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A Mode of Regulation of β-Catenin Signaling Activity in *Xenopus* Embryos Independent of Its Levels

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The signaling activity of β-catenin is thought to be regulated by phosphorylation of a cluster of N-terminal serines, putative sites for GSK3β. In the prevailing model in the literature, GSK3β-dependent phosphorylation of these sites targets β-catenin for ubiquitin-mediated degradation. Wnt signaling inhibits GSK3β activity and this blocks degradation, allowing β-catenin to accumulate and signal. We show here that β-catenin activity is not regulated solely by protein stability. Mutations in the putative GSK3β phosphorylation sites of β-catenin enhance its signaling activity, but this cannot be accounted for by accumulation of either total or cadherin-free protein. Instead, the mutant protein has a threefold higher specific activity than the wild type both *in vivo* and in an *in vitro* signaling assay. We conclude that the N-terminal serines convey a layer of regulation upon β-catenin signaling in addition to the effects these sites exert upon protein stability. © 2000 Academic Press

Key Words: **signal transduction; GSK3β; Wnts; *Xenopus.***

INTRODUCTION

β-Catenin is a pivotal player in signal transduction events involved in development and disease progression. Evidence from *Drosophila, Xenopus, Caenorhabditis elegans,* and tissue culture systems has shown that β-catenin is part of a linear signaling pathway initiated in response to a secreted protein of the Wnt family (Cadigan and Nusse, 1997). In this signaling pathway, the primary means of controlling β-catenin's activity is postulated to be the regulation of its stability. In a cell that has not received a Wnt signal, cytosolic β-catenin is thought to be phosphorylated by the serine threonine kinase zeste-white-3/glycogen synthase kinase3β (GSK3β).[2] This modification is believed to target β-catenin for ubiquitin-mediated degradation. As a consequence of a Wnt signal, GSK3β activity is down-regulated, and β-catenin becomes hypophosphorylated and stabilized and can transduce the signal to the nucleus where it interacts with members of the T-cell factor family of transcription factors to initiate expression of target genes.

This model is supported by findings from several experimental systems. In *Drosophila,* the β-catenin homologue, Armadillo (Arm) accumulates in stripes of cells corresponding directly to the areas where Wingless (Wg) signaling is taking place (Riggleman *et al.,* 1990). Also, addition of soluble Wg to cultured imaginal disks induces cytosolic accumulation of Arm (van Leeuwen *et al.,* 1994). Also, high levels of cytosolic and nuclear β-catenin are associated with elevated β-catenin signaling that occurs in some forms of cancer. Specifically, mutations in the tumor-suppressor protein adenomatous polyposis coli (APC) can lead to elevated levels of β-catenin in colon cancer cell lines (Munemitsu *et al.,* 1995). When a wild-type copy of APC is expressed in these cells, β-catenin protein levels decrease (Munemitsu *et al.,* 1995) and the expression of a β-catenin-dependent reporter gene is reduced (Korinek *et al.,* 1997; Morin *et al.,* 1997). Additionally, naturally occurring mutations within β-catenin's putative N-terminal GSK3β phosphorylation sites are linked to overaccumulation and aberrant signaling of β-catenin in melanomas and colon cancer (Rubinfeld *et al.,* 1997). When β-catenin proteins containing similar mutations are expressed in *Drosophila*

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[2] Abbreviations used: GSK3β, glycogen synthase kinase 3β; MBT, midblastula transition; CBE, cleavage-blocked embryo; Con A, concanavalin A; APC, adenomatous polyposis coli gene product; HMR, half-maximum response.

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(Pai *et al.,* 1997) or *Xenopus* embryos (Yost *et al.,* 1996), they exhibit enhanced signaling activity and accumulate to high levels. Finally, it has been shown in NIH3T3 cells that β-catenin is normally ubiquitinated, but in the presence of a Wnt signal, this modification is reduced and the protein is stabilized (Aberle *et al.,* 1997).

