The NEDD8 Pathway Is Essential for SCFβ-TrCP-mediated Ubiquitination and Processing of the NF-κB Precursor p105*

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The p50 subunit of NF-κB is generated by limited processing of the precursor p105. IkB kinase-mediated phosphorylation of the C-terminal domain of p105 recruits the SCFβ-TrCP ubiquitin ligase, resulting in rapid ubiquitination and subsequent processing/degradation of p105. NEDD8 is known to activate SCF ligases following modification of their cullin component. Here we show that NEDDylation is required for conjugation and processing of p105 by SCFβ-TrCP following phosphorylation of the molecule. In a crude extract, a dominant negative E2 enzyme, UBC12, inhibits both conjugation and processing of p105, and inhibition is alleviated by an excess of WT-UBC12. In a reconstituted cell-free system, ubiquitination of p105 was stimulated only in the presence of all three components of the NEDD8 pathway, E1, E2, and NEDD8. A Cul-1 mutant that cannot be NEDDylated could not stimulate ubiquitination and processing of p105. Similar findings were observed also in cells. It should be noted that NEDDylation is required only for the stimulated but not for basal processing of p105. Although the mechanisms that underlie processing of p105 are largely obscure, it is clear that NEDDylation and the coordinated activity of SCFβ-TrCP on both p105 and IkBα serve as an important regulatory mechanism controlling NF-κB activity.

NF-κB is a member of the Rel family of transcription factors that are known to regulate basic processes, such as the immune and proinflammatory responses, development and differentiation, malignant transformation, and apoptosis (for a recent review, see Ref. 1). NF-κB is a dimeric complex composed in many cases of p50 or p52 and p65 subunits. p50 and p52 are synthesized as inactive precursors, p105 and p100, respectively, which undergo ubiquitin-mediated limited processing that removes the C-terminal ankyrin repeat-containing domain of the molecule to yield the p50 or p52 N-terminal active subunit (2–5). Intracellular localization plays an important role in NF-κB regulation, with the inactive proteins retained in the cytosol. Regulation is achieved via two major pathways: 1) control of p105/p100 processing and 2) interaction of the p50/p65 heterodimer with inhibitory molecules, IκBs. Processing of p105 occurs under both basal and activated conditions. Several studies suggested that all the motifs that are required for basal processing or processing that may occur co-translationally are contained within the N-terminal −550 amino residues (6−9), whereas signal-induced processing requires phosphorylation of serine residues 923 and 927 (10−12). In quiescent cells, the active heterodimeric complex is retained in the cytosol bound to a member of the IκB family of inhibitory proteins. Following stimulation, specific IκB kinases (IKKs) are activated, leading to phosphorylation of the inhibitor on serine residues 32 and 36, its rapid ubiquitination by the SCFβ-TrCP ubiquitin ligase, and its subsequent degradation by the 26 S proteasome. Following degradation of IκB, the NF-κB active p50/p52/p65 heterodimeric complex translocates into the nucleus and activates target genes (for a recent review, see Ref. 13).

The ubiquitin proteolytic pathway plays key roles in regulating the levels of many proteins involved in diverse cellular processes. Proteins targeted for degradation are first tagged by a polyubiquitin chain in a three-step cascade reaction involving ubiquitin activation (catalyzed by the ubiquitin activation enzyme, E1), ubiquitin transfer (catalyzed by a ubiquitin carrier protein, E2), and ubiquitin ligation (catalyzed by a ubiquitin-protein ligase, E3). Tagged proteins are then degraded by the 26 S proteasome complex. Following degradation of IκB, the NF-κB active p50/p52/p65 heterodimeric complex translocates into the nucleus and activates target genes for a recent review, see Ref. 13).

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The abbreviations used are: NF-κB, nuclear factor κB; E1, ubiquitin-activating enzyme; E2, ubiquitin-carrier protein or ubiquitin-conjugating enzyme (UBC); E3, ubiquitin-protein ligase; IKK, IκB kinase; HIV, human immunodeficiency virus; ATPS, adenosine 5′-O-(3-thio- triphosphate); WT, wild type; DN, dominant negative; BP1, binding protein 1; APP, β-amyloid precursor protein; SUMO, small ubiquitin-related modifier; Ni-NTA, nickel-nitrilotriacetic acid.
contains three commonly shared subunits, Skp1, Cullin-1, and the RING finger protein Rbx1-Roc1-Hrt1, and an F-box protein which is a variable substrate-binding subunit involved in recognition of phosphorylated target proteins (18). The F-box protein β-TrCP recognizes the common motif DpSGdPS, where pS is phosphoserine and p is a hydrophobic residue, that is found in IκBs, β-catenin, and HIV-VPu, for example (13).

