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Direct association of pp40/IκBβ with rel/NF-κB transcription factors: Role of ankyrin repeats in the inhibition of DNA binding activity

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ABSTRACT **To understand the mechanism by which pp40/IκBβ inhibits DNA binding activity of the rel/NF-κB family of transcription factors, we have investigated the role of ankyrin repeats on the biological function of pp40 by deleting or mutating conserved residues. We show that (*i*) ankyrin repeats alone are not sufficient to manifest biological activity but require the C-terminal region of the pp40 protein; (*ii*) four out of the five ankyrin repeats are essential for inhibiting the DNA binding activity; (*iii*) pp40 mutants that do not inhibit DNA binding of rel protein also do not associate with rel; (*iv*) although pp40 can associate with the p65 and p50 subunits of NF-κB, pp40 inhibits the DNA binding activity of only the p50–p65 heterodimer and the p65 homodimer; and (*v*) pp40 inhibits the transcription of genes linked to κB site; however, mutants that do not affect DNA binding have no effect. We propose that the ankyrin repeats and the C-terminal region of pp40 form a structure that associates with the rel homology domain to inhibit DNA binding activity.**

NF-κB, rel, and dorsal are members of a family of transcription factors that share the property of being localized in the cytoplasm (1). After the appropriate signal, these transcription factors translocate to the nucleus and initiate transcription by binding to a decameric sequence generically referred to as the κB site (2). A latent form of NF-κB is detected in the cytosol that, upon treatment of the cytoplasmic extracts with ionic detergent sodium deoxycholate or agents that activate the protein kinase A or C pathway, exhibits DNA binding activity (3–6). It has been proposed that the NF-κB complex in cytosolic fractions was associated with an inhibitory protein, referred to as IκB, that, upon treatment with phorbol esters, dissociates from the complex and, thereby, allows the nuclear translocation and DNA binding activity of the NF-κB heterodimer (3, 4).

pp40, a 40-kDa phosphoprotein, was originally identified as a rel-associated protein in chicken lymphoid cells transformed with reticuloendotheliosis virus containing the v-*rel* gene. It was shown to prevent the binding of p50–p65 heterodimeric NF-κB complex and of v- and c-rel proteins to κB site (6, 7). The inhibitory activity of pp40 was modulated by the extent of its phosphorylation (6). Furthermore, avian pp40 showed functional, immunological, and structural homologies with mammalian IκB protein (6). Recently, a number of investigators have reported the identification and molecular cloning of cDNAs encoding IκB-like proteins, including the product of immediate early gene (MAD-3) in monocytes (8), chicken pp40 (7), and IκBγ, a 70-kDa protein encoded by an alternate mRNA from lymphoid cells with sequences identical to the C-terminal half of 110-kDa NF-κB protein (9–11). Analysis of the structure of MAD-3 and pp40 cDNAs revealed the presence of five ankyrin repeats previously identified in a number of transcription factors, receptors, and cytoplasmic proteins—many of which play essential roles in normal embryonic development (12–14). Furthermore, ankyrin repeats have been shown to be involved in

