Distinct molecular forms of β-catenin are targeted to adhesive or transcriptional complexes

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Introduction

β-Catenin is a cytoplasmic protein that plays essential roles in two different cellular processes: calcium-dependent intercellular adhesion and Wnt-mediated transcriptional activation (for review see Gottardi et al., 2002). For cell–cell adhesion, β-catenin binds the cytoplasmic domain of cadherin adhesion receptors along with the actin binding protein, α-catenin, to bridge the extracellular adhesive activity of cadherins with the underlying actin cytoskeleton (Rimm et al., 1995). This cadherin-bound pool of β-catenin ultimately serves to link the cytoskeletal networks of adjacent cells, which is considered essential for normal tissue architecture and morphogenesis (for review see Gumbiner, 2000). For nuclear signaling, β-catenin binds the DNA binding factor, T-cell factor (TCF)–Lef, and together this complex recruits components of the general transcriptional machinery (e.g., TATA binding protein), as well as proteins involved in chromatin modification and remodeling (e.g., the histone acetyltransferase, p300/CBP and the ATPase, Brg-1) to coordinate the activation of gene targets (Hecht et al., 1999, 2000; Barker et al., 2001; Tutter et al., 2001). How β-catenin binding to cell surface cadherins or DNA binding proteins is regulated, and the relationship between these two functions, remain to be explored.

Why might the cell use a single protein for both cell–cell adhesion and nuclear signaling? One possibility is that signaling and adhesion are tightly coordinated through competition for a common pool of β-catenin. Indeed, some evidence suggests that the signaling and adhesive pools of β-catenin are interrelated in this way. Overexpression of cadherins in Xenopus and other systems can antagonize β-catenin signaling activity (Heasman et al., 1994; Fagotto et al., 1996; Sanson et al., 1996; Orsulic et al., 1999), whereas reduction in the expression of cadherins in Drosophila embryos can enhance β-catenin signaling (Cox et al., 1996). Although experimental manipulation of cadherin levels can affect β-catenin nuclear signaling, it is not yet clear whether β-catenin signaling in vivo is actually regulated by changes in endogenous cadherin levels or function. Moreover, there is also evidence that these two processes can occur largely independently of each other. For example, cadherin loss-of-function is not often associated with enhanced β-catenin signaling (Caca et al., 1999; Vasioukhin et al., 2001), and Wnt activation does not typically alter cell–cell adhesion, although enhanced adhesive activity has been reported previously (Bradley et al., 1993; Hinck et al., 1994). Thus, how the roles of β-catenin in signaling and adhesion are controlled to be either coordinated or independent remains to be elucidated.

In the cases where expression of cadherins antagonizes β-catenin nuclear signaling activity, inhibition occurs by direct binding of β-catenin to the cytoplasmic domain of cadherins, and...
sequestration from the nuclear compartment (Fagotto et al., 1996; Orsulic et al., 1999; Shutman et al., 1999; Gottardi et al., 2001). Recent evidence argues that the signaling and adhesive forms of β-catenin may share molecularly similarities. For example, comparison of β-catenin–cadherin and β-catenin–TCF cocystal structures revealed that both β-catenin ligands bind to extensively overlapping regions along β-catenin, and in some regions engage identical residues (for review see Gottardi and Gumbiner, 2001). Given such similarity in binding mechanism between β-catenin–cadherin and β-catenin–TCF complexes, we proposed that the cadherin is such a favorable inhibitor of β-catenin nuclear signaling, not only because it sequesters β-catenin away from the nucleus, but also because it can compete directly with TCF for β-catenin binding (Gottardi and Gumbiner, 2001).

Nonetheless, the functions of β-catenin can be molecularly distinct. For example, C. elegans uses three different β-catenin gene products for adhesion and signaling functions (Korswagen et al., 2000). BAR-1 mediates Wnt signaling by forming a transcription complex with the TCF homologue, POP-1, whereas HMP-2 interacts exclusively with the cadherin gene product, HMR-1. WRM-1 is involved in a divergent Wnt pathway where it regulates POP-1 indirectly. Although this simple organism has evolved three distinct genes to segregate adhesive from signaling forms of β-catenin, there is no evidence for multiple β-catenin gene products in vertebrates. A splice variant of β-catenin lacking the COOH-terminal transactivation domain has been identified in Drosophila, however, which seems to function only in adhesion (Loureiro and Peifer, 1998). Vertebrates, therefore, must somehow be able to rely on a single β-catenin gene product for both adhesion and signaling functions.

