MOLECULAR AND CELLULAR BIOLOGY, May 1996, p. 2248–2254 Vol. 16, No. 5
0270-7306/96/$04.00+0

A Glycine-Rich Region in NF-κB p105 Functions as a Processing Signal for the Generation of the p50 Subunit

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Received 11 December 1995/Returned for modification 10 February 1996/Accepted 15 February 1996

Transcription factor NF-κB is generally considered to be a heterodimer with two subunits, p50 and p65. The p50 subunit has been suggested to be generated from its precursor, p105, via the ubiquitin-proteasome pathway. During processing, the C-terminal portion of p105 is rapidly degraded whereas the N-terminal portion (p50) is left intact. We report here that a 23-amino-acid, glycine-rich region (GRR) in p105 functions as a processing signal for the generation of p50. A GRR-dependent endoproteolytic cleavage downstream of the GRR releases p50 from p105, and this cleavage does not require any specific downstream sequences. p50 can be generated from chimeric precursor p105N-GRR-IκBα, while the C-terminal portion (IκBα) can also be recovered, suggesting that p105 processing includes two steps: a GRR-dependent endoproteolytic cleavage and the subsequent degradation of the C-terminal portion. We have also demonstrated that the GRR can direct a similar processing event when it is inserted into a protein unrelated to the NF-κB family and that it is therefore an independent signal for processing.

NF-κB was originally identified as a transcription factor that binds to the κB site in the intronic enhancer of the immunoglobulin κ light-chain gene in B lymphocytes (38). It was later found in all mammalian cells as a heterodimer whose subunits, p50 and p65, belong to the multigene Rel family (2, 40). In mature B lymphocytes and some monocytes and macrophages, NF-κB is constitutively active (38), whereas in most other cells, NF-κB is inactive, as it forms cytoplasmic ternary complexes with its inhibitors IκBα and IκBβ (1, 44). NF-κB activity can be induced by a large number of inducers, such as tumor necrosis factor alpha, lipopolysaccharide, phorbol myristate acetate, interleukin 1, and double-stranded RNA (3, 9, 12, 28, 42). Upon induction, IκB proteins are phosphorylated, ubiquitinated, and then subjected to degradation (10, 16, 20, 24, 46). The released p50-p65 complex subsequently translocates into the nucleus and initiates NF-κB-dependent transcription.

The p50 subunit of NF-κB is synthesized as a 105-kDa precursor called p105, and the N-terminal portion of p105 is p50 (17, 22). p50 consists of the Rel homology domain (RHD) shared by all members of the Rel family, a glycine-rich region (GRR) directly adjacent to the RHD, and a string of 33 to 35 amino acids after the GRR. The C-terminal portion of p105 is identical to IκBγ, which is a product of an alternatively spliced RNA found in certain mouse pre-B cells (17, 21, 25). IκBγ is homologous to other IκBs in that it has ankyrin repeats (4). The RHD is responsible for binding to DNA, dimerization, and interaction with IκB (15). The structure of the RHD in p50 resembles the structure of immunoglobulins and the core domain of tumor suppressor p53 (15, 30). Another Rel family protein, p52, shares significant homology with p50 and is also generated from a precursor, p100. The p100 protein resembles p105 in that it contains an RHD, a GRR, and the C-terminal ankyrin repeats, and it is likely that both p50 and p52 are generated from their precursors by similar mechanisms (6, 33, 37).

The processing of p105 has been proposed to be due to an ATP-dependent proteolytic event (14). The proteasome, a large multicatalytic cellular degradation machine, has been implicated in this process (34). Unprocessed full-length p105 can form a complex with p65 that is retained in the cytoplasm, presumably through the IκB-like function of the C-terminal portion of p105 (32, 35). Extracellular stimuli not only induce the degradation of IκBα and IκBβ in the p50-p65-IκB complexes but also enhance the processing of p105 in the p105-p65 complex (28, 29, 31). Upon removal of the p105 C-terminal portion, the resulting p50-p65 complex translocates to the nucleus and thus provides an additional source of active NF-κB. However, p50, as a component of the NF-κB complex, exists in all cells in the absence of external stimuli (14, 17). The in vitro translation of either full-length or C-terminal deletion fragments of p105 always results in two products (corresponding to the sizes of the full-length fragments and to p50) in an equal ratio (14, 17), and the same phenomenon has also been observed when p105 clones are expressed in vivo (32). These observations suggest that p50 can be generated without additional stimuli.

Recently, serine residues 32 and 36 in IκBα have been found to be required for phosphorylation, ubiquitination, and degradation via the 26S proteasome in response to various stimuli (7–10, 45). Although the ubiquitin-proteasome pathway has also been shown to be involved in p105 processing (34), the *cis* elements in p105 that are responsible for processing are unknown. Also, unlike IκBα and other known examples involved in proteasome-dependent degradation in which the entire target protein is degraded, p105 is processed in an unique way in which only its C-terminal portion is degraded.

We report here that a 23-amino-acid GRR in p105 functions as a processing signal for the generation of the p50 subunit. An endoproteolytic cleavage downstream of the GRR releases p50. Hence, our results provide an explanation for the incomplete processing of p105. Furthermore, we have shown that the GRR can direct the cleavage and subsequent degradation of a protein unrelated to those of the NF-κB family, and therefore, it appears to be an independent processing signal. Thus, the recognition and binding of the GRR could serve as an impor-

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tant control step in the ubiquitin-proteasome pathway for the generation of p50.

MATERIALS AND METHODS

Enzymes and antibodies. The restriction enzymes and T4 DNA ligase used for the subcloning and construction of mutants were from either New England BioLabs or Boehringer Mannheim. The *Pfu* DNA polymerase used in the PCR was from Stratagene Cloning System. The Sequenase version 2.0 DNA sequencing kit from United States Biochemical was used for DNA sequencing.

