Regulation of β-Catenin Signaling and Maintenance of Chondrocyte Differentiation by Ubiquitin-independent Proteasomal Degradation of α-Catenin*

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Accumulation of β-catenin and subsequent stimulation of β-catenin-T cell factor (Tcf)/lymphoid-enhancer-factor (Lef) transcriptional activity causes dedifferentiation of articular chondrocytes, which is characterized by decreased type II collagen expression and initiation of type I collagen expression. This study examined the role of α-catenin in β-catenin signaling, and the physiological significance of α-catenin regulation of β-catenin signaling in articular chondrocytes. We found that both α- and β-catenin accumulated during dedifferentiation of chondrocytes by escaping from proteasomal degradation. β-Catenin degradation was ubiquitination-dependent, whereas α-catenin was proteasomally degraded in a ubiquitination-independent fashion. The accumulated α- and β-catenin existed as complexes in the cytosol and nucleus. The complex formation between α- and β-catenin blocked proteasomal degradation of α-catenin and also inhibited β-catenin-Tcf/Lef transcriptional activity and the suppression of type II collagen expression associated with ectopic expression of β-catenin, the inhibition of proteasome, or Wnt signaling. Collectively, our results indicate that ubiquitin-independent degradation of α-catenin regulates β-catenin signaling and maintenance of the differentiated phenotype of articular chondrocytes.

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investigated the mechanisms of α-catenin degradation, the role of α-catenin in β-catenin signaling, and the physiological significance of α-catenin regulation of β-catenin signaling in articular chondrocytes.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The proteasome inhibitors, Z-Leu-Leu-Leu-CHO (MG132) and N-acetyl-leucyl-leucyl-norleucinal (ALLN), and the calpain inhibitor, N-acetyl-leucyl-leucyl-methioninal (ALLM), were purchased from Sigma. The peptide caspase inhibitors, CBZ-Val-Ala-Asp-fluoromethyl-ketone (Z-VAL-fmk) and CBZ-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-fmk), were purchased from Enzyme Systems Products (Dublin, CA). The synthetic peptide substrate for proteasomes (Suc-Leu-Leu-Val-Tyr-MCA) was purchased from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). Control- or Wnt-7a-conditioned medium were prepared as described previously by Hwang et al. (4), by culturing Wnt-expressing L929 cells in Dulbecco’s modified Eagle’s medium.

**Cell Culture**—Individual articular chondrocytes were prepared from joint cartilage slices from 2-week-old New Zealand White rabbits as described previously (15, 16). The cells were plated on culture dishes at a density of 5 × 10^4 cells/cm^2 and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, 100 μg/ml streptomycin, and 50 units/ml penicillin. The medium was replaced every 2 days until the cultures were tested with the indicated pharmacological reagents in each experiment. A β-catenin-negative cell line, human mesothelioma cancer cell (H28), was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics.

**Cell Fractionation**—Cytosolic and nuclear fractions were prepared as described previously (5). Briefly, nuclear fractions were prepared by homogenizing chondrocytes in 10 mM Hepes buffer (pH 7.9) containing 0.4M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease and phosphatase inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine). The homogenates were centrifuged at 13,000 g for 5 min at 4 °C to remove debris, and the supernatant (500 μl) was used for determination of cell lysates.

**Western Blot Analysis**—Chondrocytes were lysed on ice for 30 min in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease and phosphatase inhibitors. Proteins were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected using monoclonal anti-ubiquitin (Santa Cruz Biotechnology Inc.). For the detection of ubiquitin, membranes were sandwiched between several sheets of Whatman 3MM paper and submerged in deionized water, followed by washing with water, and exposed to X-ray film. The 26 S proteasome bands were excised, and the proteins were separated with 10% SDS-PAGE, transferred to a nitrocellulose membrane, and detected using polyclonal anti-ubiquitin antibody (Transduction Laboratories) and goat polyclonal anti-actin and mouse monoclonal anti-β-catenin, -α-catenin, -γ-catenin, -p120, -N-cadherin, and -ERK1 (all from BD Transduction Laboratories) and goat polyclonal anti-actin and mouse monoclonal anti-ubiquitin (Santa Cruz Biotechnology Inc.). For the detection of ubiquitin, membranes were sandwiched between several sheets of Whatman 3MM paper and submerged in deionized water, and membrane-bound ubiquitin was heat-activated by autoclaving for 30 min.
RESULTS

