Stability of the Rel Homology Domain Is Critical for Generation of NF-κB p50 Subunit*

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The NF-κB transcription factor p50 and the Rel protein-specific transcription inhibitor p105 are both encoded by the nfkb1 gene. The p50 protein is incorporated within the N-terminal portion of p105 and is a unique product of proteasomal processing. Because proteasome-mediated proteolysis generally results in complete degradation of the substrate, how p50 survives the proteasomal processing remains unknown. Survival of proteasomal processing has also been observed recently for the yeast transcription factors SPT23 and MGA2, but the mechanism is also unclear. Here we show evidence that stability of the Rel homology domain (RHD) within the N-terminal portion of the NF-κB 1 protein is required for p50 generation. We demonstrated that proteolysis initiated at an internal location of the NF-κB 1 protein, which normally generates p50, degrades the N-terminal portion of the NF-κB 1 protein when the RHD is destabilized. Our findings highlight the critical role of the unique structure of the RHD for the survival of p50 during proteasomal processing.

NF-κB transcription factors play a significant role in regulation of basic cellular functions such as immune and inflammatory responses, apoptosis, and developmental process (1). Abnormal NF-κB activities have often been implicated in many disease states such as cancers, AIDS, autoimmunity, and neurodegenerative conditions (2). Gene ablation studies in mice indicate that maintaining a normal ratio of NF-κB 1 proteins, p50 and p105, is physiologically important. Mice lacking the nfkb1 gene (hence lacking both p50 and p105), is normally except having abnormal immune responses to pathogen infections (3, 4). However, mice expressing p50 but lacking p105 (5), suggesting that p105 plays a regulatory role on p50 homodimer formation. A high p50/p105 ratio was also observed in skin carcinomas and laryngeal papilloma tissues (7, 8), linking abnormal homeostasis of p50/p105 to these pathological conditions.

Regulation of p50/p105 homeostasis includes p50 biogenesis and p105 degradation, and both processes are mediated by the proteasome. In resting cells, the ratio of p50/p105 is relatively stable, and p50 biogenesis is the major factor that determines the ratio of the two proteins (9). However, when the cell is stimulated, the cellular ratio of p50/p105 is adjusted through degradation of p105 (10, 11). This stimuli-induced p105 degradation is mediated via phosphorylation of the C terminus of p105 by the IKK kinase complex, and the subsequent recruitment of SCF<sub>TrCP</sub> ubiquitin ligase complex to this region (10–13). The binding of the SCF<sub>TrCP</sub> complex to p105 was originally attributed to enhancement of p50 generation upon stimulation (14, 15). However, recent studies have demonstrated that the level of p50 remains unchanged in the IKK-knockout lymphocytes as in the wild type counterparts (12, 16), suggesting that stimulation of the cell has little, if any, to do with p50 generation. NF-κB 1 (p105 and p50) and NF-κB 2 (p100 and p52) are closely related proteins, and p52 generation also requires the proteasome (17, 18). Unlike p50 generation, stimuli induce processing of p100 and increase p52 levels, and the C terminus of p100 was found to regulate stimuli-induced p100 processing (19).

Our previous studies (20, 21) demonstrated that p50 generation occurs during translation of the nfkb1 gene. Although this co-translational model is consistent with the kinetics pattern of p50 generation, and may explain the steady cellular ratio of p50/p105 in resting cells, it is unclear how p50 survives proteasomal processing, as the proteasome normally degrades proteins to small oligopeptides (22). It has also been observed that yeast proteins, SPT23 and MGA2, are synthesized as endoplasmic reticulum/nuclear membrane-bound precursor proteins but are processed to transcription factors by the proteasome (23). Previously, we found that nfkb1 genes carrying point mutations or small deletions affecting p50 dimerization fail to generate p50 (21). Later, Lee et al. (24) also found that mutations that destabilize the Rel homology domain (RHD) in p105 also lead to failure of p50 production. However, it is not clear how the stability of the RHD is related to p50 biogenesis. Here we demonstrate that proteasome-mediated proteolysis degrades the N-terminal RHD when it is destabilized by mutations. We show that in addition to the previously identified cis-element, a glycine-rich region (GRR), residues 400–475 of murine p105, is also involved in p50 production. By using prokaryote ATP-dependent proteases ClpAP/XP, we also recapitulate p50 generation and degradation of the destabilized RHD in vitro. Our studies establish the importance of the structure of the RHD in p50 biogenesis and may provide mechanistic insights for other proteasomal processing events.
**RESULTS**

**Detection of Proteolysis-resistant Fragments in the N-terminal Portion of p105**—Lee et al. (24) demonstrated in vitro, using purified ClpA/XP, that a sufficiently stable protein domain survives energy-dependent protease degradation as a resistant core attached with additional residues. This discovery is a reminiscence of p50 generation: proteasomal degradation, like ClpA/XP, requires ATP, and p50 can be viewed as the RHD (as the resistant core) attached with 80–90 residues.