The rabbit polyclonal antibody against the N-terminal 340 amino acids of p105 was generated by this laboratory. Rabbit polyclonal antibodies against amino acids 6 to 20 of IκBα (N terminus, C-15) and amino acids 297 to 317 of IκBα (C terminus, C-21) were from Santa Cruz Biochemical, Inc. Rabbit polyclonal antibody against the N-terminal portion of p65 (antibody A) and antibody against amino acids 471 to 490 of IκBγ (C terminus, 5177C) were also from Santa Cruz Biochemical, Inc. The mouse monoclonal antibody against the 12 amino acids in the N terminus of T7 gp10 was a gift from F. William Studier (Brookhaven National Laboratory), and the rabbit polyclonal antiserum against glutathione *S*-transferase (GST) was a gift from Richard A. Flavell (Yale University School of Medicine).

The control plasmids. Control plasmid pGL048a was made by inserting the mouse p105 coding sequence (bp 292 to 3207) (17) between a *Bam*HI site and an *Xba*I site in mammalian expression vector pEVRF (27). p105 expressed from pGL048a is a fusion protein that adds 6 amino acids (MASWGS; the last two amino acids correspond to *Bam*HI site GGATCC) from the vector to the N terminus. pGL048a was used as wild-type control for all in vivo experiments.

Control plasmid pGL015 was constructed by inserting the coding sequence of mouse IκBα (bp 95 to 1048) into T7 expression vector pET3B (41) at an *Nde*I site and a *Bam*HI site. IκBα was expressed in *Escherichia coli* BL21 (DE3)/pLysS as described by Studier et al. (41), and the bacterial lysate was used as the IκBα marker.

Control plasmid pGL114a was constructed by the insertion of the T7 gene 10 coding sequence (bp 22966 to 24001 of the T7 genome) into the pEVRF vector at a *Bam*HI site and an *Xba*I site.

Construction of p105 deletion mutants, fusions, and other hybrids. All p105 deletion mutants and fusions used in this work were derived from plasmid pGL048a by using existing restriction sites or by introducing unique or convenient restriction sites via oligonucleotide primers for PCR. Hybrids were constructed by ligating PCR fragments into the pEVRF vector. The 23-amino-acid GRR sequence used in the hybrids is flanked with a *Hin*dIII site (forward primer OGL049; the *Hin*dIII site is underlined, and the nucleotide sequence of p105 following the *Hin*dIII site starts from bp 1407) and an *Eco*RI site (reverse primer OGL057; the *Eco*RI site is underlined, and the nucleotide sequence of p105 following the *Eco*RI site starts from bp 1476). The primer sequences are 5′-CCCAAGCTTGGCGGCGGCAGTGGAGCG-3′ (OGL049) and 5′-GGAATTCGGTACTCCCTCCGCC-3′ (OGL057).

The 261-amino-acid segment from the N terminus of T7 gp10 used in the fusion (pGL083a) was a PCR product from vector pET3xA (41), and the full-length T7 gp10 used in the hybrids (pGL115a and pGL122a) was a PCR product from the T7 genome. The GST sequence used in hybrid pGL115a was a PCR product from vector pGEX-2T (Pharmacia).

The deletion mutants, fusions, and hybrids have been DNA sequenced to some extent or carefully restriction mapped to confirm their sequences and reading frames.

Expression of p105 deletion mutants, fusions, and hybrids in COS1 cells. p105 deletion mutants and fusions were transfected into COS1 cells by the standard DEAE dextran method. About 10 μg of DNA was used per transfection in a 200-by-10-mm Falcon tissue culture dish (with about 5×10^6 cells). After 3.5 h of incubation with DEAE dextran and DNA, the cells were washed twice with 1× phosphate-buffered saline (PBS) and covered with 10 ml of fresh Dulbecco modified Eagle medium (with 10% fetal calf serum, 2 μM L-glutamine, and 100 μg of penicillin-streptomycin per ml). After 48 h, the transiently transfected cells were washed several times with cold 1× PBS and then lysed with 200 μl of ELB buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) at room temperature for 5 min. The lysates were then centrifuged at 14,000 rpm for 20 min at 4°C. The supernatants were either used immediately for Western blot (immunoblot) analysis or stored at −20°C for future analysis.

Western analysis. Small amounts of cell lysates (5 to 15 μl) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 or 12% gels, transferred to an Immobilon-P membrane (Millipore) at 100 V and 4°C for 1 h, blotted with the primary antibody for 2 h and with the secondary antibody (either anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody from donkeys for polyclonal antibodies or anti-mouse immunoglobulin, horseradish peroxidase-linked whole antibody from sheep for monoclonal antibodies [Amersham Life Science]) for 1 h, and then subjected to enhanced chemiluminescence (Amersham Life Science).

RESULTS

The GRR is the key element for the generation of p50. Previous results have shown that p50 can be generated both in vitro and in vivo in the absence of most of the p105 C-terminal portion (14, 17, 34). Therefore, we assumed that the signal required for the processing was not in the C-terminal portion of p105. To identify the element critical for the generation of p50, we mapped the N-terminal portion of mouse p105 by deleting different structural blocks on the basis of information on the three-dimensional structure of p50 (RHD) (15). The principle of the mapping was to keep the C-terminal portion of p105 intact while deleting different structural blocks in the N-terminal portion. By this method, the full-length and processed products would be clearly resolved within the normal range of SDS-PAGE. The basic structure of p105 is summarized in Fig. 1a.

