Immunosuppressant FK506 Activates NF-κB through the
Proteasome-mediated Degradation of IκBα

REQUIREMENT FOR IκBα N-TERMINAL PHOSPHORYLATION BUT NOT UBIQUITINATION SITES

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Yong-kang Zhang, Xiangao Sun, Kei-ichi Muraoka, Akiko Ikeda, Shigeki Miyamoto†,
Hiroko Shimizu, Katsuji Yoshioka, and Ken-ichi Yamamoto‡
From the Department of Molecular Pathology, Cancer Research Institute, Kanazawa University,
Kanazawa 920-0934, Japan and the ‡Department of Pharmacology, University of Wisconsin-Medical School,
K4 554 Clinical Science Center, Madison, Wisconsin 53792

The immunosuppressant FK506 activates NF-κB through IκBα degradation in nonlymphoid cells. In the present study, we analyzed mechanisms by which FK506 induces IκBα degradation. We found that FK506 induces the degradation of both IκBα and IκBβ and that the time courses of the FK506-induced degradation are quite different from degradation induced by interleukin 1 (IL-1). Despite this difference, FK506-induced IκBα degradation was dependent on the N-terminal Ser-32 and Ser-36 phosphorylation sites and was mediated by proteasomes, as is the case for IL-1-induced IκBα degradation. We further showed that FK506 induces weak and slow phosphorylation of IκBα at Ser-32. However, unlike IL-1-induced degradation, IKK-1 and IKK-2 were not activated significantly nor was FK506-induced IκBα degradation dependent on the N-terminal ubiquitination sites (Lys-21 and Lys-22). These results therefore indicate that FK506 and IL-1 utilize similar but distinct mechanisms to induce the phosphorylation and degradation of IκBα.

Nuclear factor κB (NF-κB)1 is a transcription factor that plays an important role in inducing the expression of diverse cellular genes, such as for various cytokines, cell surface receptors, and acute-phase proteins. It is a heterodimer mainly composed of the p50 and RelA proteins, but there might be a considerable heterogeneity in its composition in various cell types, because of the presence of p50/RelA-related proteins (p52, c-Rel, and RelB), which share extensive homology in their activities are stimulated by IL-1 and tumor necrosis factor-α (TNF-α) activate NF-κB through the proteolytic degradation of IκB and the subsequent translocation of NF-κB to the nucleus, where it activates target genes (1–3).

The prototypic and best-studied of the IκBs is IκBα, which is phosphorylated at its N-terminal two serine residues (Ser-32 and Ser-36) prior to degradation, when cells are exposed to appropriate NF-κB activators (4–6). This phosphorylation triggers the ligation of multiple ubiquitin molecules to nearby lysine residues (Lys-21 and Lys-22), leading to the subsequent degradation of the protein by proteasomes (6–9). The signal-induced phosphorylation of IκBα is therefore a critical step in NF-κB activation and has been investigated intensively. Recently, two closely related IκB kinases (IKKs), termed IKK-1 and IKK-2, have been identified and cloned. Both kinases directly phosphorylate Ser-32 and Ser-36 of IκBα and their activities are stimulated by IL-1 and TNF-α treatment (10–14). In addition, pp90rsk kinase (15) and a kinase related to IKK-1 and IKK-2 (termed IKK-3) (16) have also been shown to phosphorylate Ser-32 and Ser-36. Thus, it remains to be established how these various IκB kinases are specifically activated in response to diverse NF-κB activators.

FK506 is a powerful immunosuppressive drug that is currently in clinical use. It exerts its major immunosuppressive effect by inhibiting transcriptional events, including the activation of several cytokine genes, particularly the interleukin-2 gene, that lead to T-cell activation (17). We previously showed that FK506 induces IκBα degradation and NF-κB activation in nonlymphoid cells such as renal mesangial cells and fibroblasts. We further showed that, as a result of NF-κB activation by FK506, interleukin-6 production is induced in the kidney, suggesting the possibility of a causal relationship between the FK506-induced NF-κB activation/IL-6 production and some FK506-induced renal abnormalities (18). However, little is known about how FK506 induces IκBα degradation in nonlymphoid cells. In the present study, we analyzed the mechanisms by which FK506 induce IκBα degradation. We found that, as in the case of IL-1-induced IκBα degradation, FK506-induced IκBα degradation is dependent on the N-terminal serine phosphorylation sites and is mediated by proteasomes. However, the N-terminal ubiquitination sites were not essential for FK506-induced IκBα degradation, and FK506 induced weak and slow phosphorylation of IκBα at Ser-32, in the absence of significant IKK activation. Thus, these results suggest that FK506 and IL-1 induce the phosphorylation and degradation of IκBα through similar but distinct mechanisms.
EXPERIMENTAL PROCEDURES

