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β-catenin is a target for the ubiquitin–proteasome pathway

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β-catenin is a central component of the cadherin cell adhesion complex and plays an essential role in the Wingless/Wnt signaling pathway. In the current model of this pathway, the amount of β-catenin (or its invertebrate homolog Armadillo) is tightly regulated and its steady-state level outside the cadherin–catenin complex is low in the absence of Wingless/Wnt signal. Here we show that the ubiquitin-dependent proteolysis system is involved in the regulation of β-catenin turnover. β-catenin, but not E-cadherin, p120[cas] or α-catenin, becomes stabilized when proteasome-mediated proteolysis is inhibited and this leads to the accumulation of multi-ubiquitinated forms of β-catenin. Mutagenesis experiments demonstrate that substitution of the serine residues in the glycogen synthase kinase 3β (GSK3β) phosphorylation consensus motif of β-catenin inhibits ubiquitination and results in stabilization of the protein. This motif in β-catenin resembles a motif in IκB (inhibitor of NFκB) which is required for the phosphorylation-dependent degradation of IκB via the ubiquitin–proteasome pathway. We show that ubiquitination of β-catenin is greatly reduced in Wnt-expressing cells, providing the first evidence that the ubiquitin–proteasome degradation pathway may act downstream of GSK3β in the regulation of β-catenin.

Keywords: β-catenin/GSK3β/proteasome/ubiquitin

Introduction

Classical cadherins are complexed via their cytoplasmic domains with three major catenins, termed α-, β- and γ-catenin (plakoglobin). β-catenin and plakoglobin play a central role in the architecture of the cadherin–catenin complex, linking cadherins to α-catenin, which in turn mediates the anchorage of the cadherin complex to the cortical actin cytoskeleton (reviewed in Kemler, 1993). Recently, many new interaction partners for β-catenin and/or plakoglobin have been identified, pointing to additional functions of β-catenin outside the cell adhesion complex (reviewed in Gumbiner, 1995; Huber *et al.*, 1996a).

The structural homology of β-catenin and plakoglobin to the *Drosophila* Armadillo (Arm) protein, a component of the Wingless (Wg) signaling pathway (McCrea *et al.*, 1991; Butz *et al.*, 1992), suggested that these proteins might also exhibit signaling function. Such a role has by now received much support from comparative biochemical and embryological studies in *Drosophila*, *Xenopus* and mouse (reviewed in Kirkpatrick and Peifer, 1995; Miller and Moon, 1996). In this pathway starting with Wg/Wnt, the inactivation of the serine/threonine kinase Zeste White 3 (ZW3) or its vertebrate homolog glycogen synthase kinase 3β (GSK3β) (Cook *et al.*, 1996) leads to the stabilization and accumulation of hypophosphorylated Arm/β-catenin (Peifer *et al.*, 1994a,b), which is believed to interact with downstream-acting transcription factors (Behrens *et al.*, 1996; Huber *et al.*, 1996b; Molenaar *et al.*, 1996). In the absence of Wg/Wnt signal, ZW3/GSK3β acts to cause Arm/β-catenin to be degraded, a process which in mammalian cells also requires the adenomatous polyposis coli (APC) tumor suppressor protein (reviewed in Peifer, 1996). It has been reported that GSK3β binds to the APC–β-catenin complex and that this interaction reduces the amount of cytoplasmic β-catenin (Munemitsu *et al.*, 1995; Rubinfeld *et al.*, 1996). However, little is known about the identity of the protease actually involved in β-catenin degradation.

Proteasomes play an essential role in the rapid elimination of short-lived key regulatory proteins, e.g. cell cycle proteins (cyclins), rate-limiting enzymes (ornithine decarboxylase) or transcriptional activators (IκB–NFκB complex, c-Jun, p53) (reviewed in Coux *et al.*, 1996). Therefore, the possibility that β-catenin could be turned over by the ubiquitin–proteasome system was examined. In most cases, targeted proteins are tagged for proteolysis by the ligation of multiple ubiquitin molecules in a multimeric chain. Ligation involves a series of enzymatic reactions: ubiquitin itself is first activated with ATP by the ubiquitin-activating enzyme (E1), and secondly transferred onto a ubiquitin carrier protein (E2), then in a third step transferred onto a ubiquitin ligase (E3), which catalyzes the covalent modification of lysine residues of the target protein with ATP-activated ubiquitin. In subsequent cycles, additional ubiquitin molecules are added to the substrate. Multi-ubiquitinated proteins are then recognized by the 19S regulatory subunit of the proteasome and rapidly degraded into short peptides (reviewed in Jentsch, 1992). Here we present results demonstrating that β-catenin is regulated by the ubiquitin–proteasome pathway. We also show that ubiquitination of β-catenin is reduced in Wnt-expressing cells and completely abolished when the GSK3β phosphorylation consensus motif in β-catenin is mutated.

