Identification of a novel 300-kDa factor termed IκBαE3-F1 that is required for ubiquitinylation of IκBα

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Abstract Destruction of IκB by ubiquitinylation is required for signal-dependent activation of NF-κB. The IκBα ubiquitin-ligase activity associated with phosphorylated IκBα (pIκBα) in HeLa cells was almost completely lost by washing under stringent conditions including 1 M NaCl; nevertheless, an $SCF^{\beta TrCP}$ complex containing Skp1, Cullin-1, and F-box/WD40 protein βTrCP was still bound to pIκBα, suggesting the existence of a putative factor that is loosely associated with pIκBα and may collaborate with $SCF^{\beta TrCP}$. The factor was named IκBαE3-F1 and was partially purified from HeLa cells. Gel filtration analysis revealed that IκBαE3-F1 has an apparent molecular mass of approximately 300 kDa.

Key words: Ubiquitin; SCF; IκB; NF-κB; Tumor necrosis factor α

1. Introduction

There is growing evidence addressing the importance of the immediate removal of an inhibitory protein termed IκB in the NF-κB signalling pathway, because IκB prevents nuclear translocation of cytoplasmic NF-κB by masking the nuclear location signal (NLS) of NF-κB [1]. The key process of the proteolysis of IκB is thought to be the ubiquitinylation reaction directed by IκBα ubiquitin (Ub)-ligase (also named IκBαE3) capable of transferring Ub to the target IκB molecule phosphorylated by IκB kinase (IKK) via E1 (Ub-activating) and E2 (Ub-conjugating) enzymes [2]. However, the molecular basis of the IκBαE3 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 IκBα as well as β-catenin, because of the similarity of the two phosphorylation sites present in both IκBα and β-catenin [5]. Before long, the $SCF^{\beta TrCP}$ complex, consisting of Skp1, Cullin-1, and βTrCP, had been identified not only as a long-sought IκBαE3 [6–10] but also as a Ub-ligase for β-catenin [7,11–13].

Based on these numerous reports, it is clear that the $SCF^{\beta TrCP}$ complex is an E3 enzyme required for the ubiquitinylation of IκBα, but it is still not clear whether the $SCF^{\beta TrCP}$ complex in combination with E1 and E2 is sufficient for the ubiquitinylation of IκBα. We found that the $SCF^{\beta TrCP}$ complex coordinately recruited to phosphorylated IκBα (pIκBα) induced by tumor necrosis factor α (TNF-α) stimulation was tightly bound to pIκBα 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 ubiquitinylating activity, suggesting the existence of other factor(s) which may collaborate with $SCF^{\beta TrCP}$ for the ubiquitinylation of pIκBα. We report here a novel factor named IκBαE3-F1 with an apparent molecular mass of 300 kDa that is loosely bound to pIκBα, differing from the tightly associated $SCF^{\beta TrCP}$ 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 μM of MG132 (Z-Leu-Leu-Leu-H, Peptide Institute, Inc., Osaka) for 45 min followed by 0.25 μM 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 IκBα

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 μg/ml each of leupeptin, aprotinin, and pepstatin A). Crude lysates were clarified by centrifugation at 15 000 rpm for 20 min. The HeLa extracts (10 mg) were incubated at 4°C for 16 h with 1 μg of a rabbit polyclonal antibody against the carboxy-terminus of IκBα (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-pIκBα(s) and IP-IκBα(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 μg/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-IκBα(m) and IP-pIκBα(m), respectively. In some experiments, the immunoprecipitates were further washed with buffer B containing 1.0 M NaCl or 1.0 M NaH_2PO_4 (pH 7.4). The

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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 ubiquitinylating activity of pIκBα immunoprecipitates by 1 M NaCl wash

