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Regulation of DNA topoisomerase IIα stability by the ECV ubiquitin ligase complex

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ABSTRACT

In this study, we attempted to elucidate the E3 ubiquitin ligase for topo IIα. When cullins and VHL were ectopically expressed in HT1080 and HEK293T cells, topo IIα was degraded most prominently in cullin 2- and VHL-expressing cells. Cullin 2 and the β domain (aa 114–123) of VHL, a subunit of the ECV (Elongin B/C-cullin 2-VHL protein) complex, specifically interact with the ATPase domain of topo IIα. We identified that topo IIα associated with endogenous Elongin C. In HT1080 cells co-transfected with deletion mutants of topo IIα GRDD (glucose-regulated destruction domain) and VHL, topo IIα was degraded by VHL expression. These results demonstrate that ECV acts as E3 ubiquitin ligase targeting GRDD-independent topo IIα to the ubiquitin–proteasome pathway.

Introduction

DNA topoisomerase IIα plays an essential role in modulating the topological structure of DNA by breakage–reunion of double-stranded DNA [1]. Topo II is the molecular target for antitumor drugs such as etoposide and doxorubicin [2,3]. The topo II-directed drugs stabilize the cleavable complex, i.e., the covalent DNA topo II intermediates [4]. Topo IIα expression is decreased and the topo II-cleavable complex is reduced in stress conditions such as glucose deprivation, hypoxia, low pH, and nutrient deprivation [5,6].

High levels of topo IIα expression have been observed in a wide variety of tumors [7]. However, malignant cells within solid tumors are often surrounded by the stress conditions mentioned above. These physiological conditions in culture can down-regulate the expression of topo IIα, resulting in growth arrest and delay at the G_1 phase of the cell cycle [8].

Recent studies have established that inhibition of proteasome attenuates stress-induced topo IIα degradation [9]. Topo IIα is degraded by UPS. In general, UPS-mediated proteolysis is targeted by ubiquitination of the substrate protein [10]. Consistent with this, topo IIα is polyubiquitinated in a cell-free system with extract of cancer cells [11]. Thus, UPS appears responsible for topo IIα degradation, but the regulatory mechanisms leading to this degradation are largely unknown.

The sequential action of three enzymes is essential to the UPS-mediated proteolysis of target protein. First, ubiquitin is activated by ATP to form thiol ester intermediate with E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin ligase catalyzes the transfer of ubiquitin to the substrate. Since E3 ubiquitin ligase binds to substrate proteins either directly or indirectly prior to conjugation, E3 ubiquitin ligase is a key player in determining the specificity of target protein. Three types of E3 ubiquitin ligase, including HECT (Homologous to the E6-AP Carboxyl Terminus) domain proteins [12], the anaphase-promoting complex [13], and SCF (Skp1-cullin 1-F-box protein) complex [14,15] have been characterized well. SCF and ECS (Elongin B/C-cullin 2-SOCS-box protein) complexes are representative cullin-based ubiquitin ligases. ECV (Elongin B/C-cullin 2-VHL protein) is also one of the families of ubiquitin ligase. The VHL protein functions as a substrate-recognition subunit in the ECV complex. This ECV complex targets hypoxia-inducible factor α (HIFα) for ubiquitination and proteolysis [16].

In this study, we identify the topo IIα-specific E3 ubiquitin ligase. We show that VHL acts as a substrate-recognition subunit to recruit topo IIα into ECV ubiquitin ligase and mediate degradation of topo IIα by UPS.

Materials and methods

Cell culture and drug treatments. HT1080 cells were maintained in RPMI 1640 medium (Hyclone, Logan, UT, USA), and HEK293T cells were maintained in Dulbecco's modified Eagle's medium

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(Hyclone), each supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Glucose deprivation was achieved by substituting glucose-free RPMI 1640 (Gibco–BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma). MG132 was purchased from Calbiochem, Inc. (La Jolla, CA, USA), dissolved in Me_2SO, and added to culture medium so that the final concentration of Me_2SO was <0.5%.

