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Regulation of the Discs Large Tumor Suppressor by a Phosphorylation-dependent Interaction with the β-TrCP Ubiquitin Ligase Receptor*

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The discs large (hDlg) tumor suppressor is intimately involved in the control of cell contact, polarity, and proliferation by interacting with several components of the epithelial junctional complex and with the APC tumor suppressor protein. In epithelial cells, hDlg protein stability is regulated through the ubiquitin-proteasome pathway: hDlg is actively degraded in isolated cells, whereas it accumulates upon cell-cell contact. During neoplastic transformation of epithelial cells, loss of the differentiated morphology and progression toward a metastatic phenotype correlate with down-regulation of hDlg levels and loss of contact-dependent stabilization. Here we show that upon hyperphosphorylation, hDlg interacts with the β-TrCP ubiquitin ligase receptor through a DSGLPS motif within its Src homology 3 domain. As a consequence, overexpression of β-TrCP enhances ubiquitination of Dlg protein and decreases its stability, whereas a dominant negative β-TrCP mutant inhibits this process. Furthermore, a mutant Dlg protein that is unable to bind β-TrCP displays a higher protein stability and is insensitive to β-TrCP. Using RNA interference, we also demonstrate that endogenous β-TrCP regulates hDlg protein levels in epithelial cells. Finally, we show that β-TrCP selectively induces the degradation of the membrane-cytoplasmic pool, without affecting the nuclear pool of hDlg.

Ubiquitin-mediated proteolysis is a highly selective, temporally controlled and tightly regulated pathway that plays crucial roles in a broad array of basic cellular processes, including regulation of the cell cycle, control of signal transduction, differentiation, and development. All of these processes involve transition states, which require the fast and irreversible destruction of specific subsets of proteins. The high specificity of the system relies mainly on the ubiquitin-protein ligases, which directly bind both the substrate and the ubiquitin-conjugating enzymes. At this step the entire degradation process can be regulated through diverse signaling pathways, depending upon the particular cellular conditions. Typical examples are represented by degradation of β-catenin and IκB, whose dynamic phosphorylation modulates their interaction with an SCF multisubunit ubiquitin ligase (1, 2). SCF (Skp1·cullin-1·F-box protein) complexes are named according to the variable F-box subunit (*e.g.* $SCF^{\beta\text{-}TrCP}$, SCF^{Skp2}), which provides the substrate specificity. The F-box protein β-TrCP (also known as Fbw1a, β-TrCP1, E3RS, and FWD1) has been demonstrated to mediate the phosphorylation-dependent degradation of IκB (3) and β-catenin (4) by specifically recognizing the serine-phosphorylated motif DSG*XX*S within these proteins. β-Catenin has a dual role: a membrane pool participates in formation of adherens junctions, whereas a short-lived soluble pool functions as signal transducer/transcription factor to promote proliferation, an activity that is tightly controlled by means of regulating its stability (5). This is achieved by a destruction complex containing the APC tumor suppressor protein, axin, the GSK3β kinase, and the F-box protein β-TrCP (4, 6). GSK3β phosphorylates β-catenin on two Ser residues within the β-TrCP binding site, leading to its ubiquitination by the $SCF^{\beta\text{-}TrCP}$ ligase (1, 4, 6). Wnt signaling blocks β-catenin ubiquitination, thereby stimulating proliferation. Besides β-catenin, several proteins are clustered at the basolateral membrane of polarized epithelial cells, where they are involved both in the structural organization of the cell junctions and in transducing signals that regulate cell proliferation. Among them is hDlg, the human homolog of the *Drosophila* tumor suppressor Discs Large (DLG), which is involved in regulation of cell adhesion, apicobasal polarity, and proliferation. Mutations causing loss of DLG function result in aberrant morphology and invasive growth of epithelial cells, causing embryonic lethality (7, 8). Recently, a murine *Dlg* truncating mutation was described, which is associated with impaired morphogenesis during development and perinatal death (9). Human Dlg is the prototype member of the MAGUK (membrane-associated guanylate kinase) family of multidomain proteins and contains an N-terminal proline-rich SH3 [1] binding domain, three PDZ domains, an SH3 domain, and a C-terminal guanylate kinase homology domain. Cell-cell adhesion mediated by E-cadherin induces the translocation of hDlg from cytoplasmic pools to the plasma membrane (10), where both proteins colocalize at the adherens junctions (10, 11). hDlg acts as a central player in the organization of these structures, being connected with both the actin cytoskeleton and the plasma membrane through binding members of the 4.1/ERM family of cytoskeletal proteins (12–14) which, in turn, form a complex with the membrane glycoprotein CD44 (12). Moreover, hDlg interacts with hCASK (15, 16), also involved in connecting the actin cytoskeleton with the membrane junctions and the extracellular matrix

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[1] The abbreviations used are: SH3, Src homology 3; CBZ, *N*-carbobenzoxy-Leu-Leu-leucinal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; RT, reverse transcription; siRNA, small interfering RNA; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; TPCK, tosylphenylalanyl chloromethyl ketone.

(17, 18). Although some hDlg isoforms are associated with the cell membrane, others show nuclear localization, possibly performing signaling functions (19). Similarly to its *Drosophila* homolog, hDlg appears to have dual roles in governing both polarity and proliferation of epithelial cells. It has been shown that hDlg binds directly to the APC tumor suppressor via its PDZ domain 2 (20) and that formation of this complex has a role in APC-mediated cell cycle arrest (21). Indeed, mutant APC proteins that are no longer able to bind hDlg appear to be less effective in this respect. We have shown previously that in differentiated epithelial cells, hDlg protein accumulates upon cell-cell contact, being rapidly degraded by the ubiquitin-proteasome pathway in isolated cells (22). However, loss of differentiated epithelial morphology and progression toward a highly transformed and metastatic phenotype of epithelial tumor cells are associated with down-regulation of hDlg protein levels and loss of its contact-dependent stabilization (22, 23).[2] Significantly, the growth suppressive activities of hDlg represent a common target for several viral transforming proteins, including high risk human papilloma virus E6 (24, 25), human T-cell lymphotrophic virus-1 Tax (26), and adenovirus 9 E4ORF1 (25). All of these bind specifically to the PDZ-2 domain of hDlg, thereby inhibiting its interaction with APC and the consequent block of cell cycle progression (26). Moreover, oncogenic human papilloma virus E6 also targets Dlg for ubiquitin-mediated degradation (27, 28). We are interested in investigating the regulation of hDlg in normal and transformed cells. The findings that hDlg forms a complex with both β-catenin and APC (20) and that hyperphosphorylated hDlg is selectively degraded by the proteasome pathway (22) prompted us to investigate the possibility that hDlg could be regulated through a pathway similar to β-catenin, involving the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase.

MATERIALS AND METHODS

Plasmids—The plasmids encoding full-length GST-Dlg fusion protein and its deletion mutant derivative GST-CT-Dlg (which contains the Dlg residues downstream from amino acids 539) were kindly provided by R. Javier and have been described previously (25, 27). GST-NT-Dlg (containing Dlg amino acid residues 1–382) and GST-ΔSH3-Dlg (bearing a deletion encompassing residues 549–617) plus the plasmids for mammalian expression of HA-tagged FL-Dlg and ΔSH3-Dlg proteins were all kindly provided by D. Gardiol and have been described previously (29). Myc epitope-tagged β-TrCP and ΔFβ-TrCP expression plasmids were kindly provided by R. Benarous and have been described previously (1, 30). HA-tagged ubiquitin expression plasmid was kindly provided by C. Kühne. Dlg point mutants FL-M1 (S597A) and FL-M2 (S597A and S601A) were constructed by site-directed mutagenesis using the Gene Tailor Kit (Invitrogen) for PCR-based mutagenesis following the manufacturer's instructions. The plasmid encoding full-length GST-Dlg fusion protein was used as a template, and the obtained mutants were verified by DNA sequencing. FL-M2 Dlg was then subcloned in *Bam*HI/*Eco*RI sites of pcDNA3-HA for mammalian expression.

