### **NEDD8** recruits E2-ubiquitin to SCF E3 ligase

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NEDD8/Rub1 is a ubiquitin (Ub)-like post-translational modifier that is covalently linked to cullin (Cul)-family proteins in a manner analogous to ubiquitylation. NEDD8 is known to enhance the ubiquitylating activity of the SCF complex (composed of Skp1, Cul-1, ROC1 and F-box protein), but the mechanistic role is largely unknown. Using an *in vitro* reconstituted system, we report here that NEDD8 modification of Cul-1 enhances recruitment of Ub-conjugating enzyme Ubc4 (E2) to the SCF complex (E3). This recruitment requires thioester linkage of Ub to Ubc4. Our findings indicate that the NEDD8-modifying system accelerates the formation of the E2–E3 complex, which stimulates protein polyubiquitylation.

*Keywords*: IκBα/NEDD8/ROC1–SCF/ubiquitylation/ubiquitin ligase

#### Introduction

Ubiquitin (Ub) is an 8.6 kDa highly conserved protein molecule. It forms a polyubiquitin chain on proteins, which becomes a degradation signal attacked by the 26S proteasome (Hershko and Ciechanover, 1998; Voges *et al.*, 1999). Protein ubiquitylation is catalysed by a cascade reaction involving three enzymes, E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme) and E3 (Ubligating enzyme) (Hershko and Ciechanover, 1998). NEDD8 (a mammalian orthologue of the budding yeast Rub1, related to Ub1) has the highest homology to Ub (57% identity) among an expanding family of Ub-like proteins in eukaryotes (Hochstrasser, 1998; Jentsch and

Pyrowolakis, 2000; Yeh et al., 2000). NEDD8 is covalently attached to target proteins via the C-terminal glycine residue in a manner analogous to ubiquitylation, which is catalysed by the APP-BP1/Uba3 heterodimer and Ubc12, similar to the E1- and E2-like enzymes, referred to here simply as 'E1L' and 'E2L', respectively (Osaka et al., 1998; Gong and Yeh, 1999). The NEDD8 conjugation system is conserved across species (Lammer et al., 1998; Liakopoulos et al., 1998) and, to date, its targets are unknown other than Cul-family proteins (Hochstrasser, 2000; Jentsch and Pyrowolakis, 2000). Cul-1 is a common subunit of Ub-protein ligases termed 'SCF', or the 'ROC1-SCF' complex, consisting of the core subunits Skp1, Cul-1/Cdc53, ROC1/Rbx1/Hrt1 and substrate recognition adaptors known as F-box proteins, which are involved in ubiquitylation of a multitude of proteins (Deshaies, 1999; Kipreos and Pagano, 2000). Recently, other Cul-family proteins have also been described to constitute a large family of distinct Ub-protein ligase complexes, since ROC1/Rbx1/Hrt1, a RING-finger protein, which is essential for the catalytic activity of SCF, interacts with all members of Cul-family proteins (Kamura et al., 1999a; Ohta et al., 1999; Seol et al., 1999). Intriguingly, it is also reported that the Rbx1 is involved in Rub1 modification of two cullins, Cdc53/Cul-1 and Cul-2, perhaps acting as an E3 for both ubiquitylation and NEDD8 modification (Kamura et al., 1999b). In addition, Furukawa et al. (2000) recently reported that ROC1 promotes nuclear accumulation of Cul-1 to facilitate its NEDD8 modification, enhancing ubiquitin ligase activity of Cul-1.

The multifunctional transcription factor, NF-κB, which consists of two distinct subunits, p50 and p65, is normally present in the cell cytoplasm as an inactive form due to association with IkB (Baldwin, 1996; Ghosh et al., 1998). IκB prevents the transportation of NF-κB into the nucleus by its nuclear localization signal. To date, it is clear that phosphorylation and subsequent proteolytic destruction of IκB result in the immediate removal of IκB (Finco and Baldwin, 1995; Maniatis, 1999). The multisubunit IkB kinase (abbreviated herein as IKK) phosphorylates two serine residues, Ser32 and Ser36, at the N-terminal region of IκBα (a major member of IκB family proteins) (May and Chosh, 1999). The ROC1–SCF<sup>βTrCP1</sup> complex, identified as an  $I\kappa B\alpha$ –E3 ligase, binds phosphorylated  $I\kappa B\alpha$ (pIκBα) to ubiquitylate, generating a polyubiquitin chain, which is finally recognized by the 26S proteasome for ultimate degradation (Yaron et al., 1998; Winston et al.,

We recently have reported that the putative IkB $\alpha$ –E3, homodimer of SCF $^{\beta TrCP1}$  or SCF $^{\beta TrCP2}$ , is recruited rapidly to bind pIkB $\alpha$  *in vivo* and *in vitro* (Suzuki *et al.*, 2000). During these studies, we noticed that Cul-1 is modified preferentially by NEDD8. More recently, it has been

demonstrated that NEDD8 modification of Cul-1 activates ubiquitylation of IkB $\alpha$  (Furukawa *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000) and p27<sup>Kip1</sup> (Morimoto *et al.*, 2000; Podust *et al.*, 2000), by SCF<sup>\betaTrCP1</sup> and SCF<sup>Skp2</sup>, respectively. However, the mechanistic role of NEDD8 in promoting SCF Ub ligase activity is to a large extent unknown.

Using a fully *in vitro* reconstituted system, we examined in the present study the mechanism by which NEDD8 modification of Cul-1 augments the function of ROC1–SCF $^{\beta TrCP1}$  as an E3–I $\kappa$ B $\alpha$  ligase. Our results showed that NEDD8-ylation of Cul-1 promotes recruitment of Ublinked E2 (Ubc4) to the ROC1–SCF $^{\beta TrCP1}$  complex, leading to efficient ubiquitylation of phosphorylated I $\kappa$ B $\alpha$ . Our results indicate that the NEDD8 system positively regulates SCF activity, possibly through a conformational change of Cul-1 that promotes the E2–E3 complex formation. These findings shed new light on the mechanism underlying the functional link between the Ub and NEDD8 systems that are highly conserved during evolution.

#### Results

# NEDD8-ylation of Cul-1 stimulates ubiquitylation of $I\kappa B\alpha$ by ROC1–SCF $^{\beta TrCP1}$

To investigate the mechanistic role of NEDD8 modification of Cul-1, we devised a fully reconstituted system for NEDD8-ylation and IκBα ubiquitylation. To this end, we produced all components involved in these systems as recombinant proteins by *Escherichia coli* or the baculovirus system (for details, see Materials and methods). Figure 1A shows dye staining patterns after separation by SDS-PAGE, indicating that all recombinant proteins were purified to near homogeneity.