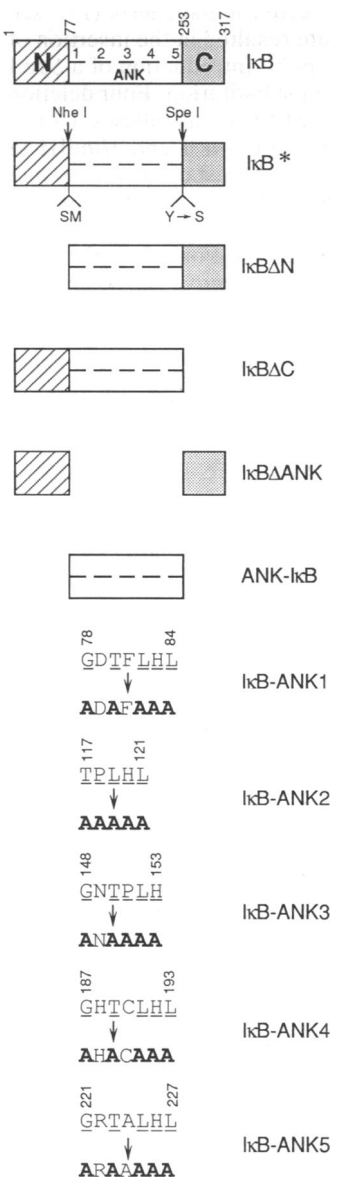

FIG. 1. pp40/IκB mutants. Structure of pp40/IκB mutants. Hatched area, N-terminal domain [amino acids (aa) 1–76]; stippled area, C-terminal domain (aa 253–317). The ankyrin-repeat region (ANK) has five ankyrin repeats indicated by dashes. IκB* has a 2-aa insertion (Ser-Met) and a 1-aa substitution (Tyr → Ser). In aa substitution ankyrin repeat mutants, only a part of aa sequences are shown and aa changed by mutation are indicated in boldface type.

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Abbreviations: aa, amino acid(s); GST, glutathionine *S*-transferase; CAT, chloramphenicol acetyltransferase.

direct protein–protein interactions (13). In this report we have explored the mechanism by which pp40/IκBβ inhibits the DNA binding activity of rel/NF-κB protein. We show that both ankyrin repeats and C-terminal domain of pp40 are required for inhibiting DNA binding activity. Furthermore, inhibition of rel DNA binding activity requires physical association with pp40.

MATERIALS AND METHODS

Construction of Plasmids. The *Eco*RI fragment of pp40 cDNA [nucleotide 1469 to *Eco*RI site in pBluescript SK− (Stratagene) (7)] was recloned into the *Eco*RI site of pBluescript SK− (Stratagene). For making various deletion mutants, pBSIκB* was first constructed. A *Nhe* I site and a *Spe* I site were introduced into the 5′ and 3′ end of ankyrin repeats region, respectively, by site-directed mutagenesis (15) (see Fig. 1). Introduction of *Nhe* I site resulted in the insertion of Ser-Met between Ala-69 and Trp-70. Introduction of a *Spe* I site resulted in the Ser-252 → Tyr substitution. Four deletion mutants were constructed using pBSIκB* as follows: (*i*) For IκBΔN, pBSIκB* was digested with *Nhe* I and *Hin*dIII to remove N-terminal domain. Translation starts from the methionine introduced by mutagenesis for the insertion of *Nhe* I site. (*ii*) For IκBΔC, pBSIκB* digested with *Spe* I was used for *in vitro* transcription as template. (*iii*) For IκBΔ, pBSIκB* was digested by *Nhe* I and *Spe* I to remove entire ankyrin repeats motif. (*iv*) For ANK-IκB, a *Nhe* I–*Spe* I fragment of IκB* was cloned into the *Spe* I site of pBluescript SK−. The first methionine is the same as that of IκBΔN. For making mutants with amino acid substitutions in ankyrin repeats shown in Fig. 1, site-directed mutagenesis was performed using appropriate oligonucleotides (15).

Protein–Protein Association Assay. ^{35}S-labeled proteins were translated *in vitro* in the presence of L-[^{35}S]methionine by using a wheat germ extract and *in vitro*-transcribed RNA from various templates indicated. pT7GT (Lynn J. Ransone, personal communication), in which the glutathione-*S*-transferase (GST) gene is under the control of the T7 promoter, was used to express GST–rel [rel466, which encodes the first 466 aa of murine c-rel (16), was inserted between the *Bam*HI and *Hin*dIII sites of pT7GT]. For the expression of GST–IκBΔN, the *Nhe* I–*Eco*RI fragment of pBSIκB*, which encodes entire ankyrin repeats and C-terminal region, was inserted into the *Cla* I site of pT7GT. For the expression of GST–ANK-IκB, the *Nhe* I–*Spe* I fragment of pBSIκB*, which encodes entire ankyrin repeats, was inserted into the *Cla* I site of pT7GT. The expression and purification of these proteins were as described (9). From 800-ml cultures, 200–800 μg of each protein was obtained. For protein–protein association assay, 5 μl of ^{35}S-labeled *in vitro*-translated protein and 10 μl of the suspension of GST fusion proteins attached to Sepharose were incubated at room temperature for 1 hr in 250 μl of 0.2% Triton X-100/20 mM Tris·HCl, pH 7.5/150 mM NaCl. Protein complex attached to Sepharose was washed four times with binding buffer and the final pellet was analyzed by SDS/PAGE on 12.5% or 10% gels.