We chose to analyze p105 processing in COS1 cells because of the high level of expression from plasmid vector pEVRF (27). Expression in vivo also avoids the problem of the incomplete and inefficient translation that occurs when long fragments are translated in vitro. After plasmids harboring the mutants were transfected into COS1 cells, small amounts of the cell lysate were analyzed by SDS-PAGE and the relevant products were visualized by Western analysis with a polyclonal antibody against the N-terminal 340 amino acids of p105. Deleting either subdomain 1 or 2 of the RHD or the short loop linking the two subdomains (15, 30) did not affect the generation of the processed product (Fig. 1a and b, lanes 3, 6, and 7 [pGL071a, pGL079b, and pGL073a, respectively]), and the migration of the processed products on SDS-PAGE gels agreed with the estimated molecular sizes. Short deletions after the C terminus of the GRR, including those after the presumed C terminus of p50 (amino acid 433 [unpublished results]), also did not affect the generation of the processed product (Fig. 1a and b, lanes 8 to 10 [pGL076a, pGL077a, and pGL078a, respectively]). However, any deletions that included a 35-amino-acid GRR (amino acids 364 to 398) completely abolished the generation of a processed product (Fig. 1a and b, lanes 4 and 5 [pGL070a and pGL075a, respectively]). Further deletions within the 35-amino-acid GRR revealed that the cluster of 8 nonglycine amino acids at the N terminus of the region (amino acids 364 to 371) (Fig. 1d) was not required (Fig. 1c, lane 4 [pGL093a]), whereas deletion of the remainder of this region (amino acids 372 to 398) (Fig. 1d) abolished the generation of p50 (Fig. 1c, lane 3 [pGL092a]). Replacement of amino acids 395 to 400 with the thrombin recognition sequence (Fig. 1d) also did not affect the generation of p50 (data not shown). Therefore, amino acids 395 to 398 are also dispensable (Fig. 1d). Hence, the remaining 23-amino-acid (372 to 394), glycine-rich peptide is defined as the critical element for the generation of p50 (Fig. 1d).

An endoproteolytic cleavage downstream of the GRR releases p50 from the p105 C-terminal portion. The generation of p50 has been suggested to be due to an ATP-dependent proteolytic degradation of the p105 C-terminal portion by the 26S proteasome (34). No C-terminal intermediate of the processing has ever been recovered (14, 19, 36). Antibodies to IκBγ do not detect any degradation products corresponding to the C-terminal portion of p105 (19), suggesting that this portion may be particularly sensitive to degradation. Therefore, it is possible that the unique structure of the C-terminal portion of p105 might determine its vulnerability in the cytoplasm. To determine how the GRR was related to the processing of the C-terminal portion of p105 and also to confirm that the entire C-terminal portion of p105 was not required for the generation

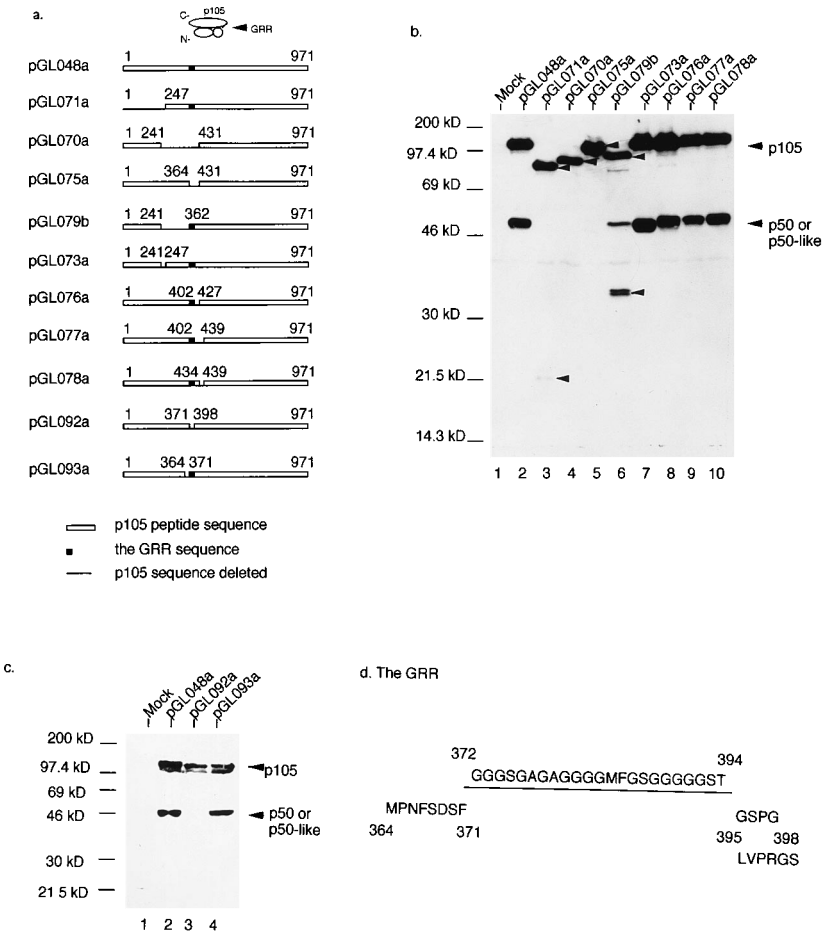

FIG. 1. Western analyses of lysates from COS1 cells transfected with p105 deletion plasmids. (a) Mouse p105 deletion constructs. The basic structure of p105 is shown at the top. The N-terminal portion is divided into two subdomains linked by a 7-amino-acid loop, and the position of the GRR is indicated. Amino acid sequences of the deleted region are labeled. (b) Deletion mapping of the p105 N-terminal portion. The lysates were resolved on an SDS–12% PAGE gel. Mock (lane 1) was the lysate from cells transfected with or without the vector plasmid. pGL048a was the wild-type p105-p50 control (lane 2). The positions of p105 and p50 are marked. The full-length deletion proteins and the processed products are marked with arrowheads, and their molecular sizes are estimated as follows: pGL071a, 80 kDa (the processed product is 21 kDa) (lane 3); pGL070a, 86 kDa (lane 4); pGL075a, 99 kDa (lane 5); and pGL079b, 94 kDa (the processed product is 35 kDa) (lane 6). The rest of the deletion mutants (pGL073a, pGL076a, pGL077a, and pGL078a [lanes 7 to 10]) have smaller deletions, and their processed products are p50-like and migrate slightly slower than the p50 control. In lanes 3 and 6, the lower amounts of the processed products in the blot might be due to either the instability of the products or the reduced efficiency of processing. For an unknown reason, in addition to the expected processed product, there is a band parallel to p50 in lane 6 (pGL079b). (c) Deletion mapping of the 35-amino-acid region. The lysates were resolved by SDS–10% PAGE. pGL048a was the wild-type p105-p50 control (lane 2). pGL092a (lane 3) lacks the 27-amino-acid GRR, and pGL093a (lane 4) lacks the 8 nonglycine amino acids. (d) The GRR peptide sequence. The 23-amino-acid functional region of the GRR is underlined.

of p50, we replaced this portion of p105 with a shortened IκBα (37 amino acids short at the C terminus). The rationale behind the design was that (i) IκBα shares homology with the C-terminal portion of p105 in that it contains ankyrin repeats, (ii) both are degraded by the 26S proteasome (34, 46), and (iii) IκBα might be more stable than the C-terminal portion of p105 since the kinetics of its degradation can be followed easily whereas the C-terminal portion of p105 has never been recovered (14, 19, 36).

