforming compounds except again by Δ N-bCTAD and Δ N-bNTAD+. Several nontransforming compounds induced increased expression of Myc as well. mRNA levels of cyclin D2 were roughly the same in all transfected cells. Cyclin D2 is not affected by a dominant negative TCF and is probably not a target of LEF/TCF (Tetsu and McCormick, 1999). These results reveal a lack of correlation in the expression patterns of the two LEF/TCF targets cyclin D1 and Myc. They also show no concordance between differential expression of these target genes and transformation of CEF, or transactivation as measured in reporter assays.

The results suggest that cyclins D1 and Myc are not likely targets that play an essential role in the transformation of CEF by β -catenin. We therefore decided to search for new candidate targets differentially regulated by the β -catenin/LEF complex in CEF.

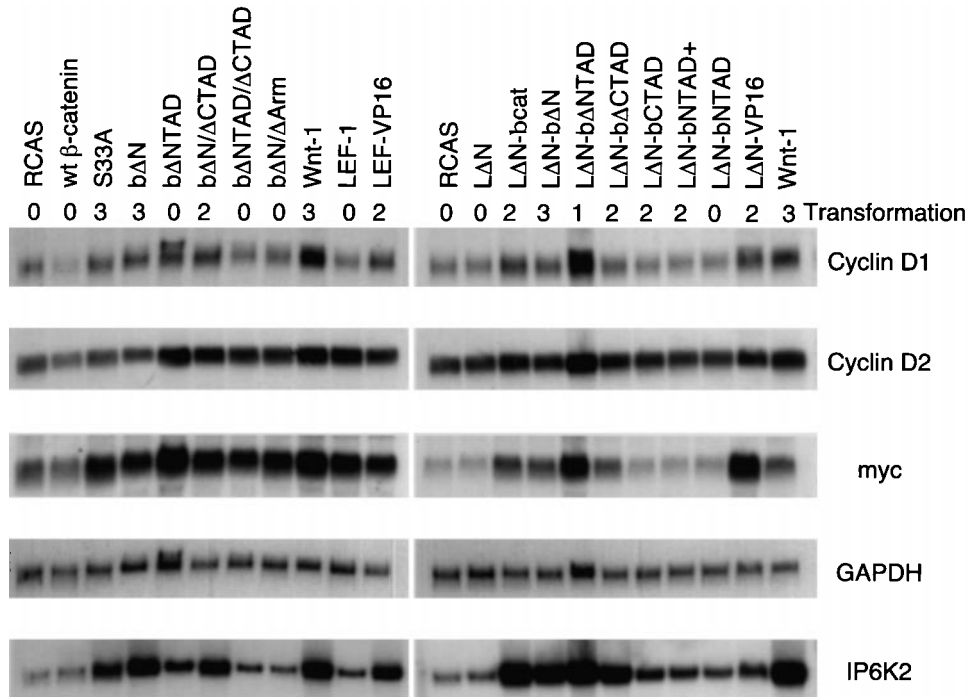


Figure 6 Expression of selected target genes of LEF/TCF in CEF transfected with various β -catenin/LEF constructs. Fifteen micrograms of total RNA from CEF transfected with various β -catenin/LEF mutants was analysed by Northern blot and probed with cDNA fragments of indicated LEF/TCF target genes. Transforming ability of the constructs is also shown. Blots for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown as loading control

Two candidate targets, IP6K2 and clone IX are regulated in concordance with the transforming activities of β -catenin constructs

Suppression subtractive hybridization was performed using mRNA from CEF infected with the empty RCAS vector as driver and mRNA from CEF transformed by Δ N-b Δ N as tester. The screen yielded six clones that appeared upregulated in the transformed cells. Of these, two could be confirmed in Northern blots. They are IP6K2, a homolog of human inositol hexakisphosphate kinase 2 and clone IX which shows no homology to a gene in the database. Both behaved identically in cells infected with the various β -catenin constructs. Figure 6 presents the data for IP6K2. There is a reasonable, albeit qualitative, correlation between transforming activity and upregulation of IP6K2. To test whether IP6K2 is a direct target gene of LEF-1, we used the hormone-inducible LEF-1 construct, LEF-ER, described previously (Aoki *et al.*, 1999). LEF-ER is a fusion of the LEF-1 coding region to the hormone-binding domain of the human estrogen receptor. The construct functions as a transactivator only in the presence of 17- β -estradiol (E2) which effects nuclear translocation and activation of the TAF2 transactivator domain contained in the ER components. LEF-ER not only transactivates in the presence of hormone, but also induces oncogenic transformation of CEF in a hormone dependent manner (Aoki *et al.*, 1999). As shown in Figure 7a, E2 treatment induced IP6K2