There is a clear correlation between β-catenin stability and signaling; however, it is not known if β-catenin must accumulate to high levels in order to signal effectively or if accumulation is sufficient for signaling. Here, we examine this question and present evidence that regulated protein stability may not be the only means for controlling β-catenin signaling in *Xenopus* embryos. In *Xenopus,* β-catenin is part of a signaling pathway required for specifying the dorsal–ventral axis of the developing embryo. Soon after fertilization, the cortex of the one-cell embryo undergoes rotation and this movement causes the asymmetric activation of an upstream component of the β-catenin signaling pathway. This somehow activates β-catenin on the dorsal side of the embryo, perhaps in part by stabilizing the β-catenin protein (Larabell *et al.,* 1997). At the midblastula transition (MBT), activated β-catenin translocates into the nucleus (Schneider *et al.,* 1996) where it initiates expression of *siamois,* a homeobox gene believed to coordinate the formation of dorsal structures (Brannon and Kimelman, 1996; Fagotto *et al.,* 1997).

Overexpression of β-catenin on the ventral side of the cleavage stage embryo can mimic this effect and lead to the formation of an ectopic axis (Funayama *et al.,* 1995). We have used this assay system and an *in vitro* assay system derived from *Xenopus* embryo extracts (Nelson and Gumbiner, 1999) to compare the activities of wild-type β-catenin and mutant β-catenin lacking the putative GSK3β phosphorylation sites.

MATERIALS AND METHODS

Embryos and microinjections. *Xenopus* were obtained from Nasco (Fort Atkinson, WI) and induced to lay by priming with 50 U of gestyl (Diosynth B.N., Oss, Netherlands) followed by injection of 700 U of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO). Eggs were fertilized *in vitro,* dejellied in 2% cysteine–HCl, pH 8.1, and cultured in 0.1× MMR (Kay and Peng, 1991). Staging of embryos was according to Nieuwkoop and Faber (1967).

mRNAs encoding for wild-type and mutant β-catenins were synthesized using Sp6 polymerase (Promega Corp, Madison, WI) and template cDNAs cloned into the pCS2-MT vector (Rupp *et al.,* 1994; Turner and Weintraub, 1994). mRNAs were dissolved in DEPC-treated water and 15 nl (containing the indicated amounts of mRNA) was injected into vegetal ventral cells of four- or eight-cell embryos.

Embryos were lysed in NP-40 buffer (1% NP-40, 10 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM EDTA, 1 mM EGTA) with protease inhibitors (200 μM PMSF, 4 μgml aprotinin, 1 μgml pepstatin A, 2 μgml leupeptin, 10 μgml antipain, 50 μgml benzamidine, 0.5 μM iodoacetamide) and phosphatase inhibitors (100 mM NaF, 100 μM sodium vanadate). Proteins were detected by Western blot using Mab 9E10.2 (anti-myc) (Evan *et al.,* 1985), goat anti mouse HRP (Bio-Rad, Richmond, CA), and the ECL system (Amersham, Buckinghamshire, England).

In the indicated experiments, proteins were separated by binding to Con A–Sepharose as follows. Ten embryos were lysed in 250 μl of RIPA buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) plus protease inhibitors and phosphatase inhibitors. The interaction between cadherins and β-catenin is normally quite strong and is retained under these conditions (Funayama *et al.,* 1995; McCrea and Gumbiner, 1991). The lysates were rotated at 4°C for 1 h with 80 μl of Con A slurry (Sigma Chemical Co.). The supernatant and beads were separated by centrifugation (5 min at 16,000*g*). Beads were washed 3× with lysis buffer, and both the beads and the supernatant were processed for Western blotting.

Gel quantitation. In order to quantitate protein expression, protein blots were developed with ECL reagent and Hyperfilm ECL autoradiography film (Amersham). Multiple timed exposures of each blot were prepared in order to ensure that at least one exposure would be below the saturation level of the film. This exposure (usually ~30 s) was scanned and quantitated using Fuji MacBas gel analysis software. A pixel density trace was generated for each lane to determine lane background and peak positions. The areas under the β-catenin peaks were calculated and assigned an arbitrary value of pixel intensity by the software. Using this method we can estimate relative protein levels and make comparisons between bands on a single given gel. These values are relative and cannot be compared between gels in different experiments. Using this quantitation method, we have found that the pixel intensity of the exogenous β-catenin protein expressed at the MBT varies linearly with the amount of input mRNA at least over the range of mRNAs tested in this study (10–500 pg) (data not shown).