Chemicals—PSI (Z-Ile-Glu(Obu)+-Ala-Leu-H aldehyde), MG132, and MG115 were from the Peptide Institute, Inc., Japan. IκB inhibitor II (Ac-Tyr-Val-Ala-Asp-chloromethyl ketone) and clasto-lactacystin β-lactone (C13H18N3O2) were from Sigma and Boston Biochemical, respectively. E64d was kindly provided by Dr. K. Tanaka. PSI (19), MG132 (9), MG112 (9), and β-lactone (21) were protease inhibitors specific for proteasomes. E64d (22) and IκB inhibitor II (23) were specific inhibitors for calpain and ICE, respectively. Stock solutions were prepared in dimethyl sulfoxide (Me2SO) (Sigma) at 10 mg/ml (MG132, MG115, E64-d) or 100 mM (PSI, lactone). ICE inhibitor II was prepared in methanol at 50 mg/ml. All of these inhibitors were stored at –20 °C. In every experiment presented, the amount of Me2SO was corrected in each sample such that the effect of Me2SO was controlled. FK506 (Fujizawa Pharmaceutical Co., Japan) was prepared in ethanol at 1 mM and diluted in growth medium when used.

Plasmid Constructions—The cDNA encoding full-length wild-type human IκBα (24) was used as a template to generate a cDNA encoding the N-terminal deletion mutant of IκBα (Fig. 2A) by PCR amplification. Various mutations of IκBα as shown in Fig. 2A were introduced by overlap PCR mutagenesis. PCR products were purified, digested with EcoRI and BamHI, and were subcloned in frame into Bluescript KS downstream of the HA epitope sequence. cDNAs encoding various mutant forms of IκBα with the HA tag sequence were excised by XbaI and inserted into the XbaI site of a mammalian expression vector (pEF-BOS) (25). The construction of mammalian expression vectors encoding IκK-2 (pFlag-IKK-2), JNK3 (pFlag-JNK3), and the truncated and constitutively active form of MEKK (pHA-δMEKK) will be described in detail elsewhere. Finally, the entire kinase domain fragment coding sequence (residues 1169–1488) with the HA tag sequence were amplified by PCR and subcloned into either the pFlag-CMV2 vector (Kodak) or the pEF-BOS vector. For the e-luciferase reporter gene construct, a synthetic NF-κB binding motif was inserted into the pGB13 basic vector (Promega). To construct a plasmid encoding the glutathione S-transferase fusion (GST) fragment (amino acids 1–54) of IκBα expression vectors, the PCR fragment encoding the N-terminal part of IκBα (1–54) was inserted into the BamHI-EcoRI fragment of the pGEX-4T3 vector, in frame.

Cell Cultures and Transfection—Murine fibroblast L-TK cells (a thymidine kinase-deficient cell line derived from L929 cells) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.), 50 units/ml of penicillin G, and 50 μg/ml streptomycin sulfate (Life Technologies, Inc.) in a 5% CO2 humidified incubator. 293 cells were grown in minimum essential medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum. L-TK and 293 cells were transfected with various plasmids using the DEAE-dextran and calcium phosphate methods, respectively. Twenty-four or 48 h after transfection, cells were left untreated or were treated with IL-1 or FK506 for various periods of time prior to harvest. In some experiments, cells were pretreated with protease inhibitors before the addition of IL-1 or FK506. Human recombinant IL-1 (Ontaka Pharmaceutical Company) was prepared in Dulbecco's modified Eagle's medium at 100 μg/ml and stored at –80°C.

Cell Lysate Preparation and Immunoblot Analysis—Cells were washed twice with ice-cold phosphate-buffered saline and lysed in an appropriate volume of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation at 4°C for 15 min at 12,000 rpm. Cell lysate samples containing 100 μg of protein were processed by polyacrylamide gel electrophoresis on 8–12% gradient gels, transferred to nitrocellulose membranes (Amerham Pharmacia Biotech), and subjected to Western blot analysis using the appropriate antibodies and an ECL detection kit (Amerham Pharmacia Biotech). IκBα proteins were detected with mouse anti-IκBα 12CA5 monoclonal or rabbit anti-IκBα (1–317) polyclonal antibodies (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology). A rabbit anti-phospho-IκBα (Ser-32) polyclonal antibody (Santa Cruz Biotechnology).