First, we tested whether the reconstituted NEDD8 system is active for modification of Cul-1. As shown in Figure 1B, when ROC1–SCF<sup>βTrCP1</sup> was incubated with E1L and E2L in the presence of His-NEDD8, two anti-HA (Cul-1)-reactive bands were detected, whereas only a single species was detected in the absence of the NEDD8 system. The fast migrating major band corresponded to unmodified Cul-1 and the species exhibiting reduced mobility on SDS-PAGE were thought to be Cul-1 conjugated to His-NEDD8, because this slow migrating band did not appear when we used ROC1-SC(K/ R)F<sup>βTrCP1</sup>, whose NEDD8-targeted lysine of Cul-1 was replaced by arginine. To confirm further that the slow migrating band corresponds to the NEDD8-modified form of Cul-1, we used GST-NEDD8. When GST-NEDD8 was used instead of His-NEDD8, the high molecular mass band corresponding to Cul-1-conjugated with GST-NEDD8 was observed in a time-dependent fashion. After 30 min incubation, 10-20% of Cul-1 was conjugated to NEDD8, but longer incubation, e.g. >2 h, resulted in modification of ~60-70% of Cul-1 (data not shown).

In the next step, we examined the role of the NEDD8 system in ubiquitylation of pI $\kappa$ B $\alpha$  mediated by ROC1–SCF $\beta$ TrCP1. As shown in Figure 1C (left), ROC1–SCF $\beta$ TrCP1 time-dependently catalysed polyubiquitylation of cell-free translated [ $^{35}$ S]I $\kappa$ B $\alpha$  that had been phosphorylated by purified I $\kappa$ B kinase (IKK). The addition of NEED8 and NEDD8-ligating enzymes resulted in

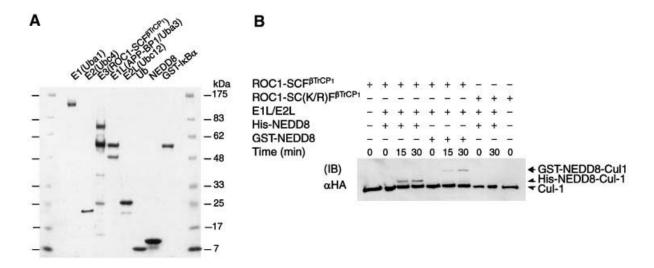
marked augmentation of  $I\kappa B\alpha$  ubiquitylation, particularly during the initial period of incubation, e.g. 30-60 min. However, less pI $\kappa$ B $\alpha$  ubiquitylation was observed in the absence of the NEDD8 system. Because reticulocyte extracts used for the synthesis of [35S]IκBα contained a significant amount of the NEDD8 system (Osaka et al., 1998; Hori et al., 1999), we next used GST-IκBα produced by E.coli as a substrate. As shown in Figure 1D (lane 2), the NEDD8 system markedly enhanced polyubiquitylation of GST-pIκBα in a phosphorylationdependent manner (Figure 1D, lane 4). However, the ubiquitylating activity was still detected in the absence of the NEDD8 system, even if GST-pIκBα was used. Thus, NEDD8-ylation of Cul-1 was not essential for the ubiquitylating reaction at least in the present in vitro system. Nonetheless, because the NEDD8 system could augment the ROC1–SCF<sup>βTrCP1</sup> activity in our *in vitro* system, we analysed its mechanistic reaction further.

We next examined whether this augmentation of ubiquitylating activity of ROC1–SCF<sup>βTrCP1</sup> is mediated solely by modification of Cul-1, but not by co-operation of the NEDD8 and Ub system. When mutant Cul-1(K/R) whose NEDD8-targeted lysine was replaced by arginine was used instead of wild-type Cul-1, the NEDD8 system did not enhance pIκBα ubiquitylation (Figure 1C, right), strongly indicating that the modification of Cul-1 by NEDD8 plays a critical role in the enhancement of ROC1– $\hat{SCF}^{\beta TrCP1}$ -dependent pI $\kappa B\alpha$  ubiquitylation. This inability of Cul-1(K/R) was not due to loss of ROC1–SC(K/R)F<sup>βTrCP1</sup> complex formation nor substrate (pIκBα) binding (see below). Moreover, when Ubc12(C/ S) defective for thioester linkage of NEDD8 was added instead of wild-type Ubc12 in the NEDD8 system, no obvious stimulatory effect on the ROC1-SCF<sup>βTrCP1</sup>dependent GST-pI\(\text{RB}\alpha\) ubiquitylating activity was observed (Figure 1D, lane 3), again suggesting the importance of NEDD8-vlation of Cul-1 for activation of IκBα-E3 ligase.

It is worth noting that the ubiquitylating activity of  $PIKB\alpha$  by ROC1– $SCF^{\beta TrCP1}$  persisted for incubation up to 2 h even when the NEDD8 system was not supplemented (Figure 1C) or ROC1– $SC(K/R)F^{\beta TrCP1}$  was used, suggesting that the ROC1– $SCF^{\beta TrCP1}$  complex is fairly stable. To confirm this finding more clearly, we tested the effect of the NEDD8 system on heat inactivation of ROC1– $SCF^{\beta TrCP1}$ . Pre-incubation of ROC1– $SCF^{\beta TrCP1}$  for 15 min at various temperatures did not alter the mode of loss of  $[^{35}S]pIKB\alpha$  ubiquitylating activity at high temperatures, irrespective of the presence or absence of the NEDD8 system (data not shown). Taken together, it is clear that the NEDD8 system does not function to protect the instability of the ROC1– $SCF^{\beta TrCP1}$  complex.

