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Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs

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ABSTRACT **RelA (p65) functions as the critical transactivating component of the heterodimeric p50–p65 NF-κB complex and contains a high-affinity binding site for its cytoplasmic inhibitor, IκBα. After cellular activation, IκBα is rapidly degraded in concert with the induced nuclear translocation of NF-κB. The present study demonstrates that tumor necrosis factor α-induced degradation of IκBα in human T cells is preceded by its rapid phosphorylation *in vivo*. However, these effects on IκBα result in nuclear mobilization of only a fraction of the entire cytoplasmic pool of RelA. Subsequent studies have revealed that (*i*) cytoplasmic RelA is stably associated not only with IκBα but also with other ankyrin motif-rich proteins including the products of the NF-κB2 (p100) and NF-κB1 (p105) genes; (*ii*) in contrast to RelA–IκBα, RelA–p100 cytoplasmic complexes are not dissociated following tumor necrosis factor α activation; (*iii*) p100 functions as a potent inhibitor of RelA-mediated transcription *in vivo*; (*iv*) the interaction of RelA and p100 involves the conserved Rel homology domain of both proteins but not the nuclear localization signal of RelA, which is required for IκBα binding; (*v*) p100 inhibition of RelA function requires the C-terminal ankyrin motif domain, which mediates cytoplasmic retention of RelA; and (*vi*) as observed with IκBα, nuclear RelA stimulates p100 mRNA and protein expression. These findings thus reveal the presence of a second inducible autoregulated inhibitory pathway that helps ensure the rapid but transient action of nuclear NF-κB.**

The mammalian transcription factor NF-κB participates in the regulation of the human immunodeficiency virus (HIV) and multiple cellular genes that contain functional κB enhancer elements (1, 2). NF-κB is an inducible heterodimeric complex composed of 50-kDa (p50) and 65-kDa (p65, now designated RelA) subunits (3–11), both of which share an ≈300-amino-acid region of N-terminal homology with the v-Rel oncoprotein (12, 13) and other members of the Rel transcription factor family (14). The RelA subunit of NF-κB contains a strong transcriptional activation domain and an intrinsic nuclear localization signal (NLS) that is recognized and masked by IκBα (previously termed MAD-3) (15, 16), a cytoplasmic inhibitor of NF-κB that contains multiple ankyrin or SW16-cdc10 motifs (17). Cellular activation appears to lead to the phosphorylation of IκBα followed by the rapid dissociation and degradation of this cytoplasmic inhibitor, concomitant with nuclear translocation of the liberated NF-κB complex (18–20). In turn, nuclear NF-κB stimulates the transcription of an array of genes involved in such diverse functions as immune activation, inflammation, and development (1, 2, 14). During this induced genetic program, NF-κB also activates expression of the IκBα gene, thus facilitating an autoregulatory feedback mechanism that serves to temporally restrict NF-κB action (18, 19).

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Recent reports have revealed that RelA can also associate with yet other ankyrin motif-rich cytoplasmic proteins distinct from IκBα, including the NF-κB1 (p105) and NF-κB2 (p100) precursor gene products, which are subsequently cleaved to produce the p50 and p52 Rel family members, respectively (21–23). However, the functional significance of these interactions *in vivo* remains unclear. In the present study, we have explored inducible phosphorylation of IκBα in human T cells and the structural basis and functional consequences of RelA interaction with the ankyrin motif-rich p100 and p105 Rel-containing precursors.

MATERIALS AND METHODS

Transient Transfection Assays. All cDNAs were subcloned into the pCMV4 expression vector (24). N- and C-terminal deletion mutants of RelA and p100 were generated by the polymerase chain reaction from the corresponding full-length cDNAs (16). The reporter plasmid κB-TATA-CAT contained the HIV-1 κB enhancer linked upstream of the *Escherichia coli* chloramphenicol acetyltransferase (CAT) reporter gene (25). Human Jurkat T cells were maintained and transfected with effector and reporter plasmids using DEAE-dextran (26) and assayed for CAT activity as described (16).