Luciferase Assay—Twenty-four hours after transfection with the e-luciferase reporter gene and IκBα expression vectors, L-TK cells were stimulated with IL-1 or FK506 for 24 h before harvesting them for the luciferase assay, which was carried out according to the manufacturer's instruction (Promega).

RESULTS

FK506 Induces the Degradation of IκBα and IκBβ—To analyze the effects of FK506 stimulation on IκB degradation, HA-tagged IκBα expression vectors were transfected into L-TK cells, which were most efficient in FK506-mediated NF-κB activation (18). Cells were then treated with IL-1 or FK506 for different periods of time. As shown in Fig. 1A and in agreement with the results of previous studies (27), treatment with IL-1 for only 2 min resulted in the appearance of a slow-migrating band, corresponding to the phosphorylated form of IκBα (27), with almost complete disappearance of the IκBα band at 10 min as a consequence of its proteolytic degradation, and then reappearance at 30 min because of resynthesis of IκBα (Fig. 1A, upper panel). By contrast, FK506 treatment resulted in only a small induction of the high molecular band at 15 min, and much slower IκBα degradation, which was detectable only after 30 min. Resynthesized IκBα bands appeared only after 240 min.
FK506-induced IκBα Degradation Requires N-terminal Phosphorylation Sites and Is Mediated by Proteasomes—The prevailing model for IκBα degradation is that IκBα becomes phosphorylated at Ser-32 and Ser-36 prior to ubiquitination and subsequent degradation in proteasomes (4–9). To determine whether FK506 also induces IκBα degradation through the same or similar mechanisms, we first constructed the expression vector encoding a truncated form of IκBα (Fig. 2A). After transfection with this expression vector, L-TK cells were treated with IL-1 or FK506. As shown in Fig. 2B, whereas wild-type IκBα was degraded both by IL-1 and FK506 stimulation (panel a), the degradation of IκBαΔN by IL-1 and FK506 was completely blocked (panel d), indicating that the N-terminal 36 amino acids are essential for degradation. This is in agreement with previous studies showing that the N-terminal region is essential for IκBα degradation (4–6). To further determine the amino acid residues required for FK506-induced IκBα degradation, various site-specific mutations were introduced into the N-terminal region of IκBα; Ser-32 and Ser-36 were replaced with alanine (S32A/S36A), and Tyr-42 was replaced with phenylalanine (Y42F) (Fig. 2A). The Tyr-42 phosphorylation was previously shown to be required for NF-κB activation induced by some atypical activators (28).

Expression vectors encoding these mutant forms of IκBα were then transfected into L-TK cells, and cells were stimulated with IL-1 or FK506. As shown in Fig. 2B, although the Y42F mutant was degraded both by IL-1 and FK506 with time courses similar to the wild-type IκBα (panel b), the S32A/S36A mutant was degraded by neither IL-1 nor FK506 (panel c). These results therefore indicate that either Ser-32 or Ser-36 are also essential for FK506-induced IκBα degradation.

To clarify whether FK506 induces IκBα degradation through proteasome-dependent mechanisms, L-TK cells transfected with the wild-type IκBα expression vector were pretreated with various protease inhibitors, including specific proteasome inhibitors before IL-1 or FK506 stimulation. As shown in Fig. 3, the IκBα degradation induced by both IL-1 (upper panel) and FK506 (middle panel) was specifically blocked by proteasome inhibitors such as MG132, MG115, and lactone, indicating that the FK506-induced IκBα degradation is mediated by proteasomes.

FK506 induces Ser-32 Phosphorylation of IκBα in the Absence of IKK Activation—The above results (Fig. 2B) indicated that Ser-32 and Ser-36 are essential for FK506-induced IκBα degradation. To further examine whether FK506 induces the phosphorylation of IκBα at Ser-32, L-TK cells transfected with the wild-type IκBα expression vector were treated with IL-1 or FK506 in the presence or absence of proteasome inhibitor (PSI), and IκBα phosphorylated at Ser-32 was detected with an anti-phospho-IκBα (Ser 32) antibody. As shown in Fig. 4, IL-1 induced rapid Ser-32 phosphorylation at 2 min, as expected, but Ser-32 phosphorylation could not be detected when the cells were treated with FK506 in the absence of PSI. However, when the cells were pretreated with PSI and then stimulated with FK506, weak Ser-32 phosphorylation was detected at 30 min (Fig. 4, lower panel, lanes 8–9).

