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*

Shai Cohen‡§¶, Amir Orian‡¶, and Aaron Ciechanover‡**

From the ‡Department of Biochemistry and the Rappaport Family Institute for Research in the Medical Sciences, the Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096 and the §Department of Internal Medicine A, Lady Davis/“Carmel” Medical Center, Haifa 34362, Israel

This paper is available online at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

1734 solely to indicate this fact.

The NF-κB1 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 recent review, see Ref. 1). Certain active subunits of NF-κB 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 (2–4). p50 is derived from the N-terminal domain of the molecule, whereas the C-terminal, ankyrin repeat-containing domain, is degraded (2). The processed subunits typically heterodimerize with members of the Rel family of regulators such as p65 (RelA), RelB, or c-Rel to generate the active heterodimeric transcription factor. According to the current model, binding of a member of the IκB family of inhibitors generates an inactive heterotrimeric complex that is sequestered in the cytosol. Following stimulation, specific IκB kinases (IKKs) are activated and phosphorylate IκB on specific Ser residues. Phosphorylation leads to recruitment of the SCF

* This work was supported in part by grants from the Israel Science Foundation founded by the Israeli Academy of Sciences and Humanities Centers of Excellence Program, the German-Israel Foundation for Research and Development, the Israel Cancer Society, the German-Israeli Project Cooperation, the United States-Israel Binational Science Foundation, a Training and Mobility of Researchers grant from the European Community, the Foundation for Promotion of Research in the Technion, and a research grant administered by the vice president of the Technion for Research (to A. C.). Purchase of the ABI 310 autosequencer was supported partially by a grant from the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ These authors contributed equally to the work.

** To whom all editorial correspondence should be addressed: Dept. of Biochemistry, Faculty of Medicine, Technion-Israel Institute of Technology, P. O. Box 9649, Haifa 31096, Israel. Tel.: 972-4-829-5356; Fax: 972-4-851-3922; E-mail: c_tzachy@netvision.net.il.

1 The abbreviations used are: NF-κB, nuclear factor κB; IKK, IκB kinase; E1, ubiquitin-activating enzyme; E2, ubiquitin-carrier protein or ubiquitin-conjugating enzyme (UBC); E3, ubiquitin-protein ligase; HIV, human immunodeficiency virus; GRR, Gly-rich region; NIK, NF-κB-inducing kinase; PAGE, polyacrylamide gel electrophoresis; ATPγS, adenosine 5′-O-(3-thiotriphosphate); ECL, enhanced chemiluminescence; WT, wild type; DHFR, dihydrofolate reductase.
proteasome. Several classes of E3s have been described, among them are the SCF complexes that recognize mostly phosphorylated proteins. The currently known tetrameric complexes are composed of Skp1, Cul1/Cdc53, and Rbx1/Roc1/Hrt1, which are common to all SCFs, and a variable F-box protein that serves as the substrate-recognizing subunit (for recent reviews on the ubiquitin system and the proteasome, see Refs. 6 and 7, respectively; for a recent review on SCF complexes, see Ref. 8). The F-box protein is involved in recognition of the phosphorylated substrate, \( \beta\)-TrCP, for example, recognizes the common motif DS\((P)\text{Gd}DS(P)\) shared by IkBo, IkB\(\beta\), \(\beta\)-catenin, and HIV-Vpu (5).

The mechanisms involved in limited processing of the p105 precursor protein have been partially elucidated. Lin and Ghosh (9) have demonstrated that the GRR that spans residues 376–404 in human p105 is essential for processing and serves as a processing stop signal for the 26 S proteasome (10). Several single residues that reside upstream to the GRR and are involved in proper folding of p50 are also essential for processing, most probably via inhibiting unfolding and entry into the proteasome (11). Processing requires also an additional adjacent downstream domain that contains Lys residues 441 and 442, which are important for ubiquitination, and an acidic region (residues 446–454) that may function as an E3 recognition motif (10). These findings suggest that processing requires at least two motifs, a physical stop signal(s) and a ubiquitination/E3 recognition site. Fan and Maniatis (2) have shown that a truncated form of p105, p60, can be processed to p50. Lin and colleagues (12) have shown that p105 can be processed cotranslationally, and synthesis of the complete molecule is not required for generation of p50. Taken together, these studies (2, 9–12) suggest that all of the motifs that are required for processing in the resting cell are contained within the first \( ~550 \) amino acid residues. Other studies have suggested a role for phosphorylation of the C-terminal domain of p105 in regulated, signal-induced processing/degradation of the molecule (13, 14). Heissmeyer and colleagues (15) have shown that IKK-mediated phosphorylation of Ser residues that reside in a sequence that spans amino acid residues 922–933 leads to rapid degradation of p105. We have recently shown that this IKK-mediated phosphorylation leads to recruitment of the SCF\(^{\beta\text{-TrCP}}\) ubiquitin ligase. Consequently, the molecule is ubiquitinated and rapidly processed. A certain proportion of the molecules are completely degraded (16). A later study by Heissmeyer and colleagues has corroborated the finding that \( \beta\)-TrCP is indeed the E3 (17). The motif DS\(^{\beta\text{-TrCP}}\)CDS\(^{\beta\text{-TrCP}}\) (17) is similar to the targeting motifs in IkBo, \(\beta\), and \(\epsilon\), and in \(\beta\)-catenin and HIV-Vpu (5).