Immunoprecipitation, Immunoblotting, and Immunofluorescence Studies. COS cells were transfected using DEAE-dextran (26). After 48 hr, recipient cells were starved for 1 hr in methionine/cysteine-deficient medium and metabolically radiolabeled for 2 hr with [^{35}S]methionine and [^{35}S]cysteine as described (16). Whole-cell extracts were prepared by lysis in a low-stringency buffer and subjected to immunoprecipitation analyses with the indicated NF-κB- and IκB-specific antisera (16).

Unlabeled subcellular protein extracts were prepared from Jurkat T cells or transfected COS cells as described (27). For phosphorylation analysis, the cell lysis buffer (27) was supplemented with 0.4% Nonidet P-40, 10 mM NaF, 0.1 mM $NaVO_4$, 2 mM EDTA, 50 μM $ZnCl_2$, and 10 μM sodium molybdate. Protein samples (20 μg per lane for Jurkat and 4 μg per lane for COS) were fractionated by SDS/PAGE and analyzed for immunoreactivity with various anti-peptide antisera using an enhanced chemiluminescence detection system (ECL; Amersham). For immunofluorescence studies, COS cells were grown on four-well chamber slides (Nunc, Naperville, IL) and then transfected using DEAE-dextran. After 48 hr, the cells were fixed, permeabilized, and sequentially incubated with rabbit anti-RelA (C terminus specific) or anti-p100 followed by donkey anti-rabbit immunoglobulin covalently coupled to Texas Red dye (16).

Abbreviations: HIV-1, type 1 human immunodeficiency virus; CAT, chloramphenicol acetyltransferase; TNF-α, tumor necrosis factor α; NLS, nuclear localization signal.

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RESULTS

Tumor Necrosis Factor α (TNF-α)-Induced Degradation of IκBα Is Preceded by IκBα Phosphorylation *in Vivo*. Prior studies have shown that nuclear NF-κB binding activity is detectable within 10 min after TNF-α stimulation of human Jurkat T cells (18, 19). In parallel, the cytoplasmic stores of IκBα are completely depleted between 10 and 40 min after TNF-α addition. To investigate whether IκBα was phosphorylated *in vivo* prior to its degradation and the nuclear import of NF-κB, cytoplasmic extracts from T cells stimulated with TNF-α for 0–10 min were immunoblotted with anti-IκBα specific antibodies (Fig. 1*A*). TNF-α stimulation led to the appearance of a low-mobility isoform of IκBα detected within 5 min after induction (lane 3). Coincubation of these TNF-α-stimulated cells with okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A (28) and an inducer of NF-κB (29), temporally accelerated the appearance of this low-mobility IκBα isoform (lanes 6 and 7), perhaps reflecting the unopposed action of an as yet unidentified kinase. Under both conditions of stimulation, this low-mobility IκBα isoform disappeared within 10 min, whereas the basally expressed IκBα isoform persisted (lanes 4 and 8). Incubation of extracts from T cells stimulated with TNF-α for 5 min with calf intestinal alkaline phosphatase revealed the selective loss of this transiently expressed IκBα species, suggesting that its altered mobility reflected phosphorylation at one or more sites.

RelA Is Associated with Multiple Cytoplasmic Proteins, Including p100 and p105. Even after the intracellular stores of IκBα were completely degraded in response to TNF-α stimulation (Fig. 1*B*, lane 3), substantial amounts of RelA remained sequestered in the cytoplasm of these induced T lymphocytes (Fig. 1*B*, lanes 3 and 5). These findings raised the possibility that RelA might be regulated by additional inhibitory molecules distinct from IκBα. To examine this possibility, control preimmune (Fig. 1*C*, lanes 1, 4, and 7) or anti-RelA (lanes 2, 5, and 8) coimmunoprecipitates were prepared from Jurkat T cells, electrophoresed, and immunoblotted with peptide-specific antisera against the ankyrin motif-containing proteins p100 (lanes 1 and 2) and p105 (lanes 4 and 5) as well as IκBα (lanes 7 and 8). These studies revealed that, like IκBα, both p100 and p105 were readily coimmunoprecipitated with RelA (Fig. 1*C*, lanes 2, 5, and 8). Further, deletion analysis showed that the physical interaction of p100 and p105 with RelA was mediated through their Rel homology domains. However, in contrast to IκBα–RelA