## The NEDD8 system has no effect on the assembly of ROC1–SCF $\beta$ TrCP1

In an *in vitro* reconstituted system, we found that Cul-1(K/R) was complexed with Skp1,  $\beta$ TrCP1 and ROC1, as is wild-type Cul-1 (see Figure 1C, right panel and data not shown), indicating that NEDD8-ylation is not required for the assembling of the ROC1–SCF $\beta$ TrCP1 complex at least *in vitro*. To confirm this in *in vivo*, we examined whether NEDD8-ylation of Cul-1 is essential for the assembly of the SCF complex. To this end, HA-Cul-1 or the HA-Cul-



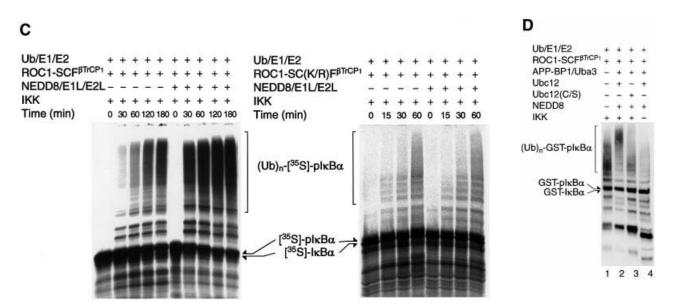
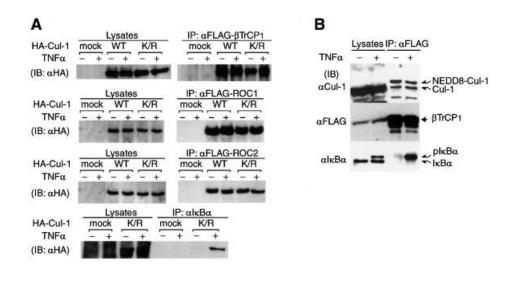


Fig. 1. NEDD8-ylation of Cul-1 stimulates ubiquitylation of IκBα by the ROC1–SCF $^{\beta TrCP1}$  complex. (A) Electrophoretic patterns of recombinant proteins consisting of the IκBα-ubiquitylating [E1 (Uba1), E2 (Ubc4) and (E3) ROC1–SCF $^{\beta TrCP1}$ ] and NEDD8-modifying [E1L (APP-BP1/Uba3) and E2L (Ubc12)] systems. A 5 μg aliquot of each affinity-purified protein, except ROC1–SCF $^{\beta TrCP1}$  (10 μg), was subjected to SDS–PAGE followed by staining with Coomassie blue R-250. (B) Modification of Cul-1 by a reconstituted NEDD8 conjugation system. The ROC1–SCF $^{\beta TrCP1}$  complex or ROC1–SC(K/R)F $^{\beta TrCP1}$  was incubated at 25°C for 15–30 min in the presence or absence of the NEDD8 system containing His-NEDD8 or GST–NEDD8 as described in Materials and methods. After incubation, the reaction was terminated by the addition of 20 μl of SDS–PAGE sample buffer. One-quarter volume of the boiled supernatant was subjected to SDS–PAGE followed by western blotting with anti-HA antibodies to detect Cul-1. Arrows indicate Cul-1, His-NEDD8-Cul-1 and GST–NEDD8-Cul-1. (C) Time course of [ $^{35}$ S]Iκβα ubiquitylation by ROC1–SCF $^{\beta TrCP1}$  (left) and ROC1–SC(K/R)F $^{\beta TrCP1}$  (right). Ubiquitylation of [ $^{35}$ S]Iκβα by ROC1–SCF $^{\beta TrCP1}$  was performed in the presence or absence of the NEDD8 system. Iκβα pre-phosphorylated by IKK was used as a substrate, unless otherwise specified. The high molecular mass ubiquitylated [ $^{35}$ S]Iκβα is shown as (Ub)n-GST–Iκβα. (D) Effect of Ubc12(C/S) on the *in vitro* ubiquitylation of phosphorylated GST–Iκβα detected by western blotting is shown as (Ub)n-GST–PIκβα detected by western blotting is shown as (Ub)n-GST–PIκβα.

1(K/R) mutant was co-expressed with FLAG-βTrCP1 in HEK 293 cells, and immunoprecipitated by anti-FLAG antibody, followed by immunoblotting with anti-HA antibody. As shown in Figure 2A (top), Cul-1(K/R) was incorporated into the SCFβTrCP1 complex, as was the wild-type Cul-1. Similar results were obtained by immunoprecipitation with FLAG-ROC1 or its isoform FLAG-ROC2 (Figure 2A, second and third rows). Thus, it was clear that NEDD8 modification is not required for the assembly of the SCFβTrCP1 complex. Furthermore, Cul-1(K/R) could

bind to phosphorylated  $I\kappa B\alpha$  ( $pI\kappa B\alpha$ ) in tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )-stimulated cells (Figure 2A, bottom), indicating that NEDD8 modification of ROC1–SCF<sup>βTrCP1</sup> is not required for substrate binding.

Curiously, we noticed very little NEDD8-ylation of overexpressed HA-tagged wild-type Cul-1. Therefore, we tested whether endogenous Cul-1 is modified by NEDD8 in living cells. After FLAG-tagged βTrCP1 was expressed in 293 cells, endogenous Cul-1 in the SCF<sup>βTrCP1</sup> complex was immunoprecipitated by anti-FLAG antibody. Western



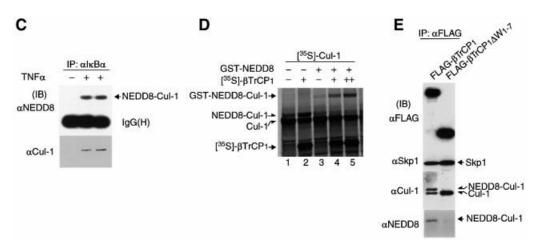


Fig. 2. Modification of Cul-1 by NEDD8 in vivo. (A) Association of Cul-1(K/R) with the SCF complex and IκBα in living cells. pcDNA3-HA-Cul-1 and HA-Cul-1(K/R) were co-expressed with FLAG-βTrCP1, FLAG-ROC1 or FLAG-ROC2 in 293 cells and either left untreated or treated with TNFα. After immunoprecipitation (IP) with anti-FLAG (upper three rows) and anti-IκBα (lower row) antibodies, immunoblotting (IB) was carried out with anti-HA antibody for cell lysates and the immunoprecipitates. (B) Co-immunoprecipitation of NEDD8-modified Cul-1 with βTrCP1 in vivo. pcDNA3-βTrCP1-FLAG was transfected into 293 cells and treated or not with TNF-α. The extracts or anti-FLAG antibody immunoprecipitates were analysed by western blotting using anti-FLAG, anti-Cul-1 and anti-IκBα antibodies. (C) NEDD8 modification of Cul-1 associated with phosphorylated IkB $\alpha$ . HEK-293 cells were either left untreated or treated with TNF- $\alpha$ . The anti-IkB $\alpha$  antibody immunoprecipitates were analysed by western blotting using anti-Cul-1 and anti-NEDD8 antibodies. Experiments were conducted in duplicate. (D) βTrCP1 augments modification of Cul-1 by NEDD8 in reticulocyte lysates. Following the synthesis of [ $^{35}$ S]Cul-1 in the presence or absence of [ $^{35}$ S] $\beta$ TrCP1 or GST–NEDD8 as indicated for 60 min at 30°C in a reticulocyte lysate transcription/translation system, samples of the resultant translational products were subjected directly to SDS-PAGE in the presence of dithiothreitol and then autoradiographed. Arrows indicate [35S]βTrCP1, [35S]-Cul1, endogenous NEDD8-modified [35S]Cul-1 and GST-NEDD8-modified [35S]Cul-1. (E) Co-immunoprecipitation of NEDD8-modified Cul-1 with \( \beta TrCP1 \) or \( \beta TrCP1 \) lacking the WD40-repeat region. Thirty-six hours after pcDNA3-FLAG-βTrCP1 or the cDNAs of FLAG-tagged βTrCP1 lacking the WD40-domains (designated FLAG-βTrCP1ΔW1-7) was transfected into 293 cells, crude extracts were prepared as described in Materials and methods. After immunoprecipitation by anti-FLAG antibody, the resulting immunoprecipitates were analysed by western blotting using anti-FLAG, anti-Skp1, anti-Cul-1 and anti-NEDD8 antibodies.