The RHD consists of two structurally similar subdomains, sd1 and sd2, linked by a short loop (25, 26). Because mutagenesis studies found that mutations affecting stability/dimerization of the RHD are all located within the sd2 (21, 24), we reasoned that an intact sd1, which is proximal to the sd2 at N terminus, might retain residual resistance to proteolysis when sd2 is destabilized by mutations (Fig. 1). To demonstrate that resistance to proteolysis by the unique structure of the RHD is the major mechanism underlying survival of the p50 protein during processing, we attempted to detect proteolysis-resistant fragments within the RHD, using an nfkb1 gene carrying composite mutations (Y267D/L269D) (27) that fails to generate p50 (21). If proteolysis leading to p50 production initiates from downstream of the RHD, such mutations may allow proteolysis to proceed into the RHD when the sd2 is destabilized. *A priori*, residual proteolysis-resistant fragments containing the sd1 may be detected in this mutant. CHO-CD14 cells transfected with the wild type nfkb1, the Y267D/L269D mutant, or a deletion mutant (∆302–310) were labeled with [35S]methionine/cystine for 1 h and chased with normal media for 1 h followed by immunoprecipitation of the lysates. Because all fragments were tagged with a T7 gp10 epitope at their N termini, anti-gp10 antibodies used for immunoprecipitation should detect all fragments that contain intact N termini. As shown in Fig. 2A (*lanes 2 and 3*), expression of either nfkb1(Y267D/L269D) or nfkb1(∆302–310) does not generate p50. A prolonged exposure of the gel to the film, however, revealed several putative proteolysis-resistant fragments from the mutants (Fig. 2B). Most notably, two fragments slightly larger than the sd1 were detected (Fig. 2B, *lanes 2 and 3*), they contain sd1 (as the proteolysis-resistant core) with additional attached residues. This is similar to the typical phenomenon of proteolysis resistance demonstrated by Lee et al. (24) in their *in vitro* studies. There were additional sd1-containing, proteolysis-resistant fragments, possibly generated by the remaining RHD structures. None of these fragments were detected in the wild type protein, suggesting that they originated from the mutants rather than being cellular proteins brought down by immunoprecipitation.
To confirm that the proteasome is required for the generation of the proteolysis-resistant fragments, we used the proteasome inhibitor MG132 in the labeling experiments. When the cells were treated with MG132, no proteolysis-resistant fragments were detected even after prolonged exposure of the gel (Fig. 2C). These results suggest that the detected fragments are products of proteasomal processing, rather than contaminants. Together, the results suggest that stability of the RHD, which is also inherently required for dimerization, is critical for p50 generation. The peculiar structure of the RHD confers its strong resistance to proteasome-mediated degradation, resulting in p50 production. When the stable structure of the RHD is abrogated by mutations, its resistance to proteolysis is significantly compromised, leading to major destruction of the N-terminal portion and generation of only low abundance of sd1-containing resistant fragments.

The RHD Also Resists Proteolysis by ClpA/XP—Our previous studies (20) showed that co-translational processing of the NF-κB 1 polypeptides is stochastic; only a portion of the NF-κB 1 polypeptides are captured by the proteasome during translation, and those that are not captured complete their synthesis to form the full-length p105 proteins. Therefore, the level of the full-length unprocessed p105 mutant proteins (i.e. p105 mutant that are not captured for processing) remains similar to that of the wild type counterpart (Fig. 2). To demonstrate that NF-κB 1 fragments, once captured, can be completely processed to p50, and that the RHD is indeed resistant to energy-dependent proteases, we established an in vitro system, using prokaryotic protease ClpAP or ClpXP. ClpAP/XP degrades proteins in a manner similar to eukaryotic proteasome. ClpA and ClpX, whose function is similar to the proteasome 19 S components, recognize a degradation signal, ssrA, attached to the C terminus of the substrate and unfold the substrate from this signal. The unfolded protein is then transported into the channel of ClpP oligomer protease for degradation (28). This in vitro system provides an experimental bypass of ubiquitination steps, which may be required for the in vivo processing of NF-κB 1 proteins, and allows us to assess the mechanism of processing directly.