Electrophoretic-Mobility-Shift Assay. A double-stranded oligonucleotide containing a κB site from the mouse κ-light chain enhancer (5′-AGCTTCAGAGGGGACTTTC-CGAGAGG-3′ and 5′-TCGACCTCTCGGAAAGTC-CCCTCTGA-3′) was used as a probe (6). Purified p50–p65 heterodimer (20 ng), 200 ng of mouse rel protein expressed in bacteria (16), and 2 μl of p50 and 10 μl of p65 translated in wheat germ extract (Promega) were used for DNA binding analysis. Five microliters of each pp40 and mutant translated *in vitro* and ≈400 ng of GST fusion proteins were used as effector proteins.

RESULTS

Ankyrin Repeats and C Terminus Are Required for pp40/IκBβ Activity. To study the mechanism by which pp40 inhibits the rel/NF-κB activity, we generated a variety of pp40 mutants capable of encoding various regions of the protein (Fig. 1). We introduced a *Nhe* I site and a *Spe* I site

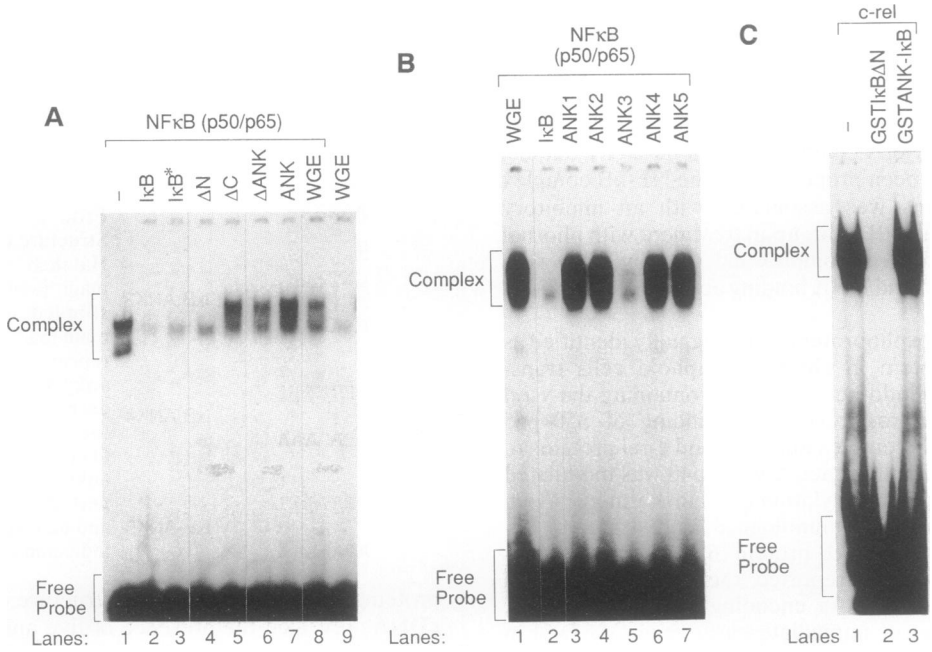

FIG. 2. Inhibitory activity of pp40/IκB mutants. (*A*) Effect of pp40 deletion mutant on the DNA binding activity of NF-κB p50–p65 heterodimer. (*B*) Effect of aa substitutions in an individual ankyrin repeat of pp40 on the DNA binding activity of p50–p65 heterodimer. In *A* and *B*, 20 ng of purified p50–p65 heterodimer was incubated with radiolabeled κB probe in the presence of 5 μl of each pp40/IκB mutant (in *A*, lanes 2–8; in *B*, lanes 2–7) or wheat germ extract (WGE; in *A*, lane 8; in *B*, lane 1). Wheat germ extract (5 μl) was incubated with κB probe (in *A*, lane 9). (*C*) Effect of pp40 deletion mutants on the DNA binding activity of c-rel. Mouse c-rel protein (200 ng) was incubated with radiolabeled κB probe in the absence (lane 1) or the presence of 400 ng of GST–IκBΔN (lane 2) or GST–ANK-IκB (lane 3). Electrophoretic-mobility-shift assays were performed.

