

Identification of a novel 300-kDa factor termed I κ B α E3-F1 that is required for ubiquitinylation of I κ B α

Hiroshi Suzuki^a, Masato Kobayashi^a, Masahiro Takeuchi^a, Kiyoshi Furuichi^a,
Tomoki Chiba^b, Keiji Tanaka^{b,*}

^aInstitute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan

^bThe Tokyo Metropolitan Institute of Medical Science, and CREST, Japan Science and Technology Corporation (JST), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan

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Abstract Destruction of I κ B by ubiquitinylation is required for signal-dependent activation of NF- κ B. The I κ B α ubiquitin-ligase activity associated with phosphorylated I κ B α (pI κ B α) in HeLa cells was almost completely lost by washing under stringent conditions including 1 M NaCl; nevertheless, an SCF ^{β TrCP} complex containing Skp1, Cullin-1, and F-box/WD40 protein β TrCP was still bound to pI κ B α , suggesting the existence of a putative factor that is loosely associated with pI κ B α and may collaborate with SCF ^{β TrCP}. The factor was named I κ B α E3-F1 and was partially purified from HeLa cells. Gel filtration analysis revealed that I κ B α E3-F1 has an apparent molecular mass of approximately 300 kDa.

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Key words: Ubiquitin; SCF; I κ B; NF- κ B;
Tumor necrosis factor α

1. Introduction

There is growing evidence addressing the importance of the immediate removal of an inhibitory protein termed I κ B in the NF- κ B signalling pathway, because I κ B prevents nuclear translocation of cytoplasmic NF- κ B by masking the nuclear location signal (NLS) of NF- κ B [1]. The key process of the proteolysis of I κ B is thought to be the ubiquitinylation reaction directed by I κ B α ubiquitin (Ub)-ligase (also named I κ B α E3) capable of transferring Ub to the target I κ B molecule phosphorylated by I κ B kinase (IKK) via E1 (Ub-activating) and E2 (Ub-conjugating) enzymes [2]. However, the molecular basis of the I κ B α E3 enzyme was only recently identified. To date, the trimer complex called SCF (Skp1, Cdc53 or Cullin-1, and F-box/WD40-repeat protein) is known to catalyze the ubiquitinylation of a number of short-lived proteins responsible for the regulation of cell cycle progression [3]. At the beginning of 1998, Jiang and Struhl [4] found the *Slimb* gene which negatively regulates the Wingless pathway and Hedgehog pathway in *Drosophila*. Intriguingly, as Slimb is an F-box/WD40-repeat protein, an SCF-like Ub-ligase was postulated to participate in the degradation of I κ B α as well as β -catenin, because of the similarity of the two phosphorylation sites present in both I κ B α and β -catenin [5]. Before long, the SCF ^{β TrCP} complex, consisting of Skp1, Cullin-1, and β TrCP, had been identified not only as a long-

sought I κ B α E3 [6–10] but also as a Ub-ligase for β -catenin [7,11–13].

Based on these numerous reports, it is clear that the SCF ^{β TrCP} complex is an E3 enzyme required for the ubiquitinylation of I κ B α , but it is still not clear whether the SCF ^{β TrCP} complex in combination with E1 and E2 is sufficient for the ubiquitinylation of I κ B α . We found that the SCF ^{β TrCP} complex coordinately recruited to phosphorylated I κ B α (pI κ B α) induced by tumor necrosis factor α (TNF- α) stimulation was tightly bound to pI κ B α that could not be released by washing with 1 M NaCl [14,15]. In the present study, however, we found that this high salt treatment resulted in almost complete loss of ubiquitinating activity, suggesting the existence of other factor(s) which may collaborate with SCF ^{β TrCP} for the ubiquitinylation of pI κ B α . We report here a novel factor named I κ B α E3-F1 with an apparent molecular mass of 300 kDa that is loosely bound to pI κ B α , differing from the tightly associated SCF ^{β TrCP} complex.

2. Materials and methods

2.1. Cell culture and TNF- α treatment

Human HeLa-S3 cells were cultured as described before [15]. After HeLa cells were pretreated with 50 μ M of MG132 (Z-Leu-Leu-Leu-H, Peptide Institute, Inc., Osaka) for 45 min followed by 0.25 μ M of okadaic acid (Wako, Tokyo) for 15 min, human TNF- α (Genzyme) was added for 15 min at a final concentration of 300 U/ml.