Antibodies—The production and purification of a polyclonal antibody against Dlg have been described previously (22). Anti-HA monoclonal antibody was from Roche Applied Science. Anti-β-galactosidase monoclonal antibody was from Promega. Rabbit polyclonal anti-HA antibody, anti-hDlg monoclonal antibody (2D11), and goat polyclonal anti-βTrCP antibodies were from Santa Cruz Biotechnology. Anti-Myc monoclonal antibody was 9E10 hybridoma supernatant (31). Biotinylated anti-mouse and anti-rabbit antibodies plus horseradish peroxidase-conjugated anti-goat antibodies and horseradish peroxidase-avidin were from DAKO. Fluorescein-conjugated goat anti-mouse and rhodamine red-conjugated goat anti-rabbit secondary antibodies were from Molecular Probes.

GST Pull-down Assays—β-TrCP was translated *in vitro* using the TNT-coupled rabbit reticulocyte system (Promega) and 10 μCi of [^{35}S]cysteine (1,000 Ci/mmol) (Amersham Biosciences), as specified by the manufacturer. For β-TrCP pull-down assays, equal amounts of different GST-fused Dlg proteins bound to glutathione-linked agarose beads were incubated with a fixed amount of the *in vitro* translated β-TrCP protein for 1 h at room temperature in a final volume of 100 μl, in a binding buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 2.5 mM EDTA. Bound proteins were washed extensively in PBS containing 1% Nonidet P-40 before analysis by SDS-PAGE and autoradiography. Alternatively, GST-Dlg beads were first incubated for 15 min at 30 °C with 10 μg of HaCaT cell extract in the presence of 200 μM ATP in a kinase buffer containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 30 mM phosphatase inhibitor 4-nitrophenyl phosphate, washed twice in binding buffer supplemented with 30 mM 4-nitrophenyl phosphate, and then used for binding assay as above. For the phosphatase assays, after incubation with the HaCaT cell extract, GST-Dlg beads were rinsed in PBS and incubated with 400 units of λ protein phosphatase (New England Biolabs) for 15 min at 30 °C prior to the binding assay. Quantitation of the binding levels was done by scanning the gels using a Packard Instant PhosphorImager.

Western Blotting—Cells were rinsed in ice-cold PBS, lysed on ice in buffer A (50 mM Hepes pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml aprotinin, 100 μM TPCK, and 50 μM TLCK) for 10 min, and then cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. Protein concentrations were determined using the Bio-Rad Protein Assay system, and equal amounts were then separated on SDS-PAGE and transferred to nitrocellulose membrane. Proteins were detected with the appropriate specific antibodies and developed with the Amersham ECL system according to the manufacturer's instructions.

Immunoprecipitations—Cells were rinsed in ice-cold PBS and lysed on ice in buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 5% glycerol, 100 μM TPCK, 50 μM TLCK, and 30 mM 4-nitrophenyl phosphate). Cell lysates were cleared by centrifugation at 13,000 rpm for 10 min, incubated with appropriate specific antibodies for times ranging from 1 h to overnight at 4 °C, and subsequently mixed with either protein A- or protein G-agarose beads (Amersham Biosciences) for 40 min. Where necessary, the anti-Dlg and anti-β-TrCP antibodies were covalently bound to the protein A or protein G using 5 mg/ml dimethylpimelimidate (Pierce) as cross-linker.

Cell Culture, Proteasome Inhibition, and Transfections—Human embryonic kidney 293 cells, human osteosarcoma U2OS cells, and HaCaT immortalized human skin keratinocytes were all maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 10% CO_2. For proteasome inhibition, growing cells were treated with CBZ (*N*-carbobenzoxy-Leu-Leu-leucinal, Sigma, MG132) at a final concentration of 50 μM for 4 h prior to harvesting for subsequent analysis. 293 cells were transiently transfected by standard calcium phosphate precipitation procedure.

Measurement of Dlg Protein Stability—At 24 h post-transfection, cells were treated with 50 μM cycloheximide for the times indicated and subsequently harvested. The amount of wild-type and mutant Dlg proteins remaining was then analyzed by Western blot as above described and subsequently estimated by densitometric scanning.

RNA Interference—Small interfering RNAs (siRNA) for human β-TrCP (32) and control scrambled siRNA were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The sequences of β-TrCP siRNA were 5′-GUGGAAUUUGUGGAACAUCTT-3′ (sense) and 5′-GAUGUUCCACAAAUUCCACTT-3′ (antisense). Transfection of 293 cells was performed using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were seeded into tissue culture plates 12–16 h prior to transfection. About 0.6 nmol of either the β-TrCP or control siRNA was transfected per 1 ml of culturing medium. 72 h later, cells were lysed, and either RNA extraction was performed, or protein extracts were prepared for PAGE analysis.

RNA Extraction and RT-PCR—The efficiency of β-TrCP gene suppression was monitored by RT-PCR, using the expression of GAPDH mRNA as a control. Total cellular RNA was isolated from cultured cells with TriZol reagent (Invitrogen) according to the manufacturer's instructions, and DNase was treated and quantified. 5 μg of total RNA was reverse transcribed for 30 min at 37 °C and then for 30 min at 42 °C using 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and either the β-TrCP-specific antisense primer 5′-AGGTATGACAGAATGTTCTCA-3′ or an oligo(dT) primer. 30 cycles of amplification were then performed using either β-TrCP primers: 5′-CACTTAGACACATACAACA-3′ and 5′-TCTGCAACATAGGTTTAAGAT-3′ or GAPDH primers 5′-CATGCCATCACTGCCACCCAG-3′ and 5′-TGGTGGACCACGAGTCACATC-3′. 309-bp β-TrCP and 307-bp GAPDH amplification products were then separated on agarose gel.

Immunofluorescence—U2OS cells were grown in glass slide chambers and transfected with Myc epitope-tagged β-TrCP expression vec-

[2] F. Mantovani, unpublished observations.

