The F-box protein β-TrCP associates with phosphorylated β-catenin and regulates its activity in the cell

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Defects in β-catenin regulation contribute to the neoplastic transformation of mammalian cells. Dysregulation of β-catenin can result from missense mutations that affect critical sites of phosphorylation by glycogen synthase kinase 3β (GSK3β). Given that phosphorylation can regulate targeted degradation of β-catenin by the proteasome, β-catenin might interact with an E3 ubiquitin ligase complex containing an F-box protein, as is the case for certain cell cycle regulators. Accordingly, disruption of the *Drosophila* F-box protein Slimb upregulates the β-catenin homolog Armadillo. We reasoned that the human homologs of Slimb – β-TrCP and its isoform β-TrCP2 (KIAA0696) – might interact with β-catenin. We found that the binding of β-TrCP to β-catenin was direct and dependent upon the WD40 repeat sequences in β-TrCP and on phosphorylation of the GSK3β sites in β-catenin. Endogenous β-catenin and β-TrCP could be coimmunoprecipitated from mammalian cells. Overexpression of wild-type β-TrCP in mammalian cells promoted the downregulation of β-catenin, whereas overexpression of a dominant-negative deletion mutant upregulated β-catenin protein levels and activated signaling dependent on the transcription factor Tcf. In contrast, β-TrCP2 did not associate with β-catenin. We conclude that β-TrCP is a component of an E3 ubiquitin ligase that is responsible for the targeted degradation of phosphorylated β-catenin.

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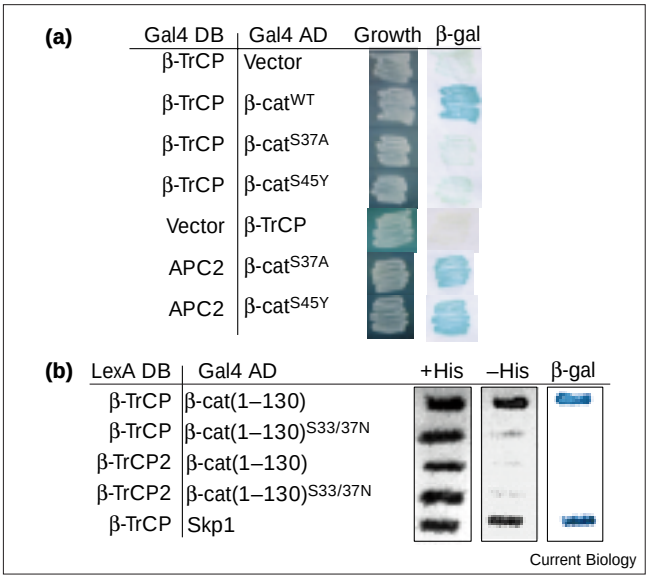
Results and discussion

Yeast were cotransformed with plasmids expressing β-TrCP [1] fused to the Gal4 DNA binding domain and β-catenin fused to the Gal4 transactivation domain. Expression of either plasmid alone did not result in transactivation of the β-galactosidase reporter gene whereas expression of both resulted in high levels of activity (Figure 1a). Mutations altering β-catenin residues Ser37 and Ser45 have been identified in human tumors [2–7] and are known to retard the targeted degradation of β-catenin in mammalian cells [8]. These mutants failed to interact with β-TrCP, but retained their ability to bind to a fragment of the adenomatous polyposis coli (APC) tumor suppressor protein (Figure 1a). To test for an interaction with the β-catenin amino-terminal region, we fused β-TrCP, or its isoform β-TrCP2, to the LexA DNA binding domain and fused amino acids 1–130 of β-catenin to the Gal4 transactivation domain. A positive interaction

