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Structural Motifs Involved in Ubiquitin-Mediated Processing of the NF-κB Precursor p105: Roles of the Glycine-Rich Region and a Downstream Ubiquitination Domain

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The ubiquitin proteolytic system plays a major role in a variety of basic cellular processes. In the majority of these processes, the target proteins are completely degraded. In one exceptional case, generation of the p50 subunit of the transcriptional regulator NF-κB, the precursor protein p105 is processed in a limited manner: the N-terminal domain yields the p50 subunit, whereas the C-terminal domain is degraded. The identity of the mechanisms involved in this unique process have remained elusive. It has been shown that a Gly-rich region (GRR) at the C-terminal domain of p50 is an important processing signal. Here we show that the GRR does not interfere with conjugation of ubiquitin to p105 but probably does interfere with the processing of the ubiquitin-tagged precursor by the 26S proteasome. Structural analysis reveals that a short sequence containing a few Gly residues and a single essential Ala is sufficient to generate p50. Mechanistically, the presence of the GRR appears to stop further degradation of p50 and to stabilize the molecule. It appears that the localization of the GRR within p105 plays an important role in directing processing: transfer of the GRR within p105 or insertion of the GRR into homologous or heterologous proteins is not sufficient to promote processing in most cases, which is probably due to the requirement for an additional specific ubiquitination and/or recognition domain(s). Indeed, we have shown that amino acid residues 441 to 454 are important for processing. In particular, both Lys 441 and Lys 442 appear to serve as major ubiquitination targets, while residues 446 to 454 are independently important for processing and may serve as the ubiquitin ligase recognition motif.

The NF-κB proteins are a group of dimeric ubiquitous eukaryotic transcription factors belonging to the Rel family. They play key roles in basic processes such as regulation of the immune and inflammatory responses, development, differentiation, malignant transformation, and apoptosis (3, 4, 17). All members of the Rel family contain a Rel homology domain within the N-terminal domain of the protein, while some members, like p105, p100, and Relish, contain ankyrin repeats at the C-terminal domain. The precursor molecules p105 and probably p100 undergo ubiquitin- and proteasome-mediated limited proteolytic processing to yield the corresponding active subunits p50 and p52 (32, 33). These subunits are derived from the N-terminal domain of the molecule. The C-terminal domain is degraded (6, 14). These subunits typically heterodimerize with members of the Rel family that do not contain ankyrin repeats such as p65, RelB, and c-Rel. In the resting cell, the heterodimer generates a ternary complex with a member of the IκB family of inhibitory proteins. IκB binding sterically hinders a nuclear localization site, and consequently, the complex is retained in the cytosol (20, 24). Following cellular stimulation by a wide array of activators, such as cytokines (tumor necrosis factor alpha and interleukin 1, for example), viral and bacterial products, UV light, and oxidants, specific IκB kinases that phosphorylate the protein on two specific Ser residues, 32 and 36 (8), are activated (30, 41, 43). This phosphorylation leads to recognition of the molecule by β-TrCP (β-transducin repeat-containing protein), which is a part of an SCF (Skp1p, Cullin1, F-box protein) ubiquitin ligase complex (see, for example, references 40 and 42); polyubiquitination on Lys residues 21 and 22 (9, 35); and subsequent degradation by the 26S proteasome (2, 9). Following degradation of IκBα, the heterodimer's nuclear localization signal is exposed and the active complex is translocated into the nucleus, where it initiates specific transcription.

Degradation of a protein by the ubiquitin system involves two successive and discrete steps: (i) formation of a polyubiquitin chain that is covalently attached to the target substrate and (ii) degradation of the tagged protein by the 26S proteasome that is composed of two 19S regulatory subunits bound to the ends of a cylindrical 20S catalytic core complex. Formation of ubiquitin conjugates of a specific protein requires the sequential actions of three enzymes: the ubiquitin-activating enzyme, E1; one of several ubiquitin carrier proteins (or ubiquitin-conjugating enzymes), E2s; and a member of the ubiquitin-protein ligase E3 family. E3s play an essential role in specific substrate recognition. The polyubiquitinated chain is probably recognized in a specific manner by the regulatory 19S subcomplex of the 26S proteasome. Following binding, the substrate moiety is unfolded and translocated into the inner core of the 20S complex, where it is proteolyzed. Free and reutilizable ubiquitin is released via the actions of isopeptidases. The ubiquitin pathway is involved in processing and

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proteolysis of many cellular regulatory proteins, including, for example, mitotic and G1 cyclins and their regulators, oncoproteins and tumor suppressors, transcriptional activators, cell surface receptors and endoplasmic reticulum membrane proteins. The system also processes major histocompatibility complex class I-restricted antigens and removes in a selective manner abnormal and mutated proteins (12, 21, 23).

It appears that limited processing of the precursor proteins p105 and p100 is also mediated by the ubiquitin system. The p50 subunit of NF-κB is generated by ATP-dependent processing of p105 in vivo and in vitro (14). Palombella and colleagues have shown that addition of ubiquitin to fraction II stimulates processing of p60, a truncated form of p105, to p50 (33). Addition of ubiquitin-Arg48, a derivative of ubiquitin that cannot generate polyubiquitin chains, inhibited processing. In a different set of experiments (33), they have shown that inhibitors of the 20S proteasome block processing of p105 in intact cells. Orian and colleagues (32) have shown that intact native p105 is processed to p50 in a reconstituted cell-free system in a process that requires prior polyubiquitination of the protein. Processing involves the E2 enzyme UBCH5a or UBCH7 (E2-F1) and a novel 320-kDa ubiquitin-ligase, E3. The multiplicity of the conjugating enzymes involved in processing has been further underscored by Coux and Goldberg (11), who showed that processing in a cell-free system can be promoted by the 25-kDa E2 and a novel 50-kDa E3.

Generation of p50 or p52 is the only known process in which the ubiquitin system is involved in limited processing rather than in complete destruction of its target molecule. The mechanisms involved in this process—the identities of the structural motifs and biochemical events—have remained elusive. Lin and Ghosh (27) demonstrated that a Gly-rich region (GRR) that spans amino acid residues 372 to 394 in the mouse NF-κB molecule is required for processing. A mutant protein in which the GRR was deleted (ΔGRR) fails to generate the p50 subunit in COS-7 cells. In a few cases, insertion of the GRR sequence into unrelated proteins was sufficient to promote processing of the chimeras. However, the mechanism of action of the GRR motif, as well as its essential characteristics, has not been determined. Interestingly, a p105-ΔGRR mutant protein was processed in *Saccharomyces cerevisiae* (36), highlighting the difference between the recognition and processing systems in yeast and mammals. A Gly-Ala repeat in Epstein-Barr nuclear antigen 1 (EBNA-1) prevents degradation of the protein by the ubiquitin system and consequently abolishes presentation of antigenic peptide epitopes to the appropriate T cells. Transfer of the repeat to EBNA-4 prevented degradation of this otherwise degradable protein (25). Sharipo and colleagues (37) have inserted a short GA repeat into IκBα and showed that it abolishes signal-induced degradation of the inhibitor. However, it should be emphasized that in both EBNA protein and IκBα, the GA repeat prevented degradation but did not promote processing, regardless of the site of its insertion in the molecules. Thus, these findings strongly suggest that an additional specific structural motif(s) within the p105 molecule renders the molecule susceptible to the unique limited processing.

