Biochemical and Biophysical Research Communications **256,** 121–126 (1999)
Article ID bbrc.1999.0296, available online at http://www.idealibrary.com on IDEAL®

In Vivo and *in Vitro* Recruitment of an IκBα-Ubiquitin Ligase to IκBα Phosphorylated by IKK, Leading to Ubiquitination

Hiroshi Suzuki,* Tomoki Chiba,† Masato Kobayashi,* Masahiro Takeuchi,*
Kiyoshi Furuichi,* and Keiji Tanaka†,[1]

**Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba-shi, Ibaraki, 305-8585, Japan; and †Tokyo Metropolitan Institute of Medical Science and CREST, Japan Science and Technology Corporation (JST), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan*

Received January 20, 1999

Activation of the transcriptional factor NF-κB is triggered by signal-dependent degradation of its inhibitor protein IκB through the ubiquitin (Ub)–proteasome pathway. We found here that a phosphorylated IκBα immunoprecipitated (IP-pIκBα) from the crude extract of HeLa cells which had been treated with tumor necrosis factor-α (TNFα) caused a dramatic ubiquitination of itself, termed autoubiquitination, when incubated with ATP, Ub, and E1-activating and E2-conjugating enzymes. IP-pIκBα also catalyzed ubiquitination of an *in vitro* synthesized ^{35}S-IκBα previously phosphorylated by IκB-kinase (IKK) which is referred to as transubiquitination. No appreciable activity of auto- and transubiquitination was observed in an unphosphorylated IP-IκBα. Moreover, the putative IκBα-Ub ligase (IκBα-E3) present in HeLa cell cytosol associated *in vitro* with an IKK-phosphorylated recombinant IκBα, a process independent of NF-κB binding to IκBα or TNFα stimulation. Replacement of the two Ser residues at positions 32 and 36 corresponding to IKK phosphorylation sites by Ala resulted in almost complete prevention of binding of an IκBα-E3 to IκBα. These results indicate that phosphorylation of IκBα is necessary and sufficient for recruitment of this IκBα-E3 to associate with IκBα. © 1999 Academic Press

NF-κB is an inducible transcriptional factor which regulates many biologically important processes, such as stress, inflammation, development and immune response (1–3). The prototypical inducible NF-κB complex is a heterodimer of the Rel protein family, consisting of p50 and p65 (also called Rel A) (1, 2). In the cytoplasmic compartment of many types of cells, NF-κB normally exists as inactive form due to association with its protein inhibitor, termed IκB. IκB prevents the transport of NF-κB into the nucleus by masking the nuclear localization signal (NLS) of NF-κB (4). This action is thought to be mediated by the multiple, tandemly-repeated ankyrin repeats present on IκB, which are thought to interact with NF-κB (2, 4). To date, seven members of the structurally and functionally related IκB protein family, IκBα, IκBβ, IκBϵ, IκBγ, Bcl-3, p100, and p105, have been identified (3).

Various external stimuli, such as cytokines including alpha-type tumor necrosis factor (TNFα), viral infection, T-cell and B-cell mitogens, and UV-stress, induce the immediate removal of IκB from the IκB-(NF-κB) complex (3, 4). This tremendous progress in delineating the molecular mechanisms of the NF-κB signaling pathway has revealed that phosphorylation of 2 serine residues (Ser-32 and Ser-36) near the N-terminus of IκBα is essential for targeting IκBα for signal-promoted destruction (5–7). This specific phosphorylation of IκBα is catalyzed by an unusually large, multi-protein kinase complex, termed IκB kinase (abbreviated IKK), with an apparent molecular mass of 700–900 kDa (8–10). The phosphorylation of IκBα by an IKK complex is necessary for its poly-ubiquitination at residues Lys-21 and Lys-22 (11–13). The ubiquitin (Ub)–proteasome system then plays the next indispensable role for down-regulating IκBα at the physiologic level (14, 15).

Ubiquitin and proteasomes are the principal components of an energy-dependent proteolytic system in eukaryotic cells (16, 17). Selective destruction of cellular proteins by this system is ensured by two sequential processes. The first is selective marking of candidate proteins for degradation by the covalent attachment of a poly-Ub chain (18). The second process is proteolytic attack of poly-ubiquitinated proteins by

[1] To whom correspondence should be addressed. Fax: (+81)-3-3823-2237. E-mail: tanakak@rinshoken.or.jp.