In vitro siamois induction assay. This assay was developed by Nelson and Gumbiner (1999). Briefly, cleavage-blocked embryos (CBEs) were prepared 40 min after fertilization by centrifuging embryos (500*g*, 10 min, 15°C) over a cushion of 50% Ficoll 0.1× MMR. CBEs were allowed to develop until MBT (as judged by the development of non-cleavage-blocked siblings). Twenty-five CBEs/sample were spun at 325*g* for 5 min at 4°C in the presence of Versilube (MIL-S-81087C) to remove buffer and were then spin-crushed (16,000*g* for 15 min at 4°C) in the presence of recombinant wild-type or mutant β-catenin and protease inhibitors. Crushed embryos were incubated at 21°C for 1.5 h. RNA was isolated using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc., Houston, TX). mRNAs were detected using RT-PCR. Primers were Siamois (upstream, 5′ TTGTCTCTACCGCACTGA 3′; downstream, 5′ TCCTGTTGACTGCAGACT 3′) and EF1 (upstream, 5′ CAGATTGGTGCTGGATATGC 3′; downstream, 5′ ACTGCCTTGATGACTCCTAG 3′). Parallel samples were processed for protein detection by extraction in NP-40 buffer plus protease inhibitors and phosphatase inhibitors.

Synthesis of recombinant proteins. Recombinant wild-type and mutant β-catenin proteins were synthesized in Sf9 cells using the Bac-N-Blue Transfection kit (Invitrogen, Carlsbad, CA) and isolated by binding to nickel agarose beads (Qiagen, Santa Clara, CA) as previously described (Fagotto *et al.,* 1998).

For protein injections, mutant and wild-type β-catenin were dialyzed into injection buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris, pH 7.5) and15 nl (containing the indicated amounts of protein) was injected into vegetal ventral cells of four-cell embryos.

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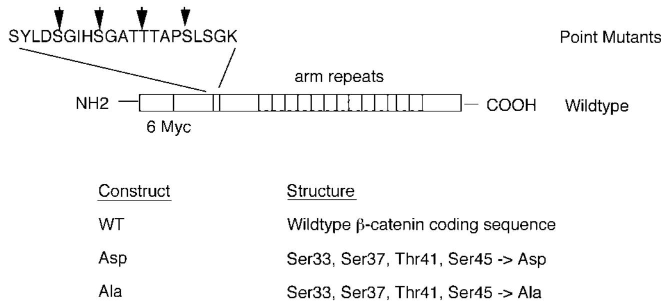

FIG. 1. Constructs used to study β-catenin signaling. Mutations were made in the indicated N-terminal serine and threonine residues.

RESULTS

Mutations in N-terminal Serines Enhance the Signaling Activity of β-Catenin

To examine the regulatory function of the N-terminus in further detail, the putative GSK3β phosphorylation sites of β-catenin were changed to alanine (to block phosphorylation) or aspartic acid residues (to try to mimic constitutive phosphorylation) (Fig. 1). Equivalent amounts of mRNA encoding wild-type or mutant β-catenin were injected into *Xenopus* embryos. Embryos were allowed to develop until neurula stage (st. 22) at which time, they were scored for the formation of an ectopic axis.

Embryos in which the Ala mutant construct was injected developed a higher percentage of axis duplications than those injected with wild type, consistent with the idea that these mutations block phosphorylation and down-regulation of β-catenin signaling (Fig. 2A). A similar construct has already been shown to have enhanced activity in *Xenopus* (Yost *et al.,* 1996). The Asp mutant was also more active than wild type in this assay, suggesting that the aspartic acid modification is unable to mimic constitutive phosphorylation and instead blocks phosphorylation (similar to the Ala mutation). This effect was similar to what has been previously reported for an Asp mutant examined in Neuro 2A cells (Aberle *et al.,* 1997).

As β-catenin signaling is thought to occur at or before the midblastula transition (Wylie *et al.,* 1996), the expression of wild-type and mutant proteins was examined in lysates prepared at this time point (Fig. 2B). Both Asp and Ala mutant constructs were more efficient than wild type at inducing the formation of an ectopic axis. However, we did not detect any difference between wild-type and mutant protein levels at MBT. We did observe enhanced accumulation of the mutant proteins in later stage (neurula) embryos, similar to what has been previously described (Yost *et al.,* 1996). However, axis formation has already occurred by this time point; and accumulation at this stage cannot be responsible for the enhanced axis-inducing activity of the mutant proteins.

Dose-Dependent Signaling Activity of Cadherin-Free Wild-Type and Mutant β-Catenin Proteins

As only the cadherin-free form of β-catenin is required for signaling (Fagotto *et al.,* 1996; Heasman *et al.,* 1994), it may not be accurate to simply compare total protein levels as described above. Hence, we have used concanavalin A to separate the ectopically expressed β-catenin proteins into cadherin-free and cadherin-bound fractions. Con A binding has been used previously to efficiently clear cadherins and their associated proteins from cell lysates (Choi and Gumbiner, 1989; Fagotto *et al.,* 1996).