Results

β-catenin is degraded by the proteasome pathway

It is well established that lactacystin and the peptide aldehyde ALLN (*N*-acetyl-Leu-Leu-norleucinal, LLnL or

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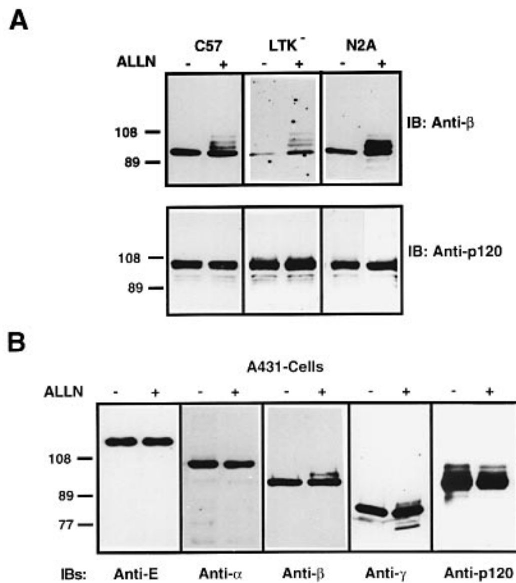

Fig. 1. Proteasome inhibitor ALLN generates higher molecular weight forms of β-catenin and plakoglobin, but not of E-cadherin, α-catenin or p120^{cas}. (**A**) Cell lysates of C57MG (C57), LTK$^-$ and Neuro2A (N2A) cells (with or without ALLN for 4 h) were immunoblotted (IB) with antibodies specific for β-catenin or p120^{cas} (control). (**B**) A431 epithelial cells were treated with ALLN as above, and components of the cadherin–catenin complex were analyzed by immunoblotting with the indicated antibodies: E, E-cadherin; α, α-catenin; β, β-catenin; γ, γ-catenin (plakoglobin). The positions of molecular weight markers are indicated on the left.

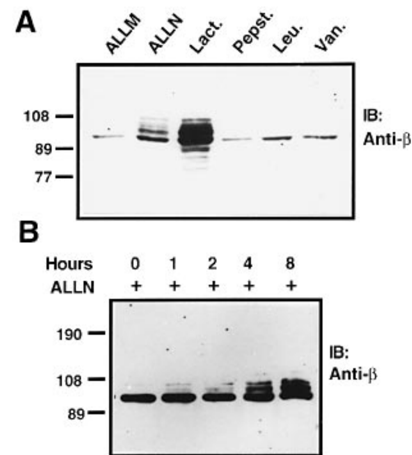

Fig. 2. Higher molecular weight forms of β-catenin are induced by proteasome-specific protease inhibitors. (**A**) LTK$^-$ cells were treated with either 25 μM ALLM or ALLN, 10 μM lactacystin, 10 μM pepstatin A, 10 μM leupeptin or 1 mM sodium vanadate. Cell lysates were immunoblotted with anti-β-catenin antibodies. (**B**) C57MG cells, incubated in ALLN-containing medium for 0, 1, 2, 4 and 8 h, were subjected to immunoblot (IB) analysis for β-catenin. Higher molecular weight β-catenin is already visible 1 h after ALLN treatment. The positions of the molecular weight markers are indicated on the left.

calpain inhibitor I) inhibit proteasome-mediated proteolysis, and that this leads to an accumulation of proteins that are usually metabolized by this pathway (Coux *et al.*, 1996). In an initial set of experiments, the effect of ALLN was tested on C57MG, LTK$^-$ and Neuro2A cells. Upon treatment with ALLN, all three cell lines clearly showed higher molecular weight forms of β-catenin, while comparable modifications were not observed when the same cell lysates were immunoblotted for p120^{cas} (Figure 1A). p120^{cas}, like β-catenin and plakoglobin, is a member of the Arm repeat family, and it is also associated with the cadherin–catenin complex (Daniel and Reynolds, 1995). A similar analysis for plakoglobin in the same fibroblastic and neural cell lines was hampered by the low amount of plakoglobin in these cells (not shown).

Using the epithelial cell line A431, which expresses a functional E-cadherin–catenin complex, the effect of ALLN on each component of the complex was investigated. As can be seen in Figure 1B, in the presence of ALLN no changes in amount or mobility were observed for E-cadherin, α-catenin, or p120^{cas}. Here again, ALLN induced slower migrating, hence larger forms of β-catenin, although the typical ladder of slower migrating bands was not as obvious as in fibroblastic cells. For the β-catenin homolog plakoglobin, higher molecular weight forms could also be detected in A431 cells in response to ALLN (Figure 1B).

Thus it appears that β-catenin modifications are much more prominent in non-epithelial cells. It is noteworthy that these cell types express considerable β-catenin mRNA but only little protein, suggesting that β-catenin is synthesized and degraded continuously. Interestingly, only components of the cadherin–catenin complex which could potentially take part in signaling are affected by the proteasome inhibitor.

Using LTK$^-$ cells, other protease inhibitors were included to demonstrate that the modified forms of β-catenin result from proteasome-specific inhibition, since ALLN also inhibits non-proteasomal proteases such as calpains and cathepsins. ALLM (*N*-acetyl-Leu-Leu-methional, LLM or calpain inhibitor II) is a structurally related peptide aldehyde and a potent inhibitor of calpains and cathepsins, but it does not inhibit the proteolytic activity of proteasomes (Rock *et al.*, 1994). In contrast, the natural compound lactacystin is a highly specific inhibitor of proteasomal activity (Fenteany *et al.*, 1995). As can be seen in Figure 2A, only ALLN and lactacystin induced slower migrating forms of β-catenin and an accumulation of the protein. ALLM and two other diffusible protease inhibitors, pepstatin and leupeptin, as well as the phosphatase inhibitor vanadate, had no effect (Figure 2A). From these results it is concluded that accumulation of post-translational modifications of β-catenins is induced only by proteasome-specific protease inhibitors. In time-course experiments, it was found that the modification can already be observed 1 h after the addition of ALLN (Figure 2B). ALLN and lactacystin gave essentially identical results in all subsequent experiments.