The notion of a single molecular form of β-catenin that participates identically in cell adhesion and gene expression seems to be over simplified. In SW480 tumor cells, a large fraction of β-catenin was found to be refractory to both cadherin- and TCF-binding in vitro (Gottardi et al., 2001). This is due, at least in part, to the interaction of β-catenin with a 9-kD polypeptide, ICAT (Tago et al., 2000), which can prevent β-catenin binding to both TCF and cadherin proteins (Gottardi and Gumbiner, 2004). The small fraction of β-catenin that can bind to TCF also can interact with the cadherin, which explains how cadherin expression inhibits β-catenin–TCF signaling and cell growth in this cell line (Gottardi et al., 2001). Thus, cells contain at least two distinct forms of β-catenin, raising the possibility that the binding properties of β-catenin may be regulated. These distinct forms were identified, however, in a tumor cell line harboring a mutation in the adenomatous polyposis coli (APC) tumor suppressor gene product, which is known to deregulate the normal degradation of β-catenin in cells. Therefore, we sought to examine the regulation of β-catenin binding properties by its physiological regulator, Wnt.

## Results

**Wnts regulate β-catenin binding specificity**

We sought to determine the binding properties of β-catenin generated by Wnt signaling. Using an in vitro pull-down assay, we find that cytosolic β-catenin from cells stably expressing Wnt1 shows preferential binding to TCF-GST compared with a cadherin cytoplasmic domain-GST fusion protein (Fig. 1 A, lanes 1–4). The selective binding activity of β-catenin does not require stable, long-term expression of Wnt in cells, as similar binding properties are observed in human embryonic kidney (HEK) 293T cells incubated with Wnt3a-conditioned media for short periods (Fig. 1 A, lanes 5 and 6). Cytosolic β-catenin from C57MG parental cells incubated with Wnt3a-conditioned media (CM) shows preferential binding to TCF-GST proteins equivalently, like the Rat1 and HEK293 controls (not depicted). The blot was probed with a pAb to β-catenin. (B) Preferential binding of β-catenin to TCF-GST over cadherin–GST is not observed with purified, recombinant β-catenin. Recombinant, purified Xenopus β-catenin (Suh and Gumbiner, 2003) and β-catenin from a C57MG/Wnt cytosolic fraction were affinity precipitated with cad-GST and TCF-GST proteins, and blotted with an antibody to β-catenin.

**β-Catenin binding selectivity is determined by its COOH-terminal region**

Several findings influenced our investigation of a mechanism that could generate a form of β-catenin that binds selectively to TCF. The COOH terminus of β-catenin can interact with the armadillo repeat region of β-catenin (Cox et al., 1999; Piedra et al., 2001) and compete with β-catenin binding to the cadherin cytoplasmic domain in vitro (Castano et al., 2002). These ob-

Figure 1. Wnt signaling generates a form of β-catenin that binds preferentially to TCF-GST compared with cadherin-GST. (A) Detergent-free supernatants were prepared from C57MG and Rat1 cells stably expressing Wnt1, and HEK293T cells incubated overnight in Wnt3a-conditioned media (CM). Samples were affinity precipitated using equimolar amounts of cad-GST or TCF-GST fusion proteins. GST gives no binding and is not depicted. A fivefold excess of parental cell lysates was required to detect a signal in lanes 5 and 6. Cytosolic β-catenin from C57MG parental cells incubated with Wnt3a-conditioned media (CM) shows preferential binding to TCF-GST proteins equivalently, like the Rat1 and HEK293 controls (not depicted). The blot was probed with a pAb to β-catenin. (B) Preferential binding of β-catenin to TCF-GST over cadherin–GST is not observed with purified, recombinant β-catenin. Recombinant, purified Xenopus β-catenin (Suh and Gumbiner, 2003) and β-catenin from a C57MG/Wnt cytosolic fraction were affinity precipitated with cad-GST and TCF-GST proteins, and blotted with an antibody to β-catenin.
To determine whether the COOH-terminal region of endogenous β-catenin exhibits a different conformation as a result of Wnt signaling, we examined whether epitopes are masked in the TCF-selective, closed form. The ability of anti–NH2- and COOH-terminal–directed mAbs to immunoprecipitate β-catenin after affinity depletion by cad-GST was examined (Fig. 2 D). Although β-catenin is immunoprecipitated similarly by the NH2- and COOH-terminal antibodies from the total starting cytosol (lanes 6 and 7), when the cadherin binding fraction is first depleted by the cadherin-GST, the remaining β-catenin is poorly immunoprecipitated by the COOH-terminal antibody compared with the NH2-terminal antibody (compare lanes 4 and 5). These antibodies, therefore, recognize distinct forms of β-catenin (see Fig. 5 C), which may differ as a result of a conformational change in the COOH terminus of β-catenin that alters β-catenin binding to cadherins. Thus, the COOH-terminal region of β-catenin is more accessible in the cadherin-binding fraction of β-catenin, and much less accessible in the nonbinding fraction.

