Serine Phosphorylation-regulated Ubiquitination and Degradation of β-Catenin*

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Several lines of evidence suggest that accumulation of cytoplasmic β-catenin transduces an oncogenic signal. We show that β-catenin is ubiquitinated and degraded by the proteasome and that β-catenin stability is regulated by a diacylglycerol-independent protein kinase C-like kinase activity, which is required for β-catenin ubiquitination. We also define a six-amino acid sequence found in both β-catenin and the NF-κB regulatory protein IκBα, which, upon phosphorylation, targets both proteins for ubiquitination. Mutation of a single serine within the ubiquitination targeting sequence prevents ubiquitination of β-catenin. Mutations within the ubiquitination targeting sequence of β-catenin may be oncogenic.

β-Catenin plays an important role in both cell-cell adhesion and growth factor signal transduction (1, 2). Consistent with these two functions within the cell, the protein is localized primarily in two intracellular pools; a membrane pool involved in cadherin-mediated cell-cell adhesion and a cytoplasmic pool important for signaling (1, 2). The role of β-catenin in cell-cell adhesion has been well studied. It links cadherins to the actin cytoskeleton, which results in the formation of the adherens junction (1, 2). Tyrosine phosphorylation of β-catenin can regulate cell-cell adhesion by disrupting particular protein-protein interactions (3, 4).

The Drosophila and Xenopus homologs of β-catenin are also known to be involved in signaling pathways that regulate embryonic patterning. The Drosophila homolog Armadillo (Arm) lies downstream of the Wingless (Wg) receptor and the serine kinase Zeste-White 3 (ZW3) in the Wingless pathway that regulates segmental pattern formation. The Xenopus pathway is comprised of the vertebrate homologs of the Drosophila proteins Wg and Zw3 (Wnt-1 and glycosyn synthase kinase 3β (GSK3β), respectively) and regulates dorsal axis formation (2).

More recently, the interaction of β-catenin with members of the LEF/TCF family of transcription factors was shown to be important in Wnt signaling and in colon cancer (5–8). β-Catenin signaling in embryogenesis and oncogenesis appears to be regulated by controlling the accumulation of cytoplasmic β-catenin. Activation of the Wnt (Wg) pathway results in the inhibition of GSK3β (ZW3) activity, which, in turn, results in stabilization of cytoplasmic β-catenin (Arm) (1, 9, 10).

The tumor suppressor protein APC appears to be required for the normal degradation of β-catenin as mutated forms of APC result in high levels of free (i.e. cytoplasmic) β-catenin with a longer half-life (11). The mechanism by which GSK3β and APC regulate β-catenin stability is unknown. Although GSK3β can directly phosphorylate APC and β-catenin in vitro, it is not clear how these interactions regulate β-catenin stability in vivo (12, 13).

In this report we show that β-catenin is normally degraded by the ubiquitin/proteasome pathway. We also show that certain protein kinase C (PKC) inhibitors cause a dramatic accumulation of cytoplasmic β-catenin by inhibiting its ubiquitination. In addition, we define a six-amino acid motif that is involved in targeting both β-catenin and the inhibitor of NF-κB, IκBα, for ubiquitination. A serine to alanine mutation within this ubiquitination targeting sequence (UTS) stabilizes the protein by inhibiting its ubiquitination.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cells—ALLN (calpain inhibitor I), ALLM (calpain inhibitor II), GF-109203X (bisindoylmalimide), and TPA were purchased from Boehringer Mannheim. Lactacystin and chlorphostin C were purchased from Calbiochem. Ro31-8220 was a gift from Dr. Robert Glazer. The monoclonal anti-β-catenin was purchased from Transduction Laboratories. The anti-ubiquitin polyclonal was raised by Dr. Weissman (23). The anti-HA antibody (monoclonal clone HA-11) was purchased from Babco. SKBR3 and HBL100 cell lines were acquired from the ATCC and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cellular Fractionation—Nonidet P-40 extracts were performed as described previously (14) except that 10 mM N-ethylmaleimide (NEM) was added to the lysis buffer. Cytoplasmic fractionation was performed by washing cells twice in phosphate-buffered saline and removing all remnants of the final wash. The cells were incubated in ice-cold hypotonic lysis buffer (10 mM Tris, pH 7.4, 0.2 mM MgCl₂, 5 mM NEM, and 10 mM ALLN) for 5 min on ice. The cells were scraped and mechanically disrupted by 30 strokes in a Dounce homogenizer. Greater than 95% of the cells were lysed as judged by light microscopy. The lysates were transferred to an ultracentrifuge tube containing a 5 × inhibitor solution (250 mM NaF, 5 mM sodium vanadate, 25 mM NEM, apronitin, leupeptin, pepstatin A, and 4-2-aminophenylbenzenesulfonyl fluoride). The insoluble components of the lysates were pelleted at 100,000 × g for 1 h. The supernatant was designated the S100 or cytoplasmic fraction, and the pellet was the P100 fraction. In some experiments the P100 fraction was extracted with Nonidet P-40 buffer. Lysates were boiled in 2 × Laemmli sample buffer with 10% β-mercaptoethanol and separated on 8% or 4–12% polyacrylamide gels (Novex).

