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Homodimer of Two F-box Proteins βTrCP1 or βTrCP2 Binds to IκBα for Signal-dependent Ubiquitination*

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Hiroshi Suzuki‡§, Tomoki Chiba§¶‖, Toshiaki Suzuki¶, Takashi Fujita¶, Tsuneo Ikenoue¶, Masao Omata**, Kiyoshi Furuichi‡, Hisataka Shikama‡, and Keiji Tanaka¶‖‡‡**

*From the ‡Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Company Ltd., 21 Miyukigaoka, Tsukuba-shi, Ibaraki, 305-8585, the ¶Tokyo Metropolitan Institute of Medical Science and ‖Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, and the **Department of Gastroenterology, Faculty of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku 113-8655, Japan*

We found previously that overexpression of an F-box protein βTrCP1 and the structurally related βTrCP2 augments ubiquitination of phosphorylated IκBα (pIκBα) induced by tumor necrosis factor-α (TNF-α), but the relationship of the two homologous βTrCP proteins remains unknown. Herein we reveal that deletion mutants of βTrCP1 and βTrCP2 lacking the F-box domain suppressed ubiquitination and destruction of pIκBα as well as transcriptional activation of NF-κB. The ectopically expressed βTrCP1 and βTrCP2 formed both homodimer and heterodimer complexes without displaying the trimer complex. Dimerization of βTrCP1 and/or βTrCP2 takes place at their conserved NH_2-terminal regions, termed a "D-domain" (for dimerization domain), located upstream of the F-box domain. The D-domain was necessary and sufficient for the dimer formation. Intriguingly, the βTrCP homodimer, but not the heterodimer, was selectively recruited to pIκBα induced by TNF-α. These results indicate that not only βTrCP1 but also βTrCP2 participates in the ubiquitination-dependent destruction of IκBα by forming $SCF^{βTrCP1\text{-}βTrCP1}$ and $SCF^{βTrCP2\text{-}βTrCP2}$ ubiquitin-ligase complexes.

NF-κB is a multifunctional transcription factor that regulates the expression of a number of genes involved in immune and inflammatory responses (1). The prototypical inducible NF-κB, consisting of two distinct subunits, p50 and p65, normally exists as an inactive form because of association with its protein inhibitor, termed IκB in the cytoplasmic compartment of many types of cells (2). IκB prevents the transportation of NF-κB into the nucleus by masking the nuclear localization signal of NF-κB (3). Therefore, major processes in regulating the NF-κB signaling pathway occur in the cytoplasm of cells in order to release negative control by IκB. The tremendous progress in delineating the molecular mechanisms of NF-κB activation has revealed that two important steps are responsible for the immediate removal of IκB associated with NF-κB: phosphorylation and subsequent proteolytic destruction of IκB. Phosphorylation of two serine residues (Ser-32 and Ser-36) near the NH_2 terminus of IκBα, one of the best characterized members of the structurally and functionally related IκB protein family, is essential for targeting IκBα for signal-promoted destruction (4, 5). This specific phosphorylation of IκBα is catalyzed by an unusually large, multiprotein kinase complex, termed IκB kinase, with an apparent molecular mass of 700–900 kDa (6–8). The phosphorylation of IκBα by an IκB kinase complex is necessary for its polyubiquitination at residues Lys-21 and Lys-22 (9, 10). The ubiquitin (Ub)[1]-proteasome system then plays the next indispensable role for down-regulating IκBα at the physiological level (6, 11).

Ub is a landmark molecule in a post-translational protein modification system which plays a central role in intracellular protein breakdown (13–15). Protein ubiquitination is initiated by the formation of a high energy thioester bond between Ub and an E1 (Ub-activating enzyme) in a reaction that requires ATP hydrolysis. The activated Ub is then transferred to an E2 (Ub-conjugating enzyme). In some cases, E2 directly transfers Ub to target proteins, but the reaction often requires the participation of an E3 (Ub-ligating enzyme). Finally, a poly-Ub chain is formed by linking the COOH terminus of one Ub to a Lys residue within another Ub. The resultant poly-Ub chain acts as a degradation signal for proteolytic attack by the 26 S proteasome, a eukaryotic ATP-dependent 2-MDa protease complex (16, 17). In this Ub-proteasome pathway, E3, the protein-Ub ligase, presumably plays the most important role in the selection of target proteins for degradation, because each distinct E3 binds the protein substrate with a degree of selectivity (14).

Accumulating evidence has revealed that activation of NF-κB is induced by signal-dependent degradation of IκB through the Ub pathway (14, 15, 18). An IκBα-Ub ligase (equivalent to IκBα-E3) is thought to be the key enzyme to control the level of IκB. However, the long sought IκBα-E3 was identified only recently. The breakthrough for its discovery was a frequent report for Slimb, an F-box/WD40-repeat protein, which negatively regulates the Wingless pathway and Hedgehog pathway (19). Based on its structural feature, Slimb was postulated to be a component of the SCF (*S*kp1-*C*dc53 or *C*ullins-*F*-box protein complex)-like Ub ligase complex which would participate in the degradation of IκBα as well as β-catenin (18). To date, SCF is known to be a large multisubunit Ub-ligase that has been identified as a ubiquitination factor acting on a number of regulatory proteins such as cyclin-dependent kinase

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§ The first two authors contributed equally to this work.

‡‡ To whom correspondence should be addressed: Tokyo Metropolitan Institute of Medical Science, Japan Science and Technology Corporation, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan. Tel. and Fax: 81-3-3823-2237; E-mail: tanakak@rinshoken.or.jp.

[1] The abbreviations used are: Ub, ubiquitin; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub-ligating enzyme; pIκBα, phosphorylated IκBα; TNF-α, tumor necrosis factor α; HA, hemagglutinin; HEK, human embryonic kidney.

inhibitors Sic1 (20, 21), p21 (22), and p27 (23–25), cyclin Cln1 (26), and cyclin D (22, 27), E2F-1 (28), and CD4 (29). Growing *in vivo* and *in vitro* evidence demonstrates that IκBα-E3 is indeed an SCF-like complex, consisting of Skp1, Cullin-1 (abbreviated Cul-1), and βTrCP (30–34). Intriguingly, the same IκBα-E3 has been found to catalyze the ubiquitination of β-catenin (30, 35–37).