2.2. Immunoprecipitation and ubiquitinylation assay of I κ B α

Crude extracts from HeLa cells treated with TNF- α or nothing were prepared by two different methods: under 'stringent' and 'mild' conditions. Under stringent conditions, HeLa cells (10^8 cells) were lysed in 1 ml buffer A (50 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 25 mM sodium β -glycerophosphate, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μ g/ml each of leupeptin, aprotinin, and pepstatin A). Crude lysates were clarified by centrifugation at 15000 rpm for 20 min. The HeLa extracts (10 mg) were incubated at 4°C for 16 h with 1 μ g of a rabbit polyclonal antibody against the carboxy-terminus of I κ B α (amino acids 297–317, c-21, Santa Cruz Biotechnology) and protein A-Sepharose. The immune complexes were then washed four times with buffer A. The resulting immunoprecipitates from TNF- α -treated or untreated HeLa cells were referred to as IP-pI κ B α (s) and IP-I κ B α (s), which are the phosphorylated and non-phosphorylated form, respectively.

Under mild conditions, HeLa cells were suspended in buffer B (50 mM Tris-HCl (pH 7.4) buffer containing 1 mM EGTA, 2 mM DTT, 25 mM sodium β -glycerophosphate, 0.1 mM PMSF, and 10 μ g/ml each of leupeptin, aprotinin, and pepstatin A) and then immediately lysed in a glass Dounce homogenizer. Immunoprecipitations were performed as previously described. The resulting immunoprecipitates from non-stimulated and TNF- α -treated HeLa cell extracts were referred to as IP-I κ B α (m) and IP-pI κ B α (m), respectively. In some experiments, the immunoprecipitates were further washed with buffer B containing 1.0 M NaCl or 1.0 M NaH₂PO₄ (pH 7.4). The

*Corresponding author. Fax: (81) (3) 3823-2237.
E-mail: tanakak@rinshoken.or.jp

slurries of these immunoprecipitates, prepared under 'mild' and 'stringent' conditions, were used for the ubiquitinylation assay as described previously [14].

3. Results

3.1. Loss of ubiquitinylation activity of pIkB α immunoprecipitates by 1 M NaCl wash

IkB α and pIkB α were prepared by immunoprecipitation (IP) using anti-IkB α antibody from non-stimulated and TNF- α -treated HeLa cell extracts under the 'mild' conditions described in Section 2. These IP-IkB α and IP-pIkB α preparations (hereafter referred to as IP-IkB α (m) and IP-pIkB α (m), respectively) were used as substrates in a ubiquitinylation assay. As previously reported [14], incubation of IP-pIkB α (m) with Ub, ATP, recombinant E1 and E2 (Ubch4) enzymes was sufficient to produce poly-ubiquitinylation of pIkB α without any other supplement (see Fig. 1, lanes indicated as 0.15 M NaCl). These ubiquitinated proteins were detected by Western analysis with anti-IkB α antibody. In contrast, Ub-ligated bands were not detected for the non-phosphorylated form of IP-IkB α (m). This reaction requires only E1, E2, and Ub, which suggests that a presumptive IkB α -Ub ligase (equivalent to IkB α E3) is tightly associated with IP-pIkB α (m) *in vivo*, as previously reported [14].

Conditions necessary for dislodging this putative IkB α E3 from the associated IP-pIkB α (m) were assessed by washing the complexes with a high-concentration salt solution. As shown in Fig. 1, when IP-IkB α (m) was washed extensively with a buffer containing 1.0 M NaCl, the poly-ubiquitinylation activity was almost completely lost. Subsequently, the materials dissociated by washing IP-pIkB α (m) with 1.0 M NaCl were collected, concentrated, and designated 1.0 M NaCl extracts. These 1.0 M NaCl extracts supported fair amounts of poly-ubiquitinylation of 1.0 M NaCl-washed IP-pIkB α (m) having no ubiquitinylation activity, but showed no

stimulating effect on the non-phosphorylated IP-IkB α (m). These reconstitution results suggest a reversible association of a putative IkB α -Ub ligase with pIkB α .

3.2. Identification of a novel 300-kDa factor required for ubiquitinylation of IkB α

To further characterize this putative IkB α -Ub ligase, we attempted to isolate it from HeLa cell extracts. Phosphorylated IkB α was prepared by immunoprecipitation from TNF- α -treated HeLa cell extracts under 'stringent' conditions as described in Section 2. The resulting immunoprecipitates, named IP-pIkB α (s), had no E3-like activity in concerted with E1, E2, and Ub, as similarly seen in 1.0 M NaCl-washed IP-pIkB α (m), and were used routinely as substrates in the IkB α ubiquitinylation assay.