IκBα and pIκBα were prepared by immunoprecipitation (IP) using anti-IκBα antibody from non-stimulated and TNF-α-treated HeLa cell extracts under the 'mild' conditions described in Section 2. These IP-IκBα and IP-pIκBα preparations (hereafter referred to as IP-IκBα(m) and IP-pIκBα(m), respectively) were used as substrates in a ubiquitinylation assay. As previously reported [14], incubation of IP-pIκBα(m) with Ub, ATP, recombinant E1 and E2 (Ubch4) enzymes was sufficient to produce poly-ubiquitinylation of pIκBα without any other supplement (see Fig. 1, lanes indicated as 0.15 M NaCl). These ubiquitinylated proteins were detected by Western analysis with anti-IκBα antibody. In contrast, Ub-ligated bands were not detected for the non-phosphorylated form of IP-IκBα(m). This reaction requires only E1, E2, and Ub, which suggests that a presumptive IκBα-Ub ligase (equivalent to IκBαE3) is tightly associated with IP-pIκBα(m) in vivo, as previously reported [14].

Conditions necessary for dislodging this putative IκBαE3 from the associated IP-pIκBα(m) were assessed by washing the complexes with a high-concentration salt solution. As shown in Fig. 1, when IP-IκBα(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-pIκBα(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-pIκBα(m) having no ubiquitinylation activity, but showed no stimulating effect on the non-phosphorylated IP-IκBα(m). These reconstitution results suggest a reversible association of a putative IκBα-Ub ligase with pIκBα.

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

To further characterize this putative IκBα-Ub ligase, we attempted to isolate it from HeLa cell extracts. Phosphorylated IκBα was prepared by immunoprecipitation from TNF-α-treated HeLa cell extracts under 'stringent' conditions as described in Section 2. The resulting immunoprecipitates, named IP-pIκBα(s), had no E3-like activity in concerted with E1, E2, and Ub, as similarly seen in 1.0 M NaCl-washed IP-pIκBα(m), and were used routinely as substrates in the IκBα 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-pIκBα(s). The presumptive IκBαE3 activity, which formed high-molecular-weight IκBα bands, eluted around fraction 28 (Fig. 2A). The size of this IκBαE3 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 IκBαE3-F1, because this factor functions as an IκBα-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 IκBαE3-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 IκBαE3-F1 without further purification.