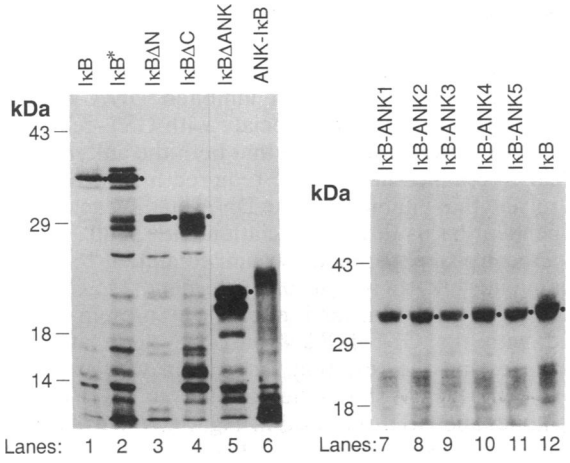

FIG. 3. Analysis of *in vitro*-translated pp40/IκB mutants. Translations were performed in the presence of L-[^{35}S]methionine using wheat germ extract. Each translated product (5 μl) was analyzed by SDS/PAGE on a 12.5% gel. Dots indicate full-length protein of each mutant. A number of shorter, presumably premature, termination products can be observed; however, only the full-length protein associates with rel (see Fig. 5).

at either end of the cluster of ankyrin repeats to generate precise deletion mutants. The resulting construct IκB* and wild-type IκBβ had identical activities (Fig. 2*A*, lane 3; the term IκB denotes wild-type and mutant pp40). We then generated mutants where the N terminus, the ankyrin repeats, the C terminus, or a combination were deleted. We also constructed mutants where the consensus aa found in ankyrin repeats from different proteins were mutated to alanine in each of the five ankyrin repeats of pp40 (Fig. 1, IκB-ANK1 to IκB-ANK5). The aa sequence -G-TPLH was mutated since this region is most conserved among ankyrin repeats in many proteins (14). Fig. 3 shows that all nine mutants, wild-type pp40 (IκB), and IκB* can be translated *in vitro* with nearly equal efficiency in wheat germ extract.

In vitro-translated wild-type or mutant proteins were used to assay the DNA binding activity of purified NF-κB (p50–p65) complex. As can be seen in Fig. 2*A*, both wild-type and IκB* (containing *Nhe* I and *Spe* I sites) proteins inhibited binding of the p50–p65 complex to DNA (compare lanes 2 and 3 to lane 1). Additionally, mutant IκBΔN where the first 77 aa were deleted also displayed nearly complete inhibitory activity (lane 4). A mutant IκBΔC (Fig. 2*A*, lane 5) that contains all the five ankyrin repeats but is missing the 65 aa at the C terminus was unable to manifest inhibitory activity. Another mutant IκBΔANK, which has intact N and C termini but no ankyrin repeats, also lacked inhibitory activity (Fig. 2*A*, lane 6). Finally, a mutant that retains only the ankyrin repeats was also unable to inhibit the activity of p50–p65 NF-κB complex (Fig. 2*A*, lane 7), leading us to conclude that both the ankyrin repeats and the C terminus of pp40 protein are required for inhibition of the DNA binding activity.