Here we show that the GRR does not interfere with conjugation of p105 but probably does interfere with its processing by the 26S proteasome. Mechanistically, the stability of p50 is dependent upon the presence of the Gly repeat. Structural analysis demonstrates that only a small number of Gly residues is required to promote processing and that efficient processing requires at least one Ala residue. Processing appears to be largely specific to p105, and the GRR does not serve as a universal transferable processing signal. It is probably a p105-specific domain that resides downstream of the GRR that contains the ubiquitination and, plausibly, the conjugation machinery recognition sites.

MATERIALS AND METHODS

Materials. Materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad. L-[^{35}S]methionine, [α-^{32}P]ATP, and [γ-^{32}P]ATP were obtained from New England Nuclear. Tissue culture sera and media were purchased from Biological Industries, Kibbutz Bet Haemek, Israel, or from Sigma. Clasto-lactacystin β-lactone and MG132 (*N*-carbobenzoxyl-Leu-Leu-leucinal) were purchased from Calbiochem. Antibody (polyclonal) against the NF-κB1 p50 subunit and MyoD were from Santa Cruz. Ubiquitin-aldehyde (UbAl) was purchased from Boston Biochemicals, Cambridge, Mass. Prestained molecular weight markers were purchased from Amersham. Ubiquitin, dithiothreitol, ATP, phosphocreatine, creatine phosphokinase, 2-deoxyglucose, Tris buffer, and poly(dI-dC) were purchased from Sigma. Hexokinase and adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS) were from Boehringer Mannheim. HEPES was purchased from Calbiochem. A wheat germ-based coupled transcription-translation kit (TNT) and κB-binding double-stranded DNA were from Promega. Restriction and modifying enzymes were from New England Biolabs. Immobilized protein A was from Pharmacia. Reagents for enhanced chemiluminescence were from Pierce. Oligonucleotides were synthesized by Biotechnology General, Rehovot, Israel. All other reagents used were of high analytical grade.

Cell lines. HeLa and COS-7 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmids and construction of mutants. Wild-type (WT) human p105 cDNA (pT7β105) used for in vitro translation was described previously (32). For expression of p105 in COS-7 cells, the entire coding region of p105 was subcloned into the pCI-neo vector (Promega) by using the *Xho*I and *Not*I sites. All mutant proteins used for in vitro translation and for expression in COS-7 cells were expressed in the same two vectors. Rat ornithine decarboxylase (ODC) and antizyme cDNAs were as described in reference 29. cDNAs coding for MyoD and c-Fos were as described previously (references 1 and 38, respectively). cDNAs coding for *Drosophila* Dorsal and Cactus and subcloned into the Bluescript vector (Stratagene) were kindly provided by Ruth Steward. Human p53 and E6 cDNAs were as described elsewhere (10).

Generation of mutant and fusion proteins. All mutant species of p105 and p50 were generated by site-directed mutagenesis with a QuikChange kit (Stratagene). The GRR coding sequence (residues 376 to 404) generated by PCR was introduced into proteins by using unique restriction sites that either existed or were generated in the target proteins and the GRR fragment. Into certain proteins we introduced an extended GRR region (residues 376 to 440), designated EGRR. The following GRR-containing proteins were generated: Dorsal(1-349)–GRR–Dorsal(350-679), c-Fos(1-313)–GRR–c-Fos(314-380), MyoD (1-174)–GRR–MyoD(175-280), MyoD(1-174)–EGRR–MyoD(175-280), ODC (1-301)–GRR–ODC(302-461), and ODC(1-301)–EGRR–ODC(302-461). Using p105-ΔGRR, we replaced amino acid residues 604 to 613 (RAGADLSLLD) with the sequence 604-GAGAGGGGGG-613 and generated another protein, designated p105–GRR(613). In addition, we generated the chimeric protein p53-GRR-ODC, which contains the GRR inserted between p53 and ODC. This chimeric protein was constructed in three steps: (i) removal of the C-terminal portion of p105 (residues 435 to 969) and in-frame insertion of the *Nco*I-*Not*I fragment containing the entire ODC sequence subcloned in these sites in a Bluescript vector (29), (ii) generation of the *Spe*I site that follows the last amino acid residue of p53, and (iii) replacement of the *Spe*I-*Spe*I fragment of the p50-GRR-ODC that contains p50 amino acid residues 1 to 342 with the *Spe*I-*Spe*I fragment that codes for full-length p53. The sequences of all constructs were confirmed by either the manual (Amersham) or the automatic (Applied Biosystems) dideoxy method using the ABI 310 autosequencer.

Generation of radiolabeled proteins. [^{35}S]methionine-labeled p105 proteins were synthesized by using the wheat germ-based coupled transcription-translation system according to the manufacturer's instructions. Unlabeled antizyme and E6 were synthesized in a similar manner in systems that contain unlabeled Met.

In vitro processing of p105. [^{35}S]methionine-labeled p105 was processed to p50 in a cell-free system essentially as described previously (32). Briefly, reaction mixtures at a final volume of 25 μl contained the following components: HeLa cytosolic extract (~100 μg of protein), 40 mM Tris-HCl (pH 7.6), 5 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 μg of ubiquitin, and ~25,000 cpm of the labeled substrate. Reaction mixtures without ATP contained 20 mM 2-deoxyglucose and 0.2 μg of hexokinase. ATP-dependent processing was monitored in the presence of a solution containing 0.5 mM ATP, 10 mM phosphocreatine, and 5 μg of creatine phosphokinase. All reaction mixtures were preincubated for 5 min at 37°C prior to the addition of the labeled substrate. Mixtures were incubated for 1 h on ice or at 37°C. Following incubation, reaction mixtures were resolved via SDS-PAGE (10% polyacrylamide). Gels were dried, and proteins were visualized with a phosphorimager (Fuji, Tokyo, Japan).

In vitro conjugation assays. Ubiquitin-p105 conjugates were generated in an assay similar to that used for processing, but with the following modifications: (i)

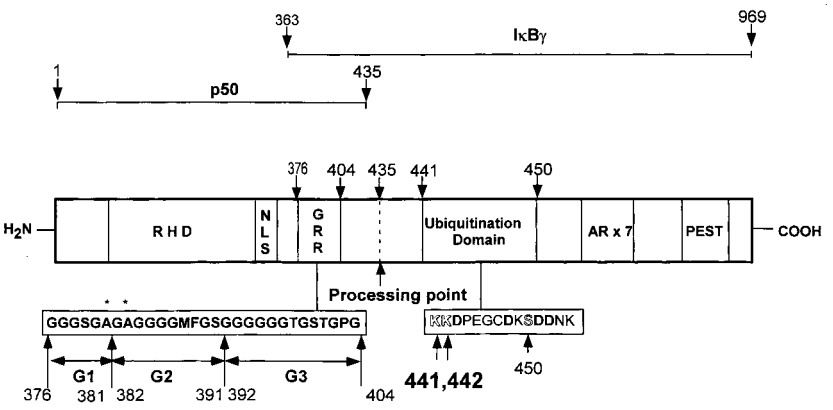

FIG. 1. Schematic diagram of the human NF-κB1 precursor p105. Domains are not shown in proportional sizes. AR, ankyrin repeat; RHD, Rel homology domain; NLS, nuclear localization signal.