To further determine the regions of β-catenin control this binding selectivity, we also examined the binding properties of a series of β-catenin deletion mutants expressed in cells. Full-length NH2-terminal myc-, or COOH-terminal flag-tagged β-catenin exhibits preferential binding to TCF-GST relative to cad-GST when transfected into HEK293 cells incubated with Wnt3a-conditioned media (Fig. 3). Importantly, the level of exogenous β-catenin expression needed to be kept low in order to detect β-catenin binding selectivity (compare 0.2 μg with 2 μg plasmid), suggesting that the cellular machinery responsible for generating binding selectivity may be easily saturable. As a control, simply diluting the sample transfected with 2 μg plasmid by 10-fold, so that the β-catenin levels were similar to those extracted transfected with 0.2 μg plasmid, did not result in differential binding (unpublished data), indicating that binding selectivity is due to an active cellular process. A construct bearing a deletion of the COOH terminus (Δβ-catΔC695) shows equivalent binding to both cadherin and TCF, consistent with a role for the COOH terminus in regulating binding selectivity. Curiously, however, deletion of both NH2- and COOH-terminal domains generates a protein that binds TCF significantly better than the cadherin, even at relatively high levels of expression (e.g., compare 3 μg plasmid for Xβ-cat arm 12 with WTX-β-cat 2 μg). It was recently proposed that the NH2-terminal region of β-catenin may be required for efficient cadherin binding, as a GST-β-catenin fusion protein missing the first 119 aa showed little cadherin binding activity in vitro (Castano et al., 2002). We also find that Δ89α-catenin binds poorly to the cadherin compared with TCF, even at our highest expression levels (Fig. 3). Thus, we suggest that the NH2-terminal region of β-catenin is required for cadherin but not TCF binding, which gives rise to apparent binding selectivity. When β-catenin is able to bind the cadherin (i.e., when the NH2 terminus is present), however, the COOH terminus is required for generating β-catenin binding selectivity.

**Cadherin preferentially binds β-catenin–α-catenin complexes**

To better characterize the molecular forms of β-catenin that bind to cad-GST and TCF-GST fusion proteins, we examined...
cytosolic fractions by gel filtration chromatography (Fig. 4). We began our analysis with Xenopus embryos, because it was easier to obtain a large amount of cytosolic β-catenin from cell lysates (Fig. 4 A). One half of each column fraction was precipitated with TCA to show the total profile of β-catenin (Fig. 4 A, top blot); the other half was subjected to cadherin-GST affinity precipitation. β-Catenin eluted across a number of fractions extending from 66 kD (BSA standard, peak fraction 37) to 232 kD (catalase standard, peak fraction 30). The major peak corresponded to ~100 kD (fraction 35), suggesting that most of the cytosolic β-catenin is monomeric. Importantly, only fractions from the higher molecular mass “shoulder” of the broad β-catenin profile were able to interact with the cadherin cytoplasmic domain (Fig. 4 A, middle blot), whereas TCF could also bind the lower molecular sized fractions corresponding to the major peak (Fig. 4 B below and not depicted).

The higher molecular size fractions of β-catenin could be due to its association with α-catenin or other possible proteins. To see what proteins associate with β-catenin and bind to cad-GST and TCF-GST fusion proteins, we performed the binding assay using cytosol prepared from [35S]methionine and cysteine-labeling cells. Metabolic labeling of Wnt-expressing cells reveals α-catenin as the major binding partner of cytosolic β-catenin (Fig. 5 A, lanes 6 and 7). No other major bands were detected between the 10–200 kD molecular mass region by

Figure 3. Differential binding activity of recombinant β-catenin as revealed by deletion analysis. (A) Schematic representation of β-catenin constructs. WT-myc-Xenopus β-catenin and GSK3β mutant (S/T>A residues 33, 37, 41, and 43) β-catenin were described previously by Guger and Gumbiner (2000). WT-human β-catenin-Flag and ΔN98-Flag constructs were described in Kolliks et al. (1999). The myc-tagged, Xenopus β-catenin construct encoding only the arm repeat region of β-catenin was described previously by Funayama et al. (1995). The myc-tagged, Xenopus-β-catenin constructs. WT-myc-β-catenin and is shown below. (B) Recombinant β-catenin binding to cad-GST versus TCF-GST proteins. HEK293T cells were transfected with decreasing amounts of β-catenin plasmid and incubated in the presence (+) of Wnt3a conditioned media (CM). Cytosolic fractions were affinity precipitated and immunoblotted with anti-myc, -flag, or β-catenin antibodies. Input amounts of wild-type β-catenin, −ΔC695, and arm 12 constructs were the same in accordance with similar expression levels (not depicted).

