

Mechanisms of ubiquitin-mediated, limited processing of the NF- κ B1 precursor protein p105

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Abstract — In most cases, target proteins of the ubiquitin system are completely degraded. In several exceptions, such as the first step in the activation of the transcriptional regulator NF- κ B, the substrate, the precursor protein p105, is processed in a limited manner to yield the active subunit p50. p50 is derived from the N-terminal domain of p105, whereas the C-terminal domain is degraded. The mechanisms involved in this unique process have remained elusive. We have shown that a Gly-rich region (GRR) at the C-terminal domain of p50 is one important processing signal and that it interferes with processing of the ubiquitinated precursor by the 26S proteasome. Also, amino acid residues 441–454 are important for processing under non-stimulated conditions. Lys 441 and 442 serve as ubiquitination targets, whereas residues 446–454 may serve as a ligase recognition motif. Following I κ B kinase (IKK)-mediated phosphorylation, the C-terminal domain of p105, residues 918–934, recruits the SCF ^{β -TrCP} ubiquitin ligase, and ubiquitination by this complex leads to accelerated processing. The two sites appear to be recognized under different physiological conditions by two different ligases, targeting two distinct recognition motifs. We have shown that ubiquitin conjugation and processing of a series of precursors of p105 that lack the C-terminal IKK phosphorylation/TrCP binding domain, is progressively inhibited with increasing number of ankyrin repeats. Inhibition is due to docking of active NF- κ B subunits to the ankyrin repeat domain in the C-terminal half of p105 (I κ B γ). Inhibition is alleviated by phosphorylation of the C-terminal domain that leads to ubiquitin-mediated degradation of the ankyrin repeat domain and release of the anchored subunits. We propose a model that may explain the requirement for two sites: a) a basal site that may be involved in co-translational processing prior to the synthesis of the ankyrin repeat domain; and b) a signal-induced site that is involved in processing/degradation of the complete molecule following cell activation, with rapid release of stored, transcriptionally active subunits. © 2001 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

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1. Introduction

The NF- κ B dimeric transcription factors play key roles in basic processes such as regulation of the immune and inflammatory responses, development and differentiation, malignant transformation and apoptosis (for a recent review on NF- κ B, see for example [1]). Certain active subunits of NF- κ B are generated from inactive precursor molecules via limited, ubiquitin- and proteasome-mediated processing. One established case is that of p50 that is generated from the p105 precursor [2, 3]. p50 is

derived from the N-terminal domain of the molecule, while the C-terminal, ankyrin repeat-containing domain (I κ B γ), is degraded [4]. The processed subunits typically heterodimerize with members of the rel family such as p65 (RelA) to generate the active heterodimeric transcription factor. In the resting cell, the heterodimer is sequestered in the cytosol following binding of a member of the I κ B family of inhibitors. Following cellular stimulation, specific IKKs are activated and phosphorylate I κ B on specific Ser residues (residues 32 and 36 in I κ B α). Phosphorylation leads to recruitment of the SCF ^{β -TrCP} ubiquitin ligase complex, polyubiquitination and subsequent degradation of the inhibitor by the 26S proteasome. Following degradation of I κ B, the heterodimer is translo-

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cated into the nucleus where it initiates specific transcription (reviewed recently in [5]).

The ubiquitin pathway is involved in regulation of broad array of basic cellular processes, such as cell cycle progression and division, differentiation and development, and the immune and inflammatory responses. Involvement of the system in these processes is mediated via specific destruction of short-lived regulatory proteins such as cyclins, transcriptional activators, and cell surface receptors, and generation of antigenic peptides presented on class I MHC molecules. Degradation of a protein by the system involves two successive steps: a) formation of a polyubiquitin chain that is covalently anchored to the target substrate; and b) degradation of the tagged protein by the 26S proteasome. Conjugation of ubiquitin involves three steps: a) activation of ubiquitin by the ubiquitin-activating enzyme, E1; b) transfer of the activated moiety to a member of the ubiquitin-carrier protein, E2, family of enzymes (also known as ubiquitin-conjugating enzymes, UBCs); and c) further transfer of the activated ubiquitin moiety from E2 to the target substrate that is specifically bound to a member of the ubiquitin-protein ligase family of proteins, E3. Subsequent processive transfer of additional activated ubiquitin molecules and their conjugation to previously attached moieties generates a polyubiquitin chain that serves as a degradation signal for the proteasome. The initial binding of the substrate to E3 plays an essential role in specific substrate targeting. Several classes of E3s have been described, among them are the SCF (Skp1, Cullin1, F-box protein) complexes that recognize phosphorylated substrates involved, for example, in cell cycle regulation and the immune and inflammatory responses. The complexes are tetrameric and are composed of three common components, Skp1, Cullin1/Cdc53 and Hrt1/Rbx1/Roc1, and a variable F-box protein that serves as the substrate-recognizing subunit, E3 (for recent reviews on the ubiquitin system see, for example, [6, 7], and for a recent review on SCF complexes, see [8]). The F-box protein involved in recognition of phosphorylated I κ B α is human β -TrCP. Recognition of the inhibitor is mediated via the sequence [DS(P)GLDS(P)]. A similar sequence is probably involved in the recognition of human β -catenin and HIV-Vpu.

