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

(Received for publication, August 12, 1999, and in revised form, October 13, 1999)

Hiroshi Suzuki‡‡, Tomoki Chiba§§, Toshiaki Suzuki††, Takashi Fujita‡, Tsuneo Ikenoue§§**, Masao Omata**, Kiyoshi Furuiichi†, 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

The Journal of Biological Chemistry Vol. 275, No. 4, Issue of January 28, pp. 2877–2884, 2000
Printed in U.S.A.

Phosphorylation of two serine residues (Ser-32 and Ser-36) near the NH2 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)-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-kB 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 (Skp1-Cdc53 or Cullins-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

---

* This work was supported in part by grants from Grants-in-aid for Scientific Research on Priority Areas (Intracellular Proteolysis) from the Ministry of Education, Science, Sports, and Culture of Japan and the Human Frontier Science Promotion Organization (to K. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ 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.

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.
Role of βTrCP1 and βTrCP2 in IkBa Proteolysis

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 IkBa-E3 is indeed an SCF-like complex, consisting of Skp1, Cul1 (abbreviated Cul-1), and βTrCP (30–34). Intriguingly, the same IkBa-E3 has been found to catalyze the ubiquitination of β-catenin (30, 35–37).

Recently we reported that the putative IkBa-E3 present in HeLa cell cytosol is recruited to be specifically associated with *in vivo* phosphorylated IkBa produced by tumor necrosis factor-α (TNF-α) stimulation and recombinant IkBa previously phosphorylated *in vitro* by IkB kinase, causing polyubiquitination of IkBa (38). We also found that the SCF complex acting as an IkBa-E3 contains not only βTrCP1 but also the structurally related βTrCP2, both of which augment ubiquitination of IkBa 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 Cul1 to form SCF complexes for ubiquitination of IkBa, 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 NH2-terminal regions, and each homodimer, but not heterodimer, was found to be recruited to phosphorylated IkBa (pIkBa) 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 IkBa, are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of materials are as follows: MG132 (carbox benzoxyl-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-IkBa 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-Skpl antibodies were described previously (39).

**Expression Plasmids**—To construct various plasmids for the expression of proteins as NH2-terminally Myc, HA, T7 and S-tagged proteins, expression plasmids for the expression of proteins as NH2-terminally Myc, HA, T7 and S-tagged proteins, were constructed. To extend further that overexpression of βTrCP1 or βTrCP2 caused marked augmentation for ubiquitination of pIkBa 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 IkBa ubiquitination. As shown in Fig. 1 (lower panel), treatment of TNF-α caused rapid loss of IkBa 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 pIkBa, in turn, accompanied by a decrease in its unphosphorylated form. This compound also resulted in abnormal accumulation of multiple polyubiquitinated bands of endogenous IkBa 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 pIkBa 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 pIkBa in a dose-dependent manner. These dominant-negative effects of βTrCP1ΔF or βTrCP2ΔF strongly indicate that both βTrCP1 and βTrCP2 direct the ubiquitination of pIkBa *in vivo*.

We examined next whether βTrCP1 and βTrCP2 are involved in regulating instability, that is, the degradation of pIkBa *in vivo* using these F-box deletion mutants. As shown in Fig. 2, the level of IkBa decreased rapidly within 20 min after TNF-α stimulation (see mock transfection). However, the immediate loss of IkBa 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 (compare with Fig. 1). Importantly, ectopic expressions of βTrCP1ΔF and βTrCP2ΔF also caused considerable accumulation of pIkBa (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 IkBa 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 IkBa (Fig. 2). Full-length βTrCP1 and βTrCP2 restored
Role of βTrCP1 and βTrCP2 in IκBα Proteolysis

![Graph](image)

**FIG.1.** 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 6 h, the cell lysates were prepared and then subjected to luciferase assay. **Upper panel**. Luciferase 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.

![Graph](image)

**FIG.2.** Effect of βTrCP1 and βTrCP2 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)k. α-IκBα, IκBα.

**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 6 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.

![Graph](image)

**FIG.4.** Effect of βTrCP1 and βTrCP2 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 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 in 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.

**Homodimerization and Heterocomplexes of βTrCP1 and βTrCP2 Which Are Associated with Skp1 and Cul1**—It is interesting to determine how the two homologous βTrCP1 and βTrCP2 interact with plκBα as an SCF complex. To test this, two different epitopes (FLAG and T7 epitopes) were tagged to the COOH terminus or NH2 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 Cul1, common partners of SCF complexes, were also evident in βTrCP immunoprecipitates from TNF-α-unstimulated 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
Role of βTrCP1 and βTrCP2 in IκBα Proteolysis

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 p16Ink4a. 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 communoprecipitated FLAG- and HA-βTrCP1 but not Myc-βTrCP1. Similarly, the anti-Myc antibody communoprecipitated 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 NH2-terminal Conserved Regions Termined the “D-domain”—We next examined how both βTrCP1 and βTrCP2 interact with each 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. 6A, S-tagged βTrCP1 and βTrCP2 copurified wild-type FLAG-βTrCP1 or deletion mutants lacking F-box or all WD40 repeats (named ΔF or ΔW1–7, respectively; see Fig. 1, upper panel), but not the NH2-terminal deletion mutant named FLAG-βTrCP1ΔNF, indicating that the F-box alone is dispensable, whereas the NH2-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 NH2-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. 6B,
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 NH2-terminal region beyond the the F-box domain is sufficient for efficient dimer formation.