expression in LEF-ER transfected CEF but not in cells transfected with a construct expressing the hormone binding domain of the human estrogen receptor. There was a slight induction of IP6K2 by overexpressed LEF-1 but this was not hormone-dependent. A time course of IP6K2 induction by E2 in LEF-ER transfected CEF (Figure 7b) shows maximal levels by 3 h and a slight decline at 24 h. Treatment of the cultures with cycloheximide did not interfere with the induction of IP6K2 by LEF-ER/E2 combination, showing that the process is independent of *de novo* protein synthesis and suggesting that IP6K2 is a direct target gene of LEF-1 in CEF.

Discussion

The literature contains inconsistent and sometimes conflicting data on the oncogenic transforming activity of β -catenin. Positive as well as negative results have been reported for NIH3T3 cells transfected with stabilized versions of β -catenin (Kolligs *et al.*, 1999; Whitehead *et al.*, 1995). L cells are transformable with such constructs (Nagasawa *et al.*, 1999), Rat-1 cells are not (Young *et al.*, 1998), although the latter can be transformed by Wnt-1 which presumably functions by activating β -catenin-LEF mediated transcriptional activation (Young *et al.*, 1998). Among epithelial cells, RK3E, rat kidney epithelial cells immortalized by E1A, are transformable by stabilized β -catenin (Kolligs *et al.*, 1999), but IEC-18 rat intestinal epithelial cells or 1811

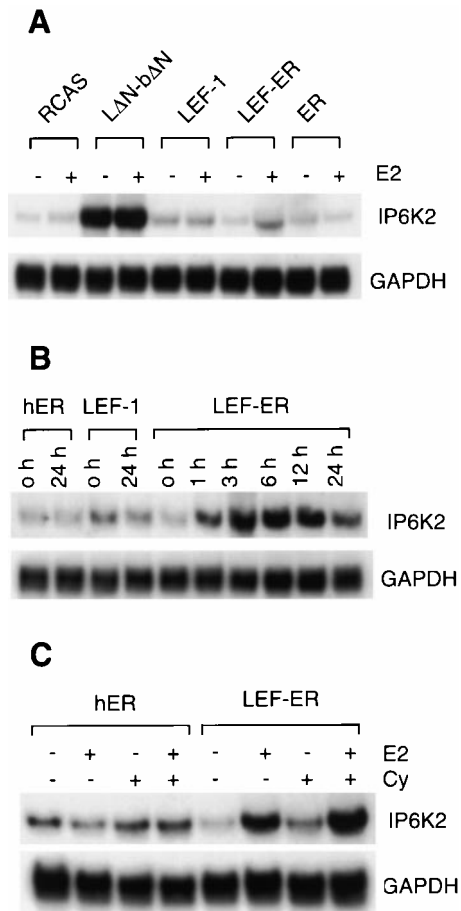


Figure 7 IP6K2 expression is regulated by LEF-1. The LEF-ER fusion protein can act as a transcriptional activator only in the presence of estrogen. (a) CEF transfected with RCAS vector, LEF Δ N-b Δ N53, LEF-1, LEF-ER or hER (human estrogen receptor) were treated or not with 2 μ M 17- β -estradiol (E2) for 6 h. Total RNA was analysed by Northern blot using 32 P-labeled IP6K2 cDNA or GAPDH as a probe. (b) Time course of IP6K2 induction by LEF-ER. CEF transfected with indicated constructs were treated or not with 2 μ M E2 for various time periods. Total RNA from each indicated time point was analysed by Northern blot as in (a). (c) LEF-ER induces IP6K2 in the absence of protein synthesis. CEF transfected with hER or LEF-ER were treated or not with E2 in the presence or absence of 50 μ g/ml cycloheximide (Cy). Total RNA was analysed by Northern blot