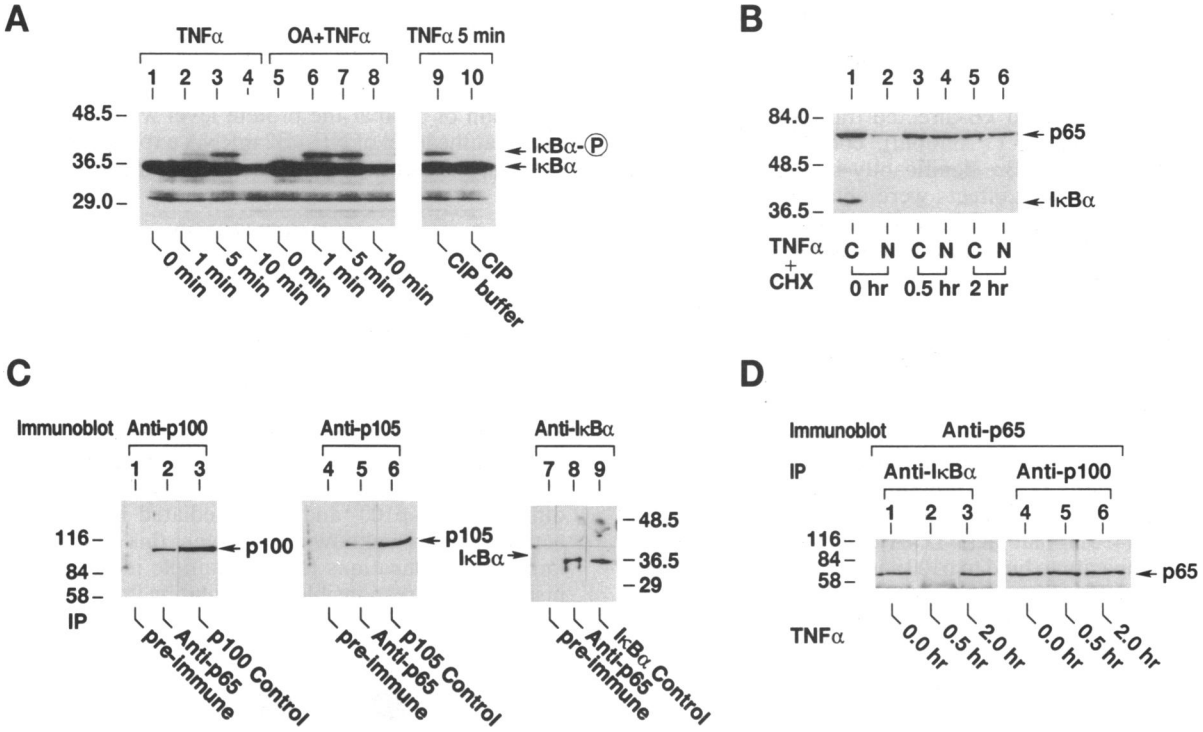

FIG. 1. (*A*) Phosphorylation of IκBα *in vivo* in TNF-α-stimulated Jurkat T cells. Cells (3×10^5 per ml) were incubated for 1 hr in the presence (lanes 5–8) or absence (lanes 1–4) of okadaic acid (OA) at 50 ng/ml and then stimulated with TNF-α (140 units/ml) for 0–10 min as indicated. The slightly faster kinetics of IκBα degradation seen in this experiment versus ref. 19 likely reflects TNF stimulation of the cells at a lower cell density. Cytoplasmic extracts prepared in the presence of phosphatase inhibitors were fractionated on an SDS/10% PAGE gel and then subjected to immunoblotting analyses with an antiserum specific for the C terminus of IκBα. For phosphatase treatment, the extract from lane 3 was incubated at 37°C for 15 min either in phosphatase buffer alone (lane 9) or in buffer containing 24 units of calf intestine alkaline phosphatase (CIP; lane 10) before analysis by SDS/PAGE. (*B*) Subcellular pattern of expression of RelA and IκBα in human Jurkat T cells stimulated with TNF-α (140 units/ml) in the presence of cycloheximide (CHX; 25 μg/ml). Cytoplasmic (C) and nuclear (N) extracts (20 μg), prepared at the indicated times after TNF-α addition, were fractionated on a SDS/8.75% PAGE, and subjected to immunoblotting analyses with a mixture of IκBα- and RelA-specific antisera. (*C*) Physical association of NF-κB RelA with p100, p105, and IκBα in the cytoplasm of unstimulated Jurkat T cells. Cytoplasmic extracts were prepared from unstimulated Jurkat T cells and incubated with either preimmune serum or RelA-specific antiserum. The immunoprecipitates were then divided into three aliquots, subjected to SDS/PAGE, and immunoblotted with peptide-specific antisera recognizing the N terminus (amino acids 1–21) of p100 (lanes 1 and 2), the N terminus (amino acids 1–21) of p105 (lanes 4 and 5), or the C terminus (amino acids 289–317) of IκBα (lanes 