blotting showed that Cul-1 was observed mainly as a single species in the 293 cell extracts, whereas two bands were evident in immunoprecipitates with anti-FLAG antibody (Figure 2B). This pattern was unaffected upon TNF- $\alpha$  stimulation, which caused association of SCF $^{\beta TrCP1}$  with pIkB $\alpha$ . The upper band reacted with anti-NEDD8 antibody (data not shown), indicating that it was NEDD8-ligated Cul-1. Thus, most Cul-1 present in cell extracts is not modified by NEDD8, while nearly half of Cul-1 in the SCF $^{\beta TrCP1}$  complex seems to be modified by NEDD8. Nonetheless, Cul-1 co-immunoprecipitated with anti-

IκBα antibody in TNF-α-treated 293 cell extracts was a single species, which was identified as a NEDD8-modified form (Figure 2C), indicating that Cul-1 in the SCF $^{\beta TrCPl}$  complex associated with pIκBα is ligated preferentially by NEDD8. However, the mechanisms involved in the efficient modification of Cul-1 by NEDD8 in the SCF complex bound to target substrate, such as pIκBα, remain unclear.

Subsequently, we investigated the influence of  $SCF^{\beta TrCP1}$  complex formation on *in vitro* NEDD8 ligation of Cul-1. For this purpose, we added F-box protein, i.e.

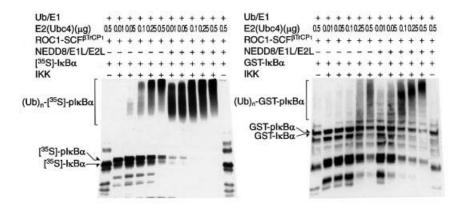


Fig. 3. Dose-dependent effect of E2 (Ubc4) on the ubiquitylation of phosphorylated IkB $\alpha$ . The ubiquitylated assays of [ $^{35}$ S]IkB $\alpha$  (left) and GST–IkB $\alpha$  (right) were performed as in Figure 1C and D, except for the use of various amounts of E2 (Ubc4) as indicated.

βTrCP1, in rabbit reticulocyte lysates, because we have already reported that NEDD8 efficiently modified all human Cul-family proteins in this in vitro system (Osaka et al., 1998; Hori et al., 1999). As shown in Figure 2D, when the cDNAs of Cul-1 were co-translated with those of βTrCP1 or none, the upper band of synthesized [35S]Cul-1 increased in a manner dependent on co-translation of [35S]βTrCP1 (lane 2). The reduced mobility band was assumed to be [35S]Cul-1 conjugated by endogenous NEDD8. To confirm this assumption, we carried out the same experiment in the presence of GST-NEDD8. The GST-NEDD8-conjugated [35S]Cul-1 increased proportionately with elevated levels of translated [35S]\(\beta\)TrCP1 (Figure 2B, lanes 3-5). Intriguingly, no obvious augmentation of the ligation of GST-NEDD8 to [35S]Cul-1 was observed following the addition of  $\beta TrCP1\Delta F$  lacking the F-box domain that cannot associate with Skp1 and thus Cul-1 (data not shown), strongly indicating that the formation of the SCF<sup>βTrCP1</sup> complex promotes NEDD8 modification of Cul-1.

Furthermore, we examined whether the substrate binding to the SCF $^{\beta TrCP1}$  complex is necessary for Cul-1 modification by NEDD8. For this purpose, we deleted all seven WD40-repeat domains (termed FLAG- $\beta TrCP1\Delta W1-7$ ) and transfected its cDNA into HEK 293 cells. As shown in Figure 2E, FLAG- $\beta TrCP1\Delta W1-7$  formed complexes with Skp1 and Cul-1, because it retains the F-box domain. However, NEDD8-modified Cul-1 was not detected in anti-FLAG-immunoprecipitates with  $\beta TrCP1\Delta W1-7$ , unlike  $\beta TrCP1$ . These results suggest that substrate binding to the SCF $^{\beta TrCP1}$  complex may be necessary for Cul-1 modification by NEDD8, at least *in vivo* (see Discussion).

# Stimulation of $I\kappa B\alpha$ ubiquitylation by NEDD8-ylation is E2 dependent

Considering the role of NEDD8-ylation in  $I\kappa B\alpha$  ubiquitylation, we next examined the effect of E2 (Ubc4). As shown in Figure 3 (left panel), augmentation of  $[^{35}S]I\kappa B\alpha$  ubiquitylation by the NEDD8 system seemed to be dependent on the amount of E2 (Ubc4). Thus, in the presence of the NEDD8 system, very little E2 enzyme is required to exhibit the same effects as with high E2 concentration in the absence of the NEDD8 system.

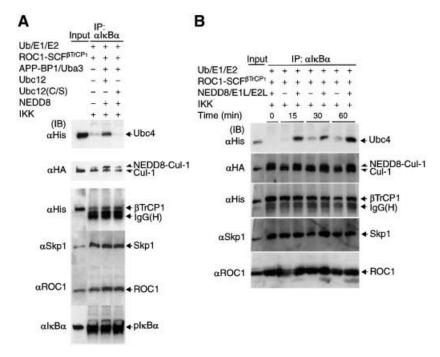
Similar dose-dependent effects of E2 on the ubiquitylation of GST–pIkB $\alpha$  were observed (Figure 3, right), suggesting that the role of the NEED8 system toward ROC1–SCF $\beta$ TrCP1 may be related to the dose of E2.

