Communication

β-TrCP Mediates the Signal-induced Ubiquitination of IκBβ*

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We have examined the role of β-TrCP (β-transducin repeat-containing protein) in the ubiquitination and degradation of IκBβ, one of the two major IκB isoforms in mammalian cells. We demonstrate that β-TrCP interacts specifically with IκBβ, and such interaction is dependent on prior phosphorylation of IκBβ on serines 19 and 23. Interaction with β-TrCP is also necessary for ubiquitination of IκBβ upon stimulation of cells, and deletion of the F-box in β-TrCP abolishes its ability to ubiquitinate IκBβ. Therefore, these results indicate that β-TrCP plays a critical role in the activation of NF-κB by assembling the ubiquitin ligase complex for both phosphorylated IκBα and IκBβ.

The transcription factor NF-κB plays a pivotal role in immune, inflammatory, and stress responses, as well as in early development (1). In nonstimulated cells, NF-κB is retained in an inactive form in the cytoplasm by its interaction with the IκB inhibitory proteins. Mammalian cells contain multiple isoforms of IκB proteins of which IκBα and IκBβ are the best studied (1). Upon stimulation of cells by various cytokines, hormones, or growth factors, a signal-transduction cascade is triggered which leads to the degradation of IκBα and release of NF-κB. The released NF-κB translocates to the nucleus where it up-regulates the transcription of specific target genes (1). The signal-induced degradation of IκB proteins is a critical point in the NF-κB activation pathway. The key step is the phosphorylation of IκB proteins at two specific N-terminal serine residues, which leads to their ubiquitination and subsequent degradation (2). The kinase responsible for specifically phosphorylating IκB is known as the IκB kinase complex (IKK) and contains two catalytic components, IKKα and IKKβ (3–9). The IKKs phosphorylate IκBα at serines 32 and 36 and mark it for degradation through the ubiquitin-proteasome system. Mutation of either serine residue makes IκBα resistant to phosphorylation and degradation (reviewed in Ref. 2).

During ubiquitin-dependent degradation, ubiquitin mole-
cules activated by ubiquitin-activating enzyme E1 are attached to specific lysine residues on the target protein by a ubiquitin-conjugating enzyme (E2), together with a ubiquitin ligase (E3) that is specific for the substrate (10). Assembly of polyubiquitin chains on the substrate protein targets it for degradation by the 26 S proteasome (10). Recently an F-box/WD40 protein called β-TrCP (β-transducin repeat-containing protein) was shown to be the substrate-recognition component of the ubiquitin ligase responsible for phosphorylation-dependent ubiquitination of IκBα (11–15). β-TrCP recognizes IκBα phosphorylated at Ser-32 and Ser-36 through its WD40 domain, whereas the F-box motif recruits additional proteins including Skp1 and Cullin to form the Skp1-cullin-F-box (SCF) ubiquitin ligase complex (16). β-TrCP belongs to a growing family of proteins containing F-boxes that are involved in assembling the SCF complex. Aside from the F-box, these proteins have another protein-protein interaction module in their C terminus, namely a WD or leucine-rich repeat (LRR) domain. These C-terminal domains mediate the interaction of SCF complexes with their substrates and determine specificity of substrate recognition. β-TrCP has been implicated in the ubiquitination of CD4 (through HIV protein Vpu) (17), IκBα (11–15), and β-catenin (14, 18–20). All these proteins share similar N-terminal inducible phosphorylation sites with the consensus sequence of DSGXXS (X represents a hydrophobic residue and S represents any amino acid.). Therefore, the inducible phosphorylation of these N-terminal serine residues is the critical step that allows recruitment of β-TrCP and subsequent ubiquitination of these proteins (16).