In p105-IκBα fusion pGL085, a shortened IκBα was fused in frame to amino acid 433, the estimated p50 C terminus (23). The fusion molecule was transfected into COS1 cells, and the cell lysate was analyzed by SDS-PAGE followed by Western analysis with different antibodies. As can be seen in Fig. 2, there were two products on the anti-p50 blot: the full-length fusion protein with a size that corresponded to the estimated molecular size of about 85 kDa and a second product with a size similar to the size of wild-type p50 (lanes 2 and 3). The IκBα antibody revealed both the full-length fusion protein and a product with the size of the shortened IκBα (Fig. 2, lane 7), suggesting that the IκBα seen on the blot was released from the fusion molecule. The recovery of the released C-terminal portion of this fusion suggests that a GRR-dependent endoproteolytic cleavage releases p50 from the p105 C-terminal portion and that the observed rapid degradation of the p105 C-terminal portion is likely to be a subsequent step instead of a prerequisite for the generation of p50.

The endoproteolytic cleavage occurs downstream of the GRR and does not require any downstream sequences. Mutants that contain short deletions downstream of the GRR (pGL076a, pGL077a, and pGL078a) generated products with molecular sizes similar to that of p50 (Fig. 1b, lanes 8 to 10), suggesting that the sequences downstream of the GRR might not be required for endoproteolysis. To further investigate this possibility, we fused either full-length IκBα or the N-terminal 261 amino acids of bacteriophage T7 gene 10 (encoding a T7

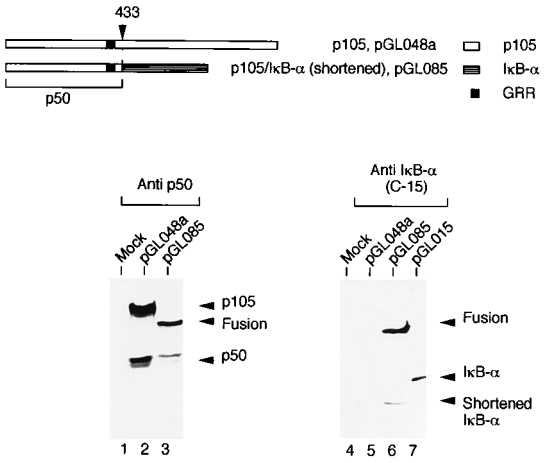

FIG. 2. Western analysis of lysates from COS1 cells transfected with fusion construct p105-IκBα (pGL085). A shortened IκBα (37 amino acids shorter at the C terminus) was fused at amino acid 433 of p105 to form fusion pGL085. pGL048a was the wild-type p105-p50 control (lane 2). The lysates were resolved by SDS–10% PAGE. Both the full-length fusion protein and p50 were detected by the p50 antibody (lane 3). The released IκBα was detected by the antibody against the N terminus of IκBα (C-15 [lane 6]). The molecular sizes of the fusion protein and the shortened IκBα were estimated as 80 and 32 kDa, respectively. The molar ratios of p50 and the shortened IκBα were not equal, suggesting that IκBα was still degraded, though not to the same extent as the C-terminal portion of p105. The control IκBα was a full-length protein (37 kDa) expressed in *E. coli* (pGL015 [lane 7]).

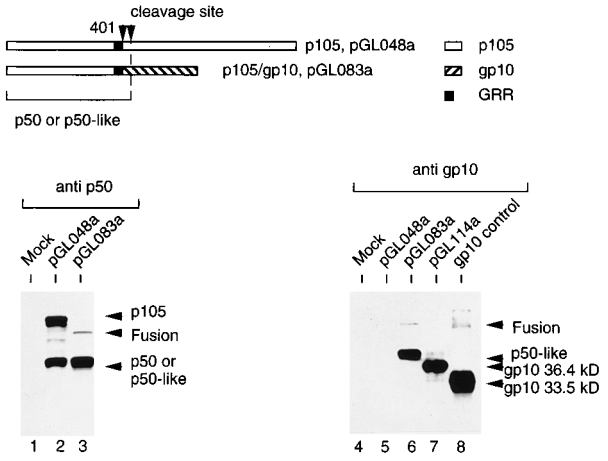

FIG. 4. Western analysis of lysates from COS1 cells transfected with fusion construct p105-gp10 (pGL083a). The N-terminal 261 amino acids from bacteriophage T7 gene 10 (capsid protein) was fused at amino acid 401 of p105 to form fusion pGL083a. The lysates were resolved by SDS–10% PAGE. pGL048a was the wild-type p105-p50 control. The p50-like product reacted with both the p50 antibody (lane 3) and the gp10 monoclonal antibody (lane 6). The processed product was generated in much higher percentage than from pGL048a (lanes 3 and 6). Both full-length gp10 (pGL114a [36.4 kDa]) and a truncated gp10 with a size of 33.5 kDa (Novagen) were used as controls (lanes 7 and 8).

capsid protein, gp10 [13]) to p105 at amino acid 401 (right after the GRR sequence). As can be seen from Fig. 3 (lanes 3 and 6 [pGL087a]) and Fig. 4 (lanes 3 and 6 [pGL083a]), both fusions generated a p50-like product that reacted to both the p50 antibody and antibodies against the fused portion (i.e., the IκBα antibody and the gp10 antibody), suggesting that the cleavage occurred within the fused IκBα and gp10 sequences. Therefore, the GRR-dependent cleavage does not require specific sequences downstream of the GRR. The small variations