In initial experiments, we performed mRNA titrations in order to determine the doses of mutant and wild-type mRNAs capable of eliciting a linear response (axis duplication) (data not shown). Based on these titrations we chose three amounts of mRNA representing low, medium, and high activity for the Asp mutant and wild-type β-catenin. The indicated amounts of mRNA (Fig. 3) were injected into embryos, lysates were prepared at MBT, and cadherin bound and unbound pools were separated by binding to Con A–Sepharose. Both the Con A supernatants (cadherin-free)

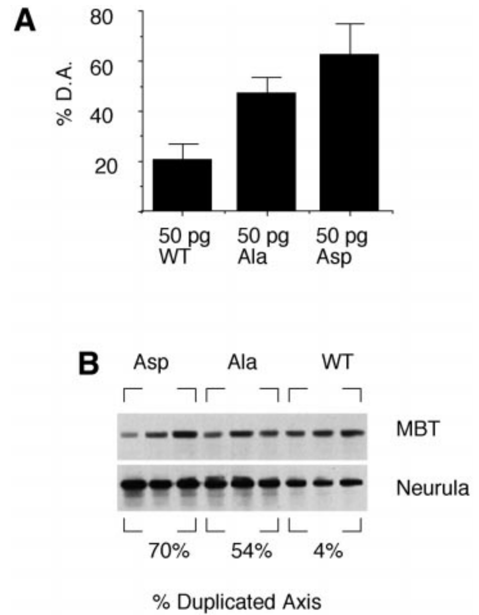

FIG. 2. β-Catenin point mutants exhibit enhanced signaling activity. (A) 50 pg of wild-type (WT) or mutant mRNAs was injected into a vegetal ventral cell of 4-cell embryos. Duplicated axes were scored at the neurula stage. Data from five experiments are shown. (B) Levels of exogenously expressed wild-type and mutant β-catenin proteins at midblastula transition (st. 8) and neurula (st. 22) stages. Embryos were injected with 50 pg of the indicated mRNA and lysed at the indicated time points. The equivalent of half an embryo of lysate was separated and blotted for Myc-tagged β-catenin. Lysates were prepared in triplicate to control for loading and/or embryo-to-embryo variability. The percentages of axis duplication that occurred in this experiment are shown at the bottom.

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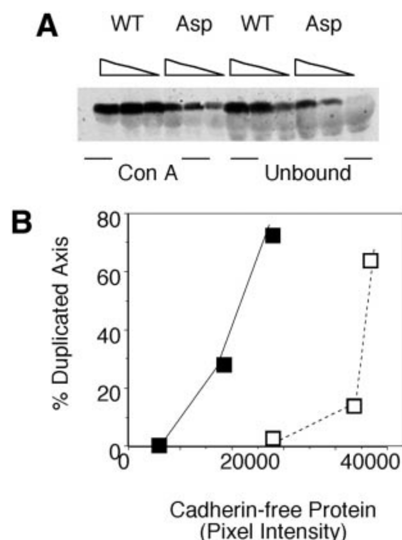

FIG. 3. Enhanced signaling of the Asp β-catenin mutant does not correlate with enhanced accumulation of cadherin-free protein. 50, 100, and 150 ng of wild-type (WT) and 10, 20, and 30 ng of Asp mRNAs were injected into a vegetal ventral blastomere of a 4-cell embryo. At midblastula transition, embryos were lysed in RIPA buffer and proteins were separated by binding to Con A–Sepharose. Remaining embryos were allowed to develop until neurula stage and were scored for axis duplication. (A) The equivalent of half an embryo of unbound and one embryo of Con A-associated protein was separated by PAGE and blotted for Myc. (B) Band intensities from the gel shown in A were quantitated using Fuji MacBas gel analysis software and the amount of axis duplication was plotted as a function of cadherin-free (unbound) protein (■, Asp; □, WT).