To determine the role of individual ankyrin repeats in the pp40 protein, constructs with consensus sequence mutations within each ankyrin repeat were translated *in vitro* and analyzed for their inhibitory effect. Fig. 2*B* shows that the proteins encoded by mutants in ankyrin repeats 1, 2, 4, and 5 (Fig. 1; IκB-ANK1, -ANK2, -ANK4, and -ANK5) were unable to inhibit the DNA binding activity of p50–p65 NF-κB complex (Fig. 2*B*, lanes 3, 4, 6, and 7, respectively). Interestingly, mutations in ankyrin repeat 3 (Fig. 1) had little effect on the inhibitory activity of pp40 (Fig. 2*B*, lane 5). Thus, it appears that the integrity of each ankyrin repeat, except ankyrin repeat 3 of pp40, needs to be maintained to inhibit DNA binding ability of p50–p65 heterodimer, suggesting a higher-order structural requirement.

We extended the results obtained with wild-type and mutant pp40 proteins on the DNA binding activity of p50–p65

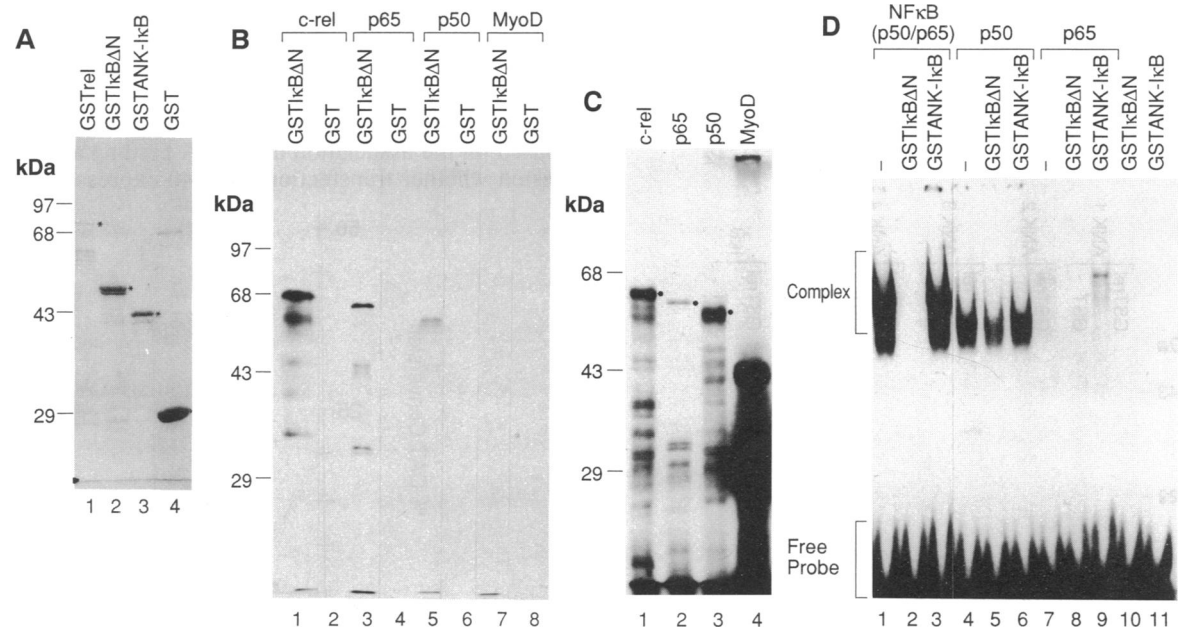

FIG. 4. Association of pp40 with rel, p65, and p50. (*A*) Analysis of GST fusion proteins. Each GST fusion protein used was analyzed by SDS/PAGE on a 10% gel. The gel was fixed and proteins were visualized by staining with Coomassie brilliant blue. Asterisks indicate full-length GST fusion proteins. (*B*) Direct association between GST–IκBΔN and rel family proteins. ^{35}S-labeled *in vitro*-translated products (5 μl) shown in *C* were incubated with 10 μl of the suspension of GST–IκBΔN or GST attached to Sepharose, and association experiments were performed. (*C*) Analysis of *in vitro*-translated products. ^{35}S-labeled *in vitro*-translated products (5 μl) were analyzed by SDS/PAGE on 10% gels. Dots indicate full-length protein. (*D*) Involvement of C-terminal domain of pp40 in the inhibition of DNA binding activity of rel family proteins. Purified p50–p65 heterodimer (20 ng; lanes 1–3), *in vitro*-translated p50 (2 μl; lanes 4–6), or *in vitro*-translated p65 (10 μl; lanes 7–9) was incubated with radiolabeled κB probe in the absence (lanes 1, 4, and 7) or presence of 400 ng of GST–IκBΔN (lanes 2, 5, and 8) or GST–ANK-IκB (lanes 3, 6, and 9) or 400 ng of GST–IκBΔN (lane 10) or GST–ANK-IκB (lane 11) was incubated with κB probe, and electrophoretic-mobility-shift assays were performed.