human epithelial cells are not (Kolligs *et al.*, 1999). MDCK (Madin-Darby canine kidney) cells are sensitive to transformation by wild-type and some stabilized β -catenin constructs (Orford *et al.*, 1999) but other β -catenin mutants known to produce a stabilized protein have failed to transform MDCK cells (Barth *et al.*, 1999). A general conclusion supported by these data would be that stabilized β -catenin can transform mammalian fibroblasts and epithelial cells but that different sublines of the same cell can vary greatly in their susceptibility to transformation suggesting the requirement for additional genetic changes not present in every cell line tested. All of the mammalian cells are from continuous lines and have undergone genetic changes leading to loss of growth regulatory mechanisms. In the RK3E cells immortalization by E1A would

have eliminated RB function. In contrast, CEF are primary, non-immortal cells that were tested here after a single subculture, a time interval that is insufficient for selection of genetic variants. They are readily transformed by β -catenin that carries a stabilizing mutation but are not affected by overexpressed wild-type β -catenin. This result suggests that the Wnt signaling pathway is effectively regulated in CEF by proteolytic cleavage of β -catenin and that elimination of this single control is sufficient to induce aberrant growth.

Oncogenic β -catenin is translocated into the nucleus where it associates with LEF/TCF proteins to form a transcriptional activator (Morin, 1999; Polakis, 1999). The deletions and chimeric constructs analysed in this study single out a few distinct domains of β -catenin as important in transformation. Both the NTAD and CTAD can mediate transformation when linked to a DNA binding domain. In deletion constructs, removal of the NTAD abolishes transforming activity, loss of the CTAD leaves a residual oncogenic potential. The data suggest that for transformation of CEF, the NTAD is the more important transactivation domain. The lack of transforming activity of b Δ NTAD might also be explained by the lower expression level of this mutant as compared to that of S33A or b Δ N (Figure 3a). However, previous reports suggest that β -catenin signaling activity is not regulated by protein level. Guger and Gumbiner (2000) demonstrated higher specific signaling activity for β -catenin with mutations in the GSK phosphorylation sites. The enhanced activity was not linked to increased levels of the mutant proteins. Young *et al.* (1998) also showed that the S37A mutant (similar to our S33A mutant) signals more effectively than wild type without accumulating to high levels. Staal *et al.* (2002) showed that Wnt signals are transduced by N-terminally dephosphorylated β -catenin and are not controlled by the absolute levels of β -catenin. The reason for the lower expression level of b Δ NTAD is unclear, but it may reflect the lack of growth advantage in the cells expressing this mutant in contrast to those expressing S33A or b Δ N. The importance of the N-terminal transactivation domain is also supported by the LAN fusion constructs. The fusion of the LEF DNA binding domain to the N-terminal transactivation domain of beta catenin (LAN-b Δ CTAD) was more potent in transformation than the fusion to the C-terminal transactivation domain (LAN-b Δ NTAD) while both constructs were expressed at similar levels (Figure 3d). Work with RK3E cells has shown greater importance of the CTAD (Kolligs *et al.*, 1999). The CTAD and NTAD bind to different domains of the co-activator CBP/p300 (Hecht *et al.*, 2000; Sun *et al.*, 2000; Takemaru and Moon, 2000). These and additional interactions may differ in CEF and immortalized rat cells. Our results also point out an obvious limitation of the deletion analysis: deletions can induce undefined conformational and hence functional changes in the residual molecule. For instance, the construct LAN-bNTAD contains the entire NTAD of β -catenin but fails to transform

probably because of such a conformational change. The low but significant oncogenicity of the armadillo repeats fused to LEF recalls earlier studies that reported two transactivation regions in this domain (Hecht *et al.*, 1999). An alternative explanation for invoking the presence of transactivation domains within the armadillo repeats would be to postulate that these repeats recruit an external transactivation domain.

Oncogenic transcription factors in general show only a very imperfect correlation between transcriptional activation as measured in reporter assays and transformation. Particularly striking examples are known from Jun (Vogt, 2001), and LEF/*β*-catenin is no exception to this rule with constructs that are highly transforming but poorly transactivating and potent transactivators that fail to transform. These data do not diminish the importance of transcriptional activation in transformation, rather they suggest that reporter assays cannot accurately reflect the transcriptional regulatory activities that are essential in transformation.

The identification of target genes is one of the central problems for the understanding of oncogenic transcription factors. Not all differentially regulated genes will have an effect on cellular growth behavior, and techniques are needed to discriminate between targets that are essential for the transformation process and innocent bystanders. Essential targets should be differentially regulated by *all* transforming constructs of an oncoprotein.