A 497-residue of NF-κB 1 N-terminal portion does not generate p50 either in vivo or in vitro, probably due to the conformation of this fragment that precludes the access of cellular processing machinery (20, 21). However, incubation of ClpXP with this 497-residue fragment containing an ssrA signal at its C terminus promotes efficient processing, and all substrates are converted to p50 (Fig. 3A). The processing is dependent upon the degradation signal, as a mutated ssrA (29) does not direct the processing (Fig. 3B). When the RHD carries the Y267DL269D composite mutations, ClpXP degrades the entire protein without generating p50 (Fig. 3, C and D). These results suggest that the RHD universally resists energy-dependent protein degradation. Similar results were obtained when ClpAP was used (data not shown). If ClpAP/XP processes NF-κB 1 proteins using a mechanism similar to that of the proteasome, it is expected that in Y267DL269D mutant, a small portion of sd1-containing N-terminal fragments will survive the proteolysis as seen in Fig. 2. To investigate whether processing of the Y267DL269D mutant by ClpAP also generates sd1-containing fragments, we immunoprecipitated in vitro translated, gp10-tagged p-497(Y267DL269D) with anti-gp10 antibodies and incubated the precipitants with ClpAP. After incubation, the bound proteins were extracted from protein A beads by boiling with SDS loading buffer. Although the majority of mutant NF-κB 1 proteins were completely degraded (Fig. 3C), a small portion of sd1-containing peptides survived and was detected by prolonged exposure of the film (Fig. 4). These results are consistent with the observations shown in Fig. 2, B and C, and further affirm that the stability of the RHD is required for its own survival during proteolytic processing by energy-dependent proteases.

Proteolysis Leading to p50 Generation Initiates at an Internal Site of the p105 Protein—Two locations within p105 sequences have been proposed to be important for p50 generation (30). One is at the vicinity of the putative C terminus of p50 (i.e. in the middle of p105) (31), and the other is at the C terminus of p105 (14). However, recent studies demonstrated that the latter location promotes complete degradation of p105 induced by stimuli, rather than p50 generation. During stimulation, p105 is degraded without generating p50 (10, 11). Additional evidence obtained from lymphocytes lacking the IkB kinases (IKKs) further supports this notion (12, 16). Unlike NF-κB 2 p52, which is induced to a higher level by stimuli through the IKK-mediated phosphorylation and subsequent processing of p100 (19), p50 expression in IKK-knockout lymphocytes is normal (16), suggesting that p50 generation is essentially a constitutive, rather than an induced, event. The C terminus of p105 contains an IKK phosphorylation target site, and phosphorylation of this consensus site leads to recruitment of SCF<sup>TRCP</sup> ubiquitin ligase complex. These features of p105 correlate with the findings that extracellular signals resulting in rapid degradation of IkB also induce degradation of p105 (10, 11) whose function in the cell is similar to that of IkB (32, 33).
To demonstrate that proteolysis leading to p50 generation initiates at the internal site of the p105 protein, we expressed the N-terminal 530 residues of p105 in the cell. Previously, we demonstrated that this 530-residue fragment is necessary and sufficient for p50 generation (20). If this portion of p105 contains all elements required for p50 generation, we should also detect proteolysis-resistant fragments from this 530-residue fragment when its sd2 harbors mutations that disrupt the stability of the protein. Both the wild type p-530 and p-530(Y267D/L269D) were expressed in the cells, and similar metabolic labeling experiments as in Fig. 2 were performed. As shown in Fig. 5, p-530(Y267D/L269D) exhibits similar proteolysis-resistant fragments as nfb1(Y267D/L369D), suggesting that the proteolysis signal is at the upstream of residue 530.

Residues 400–475 of Murine p105, in Addition to the GRR, Are Also Important for p50 Generation—Although Orian et al. (31) showed that residues 441–454 in human p105 are important for p50 generation, the sequences within this region, unlike the C-terminal IKK target region, are not well conserved among the p105 proteins from humans and mice (34, 35). Replacement of a large portion of p105 starting from the end of the GRR with either I/H9260B/H9251 or bacteriophage T7 capsid protein gp10 does not affect p50 production (36). This observation leads to the initial conclusion that the GRR is sufficient for directing processing, and the entire C-terminal portion of p105 is not required for the process (36). However, similar replacements with other proteins, such as human T cell leukemia viral protein Tax, do not lead to p50 production (Fig. 6B, lane 2), suggesting that the GRR is a necessary but insufficient element for p50 production (20, 31). One possible explanation for this phenomenon is that generation of p50 from gp10 or IkBa-containing chimeric proteins may be due to the contribution of cis-elements from these two proteins that, together with the GRR, enhance instability of the chimeric proteins. These elements may compensate for the loss of necessary cis-elements in p105 when the protein is fused to the GRR directly. Linking proteins that are relatively stable (such as Tax) to the GRR