7 and 8). Control lanes (3, 6, and 9) contained extracts from COS cells transfected with cDNA expression vectors encoding p100, p105, and IκBα, respectively. (*D*) Distinct dynamics for RelA–IκBα and RelA–p100 complex disassembly in TNF-α-stimulated Jurkat T cells. Cells were stimulated with TNF-α (140 units/ml) for the indicated times followed by the preparation of cytoplasmic extracts. These extracts (400 μg) were immunoprecipitated with antiserum specific for either the C terminus of IκBα (lanes 1–3) or the N terminus of p100 (lanes 4–6) and analyzed on SDS/8.75% PAGE followed by immunoblotting with the antiserum specific for the C terminus of RelA.

interaction, which critically involves inhibitor masking of the NLS present within the Rel homology domain of RelA (15, 16), the interactions of p100 and p105 with RelA persisted despite deletion of the NLS of RelA (data not shown).

RelA–IκBα and RelA–p100 Cytoplasmic Complexes Are Differentially Regulated After TNF-α Stimulation. To further compare and contrast the biological actions of IκBα and p100, we examined the dynamics of RelA–IκBα and RelA–p100 complex disassembly in T cells stimulated with TNF-α (Fig. 1*D*). IκBα, p100, and associated proteins were first immunoprecipitated with specific antisera against either IκBα (lanes 1–3) or p100 (lanes 4–6) and then analyzed for the presence of coimmunoprecipitated RelA by immunoblotting with RelA-specific antiserum. As predicted from the degradative fate of IκBα (Fig. 1*B*), detection of RelA was lost 30 min after TNF-α addition (lane 2), but this immunoreactive species reappeared at 2 hr (lane 3), reflecting the *de novo* synthesis of IκBα (18, 19). This dynamic change in IκBα expression precisely correlates with the transient nuclear translocation of RelA (19). In contrast, the association of RelA and p100 was not significantly altered even after 2 hr of TNF-α stimulation (lanes 4–6).