Processing/degradation of p105 is mediated by two distinct structural motifs. The first resides adjacent to the glycine-rich region and contains two lysine residues that serve as ubiquitin anchors and a downstream acidic domain that may serve as an E3-binding site. This motif is probably involved in basal constitutive and/or co-translational processing/degradation of p105, providing resting cells with the low amount of p50 required for their activity under non-stimulated conditions (8). The second motif is involved in signal-induced processing/degradation of p105 and is dependent upon IKK-mediated phosphorylation, SCFβ-TrCP-mediated ubiquitination, and limited processing/degradation of the molecule (10–12). In addition, the C-terminal domain of p105 contains seven ankyrin repeats between the glycine-rich region and the IKKβ-TrCP recognition domain. These repeats bind free p50 subunits that inhibit processing of the precursor (19–21). Following stimulation, inhibition is alleviated via phosphorylation and ubiquitination of the C-terminal domain that lead to rapid processing/degradation of p105. The released free p50 subunits are then translocated into the nucleus (22).

NEDD8 is a mammalian member of ubiquitin-like (Ubl) proteins, which modify proteins in a manner similar to ubiquitination, except that in most cases it is a single moiety of the modifying protein that is attached to the substrate (reviewed recently in Ref. 23). NEDDylation requires the coordinated action of APP-BPI/Uba3 (a heterodimeric E1-like enzyme) and UBC12 (an E2-like enzyme) (24, 25). A possible role for an E3-like enzyme has been demonstrated recently for SUMO conjugation in yeast (26), although it is not clear whether a ligase is required in all reactions involving Ubl protein modification. NEDD8 has been shown to modify members of the cullin/cdc53 family (23), which are the only known substrates of the pathway. Some of the cullins are members of ubiquitin ligase complexes; Cul-1 serves as a core subunit in the SCF ubiquitin ligase family, whereas Cul-2 and Cul-5 assemble with Elongin B and C to form similar ligase complexes (27, 28). The NEDD8 pathway is essential for cell viability in fission yeast (29), but under basal conditions it is dispensable in the budding yeast (30, 31). In mammalian cells, the NEDD8 pathway is essential for cell cycle progression and morphogenesis (32).

NEDD8 is conjugated to Cul-1 at lysine residue 720 (Ref. 33; see also below), and this modification stimulates the ubiquitin ligase activity of SCFβ-TrCP directed toward IκBα (see, for example, Ref. 34) and of SCFSkp2 directed toward p27kip1 (35, 36). Mechanistically, it appears that NEDD8 up-regulates the SCF complex activity through a conformational change of Cul-1 that may promote efficient formation of an E2-E3 complex (37, 38). Taken together, it seems that Cul-1 modification by NEDD8 is a universal regulated mechanism that has a profound effect on the ubiquitin ligase activity of SCF complexes and probably of other related cullin-containing complexes, thus affecting the levels of key cellular proteins.

Although it is recognized that NEDD8 modification stimulates the activity of SCF ubiquitin ligases, a role for the NEDD8 pathway in p105 ubiquitination that affects NF-κB activation has not been established. Here we show that NEDD8 modification is required for efficient SCFβ-TrCP-mediated ubiquitination and processing of p105 following phosphorylation of the molecule by IKKβ. Thus, NEDD8 modification serves as an additional regulatory mechanism controlling NF-κB transcriptional activity, linking together the ubiquitin and NEDD8 pathways with signal-induced NF-κB activation.
The NEDD8 pathway is required for p105 ubiquitination. In vitro translated and labeled p105 was phosphorylated in a cell-free system by IKKβ and immunoprecipitated with anti-p50 as described under “Experimental Procedures.” All samples contained purified E1 and recombinant UBCH5C. WT SCF<sup>β-TrCP</sup> complex as well as the three components of the NEDD8 pathway were added as indicated. Samples were incubated and resolved on 7.5% SDS-PAGE, and proteins were visualized using phosphorimaging as described under “Experimental Procedures.” Points of migration of p105 as well as p105-ubiquitin adducts (Conj.) are marked.