Expression plasmids and transfection. The expression plasmids for full-length topo IIα and its deletion mutants were described previously [17]. Full-length human cullin cDNAs were generated by PCR from a cDNA library of HT-29 cells. The PCR fragments were cloned into the BamHI/XhoI site of pcMYC expression vector derived from

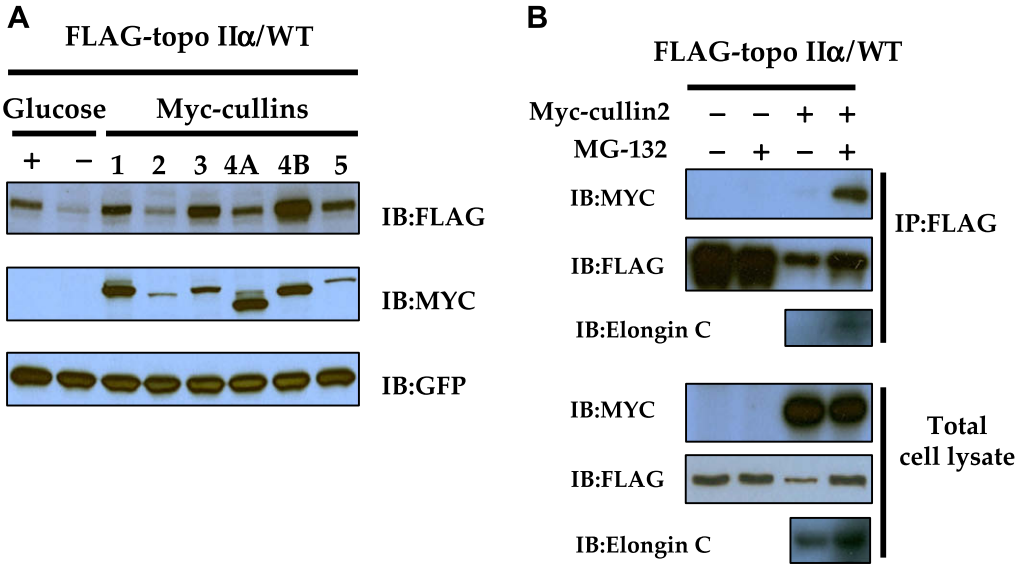

Fig. 1. Ectopic expression of cullin 2 induces degradation of topo IIα. (A) HT1080 cells were co-transfected with expression plasmids encoding full-length FLAG-topo IIα together with Myc-cullin 1, 2, 3, 4A, 4B, or 5. The cells were cultured under normal (+) or glucose-free conditions (−) for 24 h, and whole cell lysates were examined by immunoblot analysis using anti-FLAG, anti-Myc, and anti-GFP antibodies. The GFP expression levels were determined as a marker of transfection efficiency and loading control. (B) HEK293T cells were co-transfected with FLAG-tagged topo IIα together with Myc-tagged cullin 2. After transfection, the cells were cultured for 48 h and treated with 1 μM MG132 during the last 20 h.