ATPγS (5 mM) was substituted for ATP and the ATP-regenerating system and (ii) UbAl (0.5 μg), a specific inhibitor of certain isopeptidases (22), was added to the reaction mixture. Reaction mixtures were incubated at 37°C for 30 min, resolved via SDS-PAGE (7.5% polyacrylamide), and analyzed as described above.

Preparation of cell extract. HeLa cell cytosolic extract was prepared by hypotonic lysis as described previously (32). Extracts contained 8 to 10 mg of protein/ml.

Transient transfections and analysis of conjugation, processing, and degradation in intact cells. COS-7 cells were transiently transfected with the various p105 and p50 mutant cDNAs by the DEAE-dextran method (18). Processing of p105 and degradation of p50 in cells were monitored in pulse-chase labeling experiments followed by immunoprecipitation as follows. Forty h after transfection, cells were labeled with [^{35}S]methionine for 30 min (pulse). Following labeling, cells were harvested immediately (pulse; zero time) or incubation continued for the periods indicated in the figures in the presence of excess (2 mM) unlabeled methionine (chase). Following lysis (38), labeled proteins were precipitated with anti-p105 antibody. Immunocomplexes were collected with immobilized protein A. Following SDS-PAGE (10% polyacrylamide), proteins were visualized with a phosphorimager (Fuji). To increase the steady-state level of p105-ubiquitin adducts, cells were pulse-labeled for 150 min. Thirty minutes following addition of the labeling amino acid, MG132 or clasto-lactacystin β-lactone was added to a final concentration of 100 or 10 μM, respectively. Cells were harvested and lysed, p105 was immunoprecipitated, and proteins were visualized following SDS-PAGE (7.5% polyacrylamide) and phosphorimaging as described above.

Gel EMSA. COS-7 cells were transiently cotransfected with 3 μg of β-galactosidase cDNA and 10 μg of cDNA coding for the WT or the constructs of mutant p105 proteins indicated in the figures. Forty hours after transfection, cells were washed with phosphate-buffered saline and lysed by repeated cycles of freezing and thawing in 20 mM Tris-HCl (pH 7.9) buffer. β-Galactosidase activity in the high-speed supernatant was monitored as described previously (19). The amount of cell extract used in each electrophoretic mobility shift assay (EMSA) was normalized so that equal amounts of galactosidase activity were used. Parallel Western blot analyses revealed that the extracts also contained equal amounts of p105 proteins. The shift assay was preformed as described previously (1) with a γ-^{32}P-κB end-labeled probe. Proteins were resolved via PAGE (6% polyacrylamide) and detected by phosphorimaging.

RESULTS

Processing of p105 and generation of the p50 subunit of NF-κB requires the GRR. It has been reported that, in the cell, the GRR (Fig. 1) is an important structural motif required for limited processing of p105 and generation of the p50 subunit of NF-κB (27). Since limited processing of p105 is the only known case in which the ubiquitin system is involved in partial degradation and generation of an active subunit rather than in complete destruction of its target substrate, it was important to further elucidate the underlying mechanisms involved in this unique process. Towards this end, it was necessary to establish a cell-free processing system that faithfully reproduces the cellular processing events. As can be seen in Fig. 2A, processing of p105 in vitro requires an intact GRR. The initial finding describing the role of the GRR in the formation of p50 was obtained by measuring, via Western blot analysis, the steady-state levels of p50 in WT p105- and p105-ΔGRR-transfected cells (27). Since formation of p50 is a dynamic process and it is not clear whether it occurs co- (26) or posttranslationally, it was important to demonstrate kinetically the relationship between the precursor protein, p105, and its product, p50, and to establish a role for the GRR in this process. A pulse-chase experiment carried out with COS-7 cells transiently transfected with WT and p105-ΔGRR is shown in Fig. 2B. It clearly confirms that, as in the cell-free system, the GRR is essential for the formation of p50 from p105 in the intact cell as well: a ΔGRR mutant does not generate p50. It has been reported recently that p50 is generated cotranslationally from a nascent p105 polypeptide chain and not from mature p105 (26). While we have not carried out a detailed kinetic analysis of the relationship between p105 as the precursor molecule and p50 as its product, this experiment as well as several other pulse-

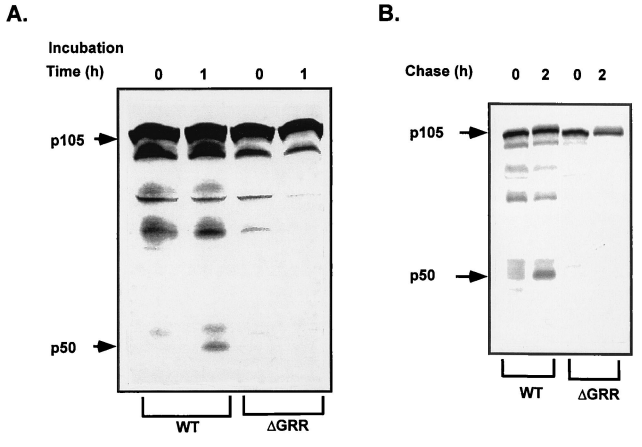

FIG. 2. Processing of WT p105 and p105-ΔGRR in vitro (A) and in vivo (B). (A) In vitro-translated WT and ΔGRR ^{35}S-labeled p105s were incubated in the presence of HeLa cytosolic extract as described in Materials and Methods. Incubation was carried out for 1 h either on ice (lanes 0) or at 37°C (lanes 1) in the presence of ATP. Reaction mixtures were resolved via SDS-PAGE, and the labeled proteins were visualized with a phosphorimager. (B) COS-7 cells transiently transfected with WT or p105-ΔGRR cDNAs were pulse-labeled with [^{35}S]methionine (lanes 0) (pulse). Following removal of the label and further incubation for 2 h (lanes 2) (chase), the labeled proteins were immunoprecipitated with anti-p50 antibody, resolved via SDS-PAGE, and visualized by phosphorimaging as described in Materials and Methods.

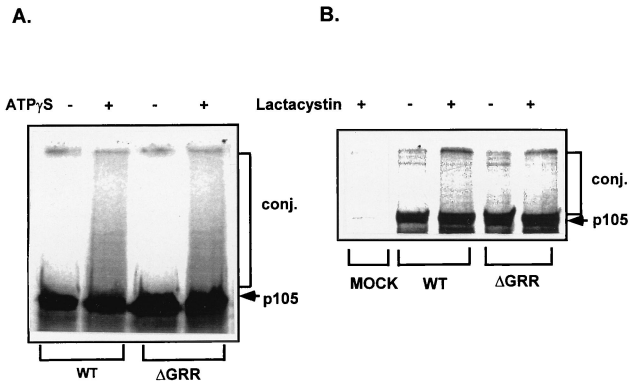

FIG. 3. Conjugation of WT p105 and p105-ΔGRR in vitro (A) and in vivo (B). (A) In vitro-translated WT and ΔGRR ^{35}S-labeled p105s were incubated in the presence of HeLa cytosolic extract, ATPγS (as indicated), ubiquitin, and UbAl, as described in Materials and Methods. Reaction mixtures were resolved via SDS-PAGE, and conjugates were visualized by exposure to a phosphorimager screen. (B) COS-7 cells were transiently transfected with the pCI-neo vector (lane MOCK) or with the same vector containing the cDNAs for WT or ΔGRR p105. Transfected cells were incubated in the presence of [^{35}S]methionine and in the presence or absence of clasto-lactacystin β-lactone as described in Materials and Methods. Following labeling, proteins were immunoprecipitated with anti-p50 antibody, resolved via SDS-PAGE, and visualized by phosphorimaging as described in Materials and Methods. Conj., ubiquitin conjugates.