It was necessary to exclude that the post-translational modifications of β-catenin do not arise from hyperphosphorylation, since Arm is known to undergo Wg-dependent alterations in its electrophoretic mobility due to changes in the phosphorylation pattern (Peifer *et al.*, 1994a). For this, cell lysates of ALLN-treated or untreated cells, randomly labeled with radioactive phosphate, were treated with phosphatases and immunoblotted for β-catenin (Figure 3). The autoradiograph in Figure 3B illustrates that the phosphatase was active under these conditions. Immunoblot analysis of the same lysates with anti-β-catenin antibodies revealed that lambda phosphatase had no effect on the slower migrating forms of β-catenin

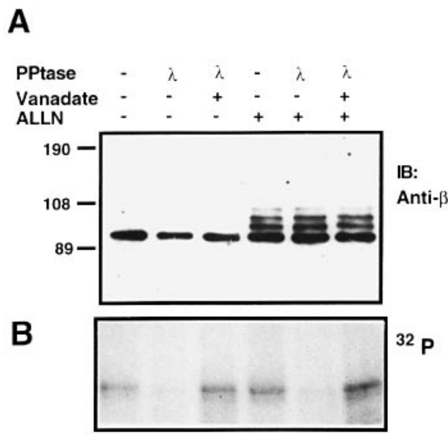

Fig. 3. Higher molecular weight forms of β-catenin are not due to hyperphosphorylation. Cell lysates of C57MG cells, with or without ALLN, were labeled *in vitro* with [γ-^{32}P]ATP (see Materials and methods) and treated with lambda phosphatase (λ) and its inhibitor vanadate, as indicated. Immunoblots were developed with anti-β-catenin antibodies. (**A**) The ALLN-induced ladder of β-catenin forms is not affected by the phosphatase. (**B**) An autoradiograph of the same lysates demonstrates that randomly labeled proteins were dephosphorylated by the phosphatase.

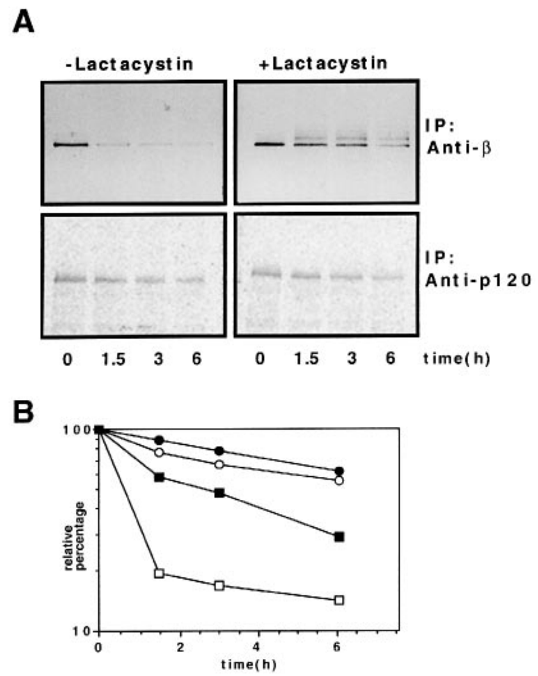

Fig. 4. Proteasome inhibitors increase the metabolic stability of β-catenin but not that of p120^{cas}. (**A**) ^{35}S-labeled C57MG cells were chased for the times indicated, and cell lysates were immunoprecipitated under denaturing conditions with anti-β-catenin or control anti-p120^{cas} antibodies. p120^{cas} had a rather long half-life (6–8 h), so only a small amount of protein was labeled during the pulse period. Therefore, to detect the radioactive signal, a PhosphoImager had to be used. In contrast, the half-life of β-catenin was <60 min in untreated cells but became 3-fold longer in response to lactacystin. (**B**) Quantitative measurements of the band intensities in (A) using MacBas image analysis software. ●, p120^{cas} + Lact.; ○, p120^{cas} – Lact.; ■, β-catenin + Lact.; □, β-catenin – Lact.

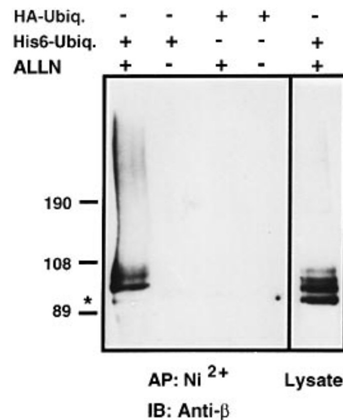

Fig. 5. His-tagged ubiquitin covalently bound to β-catenin was found only in higher molecular weight forms. His- or HA-tagged ubiquitin was transiently expressed in Neuro2A cells. Ubiquitin–substrate conjugates which accumulate after ALLN treatment were affinity precipitated (AP) by Ni^{2+}-chelate chromatography under denaturing conditions. Bound proteins were eluted and immunoblotted using anti-β-catenin antibodies. The asterisk denotes the position of the non-ubiquitinated 92 kDa form of β-catenin. Lysate: control immunoblot of whole cell lysate with anti-β-catenin antibodies. The positions of the molecular weight markers are indicated on the left.

(Figure 3A). Identical observations were made using potato acidic phosphatase (not shown). Thus, as already indicated by the results of the vanadate treatment (Figure 2A), the higher molecular weight forms of β-catenin do not arise as a result of hyperphosphorylation. To rule out that ALLN had an effect on the transcriptional regulation of β-catenin, Northern blot analysis was performed. No up-regulation of the β-catenin mRNA in response to ALLN could be detected in C57MG, LTK$^-$ or Neuro2A cells (not shown).

