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Differential interaction of plakoglobin and β-catenin with the ubiquitin-proteasome system

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β-Catenin and plakoglobin are closely related armadillo family proteins with shared and distinct properties; Both are associated with cadherins in actin-containing adherens junctions. Plakoglobin is also found in desmosomes where it anchors intermediate filaments to the desmosomal plaques. β-Catenin, on the other hand, is a component of the Wnt signaling pathway, which is involved in embryonic morphogenesis and tumorigenesis. A key step in the regulation of this pathway involves modulation of β-catenin stability. A multiprotein complex, regulated by Wnt, directs the phosphorylation of β-catenin and its degradation by the ubiquitin-proteasome system. Plakoglobin can also associate with members of this complex, but inhibition of proteasomal degradation has little effect on its levels while dramatically increasing the levels of β-catenin. β-TrCP, an F-box protein of the SCF E3 ubiquitin ligase complex, was recently shown to play a role in the turnover of β-catenin. To elucidate the basis for the apparent differences in the turnover of β-catenin and plakoglobin we compared the handling of these two proteins by the ubiquitin-proteasome system. We show here that a deletion mutant of β-TrCP, lacking the F-box, can stabilize the endogenous β-catenin leading to its nuclear translocation and induction of β-catenin/LEF-1-directed transcription, without affecting the levels of plakoglobin. However, when plakoglobin was overexpressed, it readily associated with β-TrCP, efficiently competed with β-catenin for binding to β-TrCP and became polyubiquitinated. Fractionation studies revealed that about 85% of plakoglobin in 293 cells, is Triton X-100-insoluble compared to 50% of β-catenin. These results suggest that while both plakoglobin and β-catenin can comparably interact with β-TrCP and the ubiquitination system, the sequestration of plakoglobin by the membrane-cytoskeleton system renders it inaccessible to the proteolytic machinery and stabilizes it. *Oncogene* (2000) **19,** 1992–2001.

Keywords: β-catenin; plakoglobin; β-TrCP; ubiquitination

Introduction

β-Catenin and plakoglobin (or γ-catenin) are highly homologous proteins of the armadillo family, playing a major role in the membrane anchorage of the cytoskeleton at cell-cell adherens junctions (Ozawa *et al*., 1989; Ben-Ze'ev and Geiger, 1998). In adherens junctions, β-catenin and plakoglobin independently associate with the cytoplasmic domain of adhesion receptors of the cadherin family, linking them to the actin cytoskeleton via an association with α-catenin (Takeichi, 1990; Geiger and Ayalon, 1992; Kemler, 1993; Adams and Nelson, 1998; Takeichi, 1995). In addition to this structural role in cell adhesion, β-catenin and its *Drosophila* homolog, armadillo, are key components of the wg/wnt-signaling pathway (Peifer and Wieschaus, 1990; Peifer *et al*., 1993; Wodarz and Nusse, 1998) that regulates developmental processes, including specification of the anterior-posterior segment polarity in *Drosophila* (Peifer *et al*., 1993), and axis determination in developing *Xenopus* embryos (Heasman *et al*., 1994). Signaling by β-catenin is carried out mainly by the nuclear pool of the protein, and recruitment of this protein to adherens junctions, by overexpressing cadherins inhibits its signaling activity (Fagotto *et al*., 1996; Sanson *et al*., 1996; Simcha *et al*., 1998).

Despite the close homology and colocalization in adherens junctions β-catenin and plakoglobin display clear differences in their distribution and signaling activity. Thus, plakoglobin (but not β-catenin) is a component of the submembranal plaque of desmosomes (Cowin *et al*., 1986; Franke *et al*., 1989). In addition, several lines of evidence suggest that the two proteins differ greatly in their signaling activity (Simcha *et al*., 1998; Zhurinsky, Shtutman and Ben-Ze'ev, submitted). During *Xenopus* development, a reduction in β-catenin level (Heasman *et al*., 1994), unlike in that of plakoglobin (Kofron *et al*., 1997), affects embryonic axis formation. The phenotypes of β-catenin-null (Haegel *et al*., 1995) and plakoglobin-null mice (Bierkamp *et al*., 1996; Ruiz *et al*., 1996) are also different: embryos that lack β-catenin die before gastrulation, while plakoglobin-null mice die much later due to heart failure (Bierkamp *et al*., 1996; Ruiz *et al*., 1996).

In addition to its activation of wnt signaling, non-junctional β-catenin interacts with the ubiquitin-proteasome system that regulates its degradation (Aberle *et al*., 1997; Orford *et al*., 1997; Simcha *et al*., 1998). The targeting of β-catenin for degradation involves the phosphorylation of its N-terminus by glycogen synthase kinase 3β (GSK) (Aberle *et al*., 1997; Yost *et al*., 1996) which occurs in a multi-protein complex consisting of β-catenin, GSK, the adenoma-

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tous polyposis coli (APC) tumor suppressor protein and axin/conductin (Munemitsu *et al*., 1995; Rubinfeld *et al*., 1996; Zeng *et al*., 1997; Ikeda *et al*., 1998; Behrens *et al*., 1998; Yamamoto *et al*., 1998). This complex associates with the ubiquitin ligase, β-TrCP (β-transducin repeat-containing protein) that recognizes the N-terminally phosphorylated forms of β-catenin and regulates its ubiquitination and degradation by the proteasome (Winston *et al*., 1999; Liu *et al*., 1999; Latres *et al*., 1999; Hart *et al*., 1999; Kitagawa *et al*., 1999). β-TrCP associates through its F-box with Cul1/Skp1/ROC1 forming the modular ubiquitin ligase SCF complex (Latres *et al*., 1999; Tan *et al*., 1999) and via its WD repeat motif with the phosphorylated β-catenin (Winston *et al*., 1999; Liu *et al*., 1999; Hart *et al*., 1999), thus forming a multimolecular complex consisting of β-TrCP/β-catenin/Axin/GSK/APC (Liu *et al*., 1999; Kitagawa *et al*., 1999). β-TrCP and its *Drosophila* homolog *Slimb* were implicated in the regulation of wnt/wg signaling (Jiang and Struhl, 1998) and the ΔF-β-TrCP mutant, lacking the F-box, was shown to induce axis duplication in *Xenopus* (Marikawa and Elinson, 1998; Liu *et al*., 1999), and loss of function mutations in *Slimb*, induce the accumulation of armadillo in *Drosophila* (Jiang and Struhl, 1998). Plakoglobin is also capable of associating with the GSK/APC/Axin complex (Hulsken *et al*., 1994; Rubinfeld *et al*., 1995; Kodama *et al*., 1999), but its degradation by the ubiquitin-proteasome system is less pronounced than that of β-catenin (Simcha *et al*., 1998), and its mode of interaction with the β-TrCP ubiquitin ligase was not determined.