Ubiquitin-independent Proteasomal Degradation of α-Catenin in Articular Chondrocytes—Based on previous reports that β-catenin levels were regulated by proteasomal degradation (3), we first examined whether the proteasomal degradation pathway regulates α-catenin protein level in primary cultured articular chondrocytes. As expected, α-catenin protein levels were significantly increased by 26 S proteasome inhibitors (ALLnL and MG132) but not by caspase (Z-VAD-fmk and DEVD-fmk) or calpain (ALLM) inhibitors (Fig. 1A). Protein levels of α-catenin were also significantly increased by the inhibition of the 26 S proteasome (Fig. 1A), suggesting that both α- and β-catenins are degraded via proteasomal pathways. Treatment with a translation inhibitor (cycloheximide) reduced levels of α- and β-catenin; this reduction was blocked by the inhibition of the 26 S proteasome with MG132 (Fig. 1B), confirming that both α- and β-catenins are post-translationally regulated via the proteasomal degradation pathway. Post-translational regulation of α- and β-catenin was additionally supported by our observations that their transcript levels were not affected by proteasome inhibition (Fig. 1C).

To characterize the proteasomal degradation of α-catenin, we next examined its physical association with the 26 S proteasome. For this purpose, chondrocytes were treated with ALLnL or MG132 to inhibit proteasome activity (Fig. 2A), and total cell lysates were separated by native gel electrophoresis. The 26 S proteasome band was identified by fluorogenic substrate overlay (Fig. 2B, upper panel). Proteins localized in the 26 S proteasome band were separated by SDS-PAGE. Western blotting indicated that both α- and β-catenins were located in the 26 S proteasome fraction (Fig. 2B, lower panel), suggesting that α- and β-catenins may associate with the 26 S proteasome. Proteasome inhibition with ALLnL and MG132 caused accumulation of α- and β-catenins in a dose- (Fig. 2C) and time- (Fig. 2D) dependent manner. Accumulated β-catenin was detected in the gel as a ladder form consistent with ubiquitination (3). Interestingly, we did not detect any electrophoretic mobility shift of α-catenin following proteasome inhibition (Fig. 2, C and D). Because the electrophoretic pattern of α-catenin suggested non-ubiquitination, we further tested for ubiquitination of α- and β-catenin by immunoprecipitation and Western blotting. As shown in Fig. 2E, Western blot analysis indicated that β-catenin, but not α-catenin, was ubiquitinated in chondrocytes, suggesting that α-catenin is degraded by the 26 S proteasome in a ubiquitin-independent fashion.