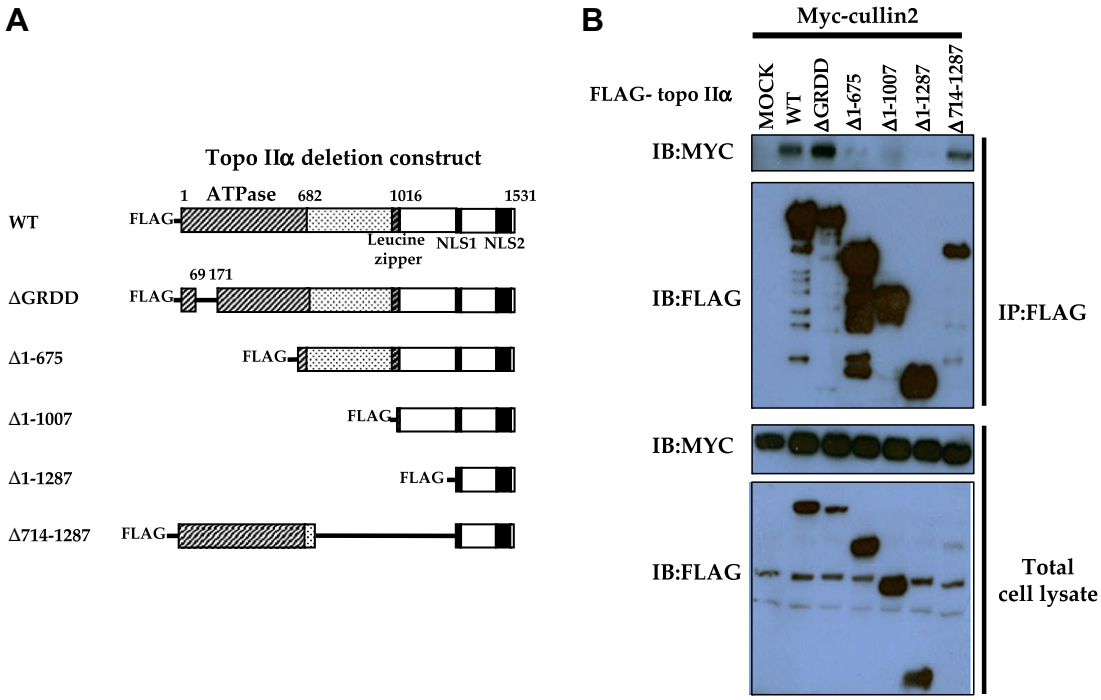

Fig. 2. The ATPase domain of topo IIα interacts with cullin 2. (A) Schematic representation of WT and deletion mutants of FLAG-tagged topo IIα. (B) HEK293T cells were co-transfected with the indicated plasmids of FLAG-tagged topo IIα and Myc-tagged cullin 2, and the cells were cultured and treated with MG132 as described in Fig. 1B. Equal amounts of total cell extracts were immunoprecipitated with ant-FLAG antibody, followed by immunoblot with anti-Myc, anti-Elongin C, and anti-FLAG antibodies.

pcDNA3 (Invitrogen, Carlsbad, CA, USA). A PCR fragment of VHL was cloned into the pcHA expression vector at the BamHI/XhoI site. VHL mutations were introduced by site-directed mutagenesis using the QuikChange XL mutagenesis kit (Stratagene, Cedar Creek, TX, USA). Transfections were performed using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) or Lipofectamine reagent (Invitrogen) for HT1080 or HEK293T cells, respectively, according to the manufacturer's protocol. pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) was co-transfected as a marker of transfection efficiency.

Immunoprecipitation and immunoblot analysis. Cells were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF, 17 μg/ml aprotinin). Cell lysates were then centrifuged and supernatants were incubated with agarose-conjugated anti-FLAG M2 antibody (Sigma). Immunocomplexes were washed five times in lysis buffer and then dissolved in 2× SDS buffer. After boiling for 5 min, the complexes were evaluated by immunoblot analysis. Cell lysates for immunoblotting were prepared with 1× SDS sample buffer (10% glycerol, 5% 2-mercaptoetanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.8) as described [2]. Protein content of the samples was determined using a Bio-Rad protein assay reagent. Equal amounts of samples were resolved by SDS–PAGE and electroblotted onto PVDF membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA). Membranes were probed with mouse monoclonal anti-human topo IIα (clone KF4, Sigma Genosys), polyclonal anti-GFP (Clontech, Palo Alto, CA, USA), anti-FLAG (Sigma), anti-HA (clone Y-11, Santa Cruz Biotechnology), anti-c-Myc (clone A-14, Santa Cruz Biotechnology), or anti-Elongin C (clone R-20, Santa Cruz Biotechnology). The immunoreactive signals were detected using ECL reagent (iNtRON Biotechnology, Seoul, Korea).