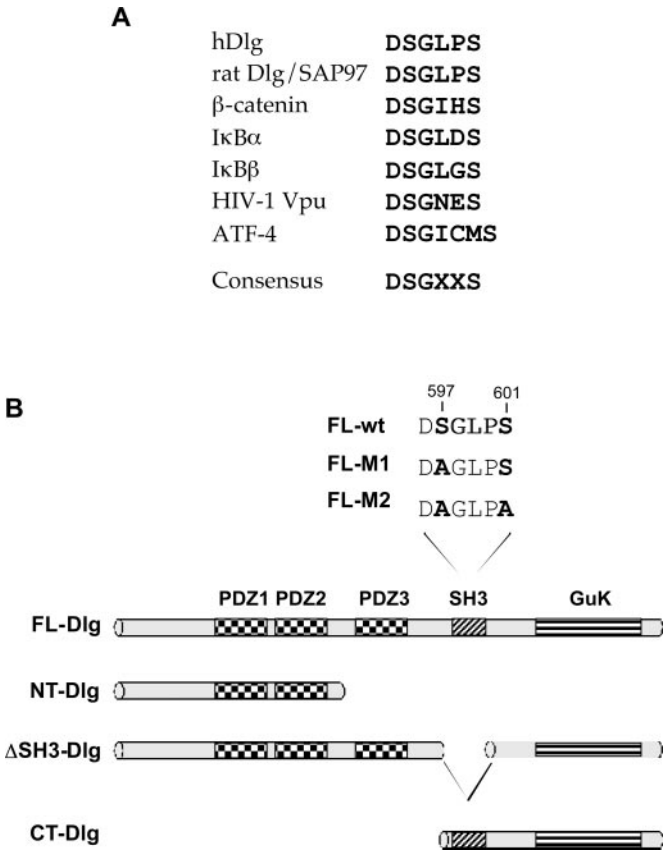


FIG. 1. **A putative consensus binding site for the F-box protein β-TrCP is present in the SH3 domain of human and rat Dlg protein.** *A*, comparison of the six-amino acid consensus motif identified within hDlg and rat Dlg/Sap97 proteins with those bound by β-TrCP in β-catenin, IκB, HIV-1 Vpu, and ATF4 proteins. *B*, schematic representation of the wild-type (*wt*) (FL-Dlg) and mutant Dlg proteins used in this study. NT-Dlg contains the N-terminal domain plus the first two PDZ domains (amino acids 1–382). ΔSH3-Dlg has a deletion encompassing the SH3 domain (amino acids 549–617). CT-Dlg contains the SH3 and GuK domains (amino acids 539–911). FL-M1 bears a Ser to Ala amino acid substitution at position 597 within the β-TrCP putative consensus binding site, whereas FL-M2 has both Ser-597 and Ser-601 mutated to Ala.

tor. After 36 h the cells were washed in PBS, fixed in 3% paraformaldehyde in PBS at room temperature for 20 min, permeabilized for 4 min in 0.1% Triton X-100 and PBS, stained with anti-Myc monoclonal antibody and with 5 μg/ml anti-Dlg antibody for 1 h at 37 °C, and detected with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody and a Rhodamine-conjugated goat anti-rabbit antibody (Molecular Probes) for 20 min at 37 °C. After extensive washing the slides were mounted with Vectashield mounting medium (Vector Laboratories) and subsequently analyzed with a Leica DMLB microscope and a Photometrics Coolsnap camera.

RESULTS

hDlg Contains a Putative Consensus Binding Site for the F-box Protein β-TrCP—It has been reported that the F-box protein β-TrCP, which is the substrate-binding component of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase complex, recognizes a serine-phosphorylated consensus sequence DSG*XX*S (in single-letter code, where *X* is any residue) within its substrates. An analysis of the primary sequence of the Dlg protein revealed a motif perfectly matching this consensus: DSGLPS involving residues 596–601, which lie within the SH3 domain. Interestingly, this sequence appears to be conserved among human and rat Dlg (Sap97). Fig. 1*A* shows a comparison of the putative β-TrCP consensus binding site of rat and human Dlg with those of some known substrates of β-TrCP, such as β-catenin, IκB, ATF4, and Vpu.

Dlg and β-TrCP Proteins Interact in Vitro—This finding prompted us to investigate whether hDlg and β-TrCP proteins could interact. We used a series of GST-fused Dlg proteins, schematically depicted in Fig. 1*B*. These are full-length FL-Dlg and its deletion derivatives: ΔSH3-Dlg, lacking the SH3 domain; NT-Dlg, which contains the N-terminal domain plus PDZ domains 1 and 2; and CT-Dlg, which contains the SH3 and GuK domains. These proteins were tested for binding to *in vitro* translated, radiolabeled β-TrCP protein. As shown in Fig. 2*A*, β-TrCP did not show any significant interaction with Dlg proteins in a standard *in vitro* binding assay. However, because β-TrCP has been shown to bind phosphorylated proteins selectively (1, 33), we repeated the assay incubating the GST fusion proteins with 10 μg of total extract of HaCaT keratinocytes as a source of cellular kinases in the presence of ATP. To monitor the efficiency of phosphate incorporation, one set of reactions was performed in the presence of [γ-^{32}P]ATP, showing that the Dlg proteins were all phosphorylated (not shown). When the pull-down assay was repeated upon incubating the beads with cell extract, both FL-Dlg and CT-Dlg, but not the Dlg mutant proteins lacking the SH3 domain, showed a greatly enhanced interaction with β-TrCP (Fig. 2*A*). To determine whether this interaction was indeed a consequence of Dlg phosphorylation, FL-Dlg was first incubated with cell extract and then either treated or not with λ phosphatase before the binding assay. As can be seen in *first four lanes* of Fig. 2*B*, phosphatase treatment almost completely abolished the binding of β-TrCP to wild-type FL-Dlg. Identical results were also obtained with the CT-Dlg protein (Fig. 2*B*, *last three lanes*). To assess directly whether the interaction of β-TrCP is mediated by the sequence DSGLPS spanning residues 596–601 of Dlg, we constructed two Dlg point mutants, by replacing either serine 597 (FL-M1) or both serines at positions 597 and 601 (FL-M2) with alanine residues. These mutations are depicted in Fig. 1*B*. As shown in Fig. 2*B*, mutation of Ser-597 (FL-M1) greatly reduced the interaction of Dlg with β-TrCP (from 28 to 7% of the input), whereas mutation of both Ser-597 and Ser-601 virtually abolished it. Interestingly, phosphatase treatment further reduced the residual binding of FL-M1 protein, whereas no difference was observed in the background level of interaction between β-TrCP and the M2 mutant protein.

In conclusion, these results show that the Dlg and β-TrCP proteins interact *in vitro*. This is strictly dependent upon the integrity of the DSGLPS consensus sequence in the SH3 domain of Dlg, whereas the N terminus and the first two PDZ domains are not required. Moreover, these experiments also suggest that phosphorylation by as yet unidentified cellular kinase(s) is necessary to allow the purified Dlg protein to interact with β-TrCP and that serines 597 and 601 of Dlg are most likely involved in this process.

Dlg and β-TrCP Proteins Interact in Vivo—To investigate whether the Dlg and β-TrCP proteins interact in cells, Myc-tagged β-TrCP was overexpressed in 293 cells with either HA-tagged FL-Dlg or ΔSH3-Dlg. After 36 h, the cells were treated for 4 h with the proteasome inhibitor CBZ (MG132), then harvested and immunoprecipitated with either anti-HA or anti-Myc antibodies. The immune complexes were subsequently analyzed by Western blot. As expected, when FL-Dlg was transfected, the anti-HA antibody immunoprecipitated two bands of ~100 kDa (Fig. 3*A*), whereas two bands of slightly lower molecular mass were immunoprecipitated from cells transfected with the ΔSH3-Dlg expression plasmid. When β-TrCP and FL-Dlg proteins were coexpressed, the anti-HA antibody also precipitated a protein of about 60 kDa, which corresponds to β-TrCP, because this also comigrates with the β-TrCP protein immunoprecipitated by the anti-Myc antibody