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

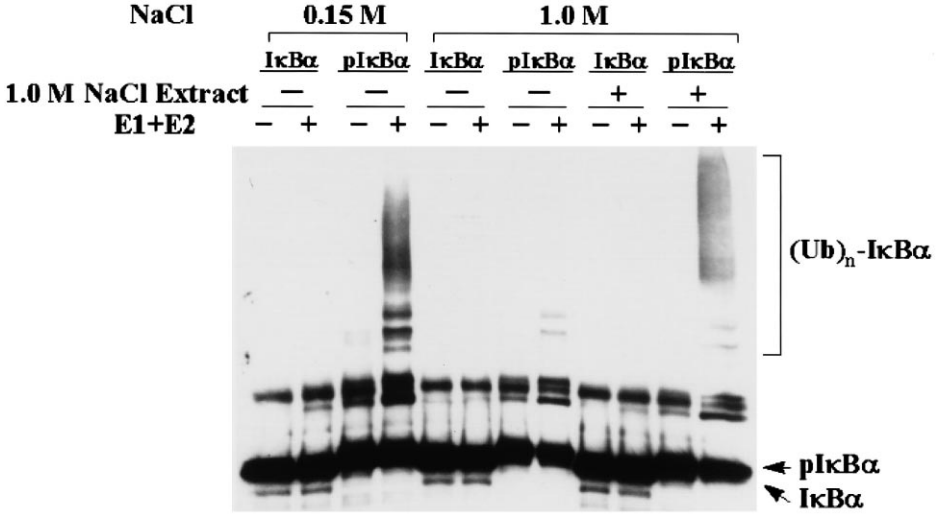

Fig. 1. Dissociation of IκBα-Ub ligase from IP-pIκBα(m) by a 1.0 M NaCl wash. IP-IκBα(m) and IP-pIκBα(m) were washed with 1.0 M NaCl. Materials dissociated were pooled, concentrated, and designated 1.0 M NaCl extracts. The ubiquitinylation assay was carried out by incubating with ATP and the indicated combinations (+/−) of E1, $(His)_6$-Ubch4 (E2), or Ub using 1.0 M NaCl-washed IP-pIκBα(m) or IP-IκBα(m) as the substrate in the presence or absence of 1.0 M NaCl extract. As controls, 0.15 M NaCl-washed IP-pIκBα and IP-IκBα 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-IκBα antibody. Multiple ubiquitinylated IκBα bands with higher molecular masses were detected by Western analysis and are designated $(Ub)_n$-IκBα.