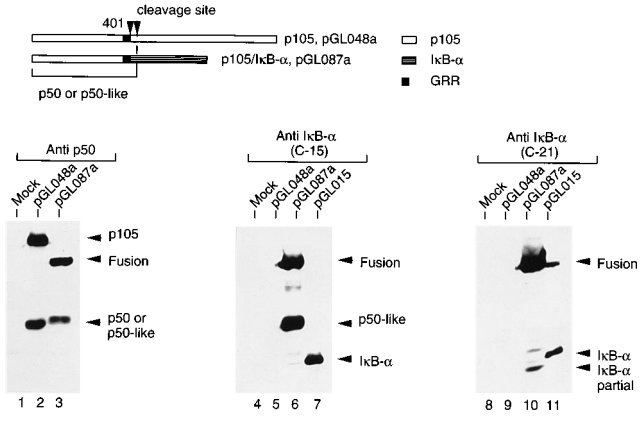

FIG. 3. Western analysis of lysates from COS1 cells transfected with fusion construct p105-IκBα (pGL087a). The full-length IκBα was fused at amino acid 401 of p105 to form fusion pGL087a. The lysates were resolved by SDS–10% PAGE. pGL048a was the wild-type p105-p50 control (lane 2). The p50-like product generated from the fusion migrated slightly slower than p50 (lane 3) and reacted with both the p50 antibody (lane 3) and the IκBα antibody (C-15 [lane 6]). The released C terminus of IκBα was recovered with the antibody against the C terminus of IκBα (lane 10). The fusion-like protein that appeared in lane 11 (control IκBα, expressed in *E. coli*) was due to overflow from the neighboring lane and did not appear in other pGL015 lysates.

in the sizes of the processed products observed in Fig. 1b (lanes 8 to 10 [pGL076a, pGL077a, and pGL078a, respectively]) and Fig. 3 (lane 3 [pGL087a]) suggest that the linear distance from the GRR where the cleavage occurs may be influenced by the secondary structure of the intervening region (see Discussion).

The C-terminal portion of fused IκBα in fusion pGL087a, as was the case with fusion pGL085 (Fig. 2, lane 7), was recovered (Fig. 3, lane 10). Although the p50-like product of p105-gp10 fusion pGL083a was generated at a much higher percentage (Fig. 4, lanes 3 and 6) and therefore should be accompanied by relatively higher amounts of the released C terminus of gp10, a polyclonal antibody against full-length gp10 failed to detect the released C terminus of gp10 (data not shown) (the gp10 antibody used in Fig. 4 was a mouse monoclonal antibody against the 12 amino acids in the N terminus of gp10). Since gp10 can be expressed as a stable protein in COS1 cells (Fig. 4, lane 7), the inherent stability of the molecules (unlike what we had presumed before) used for the fusion may not determine their kinetics of degradation following cleavage. Instead, it is possible that the stability of the released C termini is determined by other features, including those determining whether or not their sequences conform to the N-end rule (47).

The GRR functions as an independent processing signal. To investigate whether the GRR is the sole *cis* element directing the processing, the GRR sequence was placed between the IκBα sequence at its N terminus and p50 (to amino acid 363) at its C terminus (pGL108a). The IκBα-GRR-p50 hybrid was expressed in COS1 cells, and the lysate was examined with the IκBα antibody and the p50 antibody. As can be seen in Fig. 5, the hybrid protein was processed to form both an IκBα-like product (IκBα with the GRR and some sequences from the p50 N terminus) and p50. The IκBα antibody detected the full-length product and the IκBα-like product with a molecular size of about 44 kDa (Fig. 5, lane 3); the p50 antibody revealed the full-length protein, the 44-kDa IκBα-like product, and the remaining C-terminal portion of p50 (lane 6). The released p50 C-terminal portion was resistant to subsequent degradation and migrated as a doublet with an estimated molecular size of

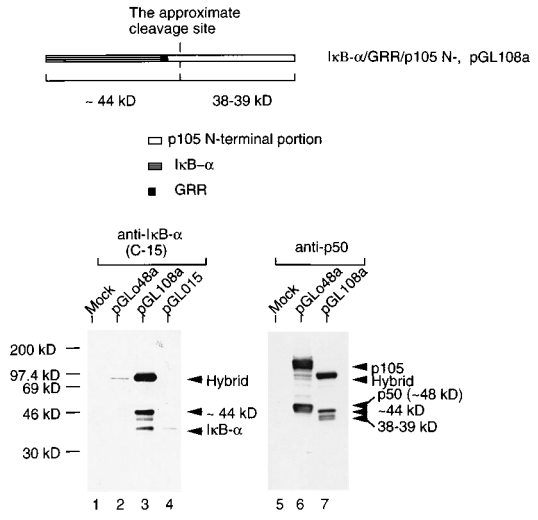

FIG. 5. Western analysis of lysates from COS1 cells transfected with hybrid construct IκBα-GRR-p50 (pGL108a). The full-length IκBα was fused at the N terminus of the GRR and p50 (RHD) was fused at its C terminus to form hybrid pGL108a. The lysates were resolved by SDS–10% PAGE. pGL048a was the wild-type p105-p50 control (lane 6), and pGL015 (expressed in *E. coli*) was the IκBα control. The hybrid generated the full-length protein, the processed IκBα (about 44 kDa [lane 3 as marked]), and the released p50 (migrated as a doublet with an estimated size of 38 to 39 kDa [lane 7 as marked]). The endogenous IκBα was somehow upregulated by the transfection of the fusion protein (lane 3). The processed IκBα with the GRR plus some of the p50 N-terminal sequence is also recognized by the p50 antibody (44 kDa [lane 7]).

around 38 to 39 kDa (Fig. 5, lane 6). The IκBα-GRR-p50 hybrid experiment demonstrated that an RHD in the N terminus of the GRR was not necessary to generate the N-terminal portion from the precursor and also that generation of the N-terminal portion did not depend on the degradation of its C-terminal portion because the released C-terminal portion (38- to 39-kDa p50) was recovered.