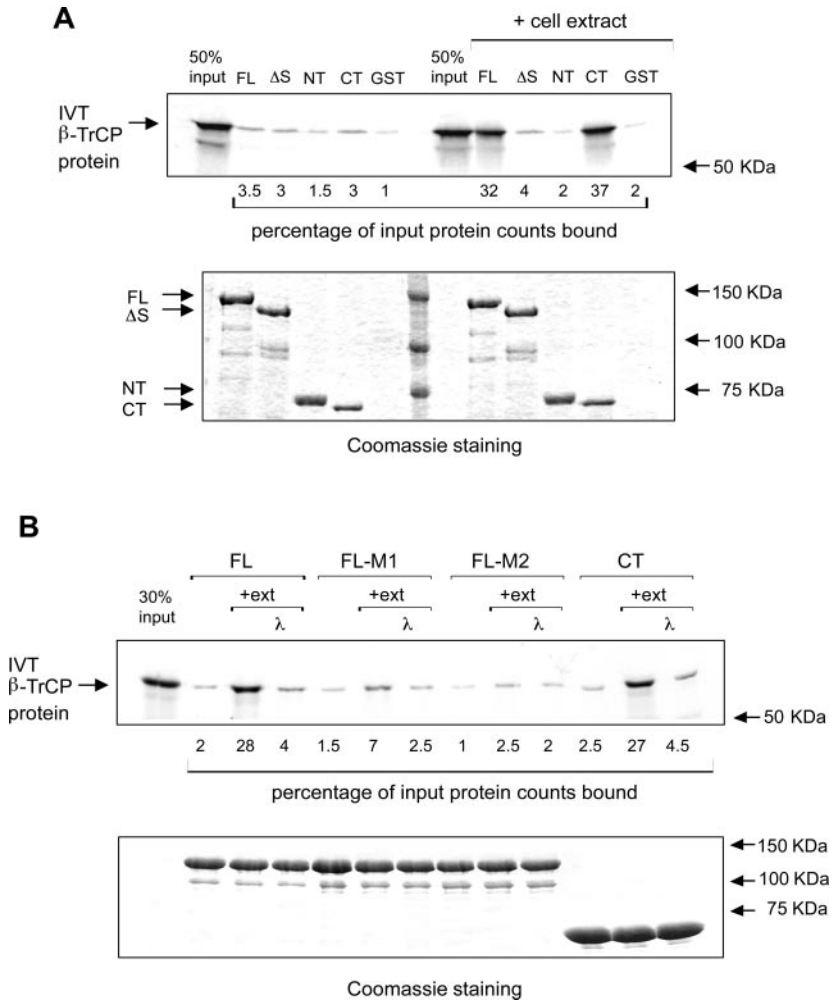


FIG. 2. **Binding to β-TrCP protein *in vitro* is mediated by the DSGLPS domain of Dlg and depends upon its phosphorylation by cellular kinases.** *A*, *upper panel*, radiolabeled *in vitro* translated (*IVT*) β-TrCP protein was incubated with purified GST-Dlg fusion proteins (FL, ΔS, NT, and CT) or with GST alone as a control. The same experiment was also performed in parallel, by preincubating the GST fusion proteins with an extract of HaCaT keratinocytes in the presence of ATP prior to mixing them with *in vitro* translated β-TrCP. After washing, bound proteins were assessed by SDS-PAGE and autoradiography and quantitated by PhosphorImager scanning. The *lower panel* represents the Coomassie-stained gel showing fusion protein levels. *B*, wild-type (GST-FL) and mutant Dlg proteins (M1, single point mutant; M2, double point mutant) were either left untreated or incubated with HaCaT cell extract and ATP as in *A* and either subsequently treated or not with λ phosphatase. Pull-down assays were then performed as in *A* with *in vitro* translated, radiolabeled β-TrCP protein.

(Fig. 3*A*). Moreover, this band was absent from the anti-Myc immune complex when β-TrCP was not transfected. Vice versa, two bands corresponding to the FL-Dlg protein were precipitated by the anti-Myc antibody when β-TrCP and FL-Dlg were cotransfected but not when only FL-Dlg was present. These results clearly show that FL-Dlg associates with β-TrCP protein in 293 cells. In agreement with the results obtained from the *in vitro* binding assay, ΔSH3-Dlg did not coimmunoprecipitate with β-TrCP (Fig. 3*A*), confirming that the SH3 domain of Dlg, containing the DSGLPS binding site, is required for the interaction of the two proteins *in vivo*. The results of the *in vitro* binding assay suggest that β-TrCP interacts specifically with phosphorylated Dlg protein. To confirm this, after immunoprecipitation with anti-Myc the immunocomplex was incubated with λ phosphatase prior to gel loading. As seen in Fig. 3*A* (*third lane*), the migration of the coimmunoprecipitated HA-Dlg protein changed significantly upon phosphatase treatment, demonstrating that the Dlg protein that interacts with β-TrCP is phosphorylated. This result, together with the observation that the SH3 domain is essential for the interaction, is consistent with the data indicating that β-TrCP forms a complex with Dlg *in vitro* when this is phosphorylated within the DSGLPS site.

Endogenous hDlg and β-TrCP Proteins Interact in HaCaT Keratinocytes—To investigate whether the endogenous hDlg and β-TrCP proteins could form a complex in human epithelial cells, immunoprecipitation of untransfected HaCaT cell lysates was performed using polyclonal anti-Dlg antiserum, and the immune complexes were then probed with anti-β-TrCP antibodies on Western blot. The experiment was also performed vice versa by immunoprecipitating the lysates with anti-β-TrCP antibodies and subsequently probing for the presence of hDlg by Western blot. As shown in Fig. 3*B*, several protein bands migrating at about 100–130 kDa and recognized by the anti-Dlg antibody were present in the anti-β-TrCP immunoprecipitate. These bands were identifiable as hDlg protein forms because they were also found in the anti-Dlg immunoprecipitate, and they could not be detected when the cell lysates were precipitated with a nonimmune serum (*PI*). The hDlg-β-TrCP protein interaction was also demonstrated using the anti-Dlg antiserum to coimmunoprecipitate a 60-kDa protein recognized by the anti-β-TrCP antibodies, which comigrates with the β-TrCP protein present in the anti-β-TrCP immunoprecipitate. From these results it can be concluded that the endogenous hDlg and β-TrCP proteins are present in the same complex within HaCaT epithelial cells.

Overexpression of β-TrCP Enhances Ubiquitin Conjugation of Wild-type FL-Dlg but Not of Mutant FL-M2 Dlg Protein in Vivo, whereas F-box-deleted ΔFβ-TrCP Inhibits the Process—The above finding prompted us to investigate the role of β-TrCP in the ubiquitination and degradation of hDlg. Therefore an *in vivo* ubiquitination assay was established, to allow detection of the short lived ubiquitin intermediates of Dlg. HA-tagged FL-Dlg was transiently overexpressed into 293 cells together with HA-tagged ubiquitin, either alone or in the presence of β-TrCP. 24 h post-transfection, the cells were treated with the proteasome inhibitor CBZ to block degradation of ubiquitin-conjugated proteins. The cells were then harvested, and cell lysates were immunoprecipitated with anti-Dlg antibody, in the presence of CBZ and the isopeptidase inhibitor