and the Con A pellets (cadherin-bound) were blotted for the presence of the exogenously expressed β-catenin proteins (Fig. 3A). Remaining embryos were allowed to develop until the neurula stage at which they were scored for the formation of an ectopic axis. Activity (axis duplication) was plotted as a function of the band intensity of the cadherin-free β-catenin protein (Fig. 3B). From such plots, we can estimate the relative amounts of cadherin-free wild-type and mutant proteins required to yield half-maximum response (HMR; 50% axis duplication). In the experiment shown, the mutant protein reached HMR at 20,000 protein units, whereas the wild type reached HMR at 45,000 protein units. From this we estimate that the cadherin-free form of the mutant protein signals efficiently at lower levels of protein than the wild-type protein and is intrinsically two to three times as active as the wild-type protein. Similar results were obtained in three independent experiments. Moreover, the enhanced signaling activity observed for the Asp mutant protein cannot be due to a reduced binding to the cadherin since similar percentages of Asp and wild-type proteins were detected in the Con A-bound fraction (Fig. 3A).

Specific Activity of the Asp Mutant Protein Is 3× Higher Than That of Wild Type in a Cell-Free Assay

We wished to compare the signaling activity of wild-type and mutant proteins using a system with a more direct readout in order to avoid potential complications of measuring protein levels in developing embryos. The homeobox gene *siamois* is the earliest known transcriptional target of β-catenin signaling in *Xenopus* (Carnac *et al.,* 1996; Brannon and Kimelman, 1996; Fagotto *et al.,* 1997). Therefore, we have examined the abilities of wild-type and mutant proteins to induce Siamois expression in an *in vitro* assay developed by Nelson and Gumbiner (1999). In this assay, recombinant proteins are added to spin-crushed embryo extracts. After incubation, mRNA is isolated from these extracts and assayed for the presence of *siamois* transcripts using RT-PCR. Figure 4 shows a representative titration of the signaling activities of wild-type and Asp mutant proteins in this assay. *siamois* was induced with as little as 200 ng of Asp protein and this induction was dosage dependent with increasing amounts of Asp protein giving higher levels of induction. The wild type did not exhibit induction until 600 ng of protein was added, indicating that the wild type is less active than the mutant in this assay. Interestingly, equivalent amounts of wild-type and mutant proteins were present at the endpoint of this experiment (Fig. 4B), demonstrating that differential degradation cannot account for the observed difference in activity.

Cadherin-Free Wild-Type and Asp Mutant Proteins Are Relatively Stable in Xenopus Embryos

The results of our *in vivo* and *in vitro* titration experiments are not consistent with the current model in which β-catenin signaling is regulated solely by changes in protein stability. However, the exogenously expressed mutant proteins accumulated to higher levels than wild type (Fig. 2B), suggesting that they may be stabilized. To address this possibility directly, we measured the stability of wild-type and mutant β-catenin proteins by injecting purified, recombinant Asp and wild-type β-catenin into *Xenopus* embryos (Fig. 5). Lysates were prepared at various time points after injection and were separated into cadherin-associated and cadherin-free fractions by Con A precipitation. The Con A-free fraction was analyzed for the presence of the recombinant proteins (Fig. 5A) and the band intensities of the cadherin-free proteins were plotted versus time (Fig. 5B). In the experiment shown, cadherin-free wild-type β-catenin had a calculated half-life of 3.6 h and the mutant protein had a half-life of 10.5 h. Consistent with previous observations, the mutant protein is more stable than the wild-type protein; however, both proteins had much longer half-lives than those previously reported for *Xenopus* (Yost *et al.,* 1996) and other systems (Aberle *et al.,* 1997; Byers *et al.,* 1996; Pai *et al.,* 1997; Rubinfeld *et al.,* 1997).

Finally, it has been proposed that stabilization of cytosolic β-catenin protein on the dorsal side of the embryo is

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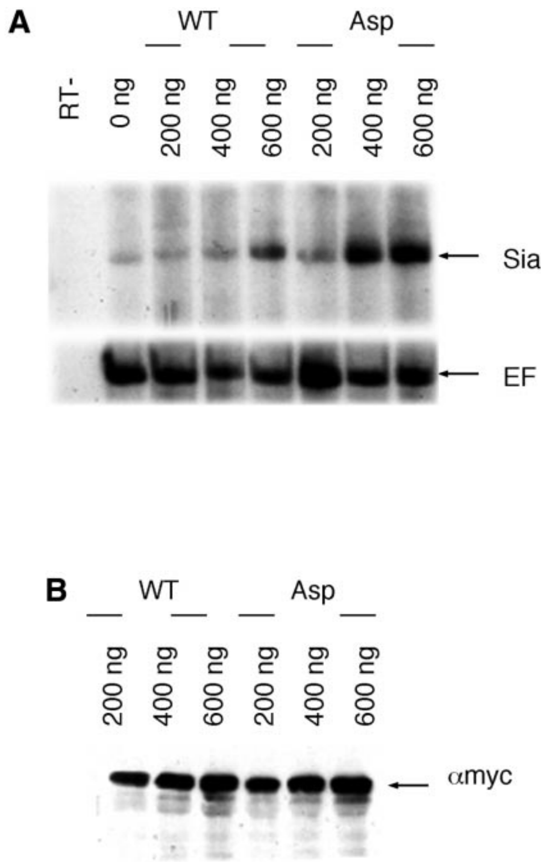

