Inducible Degradation of IκBα by the Proteasome Requires Interaction with the F-box Protein h-βTrCP*

(Received for publication, December 28, 1998, and in revised form, January 28, 1999)

Mathias Kroll†‡, Florence Margottin†, Alain Kohl†, Patricia Renard‡, Hervé Durand‡, Jean-Paul Concordet**, Françoise Bachelier†, Fernando Arenzana-Seisdedos†, and Richard Benarous†‡‡

From the †Unité d’Immunologie Virale and ||Groupe des Bunyaviridae, Unité des arbovirus et virus des fièvres hémorragiques, Institut Pasteur, 25 et 28, rue du Dr. Roux, 75724 Paris Cedex 15, France and the ‡Institut Cochin de Génétique Moléculaire (ICGM) INSERM U670, and the ¶Istitut Cochin de Génétique Moléculaire (ICGM) INSERM U670, Université Paris 7, 75014 Paris, France

NF-κB transcription factor is regulated by IκB proteins of which IκBα is the main and best characterized member (1–3). Proteasome-mediated degradation of IκBα releases NF-κB and allows its localization in the nucleus (4–9). Phosphorylation of Ser32–Ser36 residues and subsequent ubiquitination of IκBα are prerequisites to make the protein susceptible to proteasome attack (10–13). While the kinase complex accounting for IκBα phosphorylation has been recently characterized (14–20), factors necessary for ubiquitination and targeting of IκBα to the proteasome remain unknown. Covalent attachment of polyubiquitin to substrate proteins involves a cascade of ubiquitination reactions with E1, a ubiquitin-activating enzyme, and E2 a ubiquitin-conjugating enzyme that operates in conjunction with a specificity factor E3 (21–26). It has been suggested that E3 functions in substrate recognition and E2 positioning. Although E2 enzymes belonging to the Ub-ε/Ubc5 family can ubiquitinate IκBα in vitro, the E3 responsible for the signal-induced ubiquitination of IκBα remains to be identified (10, 27). A novel class of E3 is represented by the Skp1-Cullin-F-box protein complexes (SCFs) (28–33). The core component of these newly identified E3s is Skp1, which assembles with different F-box proteins and has been shown in human cells to interact selectively with CUL-1, but not with other Cullin proteins belonging to the Cdc53 family (34–36). The role of the F-box proteins in these SCF complexes is to recruit phosphorylated substrate proteins to trigger their ubiquitination (28–33). Like the other members of the F-box protein family, human βTrCP, which we recently identified (37), has a modular organization with an F-box motif involved in proteasome targeting through interaction with Skp1, and a seven WD repeats binding domain for interaction with substrate proteins (see Fig. 1A). We hypothesized that βTrCP could be the F-box adaptor protein allowing recruitment of IκBα by a SCF E3 ubiquitin-protein ligase complex that ubiquitinates IκBα and makes it a substrate for degradation by the proteasome.

**Experimental Procedures**

Cell lines, Transfections, and Infections—Subconfluent cells of the 293 human embryo kidney cell line were transfected by LipofectAMINE™ Plus (Life Technologies, Inc.) with the indicated reporter plasmids and pcDNA3 vectors expressing βTrCP proteins or with no βTrCP insert. Fluorigenic substrate luciferin served to quantify luciferase reporter gene expression in cytoplasmic extracts obtained by lysis in phosphate buffer containing 1% Nonidet P-40. The pcDNA3-βTrCP and βTrCPAF constructs are described in Ref. 37. βTrCP and βTrCPAF coding sequences were amplified by polymerase chain reaction and inserted in fusion with the Myc/His double tag in the pcDNA3.1 Myc/ Hisa vector (Invitrogen). SV5-tagged wild type or SV5-tagged S32A/S36A phosphorylation-deficient mutant IκBα were described in Ref. 11, 3Enh-κB-ConA and ConA luciferase reporter plasmids are described in Ref. 38. RSV luciferase reporter plasmid was purchased from Invitrogen. βTrCP or IκBα were inserted in fusion with the LexA DNA binding domain and the Gal4 activation domain, respectively, as described in Ref. 37. Construction and use of recombinant SFV was carried out as described before (39–42). Briefly, 293 cells were infected at a multiplicity of 5 for 6 h in serum-free medium with SFV particles carrying myc-tagged βTrCP proteins. Expression of βTrCP proteins and viral nucleocapsid protein was verified by Western blot analysis, and 100% infection efficiency was confirmed by immunofluorescence (data not shown). The same results as those shown in Fig. 3B were obtained in HeLa cells infected with SFV.