The mechanisms involved in limited processing of the p105 precursor protein have been partially elucidated. Lin and Ghosh have demonstrated that a GRR in the middle of the molecule is required for processing [9]. Fan and Maniatis have shown that a truncated form of p105, p60, can be processed to p50 [4]. Lin et al. [10] have shown that p105 can be processed co-translationally, and synthesis of the complete molecule is not required for generation of p50. Taken together, these studies suggested that all the motifs that are required for basal processing are contained within the first ~550 amino residues. Other studies have suggested a role for phosphorylation of the C-terminal domain of p105 in regulated, signal-induced processing of

the molecule (see for example [11, 12]). Heissmeyer et al. [13] have shown that IKK-mediated phosphorylation of Ser residues localized to a region that spans amino acid residues 922–933 leads to rapid degradation of p105. Thus, it appears that processing/degradation of p105 can occur under both basal and activated conditions, and that each process is mediated via distinct structural motifs, and probably catalyzed by different E2 and E3 enzymes. The enzymes and the mode of regulation of the process have remained largely obscure.

2. Results

2.1. The Gly-rich region (GRR) and processing of p105 (results of this section have been published in [14])

It has been reported that the GRR that spans amino acid residues 476–304 of p105 is an important structural motif required for the generation of the p50 subunit of NF- κ B [9], however the mechanisms involved in this unique reaction have not been dissected. Since degradation of a protein via the ubiquitin pathway involves two steps, conjugation of ubiquitin and proteasomal degradation of the tagged substrate, it was important to identify the step affected by the GRR.

We have shown that conjugation of p105 is not affected by GRR either in vitro or in vivo, and its mechanism of action appears to be interference with the function of the 26S proteasome. This function of the GRR as an essential element that governs processing predicts two related assumptions: a) that processing involves recognition of a specific motif within the C-terminal domain of p105 that is degraded; and b) that the GRR protects/stabilizes the newly formed p50 subunit. To test the second notion, we generated two p50 derivatives, one that contains the GRR and one that lacks this domain. Both proteins share the C-terminal domain of native p50 (residues 405–435). The GRR-containing protein is significantly more stable than its deleted counterpart both in vitro and in vivo. Thus, it appears that the GRR is involved in the generation of p50 by serving as processing 'stop' signal and as a stabilizing element of the cleaved fragment.

To analyze structure/function relationship of different elements within the GRR, we studied the function of different deletion and point mutants. The p105 GRR contains 19 Gly residues (out of 29 residues in total; 376–GGGSGAGAGGGGMFGSGGGGGGTGST-GPG-404; *figure 1*) and also two short GA repeat (380–GAGA-383). Analysis revealed that only six (382–GAGGGGMFGS-391) of 19 Gly residues are sufficient to promote, at least partially, generation of p50. Interestingly, the Ala residue is essential for processing.

Since a small GA-containing sequence appears to function as a processing signal, it was also important to examine its 'universality' as a transferable processing