Recently we reported that the putative IκBα-E3 present in HeLa cell cytosol is recruited to be specifically associated with *in vivo* phosphorylated IκBα produced by tumor necrosis factor-α (TNF-α) stimulation and recombinant IκBα previously phosphorylated *in vitro* by IκB kinase, causing polyubiquitination of IκBα (38). We also found that the SCF complex acting as an IκBα-E3 contains not only βTrCP1 but also the structurally related βTrCP2, both of which augment ubiquitination of IκBα when they are overexpressed in the HeLa cells (39). In the present study, we demonstrate that two homologous F-box/WD40-repeat proteins, βTrCP1 and βTrCP2, are assembled with Skp1 and Cul-1 to form SCF complexes for ubiquitination of IκBα, which would lead to signal-dependent activation of NF-κB. In addition, ectopically expressed βTrCP1 or βTrCP2 resulted in homo- and heterodimer complexes through their NH_2-terminal regions, and each homodimer, but not heterodimer, was found to be recruited to phosphorylated IκBα (pIκBα) in response to external signals. The possible roles of distinct SCF complexes, composed of two structurally related βTrCP proteins during the signal-dependent ubiquitination of IκBα, are discussed.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials are as follows: MG132 (carbobenzoxy-leucyl-leucyl-leucinal) (Peptide Institute, Inc., Osaka, Japan); okadaic acid (Wako, Tokyo, Japan); human TNF-α (Genzyme); mouse TNF-α (Roche Molecular Biochemicals); rabbit anti-IκBα antibody (c-21), rabbit anti-influenza hemagglutinin (HA) antibody (Y-11), rabbit anti-S-peptide antibody (K-14), mouse monoclonal anti-HA antibody (F-7), mouse monoclonal anti-Myc antibody (9E10) (Santa Cruz Biotechnology); mouse monoclonal anti-FLAG (M2) antibody, M2-conjugated beads, and FLAG peptide (Sigma). Rabbit polyclonal anti-Cul-1 and anti-Skp1 antibodies were described previously (39).

Expression Plasmids—To construct various plasmids for the expression of proteins as NH_2-terminally Myc, HA, T7 and S-tagged proteins, the following oligonucleotide pairs were ligated into *Kpn*I/*Bam*HI-cut pcDNA3.1(+) vector (Invitrogen): for Myc-tagged (pcDNA3.1-Myc), 5′-CACCATGGAGCAGAAACTCATCTCTGAAGAGGATCTGGG-3′ and 5′-GATCCCCAGATCCTCTTCAGAGATGAGTTTCTGCTCCATGGTG-GTAC-3′; for HA-tagged (pcDNA3.1-HA), 5′-CACCATGGCTTACCCA-TACGATGTT-CCAGATTACGCTGG-3′ and 5′-GATCCCAGCGTAATC-TGGAACATCGTATGGGTAAGCCATGGTGGTAC-3′; for T7-tagged (pcDNA3.1-T7), 5′-CACCATGGCTAGCATGACTGGTGGACAGCAAA-TGGGTGG-3′ and 5′-GATCCCACCCATTTGCTGTCCACCAGTCATG-CTAGCCATGGTGGTAC-3′; for S-tagged (pcDNA3.1-S), 5′-CACCATG-AAAGAAACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAG-CGG-3′ and 5′-GATCCCGCTGTCCATGTGCTGGCGTTCGAATTTAG-CAGCAGCGGTTTCTTTCATGGTGGTAC-3′. The cDNAs of wild-type or deletion mutants of Hs-βTrCP1 and Hs-βTrCP2 were amplified by polymerase chain reaction with appropriate primers and ligated into *Bam*HI/*Not*I-cut expression vectors. The coding residues of the Hs-βTrCP1 and Hs-βTrCP2 deletion mutants were as follows: βTrCP1ΔD (deleted 106–148), βTrCP1ΔF (deleted 148–192), βTrCP1ΔN (141–569), βTrCP1ΔNF (190–569), βTrCP1ΔW_{1-7} (1–259), βTrCP2ΔD (deleted 81–123), βTrCP2ΔF (deleted 122–167), βTrCP2ΔN (116–542), and βTrCP2ΔNF (162–542).

Transfection and Immunoblotting—Human embryonic kidney 293 cells (HEK293) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Transfections were performed using FuGENE 6 transfection reagent according to the manufacturer's instructions (Boehringer Mannheim). For coimmunoprecipitation experiments with βTrCP proteins and pIκBα, HEK293 cells were pretreated with 50 μM MG132 for 45 min followed by 0.25 μM okadaic acid for 15 min; human TNF-α was added for 15 min at a final concentration of 1 ng/ml. Cells were lysed in 20 mM Tris-HCl (pH 7.4) lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 25 mM β-glycerophosphate, 100 nM okadaic acid, 2 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml each leupeptin, aprotinin, and pepstatin A. Immunoprecipitation and immunoblotting were performed as described previously (39).

Reporter Assays—Mouse L929 cells were maintained in minimal essential medium containing 5% fetal calf serum and antibiotics. In the experiment, 2×10^5 cells were transfected by the DEAE-dextran method with a total of 2.5 μg of plasmid DNA containing 0.5 μg of H2-Luc reporter plasmid and various amounts of effector plasmid (pBOS-βTrCP1, pBOS-βTrCP2, pBOS-βTrCP1ΔF, pBOS-βTrCP2ΔF) with an empty vector to adjust to 2 μg. Cells were split into two wells at 24 h post-transfection and then stimulated with or without mouse TNF-α (1 ng/ml) for 1 h after an additional 24 h. Cells were lysed, and luciferase activity was measured with luciferase assay reagent (Promega) using a luminometer (Berthold). The relative fold activation of luciferase activities upon TNF-α treatment was calculated to normalize transfection efficiency.

RESULTS

Roles of βTrCP1 and βTrCP2 in Ubiquitination and Destruction of pIκBα Induced by TNF-α—Recently we reported that overexpression of βTrCP1 or βTrCP2 caused marked augmentation for ubiquitination of pIκBα in response to TNF-α stimulation (39). To extend these observations, we examined the effect of deletions of the F-box domain of both βTrCP1 and βTrCP2 (abbreviated βTrCP1ΔF and βTrCP2ΔF, respectively; for these mutant structures, see Fig. 1, *upper panel*) for IκBα ubiquitination. As shown in Fig. 1 (*lower panel*), treatment of TNF-α caused rapid loss of IκBα detected by Western blotting as a function of time after TNF-α stimulation in HEK293 cells. In contrast, pretreatment of proteasome inhibitor MG132 (equivalent to Suc-LLL-CHO) prior to TNF-α stimulation caused a marked increase in phosphorylated pIκBα, in turn, accompanied by a decrease in its unphosphorylated form. This compound also resulted in abnormal accumulation of multiple polyubiquitinated bands of endogenous IκBα within 5 min, and its sustained effect was observed up to 20 min (Fig. 1, *lower panel*). In contrast, in the absence of MG132, the degradation of pIκBα seemed to be so rapid that the ubiquitinated forms could not be detected to any visual extent. However, ectopic expressions of βTrCP1ΔF or βTrCP2ΔF strongly suppressed the accumulation of ubiquitinated forms of pIκBα in a dose-dependent manner. These dominant-negative effects of βTrCP1ΔF or βTrCP2ΔF strongly indicate that both βTrCP1 and βTrCP2 direct the ubiquitination of pIκBα *in vivo*.