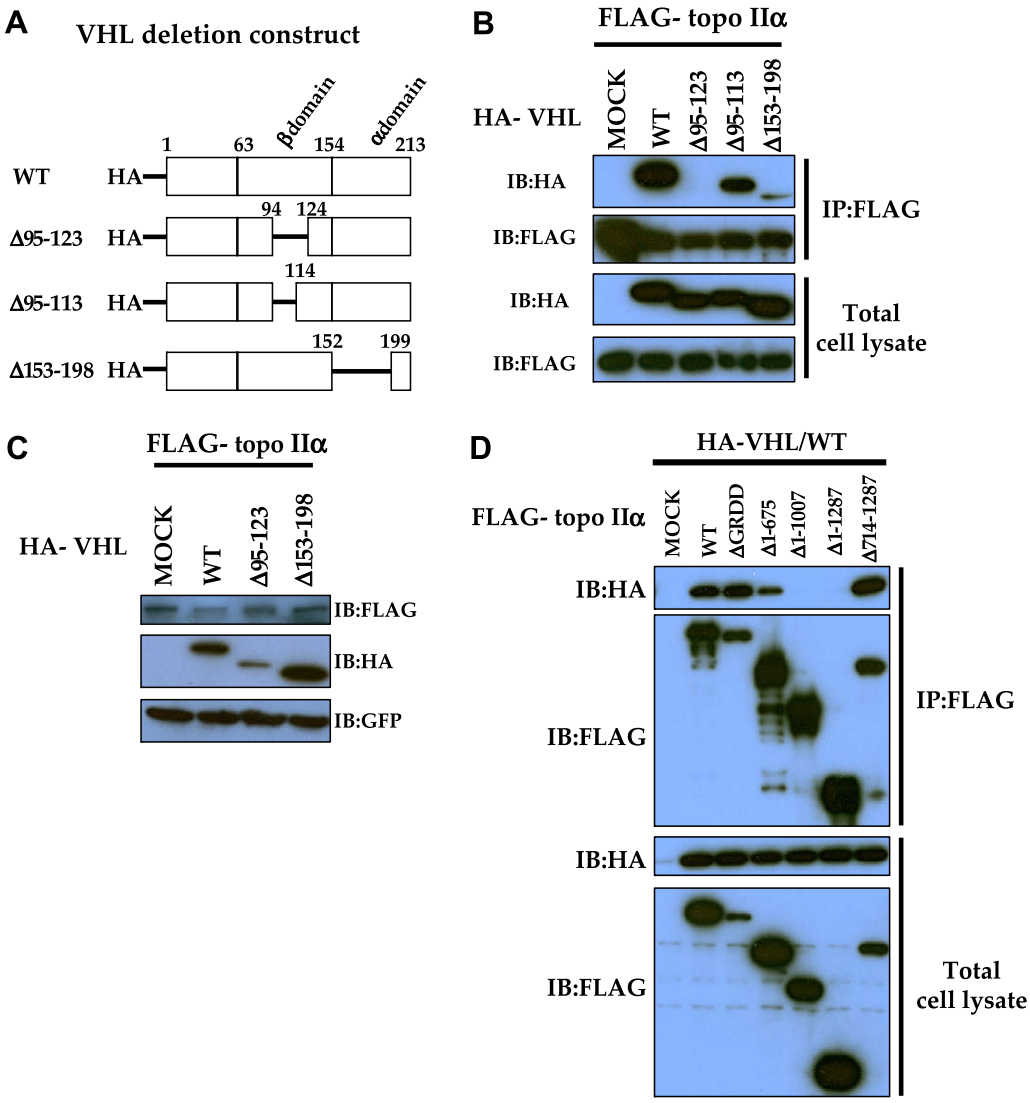

Fig. 3. Identification of the binding motif in VHL that associates with topo IIα. (A) Schematic representation of WT and deletion mutants of HA-tagged VHL. (B) HEK293T cells were co-transfected with the indicated plasmids of HA-tagged VHL and FLAG-tagged topo IIα, and the cells were cultured and treated with MG132 as described in Fig. 1B. Lysates of HEK29T cells were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting immunoprecipitates were probed with anti-HA and anti-FLAG antibodies. (C) HEK293T cells were co-transfected with the indicated expression plasmids of HA-tagged VHL together with FLAG-tagged topo IIα. (D) HEK293T cells were co-transfected with the indicated plasmids of FLAG-tagged topo IIα and HA-tagged VHL, and the cells were cultured and treated with MG132 as described in Fig. 1B. The immunoprecipitates with anti-FLAG antibody were subjected to immunoblot analysis with anti-HA and anti-FLAG antibodies. The cells were cultured and the whole cell lysates were examined by immunoblot analysis using anti-FLAG and anti-HA antibodies. As a loading control, GFP expression levels also were determined with anti-GFP antibody.