The binding of wnt to its receptor frizzled, results in the inhibition of GSK activity (Cook *et al*., 1996) and a subsequent elevation in β-catenin level (Papkoff *et al*., 1996). This is followed by the translocation of β-catenin into the nucleus and its interaction with LEF/TCF family transcription factors (Behrens *et al*., 1996; Huber *et al*., 1996; Molenaar *et al*., 1996), leading to the transactivation of LEF/TCF-responsive target genes (Molenaar *et al*., 1996; van de Wetering *et al*., 1997).

Constitutive activation of β-catenin signaling, that may result from stabilization of β-catenin in cells carrying inactivating mutations in APC or mutations in the N-terminus of β-catenin, is associated with tumors in a variety of tissues (Korinek *et al*., 1997; Morin *et al*., 1997; Rubinfeld *et al*., 1997; de La Coste *et al*., 1998; Miyoshi *et al*., 1998; Palacios and Gamallo, 1998; Sparks *et al*., 1998; Voeller *et al*., 1998; Zurawel *et al*., 1998; Chan *et al*., 1999). In line with these findings, the target genes of β-catenin/LEF/TCF in mammalian cells include oncogenes such as c-*myc* (He *et al*., 1998) and cyclin D1 (Tetsu and McCormick, 1999; Shtutman *et al*., 1999). In contrast, the level of plakoglobin is often reduced in cancer cells (Navarro *et al*., 1993; Aberle *et al*., 1995; Simcha *et al*., 1996) and the human plakoglobin gene displays loss of heterozygosity in certain tumors (Aberle *et al*., 1995). Moreover, plakoglobin was shown to suppress tumorigenicity when overexpressed in various transformed cell lines (Simcha *et al*., 1996).

Despite these differences between β-catenin and plakoglobin in tumor cells, plakoglobin was shown to contain a potent transactivation domain (Simcha *et al*., 1998) and it can bind to LEF-1 under certain conditions (Huber *et al*., 1996; Simcha *et al*., 1998; Hecht *et al*., 1999). Elevation in plakoglobin expression was shown to lead to ectopic axis formation in *Xenopus* (Karnovsky and Klymkowsky, 1995) and activation of LEF/TCF-dependent transcription in mammalian cells (Simcha *et al*., 1998). The interpretation of these results is complicated however, by the fact that increased levels of plakoglobin can also lead to compromised degradation and nuclear accumulation of the endogenous β-catenin (Miller and Moon, 1997; Simcha *et al*., 1998).

These studies on the regulation of plakoglobin and β-catenin levels, raised the question of possible differences in the interaction of the two proteins with the ubiquitin-proteasome system. In principle, a difference in the regulation of their turnover could result from intrinsic variations in their ability to interact with the ubiquitin system, or from differences in their subcellular localization and sequestration by complexing with other cellular compartments. In this study, we have compared the association of the two proteins with the recently described β-TrCP. We show that plakoglobin, when overexpressed in cells, is capable of interacting with the β-TrCP ubiquitin ligase, similar to β-catenin. However, unlike β-catenin whose level and transcriptional activity increase following overexpression of the dominant negative ΔF-β-TrCP, the endogenous plakoglobin level remains largely unaffected by this treatment. In view of the much lower levels of Triton-soluble plakoglobin, compared to β-catenin, we propose that these differences are attributable to the more strict and tight sequestration of plakoglobin by the membrane-cytoskeleton compartment, rendering it largely unavailable to the ubiquitination system.

Results

ΔF-β-TrCP increases the levels of β-catenin, induces its nuclear translocation and potentiates its transactivation capacity

CHO cells express low levels of N-cadherin and β-catenin, thus providing a sensitive system to study the modulation of β-catenin degradation (Sadot *et al*., 1998). When CHO cells are transfected with N-cadherin, β-catenin levels are increased since β-catenin is sequestered at cell-cell junctions and protected from degradation (Sadot *et al*., 1998). When β-TrCP, the ubiquitin ligase that interacts with β-catenin and regulates its activity (Polakis, 1999), or its dominant negative mutant lacking the F-box, (ΔF) were transfected into CHO cells, β-catenin levels increased six- or 11.5-fold respectively (Figure 1). To determine the subcellular distribution of β-catenin that accumulates in these cells, HA-tagged β-TrCP or ΔF were transiently transfected into CHO and 293T cells. The cells were then double stained for β-TrCP or ΔF using antibodies to HA and against β-catenin. In both cell types, the β-catenin that accumulated after transfection with β-TrCP and ΔF was primarily localized in the nuclei of the transfected cells (Figure 2b,e). Interestingly, most of the transfected β-TrCP and ΔF proteins were also localized in the nucleus (Figure 2a,d). Similar nuclear localization was also observed in other cell

types including MDCK, 3T3 and CHO (data not shown). Quantitation of the nuclear immunofluorescence, using digital microscopy, revealed strong correlation between the intensity of ΔF fluorescence (FITC) and that of β-catenin (Rhodamine) within the same nuclei (correlation coefficient $r>0.9$). The difference in the intensity of nuclear β-catenin staining between ΔF-transfected and non-transfected cells was highly significant ($P<0.0005$, Figure 2c). The intact β-TrCP, on the other hand, increased β-catenin levels only when highly overexpressed in cells (Figure 2d,e arrows), and its effect was weaker than that of ΔF (Figure 2f), in agreement with the results obtained with CHO cells (Figure 1). Thus, in such overexpressing cells, a linear correlation between the nuclear immunostaining intensities of β-TrCP (FITC) and β-catenin (Rhodamine) was observed ($r>0.9$, Figure 2d–f). The difference between β-catenin staining in the nuclei of transfected and non-transfected cells was highly significant ($P<0.005$).