# NEDD8-ylation enhances the recruitment of Ubc4 to ROC1–SCF $^{\beta TrCP1}$ without affecting the association of ROC1–SCF $^{\beta TrCP1}$ with pl $_{\kappa}$ B $_{\alpha}$

In Figure 2D, we showed that most of the endogenous  $SCF^{\beta TrCP1}$  bound to substrate (pIkB $\alpha$ ) is modified by NEDD8, although this modification is not essential for the binding. To ascertain further that the NEDD8 system does not affect substrate binding of ROC1–SCF $^{\beta TrCP1}$ , the reaction was performed in the presence or absence of the NEDD8 system and followed by immunoprecipitation of IkB $\alpha$  and western blotting against SCF components. As shown in Figure 4A, almost the same amount of each component of the ROC1–SCF $^{\beta TrCP1}$  complex was bound to pIkB $\alpha$ , irrespective of the presence or absence of the NEDD8 system, as expected.

Surprisingly, however, we found that the addition of the NEDD8 system caused a marked increase in the binding of His-tagged Ubc4 to  $pI\kappa B\alpha$  (Figure 4A), while little binding of E2 was observed in the absence of the NEDD8 system. This NEDD8-ylation-dependent binding of Ubc4 to  $pI\kappa B\alpha$  occurred in a time-dependent fashion (Figure 4B). Ubc12(C/S) had no profound effect on this Ubc4 recruitment. Moreover, UbcH7, which does not support  $pI\kappa B\alpha$  ubiquitylation, was not associated with  $pI\kappa B\alpha$  even when the NEDD8 system was also supplemented (data not shown).

We next examined whether substrate binding of ROC1–SCF $^{\beta TrCP1}$  is necessary for recruitment of E2. For this purpose, we performed immunoprecipitation analysis with anti-Cul-1 antibody in the absence of IkB $\alpha$  and IKK. As shown in Figure 5A, the NEDD8 system markedly promoted the binding of Ubc4 to ROC1–SCF $^{\beta TrCP1}$ , while no obvious binding of E2 was observed in the absence of the NEDD8 system. Furthermore, E2 was not significantly associated with the ROC1–SC(K/R)F $^{\beta TrCP1}$  complex defective for NEDD8 modification (Figure 5A). These results suggest that Ubc4 is recruited directly to ROC1–SCF $^{\beta TrCP1}$  in an NEDD8-ylation-dependent fashion without requiring the binding of the E3 (ROC1–SCF $^{\beta TrCP1}$ ) to



**Fig. 4.** Effect of the NEDD8 system on recruitment of E2 (Ubc4) onto ROC1–SCF $^{βTrCP1}$  bound to phosphorylated IκBα. The ROC1–SCF $^{βTrCP1}$  complex was incubated at 25°C for 30 min in the presence or absence of the NEDD8 system together with Ub, E1 (Uba1) and E2 (Ubc4) for ubiquitylation, IκBα and IKK at the indicated combinations. After incubation, the reaction mixtures were treated with anti-IκBα antibody and the resulting immunoprecipitates were used for western blotting with various antibodies as indicated. 'Input' denotes various input controls (1/10 the amounts added in the assay mixtures). The effects of Ubc12(C/S) (**A**) and time course (**B**) on the binding of Ubc4 to ROC1–SCF $^{βTrCP1}$  are shown.

pIκBα. However, the purified ROC1–SCF<sup>βTrCP1</sup> complex might have already associated with endogenous substrate(s) in insect cells, and substrate binding might be required for the assembly of ROC1–SCF<sup>βTrCP1</sup>. Thus, we believe that the substrate binding of ROC1–SCF<sup>βTrCP1</sup> is required for its NEDD8 modification (shown in Figure 2E) and subsequent recruitment of E2.

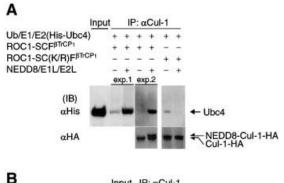
We then tested whether the thioester linkage of Ub is necessary for recruitment of E2 (Ubc4) to the ROC1- $SCF^{\beta TrCP1}$  complex. As shown in Figure 5B, in the absence of E1 (Uba1), Ubc4 did not bind to ROC1–SCF $^{\beta TrCP1}$ , even in the presence of the NEDD8 system. This finding strongly indicates that thioester linkage of Ub to E2 is required for recruitment of E2 to ROC1–SCF $^{\beta TrCP1}$ . This conclusion was confirmed further using Ubc4(C/A) and Ubc4(C/S), which cannot form a thioester linkage with Ub. Neither Ubc4(C/A) nor Ubc4(C/S) bound to ROC1–SCF<sup>βTrCP1</sup> immunoprecipitated by either anti-Cul-1 antibody (Figure 5C) or anti-IκBα antibody (Figure 5D) in the presence of the NEDD8 system. Thus, we concluded that the NEDD8 system enhances the formation of Ublinked E2–E3 complex, leading to efficient ubiquitylation of pIκBα. Finally, we examined whether Ubc4 specifically interacts with Cul-1 in living cells. For this, Cul-1-HA was co-expressed with FLAG-Ubc4 or FLAG-Ubc4(C/A) and their interaction investigated by immunoprecipitation analysis. As shown in Figure 5E, FLAG-Ubc4, but not FLAG-Ubc4(C/A), immunoprecipitated Cul-1, the band of which reacted with anti-NEDD8 antibody, suggesting that formation of a thioester linkage between Ubc4 with Ub may be necessary for the interaction with SCF whose Cul-1 is modified by NEDD8.

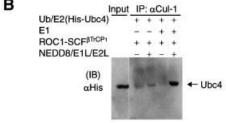
While the presence of Uba1 (E1 for ubiquitylation) was required for recruitment of E2, Uba1 was not bound to  $PIKB\alpha$  or  $ROC1-SCF^{\beta TrCP1}$  even in the presence of the NEDD8 system (data not shown). Furthermore, the binding of APP-BP1 (a regulatory subunit of E1 for NEDD8-ylation) or Ubc12 to  $ROC1-SCF^{\beta TrCP1}$  was undetectable, irrespective of the presence or absence of the NEDD8 system (data not shown), suggesting that the mechanistic actions for ubiquitylation and NEDD8-ylation are not equivalent. Taken together, these results indicate that NEDD8-ylation of Cul-1 specifically enhances recruitment of E2 (Ubc4) to a target substrate ( $PIKB\alpha$ ) through E3 (ROC1– $SCF^{\beta TrCP1}$ ), which contributes to the efficient transfer of Ub for ultimate ubiquitylation.