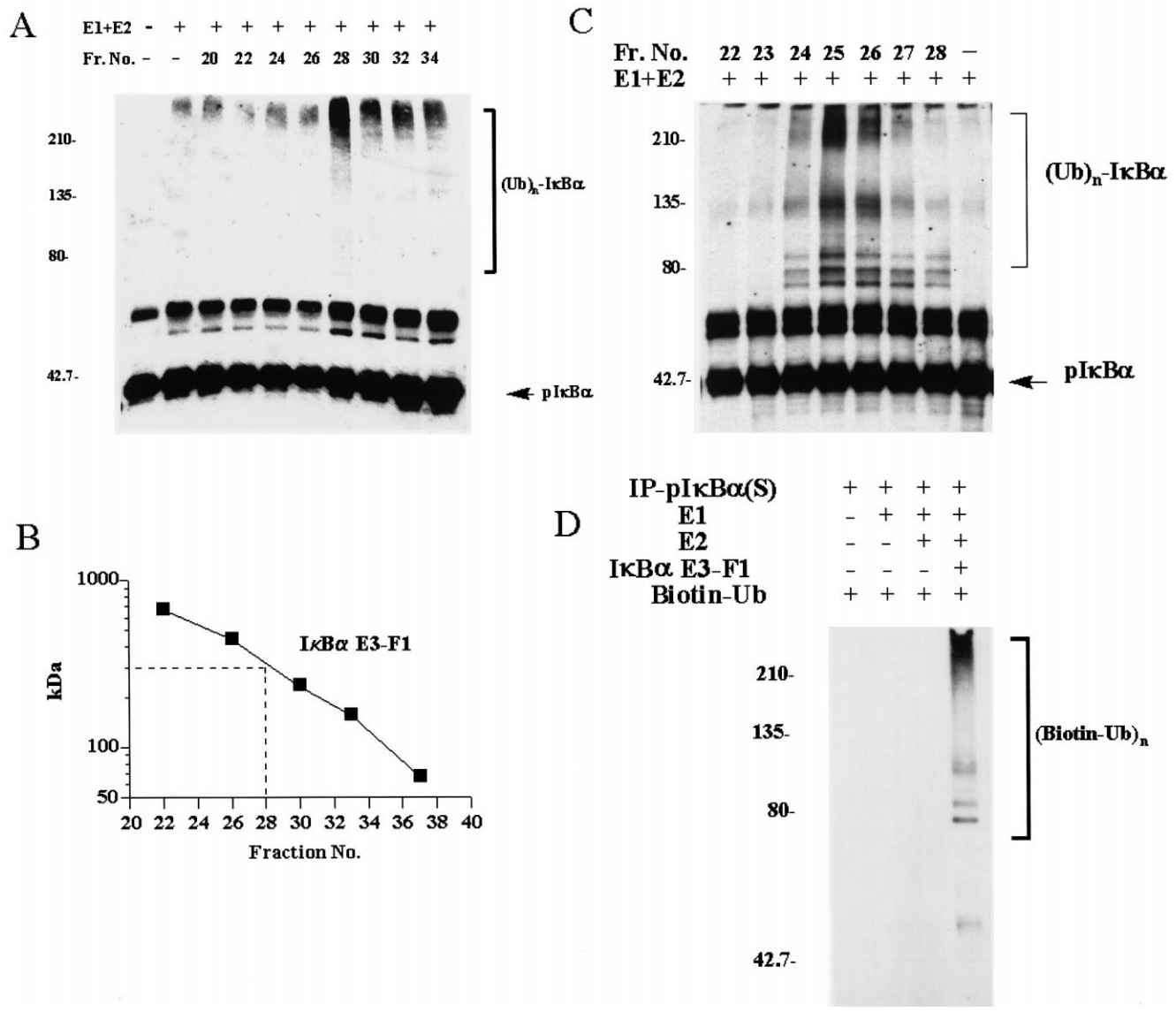

Fig. 2. Partial purification of an IκBαE3-F1 necessary for IκBα ubiquitinylation. A: Molecular sieve chromatography on a Sephacryl S-300 HR 16/60 column of HeLa cell extracts. Two-milliliter fractions were collected, and 6-μl aliquots of each fraction were used in an ubiquitinylation assay with an IP-pIκBα(s) 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 IκBαE3-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 IκBαE3-F1 activity were pooled. D: Identification of poly-ubiquitinylated IκBα. 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 $(Biotin\text{-}Ub)_n$.

Ub and assayed the ubiquitinylation reaction. As shown in Fig. 2D, larger multiple bands of biotinylated Ub-IκBα complex were detected. This reaction was defective in the absence of E1, E2, or IκBαE3-F1. Thus, we concluded that the observed high-molecular-weight bands were poly-ubiquitinylated IκBα whose formation requires E1, E2, IκBαE3-F1, ATP, and Ub.

When the IP-pIκBα(m) was washed extensively with a buffer containing 1.0 M NaCl (as described in Fig. 1) or 1.0 M NaH_2PO_4, the E3-like activity was almost completely lost (Fig. 3). This loss of E3-like activity in IP-pIκBα(m) washed with either of these high-concentration salt solutions was restored by the addition of partially purified IκBαE3-F1. Taken together, these results indicate that the enzymatically active IκBα-Ub ligase contains a certain factor(s) that is loosely associated with IP-pIκBα(m), but not IP-IκBα(m), and that the putative enzyme dissociated by washing IP-pIκBα(m) with a high-concentration salt solution is functionally equivalent to isolated IκBαE3-F1.