Next we investigated whether lactacystin might increase the metabolic half-life of β-catenin. For this, C57MG cells were incubated with or without lactacystin for 4 h, metabolically labeled for 1 h and chased for the times indicated in Figure 4A. At each time point, β-catenin (and p120^{cas} as a control) was immunoprecipitated, using denaturing conditions to avoid any non-covalent protein–protein interactions. The immunoprecipitates were separated by SDS–PAGE, processed for fluorography and subjected to quantitative measurements using a PhosphoImager (Figure 4B). In untreated cells, β-catenin had a rather short half-life of <60 min. In comparison, p120^{cas} appeared to be rather stable, with a half-life of 6–8 h, which was unchanged in the presence of inhibitors. Importantly, lactacystin increased the half-life of β-catenin by ~3-fold (150–200 min) compared with untreated cells (Figure 4A and B). These results show clearly that the accumulation of β-catenin in the presence of the inhibitor is associated with an increased metabolic stability.

Ubiquitination of β-catenin

The results presented so far strongly suggested that β-catenin is subjected to the ubiquitin–proteasome pathway. To demonstrate that the higher molecular weight forms of β-catenin indeed contain ubiquitin, a recently developed assay for the detection of ubiquitinated proteins was employed (Treier *et al.*, 1994). This experimental system allows affinity purification of His-tagged ubiquitin–substrate complexes under denaturing conditions by Ni^{2+}-chelate chromatography. DNA coding for human ubiquitin tagged with six histidine residues at its N-terminus was transfected into Neuro2A cells. His-tagged cellular proteins were affinity purified and immunoblotted for β-catenin. As can be seen in Figure 5, only higher molecular

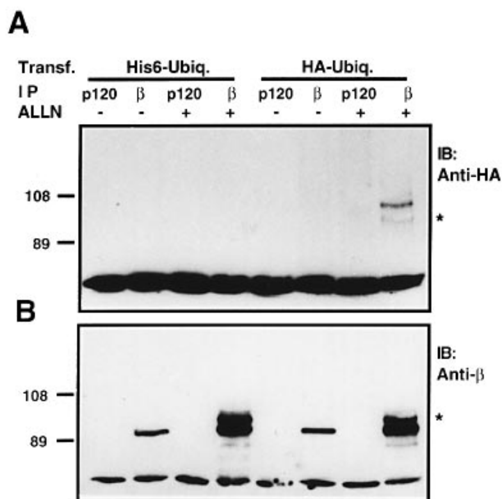

Fig. 6. Immunoprecipitated β-catenin contains HA-tagged ubiquitin in response to proteasome inhibitor ALLN. (**A**) HA- or His-tagged ubiquitin was transiently expressed in Neuro2A cells; immunoprecipitates were collected with antibodies against β-catenin or $p120^{cas}$ and blotted with antibodies against the HA tag. In (A) and (B), the asterisk denotes the first modified band, which was weakly reactive with the anti-HA antibody. (**B**) The same blot was stripped and re-probed with anti-β-catenin antibodies.

weight forms of β-catenin were eluted from the Ni^{2+}-chelate column. Little protein is detectable at the position of unmodified β-catenin, present in the whole cell lysate (Figure 5). Cells transfected with hemagglutinin (HA)-tagged ubiquitin were included as a control. In another series of experiments, HA-tagged ubiquitin (and His6-ubiquitin as a control) was transiently expressed in Neuro2A cells; immunoprecipitates were then collected with anti-β-catenin antibodies (or anti-$p120^{cas}$ as a control), and subjected to immunoblot analysis with antibodies directed against the HA epitope (Figure 6A). After stripping, the same blot was stained with anti-β-catenin antibodies (Figure 6B). HA-positive higher molecular weight proteins were only detected in β-catenin immunoprecipitates when the cells had been treated with ALLN. The differences in the intensity of staining of the HA-ubiquitinated β-catenin by the anti-HA antibody may be caused by the increased number of HA epitopes in the higher molecular weight forms. Thus, two independent experimental approaches demonstrate that inhibitors of proteasome activity lead to an accumulation of multi-ubiquitinated β-catenin.

The GSK3β consensus phosphorylation site in β-catenin is necessary for ubiquitination

It has been shown previously that inhibition of ZW3/GSK3β stabilizes Arm/β-catenin (Peifer *et al.*, 1994a) and that mutations in the GSK3β phosphorylation consensus sequence in β-catenin (amino acid positions 33–45) lead to an accumulation of mutant protein (Yost *et al.*, 1996). The results with the mutations in the GSK3β phosphorylation consensus sequence in β-catenin stimulated experiments to test whether this region may also be important for ubiquitination. To do this, several mutant β-catenin cDNAs were generated containing single or combinatorial amino acid substitutions, as underlined in the amino acid sequence depicted in Figure 7A. In mutant β.4SA, amino acid residues S33, S37, T41 and S45 were all substituted by alanine, as reported (Yost *et al.*, 1996). Mutant β.5SD had aspartates at positions S29, S33, S37, T41 and S45; mutants β.K19R and β.K49R were single point mutations with a substitution of a lysine by an arginine, while mutant β.K19,49R contained both substitutions. Wild-type and mutant β-catenins carrying a C-terminal myc tag were transiently expressed in Neuro2A cells, treated with ALLN and immunoprecipitated with anti-myc antibodies (Figure 7A). Ubiquitination was abolished completely in mutants β.4SA and β.5SD. The negatively charged aspartate residues introduced in mutant β.S5D are apparently not able to trigger ubiquitination. In contrast, myc-tagged wild-type β-catenin (β.myc) and mutant forms β.K19R and β.K49K, as well as the double mutant β.K19,49R, all still became ubiquitinated in response to ALLN, although in the double mutant ubiquitination is slightly reduced (Figure 7A). It should be noted that overexpressed β-catenin was always clearly less ubiquitinated and the ladder of higher molecular weight proteins was not as obvious as with endogenous β-catenin. One possible explanation is that the ubiquitination machinery becomes less efficient with overexpressed proteins.