Immunoprecipitations—Immunoprecipitations were performed on equal amounts of Nonidet P-40 lysates. The lysates were preclatured once with 10 μg non-immune mouse IgG and 100 μl of protein G-Sepharose (Life Technologies, Inc.) and once with 100 μl of protein G-Sepharose, both for 1.5 h in the cold. The lysates were incubated in the cold for 1.5 h with 6 μg of non-immune mouse IgG or monocalonal anti-β-catenin. Protein G-Sepharose was added for 45 min. The protein G-antibody-antigen complexes were pelleted in a cold microcentrifuge and washed six times in cold Nonidet P-40 buffer without inhibitors.

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**FIG. 1.***β*-Catenin is ubiquitinated and degraded by the proteosome.*** Immunoblots and immunoprecipitations were performed with equal amounts of Nonidet P-40 lysates. A, SKBR3 cells were treated for 4 h with increasing amounts of either the peptide aldehyde proteosomal inhibitor ALLN or the related peptide aldehyde calpain protease inhibitor ALLM. Cells were lysed in Nonidet P-40, separated by SDS-PAGE, and immunoblotted with an anti-β-catenin polyclonal antibody. B, SKBR3 cells were treated (Tx) overnight with the proteosomal inhibitors ALLN (10 μM) and lactacystin (Lact.) (100 μM) and with ALLM (10 μM) and the lactacystin vehicle ethanol (20 μL). The cells were lysed and analyzed as in A. C, HBL100 cells were treated overnight with either 10 μM ALLN or vehicle (Me2SO (DMSO)) overnight. The cells were lysed in Nonidet P-40 and immunoprecipitated (IP) with 6 μg of either anti-β-catenin monoclonal antibody (β) or non-immune mouse IgG (NI). The precipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride. The membrane was treated as in Ref. 15 and probed with an anti-ubiquitin antibody.

**FIG. 2.***Cytoplasmic β-catenin accumulates in response to bisindoylmaleimide PKC inhibitors and lithium.*** Confluent plates of HBL100 s (A, B, C, and E) were treated overnight with the PKC inhibitors GF-109203X (A), Ro31-8220 (B), and calphostin C (C), and the pluriportent inhibitor lithium chloride (E) at increasing doses. SKBR3 (D) cells were treated with increasing doses of PMA for 3 days. Cytoplasmic extracts were made from all of these cells and the proteins separated by SDS-PAGE and immunoblotted with anti-β-catenin monoclonal antibody. Equal amounts of cytoplasmic extracts were loaded per well for immunoblotting. Calphostin C was toxic to the cells at 4 μM.

The immunoprecipitated proteins were boiled for 5 min and separated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride (Millipore) and treated as described in Ref. 15.

**β-Catenin Construct and Mutagenesis**—The nine-amino acid HA-tag was added to the C terminus of β-catenin using polymerase chain reaction. The β-catenin/HA construct was cloned into the BamHI site of the pCPhA vector (Invitrogen). Mutagenesis of serine 37 was performed using the Quick-Change site-directed mutagenesis kit (Stratagene).

**RESULTS AND DISCUSSION**

**β-Catenin Is Degraded by the Ubiquitin-Proteosome Pathway**—Proteins that are to be degraded by the ubiquitin/proteosome system are first conjugated to multiple copies of the small protein ubiquitin through isopeptide linkages (17). These ubiquitinated proteins are then recognized and degraded by the 26 S proteosome. Inhibition of the proteosome results in accumulation of the ubiquitinated forms of these proteins normally degraded by the proteosome. To determine whether β-catenin is degraded by this system, human breast epithelial cell lines were treated with the peptide aldehyde proteosomal inhibitor ALLN or a related peptide aldehyde that has 50–100-fold lower potency as a proteosomal inhibitor. Anti-β-catenin immunoblots revealed that the proteosomal inhibitor at a dose as low as 10 μM inhibited the proteosome. These data demonstrate unequivocally that β-catenin is normally degraded by the proteosomal complex following ubiquitination.