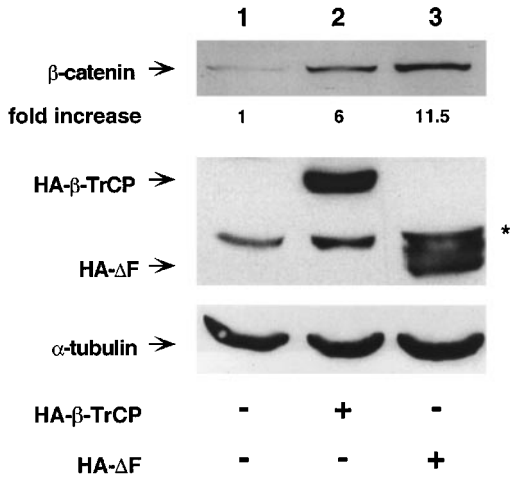

Figure 1 β-TrCP and ΔF elevate β-catenin levels in CHO cells. Cells were transiently transfected with HA-tagged β-TrCP (lane 2) or ΔF (lane 3). The levels of the endogenous β-catenin (top panel), of the HA-tagged transfected proteins (middle panel) and of α-tubulin (used as loading control, the lower panel) were determined by Western blotting. An extract of non-transfected cells was loaded in lane 1. β-Catenin levels are expressed as fold increase over its level in non-transfected cells. (*), A non-specific band recognized by the HA antibody

To test whether the β-catenin that accumulated in the nuclei of cells after transfection with β-TrCP and ΔF was transcriptionally active, 293T cells were co-transfected with a LEF-1-responsive reporter plasmid (van de Wetering *et al*., 1997). The ability to activate this reporter was compared to that of transfected wt β-catenin, or a stable mutant of β-catenin (S33Y). The results summarized in Figure 3, show that ΔF is a potent activator of the LEF-1 reporter, similar to the stable mutant of β-catenin S33Y (130 ± 22 and 185 ± 8-fold increase in luciferase activity over the level obtained with the reporter plasmid alone). In contrast, under the same experimental conditions, wt β-catenin-mediated transactivation was only about 44-fold and that of β-TrCP 15-fold higher than the basal level. Luciferase activity of the control plasmid remained unaltered when cotransfected with β-TrCP constructs.

ΔF-β-TrCP stabilizes overexpressed but not the endogenous plakoglobin

The effect of β-TrCP and ΔF on endogenous plakoglobin level in 293T cells was also tested. Cells were transfected with β-TrCP or ΔF and stained for the transfected protein and the endogenous plakoglo-

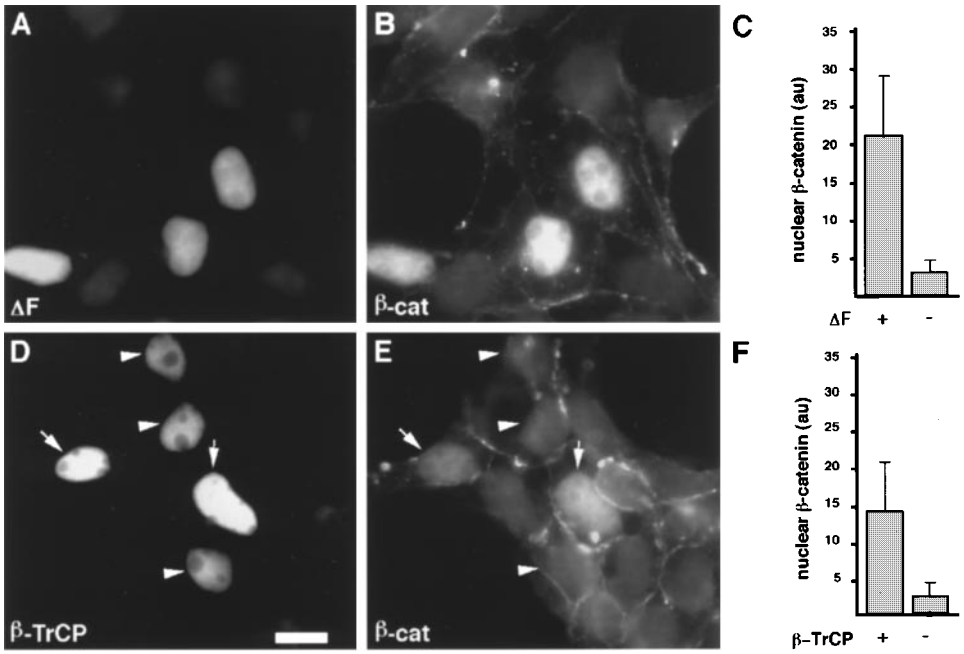

Figure 2 β-TrCP and ΔF transfection induce translocation of endogenous β-catenin into the nucleus. 293T cells were transiently transfected with HA-tagged ΔF (**a, b**) or with β-TrCP (**d, e**) and double stained with anti HA (**a, d**) and anti β-catenin (**b, e**). The quantitative analysis of nuclear staining for β-catenin in ΔF or β-TrCP-transfected as well as in non-transfected cells is shown in **c** and **f**. Note that while ΔF transfection resulted in the elevation of nuclear β-catenin in all the transfected cells, β-TrCP could only induced this effect in highly transfected cells (arrows), but not in weakly transfected cells (arrowheads). au, arbitrary units. The bar represents 10 μm

bin. The results summarized in Figure 4 demonstrate that there was no significant change in the intensity of plakoglobin immunofluorescence in nontransfected versus ΔF or β-TrCP-transfected cells (Figure 4a–f). Moreover, quantitation of the immunofluorescence using the DeltaVision digital microscope system revealed that there was no correlation between the fluorescence intensities of β-TrCP or ΔF and the low levels of plakoglobin staining within the same nuclei (Figure 4c,f). The average intensities for plakoglobin immunofluorescence in both ΔF and β-TrCP transfected and non-transfected cells did not differ significantly ($P>0.5$). Thus, in contrast to β-catenin, neither β-TrCP nor ΔF could affect the level of endogenous plakoglobin in 293T cells.

The effect of ΔF on plakoglobin was further tested in the HT1080 human fibrosarcoma cells that were stably transfected with plakoglobin under a dexamethasone-inducible MMTV promoter (Salomon *et al*., 1997). In the absence of dexamethasone, only low levels of plakoglobin are expressed in these cells, whereas upon treatment with dexamethasone the expression of plakoglobin increases dramatically (Salomon *et al*., 1997). These cells were transiently transfected with HA-tagged ΔF in the presence or absence of dexamethasone and double immunostained for the transfected protein and for plakoglobin. In the absence of dexamethasone, only a small increase in plakoglobin level was observed after transfection with ΔF (Figure 5a–c). Quantitation of the immunofluorescence revealed a linear correlation between the levels of ΔF expression and of plakoglobin ($r>0.9$). The difference between nuclear staining of plakoglobin in transfected versus non-transfected cells was statistically significant ($P<0.5$). When the cells were treated with dexamethasone, the effect of ΔF on plakoglobin was more dramatic and high levels of plakoglobin accumulated in the nuclei of the transfected cells (Figure 5d–f). Quantitation of the immunofluorescence showed a linear correlation between the intensities of ΔF and plakoglobin staining in the same nuclei ($r>0.9$).