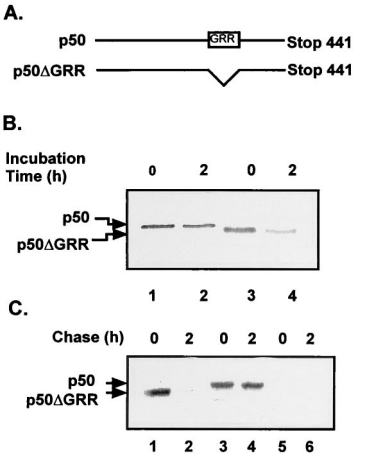

FIG. 4. Degradation of ΔGRR and GRR-containing (WT) p50s. (A) Schematic diagram of the WT and ΔGRR p50 proteins. (B) In vitro-translated and ^{35}S-labeled WT (lanes 1 and 2) and ΔGRR (lanes 3 and 4) p50s were incubated for the indicated periods in a degradation-primed cell-free system containing HeLa extract as described in Materials and Methods. Reaction mixtures were resolved via SDS-PAGE, and the proteins were visualized following exposure to a phosphorimager screen. (C) COS-7 cells were transiently transfected with the pCI-neo vector containing cDNAs for ΔGRR (lanes 1 and 2) or WT p50 (lanes 3 and 4) or with an empty vector (lanes 5 and 6). A pulse-chase immunoprecipitation experiment was carried out as described in Materials and Methods and in the legend to Fig. 2. Odd-numbered lanes contain immunoprecipitate from pulsed-labeled cells. Even-numbered lanes contain p50s recovered after a 2-h chase period.

chase experiments carried out both in vitro and in vivo (see Fig. 5, 6, 7C, and 8) shows that p50 is generated from p105 during the chase period. It appears therefore that, most probably, this problem, which is not resolved in this study, requires further investigation. The experiment shown in Fig. 2 also demonstrates that the in vitro system faithfully reproduces the cellular processing events. It should be noted that p50 was not generated from ΔGRR mutant proteins even after a longer incubation period in vitro (4 h) or following an extended period of chase in vivo (6 h) (not shown).

p105-ΔGRR is conjugated both in vitro and in vivo. Since degradation of a protein via the ubiquitin pathway involves two discrete steps, conjugation of ubiquitin and proteasomal degradation of the tagged substrate, it was important to identify the step affected by the GRR. As can be seen in Fig. 3, conjugation of p105 is not affected by GRR either in vitro (Fig. 3A) or in vivo (Fig. 3B). Since ubiquitin adducts are short-lived proteolytic intermediates, we used lactacystin, a specific inhibitor of the 20S proteasome, to increase their steady-state cellular levels. Thus, it appears that the GRR does not interfere with the conjugation machinery but that it probably does interfere with the function of the 26S proteasome.

The GRR is required to stabilize p50 both in vitro and in vivo. Although the GRR is essential for the formation of p50, its mode of action is not clear. Mechanistically, it is possible that it serves as a stop processing signal. Whether p50 is generated via progressive processing from the C terminus or following initial cleavage at the processing point (which resides downstream of the GRR), it appears that the role of GRR is to protect the newly generated p50 from further degradation. To test directly for a potential stabilizing or protective role of the GRR, we generated two p50 derivatives, one that contains the GRR and one that lacks this domain. It is important to note that both proteins share the C-terminal domain of native p50 (Fig. 4A), and the newly incorporated termination codon was inserted close to the presumed C-terminal residue of p50 (the C terminus of p50 is close to amino acid residue 435; the exact C terminus has not been identified precisely). As can be seen in Fig. 4B and C, the GRR-containing protein is significantly more stable than its mutated counterpart both in vitro and in vivo. Thus, it appears that the GRR is required not only for the generation of the p50 subunit but also for stabilizing it in the cell. It is highly likely that both formation and stabilization of p50 are mediated by the same mechanism, the inability of the 26S proteasome to further digest the molecule, most probably from its C-terminal residue (see Discussion).

Structural analysis of the GRR. To analyze the structure-function relationship of different elements within the GRR, we studied the functions of a variety of deletion and point mu-

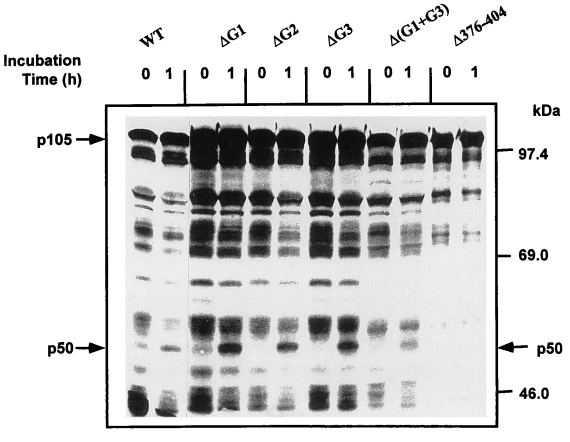

FIG. 5. Deletion analysis of the Gly clusters in the p105 GRR. The various in vitro-translated proteins were incubated in a cell-free processing system containing HeLa extract as described in Materials and Methods. The different Gly clusters are depicted in Fig. 1. ΔG1, deletion of amino acid residues 376 to 381; ΔG2, deletion of amino acid residues 382 to 391; ΔG3, deletion of amino acid residues 392 to 404; Δ(G1+G3), deletion of amino acid residues 376 to 381 and 392 to 404. Molecular mass markers are 97.4 for phosphorylase *b*, 69.0 for bovine serum albumin, and 46.0 for ovalbumin.