We examined next whether βTrCP1 and βTrCP2 are involved in regulating instability, that is, the destruction of pIκBα *in vivo* using these F-box deletion mutants. As shown in Fig. 2, the level of IκBα decreased rapidly within 20 min after TNF-α stimulation (see mock transfection). However, the immediate loss of IκBα caused by the treatment of TNF-α was markedly prevented, depending on the βTrCP1ΔF and βTrCP2ΔF added (*top panel*), even if MG132 had not been pretreated (compared with Fig. 1). Importantly, ectopic expressions of βTrCP1ΔF and βTrCP2ΔF also caused considerable accumulation of pIκBα (*top panel*). The transfections of βTrCP1ΔF and βTrCP2ΔF resulted in the accumulation of these cellular levels in proportion to the amounts of transfected plasmids (*middle panel*), whereas the level of Skp1 as an internal control was unchanged.

To extend further that βTrCP1 and βTrCP2 are involved in IκBα degradation, the release of NF-κB was measured by NF-κB-dependent transcription using a reporter plasmid that harbors NF-κB binding sites upstream of the luciferase gene. As shown in Fig. 3, ectopic expression of βTrCP1ΔF and βTrCP2ΔF significantly inhibited TNF-α-induced NF-κB activation in a dose-dependent manner, in agreement with stabilized IκBα (Fig. 2). Full-length βTrCP1 and βTrCP2 restored

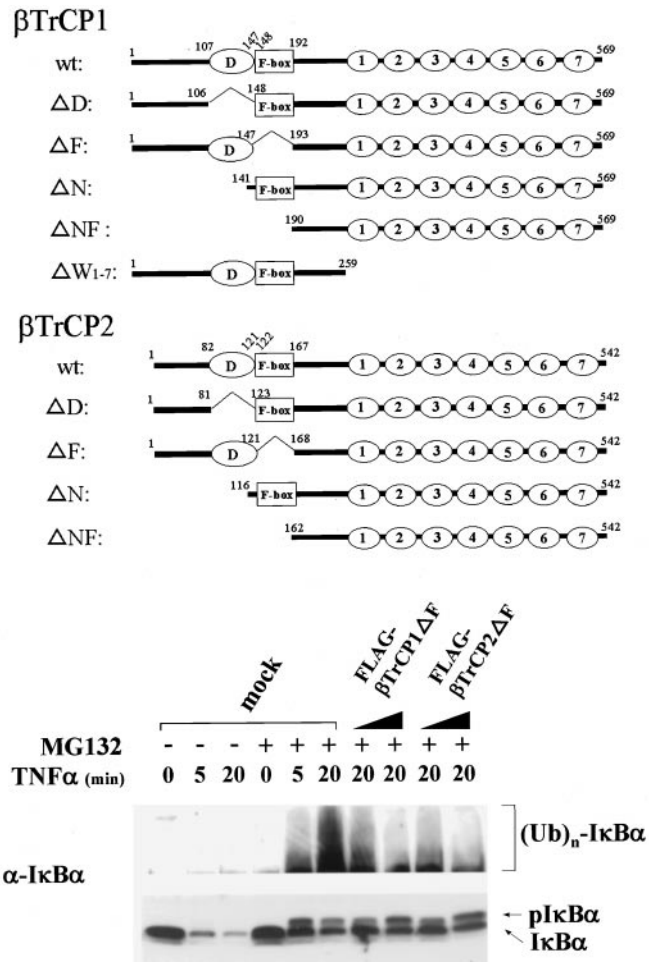

FIG. 1. **Effect of βTrCP1ΔF and βTrCP2ΔF on TNF-α-induced ubiquitination of IκBα.** HEK293 cells were transfected with pcDNA3-FLAG-βTrCP1ΔF, pcDNA3-FLAG-βTrCP2ΔF (0.08 and 2 μg/well of a six-well plate), or pcDNA3 (mock, 2 μg) and were treated for 1 h with or without MG132 (50 μM) prior to stimulation for the indicated times with TNF-α (1 ng/ml). Cell lysates were prepared in the presence of 2 mM *N*-ethylmaleimide and 0.05% SDS to inhibit isopeptidase activities and were analyzed by immunoblotting with anti-IκBα antibody. Multiple ubiquitinated IκBα bands are designated $(Ub)_n$-IκBα. The deletion mutants of βTrCP1 and βTrCP2 used in this study are represented schematically (*upper panel*). *wt*, wild-type.

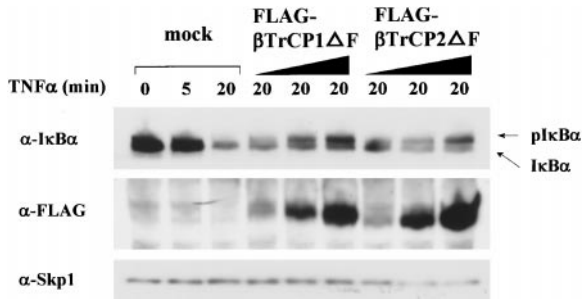

FIG. 2. **Effect of βTrCP1ΔF and βTrCP2ΔF on TNF-α-induced degradation of IκBα.** HEK293 cells were transfected with increasing amounts of pcDNA3-FLAG-βTrCP1ΔF or pcDNA3-FLAG-βTrCP2ΔF (0.08, 0.4, and 2 μg/well of a six-well plate) or pcDNA3 (mock, 2 μg) and were treated for the indicated times with TNF-α. The levels of IκBα, FLAG-βTrCP1ΔF, FLAG-βTrCP2ΔF, and Skp1 in cell lysates were analyzed by immunoblotting. The positions of phosphorylated and unphosphorylated IκBα are shown.

the transcriptional activity inhibited by βTrCP1ΔF or βTrCP2ΔF, although they did not significantly elevate the level of NF-κB activations beyond that of cells transfected by an empty vector. Paradoxically, overproduction of βTrCP resulted