Crude protein extracts prepared from a 3.0-l culture of TNF- α -treated HeLa cells were applied directly to a chromatographic column containing Sephacryl S300. Aliquots of individual fractions were tested for ubiquitinylation activity with IP-pIkB α (s). The presumptive IkB α E3 activity, which formed high-molecular-weight IkB α bands, eluted around fraction 28 (Fig. 2A). The size of this IkB α E3 enzyme was estimated to be approximately 300 kDa by comparison with molecular marker proteins (Fig. 2B). At this point, we provisionally referred to this 300-kDa factor as IkB α E3-F1, because this factor functions as an IkB α -Ub ligase E3 collaborating with E1 and E2. Pooled peak fractions from Sephacryl S-300 chromatography were then separated by DEAE-5PW anion-exchange chromatography. A single peak for the IkB α E3-F1 activity eluted around 0.25 M NaCl at fraction 25 (Fig. 2C). We used this peak fraction from DEAE-5PW chromatography as the enzyme source for the characterization of IkB α E3-F1 without further purification.

To confirm whether the multiple high-molecular-weight bands detected immunochemically are actually due to poly-ubiquitinylation of IkB α , we replaced Ub with biotin-tagged

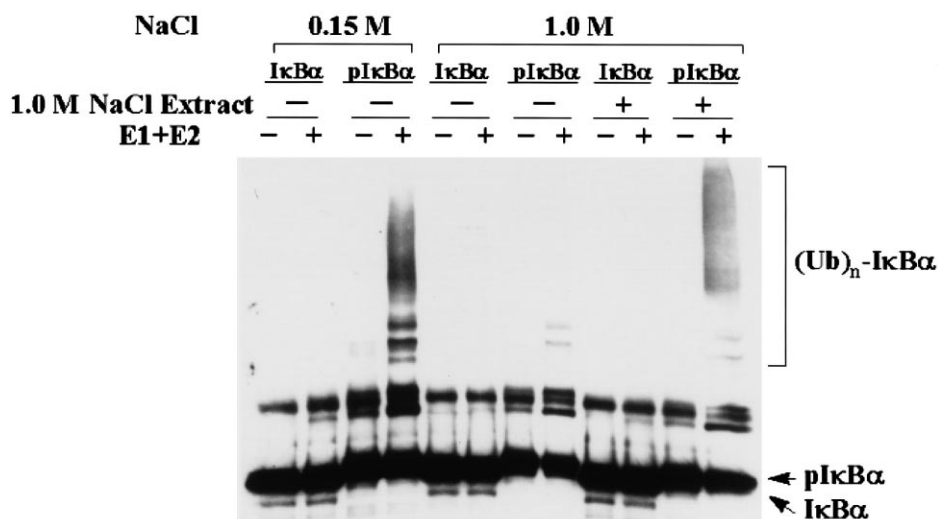


Fig. 1. Dissociation of IkB α -Ub ligase from IP-pIkB α (m) by a 1.0 M NaCl wash. IP-IkB α (m) and IP-pIkB α (m) were washed with 1.0 M NaCl. Materials dissociated were pooled, concentrated, and designated 1.0 M NaCl extracts. The ubiquitinylation assay was carried out by incubating with ATP and the indicated combinations (+/-) of E1, (His)₆-Ubch4 (E2), or Ub using 1.0 M NaCl-washed IP-pIkB α (m) or IP-IkB α (m) as the substrate in the presence or absence of 1.0 M NaCl extract. As controls, 0.15 M NaCl-washed IP-pIkB α and IP-IkB α were used. After terminating the reaction by the addition of a sample buffer for SDS-PAGE, the reaction mixtures were separated by SDS-PAGE on a 10% gel followed by Western blotting with anti-IkB α antibody. Multiple ubiquitinated IkB α bands with higher molecular masses were detected by Western analysis and are designated $(Ub)_n$ -IkB α .