Figure 4. Larger molecular size, α-catenin-containing fractions of β-catenin show preferential binding to cad-GST. (A) A cytosolic fraction from stage 12 Xenopus embryos was applied to a Sephacryl 300 gel filtration column, and fractions 28–39 were divided in two: one half of each sample was TCA-precipitated (top blot), whereas the other half was precipitated with cad-GST (middle blot). The top blot was reprobed with an antibody to α-catenin and is shown below. (B) Same as A except that starting material is an S100 fraction from Rat1/Wnt cells. Arrows refer to elution volumes of standard proteins with known molecular weight: (a) catalase (Mr = 232,000); (b) BSA (Mr = 66,000), purified mouse IgG (150 kD) eluted in fractions 31–33.
[35S]methionine/cysteine labeling or Coomassie staining (unpublished data). The cadherin-GST appears to affinity precipitate β-catenin and α-catenin bands at a ratio ~1:1, whereas the TCF-GST precipitates much more β-catenin than α-catenin (band ratios of ~3:1; Fig. 5 A, lanes 8 and 9). Given that α-catenin and β-catenin contain nearly identical numbers of cysteine and methionine residues (40 and 41, respectively), and that the labeling time is long (13 h) compared with the half lives of both proteins (7 and 5 h, respectively; unpublished data), it appears that the cadherin binds a stoichiometric complex of β-catenin–α-catenin. We conclude, therefore, that the cadherin preferentially binds β-catenin that is associated with α-catenin, whereas TCF can bind monomeric β-catenin in addition to β-catenin–α-catenin dimers.

We determined in Fig. 2 D that a COOH-terminal epitope of β-catenin was inaccessible in the fraction of β-catenin that could not interact with the cadherin. Because our experiments in Figs. 4 and 5 A showed that β-catenin–α-catenin dimers preferentially interact with the cadherin, we asked whether this COOH-terminal epitope of β-catenin was accessible in the β-catenin–α-catenin dimer fraction, and masked in the form of β-catenin that preferentially binds to TCF. To do so, we immunoprecipitated β-catenin from metabolically labeled, Wnt1-expressing cells using the NH2- and COOH-terminal monospecific antibodies previously shown to recognize distinct forms of β-catenin (Fig. 2 D). The antibody that recognizes the COOH terminus of β-catenin appears to preferentially immunoprecipitate β-catenin and α-catenin bands at a ratio of ~1:1, whereas the antibody that recognizes the NH2 terminus of β-catenin immunoprecipitates significantly more β-catenin than α-catenin (Fig. 5 B, lanes 1–4). Thus, the NH2-terminal antibody can immunoprecipitate β-catenin monomers as well as β-catenin–α-catenin dimers. Because the COOH-terminal antibody preferentially immunoprecipitates β-catenin–α-catenin dimers, the COOH terminus in most of the monomeric fraction of β-catenin is masked. To determine whether this inaccessibility of the COOH-terminal epitope was due to a more general masking of the COOH terminus, we examined the binding of β-catenin to the PDZ-containing protein, Lin7. The extreme COOH terminus of β-catenin contains a PDZ interaction motif, and has been shown previously to bind Lin7 (Perego et al., 2000). Similar to the COOH-terminal antibody findings, Lin7 affinity precipitates β-catenin and α-catenin bands at a ratio of ~1:1, suggesting that it preferentially interacts with β-catenin–α-catenin dimers. Together, these results show that the COOH-terminal region of β-catenin is accessible in the β-catenin–α-catenin dimer fraction, and is masked in the monomeric fraction, which is enriched in the TCF-selective form of β-catenin.

**Effect of APC mutations, inhibition of GSK-3β activity, NH2-terminal phosphorylation of β-catenin, and cadherin phosphorylation on β-catenin binding specificity**