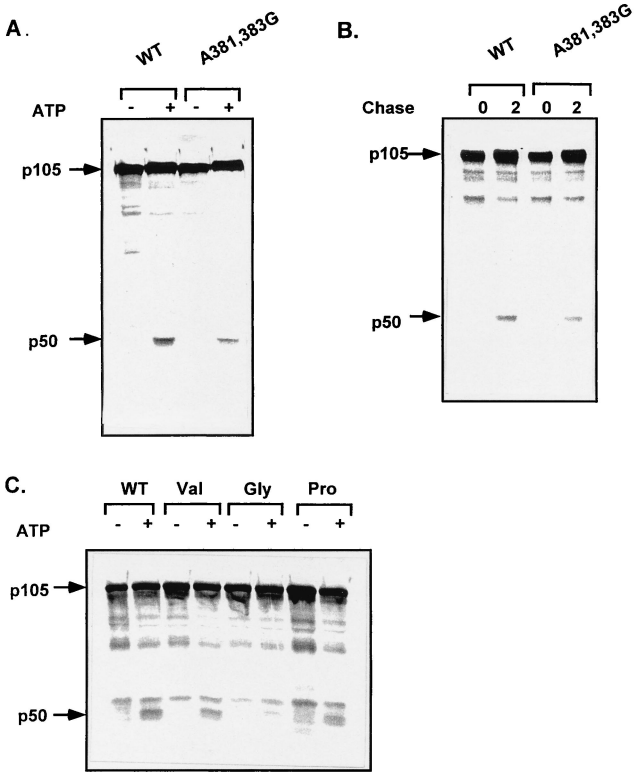

FIG. 6. Role of Ala residues 381 and 383 in the function of the GRR as a p105-processing signal. (A) In vitro-translated WT and A381,383G p105s were incubated in a cell-free processing system containing HeLa cell extract in the presence or absence of ATP, and proteins were resolved and detected as described in Materials and Methods. (B) COS-7 cells transiently transfected with cDNA encoding WT or A381,383G p105 were subjected to a pulse-chase immunoprecipitation experiment as described in Materials and Methods and in the legend to Fig. 2B. (C) In vitro-labeled and translated WT, A381,383V (Val), A381,383G (Gly), and A381,383P (Pro) p105s were incubated in a cell-free processing system containing HeLa cell extract in the presence or absence of ATP as described in Materials and Methods. Processing was monitored via SDS-PAGE and phosphorimaging of the resolved reaction mixtures.

tants. The GRR was subdivided into three clusters termed G1, G2, and G3 (Fig. 1), and deletion mutants of the different clusters were generated. As can be seen in Fig. 5, all single-cluster-deletion mutants were efficiently processed and generated the p50 subunit. This result implies that none of the clusters plays a dominant role in processing. Deletion of two clusters, G1 and G3 (protein ΔG1+G3), decreased the efficiency of processing but did not abolish it completely. It should be noted that this mutant contains only 6 (382-GAGGGGM-FGS-391) of 19 Gly residues contained in the native GRR (Fig. 1). These residues appear to be sufficient to promote, at least partially, the generation of p50.

The degradation-protective elements in EBNA-1 (25) and the artificially constructed proteolysis-resistant IκBα (37) contain GA repeats. We noted that p105-GRR contains two GA repeats, 380-GAGA-383. We also noted that in all the deletion mutants described above (Fig. 5), at least one GA repeat had been spared (Fig. 1). To investigate the role of Ala residues 381 and 383 in the processing function of the GRR, we replaced both residues with Gly. As can be seen in Fig. 6, processing of the A381,383G-p105 mutant protein was significantly reduced both in vitro (Fig. 6A) and in vivo (Fig. 6B). Notably, the substitution generated a GRR with a higher Gly content (21 versus the 19 Gly residues in the WT protein), suggesting an important role in the function of the GRR for at least one residue with a side chain. To test this notion, we replaced the two Ala residues with either Val or Pro. As can be seen in Fig. 6C, these substitutions affected only slightly the efficiency of processing. The p50 bands in Fig. 6A and C were quantified by dividing the amount of radioactivity (as determined by phosphorimager analysis) in these bands (from the reaction mixtures incubated in the presence of ATP) with the amount of radioactivity in the p105 band derived from the reaction mixture incubated in the absence of ATP. The p50/p105 ratio for WT p105 was arbitrarily designated 100%. The p50/p105 ratios for the A381,383V, A381,383P, and A381,381G mutant proteins were 78, 68, and 36%, respectively. In all cases, the total radioactivity in the reaction mixtures incubated in the presence of ATP was within 95 to 105% of the total radioactivity in the reaction mixtures incubated without ATP. Interestingly, replacement of Gly residues 385 and 387 with Ala and generation of four successive GA repeats did not increase the efficiency of processing of WT p105 (not shown). This finding suggests that a single GA, along with a few Gly residues, is sufficient to promote processing. Similar findings concerning inhibition of degradation of IκBα were also described by Sharipo and colleagues (37). However, it should be stressed again that with IκBα, the GRR and the GA repeat completely abolished degradation regardless of their sites of insertion: no processing could be observed. With p105, the native molecule, the GRR was essential for limited processing. The difference between the behavior of EBNA-1 and IκBα on one hand and p105 on the other hand is probably due to the requirement for an additional motif necessary for limited processing (see Results and Discussion).

Universality of the GRR as a transferable processing signal. Since one or a few GA repeats in the midst of a short GRR appears to constitute a sufficient processing signal for p105, it was important to study the universality of this sequence as a transferable processing element. Thus, we inserted the GRR into several proteins. Initially, we subcloned the GRR into a closely related protein, *Drosophila* Dorsal. Its N-terminal domain is homologous to the p50 domain of p105; however, its C-terminal *trans*-activation domain does not bear any homology to p105 (7). We inserted the GRR into Dorsal between amino acid residues 349 and 350, in a location similar to its position in p105, which is 9 amino acid residues downstream from the nuclear localization signal. Incubation of Dorsal-GRR in a cell-free processing system in the absence (Fig. 7B) or the presence (reference 16 and data not shown) of its inhibitory protein Cactus did not yield any detectable processing product. In addition, we inserted the GRR into several other bona fide substrates of the ubiquitin-proteasome system such as MyoD (references 1 and Fig. 7A), c-Fos (reference 38 and data not shown), and ODC (reference 31 and data not shown) (for ODC, experiments were carried out with and without antizyme; for details, see Materials and Methods). However, in none of these cases were we able to observe a processing product (Fig. 7A and B and data not shown). In the case of MyoD and ODC, insertion of an even longer fragment (residues 376 to 440) did not render the protein susceptible to processing (data not shown). These findings suggest that processing may require an additional motif that acts along with the GRR. It is also possible that folding of these proteins, in which the GRR has been inserted in the middle of the native molecule, hinders it sterically and renders it inaccessible. Therefore, it was important to test the function of GRR as a processing motif when it was inserted between two unrelated proteins that probably fold independently. To that end, we constructed a chimeric protein between p53 and ODC and inserted the GRR

