Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 345 (2006) 7–13
BBRC
www.elsevier.com/locate/ybbrc

Two distinct ubiquitin-dependent mechanisms are involved in NF-κB p105 proteolysis

Shai Cohen [a,b,*], Shirly Lahav-Baratz [a,c], Aaron Ciechanover [a]

[a] Center for Vascular and Tumor Biology, The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel
[b] Department of Internal Medicine B, Lady Davis/Carmel Medical Center, Haifa 34362, Israel
[c] Department of Obstetrics and Gynecology, Lady Davis/Carmel Medical Center, Haifa 34362, Israel

Received 30 March 2006
Available online 24 April 2006

Abstract

Generation of the p50 subunit of NF-κB is a rare case in which the ubiquitin system processes a longer precursor, p105, into a shorter active subunit: in the vast majority of cases, the target protein is completely degraded. The mechanisms involved in this process have remained elusive. It appears that a Gly rich region (GRR) in the middle of the molecule serves as a "processing stop signal", though under certain conditions, such as after stimulation, p105 can be completely degraded. Since NF-κB plays critical roles in a broad array of basic cellular processes, it is important to dissect the mechanisms that regulate its proteolysis—both destruction and processing. We have previously shown that signal-induced degradation of p105 requires ubiquitination on multiple lysines. Here we describe a novel region, a Processing Inhibitory Domain—PID, that upon its removal, the molecule is processed in high efficiency, which requires ubiquitination on a single, though non-specific, lysine.

Keywords: p105; NF-κB; Ubiquitin; Limited processing; Degradation; Proteasome

The NF-κB family of transcriptional factors plays important roles in the regulation of numerous basic cellular processes in health and disease [1]. The p50 and p52 subunits of NF-κB are generated by limited processing from the longer precursors, p105 and p100, respectively [2–4]. Processing is mediated by the ubiquitin-proteasome system and the newly generated subunits homodimerize or heterodimerize with other members of the rel family of proteins to generate the active transcription factor. A member of the IκB family of inhibitors binds to the active factor, sequestering it in the cytosol. Following stimulation, IκB kinases (IKKs) are activated and phosphorylate the inhibitors on specific serine residues. This leads to recruitment of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase, polyubiquitination, and subsequent degradation of the inhibitor. Consequently, active NF-κB is translocated into the nucleus where it initiates specific gene expression [5].

The mechanisms involved in processing of p105 have been elucidated only partially. Lin and Ghosh [6] have demonstrated that a GRR that spans residues 376–404 in human p105 functions as a "processing stop signal" for the 26S proteasome [6–8]. Several single residues upstream to the GRR that are involved in proper folding of p50 are also thought to be important for processing [8]. Processing appears to proceed via at least two different mechanisms: (i) basal-constitutive and (ii) signal-induced using a specific phosphorylation site at the C-terminal domain of p105. Fan and Maniatis have shown that a truncated form of p105, p60, can still be processed to p50 [2]. Lin and colleagues have shown that p105 can be processed co-translationally [9]. However, the same group demonstrated that the Rel homology domain in p50 undergoes co-translational dimerization with p105, and that this interaction is

* Corresponding author. Fax: +972 4 825 0837.
E-mail address: shaico@tx.technion.ac.il (S. Cohen).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.036

required for efficient production of p50 from complete p105 [10]. We and others have shown that p105 harbors in its C-terminal domain an IKK phosphorylation site [11,12]. Signal-induced modification of this site is followed by recruitment of the β-TrCP ligase which leads to both processing, but also to complete degradation of p105 [11–15]. Salmeron and colleagues [16] have identified serine 927 as essential for signal-induced p105 processing/degradation. We have shown that while C-terminal phosphorylation by IKKβ mediates both processing and degradation of p105, β-TrCP is involved only in degradation of the molecule, suggesting that an additional, yet unknown, ligase is involved in signal-induced processing [17]. Thus, processing of p105 appears to be mediated by both basal and signal-induced mechanisms, though the identity of the ligases involved is not known. While the processing of p100 to p52 appears to be mediated by a similar mechanism, there are major differences between the two processes. Processing of p100 also requires a GRR [18], IKKα phosphorylation of serine residues 865 and 869, and recruitment of the $SCF^{\beta\text{-}TrCP}$ [19]. However, in contrast to p105, following signalling, p100 undergoes only processing and not degradation [20]. Moreover, processing does not appear to occur under basal conditions. The lack of constitutive processing of p100 is attributed to a novel motif functioning as a "Processing Inhibitory Domain" (PID), which resides between the ankyrin repeat domain and the phosphorylation site of p100 (residues 754–859) [21]. Importantly, processing of p100 requires one single lysine residue, K855, which resides 10 residues upstream to the IKK/TrCP site [22]. This residue is located in a position similar to that of the critical lysine residue in IκBα that serves for anchoring of the polyubiquitin chain which mediates its destruction.