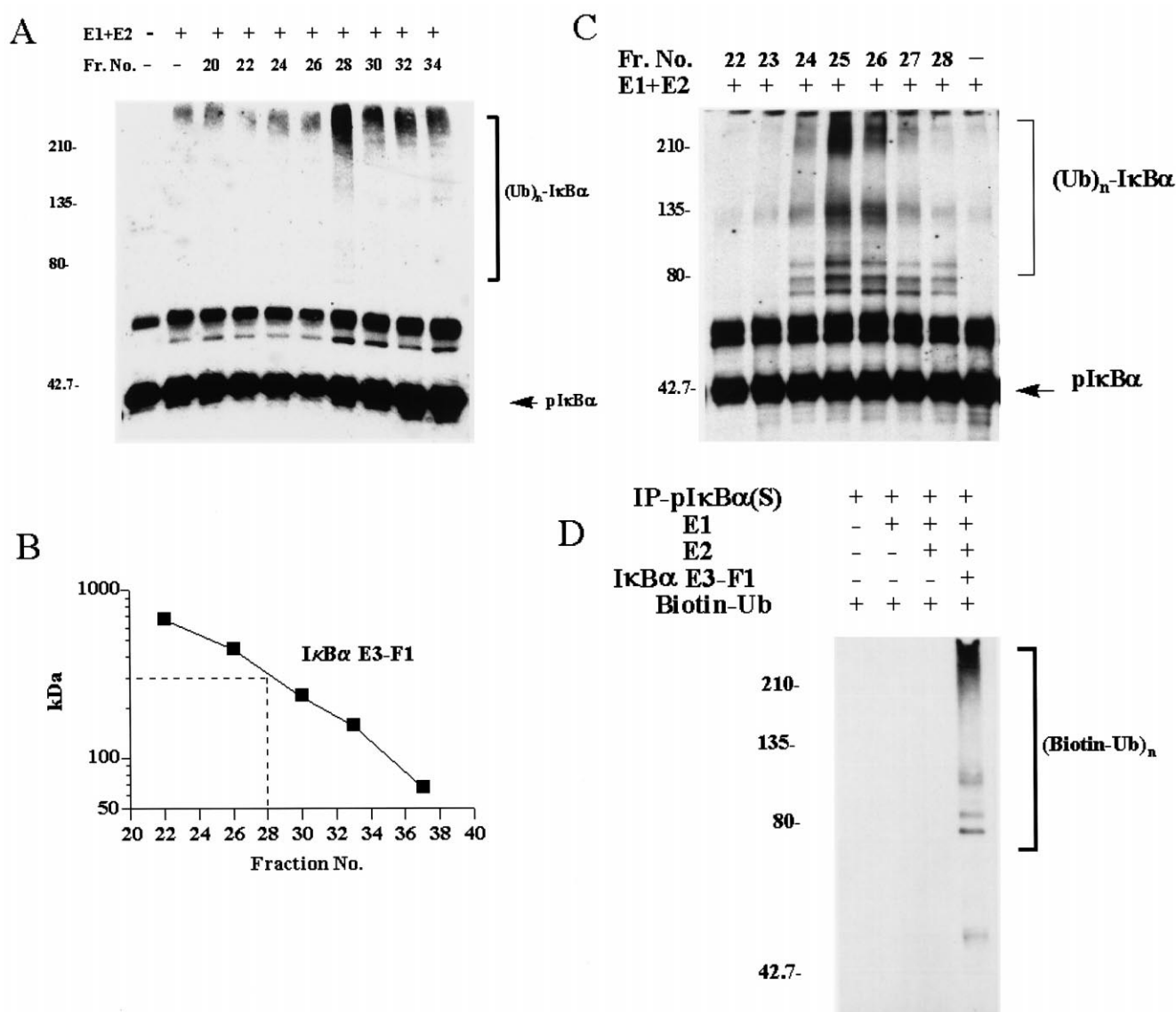


Fig. 2. Partial purification of an IkBαE3-F1 necessary for IkBα ubiquitinylation. A: Molecular sieve chromatography on a Sephacryl S-300 HR 16/60 column of HeLa cell extracts. Two-milliliter fractions were collected, and 6-μl aliquots of each fraction were used in an ubiquitinylation assay with an IP-pIkBα(s) substrate prepared from TNF-α-treated HeLa cell extracts under 'stringent' conditions. Western analysis was performed on the reaction products. The positions of molecular mass markers are shown on the left. B: Molecular mass estimation of IkBαE3-F1 (dotted line). Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), IgG (150 kDa), and bovine serum albumin (67 kDa) were used as molecular mass standards. C: DEAE-5PW anion-exchange chromatography. The pooled peak fractions from Sephacryl S300 chromatography were dialyzed against 50 mM Tris-HCl buffer (pH 7.9) containing 1 mM DTT and then loaded onto a DEAE-5PW column. The adsorbed proteins were eluted by a linear gradient of 0–500 mM NaCl in the same buffer. One-milliliter fractions were collected, and 6 μl of each fraction was assayed for ubiquitinylation activity as in A. Fractions containing IkBαE3-F1 activity were pooled. D: Identification of poly-ubiquitinated IkBα. As a substrate in the ubiquitinylation assay, biotinylated Ub was used instead of Ub in the various reaction mixtures as indicated. After incubation, the mixtures were analyzed by immunoblotting with horseradish peroxidase-conjugated streptavidin. The resulting poly-biotinylated Ubs are designated (Biotin-Ub)_n.

Ub and assayed the ubiquitinylation reaction. As shown in Fig. 2D, larger multiple bands of biotinylated Ub-IkBα complex were detected. This reaction was defective in the absence of E1, E2, or IkBαE3-F1. Thus, we concluded that the observed high-molecular-weight bands were poly-ubiquitinated IkBα whose formation requires E1, E2, IkBαE3-F1, ATP, and Ub.