Interestingly, processing of p100, the gene product of \(nfsB2\), to yield the p52 subunit is mediated by a similar mechanism. Like p105, part of it may occur cotranslationally and requires the GRR (18). A recent study has demonstrated that phosphorylation of Ser residues 867 and 870 which is mediated by NIK (NF-\(\kappa\)B-inducing kinase) is required for processing (19). Although it is not clear whether NIK phosphorylates p100 directly, it is interesting to note that the phosphorylation site is similar to that of p105, IkBo, IkB\(\beta\), \(\beta\)-catenin, and HIV-Vpu, where the two Serines are interspaced by three residues. A novel motif, processing inhibitory domain (PID), which resides between the ankyrin repeat domain and the phosphorylation site, negatively regulates processing (19).

It should be noted that the C-terminal segment of p105 which resides between the GRR and the IKK-\(\beta\)-TrCP motif contains seven ankyrin repeats. Active NF-\(\kappa\)B subunits such as p50 and p65 dock to this region, inhibit processing of the precursor molecule, and are sequestered in an inactive storage form in the cytoplasm. Thus, the ankyrin repeat domain serves as an inhibitor of NF-\(\kappa\)B activity (20–22). Harbaj and colleagues (23) have shown that processing of newly synthesized p105 molecules is more efficient compared with that of the accumulated form that is already associated with p50. Accelerated, signal-induced processing/degradation leads to release of the docked active factors (15) and probably to generation of additional active subunits from the processed precursor (16). However, the mechanisms that underlie p50/p65-mediated inhibition of p105 processing and its subsequent signal-induced alleviation have remained obscure.

**EXPERIMENTAL PROCEDURES**

**Materials**

Materials for SDS-PAGE and Bradford reagent were from Bio-Rad. L-[\(\beta\)]-35S-methionine (\(>1,000 \) Ci/mmol, \( \sim 50 \) mCi/ml) for in vitro translation and prestained molecular weight markers were obtained from Amersham Pharmacia Biotech. Tissue culture sera and media were from Biological Industries, Bet hemeke, Israel, or from Sigma. Rabbit anti-NF-\(\kappa\)B p50 antibody that recognizes both p105 and p50, was from Santa Cruz, and peroxidase-conjugated goat anti-rabbit antibody was from Jackson Immunoresearch Laboratories. Ubiquitin, dithiothreitol, ATP, and GST-phosphorylated peptide were purchased from Promega. \(\beta\)-galactosidase, glutathione, glutathione immobilized to agarose beads, isopropyl-

\[\beta\]-D-thiogalactopyranoside (IPTG), and Tris buffer were from Sigma. Hexokinase and Fugene\(^\text{TM}6\) transfection reagent were from Roche Molecular Biochemicals. HEPES buffer and protease inhibitors mixture were from Calbiochem. Reagents for enhanced chemiluminescence (ECL) were from Pierce. A wheat germ extract-based coupled transcription/translation kit was from Promega. Restriction and modifying enzymes were from New England Biolabs. Oligonucleotides were synthesized by Biotechnology General, Rehovot, Israel. All other reagents were of high analytical grade.

**Methods**

**Cell Lines—COS-7 and HeLa cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (penicillin/streptomycin).**

**Plasmids and Construction of Mutants—The human WT and \(\Delta918–934\) p105 cDNAs used for in vitro translation (p77p105) and for transient transfection (in pCI-neo) were described previously (4, 10, 16).**

**cDNAs for in vitro translation of proteins that code for the different species of truncated p105s which carry an increasing number of ankyrin repeats were generated by linearization of the cDNA that codes for p105-WT with the indicated restriction enzymes. The linear cDNAs were then incubated in the coupled transcription-translation mixture (Fig. 1; the appropriate restriction sites and enzymes are marked). To be able to linearize also the cDNA that codes for intact p105-WT, an XhoI site was inserted downstream of the stop codon of the WT molecule. For cell expression, stop codons were introduced in the place of the appropriate restriction sites by site-directed mutagenesis using the QuikChange\(^\text{TM}4\) kit (Stratagene) (see Fig. 1; the number of residues in each construct is shown in brackets). These constructs are p105-Th111 (1–545), p105-AzoII (1–677), p105-ScaI (1–715), and p105-WT. These proteins contain 0, 4, 5, and 7 ankyrin repeats, respectively. p105-WT contains also the C-terminal domain that is phosphorylated by IKK and binds \(\beta\)-TrCP. p50-XhoI (1–502) for cell expression cloned into the Rec-MV (cytomegalovirus) vector was obtained from Dr. Alain Israël, Pasteur Institute, Paris, France. For convenience, we designated proteins used for both in vitro translation and in vivo expression in cells according to the restriction site used. p105-Spe (1–340) for bacterial expression was generated by in-frame cloning of the cDNA fragment that codes for the first 340 amino acids of p105 into the pGEX3 vector (Amersham Pharmacia Biotech). A stop codon was introduced instead of the Spe site. p105-DHFR was generated by in-frame cloning of the full-length coding sequence of DHFR into the Bam1-IdeI site (653–794) after removal of the fragment that codes for residues 653–794. This resulted in removal of a sequence that contains ankyrin repeats 4–7. p105-3918–834 for both in vitro translation and cell expression, constitutively active L6 kinase \(\beta\) (IKKb-SS–EE), and AF-box human \(\beta\)-TrCP (\(\Delta\beta\)-\(\beta\)-TrCP) for cell expression were as described (16). Sequences of all constructs were confirmed using the Applied Biosystems 310 autossequencer.