To demonstrate that GRR can direct a similar processing event in a protein unrelated to those of the NF-κB family, we decided to insert the GRR between two unrelated proteins. This design stemmed from two concerns: (i) the size and fate of generated N- and C-terminal portions could be monitored separately with different antibodies, and (ii) insertion of the GRR into a protein without knowledge of its folding (i.e., the three-dimensional structure) may result in the GRR being buried and therefore not being recognized. With a hybrid in which the GRR was sandwiched by two unrelated proteins, the processing signal might have a greater chance to be exposed and recognized if the two proteins could fold relatively independently of each other. On the basis of these concerns, we inserted the GRR between gp10 at its N terminus and GST at its C terminus to form test hybrid pGL115a. Compared with control hybrid gp10-GST (Fig. 6, lane 2 [pGL122a]), gp10-GRR-GST hybrid pGL115a expressed in COS1 cells exhibited both full-length hybrid protein and the processed gp10-like product (gp10 with the GRR and some sequences from the N terminus of GST) with a molecular size of about 41 kDa (Fig. 6, lane 3). Since control hybrid gp10-GST was not processed, the GRR sequences were apparently required for the processing. Therefore, the generation of p50 in the fusions, as well as the generation of the second products in the hybrids, is unlikely to be due to a nonphysiological mechanism. An antiserum against GST was able to detect the full-length hybrid, but the C-terminal portion of GST was not detected and therefore might be degraded (data not shown). Hence, this experiment demonstrates that the GRR can function as an independent processing signal.

DISCUSSION

Specific destruction of a protein in vivo plays a critical role in regulating different bioactivities. So far, studies have shown that different targeting signals can lead proteins to degradation by the proteasome pathway (11). The "destruction box" sequences in mitotic cyclins (18, 26) and the PEST (proline-glutamate-serine-threonine) sequences in CLN3 (48) are among these examples. The GRR sequence identified in this work is distinct from these known examples in that it leads p105 and other tested proteins to an incomplete destruction that results in an intact N-terminal portion and either complete or partial degradation of the C-terminal portion. Because the processing of p105 results in the generation of a functional NF-κB p50 subunit, which in recent work has been shown to be a critical component of gene programs regulating the activation of lymphocytes and immune responses to pathogens (39), the identification of the GRR as well as its role in the p105 processing should lead to a better understanding of signal-regulated, NF-κB-dependent gene expression.

The GRR functions as a degradation signal. The major feature of the 23-amino-acid GRR is the abundance of glycines (15 among 23 amino acids). The peptide sequence was predicted to have high flexibility and to be loosely folded (5). Another obvious feature of this short peptide is that it is composed mainly of small amino acids and can be divided into two parts that are separated by two bulky amino acids, phenylalanine and methionine (Fig. 1d). At present, the implication of these features in the sequence is not clear, and further structure-function analysis will be necessary to gain a better understanding. It is highly unlikely, however, that the GRR only functions as a destruction element that makes the region

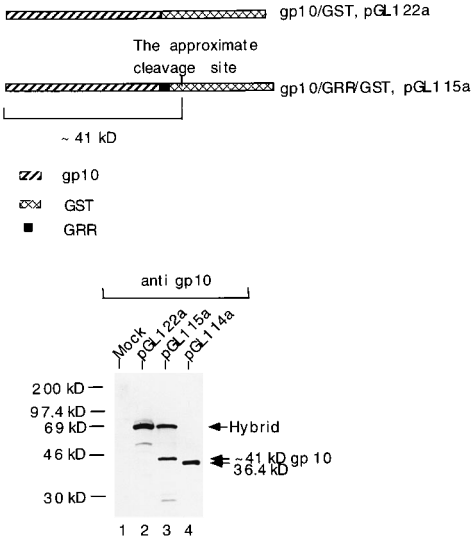

FIG. 6. Western analysis of lysates from COS1 cells transfected with hybrid construct gp10-GRR-GST (pGL115a) and its control hybrid gp10-GST (pGL122a). The full-length protein was fused at the N terminus of the GRR and GST was fused at its C terminus to form hybrid pGL115a. The control hybrid was made by fusing full-length gp10 to GST. The lysates were resolved by SDS–10% PAGE. Both the full-length protein and processed gp10 (with an estimated size of 41 kDa, with 36.4 kDa of gp10 plus the GRR and some sequences from GST) were detected by the gp10 monoclonal antibody (lane 3). gp10-GST was not processed under the same conditions (lane 2). pGL114a was the full-length gp10 (expressed in COS1) control (lane 4).

vulnerable for various proteases, because the endoproteolytic cleavage always occurs downstream of instead of within the GRR region. Also, generation of p50 in *Saccharomyces cerevisiae* is prevented by mutations on the proteasome (34), which strongly argues against the involvement of nonspecific proteases in the GRR-dependent endoproteolytic cleavage.

If the GRR can function independently as a degradation signal, any molecule that contains the GRR sequence should be potentially unstable. However, the sequence of one of the reported IκBγs (p70) overlaps with the sequence of p105 from amino acid 364 (21), and therefore this IκBγ contains the entire GRR sequence (amino acids 372 to 395). Despite containing the GRR, IκBγ can be expressed as a stable protein in some tested cell lines (21). It is possible that additional sequences in the N terminus of the GRR are necessary in order for a protease or a proteasome to sit on the GRR. Alternatively, the IκBγ sequence might actually start from the methionine at position 384, which is 20 amino acids downstream from the presumed initiating methionine at amino acid 364 (Fig. 1d). If translation actually begins at methionine 384, then the remaining portion of the GRR in the IκBγ sequence might not be functional. The latter possibility can only be tested by sequencing the N terminus of IκBγ.