When the IP-pIkBα(m) was washed extensively with a buffer containing 1.0 M NaCl (as described in Fig. 1) or 1.0 M NaH₂PO₄, the E3-like activity was almost completely

lost (Fig. 3). This loss of E3-like activity in IP-pIkBα(m) washed with either of these high-concentration salt solutions was restored by the addition of partially purified IkBαE3-F1. Taken together, these results indicate that the enzymatically active IkBα-Ub ligase contains a certain factor(s) that is loosely associated with IP-pIkBα(m), but not IP-IkBα(m), and that the putative enzyme dissociated by washing IP-pIkBα(m) with a high-concentration salt solution is functionally equivalent to isolated IkBαE3-F1.

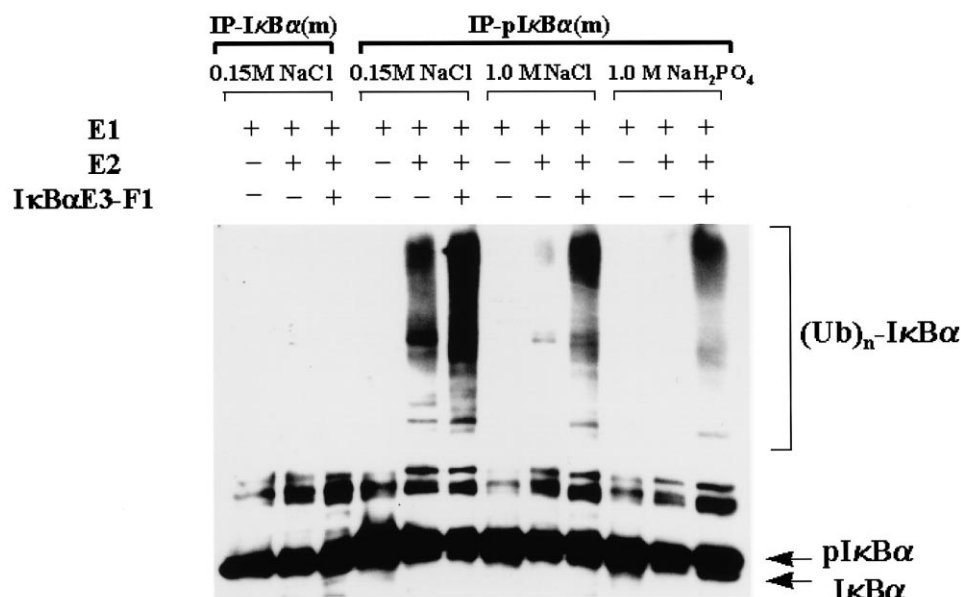


Fig. 3. IκBαE3-F1 restores the lost auto-ubiquitinating activity of IP-pIκBα(m) caused by a 1.0 M NaCl wash. IP-IκBα(m) and IP-pIκBα(m) were washed with a buffer containing 1.0 M NaCl or 1.0 M NaH₂PO₄ as indicated. These IP-IκBαs were used in ubiquitinylation assays with or without IκBαE3-F1.

3.3. IκBαE3-F1 is active for ubiquitinylation of phosphorylated IκBα irrespective of TNF-α stimulation

We next tested whether or not TNF-α stimulation is required for IκBαE3-F1 activity. An IκBαE3-F1 fraction was isolated from either TNF-α-treated or non-treated HeLa cells and assayed for ubiquitinylation activity in the presence of E1 and E2. As shown in Fig. 4, IκBαE3-F1 isolated from untreated or TNF-α-treated cells exhibited high activity on the poly-ubiquitinylation of IP-pIκBα(s). The pattern of poly-ubiquitinated bands was apparently the same when IκBαE3-F1 isolated from either TNF-α-treated or non-treated

cells was used. When non-phosphorylated IP-IκBα(s) was used as a substrate, no ubiquitinylation was observed by addition of IκBαE3-F1. These findings indicate that IκBαE3-F1 is constitutively active. Indeed, the elution profiles of crude IκBαE3-F1 extracts prepared from HeLa cells treated with either TNF-α or nothing from Sephacryl S-300 and DEAE-5PW columns were indistinguishable (data not shown).