0006-291X/99 $30.00

the 26S proteasome, a eukaryotic ATP-dependent 2-MDa protease complex (16, 19, 20). The covalent attachment of Ub through its C-terminal Gly residue to the ϵ-NH_2 group of the Lys residue on substrate proteins is known to be mediated by a cascade of three enzymes, designated E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) (18). A poly-Ub chain is formed by linking the C-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 26S proteasome.

In considering the critical role of the ubiquitination-dependent removal of IκBα in the NF-κB signaling pathway, an IκBα-Ub ligase (IκBα-E3) is believed to be the key enzyme to control the level of IκB rapidly, timely, and irreversibly, as demonstrated for many other E3s (17, 18). Currently Yaron *et al.* (21, 22) indicate the existence of the putative IκBα-E3 responsible for the ubiquitination of IκBα. However, the details of this IκBα-E3 and its physiological relevance remain to be uncovered. In the present study, we show that the putative IκBα-E3 present in HeLa cell cytosol is specifically associated with *in vivo* phosphorylated IκBα produced by TNFα stimulation and recombinant IκBα previously phosphorylated *in vitro* by IKK, causing auto- and transubiquitination of IκBα. We also show that phosphorylation at positions of Ser-32 and Ser-36 near the N-terminal region of IκBα is necessary and sufficient for the recruitment of the IκBα-E3 to bind to IκBα.

MATERIALS AND METHODS

Materials. Sources of materials are as follows: MG115 (Z-Leu-Leu-nVal-H) and MG132 (Z-Leu-Leu-Leu-H) (Peptide Institute, Inc., Osaka, Japan); okadaic acid (OA) (Wako, Tokyo, Japan); human TNFα (Genzyme); bovine Ub, creatine phosphate, creatine kinase, and inorganic pyrophosphatase (Sigma); rabbit polyclonal antibodies against IκBα (amino acids 297–317, c-21) and RelA/p65 (amino acids 531–550, c-20) (Santa Cruz Biotechnology). Recombinant mouse E1 enzyme was purified from baculovirus-infected Sf9 insect cells (provided by K. Iwai, Kyoto University). Recombinant Ubch4 was produced in *E. coli* (provided by M. Nakao, Kumamoto University). IKK was purified from HeLa cell extracts, as described by Lee *et al.* (23).

Expression plasmids, in vitro translation, and recombinant proteins. The pcDNA3FL-IκBα mammalian expression plasmid was constructed by insertion of the cDNA encoding amino acids 2-317 of human IκBα into the *Bam*HI/*Not*I sites of pcDNA3FL (24). The pcDNA3FL-N-GST-C plasmid containing glutathione S-transferase (GST) gene fused to the wild-type N- (1–72 amino acid) and C-terminal (282–317 amino acid) domains of IκBα was constructed using fragments amplified by PCR. To construct pET-N-GST-C, a fragment resulting from Bam*HI*/Not*I* digestion of pcDNA3FL-N-GST-C was cloned into *Bam*HI/*Not*I-cut pET-23c (Novagen). The N-GST-C chimera and GST-fused IκBα were cloned into the pGEX5X-1 expression vector (Amersham Pharmacia Biotech). To construct pcDNA3FL-IκBα(S32A/S36A)-, pcDNA3FL-N-GST-C(S32A/S36A)- and pcDNA3FL-GST-IκBα(S32A/S36A)-expressing plasmids, the serine codons for Ser-32 (AGC) and Ser-36 (TCC) of IκBα were changed to alanine (GCG) by PCR. IκBα and GST-IκBα proteins were produced and labeled with [^{35}S]methionine and [^{35}S]cystein using a wheat-germ lysate-based TNT transcription-translation coupled kit (Promega). GST-fused proteins were purified using GSH–Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Cell culture and TNFα treatment. Human HeLa-S3 cells were maintained in MEM for suspension cells (GIBCO) supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. After HeLa cells were pretreated with 50 μM MG132 for 45 min followed by 0.25 μM okadaic acid (OA) for 15 min, human TNFα was added for 15 min at a final concentration of 300 units/ml.