β-Catenin Inhibits α-Catenin Degradation—We next examined whether accumulation of α- and β-catenins is associated with each other in chondrocytes. For this purpose, we first used immunofluorescence microscopy and Western blot analysis to determine the expression levels of α- and β-catenins in chondrocytes transfected with wild-type α-catenin or S37A β-catenin (which cannot be ubiquitinated due to the loss of its phosphorylation site). Double immunostaining of chondrocytes (Fig. 3A) and Western blot analysis (Fig. 3B) revealed that cells overexpressing S37A β-catenin showed high levels of α-catenin protein, whereas overexpression of α-catenin did not affect β-catenin protein levels. Because the above results suggest that accumulation of α-catenin is associated with the observed increases in β-catenin protein levels, the association of α- and β-catenin accumulation was further characterized by uncoupling the degradation of α-catenin from that of β-catenin using a β-catenin-negative cell line (H28). As shown in Fig. 3C, α-catenin was accumulated in the β-catenin-negative cell line by the inhibition of proteasome, indicating that α-catenin degradation via the proteasomal pathway is independent of the existence of β-catenin protein. The role of β-catenin accumulation in α-catenin degradation was further examined by ectopic expression of S37A β-catenin in H28 cells. Ectopically expressed S37A β-catenin binds to endogenous α-catenin and caused the increase in α-catenin protein level (Fig. 3D), suggesting that the existence of β-catenin protein and its binding to α-catenin inhibit proteasomal degradation of α-catenin. This also suggests that the observed increase in α-catenin protein level in β-catenin-overexpressing chondrocytes is due to the inhibition of α-catenin degradation by β-catenin.

The inhibitory role of β-catenin in α-catenin degradation was further examined by inducing β-catenin accumulation in chondrocytes. Consistent with our previous observations that accumulation of β-catenin and subsequent stimulation of β-catenin-Tcf/Lef complex transcriptional activity caused dedifferentiation of articular chondrocytes (4–6), treatment of chondrocytes with Wnt-7a-conditioned medium, a glycogen synthase kinase-3β inhibitor (LiCl), or a proinflammatory cytokine interleukin-1β caused accumulation of β-catenin (Fig. 4A), stimulation of β-catenin-Tcf/Lef complex transcriptional activity (Fig. 4B), and suppression of type II collagen expression (Fig. 4C). The accumulation of β-catenin is accompanied by the increase in α-catenin protein level (Fig. 4A). Transcript levels of α- and β-catenin and proteasome activity were not affected by the treatment of chondrocytes with Wnt-7a, LiCl, or interleukin-1β (Fig. 4D), suggesting post-
translational regulation of α- and β-catenin protein levels.

α-Catenin Inhibits β-Catenin Signaling and β-Catenin-mediated Dedifferentiation of Articular Chondrocytes—We next examined the functional significance of the proteasomal degradation of α-catenin in articular chondrocytes. First, we examined the intracellular localization of α- and β-catenins. Immunofluorescence microscopy revealed that α-catenin was localized throughout cells, whereas β-catenin was detected mainly at areas of cell-cell contact. However, when proteasome activity was blocked by MG132 treatment, levels of α- and β-catenins were significantly increased in the nucleus (Fig. 5A). Cell fractionation experiments indicated that following proteasome inhibition, accumulated α- and β-catenins were localized in both the cytosolic fraction and the nuclear fraction (Fig. 5B).
Co-immunoprecipitation of α- and β-catenin suggested that these proteins exist as complexes in both the cytosolic and the nuclear fractions (Fig. 5C).

The functional significance of α-catenin degradation by the proteasomal pathway was next examined in terms of its role in Wnt- and β-catenin signaling. As expected, accumulation of β-catenin following proteasome inhibition by MG132 led to stimulation of β-catenin-Tcf/Lef transcriptional activity (Fig. 6A, left panel). β-Catenin accumulation also inhibited type II collagen expression (Fig. 6A, right panel), which is consistent with our previous observation (4, 5). The significance of α-catenin accumulation and association with β-catenin was determined by overexpressing α-catenin followed by proteasome inhibition. As shown in Fig. 6B, ectopic expression of α-catenin blocked the MG132-induced increase of β-catenin-Tcf/Lef transcriptional activity and also significantly blocked the inhibition of type II collagen expression. The partial recovery of type II collagen expression (Fig. 6B) may be due to low α-catenin transfection efficiency, whereas the complete inhibition of β-catenin-Tcf/Lef transcriptional activity could be due to our co-transfection of the reporter gene and α-catenin.

Because the above results suggest that α-catenin may inhibit β-catenin signaling, the role of α-catenin in β-catenin signaling was directly examined by overexpressing α- and/or β-catenin.