Homology searches of databases showed that the GRR sequence was unique for p105 and p100 species. Processing of the related Rel protein p100 results in a product with a size of 52 kDa (p52) (6, 33, 37), but the GRR of p100 is located more towards the N terminus at amino acids 343 to 373 in contrast to that of p105 at amino acids 364 to 394. Since the composition of the amino acids within the same linear distance from the GRR of both proteins cannot explain why p100 generates a product with a higher molecular weight, it is likely that the cleavage occurs at a greater linear distance from the p100 GRR to the site. Within a fixed distance, a more compactly folded sequence will contain a larger number of amino acids than the loosely folded sequence; therefore, the difference between the molecular sizes of p50 and p52 could be interpreted as a result of different secondary structures between the GRR and the cleavage site in the two proteins. Consistent with this hypothesis, in some p105 deletion mutants (pGL076a, pGL077a, and pGL078a [Fig. 1b, lanes 8 to 10]) and the p105-IκBα fusion in which the sequence after the GRR is replaced by IκBα (pGL087a [Fig. 3, lane 3]), a slightly larger p50-like product is also generated.

An endoproteolytic cleavage is the key step for releasing p50. p105 processing is different from other known proteasome-related processing events in that it results in an incomplete degradation of the precursor. The rapid degradation of the p105 C-terminal portion has been believed to be an obligatory step in the generation of p50 (34). Since the degradation by the 26S proteasome is highly processive, it is unclear how the N-terminal portion of p105 (p50) escapes degradation. The recovery of the released C-terminal portion from the processing of some chimeric molecules (p105-IκBα and IκBα-GRR-p50 [Fig. 2, 3, and 5]) strongly suggests that p105 processing includes two steps: a GRR-dependent endoproteolytic cleavage and the subsequent degradation of the C terminus. The GRR-dependent endoproteolytic cleavage, as the first step of processing, releases p50 from the rest of the p105 C-terminal portion and therefore provides a satisfactory explanation for the partial degradation of the precursor. The two-step processing probably allows the degradation of the proteasome to initiate from the newly generated N terminus.

How the GRR relates to the ubiquitin-proteasome pathway. The ubiquitin-proteasome pathway has been related to p105 processing (34). Besides the finding that ATP and Mg^{2+} were required for the processing (14), various proteasome inhibitors were found to significantly (though not completely) inhibit the processing of p105 both in vitro and in vivo. Furthermore, the generation of p50 in *S. cerevisiae* is prevented by mutations on the proteasome, and full-length p105 has been found to be ubiquitinated prior to the proteolysis (34). These results strongly indicated the involvement of the ubiquitin-proteasome pathway in the processing of p105.

The question of how the generation of p50 is regulated remains to be answered. A model in agreement with the observations described above suggests that the phosphorylation of p105, as observed in IκBα, could serve as a regulatory step for ubiquitination and the subsequent degradation by the 26S proteasome (34, 43). The observation that the signal-induced phosphorylation enhances the processing of p105 in vivo supports this model (28, 29, 31). However, the inducible phosphorylation of p105 as a necessary step for the processing of p105 is not completely convincing: p50 can be generated in vitro as well as in unstimulated cells. Also, only phosphorylated p105 was detected before or after induction and no phosphorylated p50 was detected, suggesting that both basal and induced phosphorylation occurred in the C-terminal portion of p105 (31). Our results as well as previous observations (14, 17) have clearly indicated that the entire C-terminal portion of p105 is dispensable for processing. In addition, we have demonstrated that the GRR can function as an independent signal for the processing of a non-NF-κB protein. If the basal phosphorylation is indeed in the C-terminal portion of p105, our results suggest that it is at least not directly involved in the generation of p50 in the absence of the external stimuli and therefore may not serve as a regulatory step for the generation of p50 in unstimulated cells. However, our results do not necessarily rule out the possibility of signal-induced phosphorylation as a regulatory step for p105 processing. The signal-induced phosphorylation in p105, like that in IκBα, could serve as a regulatory step to trigger ubiquitination that might unfold p105 and expose the GRR for cleavage and subsequent degradation. One possibility for the generation of p50 in the absence of the signal is the transient exposure of the GRR to the proteasome (or an unidentified factor that might recruit the proteasome) during the folding of nascent p105. However, in both stimulus-independent and stimulus-dependent situations, the recognition and binding of the GRR would serve as an important control step to regulate the generation of p50.

Although the observation that GRR functions as an independent signal to direct a two-step processing provides a more satisfactory explanation for certain phenomena involved in p105 processing, how the GRR relates to the ubiquitin-proteasome pathway remains to be determined. The generation of p50 in some yeast proteasome mutants is abolished (34), implying that the proteasome may be responsible for both the GRR-dependent endoproteolytic cleavage and subsequent degradation. Selection of a yeast mutant in which both p50 and the C-terminal portion of p105 can be generated could therefore provide genetic evidence for the two-step processing model described in this work.

ACKNOWLEDGMENTS

We are grateful to M. Biggin for his suggestions and criticisms during the course of this work. We thank J. Steitz, M. Solomom, D. Schatz, and D. Gonda for discussions and S.-P. Zhang for the computer work. Li Lin thanks her former mentors F. W. Studier and Y.-K. Sun for their inspiration.

This work was supported by the Howard Hughes Medical Institute and NIH grant R01-AI33443.

REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242:**540–545.
2. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
3. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
4. **Bennett, V.** 1992. Ankyrins. J. Biol. Chem. **267:**8703–8706.
5. **Blank, V., P. Kourilsky, and A. Israel.** 1992. NF-κB and related proteins: Rel/dorsal homologies meet ankyrin repeats. Trends Biochem. Sci. **17:**135–140.
6. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature (London) **348:**76–80.
7. **Brockman, J. A., D. C. Scherer, T. A. McKinsey, S. M. Hall, X. Qi, W. Y. Lee, and D. W. Ballard.** 1995. Coupling of a signal-response domain in IκBα to multiple pathways for NF-κB activation. Mol. Cell. Biol. **15:**2809–2818.
8. **Brown, K., S. Gerstberger, L. Carlson, G. Franzoso, and U. Siebenlist.** 1995. Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science **267:**1485–1491.
9. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
10. **Chen, Z., J. Hagler, V. Palombella, F. Melandri, D. Scherer, D. Ballard, and T. Maniatis.** 1995. Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes Dev. **9:**1586–1597.
11. **Ciechanover, A.** 1994. The ubiquitin-proteasome proteolytic pathway. Cell **79:**13–21.
12. **Cordle, S. R., R. Donald, M. A. Read, and J. Hawiger.** 1993. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-Rel and related NF-κB proteins in human monocytic THP-1 cells. J. Biol. Chem. **268:**11803–11810.
13. **Dunn, J. J., and F. W. Studier.** 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. J. Mol. Biol. **166:**477–535.
14. **Fan, C.-M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature (London) **354:**395–398.
15. **Ghosh, G., G. Van Duyne, S. Ghosh, and P. B. Sigler.** 1995. Structure of NF-κB p50 homodimer bound to a κB site. Nature (London) **373:**303–310.
16. **Ghosh, S., and D. Baltimore.** 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344:**678–682.
17. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell **62:**1019–1029.
18. **Glotzer, M., A. W. Murry, and M. W. Kirschner.** 1991. Cyclin is degraded by the ubiquitin pathway. Nature (London) **349:**132–138.
19. **Hatada, E. N., A. Nieters, F. G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T. McKeithan, and C. Scheidereit.** 1992. The ankyrin repeat domains of the NF-κB precursor p105 and the proto-oncogene bcl-3 act as specific inhibitors of NF-κB DNA binding. Proc. Natl. Acad. Sci. USA **89:**2489–2493.
20. **Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature (London) **365:**182–185.
21. **Inoue, J.-I., L. D. Kerr, A. Kakizuka, and I. M. Verma.** 1992. IκBγ, a 70 kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68:**1109–1120.
22. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product. Cell **62:**1007–1018.
23. **Lin, L., and S. Ghosh.** Unpublished results.
24. **Lin, Y. C., K. Brown, and U. Siebenlist.** 1995. Activation of NF-κB requires proteolysis of the inhibitor IκB-α: signal-induced phosphorylation of IκB-α alone does not release active NF-κB. Proc. Natl. Acad. Sci. USA **92:**552–556.
25. **Liou, H.-C., G. P. Nolan, S. Ghosh, T. Fujita, and D. Baltimore.** 1992. The NF-κB p50 precursor, p105, contains an internal IκB-like inhibitor that preferentially inhibits p50. EMBO J. **11:**3303–3309.
26. **Luca, F. C., E. K. Shibuya, C. E. Dohrman, and J. V. Ruderman.** 1991. Both cyclin AΔ60 and BΔ97 are stable and arrest cells in M-phase, but only cyclin BΔ97 turns on cyclin destruction. EMBO J. **10:**4311–4320.
27. **Mattias, P., M. M. Muller, E. Schreiber, S. Rusconi, and W. Schaffner.** 1989. Eukaryotic expression vectors for the analysis of mutant proteins. Nucleic Acids Res. **15:**6418–6419.
28. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Res. **21:**5059–5066.
29. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. Genes Dev. **7:**705–718.
30. **Muller, C. W., A. R. Felix, M. Sodeoka, G. L. Verdine, and S. C. Harrison.** 1995. Structure of the NF-κB p50 homodimer bound to DNA. Nature (London) **373:**311–317.
31. **Naumann, M., and C. Scheidereit.** 1994. Activation of NF-κB *in vivo* is regulated by multiple phosphorylations. EMBO J. **13:**4597–4607.
32. **Naumann, M., F. G. Wulczyn, and C. Scheidereit.** 1993. The NF-κB precursor p105 and the proto-oncogene product Bcl-3 are IκB molecules and control nuclear translocation of NF-κB. EMBO J. **12:**213–222.
33. **Neri, A., C.-C. Chang, L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, R. S. K. Chaganti, and R. Dalla-Favera.** 1991. B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. Cell **67:**1075–1087.
34. **Palombella, V. J., O. J. Rando, A. L. Goldbert, and T. Maniatis.** 1994. The ubiquitin-proteasome pathway is required to process the NF-κB1 precursor protein and the activation of NF-κB. Cell **78:**773–785.
35. **Rice, N. R., M. L. Mackichan, and A. Israel.** 1992. The precursor of NF-κB p50 has IB-like functions. Cell **71:**243–253.
36. **Riviere, Y., V. Blank, P. Kourilsky, and A. Israel.** 1991. Processing of the precursor of NF-κB by the HIV-1 protease during acute infection. Nature (London) **350:**625–626.
37. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352:**733–736.
38. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46:**705–716.
39. **Sha, W. C., H.-C. Liou, E. Tuomanen, and D. Baltimore.** 1995. Targeted destruction of the p50 subunit of NF-κB leads to multifocal defects in immune responses. Cell **80:**321–330.
40. **Siebenlist, U., G. Franzoso, and K. Brown.** 1994. Structure, regulation and function of NF-κB. Annu. Rev. Cell Biol. **10:**405–455.
41. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1992. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
42. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκB-α: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
43. **Thanos, D., and T. Maniatis.** 1995. NF-κB: a lesson in family values. Cell **80:**529–532.
44. **Thompson, J. E., R. J. Phillips, H. Erdjument-Bromage, P. Tempst, and S. Ghosh.** 1995. IκB-β regulates the persistent response in a biphasic activation of NF-κB. Cell **80:**573–582.
45. **Traenckner, E. B.-M., H. L. Pahl, T. Henkel, K. N. Schmidt, S. Wilk, and P. A. Baeuerle.** 1995. Phosphorylation of human IκB-α on serine 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J. **14:**2876–2883.
46. **Traenckner, E. B.-M., S. Wil, and P. A. Baeuerle.** 1994. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NF-κB. EMBO J. **13:**5433–5441.
47. **Varshavsky, A.** 1992. The N-end rule. Cell **69:**725–735.
48. **Yaglom, J., M. H. K. Linskens, S. Sadis, D. M. Rubin, B. Futcher, and D. Finley.** 1995. $p34^{Cdc28}$-mediated control of Cln3 cyclin degradation. Mol. Cell. Biol. **15:**731–741.