IκBβ, like IκBα, is also believed to undergo signal-induced phosphorylation, ubiquitination, and degradation (21–23). The critical phosphorylation sites are serines 19 and 23 because mutation of these amino acids prevents signal-induced degradation of IκBβ (21). IκBβ can also be phosphorylated in vitro by IKKα and IKKβ (7). However, unlike IκBα, direct phosphorylation of IκBβ at serines 19 and 23 in vivo is yet to be demonstrated (8). Instead, one study has reported that serines 19 and 23 of IκBβ are constitutively phosphorylated in unstimulated cells, suggesting that the regulation of IκBβ might differ more fundamentally from that of IκBα (24). Because phosphorylation of IκBβ by the IKKs does not induce a mobility shift in SDS-PAGE, and in vivo labeling experiments have been inconclusive, signal-induced phosphorylation of IκBβ remains to be unequivocally demonstrated.2 IκBβ also differs from IκBα in other aspects (1, 22, 23). For example, while IκBα responds to all NF-κB inducers, in certain cell-types IκBβ responds only to a subset of them (22, 24). Also, when stimulated by the same inducers, the kinetics of IκBβ degradation is significantly slower than IκBα (22, 24). The underlying mechanism responsible for these differences is unclear although one possible explanation for the slower kinetics of IκBβ degradation might be lower efficiency of ubiquitination of phosphorylated IκBβ. Understanding the details of the pathway by which phosphorylated IκBβ is ubiquitinated and degraded is therefore important for fully decoding the differential regulation of IκBα and IκBβ. The identification of β-TrCP as the recognition element of IκBα ubiquitin-ligase provides an opportunity to directly test whether IκBβ also undergoes signal-induced phosphorylation.

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1 The abbreviations used are: IκB, IκB kinase complex; β-TrCP, β-transducin repeat-containing protein; SCF, Skp1-cullin-F-box; HIV, human immunodeficiency virus; RT-PCR, reverse transcriptase polymerase chain reaction; TNF, tumor necrosis factor; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence.
2 S. Ghosh, unpublished results.
and degradation, and whether it is mediated through β-TrCP.

We report in this manuscript that β-TrCP specifically interacts with IκBβ in stimulated cells. This interaction requires serines 19 and 23 because mutation of these residues completely abolishes this interaction. We also demonstrate that phosphorylation-induced ubiquitination of IκBβ requires the F-box of β-TrCP, suggesting that both IκBα and IκBβ appear to be ubiquitinated and degraded through the same pathway. Therefore the differential regulation of IκBα and IκBβ is most likely because of differences in other steps in the activation pathway of NF-κB.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures, Antibodies, and Reagents**—293, HeLa, and COS cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The anti-flu mouse monoclonal antibody (12CA5) was produced and purified in this laboratory. Anti-flag monoclonal antibody M5 and anti-flag M2 affinity gel were purchased from Sigma. All other antibodies were purchased from Santa Cruz Biotechnology. Protein A-Sepharose was purchased from Amersham Pharmacia Biotech.

**Cloning of Human β-TrCP and β-TrCPΔF**—Total RNA was isolated from HeLa cells using TRIzol™ Reagent (Life Technologies, Inc.) and used for RT-PCR to amplify β-TrCP cDNA with appropriate 5' and 3' primers. A FLAG-epitope coding sequence was inserted after the starting codon. The 1.7-kilobase PCR product was subcloned into BamHI and XbaI sites of pCDNA3 (Invitrogen) and sequenced.

To delete the F-box from β-TrCP, two internal primers were designed to flank the boundary sequences outside of F-box region and used individually with the 5' or 3' primers described above to amplify the N terminus or C terminus of β-TrCP. The 500-base pair N-terminal and 1.1-kilobase C-terminal PCR products were then used as templates in the sequential PCR with 5' and 3' primers. The resulting β-TrCPΔF was subcloned into BamHI-XbaI sites of pCDNA3, and its sequence was confirmed by DNA sequencing.

**Luciferase Assay**—Subconfluent 293 cells were transfected with 500 ng of pBIIX luciferase reporter construct, along with different amounts of β-TrCP and β-TrCPΔF constructs. The total transfected DNA amounts were equalized with empty pCDNA3 vector. After 36 h, cells were treated with or without 20 ng/ml TNF-α for 4 h before harvest for luciferase assay (Promega).