Results

Ectopic expression of cullin 2 induces degradation of topo IIα through interaction with the ATPase domain of topo IIα

To determine whether cullin regulates the stability of topo IIα *in vivo*, we examined the expression levels of FLAG-tagged topo IIα in HT1080 cells co-expressing Myc-tagged cullin 1, 2, 3, 4A, 4B, or 5. The co-expression of cullin 2 specifically induced the degradation of wild-type topo IIα (Fig. 1A). To investigate the interaction between topo IIα and cullin 2, we co-transfected expression vectors encoding FLAG-tagged topo IIα and Myc-tagged cullin 2 into HEK293T cells. Co-immunoprecipitation analysis revealed that topo IIα associated with cullin 2 and endogenous Elongin C (Fig. 1B). Cullin 2 has been shown to interact with topo IIα and to prompt its degradation when these proteins are overexpressed. Next, we mapped the binding site of topo IIα on cullin 2. Deleting the ATPase domain of topo IIα (except ΔGRDD) reduced cullin 2 binding in co-transfected HEK293T cells (Fig. 2B). These results suggest that the ATPase domain of topo IIα is required for binding to cullin 2.

The β domain of VHL is required for interaction with the ATPase domain and degradation of topo IIα

We showed that topo IIα associates specifically with cullin 2 and Elongin C (Fig. 1B). These results suggest that VHL functions as receptor subunits for topo IIα in ECS ubiquitin ligase complexes. To test the interaction between topo IIα and VHL *in vivo*, topo IIα and VHL deletions were co-expressed in HEK293T cells. Topo IIα could bind to wild-type VHL but not to VHL lacking amino acids 95–123 (Δ95–123) within the β domain (Fig. 3B). VHL lacking amino acids 153–198 (Δ153–198) weakly interacted with topo IIα. To examine whether VHL stimulated degradation of topo IIα, we co-transfected HEK293T cells with expression vectors encoding FLAG-tagged topo IIα and deletion mutants of HA-tagged VHL. VHL-induced degradation of topo IIα was monitored as described in Fig. 3C. Topo IIα was degraded by overexpression of wild-type VHL. To determine which regions of topo IIα were required for interaction with VHL, we constructed expression plasmids of topo IIα deletion mutants and of VHL. We then co-transfected them into HEK293T cells. The results obtained from co-immunoprecipitation analysis correspond with that of cullin 2 (Fig. 3D). These results suggest that topo IIα binds to E3 multiprotein complexes via VHL by assembly with Elongin B/C and cullin 2, which thus mediate the polyubiquitination and degradation of topo IIα.

Degradation of topo IIα induced by VHL independent of GRDD

As shown in Figs. 2B and 3D, ΔGRDD topo IIα associated with both cullin 2 and VHL. To determine whether VHL induced GRDD-dependent topo IIα degradation, we examined the expression levels of FLAG-tagged topo IIα and its deletion mutants in HT1080 cells co-expressing HA-tagged VHL (Fig. 4). Co-expression of VHL induced the degradation of wild-type topo IIα and its deletion mutants. We conclude that VHL stimulates the degradation of topo IIα through interaction with the ATPase domain of topo IIα in a GRDD-independent manner.