heterodimer by using the rel protein and obtained essentially identical results (data not shown). Fig. 2*C* shows two representative examples where the deletion of N-terminal residues (IκBΔN, lane 2) inhibits DNA binding ability of rel, whereas a mutant synthesizing only the ankyrin repeats has no effect on the DNA binding activity (Fig. 2*C*, lane 3). We therefore conclude that inhibition of rel/NF-κB proteins by pp40 requires both intact ankyrin repeats and C-terminal domain.

Direct Association of pp40/IκBβ with rel Protein. Since pp40 does not directly bind to κB site (6) (Fig. 4*D*, lanes 10 and 11), we reasoned that it may directly form a complex with rel/NF-κB proteins and, thereby, prevent binding to DNA. We chose rel protein as a representative of the rel/NF-κB family and linked it to GST (GST–rel). ^{35}S-labeled wild-type and mutant pp40 proteins translated *in vitro* were then mixed with GST–rel and the association was analyzed by SDS/PAGE. Fig. 5 shows that both the wild-type pp40 and IκB* bound to GST–rel (Fig. 5*A*, lanes 1 and 3) but not to control GST resin (lanes 2 and 4), indicating a direct association of the two proteins. Mutant IκBΔN, which inhibited DNA binding activity of NF-κB (Fig. 2*A*, lane 4) and rel (Fig. 2*C*, lane 2), was also able to associate with GST–rel (Fig. 5*A*, lane 5), but all the mutants that did not inhibit the DNA binding activity of rel/NF-κB were also incapable of associating with GST–rel (Fig. 5*A*, lanes 7, 9, and 11). Similarly, all the mutants where consensus sequences in individual ankyrin repeats were mutated and did not inhibit DNA binding ability of NF-κB, failed to bind to GST–rel (Fig. 5*B*, lanes 1, 3, 7, and 9). Mutant IκB-ANK3, which inhibited DNA binding of NF-κB or rel, was able to associate with GST–rel (Fig. 5*B*, lane 5). We therefore conclude that both the ankyrin repeats and the C terminus of pp40 are required for the association with rel and that inhibition of the DNA binding activity of rel is dependent on its direct association with pp40.

In a reciprocal experiment, we linked mutant IκBΔN (Fig. 1, containing ankyrin repeats and C terminus) to GST (GST–IκBΔN). *In vitro*-translated murine rel protein was then mixed with the GST–IκBΔN or GST alone attached to Sepharose, and the resulting association was analyzed by SDS/PAGE. Fig. 4*A* shows the size analysis of GST–IκBΔN, GST alone, and GST–rel (used in Fig. 5). *In vitro*-translated rel protein can associate with GST–IκBΔN (Fig. 4*B*, lane 1) but not with GST alone (Fig. 4*B*, lane 2) or ankyrin repeats (data not shown). The specificity of the rel association was confirmed by using *in vitro*-translated MyoD protein, which showed no association with GST–IκBΔN or GST (Fig. 5*B*, lanes 7 and 8). Both the rel and MyoD proteins were efficiently translated in wheat germ extracts (Fig. 4*C*, lanes 1 and 4).