As shown in Fig. 7A, overexpression of α-catenin alone led to decreased basal levels of β-catenin-Tcf/Lef transcriptional activity (Fig. 7A, upper panel) and increased type II collagen expression (Fig. 7A, lower panel). Ectopic expression of S37A β-catenin led to increased β-catenin transcriptional activity and suppression of type II collagen expression (Fig. 7B). Co-expression of α-catenin and S37A β-catenin blocked the S37A β-catenin-induced increase in β-catenin-Tcf/Lef complex transcription activity and the inhibition of type II collagen expression (Fig. 7C), indicating that α-catenin inhibits β-catenin signaling and subsequent dedifferentiation of chondrocytes. The inhibitory action of α-catenin in β-catenin signaling was further confirmed by examining the role of α-catenin in Wnt signaling. Infection of cells with an adenovirus carrying Lef-1 (Fig. 8A) or treatment of cells with Wnt-7a-conditioned medium (Fig. 8B) led to increased β-catenin-Tcf/Lef transcriptional activity (left panels) and suppression of type II collagen expression (right panels). Ectopic expression of α-catenin blocked the increase of transcriptional activity and rescued type II collagen expression (Fig. 8), indicating that α-catenin inhibits Wnt signaling by disturbing the transcriptional activity of β-catenin.

In an attempt to elucidate the physiological significance of α-catenin degradation and its regulation of β-catenin signaling, we finally examined whether endogenous α-catenin can
regulate β-catenin signaling and chondrocyte differentiation by using siRNA to knock down the endogenous α-catenin in chondrocytes. As shown in Fig. 9A, down-regulation of endogenous α-catenin increased the basal level of β-catenin-Tcf/Lef transcriptional activity (left panel) and caused reduction in type II collagen expression level (right panel). Knockdown of α-catenin also increased Wnt-7a-induced activation of β-catenin-Tcf/Lef transcriptional activity and promoted the inhibition of type II collagen expression caused by Wnt-7a treatment (Fig. 9B).

Fig. 7. α-Catenin inhibits β-catenin-induced inhibition of type II collagen expression by blocking the transcriptional activity of β-catenin. A and B, chondrocytes were transfected with the indicated concentrations of α-catenin (A) or S37A α-catenin (B) expression vectors. Following incubation for 24 h, the transcriptional activity of β-catenin was determined by reporter gene assay (upper panels), and expression levels of GFP-α-catenin, β-catenin, and type II collagen were determined by Western blotting (lower panels). C, chondrocytes were transfected with S37A β-catenin with or without α-catenin expression vectors (2 μg). Following incubation for 24 h, the transcriptional activity of β-catenin was determined by reporter gene assay (upper panel), and expressions of α-catenin, β-catenin, and type II collagen were determined by Western blotting (lower panel).

Fig. 8. α-Catenin inhibits Wnt signaling and Wnt-induced inhibition of type II collagen expression. A, chondrocytes were transfected with empty vector (Control and Lef-1) or with a vector encoding α-catenin. Following incubation for 24 h, the cells were infected with empty adenovirus or with an adenovirus carrying Lef-1. The transcriptional activity of β-catenin was determined by reporter gene assay (left panel), and expression levels of α-catenin, β-catenin, type II collagen, and Lef-1 (determined with anti-hemagglutinin (HA) antibody) were determined by Western blotting (right panel). B, chondrocytes were transfected with empty vector or α-catenin expression vector. Following incubation for 24 h, the cells were treated with 200 μl of Wnt-7a-conditioned medium for 24 h. The transcriptional activity of β-catenin was determined by reporter gene assay (left panel), and expressions of α-catenin (determined with anti-GFP antibody), β-catenin, and type II collagen were determined by Western blotting (right panel). ERK was employed as a loading control.
DISCUSSION