Discussion

Overexpression of cullin 3 promotes proteasomal degradation of topoisomerase I (topo I)-DNA covalent complexes and confers resistance to topo 1-directed drugs [18]. This suggests the possibility that cullin may act as the E3 ubiquitin ligase for proteasomal topo IIα degradation. Indeed, overexpressed cullin 2 induces topo IIα degradation in co-transfected cells as in cancer cells under glucose starvation (Fig. 1A). Topo IIα associates with cullin 2 and endogenous Elongin C through the ATPase domain. Elongin C is a component of the three subunits of Elongin complex. Elongin B and C form an Elongin B/C complex that functions as a bridge to link VHL, a substrate-recognition protein, to cullin 2 in cullin-based ubiquitin ligase [19,20].

To determine the precise region within VHL protein required for this activity, we constructed deletion mutants of VHL and examined their ability to bind and degrade topo IIα. The crystal structure of the VHL-Elongin B/C complex showed that a region (amino acids 1–113) within the β domain of VHL protein might be involved in binding to substrate protein [21]. However, our sequence alignment revealed that amino acids 114–123 within the β domain of VHL played pivotal roles in topo IIα degradation and binding. The Elongin B/C box (amino acids 157–166) within the α domain of VHL was responsible for topo IIα degradation and binding functions. VHL protein has been shown to bind to Elongin B/C complex via the so-called BC-box within the α domain and to act as substrate receptor in Elongin B/C-cullin 2-Rbx1 complex [22,23]. In addition to glucose starvation, hypoxia may lead to proteasome-mediated degradation of topo IIα [24]. Our findings are consistent with the study showing that VHL is directly responsible for HIF1α ubiquitination and degradation by the proteasome. Taken together, we conclude that VHL protein acts as a substrate receptor and is indispensable for binding to and recruiting topo IIα to the ECV ubiquitin ligase.

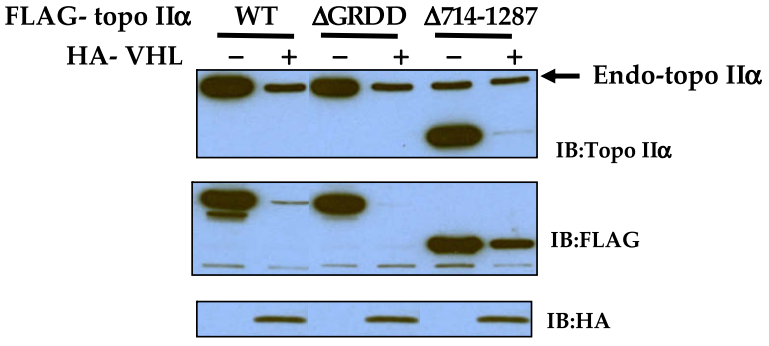

Fig. 4. GRDD-independent topo IIα degradation by VHL. HT1080 cells were co-transfected with expression plasmids encoding full-length (WT), ΔGRDD, or Δ714–2187 of FLAG-topo IIα together with HA-VHL or no insert. After transfection, the cells were cultured under normal conditions for 24 h, and the whole cell lysates were examined by immunoblot analysis using anti-topo IIα, anti-FLAG, and anti-HA antibodies.

In previous studies, we demonstrated that Jab1/CSN5-induced degradation of topo IIα is dependent on GRDD within the ATPase domain of topo IIα and that GRDD is necessary for interaction with Jab1/CSN5 [17]. Conversely, the GRDD of topo IIα had no effect on VHL-induced degradation and binding to VHL protein. These results indicate that topo IIα has a specific recognition motif for binding to VHL other than the GRDD within the ATPase domain. We conclude that a novel region within the ATPase domain of topo IIα, similar to the oxygen-dependent degradation domain (ODD) of HIF1α, serves as a recognition region for interaction with VHL and its proteasomal degradation [25]. The findings described here indicate that VHL could be a substrate-recognition protein for assembling topo IIα and subunits of cullin 2-based ubiquitin ligase in solid tumors.

Our present findings of the specific ubiquitin ligase for topo IIα could provide a means of elucidating the mechanisms of topo IIα degradation to better attack the resistance of microenvironmentally stressed solid tumors either with proteasome inhibitors used currently in the clinic or by targeting specific components of the ubiquitin system.

Acknowledgments

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