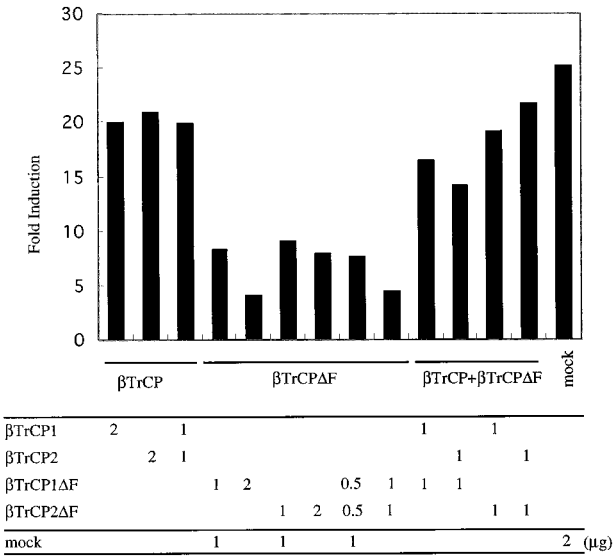

FIG. 3. **Effect of βTrCP1 and βTrCP2 on the induction of NF-κB-dependent transcription by TNF-α stimulation.** L929 cells were transfected with an NF-κB-dependent luciferase reporter construct together with various amounts of FLAG-tagged βTrCP constructs as indicated. After the cells were stimulated with or without TNF-α (1 ng/ml) for 1 h, the cell lysates were prepared and then subjected to luciferase assay. Reporter activity is expressed as fold induction of normalized luciferase activity in stimulated cells relative to that of unstimulated cells. Data show the means of four independent experiments.

in rather partial inhibition of NF-κB activation, probably because of the nature of the F-box/WD40-repeat proteins that dimerize each other with a different partner before they incorporate into the SCF complex (data not shown; see "Discussion"). Taken together, it was clearly concluded that not only βTrCP1, which had been reported by many groups (31–34), but also βTrCP2, indicated recently by us (39), are involved in the signal-dependent proteolysis of IκBα.

Homo- and Heterocomplexes of βTrCP1 and βTrCP2 Which Are Associated with Skp1 and Cul-1—It is interesting to determine how the two homologous βTrCP1 and βTrCP2 interact with pIκBα as an SCF complex. To test this, two different epitopes (FLAG and T7 epitopes) were tagged to the COOH terminus or NH_2 terminus of βTrCP1 and βTrCP2; then their interaction in transfected HEK293 cells was assessed when treated with or without TNF-α. Immunoprecipitation of FLAG-tagged βTrCP1 or βTrCP2 resulted in specific coimmunoprecipitation of phosphorylated IκBα only when the cells were stimulated by TNF-α, indicating that these FLAG-tagged proteins function in cells (Fig. 4, *right*). As shown in Fig. 4, T7-tagged βTrCP1 and βTrCP2 were evident in immunoprecipitates from both TNF-α treated and untreated cells, indicating that βTrCP1 and βTrCP2 form not only homocomplexes but also heterocomplexes, irrespective of TNF-α stimulation (Fig. 4). Skp1 and Cul-1, common partners of SCF complexes, were also evident in βTrCP immunoprecipitates from TNF-α-untreated cells, indicating that neither complex formation nor SCF complex assembly is regulated by TNF-α stimulation or substrate binding. Each homocomplex and heterocomplex seems to form a functional SCF complex because SCF components in cell lysates are efficiently coimmunoprecipitated (*left panel*).

During analysis of the SCF assembly, we noticed that the anti-Cul-1 antibody exhibits two distinct bands in the immunoprecipitates of βTrCP1 or βTrCP2 in the TNF-α-treated cell

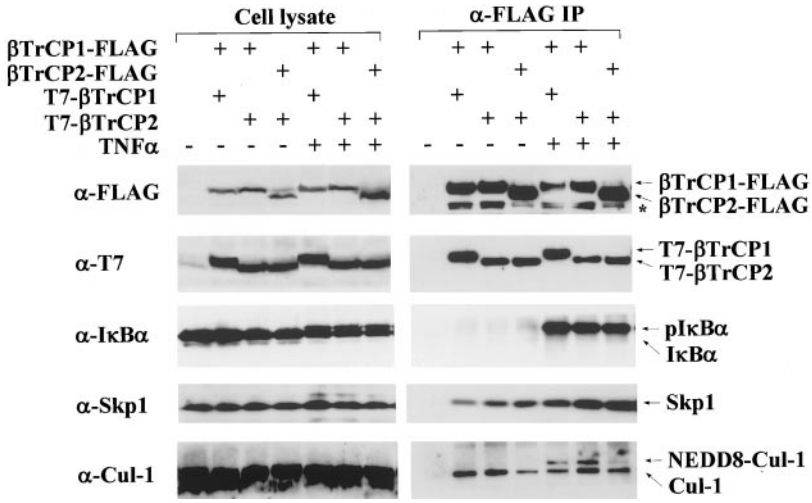

FIG. 4. **Formation of homo- and heterocomplexes of βTrCP1 and βTrCP2 which are assembled to the SCF complex.** HEK293 cells were cotransfected with the indicated combinations of pEF-Bos-βTrCP1-FLAG or pEF-Bos-βTrCP2-FLAG (10 μg each) and pcDNA3.1-T7-βTrCP1 or pcDNA3.1-T7-βTrCP2 (10 μg each). 36 h after transfection, TNF-α treatment, preparation of cell lysates, and immunoprecipitation with anti-FLAG were carried out as described under "Experimental Procedures." Cell lysates and immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. *Asterisks* indicate truncated βTrCP1-FLAG and βTrCP2-FLAG, which may be produced by proteolytic digestion or synthesized from a different translation initiation site. It is noteworthy that these faster migrating forms of βTrCP1-FLAG or βTrCP2-FLAG were not coimmunoprecipitated with T7-tagged βTrCPs (Footnote 2), supporting the importance of the NH_2-terminal region for complex formation (for details, see "Results" and Figs. 6 and 7).

extracts but exhibits a single form in those from unstimulated cells (Fig. 4, *bottom panel*). The molecular size of Cul-1 deduced from the nucleotide sequence is approximately 90 kDa, which apparently corresponds to the smaller band of the two Cul-1 bands detected, indicating that Cul-1 may be modified in response to TNF-α stimulation or binding to pIκBα. Previously, it was reported that Cdc53, a yeast homolog of Cul-1, is modified by the Ub-like protein, Rub1 (40). In addition, we recently found that human NEDD8 (yeast Rub1 homolog) was covalently ligated to Cul-4A by a novel Ub-related conjugation pathway consisting of an E1-like APP-BP1-Uba3 heterodimer and E2-like Ubc12 (41). Very recently, we also found that NEDD8 was conjugated to six Cul-family proteins *in vitro* (42). Therefore, we assume that the larger form of Cul-1 is the result of modification by NEDD8.

