Supplemental Figures

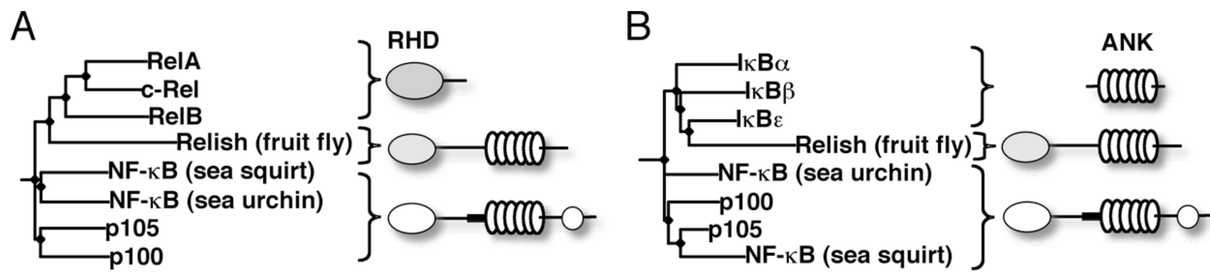

Figure S1. The evolutionarily conserved domain organization of p105 and p100

Rooted phylogenetic trees (Felsenstein, 1989) were constructed based on the sequence alignments of individual domains from human NF-κB and IκB proteins and from p105/p100 orthologs from fruit fly (*D. melanogaster*), sea urchin (*S. purpuratus*), and sea squirt (*C. intestinalis*).

(A) Rel homology domain, RHD. (B) ankyrin repeats domain, ANK.

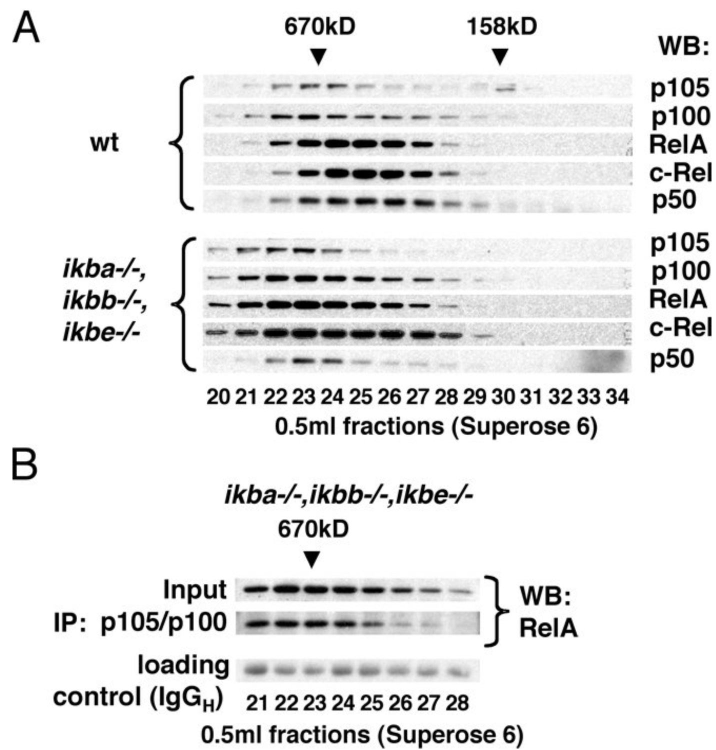

Figure S2. Endogenous RelA, c-Rel, and p50 partition into p105 and p100 complexes in the absence of classical IκB proteins

(A) Cytoplasmic extracts from wild type (wt) and *ikba-/-ikbb-/-ikbe-/-* mouse embryonic fibroblasts (MEF) were analyzed by gel filtration chromatography (GF) followed by Western blotting (WB).
(B) Cytoplasmic extract of *ikba-/-ikbb-/-ikbe-/-* MEF was fractionated by GF. Physical interaction of RelA with p105 and/or p100 in the high molecular weight (MW) gel filtration fractions was detected by immunoprecipitation (IP) using a mixture of antibodies specific for p105 and p100 followed by WB with RelA antibody.

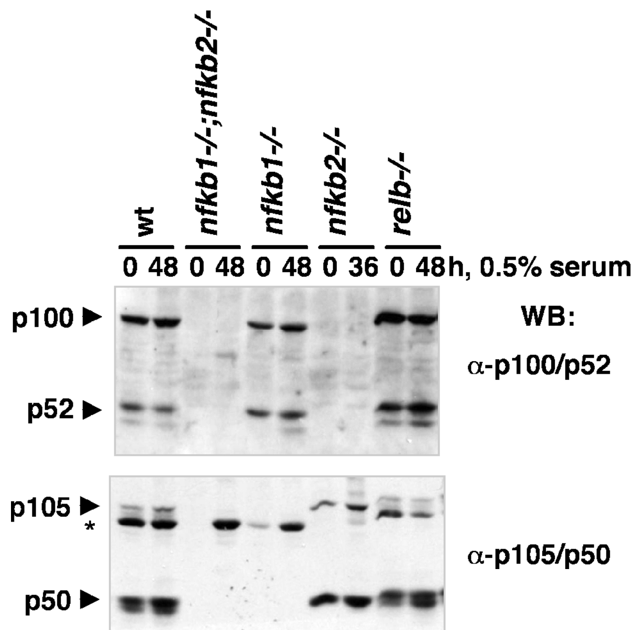

Figure S3. Two p52 and p50 isoforms are specifically detected by Western blotting