Figure 1



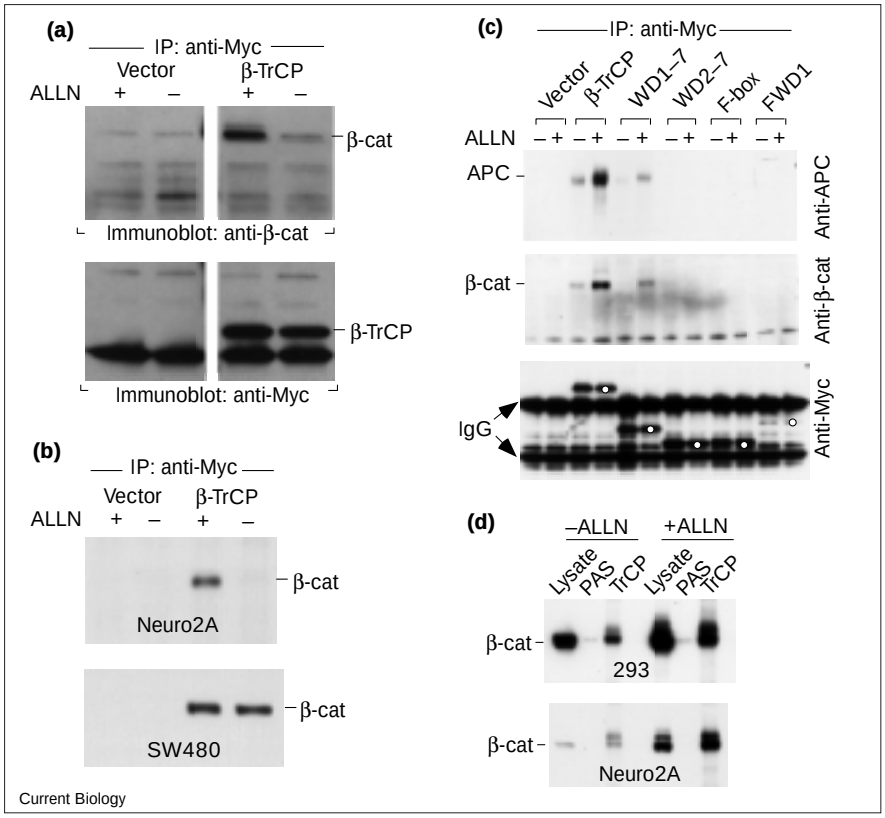
Yeast two-hybrid binding assays. (a) Yeast cotransformed with plasmids expressing the indicated proteins fused to either the Gal4 DNA binding domain (DB) or the Gal4 activation domain (AD) were maintained on media containing histidine (growth) and patches were assayed for β-galactosidase activity (β-gal). Wild-type β-catenin (β-cat^{WT}) or forms with Ser37 or Ser45 mutated were tested. APC2, a fragment of APC, was a positive control for β-catenin binding. (b) Yeast cotransformed with plasmids expressing the indicated fusion proteins were grown on synthetic media containing histidine (+His), tested for auxotrophic growth in media lacking histidine (–His) or transferred to filters for β-galactosidase assays. S33/37N indicates a double mutation of Ser33 and Ser37 to asparagine residues. Skp1 was a positive control for β-TrCP binding.

between the fusion proteins is required for the production of β-galactosidase and survival on conditional media. The β-catenin 1–130 fragment interacted with β-TrCP but a double mutation changing β-catenin residues Ser33 and Ser37 ablated the interaction (Figure 1b). However, β-catenin failed to interact with β-TrCP2 [9] even though, like β-TrCP, it contains an F-box, WD40 repeats and is 80% identical to the Slimb protein.

To examine the interaction in mammalian cells, β-TrCP with a Myc epitope tag was expressed in three different cell lines and anti-Myc immunoprecipitates were probed for β-catenin. Coimmunoprecipitation of β-catenin with β-TrCP was seen in all three cell lines and the interaction was greatly enhanced in both the Neuro2A and 293 cells by pretreatment with the proteosomal inhibitor N-acetyl-leucinyl-leucinyl-norleucinyl-H (ALLN; Figure 2a,b). It is not clear why the interaction in SW480 cells was independent of ALLN treatment, but the extremely slow turnover of β-catenin in these cells [10] might circumvent the need for a proteosomal inhibitor to establish a stable interaction. As APC is involved in the regulation of β-catenin function [10,11], we sought to determine whether APC remained associated with β-catenin in the β-TrCP complex. Accordingly, anti-Myc immunoprecipitates from 293 cells were probed for both APC and β-catenin. Wild-type APC protein was detected in the immunoprecipitates of full-length β-TrCP, and ALLN enhanced the amount that was coimmunoprecipitated (Figure 2c). We also tested deletion mutants of β-TrCP and found that a fragment lacking the F-box but containing WD40 repeats 1–7 (WD1–7) associated with β-catenin but a similar fragment lacking the first WD40 repeat (WD2–7) did not. Also, no association was detected with fragments containing the F-box without WD40 repeats (F-box) or the F-box with the first repeat (FWD1). We also immunoprecipitated endogenous β-TrCP from cells that were not transfected and detected β-catenin in the immunoprecipitates (Figure 2d).