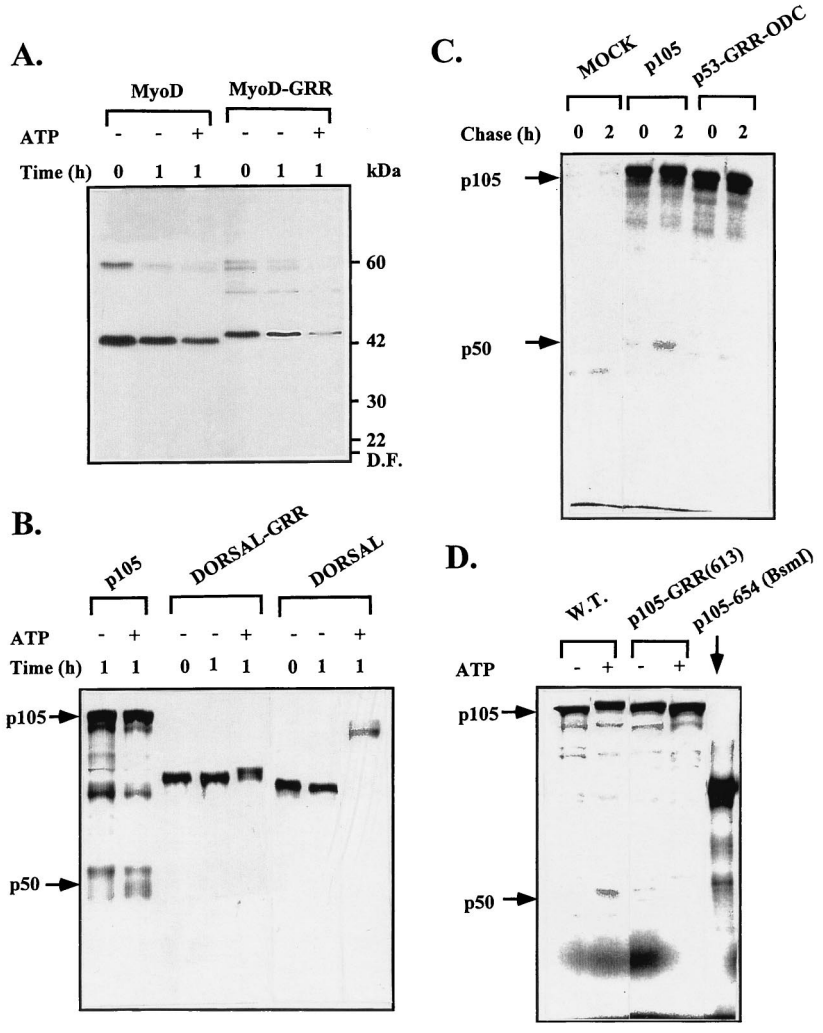

FIG. 7. Lack of processing of MyoD-GRR (A), Dorsal-GRR (B), p53-GRR-ODC (C), and p105-GRR(613) (D). The different constructs were generated as described in Materials and Methods. Dorsal and Dorsal-GRR (B) and p105-GRR(613) (D) were translated in vitro in the presence of [^{35}S]methionine, and their processing was detected as described in Materials and Methods. The MyoD proteins (A) were expressed in bacteria and detected, following a cell-free processing assay, via Western blot analysis as described previously (1). p53-GRR-ODC and WT p105 (C) were transiently expressed in COS-7 cells, and their processing was monitored in a pulse-chase experiment, followed by immunoprecipitation as described in Materials and Methods. p105-654(BsmI) is a p105 construct that was linearized with *Bsm*I prior to translation. In vitro translation of this cDNA yielded a product with a molecular mass similar to that of the putative predicted p70 product of p105-GRR(613). This construct was generated to serve as a molecular mass marker for p70. D.F., dye front.

between the two domains (p53-GRR-ODC). Here too, we were not able to detect any processing in intact cells (Fig. 7C). Similar results were obtained in vitro following incubation of the chimeric protein in the absence or presence of either E6, antizyme, or both (not shown). Interestingly, this chimeric protein contains a large fragment (residues 343 to 435) derived from p105. Nevertheless, it could not promote processing. Failure to detect processing in all of these GRR-containing proteins is probably due to the fact that the GRR is necessary but not sufficient to direct processing of p105 and cannot serve in all cases as a sole universal and transferable processing or stop signal. To further study the possible existence of an additional motif required for processing, we tested whether the GRR can be transferred within the p105 molecule. Using p105-ΔGRR, we replaced amino acid residues 604 to 613 (RAGADLSLLD) with the sequence 604-GAGAGGGGGG-613 [p105-GRR (613)]. Again, we could not detect generation of any processing product ("p70" is the predicted processing product) either in in vitro (Fig. 7D) or in vivo (not shown). This finding clearly shows that the site of insertion of the GRR within p105 is critical, which may be due to specific folding of the two domains of p105 and/or to an additional recognition or targeting motif that resides in the vicinity of, and downstream from, the GRR (see Discussion).

A region downstream from the GRR is required for processing. In an attempt to identify putative structural motifs, in addition to the GRR, that are required to promote processing, we noted a sequence that resides downstream to the GRR and that is highly homologous to the ubiquitination and E3-binding domain of IκBα (Table 1) (35, 40, 42). To test the possible role of this motif in p105 processing, we generated two groups of p105 mutants: one containing K441R, K442R, and K441,442R, which are single- or double-mutation proteins in which we replaced (with Arg) the two homologous Lys residues that in IκBα serve as ubiquitination sites (Table 1), and one containing p105-Δ446-454, which lacks the residues flanking Ser 450

TABLE 1. Comparison of the ubiquitination and recognition domains of human p105 (residues 441 to 454) and IκBα (residues 21 to 39)

Protein	Sequence[a]
Human p105	440-SKKDPEGCDKSDD-454
	||+ +
Human IκBα	20-LKKERLLDDRHDSGLDSMKD-39

[a] The pair of lysine residues that serve as ubiquitination sites in both molecules are marked by vertical lines. Two overlapping acidic residues are marked by plus signs. Ser residues are boldface, whereas all acidic residues are underlined.

and which is similar to Ser 32 of IκBα. Phosphorylation of Ser 32 and 36 in IκBα targets the molecule for conjugation and degradation (8, 9, 40, 42). It should be noted that all these mutant proteins contain intact GRRs. The proteins were tested for processing both in vitro (Fig. 8A) and in vivo, following expression in COS-7 cells (Fig. 8B). As can be clearly seen, the two Lys residues and amino acid residues 446 to 454 are independently important for processing, which is markedly reduced in their absence. Interestingly, and in striking contrast to IκBα (35), each of the Lys residues is essential for processing (see Discussion). As can be seen in Fig. 9 and in correlation

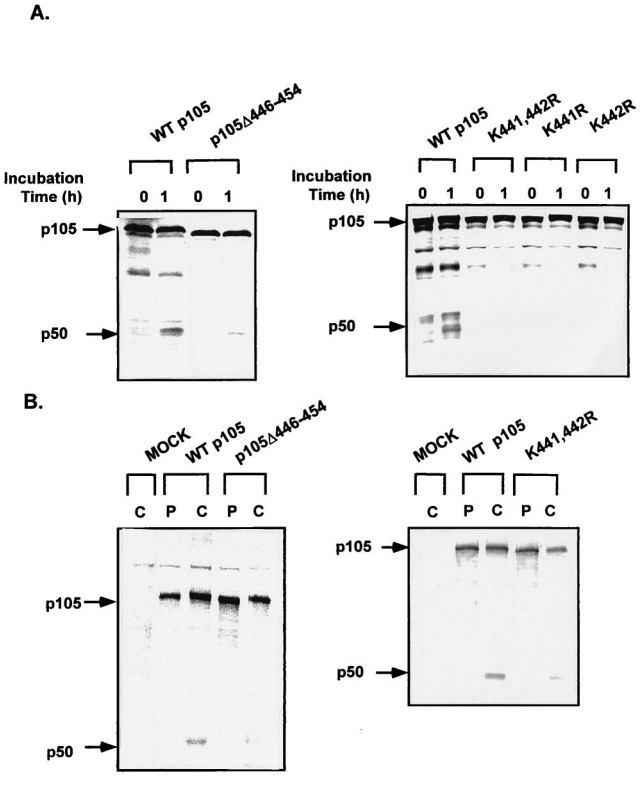

FIG. 8. A motif residing downstream of the GRR is required for processing of p105. (A) WT and Δ446-454 (left gel) and WT, K441,442R, K441R, and K442R (right gel) p105s were translated in vitro in the presence of [^{35}S]methionine and incubated in a cell-free processing system containing HeLa cell extract as described in Materials and Methods. Reaction mixtures were resolved via SDS-PAGE, and proteins were visualized following exposure to a phosphorimager screen. (B) COS-7 cells were transiently transfected with the empty pCI-neo vector (MOCK) or pCI-neo vectors containing WT (left and right gels), Δ446-454 (left gel), or K441,442R (right gel) p105. Pulse-chase and immunoprecipitation experiments were carried out as described in Materials and Methods and in the legend to Fig. 2B. P denotes 30 min of pulse labeling. C denotes 2 h of further incubation in the presence of unlabeled methionine (chase).