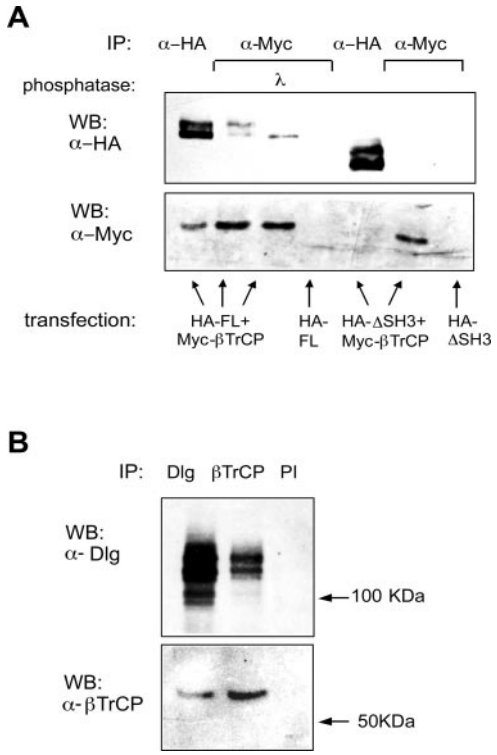


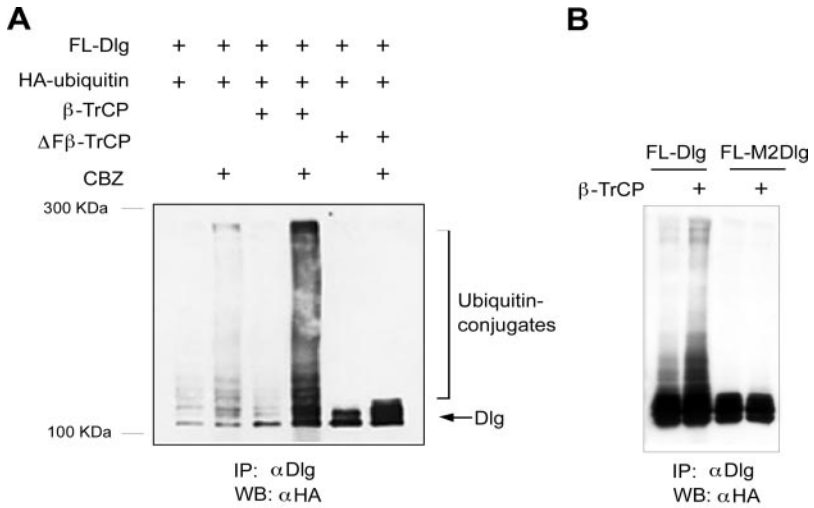
FIG. 3. **β-TrCP associates *in vivo* with phosphorylated Dlg protein, and the interaction is dependent on the presence of the SH3 domain of Dlg.** *A*, the HA-FL Dlg protein and its deletion derivative HA-ΔSH3 were transiently expressed in 293 cells, either alone or together with Myc-tagged β-TrCP protein. After 36 h the cells were treated with CBZ proteasome inhibitor for 4 h and then harvested. Cell lysates were immunoprecipitated (*IP*) with either anti-HA or anti-Myc antibodies and subsequently analyzed for the presence of HA-tagged Dlg and Myc-tagged β-TrCP proteins by Western blot (*WB*). Prior to gel loading, an aliquot of the immunoprecipitate was treated with λ phosphatase. *B*, association between endogenous hDlg and β-TrCP proteins in HaCaT keratinocytes. Untransfected HaCaT keratinocytes were treated for 4 h with CBZ proteasome inhibitor before harvesting. Cell lysates were then immunoprecipitated with either anti-Dlg or anti-βTrCP polyclonal antibodies or with nonimmune serum as a control (*PI*).

N-ethylmaleimide to inhibit destruction of the ubiquitin conjugates. Finally, ubiquitinated Dlg was revealed by anti-HA Western blot. As shown in Fig. 4*A*, proteasome inhibition causes the appearance of a ladder of high molecular mass bands in the anti-Dlg immunoprecipitate, which are barely detectable in the absence of CBZ. Appearance of these forms of Dlg was greatly enhanced upon coexpression of β-TrCP. This suggests that the high molecular mass Dlg forms stabilized by proteasome inhibition correspond to ubiquitin-conjugated Dlg molecules. To confirm this, we coexpressed HA-FL-Dlg and HA-ubiquitin together with a mutant β-TrCP protein lacking the F-box domain, ΔFβ-TrCP. ΔFβ-TrCP is unable to bind to Skp1, the core component of the SCF ubiquitin ligase complex, therefore acting as a dominant negative, inhibiting the ubiquitination of the cellular substrates of β-TrCP (30). As can be seen in the *last two lanes* of Fig. 4*A*, when ΔFβ-TrCP was expressed the high molecular mass forms of Dlg were no longer detected upon proteasome inhibition. This result demonstrates that ubiquitination of Dlg *in vivo* is inhibited by the dominant negative mutant ΔFβ-TrCP protein, suggesting that the endogenous β-TrCP protein plays a role in this process. Identical results were also obtained coexpressing untagged Dlg protein together with HA-tagged ubiquitin, to avoid the use of the HA tag to detect both Dlg and ubiquitin (data not shown). As a further control the double point mutant FL-M2 Dlg, which is unable to interact with β-TrCP, was also tested in this assay. As can be seen in Fig. 4*B*, when expressed in 293 cells this mutant protein is clearly less ubiquitinated compared with wild-type Dlg, and, importantly, the overexpression of β-TrCP does not increase its level of ubiquitin conjugation.

Expression of β-TrCP Causes Proteasome-mediated Degradation of Wild-type FL-Dlg but Not of Mutant FL-M2 Dlg Protein—The above results suggest that β-TrCP is responsible for the ubiquitin conjugation of Dlg protein *in vivo*, therefore it was logical to determine whether it can also cause a reduction in the levels of Dlg protein in cells. To do this, HA-tagged FL-Dlg was transfected into 293 cells together with increasing amounts of β-TrCP. 36 h after transfection, cells were treated with either CBZ proteasome inhibitor or with dimethyl sulfoxide as a control, and the steady-state levels of Dlg were then analyzed by Western blot. Equal transfection efficiency was assessed by analyzing the expression of cotransfected β-galactosidase. As shown in the *left panel* of Fig. 4*C*, expression of β-TrCP caused a marked reduction in the levels of FL-Dlg protein. This effect was dose-dependent and, importantly, could be rescued by treatment with the proteasome inhibitor CBZ. To confirm these results further, the same experiment was done in the presence of ΔFβ-TrCP, and this time no reduction in FL-Dlg protein levels was observed. The experiment was also performed on the FL-M2 mutant Dlg protein, which does not bind β-TrCP, and the result shows that β-TrCP has no effect on its steady-state levels (Fig. 4*C*, *right panel*). The above results lead to the conclusion that β-TrCP is able to down-regulate Dlg protein levels through the proteasome pathway and that the DSGLPS binding site within the SH3 domain of Dlg is necessary for this process.

β-TrCP Decreases the Stability of Wild-type FL-Dlg but Does Not Affect the FL-M2 Dlg Mutant Protein—We then wanted to verify that the observed decrease in the steady-state levels of Dlg protein upon β-TrCP overexpression was caused by its augmented turnover. Therefore, experiments were performed to measure the stability of FL-Dlg protein, either in the absence or presence of coexpressed β-TrCP and ΔFβ-TrCP proteins. 24 h post-transfection 293 cells were treated with 50 μM cycloheximide to block protein synthesis and either harvested immediately (time zero) or after 2, 4, or 6 h. The residual FL-Dlg protein was subsequently measured by Western blot and densitometric scanning. Three independent experiments were performed, and mean values are shown in Fig. 5*A*. FL-Dlg is relatively unstable, with a half-life of ~4.5 h, and coexpression of β-TrCP significantly accelerates its turnover, shortening the half-life down to 1.5 h. In contrast, upon coexpression of the transdominant negative ΔFβ-TrCP protein, the stability of FL-Dlg appears increased, with an estimated half-life of about 8 h. To verify that the effects of β-TrCP on Dlg protein stability were specific, the same experiment was repeated using the FL-M2 Dlg mutant protein. Interestingly, as can be seen in Fig. 5*B*, FL-M2 Dlg is much more stable compared with the wild-type FL-Dlg protein, with a half-life of ~8 h. In addition, overexpression of β-TrCP does not significantly alter the turnover of this mutant protein.