FIG. 4. Specific signaling activities of wild-type and Asp mutant proteins in a cell-free assay. (A) The indicated amount of wild-type or mutant protein was added to spin-crushed embryo extracts. mRNA was isolated from extracts after a 1.5-h incubation and subjected to RT-PCR to detect the level of induced *siamois* transcripts. EF transcripts are shown as a loading control. (B) Note that similar levels of wild-type and mutant protein remain in extracts at the end of the 1.5-h incubation. This indicates that a difference in stability cannot account for the observed difference in activity.

responsible for establishing a signaling center required for axis formation (Larabell *et al.,* 1997). To determine if there are dorsal–ventral differences in β-catenin stability, we injected recombinant wild-type protein into the vegetal ventral or vegetal dorsal cells of eight-cell embryos and calculated the half-life of the cadherin-free fractions as described above (Figs. 5C and 5D). We could detect no difference in β-catenin stability on the dorsal versus ventral side of the embryo. In both cases, β-catenin exhibited a half-life of 3.8 h.

DISCUSSION

GSK3β-dependent phosphorylation of a cluster of serinethreonine residues within the N-terminus of β-catenin is thought to regulate β-catenin signaling by stimulating turnover of the β-catenin protein. Hence, we were surprised to find that β-catenin proteins harboring mutations in these sites did not accumulate to high levels relative to wild-type protein during early *Xenopus* development (Fig. 2B). Enhanced accumulation of the mutant proteins occurred at later stages (neurula), similar to what has been previously reported (Yost *et al.,* 1996). However, as the role of β-catenin signaling in axis induction is believed to occur at or before the midblastula transition (Wylie *et al.,* 1996) such late accumulation is not likely to be relevant to its early signaling function. Similar observations that β-catenin's signaling activity does not always correlate with β-catenin levels have been reported in other studies. Young *et al.* have shown that β-catenin S37A mutant signals more effectively than wild type without accumulating to high levels (Young *et al.,* 1998). Additionally, Korinek *et al.* have demonstrated that APC can alter β-catenin signaling without affecting the amount of β-catenin protein (Korinek *et al.,* 1997) and Nelson and Gumbiner have found that β-catenin signaling can be activated by lithium in the absence of protein accumulation (Nelson and Gumbiner, 1999); therefore, β-catenin accumulation might not be the only means of regulating its signaling.

There is much evidence that only the non-cadherin-bound form of β-catenin is involved in signaling (Fagotto *et al.,* 1996; Heasman *et al.,* 1994). Hence, in order to examine cadherin-free pools of wild-type and mutant β-catenin we used Con A binding to deplete cadherin-associated proteins from lysates. This is a more accurate approach than simply comparing total protein levels as has been done in many previous studies. Additionally, in order to draw a correlation between protein levels and activity it is important to be within the linear range of the signaling response. To address this, we performed mRNA titrations in order to define the linear ranges for wild-type and mutant β-catenin and have sought to make all protein comparisons with in these ranges. In this respect, the *Xenopus* system is potentially more powerful than other systems in which expression levels can be difficult to control. From these titration experiments we obtained estimates of the relative specific activities of the wild-type and Asp mutant protein (Fig. 3). We found that the cadherin-free form of the Asp mutant protein is three times as active as wild type in axis induction.

Measuring the specific activity of a locally and transiently acting protein in a whole developing embryo is inherently complex. Hence, we compared the intrinsic activity of the mutant and wild-type proteins in a direct, short-term, cell-free assay. We found that the Asp mutant protein is three times as active as wild type at inducing *siamois* expression in spin-crushed embryo extracts (Fig. 4). Importantly, we observed no difference in the amounts of mutant and wild-type protein present at the endpoint of this assay. This demonstrates that differential stability cannot be the sole means for controlling β-catenin signaling activity.