In addition to analysing the effect on plakoglobin, we have also tested in these cells the effect of ΔF on β-catenin (without dexamethasone treatment) and found that β-catenin accumulated in the nuclei of ΔF-transfected HT1080 cells (Figure 5g–i), similar to the results obtained with 293T cells (Figure 2). Taken together, these results suggest that in HT1080 cells ΔF can induce nuclear accumulation of endogenous β-catenin and of stably transfected plakoglobin, while in 293T cells only the endogenous β-catenin was translocated into the nucleus and became active in transcription whereas plakoglobin remained unaffected.

To study the hypothesis that the observed differences in β-catenin and plakoglobin's response to ΔF transfec-

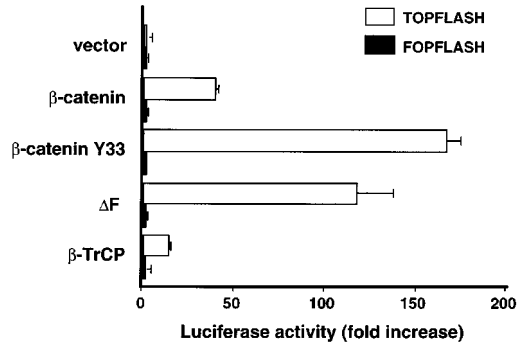

Figure 3 ΔF triggers the induction of transactivation by the β-catenin/LEF-1 complex. 293T cells were transiently transfected with a LEF-1 responsive reporter (TOPFLASH) or with a mutant reporter (FOPFLASH), together with either: wt β-catenin, mutant β-catenin Y33, ΔF, or β-TrCP. Luciferase activity was normalized to β-gal activity from co-transfected LacZ

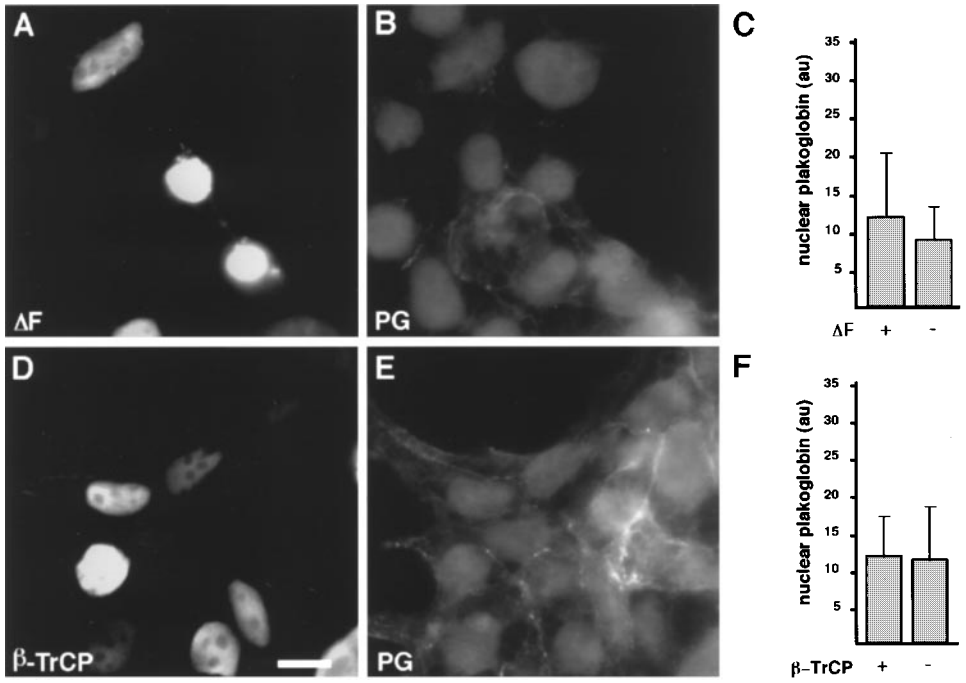

Figure 4 β-TrCP and ΔF do not affect the subcellular localization of endogenous plakoglobin in 293T cells. Cells were transiently transfected with HA-tagged ΔF (**a, b**), or β-TrCP (**d, e**) and double stained for HA (**a, d**) and for plakoglobin (**b, e**). **c** and **f** show quantitative analysis of nuclear staining for plakoglobin in ΔF or β-TrCP-transfected and non-transfected cells. au-arbitrary units. The bar represents 10 μm

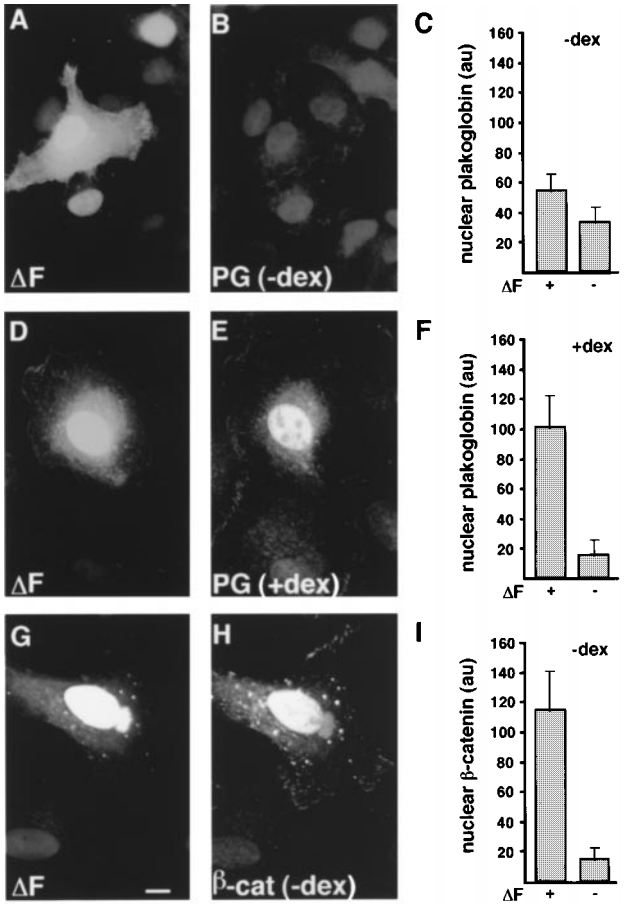

Figure 5 ΔF induces translocation of stably transfected, over-expressed plakoglobin into the nucleus. HT1080 human fibrosarcoma cells stably expressing plakoglobin under a dexamethasone-inducible promoter were transiently transfected with HA-ΔF. Untreated (**a,b,g,h**) and dexamethasone (dex) treated (**d,e**) ΔF-transfected cells were double stained with anti HA (**a,d,g**) and anti plakoglobin (**b,e**) or anti β-catenin abs (**h**). Quantitative analysis of nuclear staining for plakoglobin (**c,f**) and β-catenin (**i**) in transfected and non-transfected cells was determined as described for Figure 2. au, arbitrary units. The bar represents 10 μm