3.4. IκBαE3-F1 is different from the SCF complex

It is important to examine whether or not IκBαE3-F1 is identical to the SCF complex known as the component of a

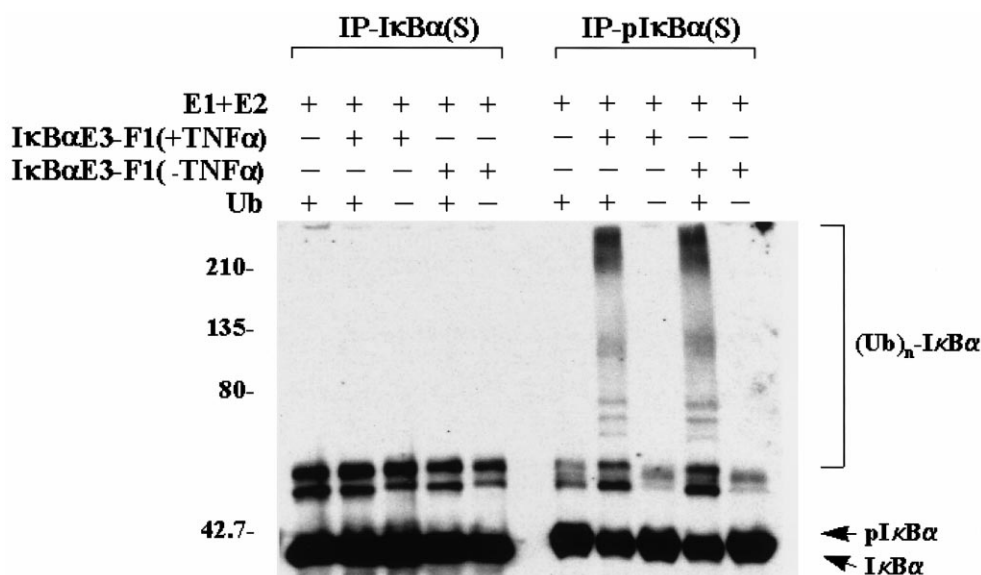


Fig. 4. Phosphorylation is required for IκBαE3-F1-dependent ubiquitinylation of IκBα, independent of TNF-α-treatment. Using the procedure described in Fig. 2A,C, IκBαE3-F1 was prepared from extracts of HeLa cells treated with either TNF-α or nothing, designated (+TNF-α) and (-TNF-α), respectively. Both IP-IκBα(s) and IP-pIκBα(s) were incubated with E1, E2, ATP, and the indicated combinations of Ub and IκBαE3-F1s. Immunoblotting was performed with an anti-IκBα antibody.