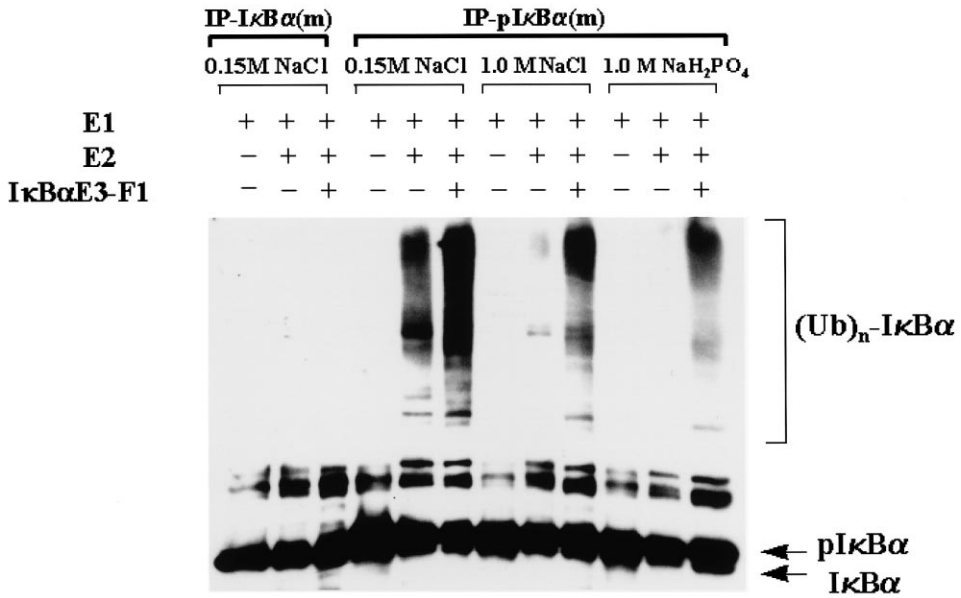

Fig. 3. IκBαE3-F1 restores the lost auto-ubiquitinylating activity of IP-pIκBα(m) caused by a 1.0 M NaCl wash. IP-IκBα(m) and IP-pIκBα(m) were washed with a buffer containing 1.0 M NaCl or 1.0 M NaH_2PO_4 as indicated. These IP-IκBαs were used in ubiquitinylation assays with or without IκBαE3-F1.

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-ubiquitinylated 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

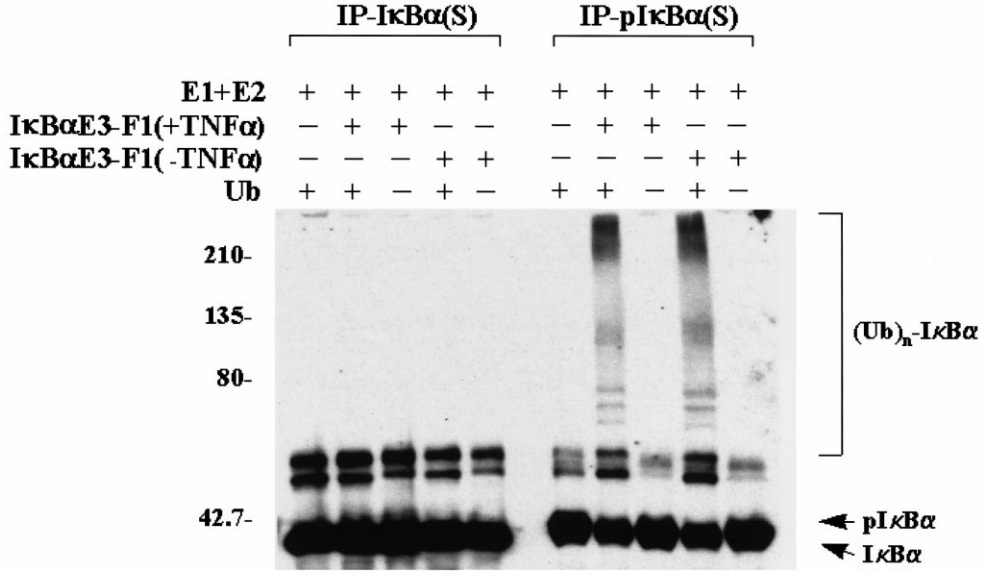

Fig. 4. Phosphorylation is required for IκBαE3-F1-dependent ubiquitinylation of IκBα, independent of TNF-α-treatment. Using the procedure described in Fig. 2A,C, IκBαE3-F1 was prepared from extracts of HeLa cells treated with either TNF-α or nothing, designated (+TNF-α) and (−TNF-α), respectively. Both 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.