tion may be due to differences in the availability of the two proteins for interaction with the proteolytic system, we examined the Triton X-100 soluble and insoluble fractions from 293T and HT1080 cells for the relative amounts of the two proteins. The results shown in Figure 6 demonstrate that while about 50% of β-catenin was Triton X-100 soluble in both 293T and HT1080 cells, most of the plakoglobin (85%) was associated with the Triton-insoluble fraction of 293T cells. In this fraction, plakoglobin is presumably protected from degradation by its interaction with membrane-cytoskeletal complexes, while the Triton X-100 soluble β-catenin may be available for ubiquitination and degradation by the proteasome. In HT1080 cells, in contrast, after dexamethasone induction, plakoglobin was mainly enriched in the Triton-soluble fraction (73%) (Figure 6) where it was available to interact with the ubiquitination and degradation machinery, similar to β-catenin.

ΔF-βTrCP can interact with both β-catenin and plakoglobin

Plakoglobin and β-catenin posses a highly homologous N-terminal sequence that contains the GSK3β phos-

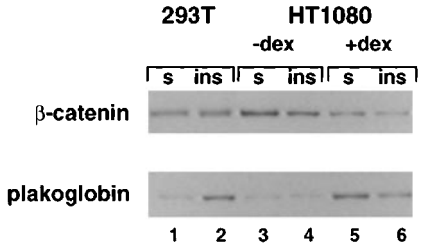

Figure 6 Different solubility in Triton X-100 of β-catenin and plakoglobin in various cell types. 293T cells (lanes 1,2) and HT1080 cells, either non-treated (lanes 3,4) or treated with dexamethasone (dex) (lanes 5,6) were extracted with Triton X-100 and the levels of β-catenin and plakoglobin in the soluble (s) and insoluble (ins) fractions were determined by Western blot analysis

phorylation site (Aberle *et al*., 1997), serving as the docking domain for β-TrCP (Winston *et al*., 1999; Hart *et al*., 1999; Liu *et al*., 1999). To test whether plakoglobin indeed interacts with β-TrCP similar to β-catenin, 293T cells were cotransfected with HA-tagged ΔF and either VSV-tagged β-catenin or plakoglobin and cell lysates were immunoprecipitated with anti HA antibody followed by immunoblotting with anti VSV antibody. As shown in Figure 7a both VSV-plakoglobin and VSV-β-catenin were comparably co-immunoprecipitated with HA-ΔF-β-TrCP (Figure 7a, lanes 3 and 4).

We have previously shown that the transfection of plakoglobin into 293T cells results in the elevation of the endogenous β-catenin, probably by the protection of β-catenin from the degradation machinery (Simcha *et al*., 1998). To determine whether plakoglobin can displace β-catenin from its complex with β-TrCP in living cells, constant amounts of DNA encoding HA-ΔF and VSV-β-catenin were co-transfected into 293T cells with increasing amounts of VSV-plakoglobin DNA. The complexes were immunoprecipitated with anti HA antibody and immunoblotted with anti VSV antibody. The results, shown in Figure 7b demonstrate that plakoglobin could effectively displace β-catenin from its complex with HA-ΔF in a dose dependent manner (Figure 7b, lanes 3 and 4). Thus, β-catenin and plakoglobin appear to bind to the same site on β-TrCP.

The ubiquitination of both β-catenin and plakoglobin is inhibited by ΔF-β-TrCP

To determine whether following their interaction with β-TrCP, both plakoglobin and β-catenin became ubiquitinated in cells, VSV-tagged plakoglobin or β-catenin were transfected into 293T cells together with HA-tagged ubiquitin, and 6xHis-tagged β-TrCP or ΔF. Immune complexes were precipitated using anti VSV antibody and blotted with anti HA antibody. SDS–PAGE analysis of the immune complexes revealed the presence of a ladder of HA-ubiquitinated proteins in cells expressing either VSV-β-catenin or VSV-plakoglobin (Figure 8a, lanes 2 and 5 respectively). Since the lower molecular weight bands in these ladders (~100 kD) are higher than that of β-catenin and plakoglobin approximately by the size of a single ubiquitin molecule (~10 kD), we propose that the ladders seen in Figure 8a correspond to the ubiquitinated forms of these molecules. It was previously reported that F-box proteins themselves can undergo ubiquitination (Zhou and Howley 1998). However, β-TrCP has a lower