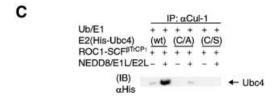
#### **Discussion**

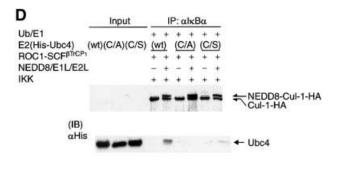
In the present study, we investigated the role of NEDD8 for Cul-1 in an *in vitro* reconstituted IkB $\alpha$  ubiquitylation system. We first showed that addition of the NEDD8 system resulted in the enhancement of polyubiquitylation of pIkB $\alpha$  phosphorylated by IKK. We also demonstrated that blockage of Cul-1 modification by NEDD8 with Cul-1(K/R) or Ubc12(C/S) did not enhance the ubiquitylation of pIkB $\alpha$ , indicating that NEDD8-ylation of Cul-1 truly activates the polyubiquitylation of pIkB $\alpha$ . Essentially similar results were reported recently by other groups for polyubiquitylation of pIkB $\alpha$  by SCF<sup> $\beta$ TrCP1</sup> (Furukawa *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000) and of p27<sup>Kip1</sup> by SCF<sup>Skp2</sup> (Morimoto *et al.*, 2000; Podust *et al.*, 2000). However, the molecular mechanism underlying the activation of SCF-Ub ligase by NEDD8 remains to be

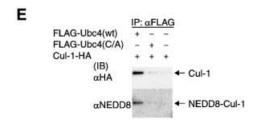
defined. To uncover the mechanistic role, we also demonstrated that the NEDD8 system enhanced the recruitment of Ub-conjugating enzyme (Ubc4) to the ROC1–SCF $^{\beta TrCP1}$  E3 ligase complex to evoke efficient ubiquitylation of pI $\kappa$ B $\alpha$ . Indeed, the stimulatory effect of NEDD8 was evident more strikingly when there was a reduced supply of E2. Moreover, we found that a thioester-linkage between E2 and Ub is necessary for its binding to E3, because free E2 without supply of Ub from E1-Ub was dysfunctional for the binding to E3 and because Ubc4(C/









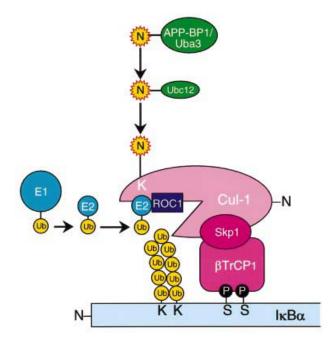


S) and Ubc4(C/A) which are unable to trap Ub were not recruited to ROC1–SCF $^{\beta TrCP1}$ . In addition to these *in vitro* experiments, we showed that ectopically expressed Ubc4, but not Ubc4(C/A), interacted with endogenous NEDD8-linked Cul-1 in living cells. The results of our study allow us to design a hypothetical model for the role of the NEDD8 system in IkB $\alpha$  ubiquitylation (Figure 6). This model can solve for the first time the mechanistic role of the NEDD8 system in regulating the ROC1–SCF $^{\beta TrCP1}$  E3 ligase complex.

However, it remains uncertain whether the effect of NEDD8 modification influences the length of the Ub chain or the efficiency of polyubiquitylation. Indeed, Wu *et al.* (2000) reported that the bacterially expressed ROC1–Cul-1 complex stimulates Ub polymerization in the absence of a substrate. This role of NEDD8-ylation resembles that mediated by a new ubiquitylation factor termed 'E4', which is involved in polyubiquitin chain assembly (Koegl *et al.*, 1999). However, we favour the notion that the NEDD8-ylation of SCF ligase promotes the efficiency of polyubiquitylation, since the addition of the NEDD8 system resulted in a significant reduction in the added substrate, namely pIκBα. Further studies are required to clarify this issue.

Recently, Lisztwan et al. (1998) reported the association of Cul-1 and Cdc34/Ubc3(E2) with the F-box protein p45Skp2. Furthermore, it is well known that several RINGfinger-type Ub-protein E3 ligases can bind specific classes of E2s (Deshaies, 1999). However, in our hands, we could not detect any obvious binding between Ubc4 and ROC1-SCF $^{\beta Tr\tilde{C}P1}$  in vitro in the absence of the NEDD8 system. Consequently, our results seem to be quite rational; the NEDD8 system apparently assists the formation of a stable complex between E2 and E3. We do not know the exact mechanism underlying this enhanced binding directed by NEDD8-ylation, but we favour the notion that NEDD8 modification of Cul-1 presumably induces conformational changes in Cul-1, which may open the binding pocket for Ub-E2 (see our model in Figure 6). However, it is still not clear whether Ubc4 binds to ROC1 and/or Cul-1, because they are hardly expressed as soluble recombinant proteins, and thus we are unable to conduct direct binding experiments in vitro. It is, however, worth

Fig. 5. Effect of the NEDD8 system on recruitment of E2 (Ubc4) to the ROC1–SCF $^{\beta TrCP1}$  complex. (A) Effect of Ubc4 binding to ROC1-SCF<sup>βTrCP1</sup> and ROC1-SC(K/R)F<sup>βTrCP1</sup>. Experiments and symbols are similar to those described in Figure  $\bar{4}$ , except that  $I\kappa B\alpha$ and IKK were not added and immunoprecipitation was carried out with anti-Cul-1 antibody. Some experiments were conducted in duplicate. (B) Only Ub-linked Ubc4 was recruited onto ROC1–SCF $^{\beta TrC\hat{P}1}$ . The experiment was similar to that described in (A) except that E1 (Uba1) was omitted from the assay mixture. (C) The NEDD8 system does not support the binding of Ubc4(C/A) and Ubc4(C/S) to ROC1–SCF  $^{\beta TrCP1}.$ The experiment was similar to that described in (A), except that Ubc4(C/A) and Ubc4(C/S) were used for wild-type Ubc4 (wt). (D) The NEDD8 system does not support the binding of Ubc4(C/A) and Ubc4(C/S) to pIκBα. The experiment was similar to that described in Figure 5 using Ubc4(C/A) and Ubc4(C/S). (E) Ubc4, but not Ubc4(C/ A), co-immunoprecipitates Cul-1 in 293 cells. Thirty-six hours after pcDNA3-Cul-1-HA with unmodified FLAG-Ubc4(wt) or FLAG-Ubc4(C/A) were transfected into 293 cells, crude extracts were prepared as described in Materials and methods. After immunoprecipitation by anti-FLAG antibody, the resulting immunoprecipitates were analysed by western blotting using anti-HA antibodies.