Immunoprecipitation of IκBα. After treatment of HeLa cells with TNFα or nothing, HeLa cells (10^8 cells) were suspended in buffer A (50 mM Tris–HCl (pH 7.4) containing 1 mM EGTA, 2 mM DTT, 25 mM sodium β-glycerophosphate, 0.1 mM PMSF (phenylmethanesulfonyl phosphate), and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A) and then immediately lysed in a glass Dounce homogenizer. Lysates were clarified by low-speed centrifugation (3000 rpm) for 10 min, followed by further centrifugation at 15,000 rpm for 20 min. Immunoprecipitations (abbreviated IP) were performed using an anti-IκBα antibody and followed by washing four times with buffer B (20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 2 mM DTT, 25 mM sodium β-glycerophosphate, 0.1 mM PMSF, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A). The resulting immunoprecipitates were referred to as IP-IκBα and IP-pIκBα, which are the nonphosphorylated and phosphorylated form, respectively. The slurries were used to assay ubiquitination.

GST pull-down with GST-IκBα. Purified recombinant GST-IκBα, GST- IκBα(S32A/S36A), N-GST-C, and N-GST-C(S32A/S36A) (each 5 μg) were incubated at room temperature for 1 h with 50 ng of IKK in the presence of 2 mM ATP. These proteins were then recovered with GSH-Sepharose followed by washing with PBS (pH 7.4). The resins were incubated at 4°C for 16 h with 5 mg of untreated and TNFα-treated HeLa cytoplasmic extract and washed 3 times with buffer B. Washed resins were suspended in 500 μl of buffer B, and 4 μl of these slurries was assayed for the ubiquitination reaction.

Ubiquitination assay of IκBα. Slurries of IP-IκBαs were incubated in a reaction volume of 20 μl that included 2 μl of 10× ATP regenerating system containing 500 mM Tris–HCl (pH 7.6), 100 mM $MgCl_2$, 20 mM ATP, 5 mM DTT, 100 mM creatine phosphate, 35 U/ml of creatine kinase, and 6 U/ml inorganic pyrophosphatase, 0.5 μM okadaic acid, 5 μM MG115, 100 ng of recombinant mouse E1, 3 μg of $(His)_6$-Ubch4, and 1 mg/ml of bovine Ub. The reactions were incubated at 37°C for 3 h. After terminating the reaction by the addition of sample buffer for SDS–PAGE, the reaction mixtures were separated by SDS–PAGE on a 10% gel followed by Western blotting with an anti-IκBα antibody.

For reactions using ^{35}S-labeled proteins as a substrate, 2 μl of *in vitro* translated ^{35}S-labeled IκBαs or ^{35}S-labeled N-GST-Cs was incubated at room temperature for 30 min with the 25 ng of IKK in the presence of the ATP regenerating system, Ub-aldehyde, okadaic acid and MG115, followed by further incubation at 37°C for 3 h in a reaction volume of 20 μl containing 100 ng of recombinant mouse E1, 3 μg of $(His)_6$-Ubch4, and 1 mg/ml of bovine Ub, and 4 μl of IP-IκBα or recombinant GST-IκBα to be tested. The reaction mixtures were separated by 10% SDS–PAGE and visualized by autoradiography.

Western blot analysis. For immunoblotting, the ubiquitination reaction mixtures and GST pull-down materials were separated by SDS–PAGE and transferred to a PVDF membrane. The membranes were probed with antibodies against IκBα and RelA and visualized with horseradish peroxidase-conjugated protein A (Bio-Rad) using the Enhanced Chemiluminescence Detection system (Amersham Pharmacia Biotech).