Because FK506 induces the phosphorylation of IκBα at Ser-

Fig. 2. Ser-32 and Ser-36 in the N-terminal region of IκBα are required for FK506-induced degradation. A, a schematic representation of HA-tagged wild-type and various mutant IκBα proteins. The wild-type N-terminal sequence encompassing amino acid residues 15–44 and various amino acid substitutions are shown: K21R/K22R (K21/22R), S32A/S36A (S32/36A), and Y42F denote Lys-21/22 substitution with arginine, Ser-32/36 substitution with alanine, and Tyr-42 substitution with phenylalanine, respectively. B, L-TK cells, transfected with expression vectors encoding wild-type (panel a) and various mutant forms of IκBα as indicated (panels b–d), were treated with IL-1 or FK506, and IκBα proteins were analyzed by Western blot with an anti-HA antibody.

*NS, nonspecific bands.

Fig. 3. FK506-mediated IκBα degradation is specifically blocked by proteasome inhibitors. L-TK cells, transfected with empty vectors (lane 1) or the wild-type IκBα expression vector (lane 2–8), were pretreated with various protease inhibitors for 60 min and then stimulated with IL-1 (upper panel), FK506 (middle panel), or were not treated (control, lower panel). The final concentrations of inhibitors used were: MG132, 10 μg/ml; MG115, 10 μg/ml; β-lactone, 10 μM; E64-D, 10 μg/ml; ICE inhibitor II, 10 μg/ml. Cell lysates (100 μg) were subjected to immunoblot analysis using an anti-HA antibody. Hyperphosphorylated IκBα (IκBα-p) appears as a distinct, more slowly migrating protein band. Lane 3 shows that IκBα was almost completely degraded with IL-1 or FK506 treatment alone, whereas the control panel shows that pretreatment with inhibitors alone did not affect the IκBα level or its phosphorylation status. *NS, nonspecific bands.
32, we next examined the effects of FK506 on the activity of IKK, the recently cloned protein kinase that preferentially phosphorylates Ser-32 and Ser-36 of IκBα (10–14). 293 and L-TK cells transfected with an IKK-2 expression vector (pFlag-IKK-2) were stimulated with IL-1 or FK506, whereas cells cotransfected with an expression vector encoding truncated and constitutively active forms of MEKK (pHA-ΔMEKK) served as positive controls, as it is known that ΔMEKK activates both IKK-1 and IKK-2 (29, 30). Flag-IKK-2 proteins were immunoprecipitated with anti-Flag antibodies and then subjected to an in vitro kinase assay using GST-IκBα (1–317) as a substrate. As shown in Fig. 5, A and B, although IL-1-stimulated IKK activity about 4-fold, no significant IKK activation by FK506 was detected either in the L-TK or 293 cells. However, FK506 was fully active in JNK activation in L-TK cells (Fig. 5C).

**FK506-induced IκBα Degradation Does Not Require N-terminal Ubiquitination Sites—**As shown in Fig. 6, substituting the N-terminal lysine residues 21 and 22 with arginine blocked IL-1-induced IκBα degradation without affecting IκBα phosphorylation. This result agrees with recent studies that show Lys-21 and Lys-22 are primary ubiquitination sites necessary for Tax- and TNF-induced IκBα degradation (6, 8). However, in FK506-stimulated cells, this mutation did not block IκBα degradation, and IκBα was degraded with a similar time course to wild-type IκBα, although this mutation did not affect the IκBα phosphorylation induced by FK506 (Fig. 6A, lanes 11–12 and Fig. 6B, lanes 6–9). In sum, both the wild-type and the K21R/K22R mutant IκBα were less effective for inhibiting the NF-κB activation induced by FK506 than the S32A/S36A IκBα mutant (Fig. 7B), which was not degraded by FK506 treatment (Fig. 2). On the other hand, the S32A/S36A and K21R/K22R mutants, neither of which were degraded by after treatment with IL-1, were equally effective in inhibiting the NF-κB activation induced by IL-1 (Fig. 7A).

**DISCUSSION**

FK506 inhibits the activation of several transcription factors involved in cytokine gene expression in T cells, including NF-κB. We previously showed that FK506 activates NF-κB through IκBα degradation in nonlymphoid cells, and this FK506-induced NF-κB activation results in the efficient induction of IL-6 production in vitro and in vivo (18). However, little is known about how FK506 induces NF-κB activation through IκBα degradation in nonlymphoid cells. In the present study, we found that FK506 induced the degradation of both IκBα and IκBβ and that the time courses of their degradation were completely different from those of the degradation mediated by IL-1 (Fig. 1). However, as in the case of IκBα degradation induced by IL-1 (4–9), FK506-induced IκBα degradation was also dependent on the N-terminal Ser-32 and Ser-36 phosphorylation sites (Fig. 2) and was mediated by proteasomes (Fig. 3). We further demonstrated that FK506 induced the weak and slow phosphorylation of Ser-32 (Fig. 4). These results therefore indicate that, whereas the time course of the FK506-mediated IκBα degradation is quite different from that induced by IL-1, FK506 and IL-1 utilize similar mechanisms for inducing IκBα degradation and hence NF-κB activation.