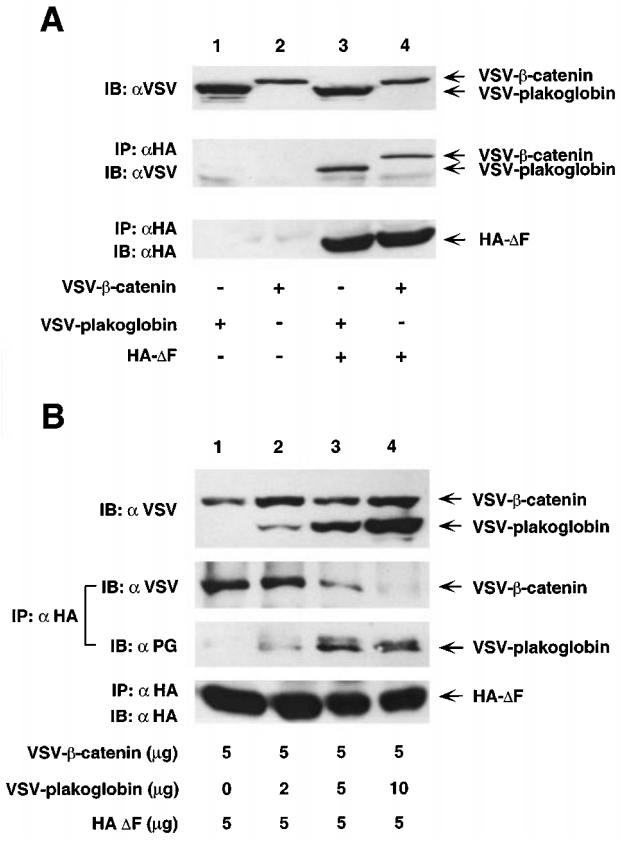

Figure 7 β-Catenin and plakoglobin can bind to ΔF. (**a**) 293T cells were transfected with VSV-tagged plakoglobin (lanes 1 and 3) or with VSV-tagged β-catenin (lanes 2 and 4) together with HA-tagged ΔF-β-TrCP (ΔF) (lanes 3 and 4). Complexes were immunoprecipitated (IP) with anti HA antibody. The levels of VSV-β-catenin and VSV-plakoglobin in cell extracts (top panel) and in the HA-immunoprecipitates (middle and lower panels) were analysed by immunoblotting (IB). (**b**) 293T cells were transfected with a constant amount of β-catenin and HA-ΔF and increasing amounts of plakoglobin as indicated. Complexes containing HA-ΔF were immunoprecipitated (IP) with anti HA antibody. The levels of VSV-β-catenin and VSV-plakoglobin in cell extracts (top panel) and the HA-immunoprecipitates were analysed by immunoblotting (IB). VSV-β-catenin and VSV-plakoglobin were detected in complex with ΔF using anti VSV and anti plakoglobin antibodies respectively (two middle panels). The lower panel shows the levels of HA-ΔF in these complexes

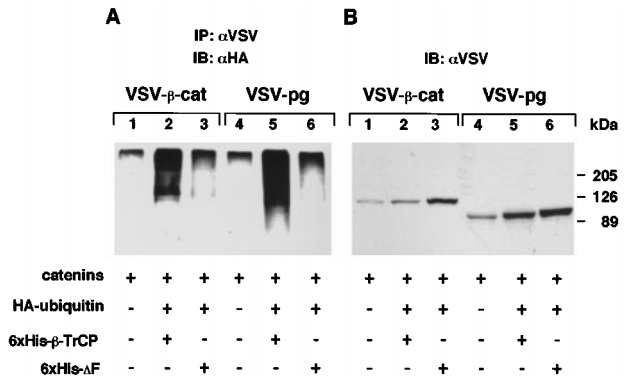

Figure 8 Plakoglobin and β-catenin are ubiquitinated in transfected cells and their ubiquitination is inhibited by ΔF. 293T cells were transfected with VSV-β-catenin (lanes 1–3) or VSV-plakoglobin (lanes 4–6) and HA-ubiquitin (lanes 2, 3, 5 and 6), and with either 6xHis-β-TrCP (lanes 2 and 5) or 6xHis-ΔF (lanes 3 and 6). Complexes were immunoprecipitated (IP) with anti VSV antibody and immunoblotted (IB) with anti HA antibody (**a**). The levels of VSV β-catenin and VSV-plakoglobin in cell lysates were detected with anti VSV antibody (**b**)

molecular weight (~60 kb) than β-catenin and plakoglobin, and thus its ubiquitination ladder is expected to run faster on the SDS gel. When ΔF was co-transfected, the ubiquitination of both β-catenin and plakoglobin was inhibited (Figure 8a, lanes 3 and 6). Interestingly, the total level of the transfected β-catenin or plakoglobin was increased five- and twofold respectively by transfection with β-TrCP, and to a greater extent (seven- and threefold respectively), by transfection with ΔF (Figure 8b). Taken together, these results indicate that both plakoglobin and β-catenin are efficiently ubiquitinated when overexpressed in 293 cells and that the ubiquitination can be inhibited by the ΔF mutant of β-TrCP.

Discussion

β-Catenin and plakoglobin display a high degree of sequence homology, especially in the armadillo repeat domain of the molecules, and were shown to fulfill similar functions in the assembly of adherens junctions. Yet, these two proteins are different in various important aspects related to their structural role in adhesion, in the mechanisms responsible for the regulation of their cellular levels, and their signaling abilities (see for review Ben-Ze'ev and Geiger, 1998). Thus, while the two proteins are associated with adherens junctions, only plakoglobin is present in desmosomes (Cowin *et al*., 1986; Franke *et al*., 1989). In addition, the elimination of β-catenin and plakoglobin genes (Haegel *et al*., 1995; Bierkamp *et al*., 1996; Ruiz *et al*., 1996), or interference with the function of maternal β-catenin (Heasman *et al*., 1994) and plakoglobin (Kofron *et al*., 1997) in *Xenopus* embryos, lead to distinct phenotypes. Furthermore, mutations in β-catenin or APC which lead to accumulation of β-catenin, are associated with oncogenic transformation (reviewed in Ben-Ze'ev and Geiger, 1998; Polakis, 1999), probably by directing the transcription of oncogenes such as c-myc and cyclin D1 (He *et al*., 1998; Tetsu and McCormick, 1999; Shtutman *et al*., 1999) and the invasion-promoting secreted protease matrilysin (Crawford *et al*., 1999). In contrast, the levels of plakoglobin are often reduced in cancer cells (Navarro *et al*., 1993; Sommers *et al*., 1994; Aberle *et al*., 1995; Simcha *et al*., 1996) and overexpression of plakoglobin may suppress tumorigenicity (Simcha *et al*., 1996).

The major aim of this study was to explore the differences in the interaction of β-catenin and plakoglobin with the ubiquitin-mediated degradation machinery that could be responsible for their different turnover and signaling activity. Previous studies have demonstrated that plakoglobin, similar to β-catenin, can interact with the GSK/APC/Axin system that regulates β-catenin stability (Hulsken *et al*., 1994; Rubinfeld *et al*., 1995; Kodama *et al*., 1999). In principle, differences in the interaction of the two catenins with the ubiquitin-proteasome system could be due to either differential phosphorylation of the two proteins by the GSK/APC/Axin complex, or to different processing by the ubiquitin system.