To explore the signaling pathways that might control these forms of β-catenin, we sought to examine the role of the APC tumor suppressor gene product and glycogen synthase kinase (GSK)-3β, two key components in the Wnt pathway, as well as the role of known phosphorylations of β-catenin in response to Wnts. We examined several colon carcinoma cell lines that contain either wild-type or mutant forms of APC (Fig. 6 A). All of these cell lines manifest constitutive β-catenin signaling due to inactivating mutations in APC (HT29, DLD1), or activating mutations within the GSK-3β regulatory region of β-catenin (HCT116). No differences in β-catenin binding to cadherin- and TCF-GST fusion proteins were observed, suggesting that β-catenin binding selectivity is not simply due to inhibition of APC-mediated destruction of β-catenin. Interestingly, the role of GSK-3β is more complex. Short-term inhibition of GSK3β by lithium chloride (LiCl; under 4 h at 10 mM) mimics the Wnt effect on β-catenin, i.e., β-catenin preferentially binds TCF-GST greater than cad-GST (Fig. 6 B, lanes 1 and 2). Therefore,
inhibition of GSK3β activity alone is sufficient to mimic the effect of Wnt on β-catenin binding activities. Curiously, however, long-term inhibition of GSK3β by LiCl (over 6 h at 10 mM) generates a pool of β-catenin that binds TCF and cadherin-GST proteins equally well (Fig. 6 B, lanes 3–6). Thus, more potent effects of LiCl do not mimic Wnt signaling, but instead result in the accumulation of high levels of β-catenin with no binding specificity, similar to tumor cells with APC mutations. One interpretation of these two types of effects is that GSK might have multiple targets besides the NH2 terminus of β-catenin (e.g., APC, Rubinfeld et al., 1996; or Axin, Jho et al., 1999). Alternatively, long-term incubation with LiCl could have pleiotropic effects on cell signaling pathways, or the cellular machinery that regulates β-catenin binding to TCF versus cadherin may be easily saturable, so that differential binding is not observed when β-catenin levels rise to unphysiological levels. This explanation is consistent with findings that total cytosolic levels of β-catenin appear to increase substantially with the duration of LiCl treatment (Fig. 6 B, compare lanes 2, 4, and 6), and because expression levels via transfection give similar results (Fig. 3).

Several well-characterized NH2-terminal GSK phosphorylation sites are known to target β-catenin for degradation by an SCF-E3-ligase complex (Winston et al., 1999), and recent work has shown that Wnt signaling is specifically mediated through forms of β-catenin that remain unphosphorylated at these NH2-terminal sites S-33, -37, and T-41 (Staal et al., 2002). We therefore asked whether the state of phosphorylation at these sites is responsible for generating the form of β-catenin selective for TCF-binding. A pAb that recognizes NH2-terminal, unphosphorylated β-catenin was shown to stain cell nuclei, whereas cell–cell contact staining was conspicuously absent (Staal et al., 2002), raising the possibility that unphosphorylated forms of β-catenin might preferentially bind to TCF in the nucleus, and be unable to bind cadherins at the plasma membrane. Contrary to this suggestion, however, we find that β-catenin that is not phosphorylated at residues 33 and 37 can interact with the cad-GST in vitro (Fig. 7 A), associate with endogenous cadherin proteins in cell lysates (not depicted), and localize to sites of cell–cell contact (not the nucleus, as originally observed in Staal et al., 2002; Fig. 7 B). Moreover, cells transfected with a form of β-catenin that cannot be phosphorylated by GSK3β and casein kinase (CK1; S/T→A point mutants at residues 33, 37, 41 and 45) exhibit binding selectivity similar to cells transfected with wild-type β-catenin (Fig. 3). Thus, the preferential binding of β-catenin to TCF over cadherin is not simply due to NH2-terminal phosphorylation status.

We also asked whether modification of the cadherin could affect β-catenin binding selectivity. A previous study showed that the serine-rich, β-catenin binding region of the cadherin is phosphorylated in vivo (Stappert and Kemler, 1994), and this phosphorylation can enhance β-catenin binding to the cadherin (Lickert et al., 2000; Huber and Weis, 2001). We therefore wished to explore whether β-catenin binding selectivity for TCF in Wnt stimulated cells would be altered by cadherin phosphorylation in our binding assay. Phosphorylation of the cadherin greatly enhances binding to β-catenin compared with unphosphorylated cadherin (Fig. 8 A and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200402153/DC1). Indeed, phospho-cadherin is able to binding the same fraction of β-catenin that binds TCF (Fig. 8, B and C), suggesting that cadherin phosphorylation allows the monomeric, closed form of β-catenin to bind the cadherin. Thus, although Wnt signaling generates a form of β-catenin that exhibits preferential binding to TCF over the cadherin, this mechanism may be overridden by extensive phosphorylation of the cadherin.