Two proteins differentially regulated by LEF/TCF have been proposed as targets relevant for oncogenesis, Myc and cyclin D1 (He *et al.*, 1998; Tetsu and McCormick, 1999). LEF/TCF binding sites are present in the promoters of the corresponding genes. In colon cancer cell lines with mutations in the APC/*β*-catenin pathway, APC downregulates Myc, and dominant negative TCF downregulates cyclin D1, accompanied by growth arrest (He *et al.*, 1998; Tetsu and McCormick, 1999). However, *in vitro* transformation studies with RK3E cells have shown that Myc is not invariably upregulated after *β*-catenin induced transformation and therefore may not be an essential target (Kolligs *et al.*, 1999). This conclusion is reinforced by our studies in CEF which show that the transforming constructs Δ N-bCTAD and Δ N-bNTAD+ fail to induce Myc. Cyclin D1 is also not upregulated in CEF by transforming constructs Δ N-bCTAD and Δ N-bNTAD+ and is only marginally upregulated by Δ N-bACTAD and the highly oncogenic S33A. A possible explanation could be derived from the fact that the constructs with no effect on Myc or cyclin D1 are only moderately (2+) transforming. For a fully transformed phenotype, upregulation of Myc and cyclin D1 may still be important. In the absence of this differential expression, a partially transformed phenotype could ensue from the action of other LEF/TCF targets.

Two novel targets of LEF/*β*-catenin are IP6K2 and the as yet unidentified clone IX. These meet the criteria

set out above for transformation relevant genes, being upregulated by all transforming constructs. IP6K2 was originally identified as a protein that affects Na⁺ uptake and is therefore also known as inorganic phosphate uptake stimulator (Norbis *et al.*, 1997). IP6K2 is responsible for the biosynthesis of diphosphoinositol polyphosphates from inositol hexakisphosphate (IP6) (Saiardi *et al.*, 1999; Schell *et al.*, 1999). These compounds may play a role in signal transduction (Safrany *et al.*, 1999). IP6 is associated with decreased iron absorption and lowered cancer rates. It reduces cell proliferation and increases expression of p53 and of p21WAF1 (Shamsuddin, 1999). IP6 has also been shown to inhibit mammary tumor development in rats (Shamsuddin and Vucenic, 1999) and to induce reversion of the human hepatocellular carcinoma cell line HepG2 (Vucenic *et al.*, 1998a,b). Overexpression of IP6K2 may reduce cellular IP6 levels, and this, in turn, may contribute to the transformed phenotype. Increased IP6K2 has also been observed in CEF transformed by the Ras, Src, Mos, Crk, and Fps oncoproteins (Aoki *et al.*, unpublished observation), but IP6K2 has also been linked to growth suppression (Morisson *et al.*, 2001).

So far all targets for LEF/*β*-catenin are 'candidate' targets. The ultimate tests for their relevance will be functional. These tests will have to demonstrate transforming activity for a target alone or in combination with other targets. Maintenance of the transformed phenotype should depend on differential expression of all essential targets, and normal level expression of a single essential target should result in a reduction or reversion of oncogenic properties.

Materials and methods

Culture of chicken embryo fibroblasts (CEF) for transfection and transformation assays

CEF cultures were prepared from White Leghorn embryos obtained from SPAFAS (Preston, CT, USA). DNA was transfected into CEF by using the dimethyl sulfoxide/polybrene method (Kawai and Nishizawa, 1984). For soft agar colony assays, the cultures were passaged three times and then seeded in nutrient agar consisting of 0.3% Sea Plaque agar in medium F-10 supplemented with 5% donor calf serum, 5% chicken serum, 3 mg/ml tryptose phosphate broth. Cells were fed with the same nutrient agar every third day. Agar colonies were scored and photographed after 3 weeks. For hormone treatment of CEF, the cultures were treated with 2 μ M 17 β -estradiol (Sigma) in the presence or absence of 50 ng/ml cycloheximide as described (Kruse *et al.*, 1997).