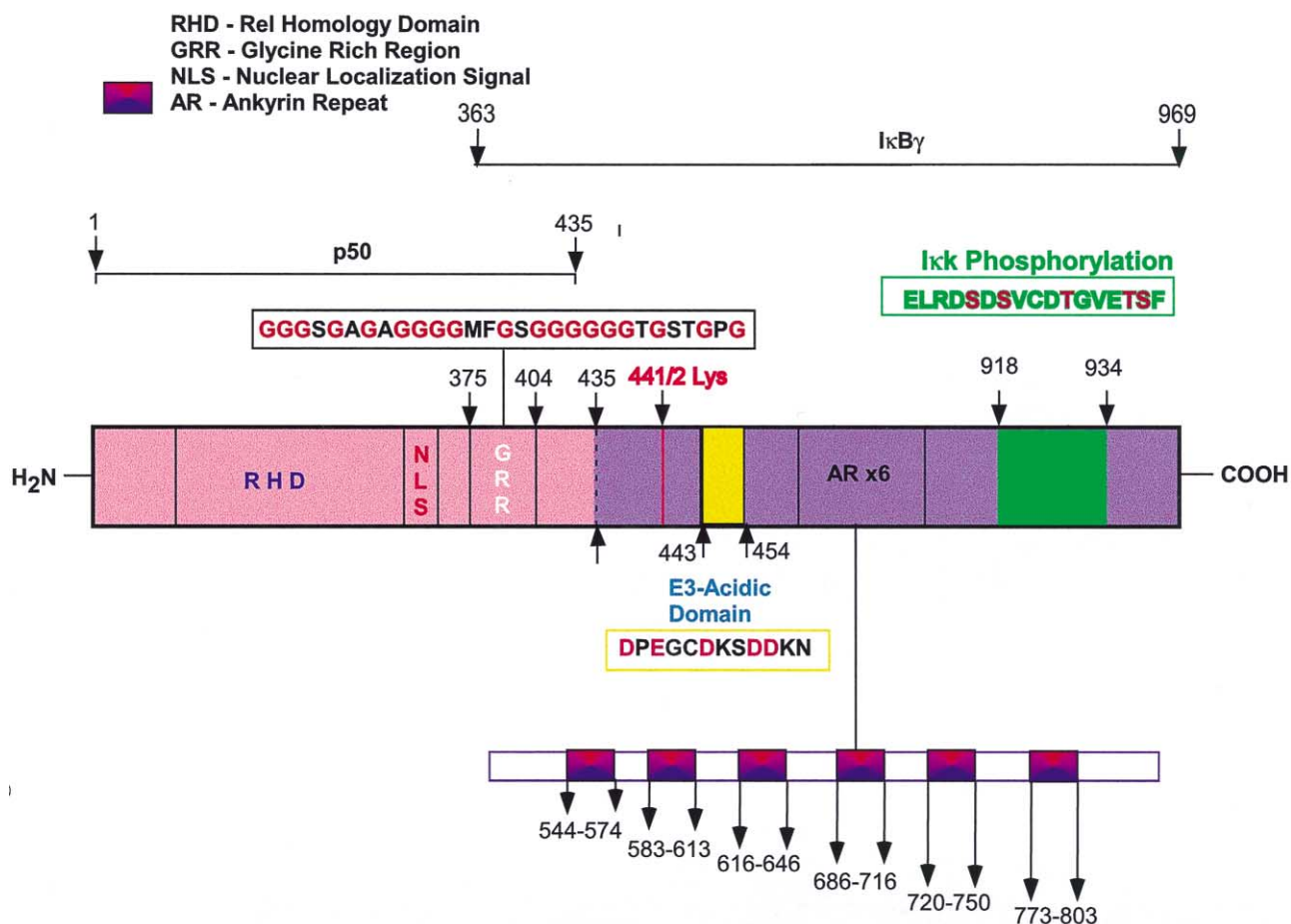


Figure 1. A schematic diagram of human p105. Shown are p50 (1–434), I κ B γ (363–969; the ankyrin repeat-containing, C-terminal domain), the GRR (376–404), the two Lys residues (441, 442) that serve as ubiquitination anchors, the acidic basal putative E3 recognition motif (443–454), the seven ankyrin repeats, and the C-terminal IKK-phosphorylation domain (918–934). Domains are not shown in proportional size.

signal. Its transfer to bona fide substrates of the ubiquitin system derived from mammalian cells, including MyoD, p53, or ODC (that is targeted by the proteasome without prior ubiquitination), and to the *D. melanogaster* Dorsal (which is closely related to p105), did not render these proteins susceptible to processing. Even its transfer within p105 (to position 604–613) did not yield a processing product. Taken together, these findings suggested that, in addition to the GRR, processing requires an additional motif(s) that resides in a defined distance, up- or downstream to the GRR, and that is involved in processing.

2.2. A region downstream to the GRR is required for processing (results of this section have been published in [14])

In our attempt to identify this putative additional motif(s), we noted a sequence that resides downstream to the GRR and that is highly homologous to the ubiquitination and E3-binding domain of I κ B α (table 1). To test the possible role of this motif in p105 processing, we generated two p105 mutants: 1) K441,442R in which we substituted with Arg the two Lys residues that are homo-

Table I. Comparison of the ubiquitination/recognition domains of human p105 (residues 441–454) and IκBα (residues 20–39).