βTrCP1 Forms a Homodimer but not a Trimer Complex—Next we examined whether βTrCP1 forms a dimer or multimer complex. When three FLAG-, HA-, or Myc-tagged βTrCP1 were coexpressed in HEK293 cells, immunoprecipitates prepared by anti-FLAG antibody contained all FLAG-, HA-, and Myc-tagged βTrCP1 (Fig. 5, *far left lane*). Similar results were observed by immunoprecipitation with anti-HA or anti-Myc antibody (data not shown). Subsequently, immunoprecipitates prepared by anti-FLAG antibody were treated with FLAG peptide. The eluates were split and subjected to a second immunoprecipitation with anti-HA or anti-Myc antibodies. The resulting immunoprecipitates were used for Western analysis using anti-FLAG, -HA, or -Myc antibodies. As shown in Fig. 5 (*right two lanes*), the anti-HA antibody coimmunoprecipitated FLAG- and HA-βTrCP1 but not Myc-βTrCP1. Similarly, the anti-Myc antibody coimmunoprecipitated FLAG- and Myc-βTrCP1, but not HA-βTrCP1. Thus it was clear that expression of three independent tagged βTrCP1 resulted in two-tagged βTrCP1 interaction, but not a three-tagged βTrCP1 complex, strongly indicating that βTrCP1 did not form a trimer or multimer complex. Thus it was concluded that βTrCP1 is assembled into the dimeric complex.

Dimerization of βTrCP1 and βTrCP2 Occurs at Their NH_2-terminal Conserved Regions Termed the "D-domain"—We next examined how both βTrCP1 and βTrCP2 interact with each

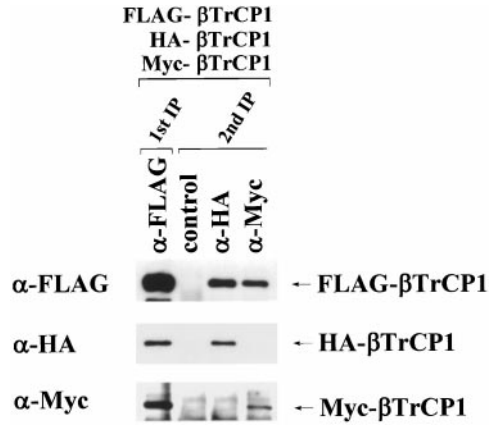

FIG. 5. **Dimer, but not multimer, formation of βTrCP1.** 36 h after cotransfection of expression plasmids encoding NH_2-terminally FLAG-, HA-, and Myc-tagged βTrCP1 into HEK293 cells, cell lysates were first subjected to immunoprecipitation (*1st IP*) with anti-FLAG (M2)-conjugated beads. The precipitated complexes were eluted by lysis buffer containing 200 μg/ml FLAG peptide. An equal volume of the eluates was again immunoprecipitated (*2nd IP*) with control mouse antibody (*control lane*), mouse anti-HA monoclonal antibody (F-7) (*α-HA lane*), or mouse anti-Myc monoclonal antibody (9E10) (*α-Myc lane*). Immunoblotting was carried out with anti-FLAG (M2), anti-HA (Y-11), and anti-Myc (9E10) antibodies and horseradish peroxidase-conjugated protein G.

other to form homo- and heterocomplexes using various deletion mutants of βTrCP1 and βTrCP2. In this experiment, the S-protein purification method was used. As shown in Fig. 6*A*, S-tagged βTrCP1 and βTrCP2 copurified wild-type FLAG-βTrCP1 or deletion mutants lacking F-box or all WD40 repeats (named ΔF or ΔW_{1-7}, respectively; see Fig. 1, *upper panel*), but not the NH_2-terminal deletion mutant named FLAG-βTrCP1ΔNF, indicating that the F-box alone is dispensable, whereas the NH_2-terminal domain together with the F-box is required for dimer formation.

To verify further that the F-box is not required and that the NH_2-terminal region is sufficient for dimer formation, S-tagged βTrCP1ΔF and βTrCP2ΔF were used to test their interactions with FLAG-tagged βTrCP constructs. As shown in Fig. 6*B*,

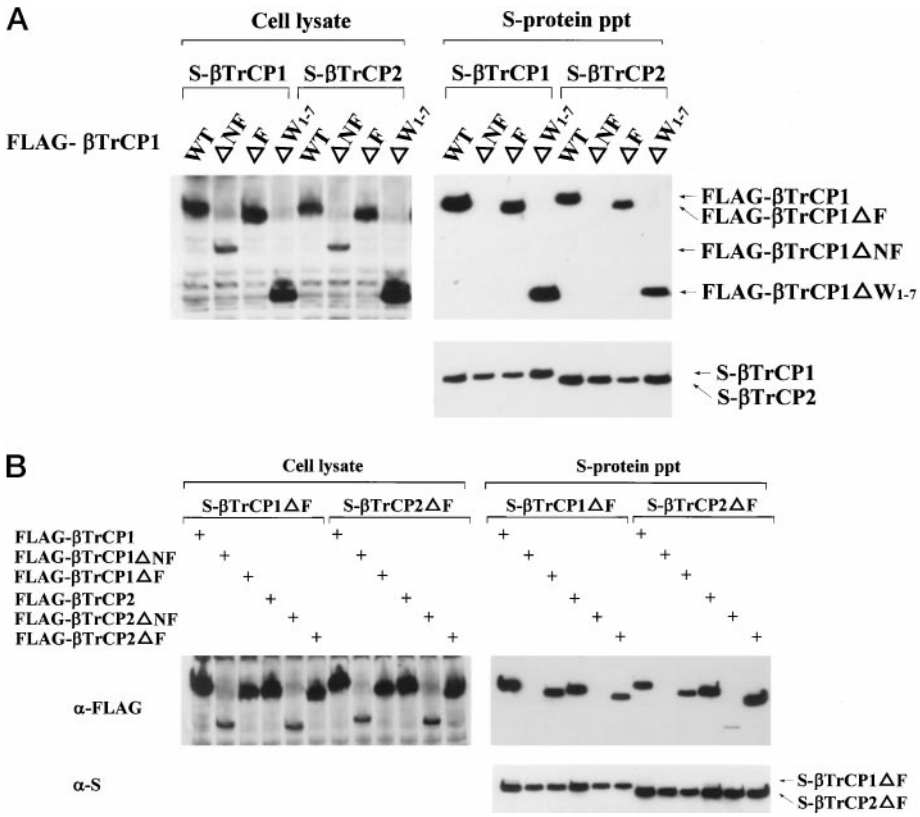

FIG. 6. **The F-box of βTrCP1 and βTrCP2 is not required, but the NH_2-terminal region is sufficient for each homo- and heterodimer formation.** S-tagged wild-type (*panel A*) or the F-box deletion mutant (*panel B*) of βTrCP1 and βTrCP2 was transfected in HEK293 cells in combination with various FLAG-tagged wild-type or deletion mutants of βTrCP (βTrCPΔF, βTrCPΔNF, and βTrCPΔ$W_{1–7}$), as indicated. 36 h post-transfection, cell lysates were prepared and subjected to the affinity purification of the S-protein complex (*S-protein ppt*) and then analyzed by immunoblotting with anti-FLAG and anti-S (K-14) antibodies.