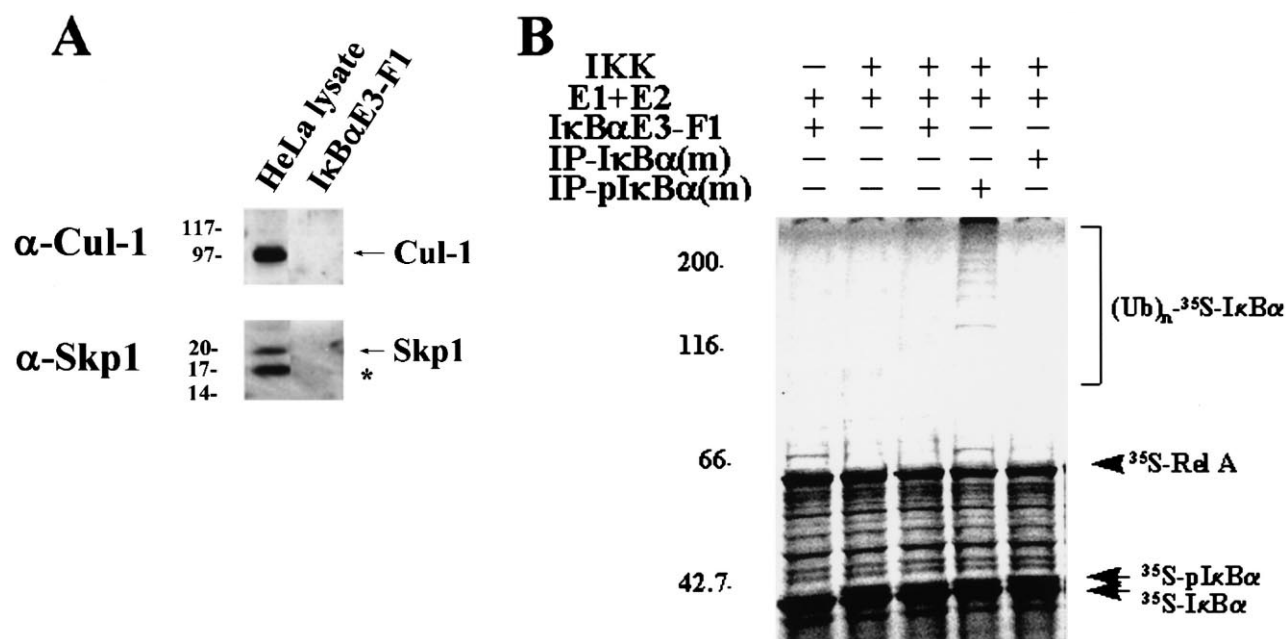


Fig. 5. IkBαE3-F1 differs from the SCF complex. A: Samples of HeLa cell extracts (8 μg) or partially purified IkBαE3-F1 (the pooled peak fractions from DEAE chromatography, see Fig. 2C) (2 μg) were used for immunoblot analysis with anti-Cullin-1 and Skp1 antibodies as indicated. An asterisk indicates an artifact band by anti-Skp1 antibody. B: IP-pIkBα(m), but not IkBαE3-F1, mediated poly-ubiquitinylation of in vitro translated ³⁵S-pIkBα. IkBα and RelA (the p65 component of NF-κB) were co-translated in vitro using a wheat-germ lysate translation system in the presence of [³⁵S]methionine and [³⁵S]cysteine. These radioactive proteins were preincubated with IKK and ATP, and assayed for ubiquitinylation after addition of IkBαE3-F1, IP-IkBα(m) and IP-pIkBα(m) as indicated (+/-). The reaction mixtures were then subjected to SDS-PAGE and autoradiographed [14]. Positions of molecular mass markers, ³⁵S-RelA, ³⁵S-IkBα and ³⁵S-pIkBα, and poly-ubiquitinated ³⁵S-IkBα are shown.

ubiquitinylation ligase of IkBα. For this, we performed immunoblot analysis using antibodies against Cullin-1 and Skp1 which are integral components of the SCF complex. As shown in Fig. 5A, no significant immunoreactive bands were detected in the isolated IkBαE3-F1 preparation, although these two antibodies reacted clearly with the crude extracts of HeLa cells, indicating that IkBαE3-F1 does not contain components of the SCF complex.

Subsequently, we examined whether or not IkBαE3-F1 is functionally equivalent to the SCF ligase. As reported previously [14], IP-pIkBα(m), but not IP-IkBα(m), caused poly-ubiquitinylation of in vitro translated ³⁵S-IkBα in the presence of IKK, E1 and E2. In contrast, IkBαE3-F1 did not exhibit any activity (Fig. 5B). These experiments were conducted in the presence of in vitro translated ³⁵S-RelA (p65 component of NF-κB) to appropriate in vivo conditions, but the same results were obtained in the absence of ³⁵S-RelA (data not shown). These results indicate that in addition to IkBαE3-F1, some other factor necessary for ubiquitinylation of pIkBα is associated firmly with IP-pIkBα(m), but not with IP-IkBα(m). This presumptive factor is clearly different from IkBαE3-F1, which is only loosely associated with pIkBα. Thus, the ubiquitinylation of pIkBα definitely requires IkBαE3-F1 and the cooperative factor bound tightly to pIkBα, which is assumed to be an SCF^{βTrCP} complex [15].

4. Discussion

Previously, we found that the very active IkBα Ub-ligase is selectively associated with pIkBα induced by treatment of TNF-α to direct its poly-ubiquitinylation [14]. The initial observation of the present work was the finding that the IkBα

Ub-ligase activity was lost almost completely upon washing IP-pIkBα(m) with high-concentration salt solutions (Figs. 1 and 3), suggesting the existence of a loosely bound factor responsible for IkBα ubiquitinylation. Currently there is accumulating evidence that SCF^{βTrCP}, consisting of Skp1, Cullin-1, and βTrCP, functions as a Ub-ligase for IkBα [6–10] and β-catenin [7,11–13], as mentioned in Section 1. However, SCF^{βTrCP} is known to be tightly associated with pIkBα without releasing by treatment with high salts [15], suggesting that there may be present some other factor(s), differing from SCF^{βTrCP}, which would collaborate with SCF^{βTrCP} ligase.

Based on these circumstances, we found a novel factor termed IkBαE3-F1 with an molecular mass of 300 kDa (Fig. 2) that may be required for signal-dependent ubiquitinylation of pIkBα. IkBαE3-F1 also is recruited to be associated with pIkBα in response to treatment with TNF-α, like SCF^{βTrCP} [14], but the activity itself is independent of the external signal, because apparently similar activity capable of assisting the poly-ubiquitinylation of pIkBα was observed for IkBαE3-F1 from both TNF-α-stimulated or unstimulated HeLa cells (Fig. 4). It is clear that IkBαE3-F1 differs from the SCF^{βTrCP} complex. First, IkBαE3-F1, but not SCF^{βTrCP}, was dissociated from pIkBα(m) by treatment with high-concentration salts, implying that it is only loosely associated with pIkBα. Second, no measurable Cullin-1 and Skp1, components of the SCF^{βTrCP} complex, were detected in the partially purified IkBαE3-F1 preparation by immunoblot analysis using anti-Cullin-1 and anti-Skp1 antibodies (Fig. 5A). In addition, IP-pIkBα(m) was assessed for the ability to ubiquitinylation in vitro translated ³⁵S-labeled IkBα, pre-phosphorylated by purified IKK, whereas IkBαE3-F1 did not cause this poly-ubiquitinylation of ³⁵S-pIkBα (Fig. 5B), implying that

I κ B α E3-F1 alone is insufficient for the Ub-ligase activity of pI κ B α , which requires another factor for full function.