In conclusion, the mutational analysis clearly demonstrated that the GSK3β consensus phosphorylation site in β-catenin is necessary for ubiquitination. This opened the possibility that an identical site in β-catenin is a target for both the Wnt signaling pathway and the ubiquitin-mediated degradation system. To test this possibility, NIH 3T3 cells and Wnt-1-transfected NIH 3T3 cells (3T3Wnt) generated by retroviral-mediated gene transfer were compared after ALLN treatment (Figure 8A). Expression of Wnt-1 in NIH 3T3 cells greatly reduced the ubiquitination of β-catenin. In addition, expression of Wnt-1 led to an increased cytoplasmic stabilization of β-catenin, as monitored by binding studies with recombinant proteins (Figure 8B). For these studies, fusion proteins containing the glutathione-*S*-transferase (GST) tag and the full-length cytoplasmic domain of E-cadherin (ECT.884, amino acid position 737–884) or, as a control, a truncated version lacking the β-catenin-binding site (ECT.823, amino acid position 737–823) were used in binding studies with cell lysates from NIH 3T3 cells or their Wnt-1 transfectant. The ECT.884 fusion protein clearly interacted with uncomplexed β-catenin in Wnt-transfected cells, whereas no β-catenin was precipitated in untransfected NIH 3T3 cells (Figure 8B). Upon ALLN treatment, ECT.884 interacted with ubiquitinated β-catenin in both cell types, indicating that ubiquitination does not interfere with binding of β-catenin to E-cadherin. Here again, in NIH 3T3 cells expressing Wnt-1, the relative amount of ubiquitinated β-catenin was considerably reduced. The faster migrating bands in Figure 8B are due to some degradation of β-catenin not seen in other cell types.

The site necessary for ubiquitination in β-catenin is similar to a motif described in the N-terminus of IκB (inhibitor of NFκB) which is required for the signal-dependent degradation of IκB via the ubiquitin–proteasome pathway (Chen *et al.*, 1995; DiDonato *et al.*, 1996). Alignment of the amino acid sequences of members of the β-catenin and IκB protein families reveals a conserved consensus motif (Figure 7B). Since this motif is conserved in these proteins, it seems likely that plako-

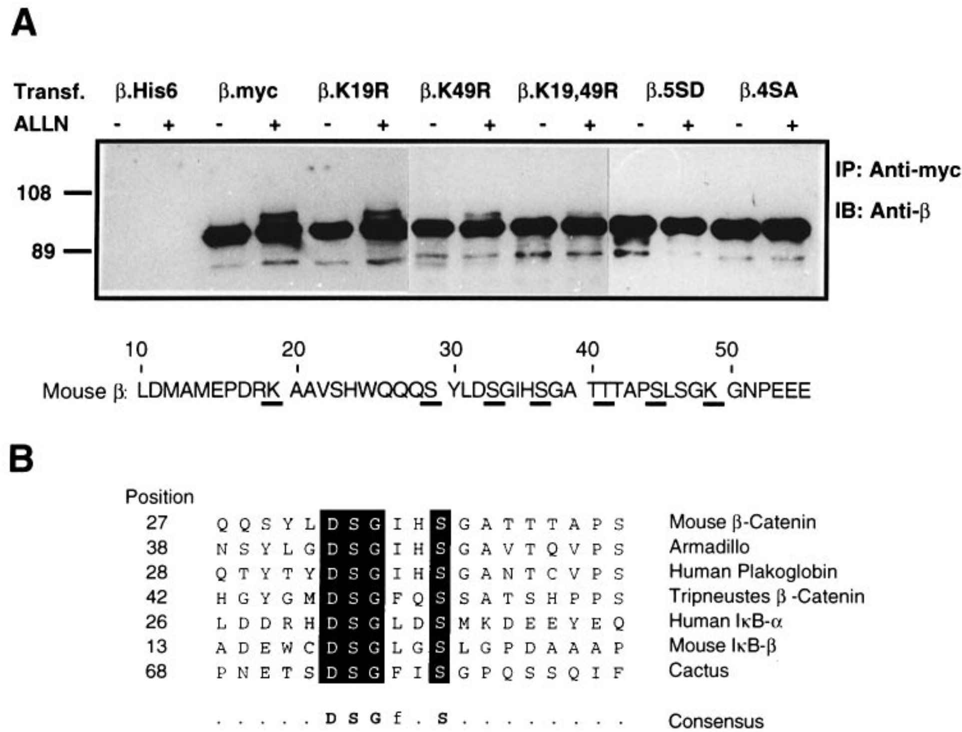

Fig. 7. Point mutations in the GSK3β consensus phosphorylation motif of β-catenin prevent ubiquitination. (**A**) Wild-type and mutated β-catenin cDNAs were transiently transfected into Neuro2A cells. All the exogenously expressed β-catenin molecules, except for β-His6, carried a C-terminal myc epitope. Cells were treated with ALLN as indicated, and lysates were then immunoprecipitated with antibodies directed against the myc epitope. Ubiquitination of β-catenin was monitored in immunoblots. For the construct β.K19,49R encoding a double mutation of both N-terminal lysines, a weak ubiquitinated band was always visible. The construct β.5SD harbors five amino acid exchanges to aspartate (S29, S33, S37, T41, S45). Construct β.4SA contains four exchanges to alanine (S33, S37, T41, S45). (**B**) Alignment of amino acid sequences necessary for ubiquitination in members of the Armadillo and IκB families. Amino acids which are identical in all family members are depicted in the consensus sequence. f = hydrophobic.

globin and Arm also become ubiquitinated; indeed, higher molecular weight modifications of plakoglobin have been found in response to ALLN (Figure 1B). Conversely, a similar consensus motif was not found in E-cadherin, α-catenin or p120^{cas}, all proteins which were not modified after ALLN treatment.