Inducing the phosphorylation of the N-terminal serines is a key step in IκB degradation and the subsequent NF-κB activation, induced by various NF-κB activators, including IL-1. Because FK506-mediated IκBα degradation is also dependent on N-terminal phosphorylation sites (Fig. 2) and FK506 induces Ser-32 phosphorylation (Fig. 4), it is of interest to study what IKB kinases are activated by FK506 and how FK506 activates them. A protein kinase complex whose activity is stimulated by IL-1 and TNF-α and which mediates IκBα phosphorylation at Ser-32 and Ser-36 was recently purified, and two of the subunits of this complex (IKK-1 and IKK-2) have now been cloned and sequenced (9–14 and 29). The results of recent mouse knockout studies indicate that whereas IKK-2 is essential for IκBα phosphorylation induced by inflammatory cytokines such as IL-1 and TNF-α, IKK-1 is dispensable for IL-1/TNF-induced...
IkBα phosphorylation and is involved in limb and skin morphogenesis (31–33). Although we detected IL-1-induced IKK-2 (Fig. 5), in agreement with the results of previous studies (9–14 and 22), we have not so far detected significant IKK-1 or IKK-2 (Fig. 5) activation with FK506. These results suggest that other recently described IkB kinases such as pp90rsk (15) and IKK-3 (16) or unidentified IkB kinases are involved in the FK506-mediated IkBα phosphorylation. However, whereas FK506 is very effective in JNK activation (Fig. 5), it did not induce a significant activation of Erk,2 which lies immediately upstream of pp90rsk in the phorbol ester and growth factor signaling pathway (34, 35). Therefore, the involvement of upstream of pp90rsk in the phorbol ester and growth factor signaling pathway (34, 35) is un-proven. Because we found that a K21R/K22R mutant IkBα was completely degraded in FK506-stimulated cells but appeared as a high molecular weight band in IL-1-treated cells, the presence of high molecular weight bands corresponding to the phosphorylated forms of the K21R/K22R mutant IkBα proteins in both IL-1- and FK506-treated cell lysates. *NS, nonspecific bands.

Another important and unresolved question is how FK506 activates the putative IkB kinase. Because we found that a nonimmunosuppressive FK506 analog (36) is inactive in NF-κB activation and competitively inhibits FK506-mediated NF-κB activation and IkBα degradation,2 it appears that cytosolic FK506-binding proteins (termed FKBP) are involved in the FK506-mediated NF-κB activation and IkBα degradation. However, it is unlikely that the inhibition of IkBα kinase degrades,2 which leads to immediate upstream of pp90rsk in the phorbol ester and growth factor signaling pathway (34, 35), the involvement of the K21R/K22R mutant IkBα protein in the ubiquitin-independent, at least at Ser-32 (Fig. 4), and these N-terminal serine residues are essential for both IL-1 and FK506-mediated IkBα degradation (Fig. 2). However, whereas the N-terminal ubiquitination sites (Lys-21 and Lys-22) are essential for both IL-1 and FK506-induced IkBα degradation (Fig. 2). In agreement with these results, the K21R/K22R mutant was less effective in inhibiting FK506-induced IkBα degradation (Fig. 6). In agreement with these results, the K21R/K22R mutant would be less effective in inhibiting FK506-induced NF-κB activation than was the S32A/S36A IkBα mutant (Fig. 7). Although the possibility that IkBα is ubiquitinated at other lysine residues in FK506-treated cells has not been completely ruled out, these results raise the possibility that IkBα is degraded by proteasomes in an ubiquitin-independent manner in FK506-treated cells. Several examples exist of proteins, including c-Jun and IkBα, being degraded in a ubiquitin-independent, proteasome-mediated manner (41–43). Thus, c-Jun and IkBα appear to be degradable by proteasomes in both ubiquitin-dependent and -independent manners. Interestingly, it was recently reported that a 450-kDa complex, whose subunits show sequence homology to those of a proteasome regulatory complex, phosphorylates c-Jun as well as IkBα (20). It is therefore possible that this regulatory complex not only phosphorylates but also presents IkBα for degradation by proteasomes in a ubiquitin-independent manner.

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\[2^\text{Y.-k. Zhang and K.-i. Yamamoto, unpublished data.}\]
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