**Expression of Recombinant p50–Spe in Bacteria—The p50–Spe protein was induced in BL21 Escherichia coli by isopropyl-\(\beta\)-D-thiogalactopyranoside. After adsorption of the cell extract onto immobilized glutathione, the purified fusion protein was eluted by free glutathione according to the manufacturer’s instructions.**
ubiquitin-mediated Processing/Degradation of NF-kB1 p105

Preparation of Cell and Nuclear and Cytoplasmic Extracts—HeLa cell extract for monitoring in vitro conjugation and processing was prepared by hypotonic lysis as described previously (10, 16). Nuclear and cytoplasmic extracts were prepared by hypotonic lysis and Nonidet P-40 extraction (cytosol) followed by treatment with a high glycerol/high salt nuclear extraction buffer as described (24).

In Vitro Processing of p105—All p105 proteins were translated in vitro using the wheat germ extract-based coupled transcription-translation kit in the presence of L-[35S]methionine according to the manufacturer's instructions. Processing of labeled p105 to p50 was monitored in HeLa cell extract as described (4, 10, 16). When p50-Spe was added, it was preincubated for 5 min at 30 °C along with the labeled substrate prior to the addition of the remaining components of the processing reaction. After incubation, reaction mixtures were resolved via SDS-PAGE (10%). Gels were dried and analyzed by PhosphorImager (Fuji, Japan).

In Vitro Conjugation Assays—Adducts of ubiquitin with p105 or the p105 derivatives were generated in crude HeLa cell extract in an assay similar to that described for processing but with the following modifications as described previously (10, 16): 0.5 μg of ubiquitin aldehyde, a specific inhibitor of certain isopeptidases, was added to the reaction mixture, and ATPγS was used instead of ATP and the ATP-regenerating system.

Transient Transfections and Processing of p105 in Cells—COS-7 cells were transiently transfected with p50 and the p105 derivatives were generated in crude HeLa cell extract in an assay similar to that described for processing but with the following modifications as described previously (10, 16): 0.5 μg of ubiquitin aldehyde, a specific inhibitor of certain isopeptidases, was added to the reaction mixture, and ATPγS was used instead of ATP and the ATP-regenerating system.

RESULTS

Processing of C-terminally Deleted p105s Is Inhibited with Increasing Number of Ankyrin Repeats—It has been shown that active NF-κB subunits dock to the C-terminal domain of p105 and probably inhibit processing (15, 20–23). Therefore, we wanted to test the hypothesis that the efficiency of processing is inversely correlated with the number of ankyrin repeats to which the active subunits dock. Further, we wanted to test whether alleviation of inhibition and supply of active subunits, derived from both the docked proteins and processed p105, requires signal-induced targeting of p105 via the C-terminal domain of the molecule. To test these hypotheses, we constructed a series of p105 deletion mutants that contain an increasing number of ankyrin repeats, yet lack the C-terminal domain (Fig. 1). We tested these constructs for both processing and conjugation in a cell-free reconstituted system. As can be clearly seen in Fig. 2, processing of the different C-terminally deleted p105 precursors is inhibited in proteins that contain more than four repeats. Thus, p105-Tdh111 that does not contain any repeat is processed efficiently (13%). Processing of p105-AatII that contains four repeats is inhibited only slightly (12%). In contrast, processing of p105-ScaI that contains five repeats is much less efficient (6%). Yet, processing of p105-WT that contains seven ankyrin repeats, but also the intact C-terminal targeting domain, proceeds in a highly efficient manner (24%). It should be noted that even under basal, nonstimulated conditions, the C-terminal domain of p105 is phosphorylated (16, 26), which leads to recruitment of β-TrCP and accelerated processing/degradation of p105 without additional stimulation (16). This may explain the high efficiency of processing of p105-WT in the HeLa extract.