either S-βTrCP1ΔF or S-βTrCP2ΔF was complexed with FLAG-tagged wild-type or deletion mutants lacking the F-box, but not with FLAG-tagged βTrCP1ΔNF, confirming again that the F-box is not required for dimer formation, but the NH_2-terminal region beyond the the F-box domain is sufficient for efficient dimer formation.

To analyze further whether the NH_2-terminal region is required for dimer formation irrespective of the F-box, two additional deletion mutants, βTrCPΔN and βTrCPΔD, which lack the NH_2-terminal entire region beyond the F-box or just the conserved region flanking the F-box, respectively, were constructed (see Fig. 1, *upper panel*). As shown in Fig. 7*A*, none of the constructs that lack the NH_2-terminal region beyond the F-box (βTrCP1ΔN, βTrCP2ΔN, βTrCP2ΔD, and βTrCP2ΔD) was complexed with S-tagged wild-type βTrCP1 or βTrCP2. Reciprocal immunoprecipitation of FLAG-tagged constructs also revealed that the S-tagged βTrCP1 or βTrCP2 cannot bind βTrCPΔN and ΔD constructs, indicating that the NH_2-terminal region is the site for dimer formation (Fig. 7*B*).

To ascertain that the F-box or the F-box-binding protein Skp1 does not play a role in dimer formation, we investigated Skp1 interaction with these FLAG-tagged βTrCP mutants. As shown in Fig. 7*B*, wild-type βTrCPs and mutants lacking the NH_2-terminal region but not the F-box (βTrCPsΔN and βTrCPsΔD) bind to Skp1. On the other hand, βTrCPsΔNF and βTrCPsΔF that lack the F-box failed to bind Skp1. The facts that the deletion of the F-box does not affect the dimerization property and that dimerization-defective mutants could still bind to Skp1 strongly indicate that Skp1 binding is not necessary and sufficient for dimer formation. Furthermore, it was indicated that the dimer formation of βTrCPs is not a prerequisite of Skp1 binding. Thus, we concluded that the NH_2-terminal region is necessary and sufficient for dimer formation and provisionally named these specific regions needed for dimerization the D-domain. Although the dimer formation of βTrCPs can occur independently of TNF stimulation or substrate binding, substrates may help the dimerization of βTrCPs. Indeed, S-βTrCP2ΔF could interact with FLAG-βTrCP2ΔNF, although the extent was not very strong (Fig. 6*B*, *right panel*). They may have indirectly interacted with each other through the WD40 repeat domain probably via the substrate. Further study is required for the clarification of this point.

βTrCP1 and βTrCP2 Form Homo- and Heterodimer Complexes, but Only the Homodimer Is Recruited to pIκBα—As shown in Fig. 4, FLAG-tagged βTrCP1 coimmunoprecipitated not only T7-βTrCP1 and T7-βTrCP2 but also pIκBα under the stimulation of TNF-α. This result indicates that βTrCP1 and βTrCP2 form a heterocomplex but does not mean that this heterodimer complex is associated with pIκBα because there is a possibility that ectopically expressed βTrCP1-FLAG or T7-βTrCP2 forms a homodimer or heterodimer with endogenous βTrCP1, βTrCP2, or other F-box proteins. To analyze whether both homodimers and the heterodimer complex are able to associate with pIκBα, two-step immunoprecipitation analysis was carried out. As shown in Fig. 8, FLAG- and HA-tagged βTrCP1 or βTrCP2 plasmids were transfected and treated with TNF-α. Immunoprecipitation was first carried out using anti-FLAG antibody, and the resulting immunoprecipitates were eluted with the FLAG peptide. The resulting eluates were split and subsequently immunoprecipitated individually with anti-HA or anti-IκBα antibodies, and the resultant immunoprecipitates were analyzed by immunoblots with anti-FLAG, anti-HA, and anti-IκBα antibodies.

When HA-tagged βTrCP plasmids were cotransfected to generate the homodimer in combination with FLAG-tagged plasmids (*i.e.* FLAG-βTrCP1-HA-βTrCP1 or FLAG-βTrCP2-HA-βTrCP2), the second immunoprecipitates obtained by anti-HA antibody contained pIκBα, and in reciprocal anti-IκBα immunoprecipitates, HA-tagged βTrCPs were observed (Fig. 8, *left*