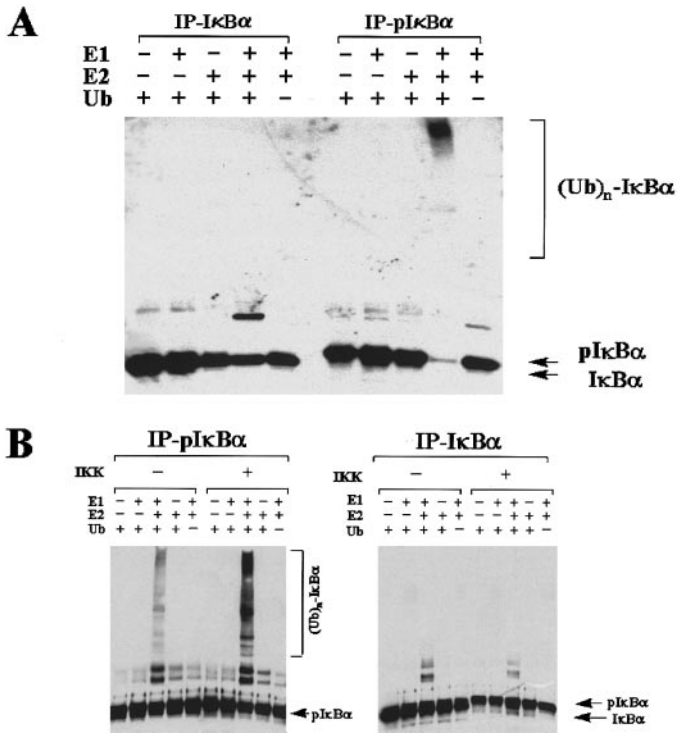

FIG. 1. Association of IκBα-Ub ligase with phosphorylated IκBα *in vivo*. (A) Detection of phosphorylation-dependent autoubiquitination activity in IP-pIκBα. IP-IκBα (left) and IP-pIκBα (right) were incubated with ATP and the indicated combinations (+/−) of E1, $(His)_6$-Ubch4 (E2), or Ub. Multiple ubiquitinated IκBα bands with higher molecular masses were detected by Western blotting and are designated $(Ub)_n$-IκBα. (B) Prerequisite of phosphorylation of IκBα for trapping of IκBα-Ub ligase. IP-pIκBα (left) and IP-IP-IκBα (right) were preincubated *in vitro* with or without 25 ng of IκBα kinase (IKK) at room temperature for 30 min in the presence of ATP to phosphorylate IP-IκBα. Auto-ubiquitination activity was then assayed as in A.

RESULTS AND DISCUSSION

Nonphosphorylated and phosphorylated IκBα (abbreviated IκBα and pIκBα, respectively) were prepared by immunoprecipitation using polyclonal antibodies raised against the C-terminal region of IκBα from nonstimulated and TNFα-treated HeLa cell extracts. These immunoprecipitated IκBα and pIκBα preparations (hereafter referred to as IP-IκBα and IP-pIκBα, respectively) were used as substrates in a ubiquitination assay. At this point, we observed a very interesting phenomenon. Incubation of IP-pIκBα with ubiquitin (Ub), ATP, recombinant mouse E1 and human E2 (Ubch4) enzymes was sufficient to produce polyubiquitination of pIκBα itself without any other supplement. These ubiquitinated proteins were detected by Western blot analysis with an anti-IκBα antibody (Fig. 1A). In contrast, Ub-ligated bands were not detected for the non-phosphorylated form of IP-IκBα. This effect was first termed "autoubiquitination." In some preparations, this autoubiquitination was very strong, leading to almost complete loss of the IP-pIκBα substrate. Furthermore, this reaction requires E1, E2, and Ub, which suggests a presumptive IκBα-Ub ligase (equivalent to IκBα-E3) is tightly associated with IP-pIκBα *in vivo*. We then attempted to clarify whether phosphorylation of IκBα plays a critical role in the trapping of this IκBα-E3. For this, we examined the effect of phosphorylation of IP-IκBα by purified IκB kinase (IKK) on the ubiquitination activity. As shown in Fig. 1B, IKK somewhat enhanced autoubiquitination in IP-pIκBα (left), whereas it had no effect on the activity in IP-IκBα (right), strongly suggesting that phosphorylation of IκBα is required prior to IP to support autoubiquitination of pIκBα and thus the phosphorylation prerequisite binding of the putative IκBα-E3.

This putative IκBα-E3 enzyme associated with pIκBα was next assessed for the ability to ubiquitinate *in vitro*-translated ^{35}S-labeled IκBα, prephosphorylated by the addition of purified IκB kinase (IKK) complex to the assay mixture. As shown in Fig. 2, ^{35}S-IκBα phosphorylated by IKK was specifically polyubiquitinated in the presence of IP-pIκBα but not IP-IκBα, implying that a putative IκBα-E3 associated with IP-pIκBα ubiquitinates exogenously added ^{35}S-IκBα pre-phosphorylated by IKK (named "transubiquitination"). Indeed, the addition of GST-Ub, instead of the native Ub, caused the appearance of very large poly-GST-ubiquitinated bands. Electrophoretic analy-