Antibodies and Reagents—The 10B monoclonal antibody directed against the amino terminus of IκBα was described in Ref. 43; the polyclonal antibody specifically recognizing IκBα phosphorylated at serine residue 32 is from New England Biolabs (9241S), and the monoclonal anti-myc antibody was from Santa Cruz Biotechnology (hybridoma 9E10, SC-40). For co-immunoprecipitation experiments (Fig. 3, A and B), 200 μg of cytoplasmic extract were incubated with anti-myc-agarose conjugates (SC-40 AC from Santa Cruz) for 60 min at 4 °C. Precipitated beads were washed 10 times in phosphate-buffered saline containing 1% Nonidet P-40 and both protease and phosphatase inhibitors. Antibody-antigen complexes were disrupted by boiling in gel loading buffer (Pierce). Precipitated proteins were fractionated by SDS-
polycrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes.

Electrophoretic Mobility Shift Assay—The electrophoretic mobility shift assay was performed with 4 μg of nuclear extract incubated for 15 min at room temperature with a [γ-32P]ATP-labeled, double-stranded oligonucleotide containing the HIV-1 long terminal repeat binding site for NF-κB (5′-ACAAAGGAGCTTCTCCTGGGACTTTCAGGGA-3′). Samples were analyzed in nondenaturing 6% polyacrylamide gels. Competition experiments were performed by adding a 40-fold molar excess of homologous, unlabeled oligonucleotide to each sample prior to analysis of cytoplasmic extracts to ensure that wild type and mutant βTrCP proteins were expressed at comparable levels (not shown). After transfection, cells were left untreated (NS) or were stimulated with either 5 ng/ml TNF or 75 ng/ml OKA for 6 h. Experiments were repeated three times in 293 cells and confirmed in HeLa cells. Results of a representative experiment are shown. Luciferase activity is expressed as relative luciferase units (RLU) per μg of protein and results from subtracting the background signal from the values obtained for each sample. Comparative electrophoretic mobility shift assay of nuclear extracts from HeLa cells expressing either βTrCP or the βTrCPΔF from SFV. A radiolabeled oligonucleotide encoding the NF-κB consensus was used as a probe to bind transcription factors. A SFV replicon without insert was used as a control. The asterisk indicates where competitor cold oligonucleotide was added to demonstrate the specificity of NF-κB/DNA interaction.

RESULTS AND DISCUSSION

To investigate the putative role of βTrCP in the regulation of NF-κB activation, we first assessed the effect of wild type (βTrCP) and a F-box deleted βTrCP (βTrCPΔF) (Fig. 1A) on the transcriptional activity of a NF-κB-dependent (3Enh-κB-ConA) promoter driving a luciferase reporter gene (10, 11). Expression of βTrCP resulted in a 2- to 3-fold increase in the activity of the 3Enh-κB-ConA promoter in cells of the 293 human embryo kidney cell line stimulated by either tumor necrosis factor (TNF) or okadaic acid (OKA), two well-characterized inducers of NF-κB (Fig. 1B, left panel). Similarly, the low level of constitutive activation of NF-κB observed in unstimulated cells (compare 3Enh-κB-ConA to ConA) is also enhanced by expression of βTrCP (Fig. 1B, left panel). In sharp contrast to βTrCP, expression of the βTrCPΔF mutant massively and consistently inhibited NF-κB-dependent transcription by more than 90% of the levels induced in TNF- or OKA-stimulated cells transfected with an insertless, control plasmid (pCDA3) (Fig. 1B, left panel). Importantly, expression of the transdominant negative mutant βTrCPΔF fully prevented localization of NF-κB to the nucleus upon cell activation. This finding is in keeping with an increased stability of inhibitor IκB proteins that anchor NF-κB in the cytoplasm in an inactive form. The failure of βTrCPΔF to modify the activity of a RSV promoter indicates that βTrCPΔF does not affect NF-κB independent mechanisms of transcription (Fig. 1B, left panel).