p65 and p50 also Associate with pp40/IκBβ. Because pp40 can prevent the DNA binding activity of p50–p65 heterodimer (Fig. 2*A*), we wanted to determine whether like rel, the two subunits also associate with pp40. ^{35}S-labeled p65 and p50 proteins were translated *in vitro* in wheat germ extract (Fig. 4*C*, lanes 2 and 3) and mixed with GST–IκBΔN. Like rel, both p65 and p50 associate with IκBΔN (Fig. 4*B*, lanes 3 and 5) and not with GST (Fig. 4*B*, lanes 4 and 6). To investigate whether association with pp40 is sufficient for the inhibition of the DNA binding activity of p65 and p50, we performed a gel-shift analysis. Fig. 4*D* shows that whereas GST–IκBΔN inhibits the DNA binding activity of both p50–p65 heterodimer and p65 (Fig. 4*D*, lanes 2 and 8), it has little effect on the DNA binding activity of p50 subunit (Fig. 4*D*, lane 5). The results with p50 subunit of NF-κB suggest that the association of pp40 with p50 is not sufficient for exerting its inhibitory activity.

pp40/IκBβ Suppresses κB-Site-Dependent Transcription. Based on the association and DNA binding assays, we next tested whether transfection by pp40 expression vector pre-

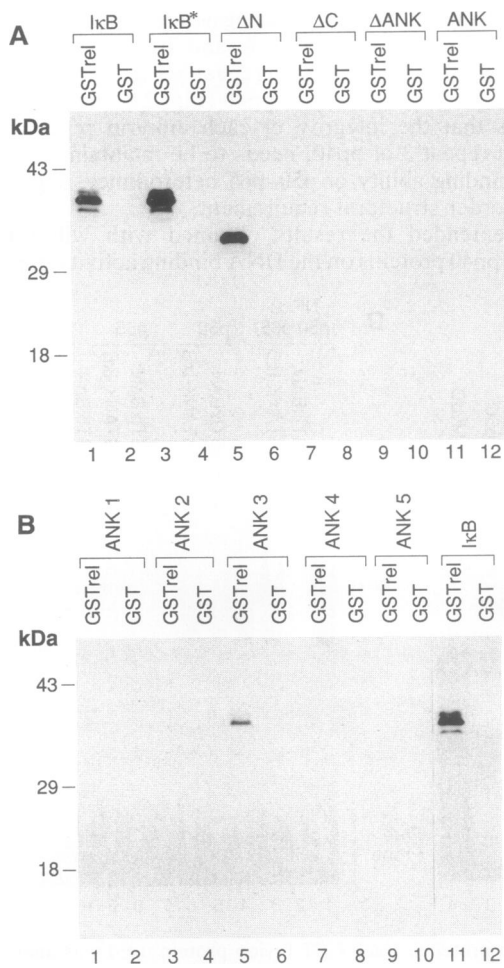

FIG. 5. Analysis of direct association between pp40/IκB and rel protein. (*A*) Ankyrin repeats and the C-terminal domain of pp40 are required for the association with rel. (*B*) Effect of the mutation in individual ankyrin repeats of pp40 on the association with rel protein. In *A* and *B*, 5 μl of ^{35}S-labeled *in vitro*-translated proteins shown in Fig. 3 was incubated with 10 μl of the suspension of GST–rel or GST protein attached to Sepharose, and protein–protein association assays were performed.

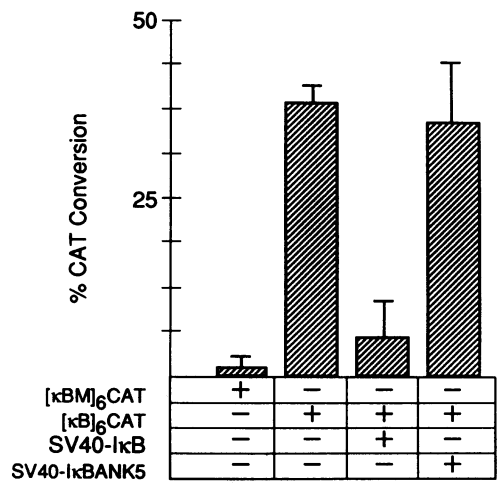

FIG. 6. Suppression of endogenous NF-κB activity by pp40. COS7 cells (5 × 10^5 cells) were transfected with 10 μg of [κB]$_6$TKCAT or [κBM]$_6$TKCAT as a reporter construct in the presence (10 μg) or absence of the pp40 or mutant pp40 (IκB-ANK5) expression plasmid. An equal quantity of protein from each sample was analyzed for CAT activity as described (17). Results are the average of three transfection experiments.