**Transfection, Immunoprecipitation, and Immunoblotting**—Cells were grown in 10 centimeter plates to 40% confluence and transfected with indicated DNA using FuGENE™ 6 (Roche Molecular Biochemistry). After incubation for 36 h, cells are treated with or without TNF-α (10 ng/ml) for 30 min before being lysed with TNT lysis buffer (200 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1% Triton-X100) supplemented with protease inhibitors. In immunoprecipitation experiments, cell lysates were incubated with 20 μl of anti-flag M2 affinity gel for 3 h or 10 μl of anti-IκBβ (C-20) along with 20 μl of protein A-Sepharose for 4 h at 4°C. Immobilized immunocomplexes were washed with TNT three times, boiled in SDS loading buffer, and resolved on 10% SDS-PAGE. Proteins were transferred to Immobilon transfer membrane (Millipore Corp.) and blotted with indicated primary antibody for 3 h at room temperature and appropriate secondary antibody for 1 h. Immunoreactive bands were visualized by ECL.

**RESULTS AND DISCUSSION**

**β-TrCP Is Involved in NF-κB Activation and β-TrCPΔF Acts As a Dominant Negative Regulator of NF-κB Activation**—Human β-TrCP was cloned using RT-PCR from HeLa cells with primers designed according to the published protein sequence. An F-box deletion mutant, β-TrCPΔF, was generated by deleting the F-box region from leucine 148 to leucine 192 (Fig. 1). Therefore the differential regulation of IκBα and IκBβ is most likely because of differences in other steps in the activation pathway of NF-κB.

We report in this manuscript that β-TrCP specifically interacts with IκBβ in stimulated cells. This interaction requires serines 19 and 23 because mutation of these residues completely abolishes this interaction. We also demonstrate that phosphorylation-induced ubiquitination of IκBβ requires the F-box of β-TrCP, suggesting that both IκBα and IκBβ appear to be ubiquitinated and degraded through the same pathway. Therefore the differential regulation of IκBα and IκBβ is most likely because of differences in other steps in the activation pathway of NF-κB.

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**β-TrCPΔF Acts As a Dominant Negative Regulator of NF-κB Activation**—In immunoprecipitation experiments, cell lysates were incubated with anti-flag M2 affinity gel for 3 h at 4°C, and the immunocomplex was analyzed on 10% SDS-PAGE and blotted with anti-IκBα (C-21) (upper panel). A fraction of cell lysate of each sample was immunoblotted against anti-IκBα as equal loading control (middle panel) or against anti-flag monoclonal antibody M5 to test β-TrCP construct expression (lower panel). Immunoprecipitation analysis of the interaction of IκBβ with β-TrCP in transfected HeLa cells (experimental procedure was similar to that for panel A). HeLa cells were transfected with wild type (wt) β-TrCP, along with wild type or mutant flu-tagged IκBβ constructs as indicated. SS/AA has two alanine residues in place of serines 19/23; SS/DD contains aspartates at serines 19/23. Cell lysates were immunoprecipitated with anti-flag gel and blotted with anti-flu monoclonal antibody (upper panel). Immunoblot analysis of the cell lysates is shown in the lower panel.

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Fig. 3. β-TrCP, but not β-TrCPΔF, promotes IKK-dependent ubiquitination of IkBβ. A, COS cells were transfected with flag-tagged wild type IkBβ, pcDNA3 (-) or β-TrCP constructs as indicated, along with flag-tagged IKKβ in lanes 2, 4, and 6. Cell lysates were incubated with anti-flag M2 affinity gel, and the immunoprecipitated complex was resolved on SDS-PAGE and blotted with anti-IκBα in upper panel or anti-ubiquitin monoclonal antibody in middle panel. IKKβ expression was checked in lower panel by Western blot with anti-flu antibody. B, the experiment was similar to that in Fig. 3A, lanes 3 and 4. COS cells were transfected as indicated, immunoprecipitated with anti-flag antibody, and immunoblotted with anti-IκBα/C-20 antibody. After ECL reaction, the blot was exposed for significantly longer time than that for panel A. C, COS cells were transfected with IkBβ, IKKβ, and β-TrCP constructs as indicated. Immunoprecipitation was carried out with protein A-Sepharose and anti-IκBβ/C-20 antibody. Bound proteins were immunoblotted with anti-ubiquitin antibody (upper panel). Protein expression in the transfected cells was analyzed, and immunoblotting results are shown in middle (β-TrCP, immunoblotted with anti-flag antibody) and lower (IkBβ, immunoblotted with anti-flu antibody) panels.