To analyze further whether the NH2-terminal region is required for dimer formation irrespective of the F-box, two additional deletion mutants, βTrCPΔN and βTrCPΔD, which lack the NH2-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. 7A, none of the constructs that lack the NH2-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 NH2-terminal region is the site for dimer formation (Fig. 7B).

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. 7B, wild-type βTrCPs and mutants lacking the NH2-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 NH2-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. 6B, 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 resultant 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 panel).
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 and 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

**FIG. 7.** The NH₂-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 (F7), and anti-IκBα antibodies.
Role of βTrCP1 and βTrCP2 in IkBa Proteolysis

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

DISCUSSION

Accumulating evidence shows that SCFβTrCP1 is responsible for the ubiquitination of pIκBa (30–34). In addition, we also reported that not only SCFβTrCP1 but also SCFβTrCP2 functions for ubiquitination, based on the findings that βTrCP2, like βTrCP1, was associated with pIκBa and that overexpression of βTrCP1 and βTrCP2 individually augmented the ubiquitination of pIκBa (39). Here we extended the original finding for the involvement of βTrCP2 in addition to βTrCP1 for ubiquitination of pIκBa and revealed that βTrCP1 and βTrCP2 are complexed with Skp1 and Cul-1 to form SCFβTrCP1 and SCFβTrCP2, respectively, which are recruited to bind to pIκBa phosphorylated in response to TNF-α stimulation. Indeed, their F-box deletion mutants prevented the ubiquitination and the subsequent degradation of pIκBa. 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 pIκBa destabilization compared with that of pIκBa degradation. Perhaps these related proteins have redundant functions to maintain the important NF-κB signaling pathway.

There are many reports that SCFβTrCP1 is involved in the ubiquitination not only of pIκBa but also of phosphorylated β-catenin (30, 35–37). This is reasonable considering the similarity of the two phosphorylation sites of IκBa and β-catenin (43), although obviously different kinases are involved, respectively i.e. IκBa 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 IκBa, 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 IκBa 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 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 pIκBa (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 IκBa 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, SCFPop1-Ste16, SCFPop1-Sud1, and SCFPop2-Ste16 (47).

We demonstrated here that dimer formation of βTrCP1 and/or βTrCP2 takes place at the D-domain in their NH2-terminal regions. This NH2-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 βTrCP1ΔF and wild-type βTrCP, raising the possibility that dimer formation is a prerequisite of Skp1 binding. However, based on the finding that both βTrCP1ΔD and βTrCP1ΔN 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, βTrCP1ΔN, in contrast to βTrCP1ΔF, did not show significant effect of pIκBa destabilization compared with that of βTrCP1ΔF (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 NH2-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 pIκBa induced by TNF-α, revealing that not only βTrCP1 but also βTrCP2 participates in the ubiquitination-dependent destruction of pIκBa by forming their homodimeric SCFβTrCP1βTrCP1 and SCFβTrCP2βTrCP2 complexes. Considering these differences, we cannot exclude the possibility that SCFβTrCP1βTrCP1 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-Us 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.

Acknowledgments—We are grateful to all other members of the Molecular Medicine Laboratory in Yamanouchi Pharmaceutical Co., Ltd., for encouragement and support.

REFERENCES

1. Baehner, P. A., and Baltimore, D. (1996) Cell 87, 13–20
2. Ghosh, S., May, M., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260

2 H. Suzuki, T. Chiba, T. Suzuki, T. Fujita, T. Ikenoue, M. Omata, K. Furuichi, H. Shikama, and R. Tanaka, unpublished data.
Role of βTrCP1 and βTrCP2 in IκBα Proteolysis