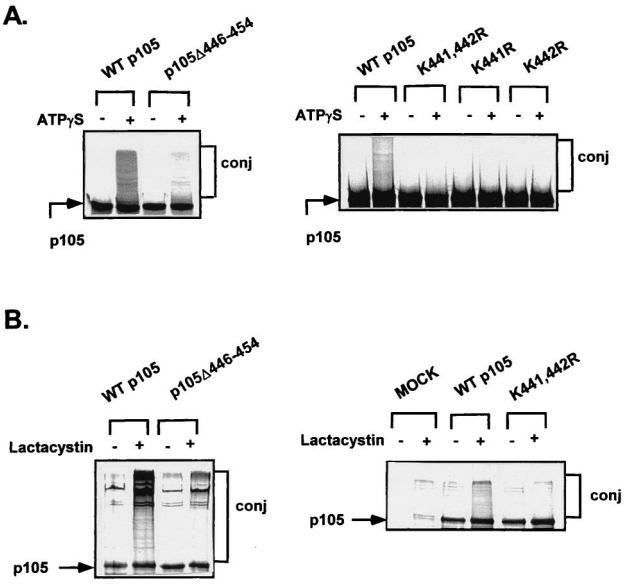

FIG. 9. Δ446-454, K441R, K442R, and K441,442R p105s fail to generate conjugates. (A) WT and Δ446-454 (left gel) and WT, K441,442R, K441R, and K442R (right gel) p105s were translated in vitro in the presence of [^{35}S]methionine and incubated in a HeLa cell extract-containing cell-free system primed for conjugation as described in Materials and Methods. Reaction mixtures were resolved via SDS-PAGE, and proteins were visualized following exposure to a phosphorimager screen. (B) COS-7 cells were transiently transfected with the pCI-neo vector alone (MOCK) (right gel only) or with the same vector containing cDNAs coding for WT (both gels), Δ446-454 (left gel), or K441,442R (right gel) p105s. Clasto-lactacystin β-lactone was added following 30 min of incubation with [^{35}S]methionine, and incubation continued for an additional 2 h as described in Materials and Methods. Cells were harvested and lysed, and following immunoprecipitation with anti-p50 antibody, proteins were resolved via SDS-PAGE and visualized by phosphorimaging. Conj., conjugates.

with inhibition of processing, conjugation of ubiquitin to the mutant proteins was also reduced significantly both in the cell-free system (Fig. 9A) and in the intact cell (Fig. 9B). To rule out, at least partially, a nonspecific effect of the alterations in the structure of p105 caused by the mutations and deletions, we removed amino acid residues 405 to 435, which are immediately adjacent to the GRR. p105-Δ405-435 was processed with the same efficiency as its WT counterpart (not shown). Attesting to the specificity of Lys 441 and 442 as ubiquitination sites is the fact that Lys residues that reside upstream (residues 426 and 432; deleted along with residues 405 to 435) or downstream (residues 449 and 453; these were present in the p105-K441,442R mutant) could not substitute for Lys 441 and 442. Interestingly, and unlike for Ser 32 of IκBα, the role of Ser 450 of p105 is not clear yet. Processing of p105-S450A was indistinguishable from that of the WT protein in vivo (not shown; see Discussion). Thus, it appears that Lys residues 441 and 442 probably serve as major ubiquitination sites and that the downstream sequence is part of the recognition domain of p105 E3 that does not have to undergo a posttranslational modification.

WT p105 but not p105-K441,442R, p105-Δ446-454, or p105-ΔGRR generates NF-κB that is active in DNA binding. It is expected that specific mutations in the recognition, ubiquitination, and processing domains that inhibit processing and reduce generation of p50 will also affect the biological function of NF-κB. To test this notion, we transiently transfected COS-7 cells with the WT, ΔGRR, K441,442R, and Δ446-454 p105s and monitored binding of labeled κB probe in EMSAs as a measure of formation of biologically active p50. As can be seen in Fig. 10, WT p105 generates active NF-κB that binds the

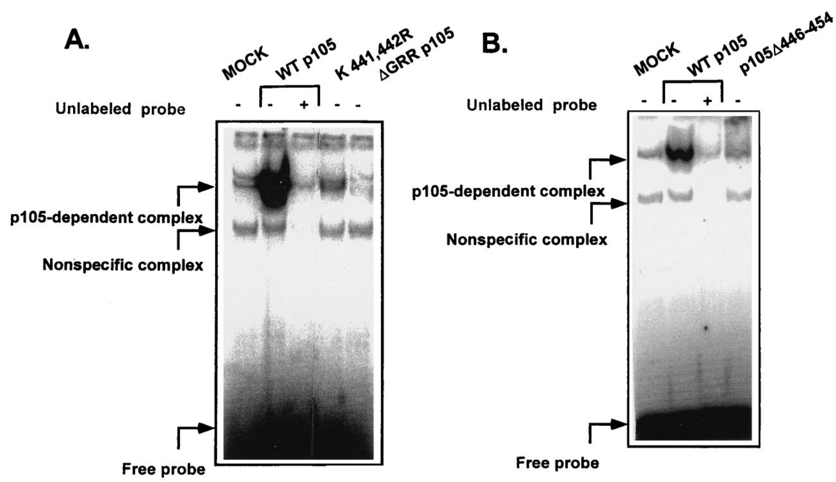

FIG. 10. WT but not K441,442R and ΔGRR (A) and Δ446-454 (B) p105s generate NF-κB active in DNA binding. (A) COS-7 cells were transiently transfected with the pCI-neo vector alone (MOCK) or with the same vector with cDNAs coding for WT, K441,442R, or ΔGRR p105. After 40 h, cells were lysed and EMSA was carried out with a ^{32}P-κB end-labeled probe in the presence or absence of excess unlabeled probe as described in Materials and Methods. (B) The same experiment was performed with WT and Δ446-454 p105s.

radiolabeled probe. The probe could be further shifted by anti-p50 antibody (not shown), and binding was abolished by an excess of unlabeled probe (Fig. 10), further confirming the specificity of the binding. In striking contrast, p105-ΔGRR did not generate any binding activity (Fig. 10A), whereas the activities generated by p105-K441,442R (Fig. 10A) and p105-Δ446-454 (Fig. 10B) were markedly reduced.

DISCUSSION

We have shown that the GRR that spans amino acid residues 376 to 404 in the p105 precursor protein is essential for limited processing of the molecule both in vitro and in vivo (Fig. 2). The GRR probably interferes with the activity of the proteasome and not with the conjugation machinery, as p105-ΔGRR is conjugated to the same extent as its WT counterpart (Fig. 3).

The mechanism of action of the GRR is not clear. One can think of two possible mechanisms. The first involves processive digestion from the C-terminal residue, while the second entails a single cleavage event at the C-terminal amino residue of p50 (approximately amino acid residue 435). In both cases, the structure of the GRR probably prevents entry into the proteasome and further degradation of p50, which subsequently falls off the protease complex. Thus, the GRR does not have only a stop processing function; the prediction is that it will protect p50 from degradation by the ubiquitin system. Figure 4 shows that this is indeed the case and that p50-ΔGRR is significantly more sensitive to degradation than its GRR-containing counterpart.