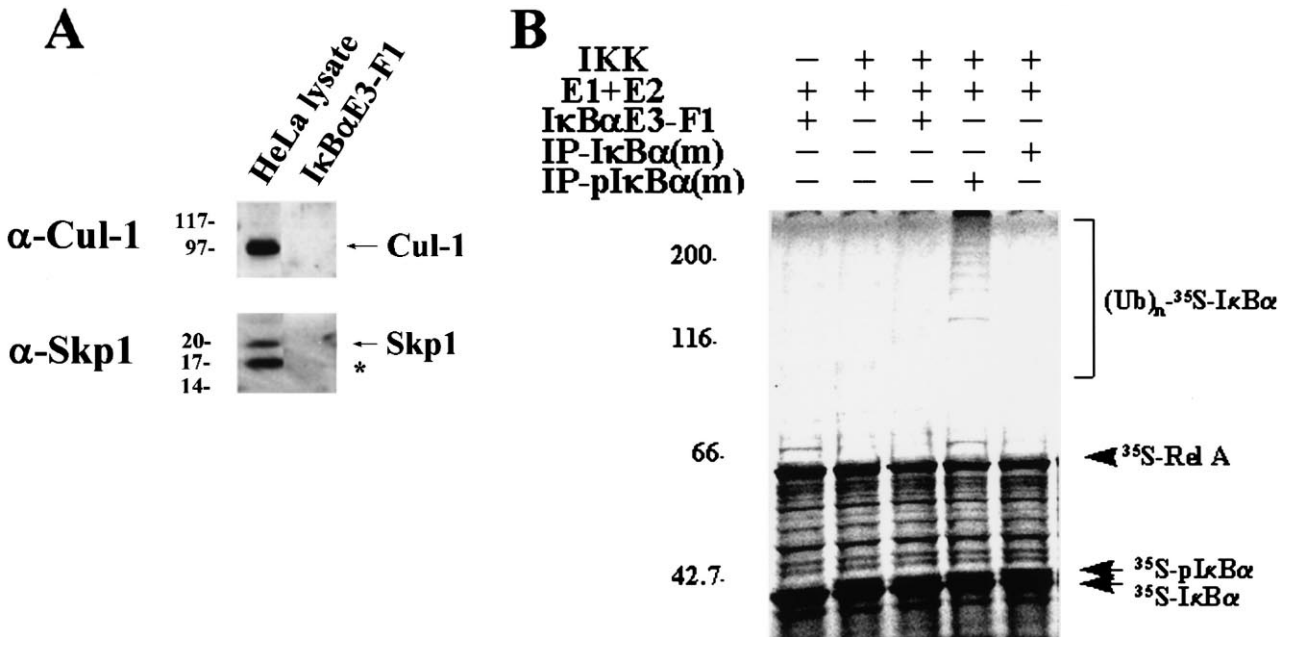

Fig. 5. IκBαE3-F1 differs from the SCF complex. A: Samples of HeLa cell extracts (8 μg) or partially purified IκBαE3-F1 (the pooled peak fractions from DEAE chromatography, see Fig. 2C) (2 μg) were used for immunoblot analysis with anti-Cullin-1 and-Skp1 antibodies as indicated. An asterisk indicates an artifact band by anti-Skp1 antibody. B: IP-pIκBα(m), but not IκBαE3-F1, mediated poly-ubiquitinylation of in vitro translated ^{35}S-pIκBα. IκBα and RelA (the p65 component of NF-κB) were co-translated in vitro using a wheat-germ lysate translation system in the presence of [^{35}S]methionine and [^{35}S]cysteine. These radioactive proteins were preincubated with IKK and ATP, and assayed for ubiquitinylation after addition of IκBαE3-F1, IP-IκBα(m) and IP-pIκBα(m) as indicated (+/−). The reaction mixtures were then subjected to SDS-PAGE and autoradiographed [14]. Positions of molecular mass markers, ^{35}S-RelA, ^{35}S-IκBα and ^{35}S-pIκBα, and poly-ubiquitinylated ^{35}S-IκBα are shown.

ubiquitinylation ligase of IκBα. 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 IκBαE3-F1 preparation, although these two antibodies reacted clearly with the crude extracts of HeLa cells, indicating that IκBαE3-F1 does not contain components of the SCF complex.