Here, we demonstrate that p105 has also a strong PID, the removal of which results in accelerated processing. Similar to processing of p100, processing of p105 also requires a single lysine residue, though its position is less critical.

Materials and methods

Materials

Materials for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Bradford reagent were from Bio-Rad. L-[^{35}S]Methionine and molecular weight markers were from Amersham Pharmacia Biotech (currently GE Healthcare). Site-directed mutagenesis was carried out using the QuickChange® kit from Stratagene. Tissue culture sera and media were from Biological Industries, Bet Haemek, Israel, or from Sigma. Mouse anti-FLAG antibody was from Sigma, and peroxidase-conjugated rabbit anti-mouse was from Jackson ImmunoResearch Laboratories. Ubiquitin, dithiothreitol (DTT), adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS), 2-deoxyglucose, glutathione, Tris buffer (Trizma® base), and N-terminal pFLAG-CMV2 plasmids were from Sigma. Protease inhibitor mixture was from Calbiochem. Hexokinase was from Roche Molecular Biochemicals. JetPEI transfection reagent was from Polyplus Transfection. Ubiquitin aldehyde (UbAl) was from BIOMOL. Reagents for enhanced chemiluminescence (ECL) were from Pierce. Wheat germ extract-based coupled transcription–translation kit was from Promega. Restriction and modifying enzymes were from New England Biolabs. Oligonucleotides were synthesized by Sigma. All other reagents were of high analytical grade.

Methods

Cultured cells. HEK293 and HeLa cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (penicillin–streptomycin).

Plasmids. Human p105-WT, p105-Δ429–654, and p105-Δ429–654/K14R cDNAs in pT7β were described previously [17] and served as platforms for generating N-terminal FLAG fusions (see also Fig. 1 under Supplementary material). To that end, the entire coding frames were subcloned into the pFLAG-CMV2 vector. Using standard molecular biological methods, these constructs served as platforms for all further manipulations. p105-Δ429–654/K14R, R683K, and p105-Δ429–654/K14R, 918K for in vitro translation were generated by site-directed mutagenesis using p105-Δ429–654/K14R in pT7β. Constitutively active IKKβ (IKKβ-SS > EE) for cell expression was as described previously [11]. The different dominant negative (DN) E2s for cell expression were as described previously [23].

In vitro conjugation. Ubiquitin adducts of [^{35}S]methionine-labeled p105s were generated in crude HeLa cell extract as described previously [7,11,24].

Preparation of nuclear and cytoplasmic extracts. Nuclear and cytoplasmic extracts were prepared as described previously [25].

Transient transfections and processing or degradation of p105 in cells. Cells growing on 35 mm dishes were transiently transfected with ~1.5 μg cDNAs coding for the different p105 derivatives. Where indicated, cells were also cotransfected with ~1.5 μg cDNAs coding for the constitutively active IKKβ and the different DN E2s. An empty vector was added, when necessary, to ascertain an equal amount of DNA in all transfections. Transfections were carried out using the JetPEI reagent following the manufacturer's instructions. Twenty-four hours after transfection, processing and degradation of p105 were monitored via Western blot analysis. For immunoblotting, we used either a whole cell extract [prepared in a buffer containing 1% Triton, 140 mM NaCl, 50 mM Tris (pH 7.2), and protease inhibitor mixture], or cytosolic and nuclear fractions that were prepared as described above. Aliquots containing equal amounts of protein were resolved by SDS–PAGE (10%), blotted onto nitrocellulose paper, and visualized using anti-FLAG antibody followed by ECL.

Protein concentration. Protein concentration was determined by the method of Bradford [26] using bovine serum albumin as standard.

Results

Deletion of a 70 amino acid segment downstream to the GRR of p105 enhances its basal processing

We previously noted [17] that p105 that lacks amino acids 429–654 is processed much more efficiently (~50%) than its WT counterpart ([17]; see also Fig. 1; compare lane 3 to lane 1). This led us to hypothesize that a regulatory domain that resides within this sequence inhibits p50 generation in resting cells. To inhibit stimulation-induced processing (that may occur to some extent even in resting cells), we replaced serine 927 in the p105-Δ429–654 with alanine. As can be seen in Fig. 1, the IKK-independent p105-Δ429–654, S927A is also processed in high efficiency (compare lanes 5 and 6 to lane 1). As we have shown previously [17] and as shown in Fig. 1 (lane 7), a mutant p105 from which we have removed all the lysine residues downstream to the GRR is processed inefficiently under basal conditions, suggesting that the enhanced basal processing is ubiquitin-dependent.