p100 Exhibits Potent IκB-Like Activity *in Vivo*. To determine whether the interaction of RelA with p100 and p105 was functionally significant *in vivo*, Jurkat T cells were transfected with an HIV κB-CAT reporter plasmid together with expression vectors encoding RelA, IκBα, p100, and p105, either alone or in various combinations (Fig. 2*A*). Whereas RelA potently activated κB-directed transcription from the HIV κB-CAT reporter (>250-fold), coexpression of RelA with either p100 or IκBα significantly inhibited activation. Only modest inhibitory effects were obtained after cotransfection of p105. Deletion of the ankyrin motif-rich domain of p100 [p100(1–455)], or coexpression of RelA with the equivalent of the posttranslationally cleaved form of p100 (p52), which also lacks the ankyrin motif-rich domain, resulted in the near complete loss of these inhibitory effects (Fig. 2*B*).

p100 Inhibition of RelA Function Is Correlated with Cytoplasmic Retention of RelA. To examine whether p100 regulated RelA activity by altering its subcellular distribution, indirect immunofluorescence studies were performed in transfected COS cells (Fig. 3). Since wild-type RelA can induce endogenous IκBα expression leading to cytoplasmic trapping of RelA (19), an N-terminal truncation mutant of RelA [p65(41–551)] that lacks DNA binding activity (16) but retains the capacity to bind to p100 and IκBα (data not shown) was used. Expression of the p65(41–551) mutant alone revealed a predominantly nuclear pattern of fluorescence (Fig. 3*a*) while coexpression of this truncated form of RelA with IκBα yielded a nuclear-excluded pattern (Fig. 3*b*). Similarly, coexpression of this RelA mutant with p100 produced a cytoplasmic pattern of RelA expression (Fig. 3*c*). However, consistent with their inability to inhibit RelA function *in vivo*, neither a truncated form of p100(1–455) nor p52 reproduced this cytoplasmic retention pattern of RelA expression (Fig. 3 *d* and *e*).

Expression of p100 and NF-κB2 mRNA Are Induced by RelA. Recent studies have shown that NF-κB is capable of regulating the expression of one of its own inhibitors, IκBα (18, 19). Since p100 is also an immediate-early activation gene product (30) and is involved in the cytoplasmic retention and functional inhibition of RelA (refs. 21 and 22; Figs. 2 and 3), immunoblotting studies were next performed to investigate whether p100 protein expression is also regulated by RelA (Fig. 4). COS cells were transfected with the control parental pCMV4 vector or vectors encoding various Rel proteins including p50, RelA, and c-Rel. As shown in Fig. 4, virtually no p100 protein was detected in extracts from cells transfected with pCMV4 (lane 1), p50 (lane 2), or c-Rel (lane 3). However, transfection of RelA markedly induced the expression of p100 protein (lane 4). Of note, this induction of p100 by RelA could be significantly inhibited by cotransfection of IκBα (lane 5). In contrast to wild-type RelA, deletion mutants of RelA lacking either the DNA-binding [p65(41–551)] or the transactivation [p65(1–312)] domains failed to induce p100 protein expression (lanes 8 and 9). These findings of RelA induction of p100 at the protein level were entirely recapitulated at the level of NF-κB2 mRNA expression as measured in RNase protection studies (data not shown).

DISCUSSION

In this report, we demonstrate an IκBα-independent mechanism of NF-κB regulation *in vivo* involving the NF-κB2 gene product, p100. In agreement with two recent studies (21, 22), we have found that RelA is physically associated with both p100 and p105 as well as IκBα in the cytoplasm of unstimulated human T cells. We further show that p100, like IκBα, potently inhibits the transcriptional activity of RelA. However, under the same conditions, p105 produces curiously less inhibition. At present, the molecular basis for these differences in p100- and p105-mediated inhibition of RelA action remains unknown. However, this finding may have important implications for the multiple intracellular mechanisms that are responsible for regulating the potent transcriptional activity of RelA.