An Atypical PKC-like Kinase Regulates Cytoplasmic β-Catenin Accumulation—GSK3β is a member of the Wnt/Wg pathway, and its activity is required to maintain low levels of cytoplasmic β-catenin (9, 12). In addition, GSK3β itself can be regulated by several other serine kinases. In vitro, p70 S6 kinase, p90RSK, Akt/protein kinase B (PKB), and certain PKC isoforms phosphorylate and regulate GSK3β activity (18–21).
Several other growth factors, including EGF and IGF also result in the inactivation of GSK3β (22). In cultured cells, inhibitor studies showed that the activity of a TPA-sensitive PKC isoform is required to inactivate GSK3β in response to the Wg signal (23). This PKC isoform appeared to be specifically involved in Wg-mediated regulation of GSK3β, as it was not involved in other GSK3β regulating signals (e.g. insulin, EGF, IGF-1, and serum) (23). No studies have determined the effect of PKC inhibition on β-catenin accumulation.

Cells were treated with different PKC inhibitors and assayed for β-catenin accumulation. In the HBL100 cell line, which has high levels of membrane-associated and low levels of cytoplasmic β-catenin, two bisindoylmaleimide-type PKC inhibitors (GF-109203X and Ro31-8220) caused a dramatic increase in the cytoplasmic pool but not the membrane pool (Fig. 2 B). The same result was seen in Madin-Darby canine kidney cells (data not shown). In SKBR3 cells, which have very low levels of β-catenin protein in both pools, there was a large increase in the cytoplasmic pool but not the membrane pool (Fig. 2 B). These results distinguish it from the DAG-dependent PKC isoform that is responsible for Wnt-dependent GSK3β regulation (23).

In addition to inhibitors of PKC, lithium chloride, which is an inhibitor of GSK3β (as well as other enzymes), was used to investigate its effects on β-catenin accumulation (24, 25). Treatment of Xenopus embryos with lithium chloride results in the formation of a secondary dorsal axis in the embryo, phenotypes that are also characteristic of ectopic Wnt or β-catenin overexpression (2, 26). Treatment with 30 μM results in the decrease in activity normally induced by Wg (23). The inhibitor profile of the PKC isoform involved in β-catenin accumulation indicates that it may be a member of the DAG-independent class of PKCs known as atypical PKCs (aPKC). These results distinguish it from the DAG-dependent PKC isoform that is responsible for Wnt-dependent GSK3β regulation (23).

**Fig. 4.** β-Catenin contains an IeBo-like six-amino acid sequence, which, when phosphorylated, targets β-catenin for ubiquitination. A, the six-amino acid motif is well conserved in both IeBo and β-catenin family members, B, either 0, 1, or 10 μg of wild-type and S37A mutant β-catenin constructs were transfected into SKBR3 cells. 36 h later, the cells were fractionated into S100 (cytoplasmic) and P100 (pellet) pools. The proteins were separated by SDS-PAGE and immunoblotted with monoclonal anti-β-catenin antibody as in Fig. 2. C, 10 μg of wild-type β-catenin or S37A mutant β-catenin were transfected into SKBR3 cells. 80 h later, cells were treated with ALLN (5 μM) and clasto-lactacycin (5 μM) overnight. The cells were fractionated into S100 and P100 pools, and the cytoplasmic proteins were separated by SDS-PAGE and immunoblotted with anti-HA antibody. Note the accumulation of HMW HA-tagged wild-type, but not mutant, β-catenin in the cytoplasmic fraction. Cells transfected with vector alone showed no anti-HA immunoreactivity (data not shown).

**Fig. 5.** A complex array of kinases regulates β-catenin stability. A, inhibitor studies from Cook et al. (23) and the present work indicate that several serine kinases are likely to be important in the regulation of β-catenin ubiquitination. A DAG-dependent classical PKC is required to mediate the inhibitory effects of Wnt/Wg on GSK3β activity. It should be noted that other serine kinases can also phosphorylate and inactivate GSK3β. Active GSK3β can phosphorylate APC and β-catenin. The present study implicates an additional DAG-independent PKC-like activity in the modulation of β-catenin ubiquitination and stability. B, conserved serine and threonine residues in the ubiquitin-targeting sequence at the N-terminal of β-catenin may also be the target of several kinases. These could act serially or in concert to prepare β-catenin for ubiquitination and/or interaction with the ubiquitin-conjugating machinery. Mutation of any one of these serines may be transforming. It is not clear if all of these serines need to be phosphorylated in order for β-catenin to be ubiquitinated, but it is clear that mutation of a single residue (S37A) is sufficient to prevent ubiquitination.
proteosomal inhibitor ALLN to generate the HMW ubiquitin-conjugated forms of β-catenin. In addition to the proteosomal inhibitor, we treated some cells with inhibitory or non-inhibitory doses of the PKC inhibitor GF-109203X and LiCl. The reduction or complete abrogation of β-catenin ubiquitination by these inhibitors demonstrates that the aPKC-like activity and GSKβ3, respectively, are required for ubiquitination of β-catenin (Fig. 3). This parallels other work regarding the role of GSKβ3 in regulating β-catenin degradation and introduces the possibility that an atypical PKC-like enzyme may also play a role in this important process.