We previously showed that stimulation of β-catenin-Tcf/Lef transcriptional activity by ectopic expression of β-catenin or Wnt-7a treatment caused dedifferentiation of articular chondrocytes (4, 5). Here, we investigated the regulatory mechanisms of α-catenin degradation in articular chondrocytes and examined the role of α-catenin in β-catenin signaling and its physiological significance in chondrocyte differentiation. We found that α-catenin was actively degraded by a ubiquitin-independent proteasomal pathway and that accumulated α-catenin inhibited the transcriptional activity of the β-catenin-Tcfl/Lef complex, thereby blocking β-catenin- or Wnt-induced dedifferentiation of primary cultured articular chondrocytes.

Ubiquitin-independent Proteasomal Degradation of α-Catenin—This is the first report that α-catenin is degraded via a proteasomal pathway, as evidenced by our observation that the inhibition of proteasome caused accumulation of α-catenin. This conclusion is further supported by our finding that α-catenin was associated with the proteasome fraction. Proteasomes play an essential role in the rapid elimination of short-lived key regulatory proteins. A major determinant for protein half-life is the presence of degradation signals, such as the ubiquitin fusion degradation signal, the PEST sequence, and the destruction box (2). It was previously shown that α-catenin, which contains a high quality PEST sequence (resides 633–651), was actively degraded in L cells (13). However, the previous report found that various proteasome and calpain inhibitors did not affect the level of endogenous α-catenin proteins in L cells (13). In contrast, under our experimental conditions, proteasome inhibition increased α-catenin protein levels not only in primary chondrocytes but also in HTB-94 chondrosarcoma, Balbc, and NIH3T3 cells (data not shown), suggesting that proteasomal degradation of α-catenin is not restricted to primary cultured articular chondrocytes.

Another interesting finding in this study is that α-catenin degradation is ubiquitination-independent. For typical proteasomal protein degradation, the rate-limiting step is the recruitment of the ubiquitin-protein isopeptide ligase (E3), which conjugates a polyubiquitin tree to the substrate (21). 26 S proteasomes preferentially degrade ubiquitinated proteins (22). However, we did not detect any evidence for the ubiquitination of α-catenin in articular chondrocytes, which suggests that α-catenin is degraded via the 26 S proteasome in a ubiquitination-independent manner. This is not unheard of; several other proteins are degraded by proteasomes in a ubiquitination-independent fashion. For instance, ornithine decarboxylase is degraded by the 26 S proteasome via a ubiquitin-independent pathway (23), and this phenomenon is conserved between animals and fungi (24). Other proteins such as calmodulin, troponin C (22), and α-synuclein (25) are also degraded by 26 S proteasomes without ubiquitination. The p53 protein can undergo proteasomal degradation by two alternative pathways; one is ubiquitin-dependent and regulated by Mdm-2, whereas the other is ubiquitin-independent and regulated by NAD(P)H quinone oxidoreductase 1 (26). Thus, it appears that ubiquitin-independent degradation by 26 S proteasomes may be more important than has generally been assumed. However, we do not yet know precisely how this occurs in the case of ubiquitin-independent degradation of α-catenin in chondrocytes. One possibility is that α-catenin degradation is dependent on the degradation of β-catenin and that ubiquitinated β-catenin accompanies α-catenin into proteasomal degradation. This is based on the observations that overexpression of S37A β-catenin or accumulation of β-catenin caused accumulation of α-catenin, whereas overexpression of α-catenin did not affect β-catenin accumulation. However, this is unlikely to happen in chondrocytes because α-catenin degradation is also observed in the β-catenin-negative cell line. Therefore, our experiments uncoupling the degradation of α-catenin from that of β-catenin using the β-catenin-negative cell line clearly indicate that α-catenin degradation is independent to the β-catenin degradation. Our results additionally indicate that accumulation of β-catenin inhibits degradation of α-catenin, as evidenced by the observation that ectopically expressed S37A β-catenin in β-catenin-negative cells binds to α-catenin and causes the increase in α-catenin protein level. This also explains the reason why the accumulation of β-catenin always causes accumulation of α-catenin in chondrocytes.