Analysis of the Gly repeat shows that, in addition to the few Gly residues, processing also requires at least one Ala or another amino acid residue with a side chain (Fig. 5 and 6). Comparison of several Gly repeats derived from p105s of several different species revealed that all of them contain two GA repeats which are not necessarily adjacent to one another (Table 2). Deletion of other residues such as M and F that are common to many GRRs does not affect processing (Fig. 5). Interestingly, Relish, the *Drosophila melanogaster* homolog of p105, contains a Ser-rich region (459-G*SSANSSSSG*TE SSNNS-475) localized to an area in the molecule homologous to that of the GRR-containing domain in p105 (13). This region also contains two separated single Gly residues (marked by asterisks) and a single Ala residue (underlined). It is not known, however, whether this protein undergoes processing. Another interesting protein is the membrane-anchored sterol-regulated transcriptional factor SREBP-1. In response to sterol deprivation, the protein is released from the endoplasmic reticulum via a mechanism that involves limited processing close to amino acid residue 585. The protease involved, S1P, is distinct from the 265 proteasome (34a), and the mechanism of cleavage have not been identified. It is interesting that the SREBP-1 protein substrate contains a short GS repeat spanning amino acid residues 444 to 457 (GSRGSGSGGSDS) (39).

Mechanistically, the GRR does not act as a specific proteasome binding domain or recognition motif. A synthetic peptide that spans the GRR domain and additional flanking sequences at both termini did not inhibit processing (data not shown). A similar conclusion was also drawn by Lin et al. (26). It is possible that the GRR generates a loose structure that cannot penetrate the proteasomal "pore" and thus constitutes a physical barrier for further processing and complete degradation of the molecule. The fact that processing occurs downstream of the C-terminal residue of the GRR at a distance that may be similar to the length between the site of entry into the 26S proteasome and the catalytic sites that reside on the inner β-subunit rings of the 20S proteasome may support this hypothesis. Also, the lack of the ability to detect the C-terminal domain of p105 suggests that processing proceeds in a processive manner from the C-terminal residue of the molecule. Obviously, a single cleavage with rapid degradation of the

TABLE 2. Comparison of GRRs in chicken, mouse, and human p105s

<table>
<tr><th>Source or protein</th><th>Sequence[a]</th></tr>
<tr><td>Human</td><td>376-GGGSGAGAGGGGMFSGGGGGGTGSTGPG-404</td></tr>
<tr><td></td><td>|||||||||||||||||||||*|||*||</td></tr>
<tr><td>Mouse</td><td>372-GGGSGAGAGGGGMFSGGGGGGSTGSPGPGYG-402</td></tr>
<tr><td></td><td>|*|*|*||||||||||*||||***|</td></tr>
<tr><td>Chicken</td><td>378-GYGGGSGAGGGGMFGGGGGGAGSG-401</td></tr>
</table>

[a] Identical residues are marked by vertical lines, while different residues are marked by asterisks.

C-terminal "leaving" polypeptide is also a plausible mechanism. In this case, the remaining p50 subunit would not be able to bind and be proteolyzed by the proteasome, as it would be protected by the C-terminal GRR.

To test whether the GRR can act alone as a universal transferable processing motif, we inserted it into Dorsal, a closely related *Drosophila* protein, as well into a series of bona fide substrates of the ubiquitin-proteasome system and a chimeric protein composed of two independent domains, p53 and ODC. In none of these cases were we able to observe processing (Fig. 7 and data not shown). It should be noted, however, that Lin and Ghosh initially reported (27) that gp10–GRR–glutathione *S*-transferase can undergo processing. However, in a later study (26) Lin et al. reported that the mirror image protein, glutathione *S*-transferase–GRR–gp10, cannot be processed. In addition, Levitskaya and colleagues (25) and Sharipo and colleagues (37) have shown that the EBNA-1 GA repeat can serve as an inhibitor for degradation but that it cannot promote processing. Thus, it appears that in the vast majority of cases, the GRR cannot act as a universal transferable endoproteolytic processing signal. Therefore, we postulated that processing requires an additional essential signal. We noted that amino acid residues 441 to 454 within the p105 molecule have homology with the ubiquitination and E3 recognition motifs of IκBα (35, 40, 42) (Table 1) and the targeting motif of β-catenin (34, 40). This result is not surprising, as the C-terminal portion of p105, also designated IκBγ, contains ankyrin repeats homologous to members of the IκB family of proteins. Indeed replacement of the Lys residues 441 and 442 reduced significantly conjugation and processing of p105 and the subsequent DNA-binding capacity of cellular NF-κB (Fig. 8 to 10). An interesting problem is why replacement of a single Lys residue, with the second Lys being retained, inhibits conjugation and processing completely. It is possible that two neighboring polyubiquitin chains must be synthesized in order to allow processing, though steric constraints may render such a modification unlikely. A remote possibility is that the Lys residues do not serve as ubiquitination sites and are part of the E3 recognition domain. Similarly, removal of residues 446 to 454 that reside downstream to the two Lys residues also inhibited conjugation, processing, and the resulting DNA-binding capacity of NF-κB (Fig. 8 to 10). It is possible that this motif serves as the E3-binding domain. However, unlike IκBα Ser residues 32 and 36 and β-catenin Ser 37, p105 Ser 450 does not appear to serve as an essential phosphorylation site. It should be noted that phosphorylation of Ser residues within the C-terminal PEST region of p105 stimulates somewhat processing of p105 (15, 28) but not of p100 (5). Thus, it is possible that E3 recognizes p105 via two distinct sites and that the abolishment of one site inhibits ubiquitination, processing, and DNA binding significantly, but not completely. We noted that Sears and colleagues (36) generated chimeric proteins between the GAL4 DNA-binding domain and the activation domain of the class II transcriptional activator CIITA, in which they inserted the GRR between the two domains. The chimeric protein was processed in COS-7 cells. Interestingly, however, the transferred GRR spanned amino acid residues 371 to 503 or 371 to 544 of p105, both of which contain the newly described ubiquitination and recognition domain. Lin et al. (26) and Sharipo et al. (37) have also proposed the existence of a downstream motif that is required along with the GRR to promote processing. However, no experimental evidence concerning its identity has been provided.

Taken together, the findings suggest a model for the generation of p50. Whether p50 is processed co- or posttranslationally, at least three successive events must occur. The precursor molecule must be recognized by an E3 via binding to a specific motif and is subsequently ubiquitinated at a unique Lys residue(s). This leads to the second step, recognition by the proteasome and processing from the C-terminal residue of p105 or from the last translated residue of the nascent polypeptide chain. Because of the structure of the proteasome where the catalytic subunits reside at a distance from the orifice of the complex, processing is halted by the GRR close to amino acid residue 435, approximately 30 residues downstream from the GRR, where the newly generated p50 falls off (third step) the proteasome. Thus, by inhibiting further insertion of the polypeptide chain into the proteasomal catalytic chamber, which results in dissociation of p50 from the proteasome, the GRR acts, albeit indirectly, to stabilize the newly formed p50.

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