A Conserved Six-amino Acid Sequence Targets β-Catenin and IxBo for Ubiquitination—Since N-terminal truncated forms of β-catenin and Arm accumulate in the cytoplasm, we analyzed the N terminus of β-catenin for serine residues that might be involved in regulating β-catenin ubiquitination (27). Five N-terminal serines that are well conserved between β-catenin, Armadillo, and plakoglobin are present between β-catenin amino acids 29 and 47. Mutation of three of these serines as well as a conserved threonine results in β-catenin accumulation and axis duplication in Xenopus (12). Interestingly, two of these serines lie within a six-amino acid region that is almost identical to a motif in the protein IxBo, which, upon serine phosphorylation, targets it for ubiquitination (see Fig. 4A) (28). Mutations of one or both of the serines in this motif stabilize IxBo by inhibiting its ubiquitination (28). Mutations of β-catenin serine 37 were recently reported to occur in several melanoma cell lines (29). To determine the role that phosphorylation of this motif within β-catenin might play in its ubiquitination and degradation, a serine to alanine mutation was made at residue 37 of β-catenin.

The wild-type and mutant β-catenin constructs were transfected into SKBR3 cells. The cytoplasmic proteins (S100) were isolated from the other cellular proteins (P100), and both fractions separated by SDS-PAGE and immunoblotted for β-catenin. The S37A mutant β-catenin accumulated to approximately 3-fold the levels of the wild-type 36 h after transfection (Fig. 4B). This differential accumulation was only detected in the cytoplasmic pool of β-catenin as the P100 fraction shows approximatively equal accumulation of the two forms of β-catenin.

To determine whether this increased accumulation was due to reduced ubiquitination of the mutant, SKBR3 cells were transfected with wild-type, the S37A mutant, or vector alone. The transfectants were lysed and assayed for the accumulation of HMW ubiquitinated β-catenin by immunoblotting with an anti-HA antibody (Fig. 4C). The wild-type β-catenin was ubiquitinated efficiently, while the mutant form was not ubiquitinated. Interestingly, ubiquitinated β-catenin only accumulated in the S100 fraction of the SKBR3 cell line (data not shown). This result strongly suggests that, like IxBo, a specific serine (residue 37, in β-catenin) must be phosphorylated prior to ubiquitination of β-catenin. Phosphorylation of this serine is probably an important mechanism by which the amount of β-catenin within the cell is regulated. Indeed, mutations of this serine and serine 33, which is also located within the UTS, occur in several melanoma and colon cancer cell lines (8, 29). In these cases, the mutant forms of β-catenin were presumed to be transforming.

These data show that β-catenin is normally degraded by the ubiquitin/proteosome system and that at least two serine/threonine kinases regulate this process, GSKβ3 and an aPKC-like enzyme. We also identify a common motif within β-catenin and IxBo, which, upon being phosphorylated, regulates the stability of these proteins by targeting them for ubiquitination. The identity of the kinase(s) that phosphorylates the IxBo UTS is unknown. The aPKC-like kinase activity that regulates β-catenin ubiquitination may directly phosphorylate the UTS, or it may be involved in the regulation of the kinase that does. Regardless of the specific interactions, it is likely that signaling through β-catenin is regulated by a complex network of kinases that regulate its ubiquitination (Fig. 5A).

The regulation of β-catenin signaling per se may well be an important mechanism by which multiple intracellular signals are integrated. We propose that several signaling pathways, such as signaling through growth factors (e.g., the Wnts, EGF, IGF, insulin), APC, and retinoic acid-induced adhesion, may converge by regulating the accumulation of cytoplasmic β-catenin (Fig. 5B) (10, 14). Several conserved serines and threonines within a small region of the N terminus of β-catenin are targets for mutation in colon cancers and melanomas. It is likely that many, if not all, of these residues must be phosphorylated to target β-catenin for degradation (8, 29). We propose that as the cellular context changes, β-catenin phosphorylation will be altered, and these alterations will determine whether or not it is ubiquitinated and degraded.

The definition of a specific region that targets an oncogenic protein for degradation may prove to be useful in the design of modalities for cancer therapy. We hypothesize that the UTS will be recognized by β-catenin-directed ubiquitin-conjugating enzymes, which may themselves constitute therapeutic targets.

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