Ankyrin Repeat-dependent Inhibition of p105 Processing in Vitro Is Probably Mediated by Anchored p50 Subunits, and Inhibition Is Alleviated by the Presence of the C-terminal Signaling Motif—We surmised that inhibition of processing which is observed with the increasing number of ankyrin repeats (Fig. 2) is caused by the presence of free p50 and other active NF-κB subunits in the HeLa cell extract in which the processing reactions are carried out. To corroborate this hypothesis directly, we added bacterially expressed p50 to a cell-free proteolytic system. As can be seen in Fig. 3A, increasing the concentration of the free subunit had no effect on processing of p105-Tdh111.

Fig. 1. Schematic structure of the different p105 truncated/mutated proteins. The different p105 proteins were constructed and designated as described under “Experimental Procedures.” Numbers in brackets denote the amino acid residues coded by the corresponding cell expression vectors. In these constructs, a stop codon was inserted after the C-terminal residue. Restriction enzymes denote the cleavage sites used for linearization of the corresponding cDNAs that were utilized for in vitro transcription/translation. For convenience, because the number of residues coded by the two groups of vectors is identical, we designated proteins used for both in vitro translation and cell expression according to the construction site used. The different domains of p105 and the sequence coding for DHFR are marked and annotated in the figure and described under “Experimental Procedures.”

- IKK-phosphorylation and β-TrCP recognition domain
  - Rel homology domain
  - GRR
  - Basal recognition/cotranslational domain?
  - Ankyrin repeat

-Dihydrofolate reductase

DHFR

p105-DHFR

[p105-Δ918-934]

[p105-50-Spe]

[p105-50-Xba]

[p105-Tdh111]

[p105-AatII]

[p105-ScaI]

[p105-WT]
Processing of p105-AatII was inhibited only slightly. In contrast, processing of p105-Scal was inhibited almost completely. Because the WT protein is processed efficiently in the presence of a complete cohort of ankyrin repeats (Fig. 2), we further hypothesized that inhibition is alleviated by signaling that is mediated by the C-terminal motif that leads to recruitment of the SCF β-TrCP E3 complex, ubiquitination, and processing/degradation of the precursor molecule. To test this hypothesis, we monitored processing of p105-WT and p105-D918–934 in the presence of an increasing concentration of exogenous p50. As can be seen in Fig. 3B, processing of the WT precursor was not affected, whereas that of the signaling motif-deleted protein was inhibited almost completely.

**The Ankyrin Repeat Domain and Anchored p50 Do Not Interfere with Conjugation of p105**—To dissect the mechanism(s) that underlies inhibition of processing by the ankyrin repeat domain and anchored p50 subunits, we examined the effect of exogenously added p50 on the efficiency of conjugation of the different p50 precursors. As can be seen in Fig. 4A, p105-Thh111, p105-ScalI, and p105-WT are equally conjugated. This similar conjugation efficiency is in striking contrast to the efficiency of processing; processing of p105-ScalI is significantly lower compared with that of its two other counterparts (Fig. 2). Similarly, conjugation of p105-ScalI was not affected by the addition of exogenous p50 (Fig. 4B). This is again in contrast to the inhibitory effect of added p50 on p105-ScalI processing (Fig. 3A). Not surprisingly, exogenous p50 had no effect on conjugation of p105-D918–934 (Fig. 4C). This is again in sharp contrast to its strong inhibitory effect on processing of the signaling motif-deleted protein (Fig. 3B). Taken together, these findings suggest that anchored p50 probably affects processing by interfering with proteasomal activity (see “Discussion”).

**p50 Inhibits Processing of p105 in Vivo, and Inhibition Is Dependent on the Presence of the Ankyrin Repeat-Containing Domain**—To test the effect of the ankyrin repeat domain and anchored p50 subunits on processing of p105 in vivo, we overexpressed p50-XhoI in cells that do not express the NF-κB cascade proteins. p50-XhoI cannot generate p50 cotranslationally because it is probably too short to allow folding of its processed tail and recognition by the proteasome (10, 12). This molecule contains 502 amino acid residues and can be resolved readily from the native p50 subunit (~435 residues) that is generated from p105. As can be seen in Fig. 5A, overexpressed p50 does not affect processing of p105-Thh111 (11%; compare lanes 1 and 2 with lanes 3 and 4). In contrast, exogenously expressed p50 inhibits processing of p105-WT by 2-fold (compare lanes 5 and 7). Interestingly, a large amount of p105-Thh111 is found in the nucleus (compare lanes 1 and 2 and lanes 3 and 4) because the molecule lacks the ankyrin repeat domain. Consequently, the p50 subunit that usually anchor to the repeat domain cannot dock which leads to exposure of the NLS and to its subsequent translocation to the nucleus. Similarly, and probably for the same reason, a large proportion of the expressed p50 (p50-Xho) also migrates to the nucleus (compare lanes 3 and 4). Finally, the p50 subunit that is generated from p105-Thh111 is also distributed between the cytosol and the nucleus (compare lane 1 with lane 2 and lane 3 with lane 4). In contrast, the vast majority of p50 that is generated from
binding and sequestering p50 in the cytosol, we generated p105 in which we replaced the last four ankyrin repeats with DHFR. The resulting protein contains only the first three repeats. The DHFR-coding sequence, which replaces the ankyrin 4–7 repeat domain, contains no ankyrin repeat, yet its insertion generates a p105 species with a molecular mass similar to that of the WT protein. Also, it enables testing of the effect of methotrexate-induced folding on processing, and examination of the possibility that the drug-induced folding can replace the docked p50 subunits in inhibiting p105 processing. As can be seen in Fig.

p105-WT is sequestered to the cytosol (compare lane 5 with lane 6) because it is probably bound to the precursor p105 molecule. Furthermore, even the exogenously expressed p50-Xba remains mostly in the cytosol, where it is also sequestered via its binding to p105 (compare lane 7 with lane 8).