Thus, on the one hand, the capacity of βTrCP to enhance both basal or signal-induced activation of NF-κB and, on the other hand, the specific inhibition of NF-κB dependent transcription promoted by βTrCPΔF, strongly suggest that βTrCP is an essential component of the NF-κB activation pathway. In support of this assumption, we found that, concomitant with the enhancement of NF-κB-dependent transcription, overexpression of βTrCP in 293 cells stimulated by TNF induced an accelerated degradation of IκBa (Fig. 2A, middle panel). In contrast, expression of the βTrCPΔF mutant either from a eukaryotic vector (Fig. 2A, right panel) or a SFV replicon (Fig. 2B, right panel), stabilized IκBa and delayed the kinetics of IκBa degradation. Moreover, the presence of βTrCPΔF promoted the accumulation of slow migrating forms of IκBa characteristic of the phosphorylation of residues Ser32 and Ser36 required for subsequent ubiquitination and degradation of IκBa by the proteasome (Fig. 2, A and B, right panel). Thus, these findings exclude an inhibitory effect of βTrCPΔF in the transduction pathway leading to phosphorylation of IκBa and suggest that stabilization of IκBa is due to the blockade of a phosphorylation event in the metabolism of the inhibitor.

Slow migrating forms of IκBa predominated following induction with TNF but could even be detected in unstimulated cells (Fig. 2, A and B, right panel, time 0). This latter phenomenon likely reflects the inhibitory effect of βTrCPΔF on basal breakdown of IκBa and is in keeping with the low and constitutive NF-κB-dependent transcription observed in 293 cells (Fig. 1B, left panel).

Characterization of the slow migrating hand of IκBa (Fig. 2A, right top panel), as a phosphorylated form of IκBa accumulated

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Inducible IκBα Degradation Involves F-box Protein h-βTrCP

A, stability of IκBα analyzed in 293 cells exposed to TNF for different lengths of time in the presence of βTrCP proteins. 293 cells were transiently transfected with βTrCP, βTrCPΔF, or control pcDNA vectors. After 36 h, cells were stimulated with TNF in the presence of 100 μg/ml cycloheximide, the protein synthesis inhibitor. Cytoplasmic proteins were separated in SDS-denaturing polyacrylamide gels, transferred onto nitrocellulose membrane, and probed with the 10B monoclonal antibody directed against the amino terminus of IκBα (top panel), a polyclonal antibody specifically recognizing IκBα phosphorylated at serine residue 32 (α-IκBα-S32-P antibody, middle panel), or a monoclonal anti-myc antibody (bottom panel) directed against the carboxyl-terminal myc tag of heterologous βTrCP proteins (see Ref. 13 for antibodies). Immunodetection was performed using an ECL chemiluminescence kit (Amer sham Pharmacia Biotec). B, stability of IκBα in TNF-treated HeLa cells infected with Semliki forest virus repli cons (SFV) (12) expressing the βTrCPΔF mutant (βTrCPΔF) or not (SFV control). After 6 h of infection, cells were cultured with cycloheximide and stimulated with TNF for the indicated times. Cytoplasmic extracts were processed as described above and probed with the 10B antibody. C, specific recognition of Ser28–Ser36 phosphorylated IκBα by the α-IκBα-S32-P antibody (13). Western blot analysis of recombinant (rIκBα, 10 ng) and cytoplasmic IκBα obtained from HeLa untreated (–) or treated with TNF for 15 min. Left, detection of IκBα proteins with 10B antibody. Right, detection with the α-IκBα-S32-P antibody.

in βTrCPΔF expressing cells, was accomplished (Fig. 2A, middle row of panels) using a polyclonal antibody that does not recognize the unphosphorylated form of IκBα but specifically reacts with Ser32–Ser36–phosphorylated IκBα which accumulates in the presence of the proteasome inhibitor z-LLL-t (Fig. 2C) (44).