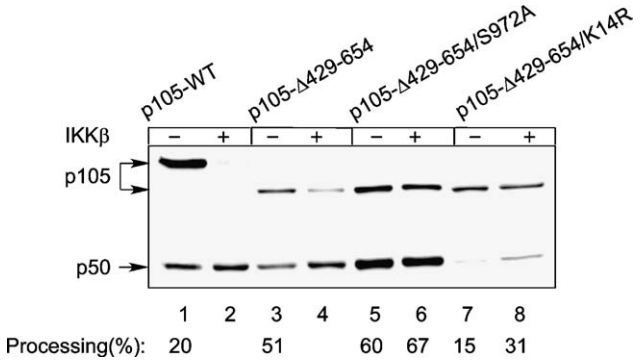

Fig. 1. p105 that lacks amino acids 429–654 is processed to p50 in high efficiency under basal conditions. Cells were transiently transfected with cDNAs coding for the following FLAG-tagged p105 proteins: WT (lanes 1 and 2), Δ429–654 (lanes 3 and 4), Δ429–654/S972A (lanes 5 and 6), or Δ429–654/K14R (lanes 7 and 8). Where indicated, a cDNA coding for constitutively active IKKβ was cotransfected. Cells were harvested and extracts were prepared as described under Materials and methods. Aliquots of extracts containing equal amounts of protein were resolved via SDS–PAGE (10%) and were blotted onto nitrocellulose paper, and proteins were visualized by using anti-FLAG antibody and ECL as described under Materials and methods.

Next, we attempted to identify more precisely the PID. As can be seen in Fig. 2A, deletion of amino acids 429–544 is sufficient to remove inhibition of processing (lane 2). In contrast, p105 lacking amino acid residues 429–474 is processed in the same low efficiency as the WT protein (lane 3). These findings suggest that the segment that spans residues 474–544 contains the PID (Fig. 2B). We were not able to further narrow this region and define it more precisely: p105 mutants with deletion of segments 474–524, 502–524, or 524–544 have all shown increased processing (Fig. 2C; compare lanes 2–4 to lane 1), making it highly likely that the entire segment (residues 474–544) is required for inhibiting processing.

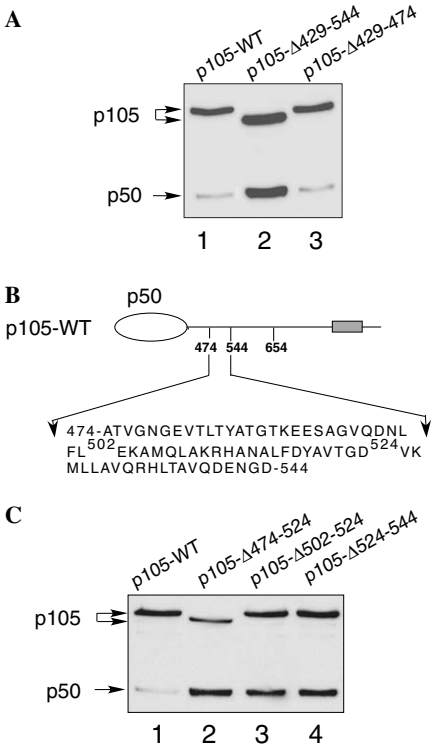

Fig. 2. p105 contains a processing inhibitory domain between residues 474–544. (A) The processing inhibitory domain spans amino acid residues 474–544. Cells were transiently transfected with cDNAs coding for the following FLAG-tagged p105 proteins: WT (lane 1), Δ429–544 (lane 2), or Δ429–474 (lane 3). Cells were harvested and proteins were visualized as described in the legend to Fig. 1. (B) Schematic representation of the p105 PID. (C) Deletion analysis of the p105 PID. Cells were transiently transfected with cDNAs coding for the following FLAG-tagged p105 proteins: WT (lane 1), Δ474–524 (lane 2), Δ502–524 (lane 3), or Δ524–544 (lane 4) (B). Cells were harvested and proteins visualized as described in the legend to Fig. 1.

Increased basal processing cannot be attributed to nuclear translocation of p105

A functional PID of ~100 residues was described in the C-terminal domain of p100 [21]. PID inhibits constitutive processing of p100, and the molecule is processed almost exclusively following stimulation. Liao and Sun [27] demonstrated that constitutive processing of p100 is enhanced by its nuclear translocation, and that the PID contributes to inhibition of processing by retention of p100 in the cytoplasm. We examined whether the mechanism of action of p105 PID also involves increased nuclear translocation of p105. As can be seen in Fig. 3A, p105-WT is found exclusively in the cytosol. As for the three p105 PID mutants that demonstrate enhanced processing (Fig. 2C), only two were translocated to the nucleus, p105-Δ429–654 and p105-Δ524–544 (the latter was translocated only partially; Fig. 3A, lanes 7 and 8), and the translocation is probably due to deletion within the ankyrin repeat domain (Fig. 3B), that when interrupted leads to retention of p105 in the cytosol (see, for example [24,28]). Notably,