To test further the role of the ankyrin repeat domain in
processing of p105 and for alleviation of inhibition by ankyrin repeat-anchored p50 subunits, which alleviates the inhibition caused by either endogenous or exogenously added p50. In COS cells that do not express the main Is Required to Alleviate p50-induced Inhibition of Processing/Degradation of p105. COS-7 cells were transfected with cDNAs coding for p50-WT (lanes 1–4) and p50-Xba (lanes 5–8) and human ΔF-β-TrCP (lanes 7 and 8). 48 h after transfection, cells were harvested, and generation and nuclear translocation of p50 were monitored using Western blot analysis as described in the legend to panel A. Quantified data are also presented (percent of processing: p50/p50 + p105).

Fig. 6. The C-terminal IKK phosphorylation and β-TrCP anchoring domain is required for in vivo stimulation-induced processing/degradation of p105 and for alleviation of inhibition of processing by ankyrin repeat-anchored p50 subunits. Panel A, IKK stimulates processing of p105 and subsequent translocation of the resulting p50 to the nucleus. COS-7 cells were transfected with cDNA coding for p105-WT and when indicated, with cDNA coding for constitutively active IKK. 48 h after transfection, cells were harvested, and nuclear and cytosolic fractions were isolated as described under “Experimental Procedures.” Protein aliquots derived from an equal number of cells were resolved via SDS-PAGE, blotted onto nitrocellulose paper, and proteins were visualized using anti-p50 antibody and ECL as described under “Experimental Procedures.” C denotes cytosolic fraction, and N denotes nuclear fraction. Sites of migration of p105-WT and p50 are indicated. Panel B, IKK alleviates p50-induced inhibition of processing of p105-WT but not of p105-Δ918-934 and allows nuclear translocation of the generated p50. COS-7 cells were transfected with cDNAs coding for p105-WT (lanes 1–6) or p105-Δ918-934 (lanes 7–12) and when indicated, with cDNAs coding for constitutively active IKK (lanes 5, 6, 11, and 12) and p50-Xba (lanes 3–6 and 9–12). 48 h after transfection, cells were harvested, and generation and nuclear translocation of p50 were monitored using Western blot analysis as described in the legend to panel A. Quantified data are also presented (percent of processing: p50/p50 + p105).

Fig. 7. β-TrCP is required along with IKK to alleviate p50-induced inhibition of processing/degradation of p105. COS-7 cells were transfected with cDNA coding for p105-WT (lanes 1–8) along with cDNAs coding for p50-Xba (lanes 3–8), constitutively active IKKβ-SS>EE (lanes 5–8), and human ΔF-β-TrCP (lanes 7 and 8). 48 h after transfection, cells were harvested, and generation and nuclear translocation of p50 were monitored using Western blot analysis as described in the legend to Fig. 6A.

We noted that in cells (Fig. 5, see also Figs. 6 and 7), unlike in the reconstituted cell-free system (Figs. 2 and 3), p50 is able to inhibit processing of p105-WT. It is possible that in the HeLa extract, p105-WT is phosphorylated by the endogenous IKK, which alleviates the inhibition caused by either endogenous or exogenously added p50. In COS cells that do not express the NF-kB activation cascade, p50 is inhibitory. The finding that processing of p105-Δth111 and p105-DHFR is not affected by overexpression of p50 (Fig. 5A, lanes 1–4; Fig. 5B, lanes 1–4) rules out the possibility that the expressed p50 interferes with processing by sequestering certain factors necessary for this proteolytic event, such as elements of the ubiquitin-conjugation machinery.