Endogenous β-TrCP Is Essential for Proteasome-mediated Degradation of hDlg in Epithelial Cells—We have demonstrated that the endogenous hDlg and β-TrCP proteins form a complex in human keratinocytes, and the transdominant negative effect of ΔFβ-TrCP on ubiquitination and turnover of Dlg protein would also suggest that the endogenous β-TrCP plays a role in these processes. To provide direct genetic evidence that β-TrCP is responsible for regulating hDlg stability, we ablated the expression of β-TrCP in human 293 cells by RNA interference (34). As expected from previous studies (32, 35), transfec-



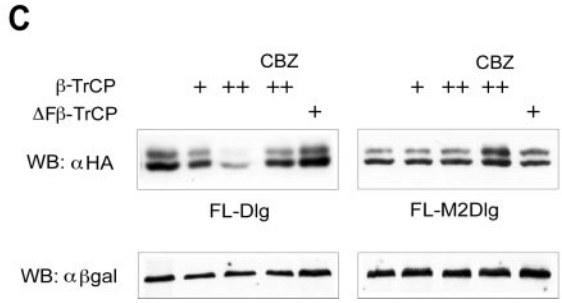


FIG. 4. **Overexpression of β-TrCP enhances ubiquitin-mediated degradation of wild-type Dlg but not of mutant FL-M2 Dlg, whereas F-box-deleted ΔFβ-TrCP inhibits this process.** *A*, β-TrCP enhances ubiquitin conjugation of wild-type Dlg, whereas F-box-deleted ΔFβ-TrCP inhibits it. HA-FL-Dlg was transfected into 293 cells together with a HA-tagged ubiquitin expression vector and with either wild-type β-TrCP, F-box-deleted mutant ΔFβ-TrCP, or empty expression plasmid. 24 h post-transfection, the cells were treated with the CBZ proteasome inhibitor for 4 h and then harvested. Cell lysates were immunoprecipitated with anti-Dlg antibody, and the ubiquitin-conjugated forms of Dlg protein were revealed by anti-HA Western blot (*WB*). *B*, the FL-M2 Dlg mutant protein is not ubiquitinated by β-TrCP. Wild-type FL-Dlg or mutant FL-M2 Dlg was transfected into 293 cells together with a HA-tagged ubiquitin expression vector and with wild-type β-TrCP where indicated. 24 h post-transfection, the cells were treated with CBZ, harvested, and Dlg proteins analyzed for ubiquitin conjugation as in *A*. *C*, *upper panels*, β-TrCP overexpression enhances proteasome-mediated degradation of wild-type Dlg, but not of the FL-M2 Dlg mutant protein. 293 cells were transiently transfected with 3 μg of HA-tagged FL-Dlg (*left panel*) and M2-Dlg (*right panel*) expression plasmids, together with increasing amounts (2.5 and 5 μg) of wild-type β-TrCP expression plasmid, or with 5 μg of mutant ΔFβ-TrCP expression plasmid, or with empty vector as a control. At 36 h post-transfection cells were either treated with the proteasome inhibitor CBZ or with dimethyl sulfoxide as a control. The remaining HA-Dlg proteins were then assessed by Western blot with anti-HA antibody. β-Galactosidase expression vector (0.2 μg) was included in each experiment to monitor for transfection efficiency, as shown by the anti-β-galactosidase (*αβgal*) Western blot in the *lower panels*.

tion of β-TrCP siRNA into 293 cells efficiently abolished the expression of β-TrCP but not of the control gene *GAPDH*, as determined by RT-PCR analysis shown in Fig. 6*A*. Most importantly, β-TrCP suppression gave rise to a stabilization of the endogenous hDlg protein in the same cells. As shown in Fig. 6*B*, in cells transfected with β-TrCP siRNA hDlg protein levels increased to those seen upon treatment with the proteasome inhibitor CBZ. We can conclude from this result that β-TrCP directly contributes to the regulation of hDlg protein turnover in epithelial cells.

β-TrCP Facilitates Degradation of the Membrane-cytoplasmic hDlg Pool without Affecting the Nuclear hDlg Protein—Because hDlg protein has been reported to localize in different cellular compartments (19), we were interested in determining whether β-TrCP preferentially targets a specific pool of hDlg. To do this, immunofluorescence assays were performed (Fig. 7). U2OS cells were transfected with Myc epitope-tagged β-TrCP, and 36 h later the cells were fixed and stained with anti-Myc antibody to identify the transfected cells (*A* and *D*), whereas the endogenous hDlg protein was detected with the anti-Dlg antibody (*B* and E). It can be seen that the overexpressed β-TrCP protein is localized both within the nucleus and the cytoplasm of the transfected cells, in agreement with previous reports (4). The endogenous hDlg protein is clearly detectable in the cytoplasm and at the membrane of the untransfected cells, whereas in the cells that overexpress β-TrCP very little or no hDlg is found at either membrane or cytoplasmic sites (*C* and F). In contrast, the nuclear pool of hDlg does not appear to be affected by overexpression of β-TrCP, although this protein is expressed abundantly in the nucleus. These results suggest that β-TrCP preferentially targets the hDlg protein localized at membrane and cytoplasmic sites, but not nuclear hDlg.

DISCUSSION

Neoplastic transformation is a multistep process, during which a precancerous cell progressively accumulates phenotyp-

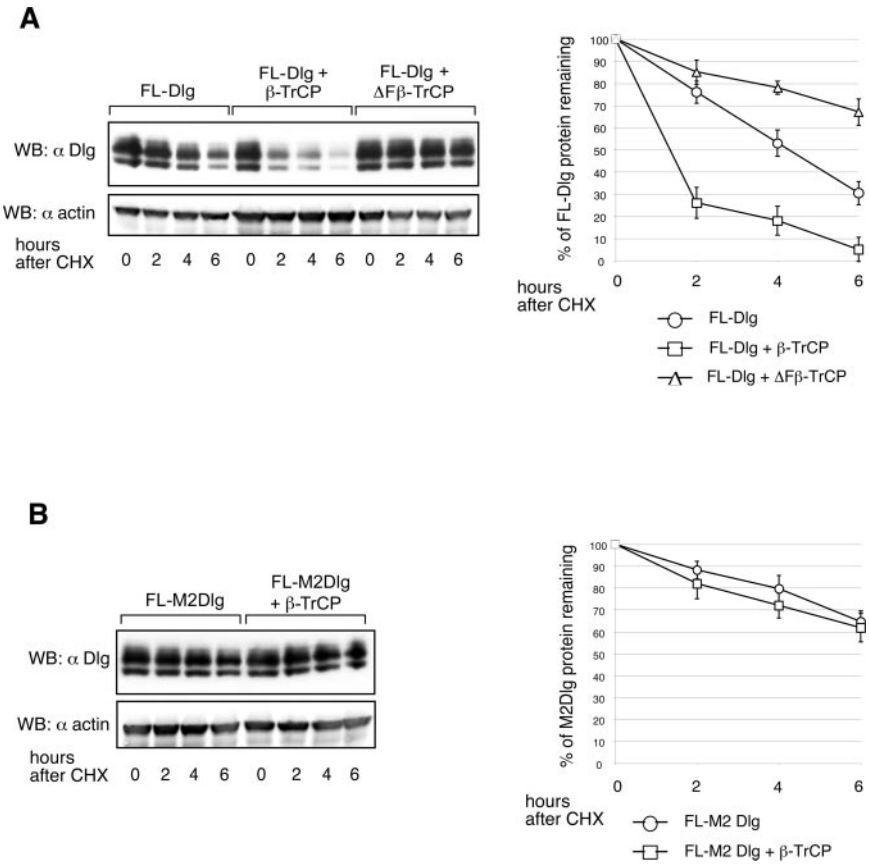


FIG. 5. **β-TrCP decreases the stability of wild-type Dlg protein but not of its mutant FL-M2.** Shown is an analysis of the turnover rate of HA-tagged wild-type FL-Dlg (*A*) and mutant FL-M2 Dlg (*B*) proteins in 293 cells. 3 μg of each Dlg expression construct was cotransfected with either empty pCDNA vector (*circles*) or with 2.5 μg of wild-type β-TrCP (*squares*) or dominant negative mutant ΔFβ-TrCP (*triangles*) expression vectors. 24 h post-transfection the cells were treated with 50 μg/ml cycloheximide (*CHX*) and harvested after the indicated times. The remaining Dlg protein was then assessed by Western blot (*WB*) with anti-HA antibody and densitometric scanning. The amount of protein at 0 h was defined as 100%, and results obtained from three independent experiments are shown in the accompanying *graphs*. Western blots were also probed with anti-actin antibody to ensure equal protein loading at different time points.