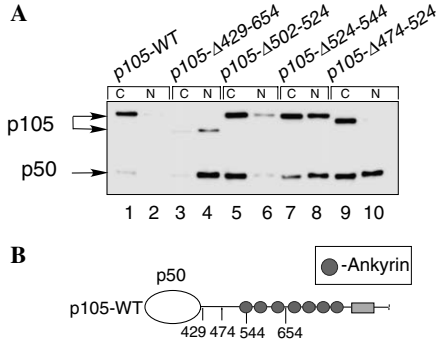

Fig. 3. The enhanced basal processing is not due to preferred sub-cellular localization. (A) HEK293 cells were transiently transfected with cDNAs coding for the following FLAG-tagged p105 proteins: WT (lanes 1 and 2), Δ429–654 (lanes 3 and 4), Δ502–524 (lanes 5 and 6), Δ524–544 (lanes 7 and 8), or Δ474–524 (lanes 9 and 10). Cells were harvested and nuclear and cytosolic fractions were prepared as described under Materials and methods. Aliquots of cytosolic and nuclear extracts representing an equal number of cells were resolved via SDS–PAGE, blotted onto nitrocellulose paper, and proteins were visualized as described in the legend to Fig. 1. C denotes cytosolic fraction; N denotes nuclear fraction. (B) Schematic representation of p105 where the relative location of the ankyrin repeats is indicated.

p105-Δ474–524, the most efficiently processed species (Fig. 2C, lane 2), is localized solely to the cytoplasm (Fig. 3, lane 9). However, since it is processed so efficiently, a large proportion of the p50 subunit that is generated from it is translocated to the nucleus. This is probably due to lack of sufficient amount of docking p105 precursor in the cytosol [24]. Thus, there is no correlation between the levels of basal processing and efficiency of nuclear translocation, suggesting that the mechanism of basal processing inhibition is not related to sub-cellular localization.

p105 fate is determined by two different ubiquitin-mediated mechanisms: multiple lysine residues are required for signal-induced degradation, while any single native lysine residue is sufficient to mediate processing

p105, p100, and IκBα share similar IKK phosphorylation and β-TrCP binding sites. However, we have shown that p105 degradation, unlike IκBα degradation [29] and p100 [22] processing, requires multiple lysine residues [17]. IκBα is ubiquitinated on lysine residues 21 and 22. K22 resides 10 residues upstream to the first phosphoacceptor site, serine 32 [29]. Similarly, p100 is ubiquitinated on lysine 855, that also resides 10 residues upstream to the first phosphoacceptor site, serine 865 [22]. In contrast, in p105 the closest lysine residue is 31 amino acids upstream to the phosphorylation site. This residue does not serve as a single polyubiquitin chain anchor that targets the protein for degradation: we had to substitute at least 19 lysines in the C-terminal domain of p105 in order to be able to observe any significant effect on signal-induced degradation ([17] and Fig. 1 in Supplementary material). To further demonstrate that p105 lacks any specific lysine residue that serves as a polyubiquitin chain anchor targeting the protein for degradation, we generated two constructs: in one we deleted a fragment containing lysine residues 2–17 in the C-terminal domain (Fig. 1B in Supplementary material), while in the other we substituted residues 18–30 in this domain (Fig. 1C in Supplementary material; for the numbers of these residues, see also Ref. [17]). Both molecules were efficiently degraded following stimulation ([17] and Fig. 1 in Supplementary material), though they harbor completely different sets of lysine residues except for lysine 1 (lysine 425). This residue does not play a role as a single ubiquitination anchor either (data not shown). Taken together, these findings support the notion that β-TrCP conjugates ubiquitin to multiple lysine residues in order to induce degradation of p105.

To further investigate the mechanism of β-TrCP-induced degradation of p105, it was important to study whether the E3 uses a unique "ruler" mechanism, and can target a single lysine residue located in a specific distance from the ligase binding site, as is the case with IκBα and p100, using this chain as an efficient degradation signal. If such a lysine does not exist naturally, as is the case in p105, the ligase uses, less efficiently, multiple other lysines. To resolve this problem, it was necessary to insert a lysine residue in the appropriate distance upstream to the phosphorylation and β-TrCP site. In that case, utilization of an exact "ruler" mechanism by the ligase would result in induction of efficient processing of p105, similar to the case of p100, or complete degradation of the molecule, similar to the case of IκBα. Towards this end, we used p105-Δ429–654/K14R in which all the lysine residues downstream to the GRR were removed (Fig. 1F in Supplementary material). This molecule lost both the enhanced basal processing and its ability to undergo signal-induced degradation ([17] and Fig. 1, lanes 7 and 8). To this molecule, we inserted several single lysine residues. The first was inserted in position 918 (p105-Δ429–654/K14R, 918K; Fig. 4A), 10 amino acids upstream to the