The C-terminal IKK Phosphorylation/β-TrCP Binding Domain Is Required to Alleviate p50-induced Inhibition of Processing of p105 in Vivo—We (16) and later Heissmeyer and colleagues (17) have shown recently that IKK-mediated phosphorylation of specific Ser residues within the C-terminal domain of p105 leads to recruitment of the SCFβ- TrCP ubiquitin ligase with subsequent polyubiquitination and rapid processing/degradation of the precursor molecule. Heissmeyer and colleagues (15) have shown that IKK-mediated degradation of p105 releases docked p50 that interacts with Bcl-3 to generate the trimeric p50/p50-Bcl-3 active transcription factor. We hypothesized that the mechanism that underlies liberation of the docked inhibitory subunits involves IKK and β-TrCP-mediated targeting of p105. As can be seen in the experiment depicted in Fig. 6A, processing of p105-WT in cells is accelerated significantly in the presence of active IKK. Furthermore, as can also
be seen in Fig. 6B, processing/degradation of most of p105 enables the generated p50 to migrate to the nucleus because it has lost its cytosolic anchor. To identify the region within p105 which mediates IKK activity, we transfected cells with either p105-WT or p105-Δ918–934, which lacks the C-terminal phosphorylation domain. As can be seen in Fig. 6B, expression of p50 inhibits processing of both p105-WT and p105-Δ918–934 (compare lane 1 with lane 3 and lane 7 with lane 9; data are also quantified). Expression of constitutively active IKKβ leads to alleviation of inhibition and translocation of the generated p50 (as well as of the exogenously expressed p50-Xba) to the nucleus (compare lanes 3 and 4 with lanes 5 and 6). In contrast, the inhibitory effect of p50 on the processing of p105-Δ918–934 (compare lane 9 with lane 7) cannot be alleviated by IKK (compare lane 11 with lane 9).

Last, it was important to examine the role of β-TrCP in the kinase-mediated alleviating effect. We used ΔF-β-TrCP, a dominant negative species of the ligase, which can bind the substrate but cannot recruit the other components of the ubiquitin conjugation machinery. Consequently, it cannot target the substrate for degradation (16). As can be clearly seen from the experiment depicted in Fig. 7, p50 inhibits processing (compare lane 1 with lane 3) of p105-WT. IKK alleviates the inhibition (compare lanes 5 and 6 with lanes 3 and 4). This alleviation was abolished, however, by concomitant expression of ΔF-β-TrCP (compare lanes 7 and 8 with lanes 5 and 6): the dominant negative ligase inhibited the stimulated IKK-mediated induced processing/degradation and generation of p50.

**DISCUSSION**

We have shown previously that p105 is targeted for processing/degradation by two distinct ubiquitin system recognition motifs. The first motif, which is adjacent to the GRR, contains two Lys 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 processing/degradation that occurs in nonstimulated, resting cells and provides the cell with the low amount of p50 required for its activity under these conditions (10). Processing of p105 under these conditions may occur cotranslationally (12, 18), and this site may be involved in targeting the molecule via this unique process. The second, C-terminal, recognition motif undergoes signal-induced IKK-mediated phosphorylation (15) with subsequent recruitment of the SCFβ-TrCP ubiquitin ligase, polyubiquitination, and accelerated processing/degradation (16). Involvement of the SCFβ-TrCP ligase complex in the process has been corroborated later by Heissmeyer and colleagues (17). We have shown that the two motifs are targeted via two different E3s (16) and most probably, via distinct E2s as well (not shown).

Several proteins have been described which are targeted via two distinct motifs and conjugation enzymes. Among them are p53, which is targeted by Mdm2 after DNA damage (27), and by the E6/E6AP ligase complex in human papillomavirus-transformed cells (28). IκBα is degraged within the context of a signaling complex following phosphorylation of Ser residues 32 and 36 (for review, see Ref. 5). The free inhibitor is probably degraded following CK II-mediated, constitutive phosphorylation of Ser293 (29). The yeast mating type transcriptional regulator MATA2 is targeted by two motifs, Deg1 and Deg2, and two E2 enzymes, Ubc6 and Ubc7 (30); however, the identity of the E3(s) has remained obscure. Similarly, the model protein lysozyme is targeted by E2–14 kDa/E3a following recognition of the N-terminal residue and also by members of the UbcH5 family and a yet to be identified E3 that recognizes a downstream motif (31). Although the distinct motifs and conjugating enzymes that govern stability of p53 and IκBα operate under different pathophysiological conditions, the physiological significance of the involvement of two sites in the degradation of MATA2 and processing/degradation of p105 has remained obscure.

In the current study we show that processing (Fig. 2) of a p105 precursor that contains more than four ankyrin repeats but lacks the C-terminal signaling domain is significantly less efficient compared with processing of p105-WT and p105 species that contain fewer than four repeats. We hypothesized that the inhibition is caused by docking of free NF-κB subunits present in the HeLa cell extract to the ankyrin repeat domain. To test this hypothesis, we studied the effect of exogenously added p50 on p105 processing in vitro. In a reconstituted cell-free system, exogenous p50 inhibits processing (Fig. 3) of precursor molecules that contain four or more ankyrin repeats (panel A) but lacks the IKK C-terminal phosphorylation and β-TrCP binding domain (panel B). Mechanistic analysis shows that the exogenous p50 does not affect conjugation (Fig. 4) but probably interferes with proteasomal processing of the conjugated proteins (Fig. 4). A similar interference had been reported for several GRR-containing proteins such as Epstein-Barr virus nuclear antigen 1 (32), p105 (9, 10), and the polyglutamine-containing mutated ataxin 3 (33). In the case of p105, interference with proteasomal degradation may result from the inability of the core protein, to which a cluster of additional large molecules is bound, to unfold in a manner that will allow entry into the proteasomal catalytic chamber. The findings in the cell-free system have been corroborated in vivo as well. As can be seen in Fig. 5, processing of the ankyrin repeat-free molecule p105-Thr111 (panel A) or of a p105 molecule that contains only three repeats and in which the remaining repeats have been replaced with DHFR (panel B), is not affected by exogenous overexpressed p50. In contrast, processing of p105-WT is inhibited (panels A and B; see also Figs. 6 and 7). As noted above, the discrepancy between the lack of p50-mediated inhibition of processing of the WT protein in the cell-free system (Fig. 3) compared with p50-induced inhibition in the cell (Figs. 5–7) can be attributed to the presence of an active kinase in the HeLa extract (16, 26 and references therein). Importantly, inhibition of processing of the WT protein in cells could be relieved by expression of a catalytically active IKKβ. Alleviation required an intact C-terminal targeting domain (Fig. 6) along with the β-TrCP ubiquitin ligase (Fig. 7).