To ascertain whether the regulatory effect of βTrCP on IκBα metabolism requires association with IκBα, carboxy-terminal c-myc-tagged variants of either βTrCP or βTrCPΔF were expressed in HeLa cells from SFV (Fig. 3A) or in 293 cells from eukaryotic expression vectors (Fig. 3B), and their co-precipitation with IκBα was investigated. Cytoplasmic extracts of cells treated or not with TNF and the proteasome inhibitor z-LLL-t, were incubated with an anti-myc tag antibody bound to protein A.

FIG. 2. Expression of the βTrCPΔF mutant inhibits degradation and promotes accumulation of Ser32–Ser36 phosphorylated IκBα. A, stability of IκBα analyzed in 293 cells exposed to TNF for different lengths of time in the presence of βTrCP proteins. 293 cells were transiently transfected with βTrCP, βTrCPΔF, or control pcDNA vectors. After 36 h, cells were stimulated with TNF in the presence of 100 μg/ml cycloheximide, the protein synthesis inhibitor. Cytoplasmic proteins were separated in SDS-denaturing polyacrylamide gels, transferred onto nitrocellulose membrane, and probed with the 10B monoclonal antibody directed against the amino terminus of IκBα (top panel), a polyclonal antibody specifically recognizing IκBα phosphorylated at serine residue 32 (α-IκBα-S32-P antibody, middle panel), or a monoclonal anti-myc antibody (bottom panel) directed against the carboxyl-terminal myc tag of heterologous βTrCP proteins (see Ref. 13 for antibodies). Immunodetection was performed using an ECL chemiluminescence kit (Amer sham Pharmacia Biotec). B, stability of IκBα in TNF-treated HeLa cells infected with Semliki forest virus repli cons (SFV) (12) expressing the βTrCPΔF mutant (βTrCPΔF) or not (SFV control). After 6 h of infection, cells were cultured with cycloheximide and stimulated with TNF for the indicated times. Cytoplasmic extracts were processed as described above and probed with the 10B antibody. C, specific recognition of Ser32–Ser36 phosphorylated IκBα by the α-IκBα-S32-P antibody (13). Western blot analysis of recombinant (rIκBα, 10 ng) and cytoplasmic IκBα obtained from HeLa untreated (–) or treated with TNF for 15 min. Left, detection of IκBα proteins with 10B antibody. Right, detection with the α-IκBα-S32-P antibody.