![Fig. 3. ClpAP/XP can process NF-κB 1 fragments and generate p50 in vitro.](image)

![Fig. 4. ClpAP/XP-mediated proteolysis of mutated RHD also generates sd1-containing fragments.](image)
exposure is shown here.

IKK and SCF sequences Does Not Significantly Enhance p50 Generation

cis the full-length p50(475)/Tax only (Fig. 6C). Anti-Tax antibodies immunoprecipitated effective production of p50 (Fig. 6B), confirming that other cis-elements, in addition to the GRR, are also important (Fig. 6). Although the sequences within this region are not well conserved among the p105 proteins of mice and humans, their function on regulation of p50 generation is likely to be similar. In addition to the chimeric protein experiments shown in Fig. 6 of this report, previous work (18) on domain swapping between p105 and p100 also helps to reach the same conclusion. NF-κB p52, unlike p50, is generated at a very low level in resting cells. However, replacing sequences downstream of the GRR in p52 with that of p50 enhances p52 production (18). These findings are consistent with previous finding in human p105 (31). The GRR, together with its downstream sequences, may serve as the processing “initiation site” where cellular factors bind and target this portion to the proteasome. Alternatively, this portion may be natively disordered and may directly interact with the proteasome, promoting its gating and the subsequent processing (20, 37, 38).

The findings that processing initiates at an internal location of the NF-κB 1 polypeptide, and that the C-terminal cis-elements do not contribute to p50 generation (Fig. 7), are consistent with our co-translational p50 biogenesis hypothesis. Eukaryotic polypeptides fold and form correct conformation co-translationally (39). Prior to completion of the p105 synthesis, the N-terminal RHD may have already folded into sufficiently stable structures that resist proteolysis. Because the cis-elements within p105 that render proteasomal processing are available before translation is completed, it is conceivable that processing may occur during translation of the NF-κB 1 polypeptide. Furthermore, the recent discovery of endoproteolytic activity of the proteasome (37) also suggests that processing a protein from an internal location is highly possible. The interaction of the N-terminal RHD and the C-terminal ankyrin repeats may block the access of cellular factors or the proteasome to this location (30). Hence, de novo processing to generate p50 from p105 must have a mechanism that allows re-

**DISCUSSION**

In this report, we used both in vivo and in vitro approaches to demonstrate that the inherent structures of the RHD are important for p50 to survive the proteasomal processing. By using NF-κB 1 proteins carrying either composite point mutations or small deletions, we showed that when the RHD is destabilized by these mutations, the processing fails to generate p50. Detection of the low abundance of sd1-containing fragments from the mutants suggests that such failure is due to degradation of the entire N-terminal portion that normally survives to become p50. Generation of p50 was also being successfully recapitulated in vitro, using the ClpAP/XP system. These studies imply that it is the unique stable structures of the RHD, rather than the specificity of the proteasome, that is critical for p50 to survive the processing. At present, it is not clear how the RHD resists proteolysis. It is possible that the structures of the RHD render its resistance to the prerequisite step of the unfolding mediated by the energy-dependent proteases. It is also possible that the RHD can be unfolded but is rapidly refolded, precluding the domain from the proteolytic core. Future studies will be directed to resolve these questions.

Our work further confirmed that proteolysis leading to p50 production initiates at an internal site, rather than at the C terminus of the p105 protein. First, a 530-residue of the N-terminal portion is sufficient to generate p50 (20), and the sd1-containing fragments are detected when this 530-residue fragment carries the Y267D/L269D mutations (Fig. 5). Second, besides the GRR, which is within the p50 protein, additional downstream sequences that span beyond the “cleavage site” are also important (Fig. 6). Although the sequences within this region are not well conserved among the p105 proteins of mice and humans, their function on regulation of p50 generation is likely to be similar. In addition to the chimeric protein experiments shown in Fig. 6 of this report, previous work (18) on domain swapping between p105 and p100 also helps to reach the same conclusion. NF-κB p52, unlike p50, is generated at a very low level in resting cells. However, replacing sequences downstream of the GRR in p52 with that of p50 enhances p52 production (18). These findings are consistent with previous finding in human p105 (31). The GRR, together with its downstream sequences, may serve as the processing “initiation site” where cellular factors bind and target this portion to the proteasome. Alternatively, this portion may be natively disordered and may directly interact with the proteasome, promoting its gating and the subsequent processing (20, 37, 38).