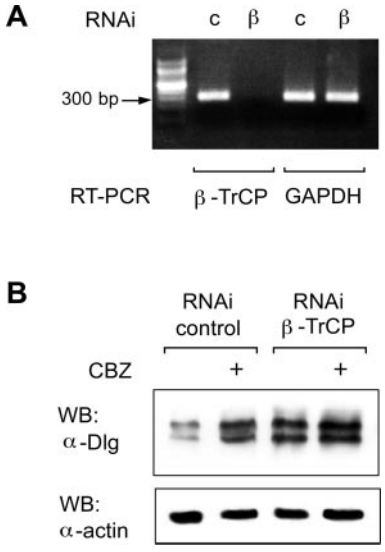


FIG. 6. **Depletion of endogenous β-TrCP by RNA interference inhibits degradation of hDlg in 293 cells.** *A*, RT-PCR analysis showing the specific depletion of β-TrCP mRNA in 293 cells transfected with either β-TrCP siRNA (*β*) or control scrambled siRNA (*c*). 72 h post-transfection total RNA was extracted from the cells and subjected to RT-PCR using either specific primers for amplifying β-TrCP (309 bp) or GAPDH (307 bp) cDNAs as a control. *B*, Western blot (*WB*) showing the levels of endogenous hDlg protein in 293 cells transfected with either β-TrCP siRNA or control scrambled siRNA. 72 h after transfection, cells were either treated with the proteasome inhibitor CBZ (+) or with dimethyl sulfoxide as control for 4 h before harvesting. The *lower panel* shows probing of the Western blot with anti-actin antibody to confirm equal protein loading.

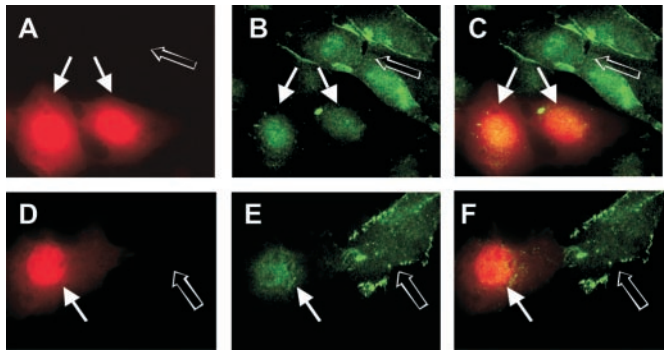


FIG. 7. **β-TrCP leads to degradation of cytoplasmic and membrane-associated hDlg protein.** U2OS cells were transfected with an expression plasmid encoding Myc epitope-tagged β-TrCP, and 36 h later the cells were fixed and stained with anti-Myc antibody to identify the transfected cells (*A* and *D*) and with anti-Dlg antibody to detect the endogenous hDlg protein (*B* and *E*). Overlaid photographs are shown (*C* and *F*). The *filled arrows* mark the transfected cells, and the *open arrows* indicate groups of untransfected cells. Note that untransfected cells show abundant hDlg protein levels both within the membrane and the cytoplasm, whereas very little or no hDlg is found in the membrane and cytoplasm of the cells overexpressing β-TrCP.

ical changes. In the course of epithelial tumor progression, crucial steps toward the acquisition of a malignant and invasive phenotype include deregulated cell proliferation, defective cell-cell adhesion, and loss of apicobasal polarity. It is not surprising that several proteins involved in cell contact and adhesion are either the product of proto-oncogenes, such as β-catenin (36), or tumor suppressor genes, such as APC (37). Recent contributions to the understanding of how the orchestration of tissue architecture and the control of cell proliferation are connected have come from studies of *Drosophila* tumor suppressor genes, which act in concert to regulate both cell growth and polarity (7, 38). Their products are the membrane-associated proteins DLG, Lgl, and Scrib, which rely on each other for the correct localization and formation of epithelial junctions. Mutations in any of these genes cause similar phenotypes: aberrant cell shape, altered apicobasal polarity, and

overproliferation of epithelial cells. There is growing evidence that these roles are conserved in mammalian cells, at least for the human homologs of DLG (22, 23) and Scrib (39). In addition, disruption of the complex between hDlg and the APC tumor suppressor has been reported to impair APC-mediated growth inhibition (21). Hence its roles in maintenance of epithelial cytoarchitecture and control of cell proliferation make hDlg a candidate tumor suppressor: its down-regulation has been clearly shown to be associated with highly malignant phenotypes in cervical neoplasia (23).

Similarly to what has been reported for β-catenin (1, 4, 40), our previous studies on the human hDlg protein have highlighted a complex regulation that involves phosphorylation and ubiquitin-mediated degradation. In differentiated epithelial cells, localization of hDlg to the basolateral membrane junctions leads to its stabilization, which appears to be promoted by cell-cell contact. When the cells do not engage stable junctions, hyperphosphorylated hDlg is degraded rapidly (22). It is also clear that hDlg degradation is enhanced in highly transformed cells showing an invasive and metastatic phenotype, which fail to form stable cell-cell junctions and have lost apicobasal polarity (22). The observation that hyperphosphorylation of hDlg is a signal for its ubiquitin-mediated degradation makes it a candidate target for an SCF ubiquitin ligase (33). Interestingly, analysis of hDlg sequence revealed a six-residue motif conserved among human, rat, and mouse proteins. This motif, DSGLPS, is homologous to a sequence found in several targets of the $SCF^{\beta\text{-}TrCP}$ ligase complex including β-catenin, IκB, Vpu, and ATF4. In all of these cases the F-box protein β-TrCP, which is the substrate-interacting subunit of the ligase, has been demonstrated to bind selectively to its consensus site when this is phosphorylated on serine residues (1, 30, 41, 42), thus inducing the ubiquitin-mediated degradation of the phosphorylated target protein.