FIG. 3. βTrCP interacts with Ser32–Ser36 phosphorylated but not with unmodified or phosphorylation-deficient S32A/S36A IκBα. A, βTrCP selectively co-precipitates phosphorylated IκBα proteins. HeLa cells were infected as described in Fig. 2B with SFV expressing a β-galactosidase (βGal), wild type (βTrCP), or ΔF (βTrCPΔF) myc-tagged proteins (11). Before harvesting, cells were exposed to TNF for 15 min in the presence of the proteasome inhibitor (z-LLL-t + TNF) or left untreated (–). Proteins from total cytoplasmic lysates were either separated in denaturing gels and probed with a α-IκBα-S32-P antibody (top panel) or immunoprecipitated (IP α-Myc) with myc antibody-agarose conjugates (13). Precipitated proteins were probed with either the α-IκBα-S32-P antibody (middle panel) or the 10B monoclonal antibody (α-IκBα, bottom panel) that recognizes both unmodified and phosphorylated IκBα. The migration pattern of anti-myc-precipitated (IP α-Myc) IκBα is compared with that of IκBα in total cytoplasmic extracts (control HeLa cell extracts, purchased from New England Biolabs) of untreated (–) or TNF-treated (+) HeLa cells detected by either α-IκBα-S32-P or α-IκBα antibodies (right, panel A). B, βTrCP proteins fail to co-precipitate with a S32A/S36A phosphorylation-deficient IκBα mutant. 293 cells were co-transfected with pcDNA-βTrCP or βTrCPΔF DNA vectors and either SV5-tagged wild type (lanes 1–6) or SV5-tagged S32A/S36A phosphorylation-deficient mutant IκBα proteins (lanes 7–10) deficient in cell activation-induced serine phosphorylation (11). A pcDNA3 plasmid expressing β-galactosidase was used as a control (lanes 1 and 2). Cytoplasmic proteins were precipitated by anti-myc antibodies and probed (WB) with α-IκBα-S32-P or α-IκBα antibodies indicated under the gels. Migration of either tagged (SV5-IκBα) or endogenous (End-IκBα) IκBα is indicated. C, interaction of βTrCP with wild type but not with the phosphorylation-deficient S32A/S36A IκBα in the two-hybrid system. βTrCP proteins or control Ras proteins were fused to the Escherichia coli LexA binding domain. IκBα proteins or control Ras proteins were fused to the Gal4 activation domain. The yeast reporter strain L40 expressing the indicated hybrid protein pairs was analyzed for histidine auxotrophy and β-galactosidase expression. D, wild type IκBα is constitutively phosphorylated in yeast. L40 strain yeast colonies expressing the Gal4 activation domain fused to Raf, IκBα wild type, or IκBα S32A/S36A were lysed, and protein extracts were blotted onto nitrocellulose. Left, detection with α-IκBα antibody. Right, detection with α-IκBα-S32-P antibody.
A-coated agarose beads. Proteins precipitated by the anti-myc antibody were probed with anti-IκBα antibodies (Fig. 3A, middle and bottom panels). In cells expressing either the wild type or the mutated counterpart of βTrCP, a single band of co-precipitated IκBα was recognized by a monoclonal antibody that detects both native and phosphorylated forms of IκBα (Fig. 3A, bottom panel, lanes 4–6). This band migrates with a pattern characteristic of the typical upshift induced by phosphorylation of IκBα Ser\textsuperscript{32} and Ser\textsuperscript{36}. Western blot analysis using the antibody specifically recognizing the phosphoserine Ser\textsuperscript{32}–Ser\textsuperscript{36} IκBα confirmed that the proteins detected are phosphorylated at the critical residues that permit subsequent ubiquitination of IκBα (Fig. 3A, middle panel, lanes 4–6). No unphosphorylated form of IκBα was detected in immunoprecipitates (Fig. 3A, lower panel). Detection of phosphorylated IκBα did not require TNF induction when the mutant βTrCP\textsuperscript{AF} was expressed (Fig. 3A, middle panel, lane 5). Moreover, larger amounts of endogenous phosphorylated IκBα co-precipitated with βTrCP\textsuperscript{AF}, compared with wild type βTrCP (Fig. 3A, compare lanes 6 with lane 4), confirming that the βTrCP\textsuperscript{AF} mutant acts as a transdominant negative regulator of both constitutive and TNF-induced proteolysis of IκBα.

To confirm the selective association of phosphorylated IκBα and βTrCP, we performed experiments using a S32A/S36A mutant of IκBα lacking the capacity to be phosphorylated by cell activation signals promoting NF-κB activation (3). The presence of a 15-amino acid SV5 carboxyl terminus tag allows distinction of endogenous from transiently expressed wild type or S32A/S36A IκBα. Despite expression of similar amounts of wild type or S32A/S36A-SV5 tagged proteins (data not shown), only the endogenous and wild type SV5-tagged IκBα (Fig. 3B, lanes 4–6), but not the SV5-S32A/S36A phosphorylation-deficient mutant (Fig. 3B, lanes 7–10) were able to associate with either βTrCP or βTrCP\textsuperscript{AF}. Expression of either wild type or phosphorylation-deficient tagged IκBα proteins from SV5 was consistently detected in more than 90% of cells (data not shown). The high infection efficiency of this system allows us to conclude that co-precipitation of the endogenous, but not the S32A/S36A-SV5 IκBα (Fig. 3B, lanes 7–10), reflects the incapacity of the phosphorylation-deficient mutant to compete for binding to IκBα.