vents the endogenous NF-κB-mediated transcription from a reporter plasmid $[\kappa B]_6$TKCAT (16). Data in Fig. 6 show that chloramphenicol acetyltransferase (CAT) activity was detected in COS7 cells transfected with $[\kappa B]_6$TKCAT constructs but not with mutant $[\kappa BM]_6$TKCAT, indicating that CAT activity was the consequence of endogenous rel/NF-κB activity. Repression of more than 5–10 times of the endogenous NF-κB activity was observed when cells were cotransfected with a construct generating wild-type pp40 but not with mutant IκB-ANK5, indicating that association of pp40 with rel/NF-κB family of proteins through ankyrin repeats is required for this suppression.

DISCUSSION

We have performed mutational analysis of pp40/IκBβ and demonstrate that four out of the five ankyrin repeats and the C terminus of pp40/IκB are required for manifesting its inhibitory activity. Mutant pp40/IκB proteins that fail to associate with rel/NF-κB also do not inhibit DNA binding activity.

The precise higher-order structure of the 33-aa ankyrin repeat motif is not known but is found in a variety of genes involved in transcription [for instance GA binding protein (GABP) (13, 14), CDC10 (18), SWI4 (19), SWI6 (20), and p110 NF-κB (10, 11)] and growth factor/receptors [for instance, Notch in the fruit fly (21) and Lin-12 and glp-1 (22, 23) in *Caenoralhlabditis elegans*]. Interestingly, ankyrin repeat motifs were found in *bcl3*, a candidate protooncogene identified in a chronic lymphocytic leukemia tumor that has t(14:19)(q32;q13.1) translocation (24). The bcl3 mRNA levels are much higher in leukemic cells than controls (24). Preliminary results in our laboratory indicate that cotransfection of bcl3 cDNA suppress endogenous NF-κB activity (L.D.K., unpublished data).

We have shown (6) that the N-terminal 51 aa of the rel protein were required for association with pp40/IκBβ. Furthermore, pp40 binds to aa 1–81 of rel and deletion of aa 51–124 of rel protein does not compromise association with pp40 (L.D.K. and J.-i.I., unpublished data). pp40 also associates with the p65 and p50 subunits of NF-κB (Fig. 4*B*). Therefore, it is likely that rel, p65, and p50 share common sequences that are involved in association with pp40. A comparison of the aa sequence of these proteins reveals a stretch of 50 aa at the N terminus that are highly conserved (25). The N-terminal half of rel and p50 has been shown to be involved in DNA binding and dimerization. Our working hypothesis is that pp40 forms a structure involving four out of the five ankyrin repeats and part of the C terminus, which then interacts with the N-terminal region of rel/NF-κB in a manner that prevents access to DNA binding domain and perhaps translocation to the nucleus. Since the nuclear localization signal of rel/NF-κB is adjacent to the DNA binding domain, it is not clear whether the primary target of IκB is to mask the DNA binding domain or nuclear localization signal.

Is association with pp40 sufficient to prevent binding of rel/NF-κB to DNA? Apparently not, because pp40 can associate with p50 (Fig. 4*B*) but does not inhibit the DNA binding activity of p50 homodimer efficiently (Fig. 4*D*). The binding affinity of pp40 to p50, p65, or rel remains to be analyzed. One explanation is that there are several members of the IκB family, but each member has a specific functional affinity to rel, p65, p50, or p49 (26). This might explain the specificity of various members of the IκB family—for instance, binding of the p50 homodimer to DNA is inhibited by IκBγ, a member of the family that contains eight ankyrin repeats (9) but not by five-ankyrin-repeat-containing pp40 or MAD-3 gene products (6). Perhaps additional ankyrin repeats join in to make a structure that alters specificity. A more detailed knowledge of the exact aa sequences involved in the interaction between IκB proteins and members of the rel/NF-κB family would be required before a definitive model can be proposed.

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