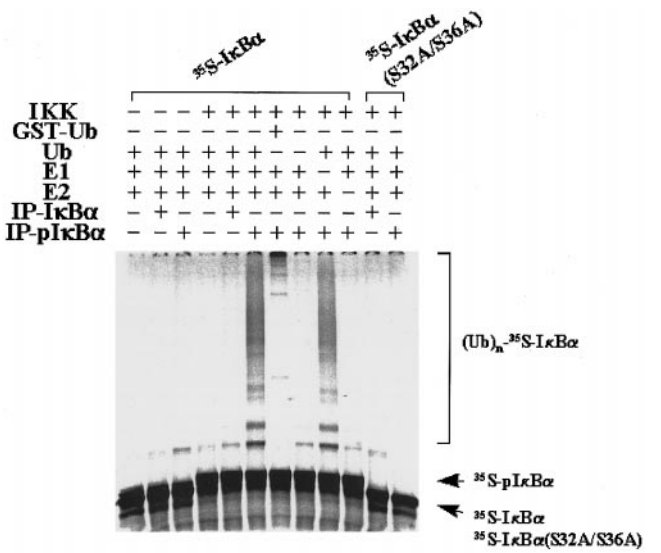

FIG. 2. IKK-dependent transubiquitination of *in vitro* translated ^{35}S-IκBα by IP-pIκBα. The suitability of ^{35}S-labeled IκBα and the mutant IκBα(S32A/S36A) as ubiquitination substrates was assessed *in vitro* using IP-IκBα or IP-pIκBα in combination with other components such as Ub, GST-Ub, E1, and $(His)_6$-Ubch4 (E2), and IKK as indicated by (+/−). After separating the reaction mixtures by 10% SDS–PAGE, multiple ubiquitinated ^{35}S-IκBα bands, designated $(Ub)_n$-^{35}S-IκBα, were visualized by autoradiography.