Human p105	440-SKKD <u>P</u> EGCDK <u>S</u> DD	-454
	+ +	
Human IκBα	20-LKKERLLD <u>D</u> RH <u>D</u> SGL <u>D</u> SMK <u>D</u> -39	

The pair of lysine residues that serve as ubiquitination sites in both molecules are marked by vertical lines. Two overlapping acidic residues are marked by +. Ser residues are in bold, whereas all acidic residues are underlined (six in IκBα and five in p105).

gous to IκBα Lys 21 and 22; and 2) p105Δ446–454 that lacks the residues that flank Ser 450 which may be homologous to Ser 32 or 36 of IκBα. Our data show that the two Lys residues are important for ubiquitination of p105, while residues 446–454 are independently important for processing, probably via binding of the E3. However, unlike Ser32 of IκBα, processing of p105-S450A is indistinguishable from that of the WT protein, suggesting that phosphorylation of this residue is not necessary for processing.

To examine the biological consequences of the alterations in the different processing signals, we transfected COS-7 cells with WT, ΔGRR, K441,442R, and Δ446–454 p105s, and monitored binding of labeled κB probe in electromobility shift assay (EMSA) as a measure for the formation of biologically active p50. While WT p105 generates active NF-κB that specifically binds the labeled probe, p105-ΔGRR does not generate any binding activity, and the activity generated by the K441,442R and the Δ446–454 species was markedly reduced.

2.3. IKK-mediated signal-induced phosphorylation of the C-terminal domain of p105 recruits the SCF^{β-TrCP} E3 complex and leads to accelerated processing/degradation of p105 (results described in this section have been published in [15])

Since deletion of residues 441–454 did not abolish processing completely [14, 15], and since signal-induced phosphorylation that could not possibly involve residues 446–454 (see above) was reported to regulate p105 processing ([11, 12]; see above), we predicted that an additional motif may be involved in recognition of p105 by the ubiquitin system. Since the C-terminal domain contains an IKK phosphorylation site that is involved in regulated processing/degradation of p105 [13], we decided to dissect the mechanism(s) that underlie its involvement in the process. Quantitative analysis revealed that deletion of residues 446–454 or residues 918–934 that comprise the IKK phosphorylation site, decreased processing by ~80%. Processing was abolished almost completely in a p105 that lacks both domains.

Since IKKs modifies the C-terminal domain of p105 [13], it was important to test whether this modification affects processing. Expression of a constitutively active IKKβ stimulated significantly processing of p105. Quantitative analysis revealed that almost all the precursor

protein disappeared. While most of it was processed to p50, a significant part was completely degraded. The kinase stimulated processing of p105-WT and p105-Δ446–454 to the same extent. In contrast, it did not have any effect on processing of p105-Δ918–936. Thus, it appears that IKKβ stimulates p105 processing via its activity on the C-terminal domain of the molecule.

To demonstrate that IKKβ phosphorylates the C-terminal domain of p105, we reconstituted the phosphorylation system in vitro. In the presence of unlabeled ATP and IKK, only ³⁵S-methionine-labeled WT but not C-terminal deleted p105 was converted into a slower migrating form which could be converted into a faster migrating form following addition of alkaline phosphatase. Similar results were obtained using [γ-³²P]ATP and unlabeled substrates, where a strongly phosphorylated form was obtained only when the WT protein was present in the reaction mixture. These in vitro experiments show that IKKβ modifies p105 on Ser and/or Thr residues in the region that spans moieties 918–934.

At this stage, it was important to identify the E3 involved in recognition of the phosphorylated C-terminal region of p105. Obvious candidates are members of the SCF family of ligases such as SCF^{β-TrCP} and SCF^{Skp2} that recognize phosphorylated substrates (reviewed recently in [8]). Transfection of cells with the dominant negative E3, ΔF-box β-TrCP1, inhibited significantly IKKβ-dependent processing of p105-WT. In contrast, processing of p105-Δ918–934 was not affected. Similarly, transfection with ΔF-box Skp2 had no effect. Based on these findings, we concluded that TrCP may serve as the E3 that recognizes the phosphorylated C-terminal domain of p105, and is involved in phosphorylation-mediated processing of the molecule. To further explore the role of β-TrCP in p105 recognition, we utilized the phosphopeptide that spans the phosphorylation domain of IκBα. This peptide binds specifically to TrCP and inhibit its activity towards IκBα and β [16]. The peptide inhibited significantly both conjugation and processing of WT and Δ446–454 p105s in a cell free reconstituted system, but had no effect on the conjugation of Δ918–934-p105. The experiments with the ΔF-box β-TrCP and the peptide provide only indirect evidence for the involvement of the enzyme in p105 processing. A more direct proof for the involvement of β-TrCP in ubiquitination of p105 came from co-immunoprecipitation experiments, where we were able to