Plasmid construction

Maps and designation of the *β*-catenin constructs are shown in Figures 1 and 4. Wild type (wt) mouse *β*-catenin and the constructs S33A, LEF-1, LEF1-ER, LEF-VP16, Δ N-VP16, Δ N-bcat, Δ N-b Δ N, Δ N-bCTAD, Δ N Δ HMG-bCTAD, and Δ N-bACTAD have been described previously (Aoki *et al.*, 1999; Vlemminckx *et al.*, 1999). The S33A mutant harbors alanine substitutions at the GSK-3 β phosphorylation sites, residues 33, 37, 41 and 45. b Δ N lacks the first 53 amino acids

of β -catenin, and was constructed by digestion of pBSFI- β -catenin with *NotI/Bsu36I* followed by ligation with adaptor oligonucleotides Δ N53-1 and Δ N53-2 (see below). bANTAD with a deletion of the first 131 amino acids of β -catenin including the N-terminal transactivation domain (NTAD) was assembled by PCR using the primers ATG Δ N131 and 3'- β cat. bANTAD/ Δ CTAD lacking both the NTAD and the C-terminal transactivation domain (CTAD) was put together by digestion of pBSFI-bANTAD with *SacI/ApaI* followed by ligation with the *SacI/ApaI* fragment of pBSFI-b Δ N/ Δ CTAD. b Δ N/ Δ Arm is b Δ N with a deletion in the armadillo repeats, and was generated by digestion of pBSFI-b Δ N with *PmlI/ApaI* followed by ligation with the *FspI/ApaI* fragment of pBSFI-b Δ N. b Δ N/ Δ CTAD is b Δ N with a deletion of the CTAD, and was prepared by digestion of pBSFI-LAN-b Δ CTAD with *NotI/SpeI* followed by ligation with the *NotI/SpeI* fragment of pBSFI- Δ N.

Construction of LAN-bcat, LAN fused to the wt β -catenin, and LAN-b Δ N, LAN fused to b Δ N, were described previously (Aoki *et al.*, 1999). LAN-bANTAD, LAN fused to amino acids 132–781 of β -catenin, was made by PCR using the AatHA Δ N131 primer and the 3'- β cat primer followed by ligation of the *AatII/StuI* fragment of the PCR products with *AatII/StuI*-digested pBSFI-LAN-bcat. LAN-b Δ CTAD with a deletion of the CTAD was described previously (Hecht *et al.*, 1999). LAN-bANTAD/ Δ CTAD was produced by digestion of pBSFI-LAN-bANTAD with *SacI/ApaI* followed by ligation with *SacI/ApaI* digested pBSFI- Δ N/ Δ CTAD. LAN-b Δ N/ Δ Arm, LAN-b Δ N without the armadillo repeats, resulted from digestion of pBSFI-LAN-b Δ N with *SphI/ApaI* followed by ligation with *SphI/ApaI* fragment of pBSFI- Δ N/ Δ Arm. LAN-bNTAD+ containing amino acids 1–131 of β -catenin was built by PCR using the AatHA1-8 primer and the 124–131RI primer followed by ligation of the *AatII/EcoRI* fragment of the PCR products with *AatII/EcoRI* digested pBSFI-LAN. LAN-bNTAD, LAN with amino acids 54–131 of β -catenin, was generated in the same manner using the AatHA54-61 primer instead of the AatHA1-8 primer. Construction of LAN-bCTAD containing the CTAD and LAN Δ HMG-bCTAD with a deletion of the HMG DNA binding domain were previously described (Hecht *et al.*, 1999). LAN-VP16 is a fusion of LAN with the VP16 transactivation domain of herpes simplex virus, and LEF-VP16 consists of full length LEF-1 fused to the VP16 transactivation domain. LEF-ER is the fusion construct of LEF-1 with the hormone-binding domain of the human estrogen receptor (ER). These constructs were previously described (Aoki *et al.*, 1999). The *SfiI* fragments carrying these constructs were subcloned into the replication-competent avian retroviral vector RCAS.Sfi as described (Aoki *et al.*, 1998). For reporter assays, the *SfiI* fragments were subcloned into pcDNA3.Sfi, which was constructed by digestion of the mammalian expression vector pcDNA3 (Invitrogen, CA, USA) with *HindIII/XbaI* followed by ligation with the adaptor oligonucleotides pc3Sfi1 and pc3Sfi2.