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![Fig. 5. The N-terminal 530 residues of NF-κB 1 are sufficient for p50 generation.](http://www.jbc.org/)

The N-terminal 530 residues of NF-κB 1 are sufficient for p50 generation. Similar experiments as Fig. 2 were performed using the wild type (wt) p-530 and p-530/Y267D/L269D. The putative sd1-containing fragments are marked with asterisks (lanes 3 and 4). For sd1, 1/20 volume of the sample was loaded (lane 5). The prolonged exposure is shown here.
access to this internal region of p105. Phosphorylation of the C terminus of p105 by the IKKs promotes mainly degradation of p105 and does not significantly contribute to p50 biogenesis. Although phosphorylation of the C terminus of p100 promotes p52 production (19), the C terminus of p100 does not serve as the initiation site for processing. p100 lacking the last 152 residues that include the binding sites for IKK and SCF/TrCP complexes exhibits elevated p52 production in resting cells (19), suggesting that the C-terminal degradation signal negatively regulates p52 production. Therefore, it appears that phosphorylation of the C terminus of p100 may induce a conformational change of the protein that allows access of the cellular machinery to its internal processing initiation site.

Although in this report we have not studied the RHD of p52, given that the NF-κB1 and 2 proteins share extensive homologies and generation of p50 and p52 both require the proteasome, it is likely that the stability of p52 RHD is also critical for p52 biogenesis.

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REFERENCES
1. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
2. Baldwin, A. S., Jr. (2001) J. Clin. Invest. 107, 3–6
3. Sha, W. C., Liu, H. C., Tuomanen, E. I., and Baltimore, D. (1995) Cell 80, 321–330
4. Snapper, C. M., Zelazowski, P., Rosas, F. R., Kehry, M. R., Tian, M., Baltimore, D., and Sha, W. C. (1996) J. Immunol. 156, 183–191
5. Ishikawa, H., Claudio, E., Dambach, D., Raventos-Suarez, C., Ryan, C., and Bravo, R. (1998) J. Exp. Med. 187, 985–996
6. Ishikawa, H., Ryseck, R. P., and Bravo, R. (1996) Oncogene 13, 255–263
7. Budunova, I. V., Perez, P., Vaden, V. R., Spiegelman, V. S., Slaga, T. J., and Jorcano, J. L. (1999) Oncogene 18, 7423–7431
8. Vancurova, I., Wu, B., Misko, V., and Sun, S. (2002) J. Virol. 76, 1533–1536
9. Naumann, M., and Scheidereit, C. (1994) EMBO J. 13, 4597–4607
10. Heissmeyer, V., Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1999)
11. Heissmeyer, V., Krappmann, D., Hatada, E. N., and Scheidereit, C. (2001) Mol. Cell. Biol. 21, 1024–1035
12. Salmeron, A., Janzen, J., Soneji, Y., Bump, N., Kamens, J., Allen, H., and Ley, S. C. (2001) J. Biol. Chem. 276, 22215–22222
13. Beinke, S., Belich, M. P., and Ley, S. C. (2002) J. Biol. Chem. 277, 24162–24168
14. Orian, A., Gonen, H., Bercovich, B., Fajerman, I., Eyting, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A. L., and Ciechanover, A. (2000) EMBO J. 19, 2580–2591
15. Cohen, S., Orian, A., and Ciechanover, A. (2001) J. Biol. Chem. 276, 22215–22222
16. Beinke, S., Belich, M. P., and Ley, S. C. (2002) J. Biol. Chem. 277, 24162–24168
17. Lin, L., and Ghosh, S. (1996) Mol. Cell. Biol. 16, 6201–6208
18. Lin, L., DeMartino, G. N., and Greene, W. C. (1998) EMBO J. 17, 4712–4722
19. Liu, C. W., Corboy, M. J., DeMartino, G. N., and Thomas, P. J. (2003) Science 299, 408–411
20. Rape, M., and Jentsch, S. (2002) Nat. Cell Biol. 4, E113–E116
21. Netzer, W. J., and Hartl, F. U. (1997) Nature 388, 343–349
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