demonstrate physical association between TrCP1 and p105 which is increased several-fold following TNF α stimulation of cells. Deletion of the IKK β phosphorylation site (residues 918–934), abrogated the enhanced binding. Only p105-WT that was phosphorylated by IKK β , could be precipitated by immobilization of TrCP. p105-WT that was incubated in the absence of the kinase or p105- Δ 918–934 that was incubated in the presence of the kinase, failed to associate with TrCP. Last, it was important to demonstrate directly that β -TrCP can conjugate ubiquitin to C-terminally phosphorylated p105 and promote its processing. Tetrameric SCF $^{\beta$ -TrCP (Skp1/Cullin1/F-box, β -TrCP/Roc1) complex conjugated phosphorylated p105-WT in a cell free system. A substrate that was not phosphorylated prior to the incubation with the ligase, was not conjugated. All these experiments firmly establish the role of β -TrCP in recognition of the phosphorylated C-terminal domain of p105. To analyze elements in the C-terminal motif of p105 that are targeted by TrCP, we initially monitored conjugation of WT, Δ 446–454, and Δ 918–934 p105s in a reconstituted cell free system. The WT and Δ 446–454 proteins are efficiently conjugated by the SCF complex. In contrast, p105- Δ 918–934 did not generate high molecular mass adducts. To specifically identify the residues that play a role in recognition of phosphorylated p105, we substituted Ser residues 922, 924, and 933 with Ala. The triple replacement abolished the ability of TrCP to catalyze conjugation, strongly suggesting that these residues are targeted by the kinase. Interestingly, a construct in which Thr residues 928 and 932 were replaced, also demonstrated reduced conjugation. While these findings clearly support the notion that IKK is mostly a Ser kinase, they suggest that the TrCP recognition motif in p105 may be novel.

While recognition of the phosphorylated C-terminal domain is mediated by SCF $^{\beta$ -TrCP, it is not clear whether this E3 is also involved in recognition of the upstream acidic domain. Since the SCF complex is involved in targeting phosphorylated substrates, its involvement in targeting the upstream domain is highly unlikely, though the many negatively charged residues in this domain may mimic the negatively charged phosphate group(s) involved in recruiting the SCF complex. In cells, dominant negative Δ F-box β -TrCP had only a minor effect on processing of p105- Δ 918–934. Also, in vitro processing of the C-terminally deleted mutant is not affected by the inhibitory phosphopeptide. Furthermore, WT and Δ 446–454 p105s are conjugated to the same extent by the SCF complex in vitro. To test a possible role for an additional, yet unidentified, E3 in targeting the acidic domain, the WT and the C-terminal deleted p105s were incubated in the presence of crude HeLa extract. Unlike the purified SCF complex that cannot conjugate p105- Δ 918–934, here both proteins were efficiently conjugated, suggesting that the HeLa extract contains an additional, acidic domain-recognizing E3. While indirect, these find-

ing strongly suggests that p105 is conjugated by at least two ligases, recognizing two distinct motifs. It is not known whether Lys 441 and 442 serve as ubiquitination sites for both E3s.

2.4. The requirement for two distinct targeting sites for processing of p105: β -TrCP targeting of the IKK phosphorylation site alleviates inhibition of processing/degradation of p105 caused by docking of NF- κ B active subunits to the ankyrin repeat domain (data presented in this section have not been published yet)

As has been clearly demonstrated, p105 has two distinct ubiquitin system targeting motifs, an acidic domain that contains also two Lys residues required for ubiquitin anchoring, and a C-terminal phosphorylation domain. An important, yet unresolved, question involves the physiological roles and evolutionary pressure that led to the development of the two sites. To resolve this problem, one has to analyze the mode of interaction of the different subunits and components of NF- κ B under different physiological conditions.

We noted that under basal conditions, the efficiency of generation of p50 from p105 in both cell free system and intact cells is low. Typically, 10–20% of the precursor molecules are processed [3, 14, 15]. It has been shown that active NF- κ B subunits such as newly generated p50 and p65 dock to the ankyrin repeat domain in the C-terminal half of p105 and inhibit its processing [17]. Therefore, we wanted to examine the hypothesis that the efficiency of processing is inversely correlated with the number of ankyrin repeats to which the active subunits dock. Furthermore, we wanted to test whether alleviation of inhibition and supply of transcriptionally active subunits requires signal-induced targeting of p105 via the C-terminal domain of the molecule. To test these hypotheses, we constructed (via utilization of unique restriction sites or introduction of stop codons) a series of p105 deletion mutants that contain an increasing number of ankyrin repeats, yet lacking the C-terminal domain. As can be seen in *figure 2*, in vitro processing of the different C-terminally deleted p105 precursors is progressively inhibited with increasing number of ankyrin repeats. Thus, p105-Tth111 that does not contain any ankyrin repeat is processed efficiently. In contrast, processing of p105-*BsmI* that contains three repeats is significantly less efficient, whereas processing of p105-*AatI* that contains four repeats is hardly detectable. It is not clear whether the inhibition is due to interference with conjugation or processing of the precursor.