**Fig. 6.** Schematic model for the role of the NEDD8 system in IκBα ubiquitylation by a ROC1–SCFβ<sup>TrCP1</sup> Ub ligase complex. For details, see text. Ub, ubiquitin; E1 (Uba1) (Ub-activating); and E2 (Ubc4) (Ub-conjugating) enzymes; N, NEDD8; p, phosphorylation of serine. APP-BP1/Uba3 and Ubc12, as E1- and E2-like enzymes, respectively, for the NEDD8 system. S and K, serine and lysine residues, respectively.

noting that a significant activity of the ROC1–SCF $^{\beta TrCP1}$  complex was detected without the NEDD8 system. In this case, E2 may be associated transiently with the E3 ligase complex during the ubiquitylating process, but the E2–E3 association was not observed appreciably in the present immunoprecipitation analysis due to the instability of the presumptive active complex.

Our results showed that the ubiquitylation reaction for pIκBα proceeded as a function of time in the absence of the NEDD8 system and that the ROC1–SCF $^{\beta TrCP1}$  complex exhibited the same thermostability in the absence of the NEDD8 system. These results imply that NEDD8-ylation of Cul-1 is not involved in preventing the inactivation of ROC1–SCF<sup>βTrCP1</sup> activity. Similar findings were reported recently by other groups (Podust et al., 2000; Read et al., 2000). Moreover, NEDD8-ylation is not involved in the formation of the ROC1–SCF<sup>βTrCP1</sup> complex, because Cul-1(K/R), which is defective for NEDD8-ylation, could assemble into the complex similarly to Cul-1 in vitro and in vivo. Importantly, Read et al. (2000) came to the same conclusions and further found that NEDD8-conjugated  $SCF^{\beta TrCP}$  and unmodified  $SCF^{\beta TrCP}$  had similar affinity, namely apparently similar  $K_{\rm m}$  values, for binding to pIκBα. Consistent with these observations, we also demonstrated that the NEDD8 system is not required for the binding of the ROC1–SCF<sup>βTrCP1</sup> complex to target pIκBα, because NEDD8-ylation-defective ROC1-SCF $^{\beta TrCP1}$  with Cul-1(K/R) could bind normally to pI $\kappa$ B $\alpha$ in vitro and in vivo. Alternatively, for the stimulatory effect of NEDD8-ylation, it is possible that NEDD8ylation of Cul-1 prevents ubiquitylation of E2 (Ubc4 in the present study), linked to the SCF ligase and consequently increases the availability of E2 for ubiquitylation. However, as in the present reconstituted system, no obvious ubiquitylation of Ubc4 was observed, making this possibility unlikely under our conditions. The above findings are not in conflict with our proposed model of the role of the NEDD8-modifying system (Figure 6). Previously, we reported that all members of the human Cul-family proteins are modified by NEDD8 (Hori *et al.*, 1999), indicating that NEDD8 conjugation to Cul-family proteins represents a general mechanism of activation of the ROC–Cul-based Ub-protein ligase.

It is of note that only a small portion of the total cellular pool of Cul-1 was modified by NEDD8. However, nearly half of endogenous Cul-1 associated with ectopically expressed  $\beta TrCP1$ , and thus  $SCF^{\beta TrCP1}$ , was modified by NEDD8, and NEDD8-modified Cul-1 was the only form detected in association with phosphorylated IkBa, the SCF substrate in vivo (Read et al., 2000; this study). These observations indicate the existence of a regulatory mechanism of the NEDD8-modifying system in vivo, but the mechanism that regulates NEDD8 conjugation to Cul-1 is unknown at present. We demonstrated in the present study that substrate binding of SCF might be required for NEDD8 modification of Cul-1, which, however, apparently is contradictory to the in vitro observation that NEDD8-ylation occurs in a substrate-independent manner (Wu et al., 2000). A similar in vivolin vitro difference was described for the requirement of ROC1 for NEDD8 conjugation to CUL-1, i.e. ROC1 is necessary for in vivo, but not in vitro. NEDD8 modification of Cul-1 (Furukawa et al., 2000). In addition, Podust et al. (2000) reported that a continuous supply of the NEDD8 system is required for p27<sup>Kip1</sup> ubiquitylation in crude cell lysates, indicating the existence of certain isopeptidase(s) capable of hydrolysing NEDD8 from the NEDD8-Cul-1 conjugate, leading to cessation of activation of SCFSkip2 ligase activity. Taken together, it seems that Cul modification by NEDD8 is a regulated process, which may be tightly linked to the SCF function, although the underlying mechanism(s) remains to be identified.

It has been reported that deletion of the Rub1-NEDD8 ligation pathway in the budding yeast is viable and that modification of Cdc53 by Rub1 does not seem to be strictly essential for the function of SCF<sup>CDC4</sup> in budding yeast (Lammer et al., 1998; Liakopoulos et al., 1998). In contrast, in fission yeast, we recently concluded that the NEDD8-modifying pathway is essential for cell viability and function of Pcu1 (Cul-1 orthologue), and thereby SCF. This conclusion was based on the finding that Pcu1K713R defective for NEDD8-ylation could not rescue growth arrest caused by deletion of pcu1+ and that forced expression of Pcu1K713R or depletion of NEDD8 in cells resulted in impaired cell proliferation and marked stabilization of the cyclin-dependent kinase (CDK) inhibitor Rum1, which is a substrate of the SCF complex (Osaka et al., 2000). In Arabidopsis thaliana, Axr1, an APP-BP1 homologue, plays a pivotal role in auxin response in synergy with SCF, but axr1 mutant cells remain viable; however, it is not clear at present whether its null mutation is lethal or not (Pozo et al., 1998; Gray and Estelle, 2000). In addition, the mutation of hamster SMC, encoding a protein almost identical to APP-BP1, is responsible for cell cycle defects in the hamster ts41 cell line (Chen et al., 2000). Recently, we also found that the NEDD8-conjugating pathway is essential for proliferation of mammalian cells, because deletion of the *Uba3* gene encoding a catalytic subunit of NEDD8-activating enzyme in mice resulted in impaired cullin function and early embryonic death (K.Tateishi, M.Omata, K.Tanaka and T.Chiba, unpublished results). Taken together, these findings suggest that the NEDD8-Rub1-ligating pathway plays a critical role for the SCF complex in a variety of eukaryotic cells.