3. Baldwin, A. S. (1996) Annu. Rev. Immunol. 14, 649–681
4. Brown, K., Geratberger, S., Carlson, L., Francesz, G., and Siebenlist, U. (1995) Science 267, 1485–1488
5. Chen, Z. J., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) Genes Dev. 9, 1598–1597
6. Chen, Z. J., Parent, L., and Maniatis, T. (1996) Cell 84, 855–862
7. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554
8. Mercurio, F., Zhu, H., Murray, B. W., Scherer, D., Ballard, D., and Maniatis, T. (1995) Genes Dev. 9, 1598–1597
9. Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11259–11263
10. Baldi, L., Brown, K., Francesz, G., and Siebenlist, U. (1996) J. Biol. Chem. 271, 376–379
11. Traenckner, E. B.-M., Wilk, S., and Baeuerle, P. A. (1994) EMBO J. 13, 5433–5441
12. Lagna, G., Carnevali, F., Marchioni, M., and Hemmati-Brivanlou, A. (1999) Mech. Dev. 80, 101–106
13. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–429
14. Herschko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
15. Ciechanover, A. (1998) EMBO J. 17, 7151–7160
16. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
17. Baumeister, W., Walz, J., Zühl, F., and Seemüller, E. (1998) Cell 92, 367–380
18. Maniatis, T. (1999) Genes Dev. 13, 505–510
19. Jiang, J., and Struhl, K. (1998) Science 281, 493–496
20. Feldman, R. M., Correll, C. G., Kaplan, K. B., and Deshaies, R. J. (1997) Cell 91, 221–230
21. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997) Cell 91, 209–219
22. Yu, Z. K., Gervais, J. L., and Zhang, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11324–11329
23. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, H. (1999) Nat. Cell Biol. 1, 193–199
24. Sutterlin, H., Chatelain, E., Marti, A., Wirbelauer, C., Senftlen, M., Müller, Y., and Ronai, Z. (1999) Nature 401, 9–14
25. Tsvetkov, L. M., Ye, K.-H., Lee, S.-J., Sun, H., and Zhang, H. (1999) Curr. Biol. 9, 661–664
26. Skowyra, D., Depp, K., Kamura, T., Conrad, M. N., Conaway, R. C., Conaway, J. W., Elledge, S. J., and Harper, J. W. (1999) Science 284, 662–665
27. Russell, A., Thompson, M. A., Hendley, J., Trute, L., Armes, J., and Germain, D. (1999) Oncogene 18, 1983–1991
28. Marti, A., Wirbelauer, C., Scheffner, M., and Kreck, W. (1999) Nat. Cell Biol. 1, 14–19
29. Margottin, F., Bour, S. P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K., and Benarous, R. (1998) Mol. Cell 1, 565–574
30. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Anderson, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) Nature 396, 590–594
31. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) Genes Dev. 13, 270–283
32. Spence, E., Jiang, J., and Chen, Z. J. (1998) Genes Dev. 12, 284–294
33. Kroll, M., Margottin, F., Kohl, A., Renard, P., Durand, H., Concordet, J. P., Bacherie, F., Areznaza-Seisdedos, F., and Benarous, R. (1999) J. Biol. Chem. 274, 7941–7945
34. Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, K., Higashi, H., Nakano, H., Okumura, K., Onoe, K., Good, R. A., and Nakayama, K. I. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3839–3843
35. Latres, E., Chiaur, D. S., and Pagano, M. (1999) Oncogene 18, 849–854
36. Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfield, B., Margottin, F., Benarous, R., and Polakis, P. (1999) Curr. Biol. 9, 207–210
37. Kitaoka, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K. I., and Nakayama, K. (1999) EMBO J. 18, 2401–2410
38. Suzuki, H., Chiba, T., Kehayashi, M., Takeuchi, M., Suzuki, T., Ichihara, A., Ikemune, T., Omata, M., Furushiki, K., and Tanaka, K. (1999) Biochem. Biophys. Res. Commun. 256, 121–126
39. Suzuki, H., Chiba, T., Kehayashi, M., Takeuchi, M., Suzuki, T., Ichihara, A., Ikemune, T., Omata, M., Furushiki, K., and Tanaka, K. (1999) Biochem. Biophys. Res. Commun. 256, 127–132
40. Hochstrasser, M. (1996) Genes Dev. 10, 901–907
41. Osaka, F., Kawasaki, H., Aida, N., Sasaki, M., Chiba, T., Kawashima, S., Tanaka, K., and Kato, S. (1998) Genes Cells 3, 221–235
42. Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, K. (1999) Oncogene 18, 6609–6634
43. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) EMBO J. 16, 3797–3804
44. Frey, S. Y., Chen, A., Xiong, Y., Pan, Z.-Q., and Ronai, Z. (1999) Oncogene 18, 2039–2046
45. Kominami, K., and Toda, T. (1997) Genes Dev. 11, 1548–1560
46. Maekawa, H., Kitamura, K., and Shimoda, C. (1998) Curr. Genet. 33, 29–37
47. Kominami, K., Ochotorena, I., and Toda, T. (1998) Genes Cells 3, 721–735
48. Wolf, D. A, McKean, F., and Jackson, P. K. (1999) Curr. Biol. 9, 373–376
49. Patton, E. W., Willems, A. R., and Tyers, M. (1998) Trends Genet. 14, 236–243