Based on these results, we propose a model trying to explain the requirement for two distinct recognition signals for processing/degradation of p105 under different physiological conditions. According to the model, a nascent p105 polypeptide chain can be initially processed cotranslationally. Processing may require the basal/constitutive recognition motif. The p50 that was generated under these conditions is docked to an emerging ankyrin repeat domain in a p105 molecule that has not been processed yet. Docking of several additional free NF-κB subunits hinders recognition of these p105 molecules by the proteasome and may inhibit cotranslational processing. The completely synthesized p105 along with the docked subunits serve as an inactive storage for these subunits. After stimulation, the C-terminal domain is phosphorylated. This modification leads to recruitment of the SCFβ-TrCP ubiquitin ligase, 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. These subunits serve as a source for an active transcriptional regulator in stimulated cells. Several experimental lines of evidence lend support this model, including progressive inhibition of processing with increasing number of ankyrin repeats and alleviation of inhibition that requires an intact C-terminal domain, IKK,
and β-TrCP E3. However, several other important problems remain unsolved. The mechanism(s) that underlies selection of p105 molecules that undergo cotranslational processing from those that are synthesized to completion is not known. It is possible that cotranslational processing is the default mechanism that provides the resting cell with the small amount of p50 required for its basal needs. This occurs after stress/stimulation when all of 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 which will occur now only after stimulation and targeting of the C-terminal domain.

An important observation relates to the distribution of p105 and p50 in the cytosol and nucleus. p105 WT appears to be sequestered solely in the cytosol (see. e.g. Fig. 5A, lanes 5 and 7). In contrast, p105 that lacks the last four ankyrin repeats is translocated to the nucleus (Fig. 5B, lanes 2 and 4). It is possible that the docked p50 subunits do not allow migration to the nucleus, possibly by sterically hindering the NLS. Thus, docking of active NF-κB subunits to p105 not only sequesters them in an inactive storage form, but also ensures that they will not be translocated inadvertently to the nucleus.

Although clearly not the main focus of this study, it was suggested by Heissmeyer and colleagues (15, 17) that phosphorylation of the C-terminal domain and recruitment of the SCFβ-TrCP E3 complex leads to complete degradation of p105 and not to increased processing. The researchers argue, although they do not provide direct experimental evidence, that the increased amount of p50 observed under these conditions is attributed to increased transcription of p105 induced by IKK. Our experiments indicate that whereas stimulation leads also to complete degradation of a certain proportion of p105, accelerated processing accompanied by a significant increase in generation of p50 from p105 also occurs (Ref. 16 and Figs. 6 and 7). (i) In pulse-chase experiments, we could not detect increased synthesis of p50 from p105 also occurs (Ref. 16 and Figs. 6 and 7). (ii) In a cell-free reconstituted system, increased transcription of p105 induced by IKK. Our experiments strongly support the notion that IKK/TrCP also stimulates processing. (iii) In a recent study, Xiao and colleagues (19) have shown that p100 is processed in a NIK-mediated manner (although NIK is not necessarily the p100 kinase), following phosphorylation of Ser residues 867 and 870, which are similar to p105 Ser residues 824 and 827 targeted by IKK (17). Although neither the sites nor the kinase in the two molecules is identical, it is likely that they are processed in a similar manner. (iv) Last, it is hard to conceive that the degradation machinery that acts under stimulation is completely different from the one that acts under basal conditions and can overcome completely the GRR barrier/processing stop signal (9, 10) and the upstream residues that confer tight folding upon the p50 domain (11). The GRR physically interferes with proteasomal degradation regardless of the molecule in which it is inserted. For example, it can completely protect IκBα from the rapid and efficient tumor necrosis factor-α-induced degradation (34). As noted, it will be also difficult to overcome the tight folding of p50 which is dependent on structural characteristics distinct from the GRR (11). It appears that the property of limited degradation/processing is intrinsic to p100/p105 and not to the enzymatic machinery involved. It is possible, however, that after stimulation and utilization of the C-terminal phosphorylation site, the GRR/p50 folding does not function efficiently, and complete proteolysis of p105 can “overcome” partially these barriers.