~1.5 μg of each of the cDNAs coding for the constitutively active IKKβ and WT-UBC12, DN-UBC12(C111S), or Cul-1(K696R) as indicated. An empty vector was added when necessary to maintain an equal amount of DNA in all transfections. Transfections were carried out in 25-cm<sup>2</sup> flasks according to the manufacturer’s instructions. Labeled p105 was phosphorylated in a cell-free system in a 25-μl reaction mixture that contained 5 mM MgCl<sub>2</sub>, 0.5 mM ATP, and 0.4 μg of baculovirus-expressed IKKβ. The reaction mixture was incubated at 30 °C for 20 min.

Effect of the NEDD8 Pathway on In Vitro Ubiquitination and Processing of p105—In vitro ubiquitination and processing of p105 were monitored in crude HeLa extract as described (4, 8, 11). Ubiquitin-p105 conjugates were generated also in a reconstituted cell-free system as described (11). Briefly, phosphorylated [35S]methionine-labeled p105 was incubated in a reaction mixture containing 5 mM MgCl<sub>2</sub>, 0.5 mM ATP, and 0.4 μg of baculovirus-expressed IKKβ. The reaction mixture was incubated for 30 min at 30 °C.

RESULTS

The NEDD8 Conjugation Pathway Is Required for p105 Ubiquitination—Ubiquitination of p105 appears to require the activity of the SCF<sup>β-TrCP</sup> E3 ligase complex that is preceded by IKKβ-mediated phosphorylation of specific serine residues at the C-terminal domain of the molecule (10–12). It has been recently shown that NEDD8 modification of Cul-1 activates the ubiquitin ligase activity of SCF complexes (33–38). Because a role for SCF<sup>β-TrCP</sup> in p105 processing has been established only recently, and because processing of p105 is only weakly dependent on signaling and occurs also under basal conditions (8), we wanted to clarify the role of NEDDylation in SCF<sup>β-TrCP</sup>-mediated p105 ubiquitination and processing. Ubiquitin conjugation assays were carried out in a cell-free system containing phosphorylated p105, E1, UBCH5C, and SCF<sup>β-TrCP</sup>. Labeled p105 was immunoprecipitated from the mixture in which it was synthesized to isolate it from endogenous components of the NEDDylation machinery. As can be seen in Fig. 1 (lane 2), the three ubiquitin conjugating enzymes, E1, UBCH5C, and SCF<sup>β-TrCP</sup>, are not sufficient to promote conjugation of phosphorylated p105. It is the addition of the three components of the NEDDylation machinery, APP-BP1/Uba3, UBC12, and NEDD8, that stimulated conjugation significantly and resulted in conversion of almost all the labeled substrate into high molecular mass ubiquitin adducts (lane 3). Addition of either APP-BP1/Uba3 alone (lane 4) or along with UBC12 (lane 5) had only a slight effect on conjugation.

SCF<sup>β-TrCP</sup> Ubiquitin Ligase Complex That Contains Cul-1(K696R) Fails to Ubiquitate p105—To further dissect the involvement of NEDD8 modification on p105 processing, we tested the effect of different mutated components of the
NEDD8 pathway on p105 ubiquitination and processing in vitro and in vivo. Cul-1 is conjugated to NEDD8 at lysine 696 (or lysine 720 depending on the Cul-1 protein species used; see above and Refs. 33–35 and 37). We generated a point mutant Cul-1 in which this lysine is substituted with arginine and therefore cannot be conjugated to NEDD8. Ubiquitin conjugation of phosphorylated p105 was monitored in a reconstituted cell-free system that contains either the WT or the mutant Cul-1(K696R) SCFβ-TrCP complex. Only the WT enzyme was able to catalyze conjugation of p105, whereas the enzyme that contains the mutant Cul-1 was inactive (Fig. 2; compare lane 2 to lane 3). In this experiment, the NEDD8 pathway components were provided by the wheat germ cell extract in which p105 was translated. It is clear that the conjugates are generated from p105, as its concentration diminishes in the presence of the WT enzyme but not in that of the mutant one.