**Discussion**

We show that Wnt signaling generates a monomeric form of β-catenin that binds TCF selectively compared with the cadherin. In contrast, the cadherin preferentially binds a β-catenin–α-catenin dimer. This selective targeting of distinct molecular forms of β-catenin provides a mechanism by which cells could potentially separate the adhesion and signaling functions of β-catenin. We propose that segregation of these functions of β-catenin may be necessary for two reasons. First, selective targeting of β-catenin to transcriptional complexes would prevent the cadherin from competing with Wnt signaling activity, which may be important during low or transient Wnt activation where signaling may need to be especially efficient. Second, such a mechanism would ensure that cell–cell adhesion is maintained during Wnt inductions throughout development. Loading the cadherin with β-catenin monomers generated by strong Wnt signals might have undesired consequences for cell adhesion, because cadherins bound to β-catenin without α-catenin would be unable to contribute to adhesion. Thus, generation and targeting of distinct molecular forms of β-catenin could ensure that adhesion and signaling are not always coupled, and when necessary, can be regulated independently of one another.
We provide evidence that the Wnt-stimulated, TCF-binding selectivity of H9252-catenin is mediated by the COOH-terminal region of H9252-catenin. First, COOH-terminal epitopes of H9252-catenin are masked in the fraction of H9252-catenin that is unable bind the cadherin. Second, a COOH-terminal peptide of H9252-catenin can compete H9252-catenin binding to cadherin, but not to TCF. Third, deletion of the COOH terminus of H9252-catenin results in a loss of binding selectivity. Together with previously published data showing that the COOH-terminal region of H9252-catenin can bind directly to the armadillo repeat region of H9252-catenin (Cox et al., 1999; Piedra et al., 2001) and restrict cadherin binding in vitro (Castano et al., 2002), we propose that in vivo, the COOH terminus of H9252-catenin adopts a folded-over conformation which controls H9252-catenin binding selectivity by restricting cadherin but not TCF binding. Thus, Wnts may activate H9252-catenin signaling not only by increasing its cytosolic levels, but by regulating the conformation of its COOH terminus.

The existence of a form of β-catenin that distinguishes between cadherins and TCF was not anticipated, given the overall structural similarity between the β-catenin–cadherin and β-catenin–TCF binding interfaces revealed by X-ray crystallography (Graham et al., 2000; Huber and Weis, 2001). Upon closer examination, however, the β-catenin–TCF binding interface is less extensive than the β-catenin–cadherin binding interface, spanning arm repeats 3–10 compared with all 12 armadillo repeats for the cadherin. Thus, it is possible that the COOH-terminal region of β-catenin may fold-back over the last two armadillo repeats of β-catenin, which could have consequences for cadherin but not TCF binding. Indeed, alteration of a single residue in the 12th arm repeat of β-catenin decreases β-catenin binding to the cadherin by a factor of four (Roura et al., 1999), further arguing that small perturbations in the β-catenin–cadherin interface can have significant consequences for binding.

It has also been shown that phosphorylation of E-cadherin increases cadherin–β-catenin complex formation (Lickert et al., 2000). In crystal structures, this phosphorylation results in interactions with β-catenin that appear to mimic TCF binding (Huber and Weis, 2001). Indeed, we find that cadherin phosphorylation allows the cadherin to bind the monomeric, closed form of β-catenin that otherwise would be TCF selective. The fact that cadherin phosphorylation can reverse Wnt-mediated β-catenin binding selectivity suggests a mechanism by which cadherins compete for the Wnt-activated form of β-catenin. It will be important, therefore, to determine when and where cadherin modification occurs to better understand the relationship between adhesion and signaling.
Our observation that the cadherin binds preferentially to β-catenin–α-catenin dimers compared with β-catenin monomers raises the possibility that α-catenin plays a positive role in β-catenin binding to cadherin. Indeed, one study showed that preassociation of recombinant α-catenin with β-catenin increases β-catenin binding to cadherin, suggesting that α-catenin induces an open conformation of β-catenin (Castano et al., 2002). Other evidence, however, argues that α-catenin is not required for β-catenin binding to cadherin. For example, recombinant cadherin–β-catenin complexes are readily formed in vitro (Huber et al., 2001), and cells lacking α-catenin still form cadherin–β-catenin complexes (Bullions et al., 1997; Vasioukhin et al., 2001). We suggest that the form of β-catenin that binds preferentially to cadherin, also binds α-catenin.

Based on our findings from this and previous reports, we propose that cells contain a number of distinct molecular forms of β-catenin (Fig. 9). Thus, although an organism like C. elegans controls the adhesive and signaling functions of β-catenin through expression of a multi-gene family, vertebrates regulate β-catenin functions by generating distinct molecular forms at the protein level. First, there is the well-known form of β-catenin that is phosphorylated at the NH₂ terminus and is targeted for degradation (Fig. 9, phosphorylated; for review see Polakis, 1999). We reported previously a large pool of β-catenin in the SW480 tumor cell line that cannot bind to either TCF or cadherin, and provided evidence that this was an “inactive” form for both adhesion and signaling (Fig. 9; Gottardi et al., 2001). This form may be due, at least in part, to ICAT, a small 9-kD polypeptide that inhibits β-catenin binding to both TCF and cadherin (Gottardi and Gumbiner, 2004; Tago et al., 2000).