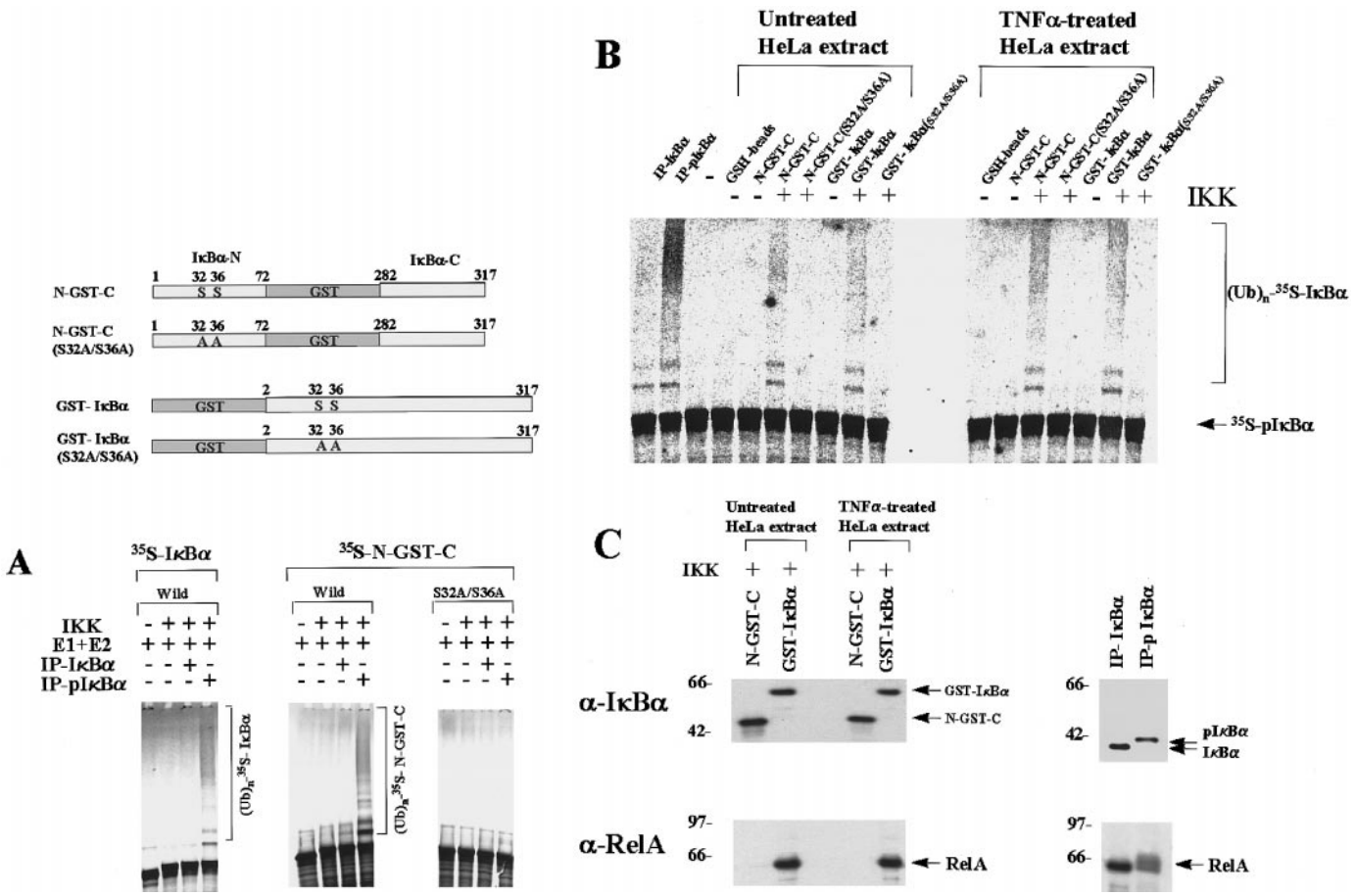

FIG. 3. *In vitro* binding of IκBα-Ub ligase with the N-terminal region of IKK-phosphorylated IκBα, independent of NF-κB association or TNFα stimulation. (Top) Structures of IκBα chimeras and GST-fused IκBα. Numbers indicate amino acid positions in the sequence of IκBα. The ankyrin-repeat domain of IκBα was replaced by the GST sequence, and the resulting protein was named N-GST-C. The GST-fused IκBα protein was named GST-IκBα. The two Ser residues at positions 32 and 36 corresponding to IKK phosphorylation sites were replaced by Ala and named N-GST-C(S32A/S36A) and GST-IκBα(S32A/S36A). (A) Transubiquitination of ^{35}S-N-GST-C and ^{35}S-GST-fused IκBα by IP-pIκBα. The *in vitro* translated ^{35}S-IκBα, ^{35}S-N- GST-C, and ^{35}S-N-GST-C(S32A/S36A) were used for substrates for *in vitro* ubiquitination assay with IP-IκBα or IP-pIκBα as described in the legend to Fig. 2. (B) Association of active Ub-IκBα ligase with phosphorylated N-GST-C IκBα and phosphorylated GST-IκBα. Recombinant N-GST-C, N-GST-C(S32A/S36A), GST-IκBα and GST-IκBα(S32A/S36A) (5 μg each) were incubated with or without 50 ng IKK (as indicated). These proteins were then recovered with the GSH-Sepharose and incubated at 4°C for 16 h with 5 mg of untreated or TNFα-treated HeLa extracts, before collection. These GST-pull-down products were assayed for their ability to support ubiquitination of *in vitro* translated ^{35}S-pIκBα phosphorylated by IKK under conditions described in the legend to Fig. 2. (C) Association of Rel A with GST-IκBα but not N-GST-C IκBα. The GST-pull-down products by IKK-treated N-GST-C and GST-IκBα from crude extracts from HeLa cells treated with TNFα or nothing (left), in which conditions were the same where poly-ubiquitination was observed as in B, and IP-IκBα or IP-pIκBα (right) were subjected to SDS–PAGE followed by Western blotting with an anti-IκBα antibody (top) and with an anti-Rel A antibody (bottom).

sis revealed that IKK caused the electrophoretic mobility of ^{35}S-IκBα to shift, indicating that IKK actively phosphorylated IκBα (Fig. 2). Two Ser residues (positions 32 and 36) appear to be responsible for inducing ubiquitination upon their phosphorylation (5–7). We replaced these Ser residues with Ala, yielding the mutant IκBα(S32A/S36A). This mutant protein was not a substrate in the ubiquitination assay, even if IP-pIκBα or IKK was added. It is also important to note that no poly-ubiquitination of phosphorylated ^{35}S-IκBα was observed if either Ub or E2 was omitted. In contrast, the omission of purified E1 caused a slight effect, because E1 might be supplied by the wheat-germ lysate used for *in vitro* translation of ^{35}S-IκBα as previously reported (6).