We decided to investigate whether Dlg was a substrate for β-TrCP. We demonstrated that, although purified Dlg protein is unable to interact with β-TrCP *in vitro*, it acquires this ability upon incubation with epithelial cell extract. We then proved that the interaction with β-TrCP requires the phosphorylation of Dlg. Moreover, mutation of the two serines at positions 597 and 601 within the DSGLPS sequence in the SH3 domain of Dlg reduced the binding to β-TrCP to background levels, demonstrating that the DSGLPS sequence is indeed the β-TrCP recognition site. Complex formation between Dlg and β-TrCP proteins *in vivo* was then confirmed by their coexpression in epithelial cells, and the SH3 domain of Dlg, which contains the binding site, was again found to be required for complex formation. Interaction between the endogenous hDlg and β-TrCP proteins was also detected in untransfected HaCaT keratinocytes, where both proteins are present at physiological levels in their normal cellular location. Moreover, we also demonstrated that the Dlg protein that interacts with β-TrCP is hyperphosphorylated, further supporting the results from the *in vitro* interaction studies. These results are consistent with the hypothesis that, similarly to what described for β-catenin, β-TrCP binds to its phosphorylated recognition site on hDlg, thereby linking it to the core subunit of the SCF ubiquitin ligase complex, and indeed it is clear from our experiments that complex formation with β-TrCP leads to ubiquitination of hDlg protein *in vivo*. Upon expression of β-TrCP, the amount of ubiquitin-conjugated intermediates of wild-type Dlg protein was increased consistently. This was, however, not observed for the mutant FL-M2 Dlg protein, which does not interact with β-TrCP because of disruption of the DSGLPS binding motif. Interestingly, expression of a mutant β-TrCP protein deleted for its F-box domain, ΔFβ-TrCP, inhibited the ubiquitination of Dlg. It has been reported that the F-box domain is necessary for anchoring β-TrCP to Skp1, the core component of the ligase complex (30). ΔFβ-TrCP protein is therefore unable to bind to Skp1; however, it can still interact with its substrates through the C-terminal WD repeats (30), and thereby ΔFβ-TrCP acts as a dominant negative mutant by sequestering the substrates of wild-type β-TrCP and preventing their ubiquitination and proteasome degradation (2, 4, 30, 41). Therefore, because ΔFβ-TrCP is also able to inhibit the ubiquitination of Dlg *in vivo*, this further supports a role for endogenous β-TrCP in this process. This was directly confirmed by the demonstration that inhibition of endogenous β-TrCP expression by RNA interference resulted in significant increase of hDlg protein levels in epithelial cells, similar to what was observed upon proteasome inhibition. On the other hand, overexpression of β-TrCP caused a dose-dependent reduction in the steady-state levels of Dlg, which could be rescued by treating the cells with a proteasome inhibitor. Accordingly, we also observed an increased turnover rate of wild-type FL-Dlg protein *in vivo* upon overexpression of β-TrCP, whereas expression of the dominant negative ΔFβ-TrCP resulted in Dlg stabilization. Notably, the FL-M2 Dlg mutant protein appeared to be significantly more stable compared with wild-type Dlg, and moreover its turnover rate was not affected by the expression of β-TrCP, consistent with the fact that β-TrCP fails to ubiquitinate this mutant.

It has to be noted, however, that in our assays the FL-M2 Dlg mutant protein also appears to be ubiquitinated, although to a much lower extent compared with wild-type Dlg (Fig. 4*B*), and its steady-state levels also increase upon proteasome inhibition (Fig. 4*C*). These observations are not surprising because we have found previously that some Dlg deletion mutants that cannot interact with β-TrCP, such as ΔSH3-Dlg, NT-Dlg, and PDZ2-Dlg, are nonetheless degraded through the proteasome pathway (27, 29). It is therefore likely that in addition to β-TrCP, other ubiquitin ligase(s) also target Dlg, binding within the N-terminal half of the protein. It has been reported that other substrates of the $SCF^{\beta\text{-}TrCP}$ ligase, such as the nuclear factor-κB precursor p105 and β-catenin, are also targeted by different ubiquitin ligases under specific conditions, and these interact with mutant p105 and β-catenin proteins that fail to bind β-TrCP (43–45). It could be speculated that specific ligases are responsible for regulating the stability of separate pools of hDlg protein, which play distinct functions within the cell. Interestingly, in our immunofluorescence analysis we have observed that β-TrCP can efficiently promote the degradation of the hDlg protein localized at the membrane and in the cytoplasm of U2OS cells, whereas the stability of the nuclear pool of hDlg is not affected. Although it has been reported that β-TrCP/E3RS protein is retained mainly in the nucleus, by virtue of its interaction with a hnRNP-U pseudosubstrate that stabilizes it, this interaction is however displaced by high affinity binding of β-TrCP to its targets (46), and indeed true β-TrCP substrates such as IκB and β-catenin appear to be ubiquitinated and degraded in the cytoplasm (47, 48). A nuclear substrate, the transcription factor ATF4, has also been described for β-TrCP, although it is not clear whether ATF4 is then ubiquitinated in the nucleus or in the cytoplasm by the $SCF^{\beta\text{-}TrCP}$ ligase complex (41). Although both hDlg and β-TrCP proteins are present within the nucleus, their interaction might not take place in this particular location, possibly because phosphorylation of the binding site on hDlg occurs only at membrane-proximal sites, as a consequence of extracellular stimuli. Interestingly, normal squamous epithelial basal cells, which are actively proliferating, show extremely low levels of hDlg protein at their membrane sites compared with the differentiated keratinocytes of the upper epithelial layers (23).

Moreover, loss of hDlg at sites of intercellular contact is thought to represent a crucial step in the progression of cervical cancer, being associated with high grade neoplasias and invasive carcinomas (23), and malignant progression of prostate cancers also appears to correlate with increased degradation of Dlg protein.[2] It has been reported recently that β-TrCP is overexpressed in primary colon cancers (49), and it would be extremely interesting to evaluate whether hDlg degradation is deregulated also in these malignancies.

Based on the above information it is tempting to propose the following model. hDlg performs both structural and signaling functions at the membrane-cytoplasm interface of epithelial cells, controlling both cell polarity and proliferation, *e.g.* assisting the growth inhibitory activity of APC (21). Once the cell is induced to proliferate, however, these functions need to be inhibited. One possible mechanism could involve phosphorylation of hDlg on the DSGLPS motif as a result of proliferative stimuli, causing β-TrCP binding and subsequent degradation of hDlg. Although β-TrCP protein levels are very low in normal cells and tissues (49, 50) it has been shown recently that Wnt signaling, which stimulates cell proliferation by inhibiting β-TrCP-mediated degradation of β-catenin, also induces the expression of β-TrCP (49). This would be expected to accelerate the turnover of hDlg. Mitogenic stimuli have also been shown to induce the expression of β-TrCP2/HOS (51), the closest homolog of β-TrCP, which is mainly localized in the cytoplasm (46) and which could also play a role in degrading hDlg, similarly to what has been reported for IκB (52, 53). Indeed our data cannot exclude, at present, the intriguing possibility that β-TrCP and HOS play redundant roles in degradation of endogenous hDlg because the ΔFβ-TrCP dominant negative can be expected to interfere with both proteins, which recognize the same motif on their substrates. Moreover, comparison of the gene sequences of β-TrCP and HOS would also predict that the siRNA-mediated depletion of β-TrCP could also block the expression of HOS because of the homology in this portion of their mRNAs. Therefore, the increase of hDlg protein levels observed in these experiments could be partly the result of interference with the activity and the expression of HOS. However, we are presently lacking any direct evidence that implicates HOS in the regulation of hDlg, and additional experiments are required to address this issue further. Clearly other pieces are still missing from the puzzle, including the identification of the protein kinase responsible for β-TrCP binding. hDlg has been reported to interact with $p56^{lck}$ tyrosine kinase in human T lymphocytes (54), and it has also been found to be phosphorylated at mitosis in HeLa cells, possibly by a PDZ-binding kinase (55). An appealing candidate for phosphorylating hDlg on its β-TrCP binding site would be the GSK3β Ser/Thr kinase, whose consensus site overlaps with the DSGLPS β-TrCP binding site within the SH3 domain of hDlg and which phosphorylates a very similar site on β-catenin, thus leading to β-TrCP binding (1, 6). Preliminary results, however, indicate that ubiquitination of hDlg is independent of the presence and activity of GSK3β and moreover that phosphorylation of hDlg by GSK3β does not increase its affinity for β-TrCP.[3] Experiments are now in progress to evaluate the contribution of other kinases to this process.

In conclusion, although the precise roles of hDlg in the cellular pathways regulating cell growth and polarity are still far from being elucidated, the number of reports describing new cellular partners for this protein is rapidly growing. After having determined that hDlg stability is dynamically regulated by the SCF $^{\beta\text{-TrCP}}$ ubiquitin ligase complex, it is now imperative to unravel the cellular pathways responsible for inducing its degradation. Changes in hDlg expression and regulation are associated with cell transformation and therefore this can be expected to remain a very interesting topic in the forthcoming years.

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[3] F. Mantovani, personal observations.

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