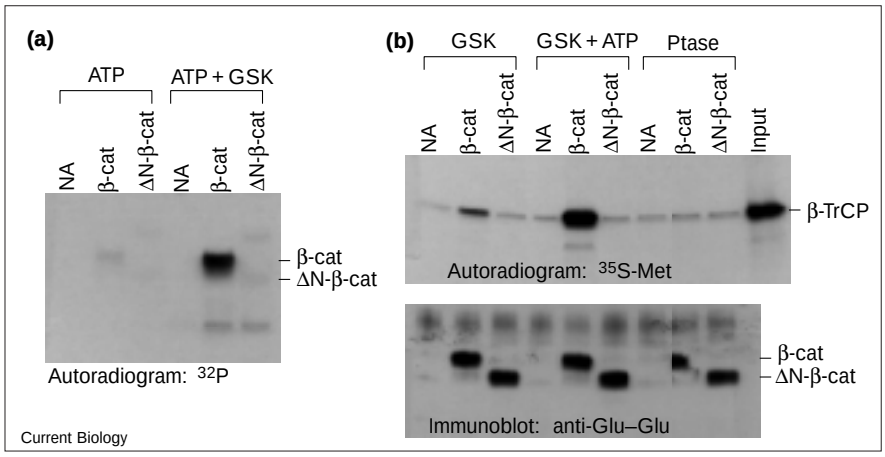
These results suggested that a modification of β-catenin that occurred in response to ALLN was responsible for the enhanced binding to β-TrCP. Previous reports demonstrating phosphorylation-dependent interaction of substrates to their F-box proteins [12–15], and our finding that mutations in β-catenin serine residues abrogate its binding to β-TrCP, suggested that the modification might involve phosphorylation. We examined this by phosphorylating purified recombinant β-catenin with GSK3β *in vitro* and then tested its ability to bind to *in vitro* translated β-TrCP. As a control we also tested a deletion mutant of β-catenin that lacks 89 amino-terminal amino acids (ΔN-β-catenin) and therefore does not contain the critical

Figure 2



Association of β-TrCP with β-catenin and APC in mammalian cells. (a) An empty vector or plasmid expressing Myc–βTrCP were transfected into 293 cells that were left untreated (–) or incubated for 4 h in 25 μM ALLN (+). Anti-Myc immunoprecipitates were analyzed for β-catenin (β-cat; top panels) or Myc–βTrCP (bottom panels). (b) The interaction between β-TrCP and β-catenin was also tested in Neuro2A and SW480 cells. (c) Myc-tagged full-length βTrCP and the indicated deletion mutants were expressed in 293 cells and anti-Myc immunoprecipitates were analyzed for APC (top panel), β-catenin (middle panel), or the Myc epitope (bottom panel). The positions of the Myc-tagged fragments are indicated by the white dots. (d) Lysates from untransfected 293 and Neuro2A cells were subjected to immunoprecipitation with a rabbit polyclonal antibody to β-TrCP (TrCP) or, as a control, protein-A–Sepharose alone (PAS), and the immunoprecipitates were analyzed for β-catenin. The protein fragments of β-TrCP contain the following amino acids: full-length β-TrCP, 1–569; WD1–7, 241–569; WD2–7, 291–569; F-box, 1–247; FWD1, 1–306.

Figure 3

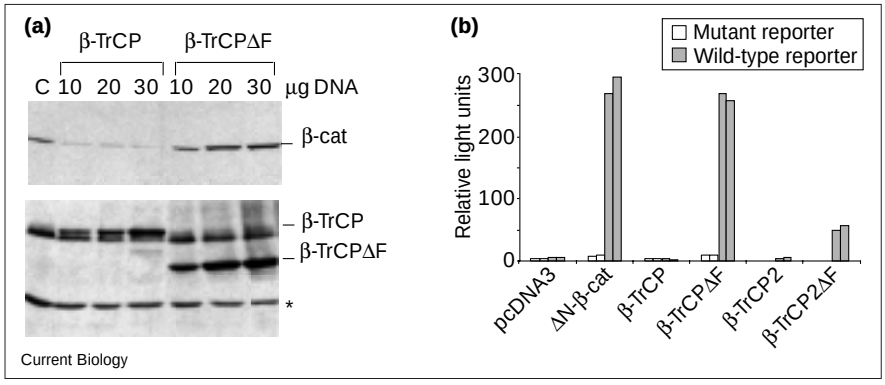


Association of β-TrCP and phosphorylated β-catenin *in vitro*. (a) Kinase reactions were performed with purified Glu–Glu-tagged full-length β-catenin (β-cat), the amino-terminal deletion mutant (ΔN-β-cat) or no protein (NA) in the presence of [γ-^{32}P]ATP with or without purified GSK3β (GSK). Phosphorylation was examined by autoradiography. (b) Parallel reactions were performed with GSK3β with or without nonradioactive ATP, or with phosphatase (Ptase), and the β-catenin recovered by Glu–Glu immunoprecipitation was tested for binding to *in vitro* translated radiolabeled β-TrCP as assessed by fluorography (top panel). The recovery of β-catenin was assessed by immunoblotting with anti-Glu–Glu antibody (bottom panel).