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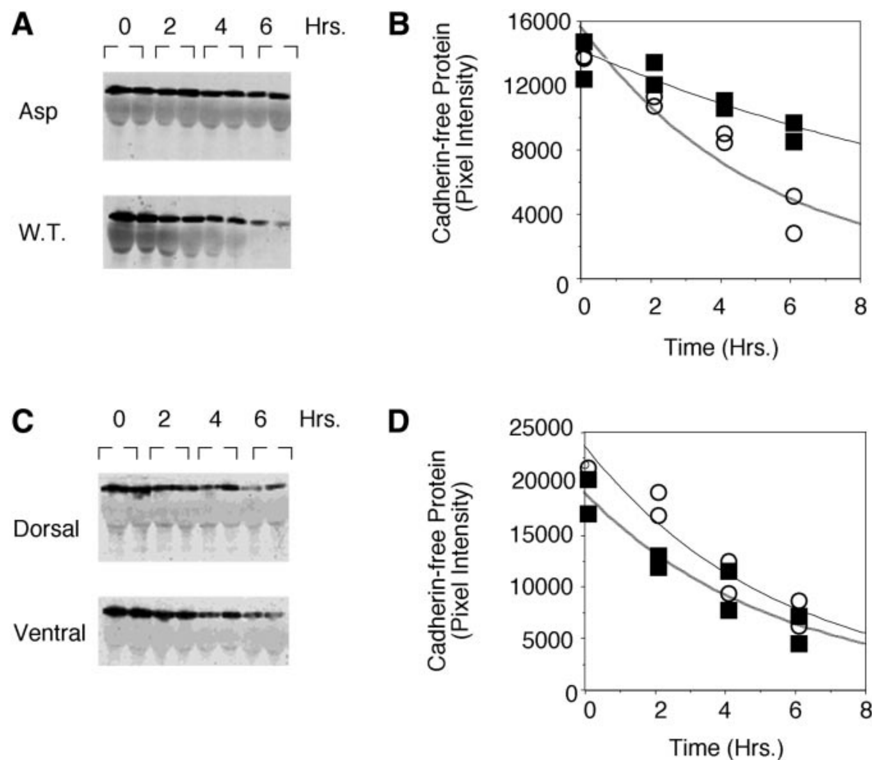

FIG. 5. Rates of turnover of wild-type and mutant β-catenin proteins. (A) The stability of the Asp mutant and wild-type β-catenin proteins was examined by injecting 2.5 ng of Myc-tagged recombinant protein into the vegetal ventral cells of 8-cell embryos. Lysates from 10 embryos were prepared in duplicate at 0, 2, 4, and 6 h after injection and separated into cadherin bound and unbound fractions by binding to Con A–Sepharose. Volumes equivalent to 1/4 embryo of the Con A supernatants were separated by PAGE and recombinant proteins were detected by blotting for the Myc-tag. (B) Band intensities shown in A were quantitated using Fuji MacBas gel analysis software and plotted versus time using KaleidaGraph (Synergy Software, Reading, PA) (■, Asp; ○, WT). The estimated half-lives are 3.6 h for the wild-type protein and 10.5 h for the Asp mutant protein. Embryos remaining after the time course was taken were allowed to develop to the neurula stage and were scored for axis duplication. 33% (8/24) of Asp-injected and 0% (0/39) of wild-type-injected embryos exhibited a duplicated axis. (C) β-Catenin stability was compared on the dorsal and ventral sides of *Xenopus* embryos. 2.5 ng of Myc-tagged recombinant wild-type β-catenin was injected into the dorsal vegetal or ventral vegetal cells of 8-cell embryos. Lysates were prepared as described above. Volumes equivalent to 1/2 embryo of the Con A supernatants were separated and blotted for Myc. (D) Band intensities shown in C were quantitated and plotted versus time (■, dorsal; ○, ventral). There was no difference in β-catenin stability between the dorsal (t_{12} = 3.8 h) and the ventral (t_{12} = 3.8 h) sides of the embryo.