To link p105 phosphorylation and Cul-1 NEDDylation, we demonstrated that only a phosphorylated substrate that is incubated in the presence of the WT SCF complex and the three components of the NEDD8 pathway can be conjugated (Fig. 3, lane 3). Non-phosphorylated p105 (lane 7) or phosphorylated p105 that is incubated in the presence of mutant Cul-1-containing SCF complex (lane 4) cannot be conjugated.

**Mutant UBC12 Exerts a Dominant Negative Effect on p105 Ubiquitination**—The requirement for NEDD8 modification in p105 ubiquitination was further studied by using a point mutant UBC12 in which the active site cysteine 111 was substituted with serine. The thiol group of this cysteine residue serves as a transient acceptor during the transfer of NEDD8 from the E1 (APP-BP1/Uba3) to the target substrate. A serine residue in this position forms a stable ester with glycine 76 of NEDD8 that is not cleaved to transfer activated NEDD8 to the target protein. This results in sequestration of NEDD8 and a dominant negative inhibition of NEDD8 conjugation (44). Addition of the mutant UBC12 to ubiquitination assays carried out in crude HeLa cell extract blocked p105 conjugate formation in a dose-dependent manner (Fig. 4A, compare lanes 4 and 5 to lane 2; Fig. 4B, compare lanes 3–5 to lane 2). Inhibition was not complete, as ubiquitination proceeds via the basal site of p105 even in the presence of a high concentration of DN-UBC12 (Fig. 4, A and B, lanes 5). It should be noted that the cell extract contains a sufficient amount of UBC12 (as well as of all other components of the NEDD8 pathway) to support maximal conjugation, and addition of exogenous WT-UBC12 does not stimulate conjugation further (and even inhibits it slightly; Fig. 4A, compare lane 3 to lane 2). Importantly, inhibition of conjugation by DN-UBC12 could be alleviated by the addition of WT-UBC12 (Fig. 4B, compare lanes 6–8 to lane 5). As expected, the dominant negative UBC12 had no effect on conjugation of

**FIG. 3.** Ubiquitination of p105 by SCFβ-TrCP requires phosphorylation followed by the activity of the NEDD8 pathway. *In vitro* translated and labeled p105 was phosphorylated, immunoprecipitated, and subjected to ubiquitination (lanes 1-4) as described in the legend to Fig. 1 and under “Experimental Procedures.” Parallel samples were incubated with non-phosphorylated p105 (lanes 5-8). All samples contained purified E1 and recombinant UBCH5C. WT and Cul-1(K696R) SCFβ-TrCP complexes, as well as all three components of the NEDD8 pathway (APP-BP1/Uba3, UBC12, and NEDD8) were added as indicated. Samples were incubated and resolved on 7.5% SDS-PAGE, and proteins were visualized via phosphorimaging as described under “Experimental Procedures.” Points of migration of p105 and p105-ubiquitin adducts (Conj.) are marked.

**FIG. 4.** A catalytic site mutant UBC12(C111S) exerts a dominant negative effect on p105 ubiquitination. *In vitro* translated and labeled p105 was phosphorylated as described in the legend to Fig. 1, and the crude translation mixture was used as a source for the substrate as described in the legend to Fig. 2. Ubiquitination of labeled p105 was carried out in crude HeLa cell extract as described under “Experimental Procedures,” and the different enzymes were added as indicated. Panel A, a mutant UBC12 (C111S) inhibits in vitro ubiquitination of p105. WT- (0.8 μg; lane 3) and DN-UBC12 (1.2 μg and 4.8 μg; lanes 4 and 5, respectively; marked by a wedge) were added as indicated. Panel B, inhibition of p105 ubiquitination by DN-UBC12 is alleviated by an excess of the WT enzyme. The DN-UBC12 was added at 1.2, 3, and 4.8 μg (lanes 3–5), whereas the WT-UBC12 was added at 0.8, 1.6, and 2.4 μg (lanes 6–8) to reaction mixtures that already contain 4.8 μg of DN-UBC12. Results presented in panels A and B were quantified where 100% represents conjugate formation in the crude extract in the absence of WT- or DN-UBC12. Numbers represent % inhibition relative to this value. Panel C, conjugation of p105 that lacks the β-TrCP-binding site is not affected by DN-UBC12. WT- and DN-UBC12 were added at 0.8 and 4.8 μg, respectively, as indicated. The p105 protein used in this experiment lacks the β-TrCP-binding site (p105Δ918–934). Samples were incubated and resolved on 7.5% SDS-PAGE, and proteins were visualized via phosphorimaging as described under “Experimental Procedures.” Points of migration of p105 as well as p105-ubiquitin adducts (Conj.) are marked.
In Vivo Processing of p105 Requires the NEDD8 Pathway—