The binding of this putative IκBα-Ub ligase to pIκBα was next examined *in vitro* to identify which region of IκBα interacts with the putative IκBα-E3. To accomplish this, we constructed two general types of IκBα derivatives. The first were chimera IκBαs in which the middle ankyrin-repeat domain, responsible for association with NF-κB (p50–p65 complex), was replaced by glutathione-S-transferase (GST) (see Fig. 3, top). The resulting chimeric IκBαs, named N-GST-C, and

N-GST-C(S32A/S36A) harbor GST flanked by both the N- and C-termini of IκBα. A similar GST-IκBα chimera was recently used to determine the role of signal-induced phosphorylation in IκBα degradation in EL-4 cells (25). The other types were GST fused to the N-terminal end of an IκBα (named GST-IκBα) and GST-IκBα(S32A/S36A).

The *in vitro* translated ^{35}S-IκBα and ^{35}S-N-GST-C were poly-ubiquitinated by IP-pIκBα after phosphorylation by IKK, indicating strongly that the ankyrin-repeat domain of IκBα is not required for ubiquitination of pIκBα (Fig. 3A). Moreover, *in vitro* translated ^{35}S-N-GST-C(S32A/S36A) was not ubiquitinated by a complete assay mixture containing IP-pIκBα. These results indicate the critical role of the N-terminal phosphorylation sites (Set-32 and Ser-36) in ubiquitination.

Subsequently, we examined whether the putative IκBα-E3 binds directly to recombinant IκBα phosphorylated *in vitro* by IKK. The N-GST-C or GST-IκBα fused proteins (Fig. 3, top) were incubated at room temperature for 1 hr with or without IKK in the presence of 2 mM ATP. These proteins were then bound to GSH-Sepharose and washed with phosphate buffer saline (PBS). The resins were incubated at 4°C for 16 h with 5 mg of untreated or TNFα-treated HeLa cytoplasmic extracts and then washed 3 times with buffer containing 150 mM NaCl. These GST-pull-down products were used in a ubiquitination assay with IKK-pretreated ^{35}S-IκBα as a substrate. As shown in Fig. 3B (right), pull-down materials from TNFα-treated HeLa cell extracts with both phosphorylated N-GST-C and GST-IκBα supported the poly-ubiquitination of phosphorylated ^{35}S-IκBα. In contrast, pull-down materials from unphosphorylated N-GST-C or GST-IκBα or from N-GST-C(S32A/S36A) and GST-IκBα(S32A/S36A) pre-incubated with IKK had no effect, strongly indicating that this putative IκBα-E3 binds the N-terminal region of IκBα previously phosphorylated by IKK. Unexpectedly, similar results were obtained from TNFα-untreated HeLa cell extracts (Fig. 3B, left), implying that activation of the NF-κB pathway does not affect the association of active IκBα-E3 with previously phosphorylated pIκBα.

These results also indicate that the binding of IκBα to NF-κB is not required for the recruitment of IκBα-E3 to pIκBα. To confirm this, the direct interaction of N-GST-C or GST-IκBα with NF-κB was examined. IκBα fusion proteins were incubated with the crude extracts of HeLa cells that had or had not been treated with TNFα. The presence of RelA (the p65 component of NF-κB) in the pull-down cocktail was then assayed by Western blotting with an anti-RelA antibody. As shown in the Western blots in Fig. 3C (left), N-GST-C was not associated with RelA while GST-IκBα was, indicating that NF-κB binds to the ankyrin-repeat region of IκBα. Additionally, RelA was co-immunoprecipitated with IP-IκBα or IP-pIκBα (Fig. 3C, right), indicating that phosphorylation of IκBα produced by TNFα stimulation does not appear to affect the binding of IκBα to NF-κB. Taken together, we concluded that the attachment of NF-κB to IκBα is not needed for the association of the active IκBα-Ub ligase complex with pIκBα.

Various external stimuli are known to activate IKK, which in turn phosphorylates 2 sites near the N-terminal region of IκBα, the residues Ser-32 and Ser-36, leading to *in vivo* poly-ubiquitination of IκBα (5–7). It was recently reported that the putative IκBα-Ub ligase is associated with phosphorylated IκBα, based on the findings that phosphopeptides corresponding to IKK-phosphorylation sites specifically inhibit Ub conjugation of IκBα and its subsequent degradation (21) and that an F-box/WD-domain protein belonging to a family of β-TrCP/Slimb is incorporated into the immunopurified pIκBα/NF-κB complex (22). These findings are fairly consistent with the results presented in this work. Here we demonstrated directly that the active IκBα-Ub ligase was associated with IP-pIκBα and phosphorylation at positions Ser-32 and Ser-36 corresponding to the IKK phosphorylation sites of IκBα is necessary and sufficient for *in vitro* recruitment of this IκBα-E3 complex to associate with IκBα. We presume that the putative IκBα-Ub ligase is active irrespective of TNFα stimulation, because factors from both stimulated and unstimulated HeLa cell extracts similarly associate with recombinant GST-fused IκBα protein which had been phosphorylated by purified IKK. These results also suggest that phosphorylation of IκBα by IKK is apparently sufficient for the recruitment of IκBα-Ub ligase induced by TNFα signaling.