We surmised that the progressive inhibition in processing that has been observed with increasing number of ankyrin repeats, is due to the presence of free p50 and other active NF- κ B subunits in the HeLa cell extract in which the reactions were carried out. To corroborate this assumption directly, we overexpressed in cells p50-*XbaI*.

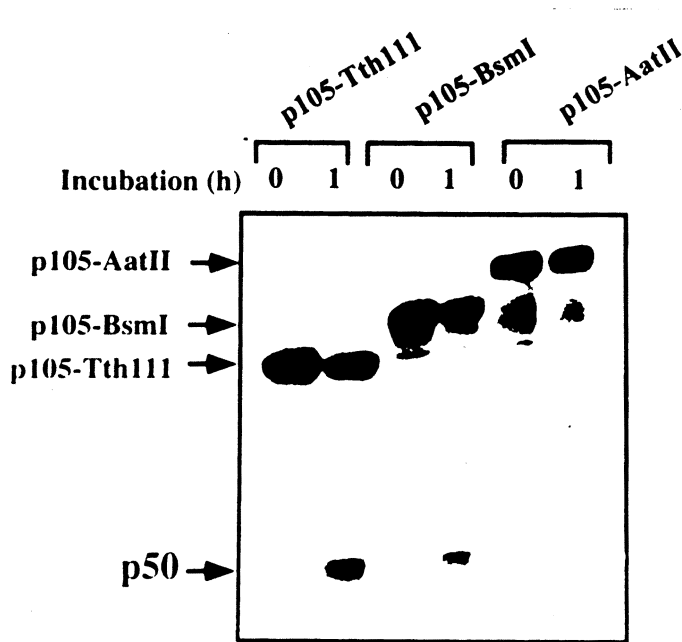


Figure 2. Efficiency of processing of p105 in a cell-free system is inversely related to the number of ankyrin repeats. [^{35}S]methionine-labeled and in vitro translated p105-Tth111 (no ankyrin repeats), p105-BsmI (three ankyrin repeats), and p105-AatII (4 ankyrin repeats) were subjected to processing in HeLa cell extract-based cell free system and generation of p50 was monitored.

This molecule, that has a molecular mass which is slightly larger than that of p50, cannot be processed as it does not contain the required downstream Lys residues and acidic domain [14]. As can be seen in *figure 3A*, overexpressed p50 inhibited processing of WT-p105 (compare lane 3 to lane 2). Similar to the in vivo experiment, addition of exogenous bacterially expressed p50 to a cell-free proteolytic system also resulted in inhibition of processing of WT-p105 (not shown). Not surprisingly, processing of p105-Tth111, that is an ankyrin repeat-free precursor, is not inhibited by increasing concentration of exogenous p50 both in vitro and in vivo (not shown).

We have shown that IKK-mediated phosphorylation of specific Ser residues within the C-terminal domain of p105 leads to recruitment of the SCF $^{\beta\text{-TrCP}}$ ubiquitin ligase with subsequent polyubiquitination and rapid processing/degradation of the precursor molecule [15]. Heissmeyer et al. [13] have shown that IKK-mediated degradation of p105 probably releases docked p50 that interacts with Bcl-3 to generate the trimeric p50•p50•Bcl-3 active transcription factor [13]. We hypothesized that the mechanism that underlies liberation of the docked inhibitory subunits involves the C-terminal domain, IKK and $\beta\text{-TrCP}$ -mediated targeting of p105. To corroborate this hypothesis, we transfected cells with p105-WT in the absence or presence of p50 along with active or inactive IKK β . The p50-induced inhibition is partially alleviated by expression of a constitutively active IKK β (*figure 3A*, lane 4), but not by expression of a catalytically inactive enzyme (*figure 3A*, lane 5; compare also *figure 3B*, lane 3 to lane

2). Not surprisingly, the kinase had no effect on inhibition of processing of the C-terminally deleted p105 (*figure 3B*, lanes 4–6). Last, it was important to examine the role of $\beta\text{-TrCP}$ in the kinase-mediated alleviating effect. Towards that end, we made use of the dominant negative species of the ligase, $\Delta\text{F-box } \beta\text{-TrCP}$ (see above). We have clearly shown that while IKK expression was able to relieve p50-mediated inhibition of p105 processing (see also above), this alleviation was abolished by a concomitant expression of $\Delta\text{F-box } \beta\text{-TrCP}$ (not shown).