α-Catenin Regulation of β-Catenin Signaling and Chondrocyte Differentiation—Our current results clearly indicated that α-catenin inhibits nuclear signaling of β-catenin and thereby blocks β-catenin- or Wnt-induced dedifferentiation of chondrocytes. The observations that 1) α-catenin inhibits β-catenin signaling and that 2) Wnt signaling increased not only β-catenin but also α-catenin levels appear to be paradoxical for the maximum effects of Wnt signaling, i.e. stimulation of β-catenin-Tcf/Lef transcriptional activity and resulting dedifferentiation of chondrocytes. However, our results suggested that the increase in α-catenin by Wnt signaling is to assure fine-tuning of the transcriptional activity of β-catenin-Tcf/Lef complex. This conclusion is based on the observations that 1) knockdown of endogenous α-catenin by siRNA increased the basal level of β-catenin-Tcf/Lef transcriptional activity and increased Wnt-7a-induced activation of β-catenin-Tcf/Lef transcriptional ac-
activity and 2) binding of α-catenin to β-catenin inhibited degradation of α-catenin and thereby inhibited β-catenin signaling. Our previous study (16) indicated that chondrogenic differentiation of mesenchymal cells accompanied decreased expression of α- and β-catenin. We also reported that down-regulation of β-catenin is necessary for chondrogenesis and accumulation of β-catenin inhibited chondrogenesis by stabilizing cell-cell adhesion (5). Therefore, β-catenin inhibits chondrogenesis by acting as a cytoskeletal component, whereas it causes dedifferentiation of chondrocytes by acting as a transcriptional co-activator. Therefore, it is likely that down-regulation of both α- and β-catenin during chondrogenesis is necessary for the disruption of cell-cell adhesion, which is necessary for chondrocyte differentiation, and the increase of α-catenin during dedifferentiation is to assure fine-tuning of the β-catenin-Tcf/Lef transcriptional activity.

Consistent with our observations, several previous studies have indicated that the inhibition of β-catenin signaling by α-catenin occurs via direct binding to β-catenin (9, 12–14, 27). It is thought that α-catenin overexpression inhibits β-catenin-Tcf/Lef-dependent transcription by sequestering β-catenin in the cytoplasm (9, 12, 13). In contrast, endogenous or exogenously expressed α-catenin is found in the nuclei of various cell types such as SW480, DLD-1, and Cos cells but not in the nuclei of HCT116 cells (14, 27). The reason for this differential nuclear localization of α-catenin is presently unknown but may suggest that β-catenin is not the only factor involved in nuclear localization of α-catenin. It was further demonstrated that α-catenin binding to β-catenin does not affect entry of α-catenin into the nucleus but rather inhibits the interaction of the β-catenin-Tcf/Lef complex with target DNA (27). In articular chondrocytes, α- and β-catenin complexes are localized in both the cytosol and the nucleus, indicating that sequestration of β-catenin in the cytoplasm is not the mechanism by which β-catenin signaling is inhibited by α-catenin. Instead, it is more likely that association of α-catenin with β-catenin blocks their interaction with target DNA. However, this hypothesis will await further study.

In summary, we have demonstrated that both α- and β-catenins are actively degraded by 26S proteasomes in articular chondrocytes. The degradation of β-catenin is ubiquitination-dependent, whereas α-catenin is degraded independent of detectable ubiquitination. We also demonstrated that accumulated α-catenin associates with β-catenin, resulting in the inhibition of β-catenin-Tcf/Lef transcriptional activity and subsequent blockade of β-catenin- or Wnt-induced the inhibition of type II collagen expression. Our results collectively suggest that α-catenin functions in chondrocytes not only as a cytoskeletal component but also as a modulator of β-catenin signaling.

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