Having established a role for NEDD8 modification in the cell-free assays in vitro, it was important to assess the requirement for NEDDylation of Cul-1 on p105 processing in vivo. We transfected COS-7 cells with cDNAs coding for p105, IKKβ, and either dominant negative UBC12 or mutant Cul-1(K696R). Because p105 processing proceeds via independent mechanisms under basal and stimulated conditions, and because NEDDylation is expected to have an effect only on the stimuli-mediated processing of p105 but not basal processing as defined as described under “Experimental Procedures.” Expression of p105 and p50 was monitored via Western blot analysis with anti-p50 antibody and ECL as described under “Experimental Procedures.” Panel A, IKKβ-mediated processing/degradation of p105 was monitored after 48 h. Data were quantified and presented as described under panel A. Here too the effect was completely dependent on co-transfection with IKK (not shown).

FIG. 5. Effect of dominant negative UBC12(C111S) on p105 processing.

**Panel A** represents inhibition of processing. Samples were incubated and resolved on 10% SDS-PAGE, and proteins were visualized via phosphorimaging as described under “Experimental Procedures.” Points of migration of p105 and p50 are marked.

**Panel B** represents inhibition of processing. Samples were incubated and resolved on 10% SDS-PAGE, and proteins were visualized via phosphorimaging as described under “Experimental Procedures.” Points of migration of p105 and p50 are marked.