The sequences of the primers are as follows: Δ N53-1, 5'-GGCCACCATGGC; Δ N53-2, 5'-TCAGCCATGGT; ATG Δ N131, 5'-GCTCGCGCCGCCATGTTGAAACATGCAGTTGTC; 3'- β cat, 5'-CCCAGAAGCTGCACTAGAGTCC; AatHA1-8, 5'-GCTCGACGTCCCTGACTATGCCAGCCTGGCTACTCAAGTGTGACCTGATG; AatHA54-61, 5'-GCTCGACTGCCCTGACTATGCCAGCCTGGAGGAGAAGATGTTGACACCTCC; 124-131RI, 5'-GCTCGAATTCTCACATCTGTGATGGTTTCAGCCAAGC; AatHA132-139, 5'-GCTGGACGTCCCGTACTATGCCAGCCTGTTGAAACATGCAGTTGTCAATTTG; pc3Sfi1, 5'-

AGCTTGGCCATTAGGGCCGATGATGACGACGGCC-GCCTCGGCCT; pc3Sfi2, 5'-CTAGAGGCCGAGGCGGCCGTCGTCATCATCGGCCCTAATGGCCA.

Transcriptional activation

Reporter assays were performed using the human kidney cell line 293 (ATCC) maintained in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (Intergen). Cells were seeded into MP-12 tissue culture plates at 2.5×10^5 cells per well. On the next day, the cultures were transfected with 0.4 μ g of TOPFLASH firefly-luciferase reporter, 1.6 μ g of pcDNA3 expression vector carrying β -catenin or LEF constructs, and 20 ng of pRL-CMV, a Renilla-luciferase construct, as internal control. After 40 h incubation, the cultures were washed once with PBS, and then lysed in 250 ml of Passive Lysis Buffer (Promega) according to the manufacturer's protocol. Firefly luciferase activities and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) with a Berthold Biolumat model LB 9501. Firefly luciferase activities were normalized against the Renilla luciferase activities.

Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5/150 mM NaCl/10% glycerol/1% Nonidet P-40/10 mM NaF/1 mM sodium pyrophosphate/1 mM sodium orthovanadate) containing protease inhibitors (Complete, Boehringer Mannheim). Lysates containing 60 μ g of protein were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore). The membranes were blocked with 5% nonfat dry milk/Tris-buffered saline/0.05% Tween-20 and then probed with anti- β -catenin (Clone 14, Transduction Laboratories or Clone 9G10, Calbiochem) to detect β -catenin, and anti-HA (HA.11, Covance) or anti-TCF (Clone REMB6, Exalpha) to detect LEF Δ N fusion constructs. After incubation with horseradish peroxidase-conjugated secondary antibody (Pierce), bound proteins were detected by incubation with a chemiluminescent substrate (Pierce) according to the manufacturer's protocol.

Northern blot analysis

Transfected CEF were lysed with the RNA STAT-60 reagent (Tel-Test, Friendswood, TX, USA), and total RNA was isolated following the manufacturer's protocol. Fifteen micrograms of total RNA was separated by gel electrophoresis and then transferred onto Hybond-N membranes (Amersham) as described previously (Fu *et al.*, 1999). Hybridization was carried out at 42°C overnight in Hood buffer containing 50% formaldehyde/5 \times SSC/20 mM Na₂HPO₄/NaH₂PO₄, pH 6.7/7% SDS/1% polyethylene glycol (molecular weight, 20000) and 0.5% bovine albumin. The filters were then washed three times for 15 min with 0.2 \times SSC/0.1% SDS at 55°C, and autoradiographed. Hybridization probes were prepared from purified DNA fragments that were labeled with [³²P]dCTP by using a random primed DNA labeling kit (Boehringer Mannheim).

Suppression subtractive hybridization

Poly-A mRNA was isolated from total RNA prepared from CEF transfected with LEF Δ N- Δ N53 or vector alone using the Oligotex mRNA Midi Kit (Qiagen). cDNA was synthesized with TimeSaver cDNA synthesis kit (Pharmacia). Suppression subtractive hybridization was performed using

the PCR-select cDNA Subtraction Kit following the manufacturer's protocol (Clontech). For the forward subtraction, cDNA from LEFAN- Δ N53 transfected cells was used as tester and cDNA from vector transfected cells as driver, and for the reverse subtraction, cDNA from vector transfected cells was used as driver and cDNA from LEFAN- Δ N53 transfectants as tester. Two hundred and four colonies were randomly picked from the forward-subtracted cDNA library, and colony PCR was performed. The PCR products were subjected to slot blot analysis using the forward-subtracted and the reverse-subtracted probes. The clones that hybridized

with the forward-subtracted probe but not with the reverse-subtracted probe were analysed further by Northern blot analysis.

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