serine and threonine residues that affect β-catenin turnover in cells [16]. One set of kinase reactions contained [γ-^{32}P]ATP to account for phosphate incorporation and a second set of reactions, without radioactive ATP, was used to assess subsequent binding of β-catenin to β-TrCP. Only when GSK3β was included in the reaction with full-length β-catenin was significant radiolabeling observed following the kinase reaction (Figure 3). When β-catenin was recovered from the nonradioactive kinase reaction and tested for binding to β-TrCP, only the full-length protein bound, and binding was greatly enhanced by prior phosphorylation by GSK3β. Moreover, the low level of binding observed with the nonphosphorylated full-length β-catenin was reduced to background levels following its pretreatment with phosphatase.

The interaction of phosphorylated β-catenin with β-TrCP suggested that β-TrCP might be involved in the regulation of β-catenin levels in the cell. The amount of β-catenin was examined by immunoblotting following overexpression of β-TrCP or the F-box deletion mutant β-TrCPΔF, which acts as a dominant negative in CD4 degradation mediated by the HIV-1 protein Vpu [1]. Expression of increasing amounts of full-length β-TrCP resulted in a concomitant reduction in β-catenin, whereas expression of β-TrCPΔF promoted β-catenin accumulation (Figure 4a). One of the consequences of β-catenin stabilization is the enhancement of signaling that occurs through its interaction with the lymphocyte enhancer factor/T-cell factor (Lef/Tcf) family of transcription factors [11]. To measure Lef/Tcf-dependent transcription, a plasmid containing a multimerized synthetic Tcf binding site fused to the luciferase reporter gene was cotransfected into 293 cells with β-TrCP constructs or, as a positive control, the amino-terminal deletion mutant of β-catenin. Very little transactivation occurred with vector alone, whereas cotransfection of ΔN-β-catenin

Figure 4



Effect of β-TrCP on β-catenin levels and Tcf-dependent transcriptional activation. (a) Approximately 8×10^6 Hela cells were transfected with the indicated amounts of Myc–β-TrCP or the dominant-negative mutant Myc–β-TrCPΔF or were not transfected (C). After 24 h, cells were lysed and 50 μg total protein from each lysate was immunoblotted with antibody to β-catenin (β-cat; top panel) or to Myc (lower panel). Staining of a nonspecific protein (asterisk) indicates comparable loading of total protein in each lane. (b) Activation of Lef/Tcf-dependent transcription was determined by cotransfection of 293 cells with the wild-type reporter Top-tk-Luci, or the mutant control reporter Fop-tk-Luci, and either empty vector (pcDNA3), full-length β-TrCP, or β-TrCPΔF. ΔN-β-catenin was used as a positive control, and β-TrCP2 and the corresponding F-box deletion mutant β-TrCP2ΔF (generated by deletion of amino acids 1–170) were also tested. Luciferase activity was normalized to Renilla luciferase activity produced by cotransfection with RSV-Renilla luciferase. Representative data from three independent experiments are shown.

resulted in a strong activation of luciferase transcription from the wild-type promotor, but not from the mutant promotor (Figure 4b). Full-length β-TrCP had no effect on the already low level of basal signaling, but the dominant-negative mutant β-TrCPΔF elicited a strong response similar to that of ΔN-β-catenin. β-TrCP had no effect on basal signaling although a mutant β-TrCP2 lacking the F-box domain promoted a low level of luciferase transcription.

It is now apparent that β-catenin possesses signaling capabilities that have consequences for cell growth control [17–22]. Mutations that affect the sites of phosphorylation by GSK3β [23] stabilize β-catenin *in vivo*, and these types of mutations have recently been identified in a wide variety of primary human cancers [2–8]. The crucial Ser33 and Ser37 residues in β-catenin are located in a DSGXXS motif (in single-letter amino-acid code where X is any amino acid) identical to that of the phosphorylated Ser52/Ser56 Vpu residues required for Vpu-mediated CD4 degradation by the proteasome [1]. This same motif is also present in the NFκB inhibitor IκB where phosphorylation of these serines initiates the rapid degradation of IκB [24]. It was recently reported that β-TrCP is a component of the E3 ubiquitin ligase that targets IκB for ubiquitination [25]. Therefore, the DSGXXS motif might represent a signal sequence that is common to a given subset of substrates that rely upon β-TrCP for their targeted degradation. Here, we have shown that these sites of phosphorylation are required for the association of β-catenin with β-TrCP. Our previous finding that β-TrCP interacts with a component of the SCF ubiquitin ligase complex, Skp1 [1], suggests that β-TrCP is part of the multimeric E3 ubiquitin ligase belonging to the SCF family [12]. We propose that $SCF^{\beta\text{-}TrCP}$ is responsible for targeting phosphorylated β-catenin for ubiquitination and thereby promoting its degradation in the proteosome. The enhanced stability and signaling output that is observed with oncogenic mutants of β-catenin might be explained by their failure to interact with the $SCF^{\beta\text{-}TrCP}$ complex.