Here, we provide evidence for a TCF-selective form of β-catenin that is targeted to transcription complexes (closed conformation), and a form that can target to adhesive complexes (β-catenin–α-catenin dimer). Although the latter form can interact with both the cadherin and TCF, there is evidence that α-catenin inhibits the transcriptional activity of β-catenin in the nucleus (Giannini et al., 2000), suggesting that this form is specific for adhesion functions. Finally, we postulate that cells can contain a form of β-catenin that is competent for both signaling and cadherin binding (open conformation) which is observed, for example, under long-term LiCl treatment (Fig. 5 A, lanes 14 and 15), and would explain the many cases in which cadherin expression inhibits the transcriptional activity of β-catenin (Heasman et al., 1994; Fagotto et al., 1996; Sanson et al., 1996; Orsulic et al., 1999; Shuttman et al., 1999; Gottardi et al., 2001).

Other than the targeting of β-catenin for degradation, the modifications and machinery that regulate these various forms of β-catenin are presently unknown. It is not clear, for example, whether the formation of the inactive ICAT complex is simply controlled by levels of ICAT expression, or is regulated in another way (Gottardi and Gumbiner, 2004). Also, it is not understood what controls the formation of the β-catenin–α-catenin dimer, although it is clear that β-catenin monomers and α-catenin can coexist in the cytosol without forming complexes, even though they readily bind with high affinity in vitro (Koslov et al., 1997). Nor is the time and place of cadherin phosphorylation known. We show that the TCF-selective,
closed form of β-catenin is regulated by the Wnt pathway, and that the binding selectivity is observed after short-term LiCl treatment suggests that GSK3β may be involved. However, the mechanism must be distinct from the pathway that regulates β-catenin levels in the cytosol, because the absence of GSK3β-dependent NH2-terminal phosphorylation does not account for its binding properties, and long-term LiCl treatments and APC mutations lead to β-catenin accumulation without generating the TCF-selective form. It is tempting to speculate that the APC-axin-GSK3β–containing complex regulates the generation of these various forms of β-catenin by post-translational modifications, in addition to the targeting of β-catenin for degradation. Indeed APC mutations have been found to affect adhesive functions of β-catenin as well as Wnt signaling in Droso-philid (Hamada and Bienz, 2002).

If we propose that Wnt signaling generates a TCF-selective form of β-catenin that is resistant to cadherin binding, how do we explain the fact that cadherin expression has been found to antagonize Wnt signaling in numerous model systems (Heasman et al., 1994; Fagotto et al., 1996; Gottardi et al., 2001)? One possibility is that cadherin overexpression of cadherin drives the formation of complexes that do not occur under normal physiological conditions. Although the various molecular forms of β-catenin seem fairly stable in our experiments, it is possible that they are more interconvertible in the cell, or that one form is an intermediate for the other, and can be depleted during its generation. A more interesting possibility is that the relationship between cadherins and Wnt signaling may depend on the specific situation faced by each cell responding to a Wnt signal. For example, our finding that cadherin phosphorylation increases β-catenin binding to cadherin, reversing the differential binding activity observed during Wnt signaling, suggests that variations in cadherin phosphorylation may alter the extent to which adhesion and signaling are coupled. We also hypothesize that the cell can potentially generate either the open form of β-catenin, which binds to both cadherin and TCF, or the closed, TCF-selective form, and the relative proportion of these two forms may differ between different cells responding to Wnt signaling, or different strengths of Wnt signaling.

Indeed, consideration of the various findings suggests a model in which the extent of coupling between the adhesive and nuclear signaling functions of β-catenin is regulated differentially in different cell types, depending on the biological needs of the cells and tissues responding to a Wnt signal. Elucidating the cellular and biochemical mechanisms regulating the generation of the different forms of β-catenin and determining when and where they occur should provide insight into the relationship between the adhesive and signaling functions of β-catenin.

Materials and methods

Cell culture
C57MG and Rat1 parental and Wnt1-expressing cell lines were provided by J. Kitajewski (Columbia University, New York, NY). Wnt3a-expressing l cells were provided by L. Schweizer and H. Varmus (Sloan-Kettering Institute, New York, NY). HEK293T, SW480, HCT116, DLD1, and HT29 cell lines were purchased from American Type Culture Collection.

Antibody and plasmid reagents
Rabbit pAbs to α-catenin, and the NH2-terminal region of β-catenin have been described elsewhere (McGree et al., 1993); 1:5,000 IP. The NH2-terminal and COOH-terminal monoclonal anti–β-catenin antibodies (1:1.1 and M5.2, respectively) were provided by N. Gruel, V. Choumet, and J. Luc Teillard (Pasteur Institute, Paris, France). Other antibodies used in this study: anti–NH2-terminal dephospho–β-catenin pAb (BE4; A.G. Scientific), anti–β-catenin COOH-terminal mAb (C19220; Transduction Laboratories), anti–α-catenin mAb (BD Transduction Laboratories), anti–human E-cadherin (HECD-1 mAb; Zymed Laboratories; 1:500 IP), anti–TCF-4 (1:500 IP, H95-3; Upstate Biotechnology), anti–FLAG epitope (1:5,000 WB, M2 mAb, Sigma-Aldrich) and anti–MyC (1:5,000 WB, 9E10 epitope). The mouse Lin7 GST fusion construct was provided by S. Straight and B. Margolis (University of Michigan, Ann Arbor, MI). Xenopus C-cadherin cytoplasmic domain (cad-GST) and Xenopus TCF-β-catenin binding region (TCF-GST) fusion proteins have been described previously (Gottardi et al., 2001).