Discussion

Our interest in the role of β-catenin in cell adhesion and signal transduction stimulated the analysis presented here on the regulation of β-catenin stability. Previous work has established the central role of β-catenin in the E-cadherin–catenin complex (Aberle *et al.*, 1994). A significant amount of β-catenin associates immediately after synthesis of E-cadherin, most likely already co-translationally at the level of the endoplasmatic reticulum (Ozawa and Kemler, 1992). It had been noted by us and others that many cadherin-negative cell lines accumulate a normal amount of β-catenin mRNA but only a very low level of β-catenin protein (Nagafuchi and Takeichi, 1989; Ozawa *et al.*, 1989). The introduction of E-cadherin into these cells clearly stabilized β-catenin protein, which had already suggested to us that in non-epithelial cells β-catenin might be synthesized and degraded continuously. The degradation of β-catenin by the ubiquitin–proteasome pathway reported here could, therefore, represent a general mechanism to regulate the turnover of β-catenin.

There is, however, another molecular mechanism which results in a cytoplasmic stabilization of β-catenin, namely in response to Wg/Wnt signaling. The stabilization of hypophosphorylated Arm/β-catenin by the inhibition of ZW3/GSK3β after Wg/Wnt signaling represents one major event in this pathway (Peifer *et al.*, 1994a). Mutations in the GSK3β consensus motif in β-catenin also lead to protein stability (Yost *et al.*, 1996). It is not known as yet whether β-catenin becomes phosphorylated within the GSK3β consensus motif itself, or whether this motif is only required for the phosphorylation of other residues in the protein. In any case, it appears very likely that ZW3/GSK3β uses Arm/β-catenin as a substrate. Another direct substrate for GSK3β is APC, which must be phosphorylated for the increased binding of APC to β-catenin. This binding results in a reduced stability of β-catenin (Munemitsu *et al.*, 1995; Rubinfeld *et al.*, 1996). At present, it appears that the intracellular pool of β-catenin is regulated by an active GSK3β which recognizes an APC–β-catenin complex. We show here that expression of WnT-1 in NIH 3T3 cells results in an increased cytoplasmic pool of β-catenin, in agreement with what occurs in C57MG cells (Papkoff *et al.*, 1996). More importantly the expression of Wnt-1 reduced the amount of β-catenin available for ubiquitination. Our results indicate that GSK3β and the ubiquitination machinery use essentially the same site in β-catenin. We also provide the first evidence on a molecular link between the Wg/Wnt signaling pathway and the ubiquitin–proteasome degradation pathway. Thus, in our present working model,

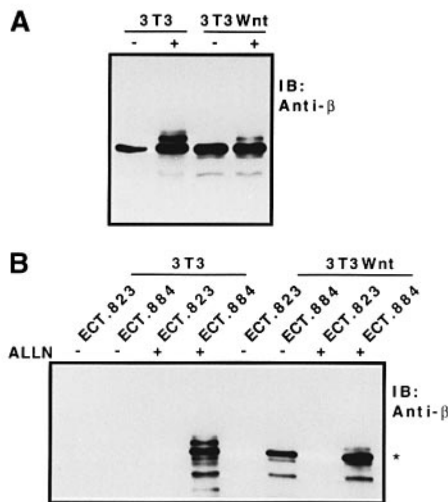

Fig. 8. Ubiquitination of β-catenin is reduced in Wnt-expressing cells. (**A**) Upon ALLN treatment, the relative amount of ubiquitinated β-catenin was greatly reduced in NIH 3T3 cells expressing Wnt-1 (3T3Wnt) as compared with untransfected NIH 3T3 cells. (**B**) Ubiquitinated β-catenin is able to bind to the cytoplasmic domain of E-cadherin. Recombinant GST fusion protein ECT.884 containing the entire cytoplasmic region of E-cadherin (ECT.823 lacking the β-catenin-binding site as a control) was used in binding studies with cell lysates from NIH 3T3 and Wnt-expressing NIH 3T3 cells. ECT.884 efficiently precipitated β-catenin from 3T3Wnt but not from untransfected 3T3 cells, demonstrating that Wnt-1 increased the cytoplasmic pool of β-catenin. Upon ALLN treatment, the amount of ubiquitinated β-catenin is reduced in 3T3Wnt cells. The faster migrating bands represent some degradation of β-catenin not seen in other cell types. The asterisk denotes the position of non-ubiquitinated β-catenin.

the phosphorylation of β-catenin by GSK3β in the absence of Wg/Wnt signal would represent the initial event for ubiquitination and degradation of β-catenin. The inactivation of ZW3/GSK3β in response to Wg/Wnt signal would then make a hypophosphorylated β-catenin unavailable for the ubiquitin–proteasome degradation machinery. Similarly, the increased stability of *Xenopus* β-catenin with mutated serine residues in the GSK3β consensus motif could also be explained if the mutant protein can no longer be ubiquitinated. The role of APC in this molecular interaction is at present less well understood. APC is a multi-domain protein, and the interaction sites for β-catenin represent only a small portion of the protein. Other interaction partners for APC are known, and still more are likely to be identified (Kinzler and Vogelstein, 1996; Peifer, 1996). In the present context, it is tempting to speculate that APC may be involved in directing β-catenin to proteasomes.