Subsequently, we examined whether or not IκBαE3-F1 is functionally equivalent to the SCF ligase. As reported previously [14], IP-pIκBα(m), but not IP-IκBα(m), caused poly-ubiquitinylation of in vitro translated ^{35}S-IκBα in the presence of IKK, E1 and E2. In contrast, IκBαE3-F1 did not exhibit any activity (Fig. 5B). These experiments were conducted in the presence of in vitro translated ^{35}S-RelA (p65 component of NF-κB) to appropriate in vivo conditions, but the same results were obtained in the absence of ^{35}S-RelA (data not shown). These results indicate that in addition to IκBαE3-F1, some other factor necessary for ubiquitinylation of pIκBα is associated firmly with IP-pIκBα(m), but not with IP-IκBα(m). This presumptive factor is clearly different from IκBαE3-F1, which is only loosely associated with pIκBα. Thus, the ubiquitinylation of pIκBα definitely requires IκBαE3-F1 and the cooperative factor bound tightly to pIκBα, which is assumed to be an SCF$^{\beta TrCP}$ complex [15].

4. Discussion

Previously, we found that the very active IκBα Ub-ligase is selectively associated with pIκBα induced by treatment of TNF-α to direct its poly-ubiquitinylation [14]. The initial observation of the present work was the finding that the IκBα Ub-ligase activity was lost almost completely upon washing IP-pIκBα(m) with high-concentration salt solutions (Figs. 1 and 3), suggesting the existence of a loosely bound factor responsible for IκBα ubiquitinylation. Currently there is accumulating evidence that SCF$^{\beta TrCP}$, consisting of Skp1, Cullin-1, and βTrCP, functions as a Ub-ligase for IκBα [6–10] and β-catenin [7,11–13], as mentioned in Section 1. However, SCF$^{\beta TrCP}$ is known to be tightly associated with pIκBα without releasing by treatment with high salts [15], suggesting that there may be present some other factor(s), differing from SCF$^{\beta TrCP}$, which would collaborate with SCF$^{\beta TrCP}$ ligase.

Based on these circumstances, we found a novel factor termed IκBαE3-F1 with an molecular mass of 300 kDa (Fig. 2) that may be required for signal-dependent ubiquitinylation of pIκBα. IκBαE3-F1 also is recruited to be associated with pIκBα in response to treatment with TNF-α, like SCF$^{\beta TrCP}$ [14], but the activity itself is independent of the external signal, because apparently similar activity capable of assisting the poly-ubiquitinylation of pIκBα was observed for IκBαE3-F1 from both TNF-α-stimulated or unstimulated HeLa cells (Fig. 4). It is clear that IκBαE3-F1 differs from the SCF$^{\beta TrCP}$ complex. First, IκBαE3-F1, but not SCF$^{\beta TrCP}$, was dissociated from pIκBα(m) by treatment with high-concentration salts, implying that it is only loosely associated with pIκBα. Second, no measurable Cullin-1 and Skp1, components of the SCF$^{\beta TrCP}$ complex, were detected in the partially purified IκBαE3-F1 preparation by immunoblot analysis using anti-Cullin-1 and anti-Skp1 antibodies (Fig. 5A). In addition, IP-pIκBα(m) was assessed for the ability to ubiquitinylate in vitro translated ^{35}S-labeled IκBα, pre-phosphorylated by purified IKK, whereas IκBαE3-F1 did not cause this poly-ubiquitinylation of ^{35}S-pIκBα (Fig. 5B), implying that

IκBαE3-F1 alone is insufficient for the Ub-ligase activity of pIκBα, which requires another factor for full function.

Consistent with this notion, we assume that IκBαE3-F1 plays an essential role for the IκB degrading pathway by cooperating with $SCF^{\beta TrCP}$ to form the ultimate active Ub-IκBα 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 IκBα. The relationship between these newly identified factors and the presently described IκBαE3-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 IκBαE3-F1, which is in progress, is required to clarify this issue. It is also unknown whether IκBαE3-F1 is involved in the ubiquitinylation of β-catenin which is known to be ubiquitinylated by the same IκBαE3-$SCF^{\beta TrCP}$ complex [7,11–13]. Whether the presently described IκBαE3-F1 has a common function for various SCF species containing many types of F-box protein ligases (reviewed in [20–22]) awaits further study.

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