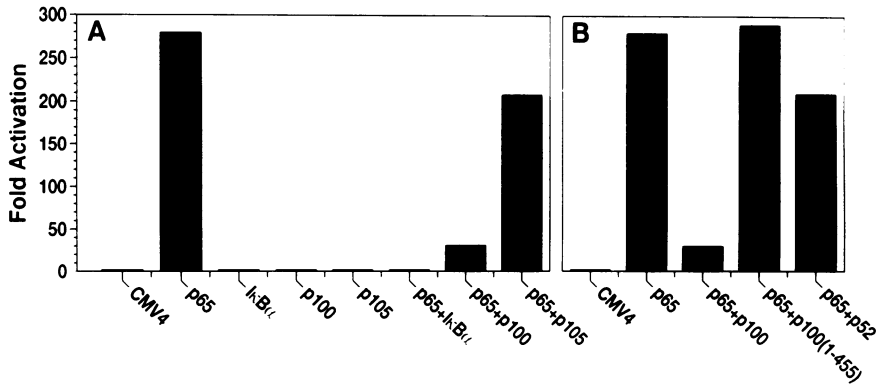

FIG. 2. Inhibition of NF-κB RelA-mediated transcriptional activation by p100 *in vivo*. (*A*) Human Jurkat T cells were transfected with the indicated expression vectors alone (2 μg) or in combination (2 μg of each) together with a CAT reporter plasmid containing the HIV-1 κB enhancer (κB-TATA-CAT). (*B*) RelA was cotransfected with various truncated forms of p100 including p100(1–455), which contains the N-terminal 455 amino acids and lacks the C-terminal ankyrin motif repeat domain, and p52, which encodes a 447-amino-acid-long peptide that approximates the physiologically cleaved form of p100. CAT activities were measured 48 hr after transfection and are presented as fold induction relative to the basal level of enzymatic activity obtained with the parental pCMV4 expression vector.

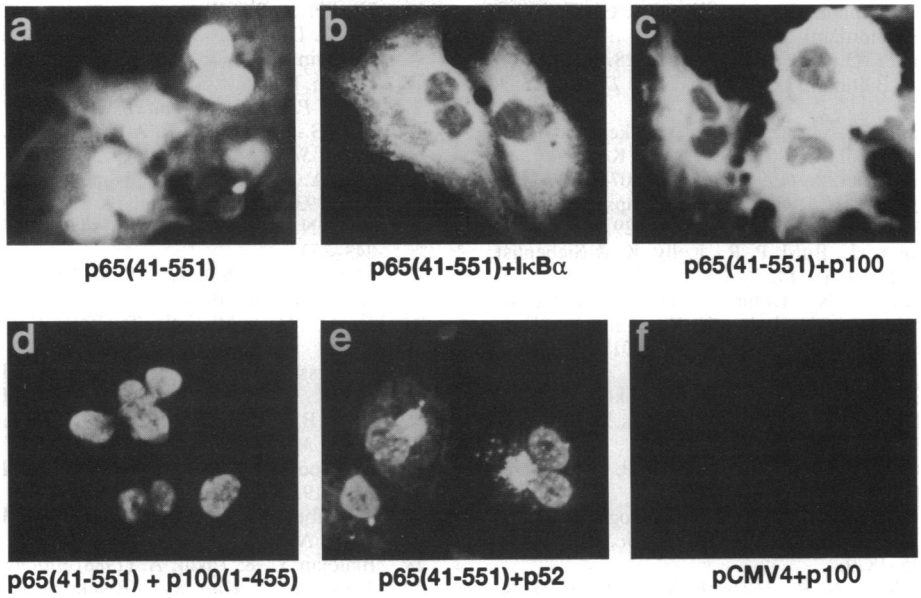

FIG. 3. p100 inhibits nuclear expression of RelA. COS cells were transfected with a N-terminal truncation mutant of RelA, p65(41–551), alone (*a*) or with IκBα (*b*), p100 (*c*), p100(1–455) (*d*), or p52 (*e*) or, as an antibody specificity control, with p100 alone (*f*). Forty-eight hours after transfection, the cells were fixed, incubated with the antiserum against the C terminus of RelA, and then subjected to indirect immunofluorescence staining using Texas Red-conjugated donkey anti-rabbit IgG (Amersham).