In this study, we have compared the interaction of the two proteins with the recently discovered SCF component β-TrCP (Margottin *et al*., 1998). This protein contains two main domains, an F-box motif that binds to Skp1 and enables its assembly with the Skp1/Cul1 complex, and the WD domain that specifically binds to the target protein (for reviews see Hershko and Ciechanover, 1998; Ciechanover, 1998; Elledge and Harper, 1998). Cdc53/Cul1 interacts with the E2 ubiquitin-conjugating enzyme that functions in conjunction with the Skp1-β-TrCP complex to link ubiquitin to lysine residues in the target protein (Patton *et al*., 1998). The importance of β-TrCP in regulating various signaling pathways, by controlling specific protein turnover, was recently highlighted in studies demonstrating that β-TrCP and its *Drosophila* homolog *Slimb* regulate three different signaling pathways (see for review Maniatis, 1999); namely, NF-κB, Wnt/Wingless and Hedgehog (Yaron *et al*., 1998; Jiang and Struhl, 1998). The regulation of NF-κB signaling is achieved by controlling the turnover of IκBα (Yaron *et al*., 1998; Spencer *et al*., 1999; Fuchs *et al*., 1999), while the stabilization of β-catenin by a dominant negative ΔF-β-TrCP mutant, lacking the F-box, induces axis duplication in *Xenopus* (Marikawa and Elinson, 1998; Liu *et al*., 1999), and loss of function mutations in *Drosophila Slimb*, induce accumulation of armadillo and cubitus interuptus (Jiang and Struhl, 1998).

We showed here that both β-catenin and plakoglobin are capable of interacting with β-TrCP when overexpressed in cells, and are ubiquitinated in the transfected cells, and that plakoglobin can compete with β-catenin for β-TrCP binding. The endogenous β-catenin and plakoglobin however, differed significantly in their response to overexpression of the dominant negative ΔF mutant. While the level of endogenous β-catenin was elevated, most probably by its binding to ΔF that sequestered it away from the degradation system, in agreement with other studies (Liu *et al*., 1999; Kitagawa *et al*., 1999; Hart *et al*., 1999), the level of plakoglobin remained unaffected in such cells. Furthermore, the increase in β-catenin levels was associated with its nuclear accumulation resulting in a very efficient activation of a LEF/TCF-responsive reporter, further supporting the importance of the regulation of β-catenin stability in the control of its signaling activity. Interestingly, overexpression, of intact β-TrCP also resulted in the stabilization of β-catenin in both CHO and 293T cells, but to a lesser extent than with ΔF. This observation can be attributed to the sequestration of the other SCF components by the excess β-TrCP (Spencer *et al*., 1999). Alternatively, β-TrCP may bind to β-catenin but fail to efficiently ubiquitinate it (at least in the cells tested here) and thereby block the degradation of β-catenin and plakoglobin via an alternative, yet unidentified protein. It is noteworthy, however, that when overexpressed in HeLa cells, β-TrCP reduces β-catenin levels (Hart *et al*., 1999).

Since both overexpressed β-catenin and plakoglobin bind to β-TrCP in cells and become ubiquitinated, we propose that the lack of ΔF effect on plakoglobin results from the unavailability of the endogenous plakoglobin molecules to interact with ΔF in the cells used in this study. This is supported by our finding that in cells overexpressing plakoglobin under an inducible promoter the effect of ΔF on plakoglobin is similar to that on β-catenin, i.e. it induces the accumulation of the protein and its nuclear translocation. In addition, while β-catenin is about equally distributed between the Triton X-100-soluble and -insoluble fractions, most of the plakoglobin is Triton X-100-insoluble in nontransfected cells. In contrast, in cells overexpressing plakoglobin, the distribution of plakoglobin and that of β-catenin in these two subcellular fractions is similar.

A differential interaction of the two molecules with their different molecular partners was also suggested to explain their different association with LEF-1 (Simcha *et al*., 1998). While transfection of LEF-1 can readily bring about the accumulation and nuclear translocation of β-catenin in MDCK cells (Behrens *et al*., 1996; Simcha *et al*., 1998), it has no effect on plakoglobin localization in the same cells (Simcha *et al*., 1998). Nevertheless, when plakoglobin is overexpressed along with LEF-1, it colocalizes with LEF-1 in the nuclei of the transfected cells (Simcha *et al*., 1998). It is noteworthy, that in MDCK cells, similar to 293T and HT1080 cells (Figure 6), the Triton X-100-soluble pool of β-catenin is much larger than that of plakoglobin, again pointing to a differential availability of the two catenins to other partner molecules.

The basis for the reduced levels of Triton-soluble plakoglobin is not yet clear. Nevertheless, it is well established that while both molecules are part of the cytoplasmic plaque of adherens junctions, only plakoglobin can interact with desmosomes (Cowin *et al*., 1986; see for review Ben-Ze'ev and Geiger, 1998). This additional structural interaction of plakoglobin may further reduce the free plakoglobin pool. Indeed, the rate of plakoglobin degradation in transfected cells decreased 15–20-fold when plakoglobin is co-transfected with its desmosomal partners, desmoglein or desmocollin (Kowalczyk *et al*., 1994). It is noteworthy that both MDCK and 293T cells, that were used in the present and previous studies (Simcha *et al*., 1998), are epithelial and contain desmosomes.

Regulation of the signaling function of catenins depends, to a large extent, on the equilibrium between the free and junctional associated catenins. An increase in the expression of the junctional partners of β-catenin (for example cadherin and α-catenin) results in a significantly reduced β-catenin signaling (Fagotto *et al*., 1996; Sanson *et al*., 1996; Simcha *et al*., 1998; Sadot *et al*., 1998). Moreover, changes in the level of plakoglobin were also shown to influence this balance in β-catenin's availability, by affecting β-catenin's stability in the cell (Miller and Moon, 1997; Salomon *et al*., 1997; Simcha *et al*., 1998).

While the results of this study imply a difference in availability of the two proteins for interaction with β-TrCP, an intrinsic difference in the abilities of the two molecules to interact with β-TrCP, and other components of the ubiquitin-proteasomal system, cannot be entirely excluded. It was recently suggested that an intrinsic difference in the capacity of the two molecules to complex with transcriptional partners may explain the weak potential of plakoglobin, in contrast to β-catenin, to function in transactivation (Simcha *et al*., 1998). This is supported by our finding that the ability of the two molecules to form a ternary complex consisting of catenin/LEF/DNA is different *in vitro* and in cells transfected with the two molecules (J Zhurinsky, M Shtutman and A Ben-Ze'ev, manuscript submitted).

Taken together, these results imply that a fine-tuned balance, regulated at multiple levels, has evolved in mammalian cells to control the level and availability and thus the signaling by β-catenin. This includes the regulation of junction assembly, the activation or inactivation of the GSK/APC/Axin system and the availability to the ubiquitination machinery. The ability of excess plakoglobin to interact with some of these systems can exert complex effects on β-catenin levels and signaling. Thus, competition on the degradation pathway may lead to protection followed by accumulation and an increase in β-catenin's transactivation potential. Competition on cadherin binding, on the other hand, may release β-catenin from a protected junctional pool making it available for transactivation, degradation, or both (Miller and Moon, 1997; Salomon *et al*., 1997; Simcha *et al*., 1998). Such conditions most probably prevail during embryonal development when cells extensively modulate their junctions, when Wnt signaling is activated (Klymkowsky *et al*., 1999), or during epithelial mesenchymal transition that is associated with dramatic changes in junctional assembly (Eger *et al*., 2000). Future studies will have to unravel the details of this very complex, multimolecular regulation of β-catenin level and its signaling role in mammalian cell function.