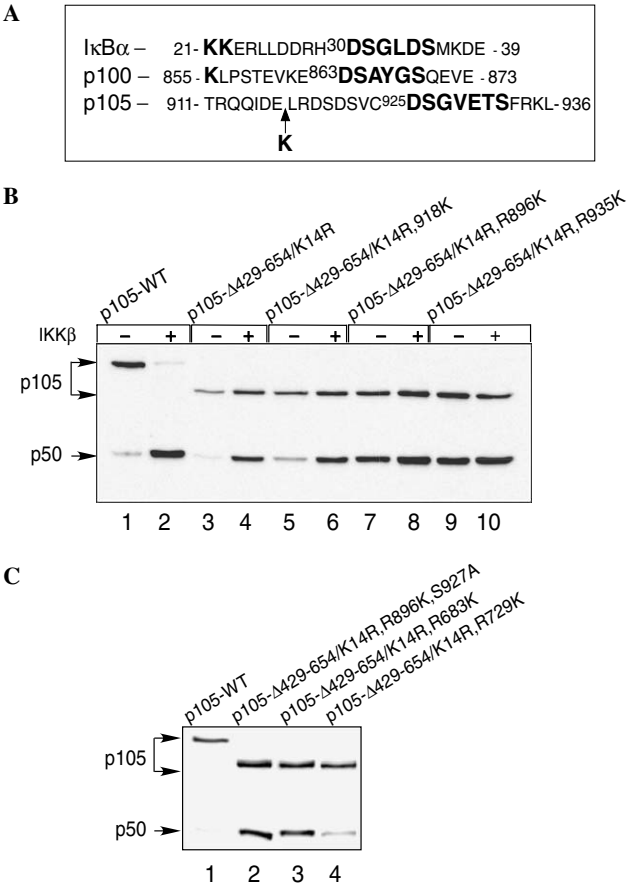

Fig. 4. Any single lysine residue, preferably in a native site, is sufficient to promote processing of p105. (A) Phosphorylation and ubiquitination sites of IκBα and p100. p105 has a similar phosphorylation but not ubiquitination site. A lysine residue was inserted artificially between residues 917 and 918. (B) Cells were transiently transfected with cDNAs coding for the following FLAG-tagged p105 proteins: WT (lanes 1 and 2), Δ429–654/K14R (lanes 3 and 4), Δ429–654/K14R, 918K (lanes 5 and 6), Δ429–654/K14R, R896K (lanes 7 and 8), or Δ429–654/K14R, R935K (lanes 9 and 10). Where indicated, a cDNA coding for constitutively active IKKβ was cotransfected. (C) Cells were transiently transfected with cDNAs coding for the following FLAG-tagged p105 proteins: WT (lane 1), Δ429–654/K14R, R896K, S927A (lane 2), Δ429–654/K14R, R683K (lane 3), or Δ429–654/K14R, R729K (lane 4). Cells were harvested and proteins visualized as described in the legend to Fig. 1.

IKK phosphorylation and β-TrCP binding site, in a position homologous to the native lysine residue that serves as a ubiquitin chain anchor in both IκBα and p100. In two other mutants we inserted single lysine residues in their native sites, one in position 896, upstream to the IKK and TrCP site (p105-Δ429–654/K14R, R896K) and one in position 935, downstream to this site (p105-Δ429–654, K14R, R935K; Fig. 4A). As can be seen in Fig. 4B, insertion of a lysine residue at the presumed optimal site for β-TrCP ubiquitination restored only partially basal processing, but had no effect on signal-induced degradation (Fig. 4B, lanes 5 and 6). Even insertion of a stretch of three lysine residues in positions 10–12 upstream to the IKK and TrCP site could not restore signal-induced degradation (data not shown). As for processing, reinstatement of either one of the two native lysine residues (896 or 935) restored the high efficiency of basal processing to the PID-deleted mutant, but as expected, had no effect on signal-induced degradation that requires, as noted, multiple lysines in this region (Fig. 4B, lanes 7–10). It should be noted that we could not detect any effect of the distance of the inserted lysine residues from the TrCP site on their ability to restore processing. As can be seen in Fig. 4C, reinstatement of a native lysine residue in position 683, which resides 250 residues upstream to the IKK and TrCP site, restored processing completely. In contrast, substitution of an arginine with lysine in position 729 (non-native lysine) had a small effect on processing, similar to the effect of K918, the single lysine that was artificially inserted much closer to the IKK and TrCP site (Fig. 4B, lane 5). Thus, it appears that unlike degradation, processing requires only a single residue, preferably in a native position, but unrelated to its distance, upstream or downstream, close or far, from the IKK and TrCP site. That, provided that p105 is not under the inhibition of the PID. Interestingly, the efficiency of processing does not correlate with the efficiency of general conjugation of the relevant p105 protein. As can be seen in Fig. 5, p105s that are processed in high efficiency (p105-Δ429–654, and p105-Δ429–654/K14R, R683K; lanes 4 and 6) are conjugated significantly less efficiently compared with the WT protein (lane 2). The WT protein, despite having a complete cohort of lysines which are efficiently ubiquitinated, is nevertheless poorly processed because of the presence of the PID. This result suggests that PID may exert its effect at the level of the proteasome and not at the level of the E3 ligase.