3. Discussion

We have shown that p105 is targeted for processing/degradation by two distinct ubiquitin system recognition motifs. The first motif, which resides adjacently and downstream to the GRR, contains two Lys residues that serve as ubiquitin anchors and a downstream acidic domain that serves, most probably, as an E3 binding site. This motif is involved, most probably, in basal/constitutive processing/degradation that occurs in resting cells under non-stimulated conditions and provides the cell with the low amount of p50 required for its activity under these conditions. Processing of p105 under these conditions may occur co-translationally prior to completion of synthesis of the entire molecule. The second, C-terminal recognition motif, undergoes signal-induced IKK-mediated phosphorylation with subsequent recruitment of the SCF $^{\beta\text{-TrCP}}$ ubiquitin ligase, polyubiquitination

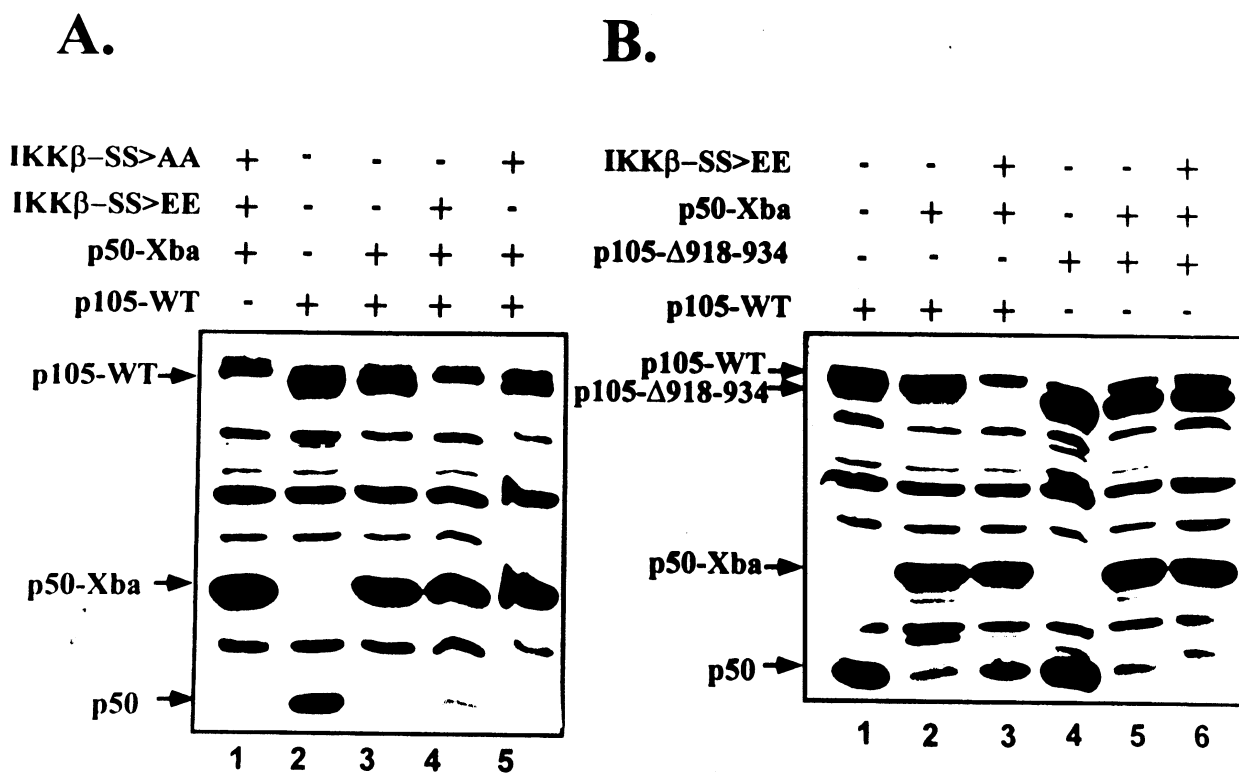


Figure 3. IKK β alleviates p50-mediated inhibition of p105 processing/degradation via phosphorylation of the precursor's C-terminal domain. **A.** Active IKK β is required to alleviate p50-mediated inhibition of WT-p105 processing. COS-7 cells were transiently transfected with cDNAs coding for p105-WT, p50-XbaI, and for either active (SS > EE) or inactive (SS > AA) IKK β as indicated. Generation of p50 was monitored via Western blot analysis. Sites of migration of p105, the p105-derived p50, and p50-XbaI, are indicated by arrows. **B.** The C-terminal IKK phosphorylation/ β -TrCP binding domain of p105 is required for IKK β -mediated alleviation of p50-induced inhibition of p105 processing. COS-7 cells were transiently transfected with cDNAs coding for p105-WT (lanes 1–3) or 105- Δ 918–934 (lanes 4–6), in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5, and 6) of p50-XbaI, and in absence (lanes 1, 2, 4, and 5), or presence (lanes 3 and 6) of constitutively active IKK β . Generation of p50 was monitored via Western blot analysis. Sites of migration of the different p105 species, the p105-derived p50, and p50-XbaI, are indicated by arrows.