The GSK3β consensus motif in β-catenin is necessary for ubiquitination. Since ubiquitination generally occurs on lysine residues (Hershko and Ciechanover, 1992), we have mutagenized lysine residues near the consensus site, but this did not affect ubiquitination of β-catenin. We cannot at present rule out that more distantly located lysine residues may serve as acceptor sites for ubiquitin. However, as noted above, there is an interesting homology between the GSK3β consensus motif in β-catenin and a similar motif at the N-terminus of the inhibitor of NFκB (IκB) which also mediates a phosphorylation-dependent ubiquitination and degradation of IκBα (Chen *et al.*, 1995; DiDonato, 1996). It has been shown that serine residues at positions 32 and 36 in IκBα are signal-dependently phosphorylated, this being a prerequisite for targeting the protein to the ubiquitin–proteasome pathway. We show here that mutations of the equivalent serine residues in β-catenin also abolish ubiquitination. The homology between the respective motifs in β-catenin and IκB suggests that the mechanism for degradation by the ubiquitin–proteasome pathway may be similar for both proteins.

Materials and methods

Chemicals and reagents

The calpain inhibitors ALLN and ALLM and other protease inhibitors were purchased from Sigma (Deisenhofen, Germany). Lactacystin was purchased from E.J.Corey (Harvard University, Cambridge, MA). ALLM, ALLN [10 mg/ml (25 mM) in ethanol] and lactacystin [4 mg/ml (10 mM) in water] were diluted 1:1000 into the cell culture medium to stimulate cells for 4–8 h.

Plasmids and constructs

The prokaryotic expression vector pGEXUCI encoding a GST fusion protein of the entire cytoplasmic domain of mouse E-cadherin (GSTECT884) has been described (Aberle *et al.*, 1996). GSTECT823 was constructed by PCR using primers ECT.F.B (5′ATAGGATCCA-GAACGGTGGTCAAAGAG) and E2537r (5′ACTGTCGACTCAGT-CGCTGTCGGCTGCCTT). The PCR fragment was inserted into the *Bam*HI–*Sal*I sites of pGEX4T1 (Pharmacia, Freiburg, Germany). For all PCR reactions, *Pwo* polymerase (Boehringer, Mannheim, Germany), which exhibits proofreading activity, was used. All PCR-derived constructs were sequenced using Sequenase 2.0 (US Biochemical Corp., Cleveland, OH).

A eukaryotic expression vector for β-catenin carrying six histidine residues at its C-terminus (pcDNA3β-His6) was constructed by PCR, using primers β1678 (5′gcgggaacagggtgctattc) and β2440r (5′tcag-gatcccaggtcagtatcaaac). The PCR fragment was cut with *Bam*HI and *Spe*I and ligated simultaneously with an N-terminal *Bam*HI–*Spe*I fragment from pGEXβtot (Aberle *et al.*, 1994) into the *Bam*HI site of pQE60 (Qiagen, Hilden, Germany). The resulting vector, pQE60βHis6, was cut with *Bam*HI–*Sst*I and *Sst*I–*Nhe*I, and the two fragments were subcloned into the *Bam*HI–*Xba*I-digested cytomegalovirus (CMV)-based expression vector pcDNA3 (Clontech, Heidelberg, Germany). An expression vector for untagged β-catenin ($pCS2^{+}β$) was constructed by inserting the *Bam*HI fragment of pGEXβtot into the *Bam*HI site of $pCS2^{+}$ (kindly provided by R.Rupp, Tübingen, Germany). For the construction of a cDNA encoding myc-tagged mouse β-catenin, two complementary oligonucleotides, 5′GATCCACTAGTGAACAGAAGCTCATCTCTGA-AGAAGATCTGTGAGATCTC and 5′TCGAGAGATCTCACAGATC-CTCTTCAGAGATGAGCTTCTGTTCACTAGTG, encoding the myc epitope (Evan *et al.*, 1985) flanked by a stop codon and restriction sites, were cloned into the prokaryotic vector pSP72 (Promega, Southampton, UK) using *Bam*HI (5′) and *Xho*I (3′). The *Bam*HI site of pSP72myc tag was used to insert the *Bam*HI fragment of pQE60βHis6 fusing the β-catenin cDNA missing a stop codon with the myc tag (pSPMMBCmyc tag, *Mus musculus* β-catenin). For expression in eukaryotic cells, a *Sac*I–*Xho*I fragment of pSPMMBCmyc tag containing the myc-tagged C-terminus of β-catenin was inserted into a *Sac*I–*Xho*I-digested $pCS2^{+}β$ ($pCS2^{+}$MMBCmyc tag).

Site-directed mutagenesis was performed exactly as described (Aberle *et al.*, 1996). Mutagenic primers were: K19R, TGACAGCA-GCGCGCCTGTCCGGCT (*Bss*HII); K49R, AGGGTTGCCCCGGCCG-CTCAGGGAA (*Eag*I); S29D, GCCACTCAGGTCAGGAGCTGTG-TCGGTGGCACCATCATGAATTCCATCATCCAAGTAATCCTGCT-GCTGCC (*Eco*RI, *Bsp*HI); S33A, CTTGCCACTCAGCGCTGG-AGCTGTGGCGGTTGCACCAGCATGGATTCCAGCATCCAAGTA-AGACTG (*Eco*47III). Mutated N-termini of β-catenin were excised from pGEXMβX with *Sma*I (5′) and *Sac*I (3′) and inserted into *Sma*I–*Sac*I-digested $pCS2^{+}$MMBCmyc tag.