Since we could not detect a correlation between the efficiency of processing in cells and conjugation in a cell free system, it was important to study whether the enhanced basal processing we observed in the PID-deleted p105 mutant or the single lysine-containing p105s is dependent on an active ubiquitin system. By using different dominant negative (DN) E2s, we were able to show that the ubiquitin system is indeed involved in the high levels of basal processing observed in the different p105 PID-deleted proteins. As can be seen in Fig. 6, UbcH5a inhibited the processing

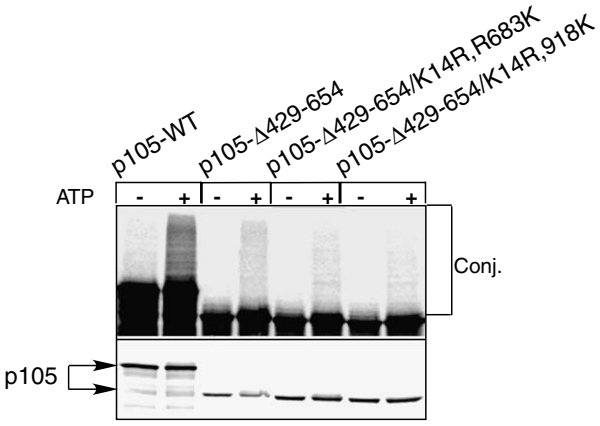

Fig. 5. Enhanced basal processing, being dependent on a single lysine residue, is not necessarily dependent on high level of ubiquitination. In vitro-translated and [^{35}S]methionine-labeled WT and p105 mutants Δ429–654, Δ429–654/K14R, R683K, and Δ429–654/K14R, 918K were subjected to conjugation in a cell free reconstituted system as described under Materials and methods. Proteins were resolved by SDS–PAGE and were visualized by exposure to PhosphorImager. The lower panel shows the different p105 proteins. Conj. denotes conjugates.

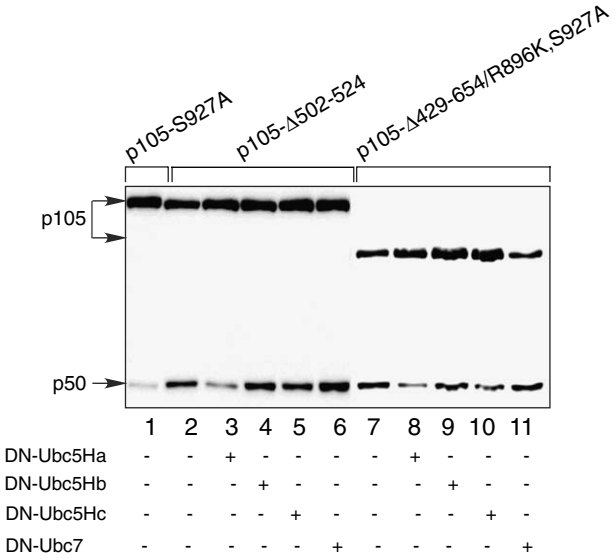

Fig. 6. Enhanced basal processing in PID-deleted p105 mutants is inhibited by dominant negative UbcH5a. Cells were transiently transfected with cDNAs coding for the following FLAG-tagged p105 proteins: S927A (lane 1), Δ502–524 (lanes 2–6), and Δ429–654/K14R, R896K, S927A (lanes 7–11). Where indicated, cDNAs coding for DN-UbcH5a, DN-UbcH5b, DN-UbcH5c, or DN-Ubc7 were cotransfected. Cells were harvested and proteins were visualized as described in the legend to Fig. 1.

of both p105-Δ502–524 (lane 3) and p105-Δ429–654/K14R, R896K, S927A (lane 8). Other E2s were inactive.

Discussion

Most studies in the area of NF-κB activation have focused on signal-induced activation of the IKK complex and phosphorylation and degradation of the inhibitor IκBα. However, an important regulatory step must involve

also in processing of the p105 precursor to the active p50 subunit, a process which is poorly understood mechanistically. Processing requires a GRR in the middle of the molecule that serves a "processing stop signal" [6,7,18]. p50 is generated in a small amount under basal conditions [17], and signalling leads only to a slight, ~2- to 3-fold, stimulation of p50 generation [15]. Following signalling, the ubiquitin ligase β-TrCP is recruited and binds to the phosphorylated serine residues in the IKKβ phosphorylation domain [11,12], which following ubiquitination, is completely degraded. We have shown that IKK has a dual effect on p105 fate, degradation which is dependent on β-TrCP, and processing which is independent of the activity of the ligase ([17] for a model depicting the different fates of p105, see Fig. 2 in Supplementary material and below). Importantly, p100, the homologous molecule of p105 that serves as a precursor to the p52 subunit, behaves completely differently. In contrast to p105 that is mostly degraded following signalling, p100 is processed in high efficiency. Interestingly, degradation of p105 and processing of p100 are mediated by the same ligase—β-TrCP. We have shown that in p100, the ligase targets a single specific lysine residue [22]. A lysine residue located in an exactly similar position in IκBα is involved in complete degradation of the molecule following signalling [29]. Yet, such a lysine does not exist in p105 and post-signalling degradation of p105 requires ubiquitin modification of multiple lysines [17].