It is of note that IP-pIκBα is capable of catalyzing the transubiquitination of exogenously added ^{35}S-IκBα pre-phosphorylated by IKK, indicating that the IκBα-E3 previously bound pIκBα can be transferred to another pIκBα, perhaps after completion of the poly-ubiquitination of pIκBα. As transubiquitination of IκBα by IP-pIκBα occurs in the presence of the proteasome inhibitor, the successive binding of IκBα-E3 from ubiquitinated pIκBα to another non-ubiquitinated one is thought to proceed without the degradation of poly-ubiquitinated IκBα by the 26S proteasome. Taken together, the binding of IκBα-E3 to phosphorylated IκBα is reversible, this process must proceed rapidly for the progressive ubiquitination of many pIκBα molecules. This poly-ubiquitinated IκBα is then targeted for ATP-dependent destruction via the 26S proteasome, as previously reported (14, 15). Further study is required for the clarification of the molecular mechanism of this process.

ACKNOWLEDGMENTS

The authors thank Steven E. Johnson for editing the manuscript. We are also grateful to Chinami Terazaki for plasmid constructs and

all other members of MML in Yamanouchi Pharmaceutical Co., Ltd., for encouragement and support.

REFERENCES

1. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20.
2. Baldwin, A. S. (1996) *Annu. Rev. Immunol.* **14,** 649–681.
3. May, M. J., and Ghosh, S. (1998) *Immunol. Today* **19,** 80–88.
4. Finco, T. S., and Baldwin, A. S. (1995) *Immunity* **3,** 253–272.
5. Traenckner, E. B.-M., Pahl, H. L., Henkel, T., Schmidt, K..N., Wilk, S., and Baeuerle, P. A. (1995) *EMBO J.* **14,** 2876–2883.
6. Chen, Z. J., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) *Genes Dev.* **9,** 1586–1597.
7. DiDonato, J. A., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996) *Mol. Cell. Biol.* **16,** 1295–1304.
8. Maniatis, T. (1997) *Science* **278,** 818–819.
9. Israél, A. (1997) *Nature* **388,** 519–521.
10. Scheiderelt, C. (1998) *Nature* **395,** 225–226.
11. Scherer, D. C., Brockman, J. A, Chen, Z., Maniatis, T., and Ballard, D. W. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11259–11263.
12. Baldi, L., Brown, K., Franzoso, G., and Siebenlist, U. (1996) *J. Biol. Chem.* **271,** 376–379.
13. Rodriguez, M. S., Wright, J., Thompson, J., Thomas, D., Baleux, F., Virelizier, J. L., Hay, R. T., and Arenzana-Seisdedos, F. (1996) *Oncogene* **12,** 2425–2435.
14. Traenckner, E. B.-M., Wilk, S., and Baeuerle, P. A. (1994) *EMBO J.* **13,** 5433–5441.
15. Chen, Z. J., Parent, L., and Maniatis, T. (1996) *Cell* **84,** 853–862.
16. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65,** 801–847.
17. Ciechanover, A. (1998) *EMBO J.* **17,** 7151–7160.
18. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479.
19. Baumeister, W., Walz, J., Zühl, F., and Seem,ller, E. (1998) *Cell* **92,** 367–380.
20. Tanaka, K., and Chiba, T. (1998) *Genes Cells* **3,** 485–498.
21. Yaron, A., Gonen, H., Alkalay, I., Hatzubai, A., Jung, S., Beyth, S., Mercurio, F., Manning, A. M., Ciechanover, A., and Ben-Neriah, Y. (1997) *EMBO J.* **16,** 5486–6494.
22. 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.
23. Lee, F. S., Hagler, J., Chen, Z. J., and Maniatis, T. (1997) *Cell* **88,** 213–222.
24. Takeuchi, M., Rothe, M., and Goeddel, D. V. (1996) *J. Biol. Chem.* **271,** 19935–19942.
25. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) *Science* **267,** 1485–1488.