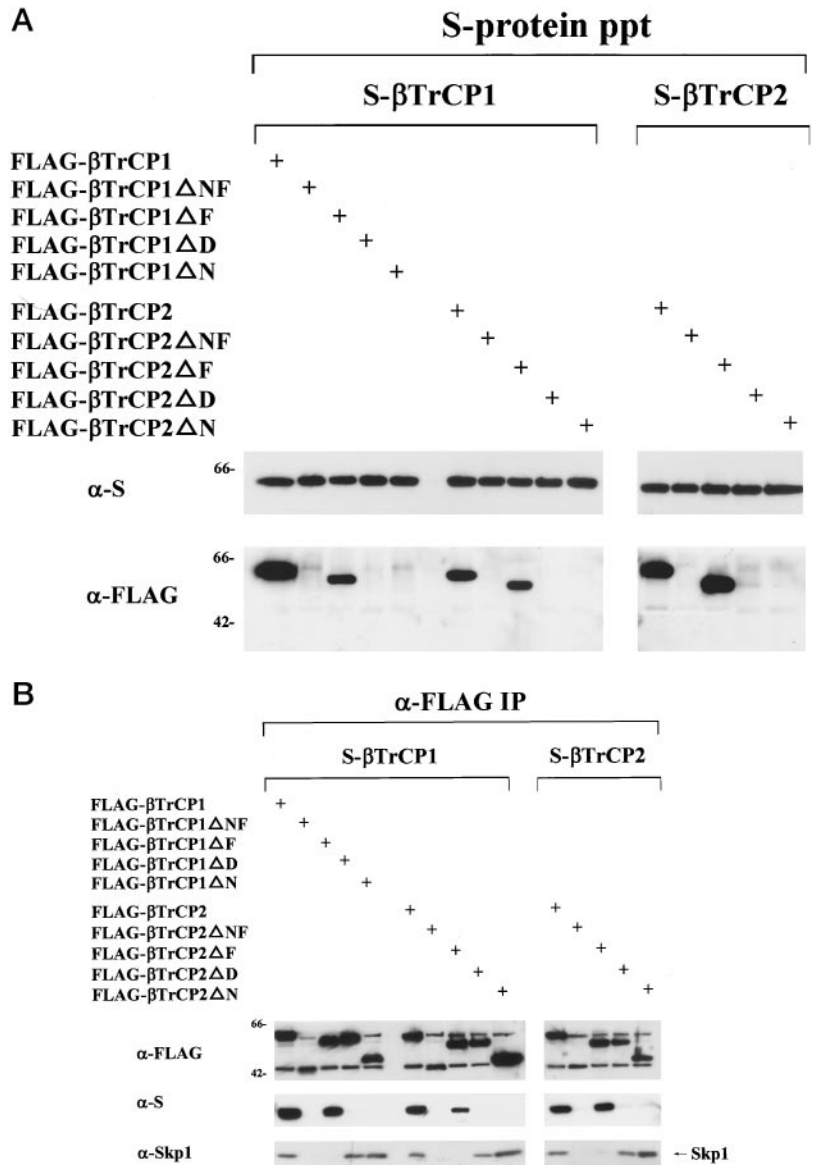

FIG. 7. **The NH_2-terminal region of βTrCP is responsible for homo- and heterodimer formation with βTrCP1 and βTrCP2.** S-tagged βTrCP1 or βTrCP2 was cotransfected with various FLAG-tagged βTrCP1 and βTrCP2 constructs as indicated, and then affinity purification by S-protein (*panel A*) or anti-FLAG (M2)-conjugated beads (*panel B*) was carried out. Purified S-protein precipitates (*ppt*, *panel A*) and anti-FLAG immunoprecipitates (*panel B*) were subjected to Western blotting using anti-FLAG-, anti-S-, or anti-Skp1 antibodies.

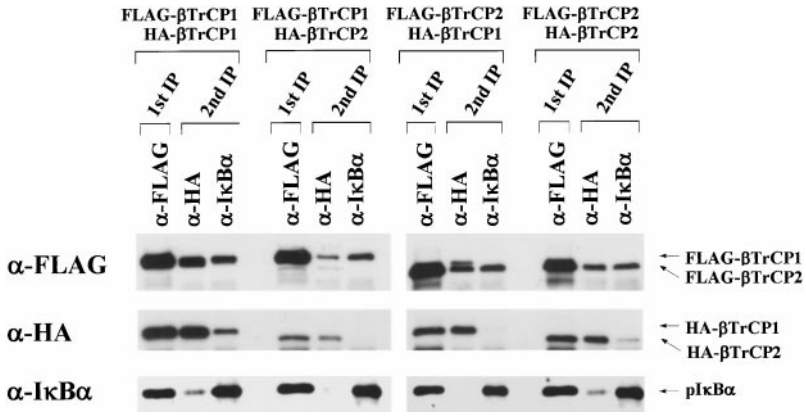

FIG. 8. **Binding of pIκBα with the homodimers of βTrCP1 and βTrCP2.** HEK293 cells were cotransfected with the indicated combinations of plasmids encoding FLAG-tagged or HA-tagged βTrCP1 and βTrCP2. After TNF-α treatment, cell lysates were prepared, followed by immunoprecipitation (*1st IP*) with anti-FLAG antibody. The precipitated complexes were eluted by lysis buffer containing 200 μg/ml FLAG peptide. Equal volumes of eluates were immunoprecipitated again (*2nd IP*) with rabbit anti-HA (Y-11) (*α-HA lane*) or rabbit anti-IκBα antibodies (*α-IκBα lane*). Immunoblotting was carried out with anti-FLAG (M2), anti-HA (F-7), and anti-IκBα antibodies.

and *right panels*), strongly indicating that the homodimer of βTrCP1 or βTrCP2 is associated with pIκBα. The reason that the extent of pIκBα obtained by immunoprecipitation with the anti-HA antibody was considerably less than that obtained by the anti-IκBα antibody may be in part because FLAG-tagged βTrCP1 or βTrCP2 can homodimerize with itself or with endogenous βTrCPs. On the other hand, when plasmids were cotransfected to produce heterodimers (*i.e.* FLAG-βTrCP1-HA-βTrCP2 or FLAG-βTrCP2-HA-βTrCP1), the second immunoprecipitates obtained by anti-HA antibody contained no pIκBα, and HA-tagged βTrCPs were not seen in reciprocal anti-IκBα immunoprecipitates (Fig. 8, *middle two panels*), strongly indicating that the heterodimers of βTrCP1 and βTrCP2 do not bind to pIκBα. Therefore, it is clear that the heterodimeric complex of βTrCP1 and βTrCP2 cannot be recruited to associate with pIκBα, and in turn, the homodimers of βTrCP1 and

βTrCP2 complexed with Skp1 and the Cul-1 function as an SCF Ub-ligase complex responsible for the ubiquitination of pIκBα.

DISCUSSION

Accumulating evidence shows that $SCF^{\beta TrCP1}$ is responsible for the ubiquitination of pIκBα (30–34). In addition, we also reported that not only $SCF^{\beta TrCP1}$ but also $SCF^{\beta TrCP2}$ functions for ubiquitination, based on the findings that βTrCP2, like βTrCP1, was associated with pIκBα and that overexpression of βTrCP1 and βTrCP2 individually augmented the ubiquitination of pIκBα (39). Here we extended the original finding for the involvement of βTrCP2 in addition to βTrCP1 for ubiquitination of pIκBα and revealed that βTrCP1 and βTrCP2 are complexed with Skp1 and Cul-1 to form $SCF^{\beta TrCP1}$ and $SCF^{\beta TrCP2}$, respectively, which are recruited to bind to pIκBα phosphorylated in response to TNF-α stimulation. Indeed, their F-box deletion mutants prevented the ubiquitination and the subsequent degradation of pIκBα. Moreover, these two mutant βTrCP expressions caused significant prevention of signal-dependent transcriptional activation of NF-κB. However, it is unknown why two homologous F-box/WD40-repeat proteins display apparently similar roles for pIκBα destruction. Perhaps these related proteins have redundant functions to maintain the important NF-κB signaling pathway.