In this study, we have shown that, similar to p100 [21], p105 also contains a PID, that may regulate formation of p50 (Figs. 1 and 2). The p105 PID is located upstream to its p100 functional homologue, between the GRR and the ankyrin repeat domains: in p100 it is located in the Death Domain (DD), downstream to the ankyrin repeat [21,27]. The p105 PID does not harbor any structural similarity to the p100 domain and it appears to act via a different mechanism: it does not affect nuclear shuttling of the molecule ([17] and Fig. 3). In an attempt to generate a highly "processable" p105, we inserted a lysine residue in a location identical to the β-TrCP ubiquitination site in p100 and IκBα. To inhibit degradation and to study only processing, examination of the role of this and additional lysine residues was carried out in mutant p105 in which we removed all lysine residues in the C-terminal domain that can be ubiquitinated and lead to degradation of the molecule. Obviously, in all these experiments we used a PID-deleted p105. As can be seen in Fig. 4, insertion of a lysine residue in position 918, 10 residues upstream to the IKK and TrCP serine target, increased processing slightly. Needless to say that this mutant was not degraded following signalling. However, insertion of a single lysine residue into native sites that reside either in proximity or far from the IKK and TrCP site, resulted in a p105 species that is processed with high efficiency. Thus, similar to p100, it appears that a single lysine residue directs processing. However, unlike in p100, its location in p105 is less restricted, though a native residue appears to be more effective than an artificially inserted one. The involvement of a single lysine residue in processing does not necessitate a strong ubiquitination signal, especially not in a molecule in which multiple residues must be modified in order to target it for destruction (Fig. 5). While involvement of lysine residues in the enhanced processing strongly suggests that the process is mediated by the ubiquitin system, we have gone to show it more directly (Fig. 6). It should be noted that UbcH5a was also the only E2 that inhibited signal-induced, yet TrCP-independent, processing of p105 [17]. Thus, it is possible that IKK signalling leads to functional removal of PID in a TrCP-independent manner and is mediated by an as yet to be identified E3 (Fig. 2 in Supplementary material). While the E3 that mediates processing under basal conditions is probably distinct from the one that mediates processing following signalling, the two processes can be possibly mediated by the same ligase: under basal conditions it acts inefficiently, but can act more effectively following an appropriate alteration in the PID.

Acknowledgments

S.C. is supported by a Bat-Sheba De Rotschild Fund for the Advancement of Science in Israel. Research in the laboratory of A.C. is supported by grants from the Prostate Cancer Foundation Israel—Centers of Excellence Program, Israel Science Foundation (ISF), the European Union (EU) Networks of Excellence (NeOEs) NeuroNE and Rubicon, a Professorship from the Israel Cancer Research Fund (ICRF) USA, and a grant from the Foundation for Promotion of Research in the Technion. Infrastructural equipment supporting this study has been purchased with the support of the Wolfson Charitable Fund Center of Excellence for studies on Turnover of Cellular Proteins and its Implications to Human Diseases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.04.036.

References

[1] M. Karin, F.R. Greten, NF-κB: linking inflammation and immunity to cancer development and progression, Nat. Rev. Immunol. 5 (2005) 749–759.
[2] C.M. Fan, T. Maniatis, Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway, Nature 354 (1991) 395–398.
[3] V.J. Palombella, O.J. Rando, A.L. Goldberg, T. Maniatis, The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB, Cell 78 (1994) 773–785.
[4] A. Orian, S. Whiteside, A. Israel, I. Stancovski, A.L. Schwartz, A. Ciechanover, Ubiquitin-mediated processing of NF-κB transcriptional activator precursor p105. Reconstitution of a cell-free system and identification of the ubiquitin-carrier protein, E2, and a novel