Further evidence of IκBα interaction with βTrCP was provided by the yeast two-hybrid system (carried out as described previously (37)). We observed that βTrCP fused to the LexA DNA binding domain (LexA-βTrCP) associates specifically with IκBα fused to the Gal4 activation domain (Gal4AD-IκBα), as detected by histidine auxotrophy or β-galactosidase expression (Fig. 3C). The interaction between IκBα and βTrCP is likely accounted for by the existence in yeast of phosphorylated IκBα as shown by the recognition of the Gal4AD-IκBα hybrid by the antibody that specifically reacts with Ser\textsuperscript{32}–Ser\textsuperscript{36} phosphorylated IκBα (Fig. 3D). This hypothesis is reinforced by the fact that the Gal4AD-IκBα S32A/S36A mutant, which is not recognized by the anti-phosphoserine IκBα antibody (Fig. 3D), did not associate with LexA-βTrCP (Fig. 3C).

We have previously documented that βTrCP is a component of a SCF complex involved in HIV-1 Vpu-mediated CD4 degradation. The WD domain of βTrCP is responsible for the interaction with Vpu (37). Although βTrCP is involved in both Vpu-mediated CD4 and IκBα proteolysis, it should be stressed that important differences between the two degradation pathways exist. Indeed, if both Vpu and IκBα can be phosphorylated at serine in DGSGXXS motifs, phosphorylation of Vpu occurs constitutively (45–46) while that of IκBα requires activation of cell signaling. Furthermore, and in contrast to IκBα, Vpu has not yet been characterized as a substrate for ubiquitination or degradation by the proteasome. No human protein recognized as ubiquitination substrate by an SCF complex and undergoing degradation by the proteasome has been documented so far. IκBα phosphorylated at critical serine residues represents the first example of this kind of substrate (8).

In the SCF complexes with E3-ubiquitin-protein ligase activity, the F-box component allows specific recognition of substrates (28–33). The existence of a large number of F-box-containing proteins revealed by genome sequencing and the combinatorial interactions of SCF components that belong to different protein families (Cullin, E2 ubiquitin-protein conjugating enzymes) suggest that the growing family of E3 ligases is composed of a large number of different SCF complexes. This diversity has likely hampered the identification of the E3 ligase responsible for ubiquitin conjugation of IκBα required for IκBα degradation and NF-κB activation. While precise characterization of the ubiquitin conjugating activity associated with SCF\textsuperscript{βTrCP} is still missing, our findings provide evidence that βTrCP is ultimately responsible for recognition of phosphorylated IκBα by the SCF complex.

As shown for other F-box proteins in yeast, βTrCP could target different substrates as well as IκBα to the proteasome.
This hypothesis is sustained by the recent discovery that, Slimb, the Drosophila homolog of βTrCP, may be involved in an as yet unidentified step of regulation of the wingless and hedgehog pathways (47). However, it cannot be assumed from this that βTrCP is a broad, universal adaptor for ubiquitinated substrates. Indeed, in the SCF complex, F-box proteins determine and restrict substrate recognition by the proteasome. Thus, the yeast F-box protein Cdc4 is able to selectively bind phosphorylated Scl1, but not phosphorylated Cln1 or Cln2, whereas Grr1, another yeast F-box protein, shows selective association with the latter substrates but not with Scl1 (30–32).

In conclusion, our findings characterize βTrCP as a F-box protein, which selectively associates with Ser\(^{32}\)–Ser\(^{36}\) phosphorylated, but not unmodified, IκB. βTrCP represents the SCF adaptor which ultimately accounts for recognition of phosphorylated IκBa by the ubiquitination machinery and allows targeting of the ubiquitinated inhibitor to the proteasome (see model in Fig. 4). While this manuscript was completed and sent for review, a report by Yaron et al. (48) was published that supports the involvement of βTrCP in IκBa degradation and NF-κB activation. Thus, βTrCP can be considered as a new target for pharmacological intervention in the physiopathological processes regulated by NF-κB.

Acknowledgments—We thank Michèle Bouloy and Agnès Billecocq of the Bunyaviridae research group at Institut Pasteur for their generous support to this project. S. Michelson is acknowledged for critical reading of the manuscript.

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