Consistent with this notion, we assume that I κ B α E3-F1 plays an essential role for the I κ B degrading pathway by cooperating with SCF^{βTrCP} to form the ultimate active Ub-I κ B α ligase complex. Quite recently, another factor named yeast Rbx1 [16,17] and its mammalian homologue ROC1 [18,19] have been found as a fourth component of the SCF complex, which plays an indispensable role for poly-ubiquitinylation of specific targets, such as G1 cyclin and I κ B α . The relationship between these newly identified factors and the presently described I κ B α E3-F1 is unknown, but they are unlikely to be identical, because the new factor was reported to be tightly associated with Cullin-1. Further purification of I κ B α E3-F1, which is in progress, is required to clarify this issue. It is also unknown whether I κ B α E3-F1 is involved in the ubiquitinylation of β -catenin which is known to be ubiquitinated by the same I κ B α E3-SCF^{βTrCP} complex [7,11–13]. Whether the presently described I κ B α E3-F1 has a common function for various SCF species containing many types of F-box protein ligases (reviewed in [20–22]) awaits further study.

References

- [1] Ghosh, S., May, M.J. and Kopp, E.B. (1998) *Annu. Rev. Immunol.* 16, 225–260.
- [2] Maniatis, T. (1999) *Genes Dev.* 13, 505–510.
- [3] Elledge, S.J. and Harper, J.W. (1998) *Biochim. Biophys. Acta* 1377, M61–M70.
- [4] Jiang, J. and Struhl, G. (1998) *Nature* 391, 493–496.
- [5] Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997) *EMBO J.* 16, 3797–3804.
- [6] 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.
- [7] Winston, J.T., Strack, P., Beer-Romero, P., Chu, C.Y., Elledge, S.J. and Harper, J.W. (1999) *Genes Dev.* 13, 270–283.
- [8] Spencer, E., Jiang, J. and Chen, Z.J. (1999) *Genes Dev.* 13, 284–294.
- [9] Kroll, M., Margottin, F., Kohl, A., Renard, P., Durand, H., Concordet, J.P., Bachelier, F., Arenzana-Seisdedos, F. and Benarous, R. (1999) *J. Biol. Chem.* 274, 7941–7945.
- [10] Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, K., Higashi, H., Nakano, H., Okumura, K., Onoe, K., Good, R.A. and Nakayama, K. (1999) *Proc. Natl. Acad. Sci. USA* 96, 3859–3863.
- [11] Latres, E., Chiaur, D.S. and Pagano, M. (1999) *Oncogene* 18, 849–854.
- [12] Hart, M., Concordet, J.P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R. and Polakis, P. (1999) *Curr. Biol.* 9, 207–210.
- [13] Kitagawa, K., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K. and Nakayama, K. (1999) *EMBO J.* 18, 2401–2410.
- [14] Suzuki, H., Chiba, T., Kobayashi, M., Takeuchi, M., Furuichi, K. and Tanaka, K. (1999) *Biochem. Biophys. Res. Commun.* 256, 121–126.
- [15] Suzuki, H., Chiba, T., Kobayashi, M., Takeuchi, M., Suzuki, T., Ichiyama, A., Ikenoue, T., Omata, M., Furuichi, K. and Tanaka, K. (1999) *Biochem. Biophys. Res. Commun.* 256, 127–132.
- [16] Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin Jr., W.G., Elledge, S.J., Conaway, R.C., Harper, J.W. and Conaway, J.W. (1999) *Science* 284, 657–661.
- [17] Skowyra, D., Koepp, D.M., Kamura, T., Conrad, M.N., Conaway, R.C., Conaway, J.W., Elledge, S.J. and Harper, J.W. (1999) *Science* 284, 662–665.
- [18] Ohta, T., Michel, J.J., Schottelius, A.J. and Xiong, Y. (1999) *Mol. Cell* 3, 535–541.
- [19] Tan, P., Fuchs, S.Y., Chen, A., Wu, K., Gomez, C., Ronai, Z. and Pan, Z.-Q. (1999) *Mol. Cell* 3, 527–533.
- [20] Harper, J.W. and Elledge, S.J. (1999) *Nature Cell Biol.* 1, 5–7.
- [21] Tyers, M. and Williams, A.R. (1999) *Science* 284, 601–604.
- [22] Patton, E.E., Willems, A.R. and Tyers, M. (1998) *Trends Genet.* 14, 236–243.