Supplementary material

A detailed description of cDNA constructs and additional methodological details are published with this paper on the internet.

Acknowledgements

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Supplementary material

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Supplementary materials and methods

cDNA cloning and construction

A *Bgl*II–*Not*I fragment (nucleotides 940–1779) of a human β-TrCP cDNA (accession number AA478504) was cloned into a modified pcDNA3 vector (Invitrogen) containing a Myc epitope tag (pCAN-Myc). This construct was designated WD2–7 (amino acids 291–569). A *Cla*I–*Not*I fragment was isolated from a β-TrCP PCR fragment (nucleotides 70–989) and subcloned into the pCAN-Myc vector to generate FWD1 (amino acids 1–306). A full-length β-TrCP (amino acids 1–569) was generated from WD2–7 and FWD1 and subcloned as a *Cla*I–*Not*I fragment into pCAN-Myc. The WD1–7 (amino acids 241–569) and F-box (amino acids 1–247) constructs were PCR products (nucleotides 790–1779 and 70–810, respectively) that were subcloned as *Cla*I–*Xba*I fragments into the *Cla*I and *Xba*I sites of pCAN-Myc. The β-catenin constructs have been previously described [S1]. pcDNA3 β-TrCP and pcDNA3 β-TrCPΔF have been described elsewhere [S2], and β-TrCP2ΔF was generated by deletion of amino acids 1–170 by PCR and cloned into pcDNA3.

Expression and immunoprecipitation of β-TrCP

Transfections of the indicated plasmids into the human embryonic kidney cell line 293 and the SW480 colorectal cell line were performed as previously described [S3]. Neuro2A cells were transfected according to the protocol for 293 cells. Cells were lysed 48 h post-transfection with 0.4 ml Triton lysis buffer (20 mM Tris-HCl, pH 8.0, 1.0% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM NaF, 1 mM pefabloc, 10 μg/ml each of aprotinin, pepstatin and leupeptin) per 35 mm well of confluent cells and the β-TrCP proteins were immunoprecipitated with antibody specific to the Myc epitope as described previously [S4]. Rabbit polyclonal antibodies to β-catenin and APC [S4] were used in all experiments except for the use of polyclonal anti-β-catenin antibody C2206 (Sigma) for the experiment in Figure 4a. The protease inhibitor ALLN was from Sigma. Modulation of β-catenin levels was monitored after transfection of HeLa cells with pcDNA3.1 Myc/HisA vector (InVitrogen) containing β-TrCP or β-TrCPΔF.

Binding studies

Yeast two-hybrid binding analysis has been described previously [S2,S4]. *In vitro* binding of purified Glu–Glu tagged β-catenin to *in vitro* translated β-TrCP was carried out in 150 μl Triton lysis buffer containing 1 μg β-catenin and 10 μl reticulocyte lysate in which radiolabeled β-TrCP was produced by *in vitro* transcription and translation according to the manufacturer's instructions (Promega). Following a 2 h incubation, β-catenin was recovered by immunoprecipitation with antibody to the Glu–Glu epitope and the immunoprecipitates were washed three times and analyzed for β-TrCP by fluorography of SDS–polyacrylamide gels. The phosphorylation of β-catenin by GSK3β was performed at 30°C for 15 min in 20 μl kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, 25 mM NaCl, 50 mM ATP, 10% glycerol) containing 2.5 μg/ml recombinant GSK3β. Phosphatase treatment of β-catenin was performed with a 1:100 dilution of lambda phosphatase (New England Biolabs) for 15 min at 30°C.

Transactivation studies

The 293T cells were seeded in 12-well plates (1.50×10^5 cells per well) and transfected with 0.5 μg Top-tk-Luci or Fop-tk-Luci plasmids together with 0.5 μg of the indicated pcDNA3 expression vectors and 10 ng of an RSV-Renilla luciferase plasmid. Fugene reagent was used for transfection (Boehringer Mannheim). Luciferase activity was measured 24 h after transfection and normalized to transfection efficiency by measuring Renilla luciferase activity (Promega Dual-Luciferase reporter assay system).

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