Although both IκBα and p100 function as important cytoplasmic inhibitors of RelA, several fundamental differences exist regarding their mechanism of action and regulation. Our present studies coupled with a recent report (20) have shown that a fraction of IκBα is rapidly phosphorylated *in vivo* after cellular activation with TNF-α. This phosphorylation event appears to lead to enhanced degradation of the IκBα. However, it remains unclear whether the dissociation of IκBα from NF-κB forms an intermediate step between phosphorylation and degradation or whether degradation can occur in the absence of release, or even perhaps phosphorylation, given the incompleteness of the latter modification.

In contrast to TNF-α-induced release of IκBα from NF-κB, this cytokine does not promote the dissociation of p100 from RelA. These findings suggest that the cytoplasmic p100–RelA complex is subject to an alternate form of regulation. Since p52, the processed form of p100, continues to associate with p65, we hypothesize that one mechanism for activation of the p100–RelA complex may involve physiological processing of p100 to p52. This processing results in removal of the C-terminal ankyrin motifs that are essential for p100 inhibition of RelA function but not its assembly with RelA. In the case of IκBα, its ankyrin motifs are needed both for functional inhibition and for physical assembly with RelA (17, 32). The ankyrin repeat motifs present in both IκBα and p100 likely mediate protein–protein interactions that serve to sequester RelA in the cytoplasmic compartment. The regulated partitioning of factors between the cytoplasm and nucleus by such ankyrin motif-containing inhibitors has emerged as a hallmark of the Rel family of transcription factors.

Finally, the interaction of IκBα with RelA involves the binding of this inhibitor to the NLS of RelA. Deletion of the NLS in RelA results in sharply diminished IκBα binding and IκBα-mediated function (15, 16). In contrast, NLS deletion in RelA has no effect on its interaction with p100 (data not shown). Rather, distinct sequences within the Rel homology domain of both RelA and p100, likely those responsible for dimerization with other Rel family members, mediate this specific factor–inhibitor interaction.

Of note, our current studies indicate that RelA autoregulates the expression of not only IκBα (19) but also p100. Specifically, transfection of RelA induces increased expression of NF-κB2 mRNA and the corresponding p100 protein. The NF-κB1 gene also appears to be similarly regulated (31), and the p105 precursor product of this gene also antagonizes RelA function (21, 22), albeit weakly in our studies compared to p100. These findings thus highlight a redundant system of inducible ankyrin-rich cytoplasmic inhibitors that provides for yet additional plasticity in the regulation of NF-κB induction occurring in response to various immune, inflammatory, or developmental stimuli.

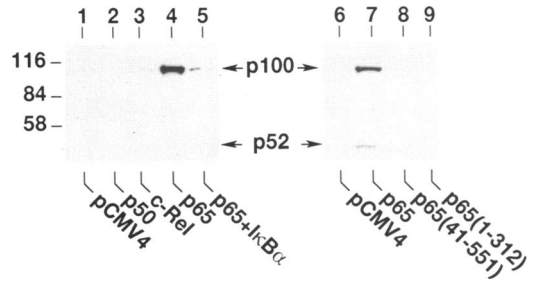

FIG. 4. NF-κB induces the expression of endogenous p100 protein. The effects of various Rel-related proteins on p100 protein expression are shown. COS cells were transfected with either the parental pCMV4 vector (lanes 1 and 6) or the expression vectors encoding the indicated Rel-related polypeptides. Whole-cell extracts were prepared 48 hr after transfection and subjected to SDS/PAGE and immunoblotting analysis with peptide-specific antiserum recognizing the N terminus of human p100. The p100 polypeptide and its processed form, p52, are indicated.

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