previously (11–15), introduction of β-TrCPΔF into the cells significantly inhibited NF-κB activation in a concentration-dependent manner (data not shown). The F-box motif has been found to be important for associating with Skp1, which in turn binds to Cullin and an E2 enzyme to form a functional ubiquitin-conjugating complex. Therefore the β-TrCPΔF construct would bind to phosphorylated IkB but fail to recruit the other components, thus inhibiting the degradation of IkB and activation of NF-κB. Surprisingly, we also found that transfection of wild type β-TrCP also inhibited NF-κB activation, although to a lesser extent than the F-box deletion mutant (data not shown). The explanation for this observation is unclear, but one possibility is that overexpression of β-TrCP results in the accelerated degradation of some other component that is required for activation and nuclear translocation of NF-κB.

β-TrCP bind to Phosphorylated IkBβ—To examine whether β-TrCP directly interacts with phosphorylated IkBβ, we conducted immunoprecipitation experiments in transfected cells. IkBα and IkBβ were transfected into 293 and HeLa cells respectively, along with either FLAG-tagged wild type β-TrCP or FLAG-tagged TrCPΔF construct. Cells were incubated with the proteasome inhibitor, calpain inhibitor 1, before treatment with TNF-α. Cell lysates were precipitated with anti-flag affinity gel, and the immobilized immunocomplex was immunoblotted for IkBα or IkBβ. The experiment confirmed that interaction between IkBα and β-TrCP is only observed in TNF-α stimulated cells (Fig. 2A). Similarly, IkBβ failed to associate with β-TrCP in unstimulated cells, but bound efficiently to β-TrCP in TNF-α stimulated cells (Fig. 2B). In both instances, deletion of the F-box deletion did not affect the ability of β-TrCP to interact with IkBα or IkBβ (Fig. 2, A and B). This result is therefore consistent with the notion that β-TrCP interacts with its phosphorylated substrate through its WD domain, and this binding is independent of the F-box.

Treatment with TNF-α has been shown to cause the phosphorylation of IkBα at the N-terminal serine residues 32 and 36 (21). It has been implied, but not directly demonstrated, that IkBβ is also inducibly phosphorylated on serines 19 and 23 (2). To ascertain whether β-TrCP binds only to IkBα phosphorylated at serines 19 and 23, we used an IkBβ mutant, IkBβ19/23 SS/AA, in which serines 19 and 23 were replaced with nonphosphorylatable alanines. As shown in Fig. 2C, IkBβ19/23 SS/AA mutant failed to associate with β-TrCP even upon TNF-α treatment. This confirmed that the interaction between IkBβ and β-TrCP is contingent upon prior phosphorylation of IkBβ at the N terminus. Interestingly, an IkBβ19/23 SS/DD mutant in which the two serines were substituted by aspartic acid, to mimic the phosphorylated state, also failed to bind to β-TrCP or TrCPΔF. This experiment demonstrates the stringent substrate specificity of β-TrCP for phosphate groups in the IkB proteins. Similar specificity of interaction had been observed in earlier studies where it was demonstrated that only a phosphopeptide encompassing the IkBα degradation motif, but not a serine-to-glutamate-substituted peptide, could compete with intact IκBα for ubiquitin conjugation (15).