ubiquitin-protein ligase, E3, involved in conjugation, J. Biol. Chem. 270 (1995) 21707–21714.
[5] M. Karin, Y. Ben-Neriah, Phosphorylation meets ubiquitination: the control of NF-κB activity, Annu. Rev. Immunol. 18 (2000) 621–663.
[6] L. Lin, S. Ghosh, A glycine-rich region in NF-κB p105 functions as a processing signal for the generation of the p50 subunit, Mol. Cell. Biol. 16 (1996) 2248–2254.
[7] A. Orian, A.L. Schwartz, A. Israel, S. Whiteside, C. Kahana, A. Ciechanover, Structural motifs involved in ubiquitin-mediated processing of the NF-κB precursor p105: roles of the glycine-rich region and a downstream ubiquitination domain, Mol. Cell. Biol. 19 (1999) 3664–3673.
[8] C. Lee, M.P. Schwartz, S. Prakash, M. Iwakura, A. Matouschek, ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal, Mol. Cell 7 (2001) 627–637.
[9] L. Lin, G.N. DeMartino, W.C. Greene, Cotranslational biogenesis of NF-κB p50 by the 26S proteasome, Cell 92 (1998) 819–828.
[10] L. Lin, M. Kobayashi, Stability of the Rel homology domain is critical for generation of NF-κB p50 subunit, J. Biol. Chem. 278 (2003) 31479–31485.
[11] A. Orian, H. Gonen, B. Bercovich, I. Fajerman, E. Eytan, A. Israel, F. Mercurio, K. Iwai, A.L. Schwartz, A. Ciechanover, SCF(β-TrCP) ubiquitin ligase-mediated processing of NF-κB p105 requires phosphorylation of its C-terminus by IκB kinase, EMBO J. 19 (2000) 2580–2591.
[12] V. Heissmeyer, D. Krappmann, E.N. Hatada, C. Scheidereit, Shared pathways of IκB kinase-induced SCF(βTrCP)-mediated ubiquitination and degradation for the NF-κB precursor p105 and IκBα, Mol. Cell. Biol. 21 (2001) 1024–1035.
[13] K. Fujimoto, H. Yasuda, Y. Sato, K. Yamamoto, A role for phosphorylation in the proteolytic processing of the human NF-κB1 precursor, Gene 165 (1995) 183–189.
[14] K.H. Mellits, R.T. Hay, S. Goodbourn, Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB, Nucleic Acids Res. 21 (1993) 5059–5066.
[15] M.L. MacKichan, F. Logeat, A. Israel, Phosphorylation of p105 PEST sequence via a redox-insensitive pathway up-regulates processing of p50 NF-κB, J. Biol. Chem. 271 (1996) 6084–6091.
[16] A. Salmeron, J. Janzen, Y. Soneji, N. Bump, J. Kamens, H. Allen, S.C. Ley, Direct phosphorylation of NF-κB1 p105 by the IκB kinase complex on serine 927 is essential for signal-induced p105 proteolysis, J. Biol. Chem. 276 (2001) 22215–22222.
[17] S. Cohen, H. Achbert-Weiner, A. Ciechanover, Dual effects of IκB kinase β-mediated phosphorylation on p105 Fate: SCF(β-TrCP)-dependent degradation and SCF(β-TrCP)-independent processing, Mol. Cell. Biol. 24 (2004) 475–486.
[18] M. Heusch, L. Lin, R. Geleziunas, W.C. Greene, The generation of *nfkb2* p52: mechanism and efficiency, Oncogene 18 (1999) 6201–6208.
[19] U. Senftleben, Y. Cao, G. Xiao, F.R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S.-C. Sun, M. Karin, Activation by IKKα of a second, evolutionary conserved, NF-κB signaling pathway, Science 293 (2001) 1495–1499.
[20] A. Fong, S.C. Sun, Genetic evidence for the essential role of β-transducin repeat-containing protein in the inducible processing of NF-κB2/p100, J. Biol. Chem. 277 (2002) 22111–22114.
[21] G. Xiao, E.W. Harhaj, S.C. Sun, NF-κB-inducing kinase regulates the processing of NF-κB2 p100, Mol. Cell 7 (2001) 401–409.
[22] R.E. Amir, H. Haecker, M. Karin, A. Ciechanover, Mechanism of processing of the NF-κB2 p100 precursor: identification of the specific polyubiquitin chain-anchoring lysine residue and analysis of the role of NEDD8-modification on the SCF(β-TrCP) ubiquitin ligase, Oncogene 23 (2004) 2540–2547.
[23] H. Gonen, B. Bercovich, A. Orian, A. Carrano, C. Takizawa, K. Yamanaka, M. Pagano, K. Iwai, A. Ciechanover, Identification of the ubiquitin carrier proteins, E2s, involved in signal-induced conjugation and subsequent degradation of IκBα, J. Biol. Chem. 274 (1999) 14823–14830.
[24] S. Cohen, A. Orian, A. Ciechanover, Processing of p105 is inhibited by docking of p50 active subunits to the ankyrin repeat domain, and inhibition is alleviated by signaling via the carboxyl-terminal phosphorylation/ubiquitin-ligase binding domain, J. Biol. Chem. 276 (2001) 26769–26776.
[25] I. Alkalay, A. Yaron, A. Hatzubai, S. Jung, A. Avraham, O. Gerlitz, I. Pashut-Lavon, Y. Ben-Neriah, In vivo stimulation of IκB phosphorylation is not sufficient to activate NF-κB, Mol. Cell. Biol. 15 (1995) 1294–1301.
[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[27] G. Liao, S.C. Sun, Regulation of NF-κB2/p100 processing by its nuclear shuttling, Oncogene 22 (2003) 4868–4874.
[28] N.R. Rice, M.K. Ernst, In vivo control of NF-κB activation by IκBα, EMBO J. 12 (1993) 4685–4695.
[29] D.C. Scherer, J.A. Brockman, Z. Chen, T. Maniatis, D.W. Ballard, Signal-induced degradation of IκBα requires site-specific ubiquitination, Proc. Natl. Acad. Sci. USA 92 (1995) 11259–11263.