Affinity precipitation experiments
Cells were grown to in 14-cm tissue culture dishes and a detergent-Free, cytosolic fraction was generated by centrifugation at 100,000 g accord-
ing to Gottardi et al. (2001). Metabolic labeling of proteins with \[^{[35]S}\]methionine/cysteine was done according to Gottardi and Gumbiner (2004). Affinity precipitations were performed with recombinant GST-cad, GST-Tcf and GST-Mlin7 fusion proteins. For cadherin/Tcf-GST binding experiments, 100–500 µg of a cytosolic fraction was subjected to affinity precipitation with \(\sim 40\) pmol of cad-GST or Tcf-GST prebound to glutathione-coupled agarose beads (Sigma-Aldrich) for \(60'\) at \(4'\). Each precipitation was washed in a detergent buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl and 0.1% NP-40), and bound protein complexes were analyzed by Western analysis for in vitro competition of the \(\beta\)-cat COOH-terminal peptide (amino acids 669–781; provided by A. Garcia de Herreros, Universitat Pompeu Fabra, Barcelona, Spain; Costanzo et al., 2002), the \(\beta\)-catenin COOH terminus was cleaved from GST with PreScission protease (Amersham Biosciences). \(\beta\)-Catenin \((\sim 17\) pmol), produced by the baculovirus system, cadherin-GST (51 pmol), or TCF-GST (51 pmol) were incubated in 150 ml of buffer \((10\) mM Tris, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% NP-40, 10 µg/ml leupeptin and aprotonin) for \(60'\) at \(4'\) with shaking in the presence or absence of increasing amounts of the \(\beta\)-catenin COOH-terminal peptide \((0, 1, 1.0, 5.0, 15.0 \mu g \left[1.6\right] \text{nmmol peptide})\). \(\beta\)-Catenin that was affinity precipitated by cadherin-GST and TCF-GST immobilized to glutathione-coupled agarose was washed and subjected to SDS-PAGE and Western analysis.

**Gel filtration chromatography**

The cytosolic fraction was separated on a HiPrep 16/60 Sephacryl S-300 sizing column (Amersham Biosciences; High Resolution Code 17–116701, 10–1500 kDa inclusion range) equilibrated with buffer containing 30 mM Hepes, pH 7.5, and 150 mM KCl and developed at 0.4 ml/min. 75 2.0-ml fractions were collected.

**Online supplemental material**

Fig. S1 depicts in vitro phosphorylation of cadherin-GST. (A) Purification of cad-GST and TCF-GST proteins and detection by Coomassie. (B and C) Fig. S1 depicts in vitro phosphorylation of cadherin-GST. (A) Purification of cad-GST and TCF-GST proteins and detection by Coomassie. (B) and GST-mLin7 fusion proteins. For cadherin/TCF-GST binding experiments, 100–500 µg of a cytosolic fraction was subjected to affinity precipitation with \(\sim 40\) pmol of cad-GST or TCF-GST prebound to glutathione-coupled agarose beads (Sigma-Aldrich) for \(60'\) at \(4'\). Each precipitation was washed in a detergent buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl and 0.1% NP-40), and bound protein complexes were analyzed by Western analysis for in vitro competition of the \(\beta\)-catenin COOH-terminal peptide (amino acids 669–781; provided by A. Garcia de Herreros, Universitat Pompeu Fabra, Barcelona, Spain; Costanzo et al., 2002), the \(\beta\)-catenin COOH terminus was cleaved from GST with PreScission protease (Amersham Biosciences). \(\beta\)-Catenin \((\sim 17\) pmol), produced by the baculovirus system, cadherin-GST (51 pmol), or TCF-GST (51 pmol) were incubated in 150 ml of buffer \((10\) mM Tris, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% NP-40, 10 µg/ml leupeptin and aprotonin) for \(60'\) at \(4'\) with shaking in the presence or absence of increasing amounts of the \(\beta\)-catenin COOH-terminal peptide \((0, 1, 1.0, 5.0, 15.0 \mu g \left[1.6\right] \text{nmmol peptide})\). \(\beta\)-Catenin that was affinity precipitated by cadherin-GST and TCF-GST immobilized to glutathione-coupled agarose was washed and subjected to SDS-PAGE and Western analysis.

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