FIG. 6. The NEDD8 pathway is required for processing of p105 in vivo. cDNAs coding for p105, IKKβ, DN-UBC12, and DN-Cul-1(K696R) were transfected into COS-7 cells as indicated and as described under “Experimental Procedures.” Expression of p105 and p50 was monitored using Western blot analysis with anti-p50 antibody and ECL as described under “Experimental Procedures.” Panel A, IKKβ-mediated processing/degradation of p105 was monitored after 48 h. Data were quantified and presented as described under panel A. Here too the effect was completely dependent on co-transfection with IKK (not shown).
These findings strongly support the notion that NEDDylation
nally, our findings have been corroborated
mutant UBC12(C111S) inhibited, in a cell-free assay, p105
IKK
Cul-1, blocked completely phosphorylation-mediated p105
the NEDD8 pathway, either by using a DN-UBC12 or a mutant
(Fig. 3) or lacks the IKK
in a cell-free system, the addition of all three NEDD8 pathway
Several lines of evidence support our conclusion. We show that
scoring the role of SCF
ankyrin repeat domain at the C-terminal half of p105 and
ing (7), using the basal/constitutive recognition motif. The p50
p105 molecules may undergo co- or post-translational process-
cording to our proposed model (8, 11, 22), following stimulation
under different physiological conditions. The two motifs are
sor) and not to the basal processing.
We have recently identified SCF
/TrCP following phosphorylation of the precursor by IKK
is why p105 processing requires such an elaborate mechanism.
Two motifs acting under basal conditions that allows the
complex cycle involving control of the extent of NEDD8 modi-
NEDDYlation clearly adds an additional layer of control to
We thank Dr. Keiji Tanaka from the Tokyo Metrop-
Alization of the Cul-1 component of the SCF complex is required
requirement of the immature SCF complex, and ubiquitination of p105 at
but rather at p100. p52, which is derived from p100, is required
NF-
B transcriptional activator required for many of its basic
Be that the evolution of the cumbersome
We have recently identified SCF
/TrCP-cata-
DISCUSSION
We have identified SCP6-TrCP as the ubiquitin ligase
complex mediating phosphorylation-dependent p105 processing
(11). In the current study we show that NEDD8 modification
of the Cul-1 component of the SCF complex is required for
efficient ubiquitination and processing of p105 by the
SCP6-TrCP following phosphorylation of the precursor by IKKβ.
Several lines of evidence support our conclusion. We show that
in a cell-free system, the addition of all three NEDD8 pathway
components is required to reconstitute p105 ubiquitination
(Figs. 1 and 3). Not surprisingly, the NEDD8 pathway could
not support conjugation of p105 that was not phosphorylated
(Fig. 3) or lacks the IKKβ and β-TrCP recognition site (Fig. 4C).
These findings strongly support the notion that NEDDylation
is required specifically for p105 processing which is dependent
on IKK-mediated phosphorylation followed by SCP6-TrCP-cata-
yzed ubiquitination. Consistent with these results, a mutant
Cul-1(K69R)-containing the SCP6-TrCP complex that cannot
be modified by NEDD8 is completely inactive and cannot cate-
yze ubiquitination (Figs. 2 and 3). Furthermore, a catalytic site
mutant UBC12(C111S) inhibited, in a cell-free assay, p105
ubiquitination (Fig. 4) and subsequent processing (Fig. 5). Fi-
ally, our findings have been corroborated in vivo: inhibition of
the NEDD8 pathway, either by using a DN-UBC12 or a mutant
Cul-1, blocked completely phosphorylation-mediated p105
processing (Fig. 6). Here too, inhibition was limited to the
IKKβ-stimulated processing (and the phosphorylated precer-
not to the basal processing.
We have previously reported that p105 is targeted for ubiqui-
utination and processing by two distinct, basal/constitutive (8)
and signal-induced (11), recognition motifs that are utilized
under different physiological conditions. The two motifs are
probably recognized by distinct E2 and E3 enzymes (11). Ac-
cording to our proposed model (8, 11, 22), following stimulation
and consumption of cell stores of NF-κB, newly synthesized
p105 molecules may undergo co- or post-translational processing
(7), using the basal/constitutive recognition motif. The p50
subunits generated during this process dock to the emerging
ankylin repeat domain at the C-terminal half of p105 and
inhibit processing of these precursors (22). The completely syn-
thesized p105 with the docked p50 subunits serves as an inac-
in storage by sequestering these subunits in the cytosol and
hibiting their translocation into the nucleus. Following stimu-
lation, the SCP6-TrCP ubiquitin ligase is recruited to the IKK-
mediated phosphorylation motif at the C-terminal domain,
leading to rapid polyubiquitination and subsequent processing/deg-
pagation of p105 with release of the docked p50 molecules
and an additional p50 subunit released from processed p105
(11, 12, 22). Our results further support this model by under-
scoring the role of SCP6-TrCP as the ligase involved in phospho-
ylated p105 processing.
NEDDylation clearly adds an additional layer of control to
an already tightly regulated, signal-induced pathway. It is a
complex cycle involving control of the extent of NEDD8 modi-
fication (34, 37), regulated deNEDDylation by the COP9 sig-
nalsome (45, 46) and possibly other C-terminal hydroxylases
(47), and down-regulation of NEDD8 by its specific recruit-
to the proteasome (48). An interesting question in this context
is why p105 processing requires such an elaborate mechanism.
Processing of p105 is mostly constitutive and is stimulated
–2.0-fold following stimulation. This is due, most probably, to
the presence of two sites of recognition in p105, one operating
under non-stimulated conditions and the other following sig-
naling (see above). Even this site was found to be partially
phosphorylated in resting cells. In contrast, processing of p100
to yield p52 is mostly signal-induced (49, 50), although we
know little on this process. Because the two molecules are
homologous, it is possible that the evolution of the cumbersome
regulatory mechanism that involves signaling, IKK activation,
IKK-mediated phosphorylation, NEDDylation of the Cul-1
component of the SCF complex, and ubiquitination of p105 at
the second, signal-induced site, was not directed toward p105
but rather at p100. p52, which is derived from p100, is required
for lymphoid tissue development for example (see Ref. 49) and
cannot be replaced by p50. p105 that provides the cell with the
NF-κB transcriptional activator required for many of its basic
needs, some independent on one another, had to evolve addi-
tional motifs acting under basal conditions that allows the
molecule to be relieved from its tight signaling control and
processed mostly in a constitutive manner.
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