Material and methods

Plasmids

β-TrCP cDNA was isolated from 293 cells by RT–PCR using the following primers: 5′-TGGCCTCGGCGATTATG-GAC-3′ and 5′-GGGTCCTGGGCAAGTATGAG-3′. The β-TrCP PCR product was inserted into pCR-TOPO2.1 (Invitrogen) by TA cloning. An F-box deletion mutant of β-TrCP (ΔF) in which nucleotides 93–536 were removed was generated by two step PCR as described (Higuchi *et al*., 1988) using the following primers: inside 5′-TAATGGCGAAC-CCCCTAGGTACCGAGTGACCTTTGA-3′ and 5′-TCA-GA GGTCACTCGGTACCTAGGGGGTTCGCC AT TA-3′, outside: a T7 primer (a 17 oligomer), and 5′-AGGACT-GAACCTGTATGG-3′. The β-TrCP and ΔF mutant were cloned into the pCAGGS expression vector (Niwa *et al*., 1991) containing a 6xHis tag between the *Xho*I and *Eco*RI sites. HA tagged β-TrCP and ΔF were generated by subcloning their cDNAs into the pCGN-HA vector. In brief, the N-termini of the full-length β-TrCP and that of ΔF were generated by PCR using the following primers; 5′-CGTCTA-GAATGGACCCGGCCGAGGCGGT-3′ and 5′-CTCTCG-ATAAGCTTCTTCCAC-3′. The *Xba*I–*Hind*III PCR fragments were joined to the *Hind*III–*Bam*HI C-terminal part of β-TrCP and cloned into *Xba*I–*Bam*HI cleaved pCGN-HA. VSV-tagged β-catenin and plakoglobin (Simcha *et al*., 1998), the pCGN-HA containing the S33Y β-catenin mutant (Shtutman *et al*., 1999), the TOPFLASH and FOPFLASH luciferase reporter vectors (van de Wetering *et al*., 1997) and the HA-ubiquitin expressing vector (Treier *et al*., 1994) were described.

Cell lines and transfections

CHO, 293T and HT1080 cells were maintained in DMEM with 10% calf serum. HT1080 stably expressing an inducible plakoglobin from the MMTV promoter (Salomon *et al*., 1997) were treated with 0.1 μM dexamethasone. Transient transfections were performed using the calcium phosphate precipitation methods for 293T and HT1080 cells and by Lipofectamine (GIBCO) in CHO cells. A β-galactosidase-expressing vector was co-transfected in the transactivation assays as an internal control for transfection efficiency. After 24 h, the cells were lysed and both luciferase and β-galactosidase activities were determined by enzyme assay kits (Promega). For Western blots and immunoprecipitations (IP) cells were harvested 24 h after transfection and lysed in either Laemmli's sample buffer or IP buffer (see below), respectively.

Immunoblotting and immunoprecipitations

Equal amounts of total protein from the different transfected cells were separated by SDS–PAGE, and subjected to Western blotting using the following antibodies: monoclonal anti HA (clone 12CA5 Boehringer Mannheim), polyclonal anti HA (a gift from M Oren, Weizmann Institute of Science, Rehovot, Israel), polyclonal anti VSV (a gift from JC Perriard, Swiss Federal Institute of Technology, Zurich Switzerland), polyclonal anti β-catenin and monoclonal anti α-tubulin (both from Sigma) and monoclonal anti plakoglobin (Salomon *et al*., 1997). For immunoprecipitation, cells were lysed in IP buffer containing 20 mM Tris HCl pH 8.0, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium vanadate and 50 μg/ml PMSF. Equal amounts of protein were incubated with 2 μl of the antibody and 20 μl of protein A/G-agarose beads (Santa Cruz) for 4 h at 4°C. The beads were washed five times with 20 mM Tris HCl buffer pH 8.0 containing 150 mM NaCl and 0.5% NP40, and the immune complexes recovered by boiling in Laemmli's sample buffer and resolved by SDS–PAGE. Blots were developed using the ECL method (Amersham). Autoradiograms were scanned by a GS-700 imaging densitometer (Bio Rad) and quantitated using the FotoLook PS 2.07.2 software. The intensity of the bands was quantitated using NIH image 1.61 software.

Cell fractionation

Fractionation into Triton X-100-soluble and -insoluble fractions was carried out with cells cultured on 35 mm tissue culture plates that were extracted, at room temperature, with 0.5 ml of 50 mM MES buffer pH 6.8 containing 2.5 mM EGTA, 5 mM $MgCl_2$ and 0.5% Triton X-100, for 3 min. The Triton X-100-soluble fraction was removed and the insoluble fraction was scraped into 0.5 ml of the same buffer. Equal volumes of the two fractions were analysed by SDS–PAGE.

Immunofluorescence microscopy

Cells were cultured on glass coverslips, fixed with 3% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.5% Triton X-100. Monoclonal or polyclonal antibodies to HA were used to label the transfected HA-tagged β-TrCP or ΔF. The secondary antibodies were Alexa 488 goat anti mouse or anti rabbit IgG (Molecular Probes). Plakoglobin was detected by a monoclonal antibody 11E4 (Salomon *et al*., 1997) and β-catenin by a polyclonal antibody (Sigma). The secondary antibodies were Cy3 goat anti mouse or anti rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Image acquisition was performed using the DeltaVision system (Applied Precision, Issaquah WA, USA), equipped with a Zeiss Axiovert 100 microscope (Oberkochen, Germany) and Photometrics 300 series scientific-grade cooled CCD camera (Tucson, AZ, USA) reading 12 bit images using a 100×1.3 NA plan-Neofluar objective (Zeiss, Oberkochen, Germany). For quantitative evaluation of the immunofluorescence, nuclei identified by the β-TrCP/ΔF labeling, or by phase contrast, were enclosed in a polygon and marked using the Priism software (Kam *et al*., 1993). The fluorescence intensity values were calculated for both β-TrCP, or ΔF and β-catenin, or plakoglobin in the corresponding

nuclei. At least 30 transfected and 30 non-transfected cells were examined in each experiment.

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