and accelerated processing/degradation of p105. The two motifs are targeted via two different E3s, and most probably, via distinct E2s as well.

Several proteins have been described that are targeted following recognition of two distinct motifs and conjugation enzymes. Among them are p53 that is targeted by Mdm2 following DNA damage [18] and by the E6/E6-AP ligase complex in high risk, human papillomavirus-infected/transformed cells [19]. The yeast mating type transcriptional regulator MAT α 2 is targeted by two motifs, Deg1 and Deg2, and two E2 enzymes, Ubc6 and Ubc7 [20], however the identity of the E3(s) and the physiological significance of the two signals have remained obscure. Similarly, the model protein lysozyme is targeted by E2-14kDa/E3 α following recognition of the N-terminal residue, but also by members of the UbcH5

family and a yet to be identified E3 that recognizes, most probably, a mid-molecule, downstream signal [21].

To resolve the problem of the physiological relevance of the two sites in p105, we have shown that processing is regulated by endogenous free NF- κ B subunits that dock to the ankyrin repeat domain of the molecule and inhibit processing of the precursor. Inhibition is alleviated by IKK-mediated phosphorylation that leads to SCF $^{\beta$ -TrCP recruitment, rapid ubiquitination, and efficient degradation of C-terminal domain with subsequent release of the docked subunits and probably an additional p50 subunit that originates from the processed precursor.

Based on these results, we propose a model according to which a nascent p105 polypeptide chain is processed initially co-translationally to generate p50. Processing requires the basal/constitutive acidic recognition motif.

The p50 that is generated under these conditions is docked to an emerging ankyrin repeat domain in a p105 molecule that has not been processed yet (co-translationally). Docking of several p50 and/or other free NF- κ B subunits sterically hinders either the basal recognition site or processing of the ubiquitinated precursor by the 26S proteasome. The completely synthesized p105 molecule along with the docked subunits serve as an inactive storage for these subunits in case rapid supply of a large quantity is required following cell activation. Upon stimulation, the C-terminal domain is phosphorylated. This modification leads to recruitment of the SCF ^{β -TrCP} ubiquitin ligase complex which results in polyubiquitination and subsequent processing/degradation of p105 with release of the docked molecules and an additional p50 subunit generated from the precursor.

While several experimental lines of evidence lend support to this model, including progressive inhibition of processing with increasing number of ankyrin repeats, inhibition of processing by p50, alleviation of inhibition that requires an intact C-terminal domain, phosphorylation by IKK, and the requirement for β -TrCP E3, several important problems have remained unsolved. The mechanism(s) that selects p105 molecules for co-translational processing from those that will generate the complete, 969 residues, species is not known. Co-translational processing maybe the default mechanism that provides the resting cell with the small amount of p50 required for its basal activities. This may occur in the post-stimulation resting cell when all the endogenous p50 has been depleted. Once a small amount of p50 is synthesized, it binds to the emerging nascent p105 chain and inhibits further generation of p50 (via processing of p105 or via a cotranslational mechanism) which will occur now only following stimulation and targeting of the C-terminal domain. Also, the mechanism(s) that spares the anchored subunits from degradation, while allowing complete proteolysis of the ankyrin repeat domain and a large proportion of the N-terminal p50 domain of p105, is still obscure. It should be noted that the N-terminal domain contains the 'protective' Gly rich region (GRR) that may not function efficiently. Following stimulation, proteolysis can 'overcome' the protective barrier of the GRR, at least partially. Therefore, stimulation may lead not only to processing, but also to partial degradation of p105. Also, the mechanism that underlies p50-induced inhibition of processing is not known. It can result from an intra-molecular mechanism involving folding of the ankyrin repeat domain with its docked sub-units [22] or from steric hindrance by the docked p50 subunits, so that the acidic E3 site, the ubiquitin anchoring Lys residues, or the proteasome recognition motif, are not accessible. It is this *cis* inhibitory mechanism that may have played a role in the evolution of the second, signal-induced C-terminal phosphorylation domain under conditions where the first site is sterically hindered.

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