There are many reports that $SCF^{\beta TrCP1}$ is involved in the ubiquitination not only of pIκBα but also of phosphorylated β-catenin (30, 35–37). This is reasonable considering the similarity of the two phosphorylation sites of IκBα and β-catenin (43), although obviously different kinases are involved, respectively *i.e.* IκBα kinase and glycogen synthase kinase 3β (18). Moreover, Fuchs *et al.* (44) reported that βTrCP2 is also responsible for the ubiquitination-dependent breakdown of β-catenin as well as IκBα, which is in contrast to the finding that βTrCP2, unlike βTrCP1, seems not to interact directly with β-catenin based on yeast two-hybrid analysis (36). Whether βTrCP2 is involved in the destruction of β-catenin requires further study. Fuchs *et al.* (44) did not show the effect of βTrCP1 and βTrCP2 for the degradation of IκBα and β-catenin simultaneously; therefore, it is unknown whether the role of βTrCP2 for ubiquitination of β-catenin occurs physiologically. The similarity between βTrCP1 and βTrCP2 is very close, displaying an overall identity of 76%, with almost an identical WD40 repeat domain that is capable of binding phosphorylated IκBα and β-catenin. Hence, overproduction of the related family of the βTrCP protein may bind directly to the substrates or indirectly through dimerization via the D-domain under non-physiological conditions. This assumption is also supported by the results in which excess production of wild-type βTrCP proteins leads to inhibition of the NF-κB-dependent transcriptional activation (data not shown). Finally, we found that overexpression of not only βTrCP1 but also βTrCP2 caused coimmunoprecipitation of β-catenin; however, the homodimer of βTrCP2 or the heterodimer of βTrCP1 and βTrCP2 does not associate with β-catenin, although the homodimer of βTrCP1 is firmly bound,[2] which clearly differs from their effect for pIκBα (see below). Therefore, we would favor a hypothesis that these two F-box proteins do not simply have redundant functions, rather they confer substrate specificity; *i.e.* βTrCP1, compared with βTrCP2, has a dominant effect on the ubiquitination of β-catenin. However, it remains unknown whether a similar substrate specificity is present for the ubiquitination of IκBα because βTrCP1 and βTrCP2 appear to have equivalent activity as shown by the present study. Further investigation is needed for the demonstration of this interesting hypothesis.

For the first time, we also present direct evidence that ectopically expressed βTrCP1 and βTrCP2 form both a homodimer and a heterodimer, irrespective of the treatment of TNF-α, without displaying the multimer complex. This finding is consistent with recent observations that fission yeast SCF, which regulates the level of the cyclin-dependent kinase inhibitor Rum1 and the S-phase regulator Cdc18 (45, 46), is composed of two related F-box-WD40 repeat proteins termed Pop1-Ste16 and Pop2-Sud1, forming three distinct complexes, $SCF^{Pop1\text{-}Pop1}$, $SCF^{Pop1\text{-}Pop2}$, and $SCF^{Pop2\text{-}Pop2}$ (47).

We demonstrated here that dimer formation of βTrCP1 and/or βTrCP2 takes place at the D-domain in their NH_2-terminal regions. This NH_2-terminal domain beyond the F-box is necessary and sufficient for their dimerization. Unexpectedly, Skp1 binding to the F-box did not require the dimer formation of F-box proteins, and vice versa. Initially, we thought that two F-box interfaces are required for the Skp1 binding because Skp1 was not associated with the heterodimer consisting of βTrCPΔF and wild-type βTrCP, raising the possibility that dimer formation is a prerequisite of Skp1 binding. However, based on the finding that both βTrCPΔD and βTrCPΔN could bind to Skp1, we concluded that dimer formation is not essential for Skp1 binding, and hence SCF complex formation occurs (data not shown). Indeed, βTrCPΔN, in contrast to βTrCPΔF, did not show significant effect of pIκBα destabilization compared with that of βTrCPΔF (data not shown), probably because of its functional SCF complex assembly or in part because of the lack of dimerization property with endogenous F-box proteins. Because F-box proteins bind the substrate, the combination of the F-box would regulate the affinity of the SCF complexes for the substrates. From this point of view, it is tempting to determine whether dimerization via the D-domain is regulated by other factors.

Consistent with our findings, Pop1-Ste16 and Pop2-Sud1 are associated with each other at their NH_2-terminal regions (48). Moreover, based on genetic evidence, Wolf *et al.* (48) reported that only the heterocomplex of Pop1 and Pop2 mediated Cdc18 and Rum1 proteolysis, even though Pop1 and Pop2 formed both homo- and heterocomplexes (47). They also showed that the Pop genes failed to complement the deleted phenotypes of each other, indicating that the functions of Pop1 and Pop2 are not redundant. On the other hand, we demonstrated that the homodimers, but not the heterodimers, of βTrCP1 and βTrCP2 were selectively recruited to pIκBα induced by TNF-α, revealing that not only βTrCP1 but also βTrCP2 participates in the ubiquitination-dependent destruction of pIκBα by forming their homodimeric $SCF^{\beta TrCP1\text{-}\beta TrCP1}$ and $SCF^{\beta TrCP2\text{-}\beta TrCP2}$ complexes. Considering these differences, we cannot exclude the possibility that $SCF^{\beta TrCP1\text{-}\beta TrCP2}$ is responsible for other function(s) so far unknown because it is possible that βTrCP1 or βTrCP2 may have interacted with other factors that would form the functional complexes to contribute as protein-Ub ligases at physiological levels. It is notable that the F-box/WD40-repeat proteins consist of a large gene family in various eukaryotic cells (49), and it is known that another βTrCP protein with a close similarity to βTrCP1, displaying 97% identity, exists in *Xenopus laevis* (12). If the homolog exists in mammalian cells, the protein may interact with βTrCP1 or βTrCP2 to form different SCF complexes that function for the ubiquitination of other targets.

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[2] H. Suzuki, T. Chiba, T. Suzuki, T. Fujita, T. Ikenoue, M. Omata, K. Furuichi, H. Shikama, and K. Tanaka, unpublished data.

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CELL BIOLOGY AND METABOLISM:

Homodimer of Two F-box Proteins βTrCP1 or βTrCP2 Binds to IκBα for Signal-dependent Ubiquitination

Hiroshi Suzuki, Tomoki Chiba, Toshiaki Suzuki, Takashi Fujita, Tsuneo Ikenoue, Masao Omata, Kiyoshi Furuichi, Hisataka Shikama and Keiji Tanaka

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