The CMV-driven ubiquitin expression constructs pMT107 (His6-ubiquitin) and pMT123 (HA-ubiquitin) have been described (Treier *et al.*, 1994). and were kindly provided by D.Bohmann (EMBL, Heidelberg, Germany).

Cell culture and immunological procedures

Human epidermoid carcinoma cells A431 (ATCC, CRL1555), mouse neuroblastoma Neuro2A cells (ATCC, CCL-131), mouse mammary C57MG cells and mouse fibroblastic LTK^- cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). NIH 3T3 cells stably expressing Wnt-1 were produced by retroviral infection according to Pear *et al.* (1993). Briefly, DNA of the retroviral vector pLNCX.wnt1 (a kind gift of Jan Kitajewski), which harbors a full-length Wnt-1 cDNA under the control of the CMV promotor (Miller and Rosman, 1989; Parkin *et al.*, 1993), was transfected into the packaging cell line Bosc23 using the calcium phosphate co-precipitation method. Supernatant containing viral particles was harvested after 2–4 days and used to infect subconfluent NIH 3T3 cells. Stably infected clones were selected with 1 mg/ml G418 and used as pools in subsequent experiments.

Cells grown to 80% confluency were washed twice with phosphate-buffered saline (PBS) and lysed in 1 ml of PSK buffer [10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM KCl, 2 mM $MgCl_2$, 4 mM EGTA, 1 mM NaF, 1 mM sodium vanadate, 0.25% (v/v) Triton X-100 (TX-100), 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 0.1 μg/ml α_2-macroglobulin]. Cell lysates were subjected to immunoprecipitation experiments as described (Hoschützky *et al.*, 1994).

For pulse–chase experiments, cells were grown in 3.5 cm plates. Proteasomes were inhibited by the presence of lactacystin (10 μM), starting 4 h before and during the whole experimental procedure. Treated or untreated cells were washed twice with PBS and starved for 60 min in methionine- and cysteine-free medium. After a labeling period of 60 min with [^{35}S]methionine/[^{35}S]cysteine (150 μCi/ml), cells were chased for 0, 1.5, 3.0 and 6.0 h. At each time point, cells were lysed in PSK buffer containing 0.5% SDS and heated for 5 min to 100°C. The soluble fractions were diluted 5-fold with PSK buffer and subjected to immunoprecipitation experiments. Immunoblots were carried out as described (Aberle *et al.*, 1994). Gels and blots were calibrated with pre-stained molecular weight markers (Sigma).

Polyclonal antibodies against the C-terminus of β-catenin (P14L) have been described (Butz and Kemler, 1994). Monoclonal antibodies against human E-cadherin (HECD-1) were purchased from Takara (Shiga, Japan). Monoclonal antibodies against the cytoplasmic domain of human E-cadherin, the C-termini of α-, β-, γ-catenin and $p120^{cas}$ were purchased from Transduction Laboratories (Lexington, KY). Antibodies against the HA epitope were obtained from Boehringer. Anti-myc antibodies (Evan *et al.*, 1985) were purified from supernatants of hybridoma clone 9E10 grown in DMEM containing 3% FCS.

Affinity precipitation

Purified GST-tagged cytoplasmic domain of mouse E-cadherin (GST.ECT) was used for affinity precipitation of whole cell lysates (Aberle *et al.*, 1996).

Purification of His-tagged ubiquitin transiently expressed in Neuro2A cells was done following the method of Treier *et al.* (1994) with the following modifications. Cells were lysed with 1 ml of GTN buffer per 100 mm dish (6 M guanidinium-HCl, 20 mM Tris–HCl, pH 8.0, 200 mM NaCl, 10 mM imidazole, 0.1% TX-100). Ni^{2+}-NTA–agarose beads (Qiagen) (50 μl, 50% slurry) were successively washed with the following solutions (pH 7.5–8.0): 1 ml of GTN buffer; 1 ml of 8 M urea, 20 mM Tris–HCl, 200 mM NaCl, 0.1% TX-100; 1 ml of 8 M urea, 20 mM Tris–HCl, 1 M NaCl, 0.1% TX-100; 1 ml of 4 M urea, 20 mM Tris–HCl, 200 mM NaCl, 0.1% TX-100; 1 ml of 1 M urea, 20 mM Tris–HCl, 200 mM NaCl, 0.1% TX-100; and 1 ml of 20 mM Tris–HCl, 200 mM NaCl, 10 mM imidazole, 0.1% TX-100.

Phosphatase treatment

Cells were lysed in 50 mM Tris–HCl, pH 8.0, 5 mM dithiothreitol, 2 mM $MgCl_2$, 0.1% TX-100, with protease inhibitors, as described above. Then 50 μl aliquots of the TX-100-soluble fraction containing no phosphatase inhibitors were incubated with 0.1 μl of [γ-^{32}P]ATP (3000 Ci/mmol) at 37°C for 15 min. Subsequent dephosphorylation of cellular proteins was carried out for 45 min at 30°C by adding $MnCl_2$, bovine serum albumin and 400 U of lambda protein phosphatase (λ PPtase), according to the instructions of the manufacturer (Biolabs, Hitchin, UK). In some samples, sodium vanadate (2 mM final concentration) was added to inhibit the phosphatase. Dephosphorylation was stopped by adding an equal volume of Laemmli buffer and monitored by autoradiography or immunoblotting.

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