REFERENCES

1. Foo, S. Y., and Nolan, G. P. (1999) Trends Genet. 15, 229–235
2. Fan, C. M., and Maniatis, T. (1991) Nature 354, 395–398
3. Faundez, Z., Hershko, A., Pines, O., and Ciechanover, A. (1997) Trends Biochem. Sci. 22, 115–122
4. Orian, A., Whiteside, S., Israel, A., Stanovski, I., Schwartz, A. L., and Ciechanover, A. (1995) J. Biol. Chem. 270, 21707–21714
5. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
6. Ciechanover, A., Orian, A., and Schwartz, A. L. (2000) BioEssays 22, 442–451
7. Voges, D., Zwickl, P., and Baumeister, W. (1999) Annu. Rev. Biochem. 68, 1015–1068
8. Deshayes, B. J. (1999) Annu. Rev. Cell Dev. Biol. 15, 435–467
9. Lin, L., and Ghosh, S. (1999) Mol. Cell Biol. 19, 2248–2254
10. Orian, A., Schwartz, A. L., Israel, I., Whiteside, S., Kahan, C., and Ciechanover, A. (1999) Mol. Cell 3, 3664–3673
11. Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M., and Matouschek, A. (2001) Mol. Cell 7, 627–637
12. Lin, L., DeMartino, G. N., and Greene, W. C. (1998) Cell 92, 819–828
13. Fujimoto, K., Yasuda, H., Sato, Y., and Yamamoto, R. (1995) Gene (Amst.) 165, 183–189
14. MacKichan, M. L., Logeat, F., and Israel, A. (1996) J. Biol. Chem. 271, 6084–6091
15. Heissmeyer, V., Krappmann, D., Wuylex, F. N., and Scheidereit, C. (1999) EMBO J. 18, 4766–4778
16. Orian, A., Gonen, H., Bercovich, B., Fajerwman, I., Eytan, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A. L., and Ciechanover, A. (2000) EMBO J. 19, 2560–2591
17. Heissmeyer, V., Krappmann, D., Hadana, E. N., and Scheidereit, C. (2001) Mol. Cell. Biol. 21, 1024–1035
18. Heusch, M., Lin, L., Geletjans, R., and Greene, W. C. (1999) Oncogene 18, 6201–6208
19. Xiao, G., Harhaj, E. W., and Sun, S.-C. (2001) Mol. Cell 7, 401–409
20. Harada, E. N., Nieters, A., Wuylex, F. N., Gruenberg, J., McKeithan, T. W., and Scheidereit, C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2489–2493
21. Rice, N. R., MacKichan, M. L., and Israel, A. (1992) Cell 71, 243–253
22. Sun, P. C., Ganchi, P. A., Beraud, C., Ballard, D. W., and Greene, W. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1346–1350
23. Harhaj, E. W., Magirwar, S. B., and Sun, S.-C. (1996) Oncogene 12, 6084–6092
24. Alkalay, I., Yaron, A., Hatzebui, A., Jung, S., Avraham, A., Gerlitz, O., Pashut-Lavin, I., and Ben-Neriah, Y. (1995) Mol. Cell. Biol. 15, 1294–1301
25. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
26. Li, C. C., Korner, M., Ferris, D. K., Chen, E., Dai, R. M., and Longo, D. L. (1994) Biochem. J. 303, 499–506
27. Honda, R., Takanaka, H., and Yashuda, H. (1997) FEMS Lett. 420, 25–27
28. Scheffner, M., Huijbregts, J. M., Vierstra, R. D., and Howley, P. M. (1993) Cell 73, 485–495
29. Schwarzb, E. M., Van Antwerp, D., and Verma, I. M. (1996) Mol. Cell. Biol. 16, 3554–3559
30. Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993) Cell 74, 357–369
31. Gonen, H., Stanovski, I., Shkedya, D., Hadari, T., Bercovich, B., Bengal, E., Meslati, S., Abu-Chatoum, O., Schwartz, A. L., and Ciechanover, A. (1996) J. Biol. Chem. 271, 302–310
32. Levitskaya, J., Sharipo, A., Leonicheks, A., Ciechanover, A., and Masucci, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12616–12621
33. Cummings, C. J., Reinstein, E., Sun, Y., Antalffy, B., Jiang J.-B., Ciechanover, A., Orr, H. T., Beaudet, A. L., and Zoghbi, H. Y. (1999) Neuro 24, 879–892
34. Sharipo, A., Imreh, M., Leonicheks, A., Imreh, S., and Masucci, M. G. (1998) Nat. Med. 4, 939–944
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
Shai Cohen, Amir Orian and Aaron Ciechanover

J. Biol. Chem. 2001, 276:26769-26776.
doi: 10.1074/jbc.M102448200 originally published online May 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102448200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 14 of which can be accessed free at http://www.jbc.org/content/276/29/26769.full.html#ref-list-1