We propose that there may be two components of β-catenin regulation in *Xenopus,* one that is independent of protein levels and one that may require protein accumulation. These two aspects of signaling are separable in *Xenopus* because the β-catenin protein is relatively stable in this system. In *Drosophila* embryos and tissue culture systems the t_{12} of total β-catenin was determined to be 16 and 40 min, respectively (Pai *et al.,* 1997; Rubinfeld *et al.,* 1997). In these systems, mutations in the GSK3β sites produce a dramatic effect on β-catenin protein accumulation and this effect might be sufficient to account for the enhanced activity of the mutant proteins. In *Xenopus* embryos, the half-life of cadherin-free β-catenin is 3.6 h (Fig. 5). Mutations in the putative GSK3β phosphorylation sites stabilize the cadherin-free form of the protein even further (Fig. 5), but obvious accumulation of such mutant proteins is detected only late in development (Figs. 2 and 5). This slow accumulation of mutant β-catenin does not seem likely to contribute significantly to its early signaling function at the MBT. Additionally, we were unable to detect differences in β-catenin stability on the dorsal versus ventral side of the embryo. It has been proposed that β-catenin accumulates on the dorsal side of the *Xenopus* embryo even by the time of the two-cell cleavage stage (Larabell *et al.,* 1997). However, the biochemically measured dorsal–ventral differences in β-catenin levels in the blastula were subtle, and such differences were not observed in previous studies (Fagotto and Gumbiner, 1994; DeMarais and Moon, 1992). Furthermore, although dorsal accumulation of β-catenin at the two-cell stage was observed using whole-mount immunocytochemistry (Larabell *et al.,* 1997), it was not possible to distinguish a soluble signaling pool of β-catenin from the

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large cytoplasmic vesicle fraction containing cadherin–catenin complexes (Fagotto and Gumbiner, 1994). It remains unclear to what extent β-catenin accumulation contributes to endogenous β-catenin signaling in this system.

Our evidence suggests that the N-terminus of β-catenin can regulate the specific activity of the protein in addition to its stability. The molecular nature of this additional form of regulation remains unknown. We speculate that the putative GSK3β sites may directly control import and/or export of β-catenin to/from the nucleus, or that they regulate β-catenin's transcriptional activity once it is inside the nucleus. In many previous studies, nuclear localization of β-catenin often correlated with an increase in the amount of cytosolic β-catenin protein. Hence it maybe assumed that the import of β-catenin into the nucleus is regulated by the amount of available (cadherin-free) β-catenin. However, Fagotto *et al.* have demonstrated that the import of β-catenin into the nucleus occurs by a novel pathway and can be inhibited by factors in the cytosol without affecting stability of the β-catenin protein (Fagotto *et al.,* 1998). Additionally, GSK3β has been demonstrated to regulate the nuclear localization of NF-ATc (Beals *et al.,* 1997) and both the nuclear localization and the stability of cyclin D1 (Diehl *et al.,* 1998). With regard to cyclin D1, the effect on nuclear localization appears to be an early regulatory step and destabilization of the protein happens later in the cell cycle. A similar biphasic form of regulation might occur during β-catenin signaling and could be a way of allowing a quick response to a signal followed by long-term "memory" of the response.

We have attempted to examine the nuclear localization of ectopically expressed mutant and wild-type proteins *in vivo* using a whole-mount protocol (Schneider *et al.,* 1996). Unfortunately, this protocol was not sensitive enough to detect wild-type and mutant proteins at levels at which the signaling response is linear. We have also tried to examine the activity of wild-type and mutant proteins in an *in vitro* nuclear import assay (Fagotto *et al.,* 1998) and, to date, have observed no obvious differences between them in this system (data not shown). However, this may be due to the loss of regulatory modifications (phosphorylation) during isolation of β-catenin proteins from Sf9 cells. In support of this, we observe that both mutant and wild-type proteins isolated from Sf9 cells are phosphorylated to a similar extent (data not shown). Alternatively, it is possible that these sites do not regulate import of β-catenin but instead control the activity of the protein once it is in the nucleus (perhaps by regulating its binding to components of the transcriptional machinery). In support of this, the N-terminus of β-catenin has been found to have transactivation activity (Hsu *et al.,* 1998).

In conclusion, we have shown that the N-terminal serines of β-catenin can regulate its signaling activity independent of controlling protein stability. How this activity of β-catenin is controlled by these sites is not yet clear. Modification of the N-terminus of β-catenin probably regulates its binding to a protein that influences nuclear import, nuclear export, or the transcriptional activity of the protein. Further work will be required to determine what protein–protein interactions are regulated by these sites, how these interactions affect β-catenin's signaling function, and whether this form of regulation occurs in other systems.

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