β-TrCP Promotes Ubiquitination of Phosphorylated IkBβ in Vivo—To directly assess whether β-TrCP is a component of the ubiquitin ligase for IkBβ, we transfected COS cells with wild type or ΔF mutant β-TrCP along with HA(flu)-tagged IKKβ. COS cells appear to lack certain components in the signaling
pathways leading to NF-κB activation and hence do not respond to traditional inducers of NF-κB. Therefore to help bypass this difficulty, we co-transfected HA(flu)-tagged IKKβ, FLAG-tagged wild type or ΔF mutant β-TRCP, and IκB. The IκB binding to β-TRCP was analyzed by immunoprecipitation of β-TRCP with anti-FLAG antibody, followed by immunoblotting with IκB antisera. Under these conditions, where transfection of IKKβ presumably led to continuous phosphorylation of IκB proteins, multiple IκB bands with increasing molecular weights were detected in β-TRCP transfected cells. (Fig. 3A, upper panel, lane 4). In contrast, only a single band corresponding to IκBβ was observed in cells transfected with the dominant negative β-TRCPΔF construct (Fig. 3A, upper panel, lane 6). Although we could detect forms of IκBβ containing one or two ubiquitin molecules using the IκBβ antibody, we did not observe polyubiquitinated forms under these experimental conditions. Because the levels of polyubiquitinated IκB forms are very low, probably because they are rapidly degraded, we repeated the experiment and exposed the ECL blot for significantly longer periods. Under these conditions, we could detect low amounts of higher molecular weight forms of IκBβ that probably represent polyubiquitinated forms of the protein (Fig. 3B). To further characterize the higher molecular weight IκBβ immunoreactive bands, we immunoblotted the anti-flag-immunoprecipitated complexes with ubiquitin antibody. In contrast to the immunoblot with the IκBβ antibody, the slower migrating bands were readily detected with the ubiquitin antibody (Fig. 3A, middle panel, lane 4). The explanation for why the ubiquitin antibody detects the higher molecular weight species more readily is probably because of the far greater number of epitopes that are presented by the polyubiquitinated forms. As expected, deletion of the F-box in β-TRCP (β-TRCPΔF) almost completely blocked the formation of ubiquitin-IκBβ conjugates (Fig. 3A, middle panel, lane 6). Therefore in cells transfected with IKKβ, β-TRCP is directly involved in IκBβ ubiquitination.

To further confirm that β-TRCP promotes the ubiquitination of IκB, whereas the β-TRCPΔF suppresses it, we examined the state of IκBβ in β-TRCP and β-TRCPΔF transfected cells by directly immunoprecipitating IκBβ itself. Transfection of either IκBβ or IκBβ along with β-TRCP does not lead to significant ubiquitination of IκBβ (Fig. 3C, upper panel, lanes 1 and 2). However, upon activation by IKKβ transfection, IκBβ is polyubiquitinated (Fig. 3C, upper panel lane 3). Transfection of wild type β-TRCP significantly increased the level of ubiquitination of IκBβ (Fig. 3C, lane 4), whereas cells transfected with β-TRCPΔF failed to generate ubiquitinated forms of IκBβ (Fig. 3C, lane 5). The ubiquitination of IκBβ is dependent on serines 19 and 23 because a mutant IκBβ containing alanines in these positions could not be ubiquitinated (Fig. 3C, lane 6). Therefore these observations are in agreement with earlier results examining the binding of mutant IκBβ with β-TRCP (Fig. 2, B and C).

In summary, we report that β-TRCP binds specifically to the inducibly phosphorylated IκBβ and promotes its ubiquitination. Our findings further help establish the role of β-TRCP as a component of the ubiquitin ligase for IκB proteins, and demonstrate that the signal-induced phosphorylation of IκBβ by IKKs is a critical step that precedes their ubiquitination and degradation. Therefore, differences in the regulation of IκBa and IκBβ must be because of differences in other steps in the pathway. For example, it is possible that IκBβ complexes contain an additional regulatory component that determines the rate of degradation of ubiquitinated IκBβ proteins, thus explaining their slower rate of degradation. Alternatively, such an associated regulatory protein may influence the ability of IκBβ to be efficiently phosphorylated by IKK. Finding the answers to these questions remains a challenge for the future.

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