However, the reason why the NEDD8 system is essential in mammals and fission yeast but not in budding yeast is obscure (Lammer et al., 1998; Liakopoulos et al., 1998; Osaka et al., 2000). One possible explanation is that Cdc53 binds efficiently to E2 without NEDD8-ylation. This may be due to a structural difference between Cdc53 and Cul-1 or budding yeast may have other mechanism(s) to compensate for the loss of the NEDD8 system. Alternatively, it may be possible that the level of E2 in cells may differ among different organisms or, although unlikely, degradation of certain substrates by Cul-based E3 may be essential in some organisms but not in others. Genetic analyses have revealed an indispensable role for NEDD8-ylation for in vivo SCF function in various eukaryotes, except budding yeast, but biochemical analyses revealed that ROC1–SCF<sup>βTrCP1</sup> or SCF<sup>Skp2</sup> still retain significant activity without NEDD8-ylation (Morimoto et al., 2000; Podust et al., 2000; Read et al., 2000; Wu et al., 2000; this study). The reason is unknown at present, but it is possible that the level of thioester linkage of Ub to E2 is quite low in living cells, compared with an *in vitro* reconstitution system. Otherwise, it may be possible that another unknown protein(s), which is essential for cell viability, interacts with SCF in a NEDD8-dependent manner as E2-Ub does. For example, the essential gene named Sgt1 may be involved in the regulation of SCF activity in living cells (Kitagawa et al., 1999) although the addition of the human orthologue of Sgt1 had no effect on the  $I\kappa B\alpha$  ubiquitylation mediated by ROC1–SCF $^{\beta TrCP1}$ (data not shown). Moreover, it was reported recently that Cks1/Suc1 is required for SCFSkp2-mediated ubiquitylation of p27<sup>Kip1</sup> (Ganoth et al., 2001). Further studies nevertheless will help to clarify these unanswered issues.

#### Materials and methods

#### Construction of plasmids

Cul-1(K/R) (Lys696 in Cul-1 replaced by arginine), Ubc12(C/S) (Cys111 replaced by serine) and Ubc4(C/A) or (C/S) (Cys85 replaced by alanine or serine) mutants were synthesized by a PCR-assisted method using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). For PCR amplification of human Ubc4, we used the sequence described by Rolfe *et al.* (1995). Note that there are two Cul-1 proteins; the longer one has a short stretch with an extra 24 amino acids near the N-terminal region of the shorter one, and, therefore, the NEDD8-ylated K696 of the latter Cul-1 corresponds to K720 of the former, as reported (see Morimoto *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000). The deletion mutant FLAG-βTrCP1ΔW1–7 (reisdues 260–569 deleted; see Suzuki *et al.*, 2000) was amplified by PCR with appropriate primers. Plasmids for the expression of N- and C-terminally HA- or FLAG-tagged proteins were ligated into pcDNA3.1(+) vector (Invitrogen).

## Cell cultures, transfections, immunoprecipitation and western blotting

The method used for culture of human HEK 239 cells, which subsequently were used for transfections, has been described previously (Suzuki *et al.*, 2000). In brief, transfections were performed using the

FuGENE 6 transfection reagent. MG132 (Z-Leu-Leu-Hu) at 50  $\mu$ M and okadaic acid at 0.25  $\mu$ M were pre-treated prior to TNF- $\alpha$  stimulation. Preparation of cell lysates and immunoprecipitation with various antibodies were performed as described before (Suzuki *et al.*, 2000). For western blotting, the reaction mixtures were separated by SDS-PAGE and transferred to a PVDF membrane. The membranes were probed with various antibodies and visualized with horseradish peroxidase-conjugated second antibody using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The antibodies used this study were as described previously (Suzuki *et al.*, 2000).

#### Preparation of recombinant proteins

Recombinant His-Ubc4, His-Ubc12, His-Ubc12(C/S), His-NEDD8, His-Ubc4(C/A), Ubc4(C/S) and GST-IkB $\alpha$  were produced in *E.coli*. Recombinant E1 (Uba1), His-APP-BP1/T7-Uba3, ROC1–SCF $^{\beta TrCP1}$  and ROC1–SC(K/R)F $^{\beta TrCP1}$  [T7-ROC1/FLAG-Skp1/HA-Cul-1 or HA-Cul-1(K/R)/His- $^{\beta TrCP1}$ ] were produced from baculovirus-infected HiFive insect cells. These proteins, which are all human species except mouse E1, were affinity purified using a His Trap column (Pharmacia Biotech). Of these, APP-BP1/Uba3 acting as an E1 for NEDD8-ylation and the ROC1–SCF $^{\beta TrCP1}$  complex functioning as an E3 for IkB $\alpha$  ubiquitylation were generated by simultaneously infecting two or four baculoviruses, respectively.

## In vitro $I_K B \alpha$ ubiquitylation and immunoprecipitation analysis

A 2  $\mu l$  aliquot of in vitro translated  $^{35}S$ -labelled IkB $\alpha$  or 0.5  $\mu g$  of GST-IkBa were pre-incubated at room temperature for 30 min with 25 ng of IKK in the presence of an ATP-regenerating system, followed by further incubation at 37°C for 0.5-3 h in a reaction volume of 20  $\mu$ l containing 100 ng of recombinant mouse E1, 0.01-0.5 µg of Ubc4, 1 µg of ROC1–SCF  $^{\beta TrCP1}$  and 10  $\mu g$  of bovine Ub. The reaction was carried out in the presence or absence of the NEDD8 system consisting of NEDD8 (10  $\mu g$ ), APP-BP1/Uba3 (0.5  $\mu g$ ) and Ubc12 (0.5  $\mu g$ ) that had been preincubated with ROC1–SCF<sup>\(\beta\)TrCP1</sup> for 15 min at 25°C. After terminating the reaction by the addition of 8 µl of SDS-PAGE sample buffer, the boiled supernatant was separated by 10-20% SDS-PAGE and visualized by autoradiography or western blotting with anti-IkB $\alpha$  (mouse monoclonal IgG1, Santa Cruz Biotechnology, Santa Cruz, CA) (Chemiluminescence). The reaction mixtures were also used for immunoprecipitation with anti-IκBα antibodies (rabbit polyclonal IgG, Santa Cruz Biotechnology) or anti-Cul-1 serum raised in rabbits (Suzuki et al., 2000), followed by western blotting with various antibodies. IKK was purified from 293 cell extracts, as described by Lee et al. (1997).

#### Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (Intracellular Proteolysis) from the Ministry of Education, Science, Sports and Culture of Japan.

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Received October 23, 2000; revised March 23, 2001; accepted June 5, 2001