MEF from wild type animals (wt) and from mice with the individual or combined genetic deficiency in *nfkb1* and *nfkb2* genes; or in *relb* gene, were cultured in low (0.5%) serum-containing media for indicated times (0, 36 or 48 hours). Whole cell extracts were prepared and analyzed by Western blotting with the antibodies specific for p100/p52 (top panel) or p105/p50 (bottom panel). Star (*) indicates the non-specific band recognized by p105/p50 antibody.

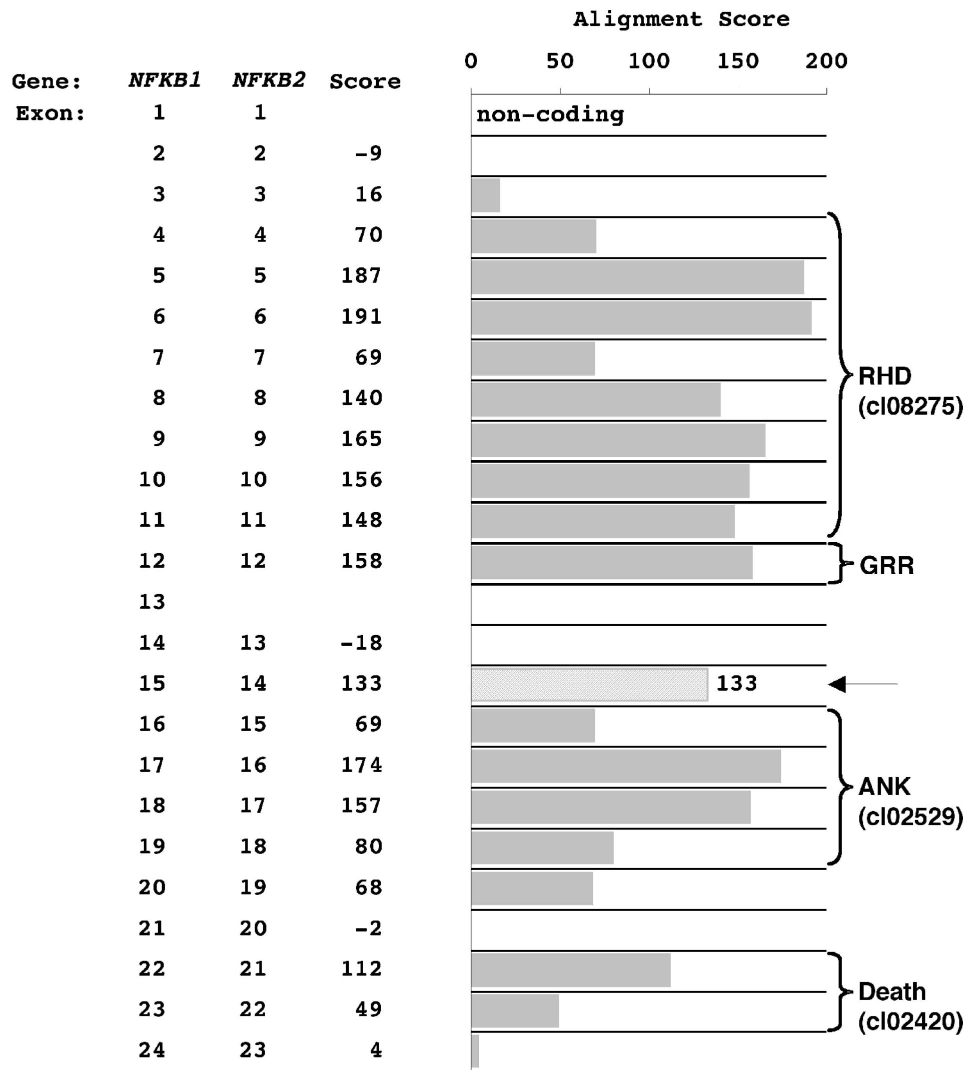

Figure S4. Sequence conservation between p105 and p100 proteins

Pairwise alignment of the translated protein sequences derived from the homologous exons of human *NFKB1* and *NFKB2* genes were performed using the sequence alignment tool ClustalW (Thompson et al., 1994). The alignment scores, which measure the sequence conservation, were plotted against *NFKB1* exon number. The numbers for corresponding exons of *NFKB2* gene and the numeric values for the alignment scores are also given. Exons encoding conserved protein domains (according to NCBI domain classification) and the glycine rich region (GRR) are indicated with the brackets next to the bar graph. Arrow points to the region (alignment score=133) in p105 and p100 proteins that serves as C-terminal dimerization domain.

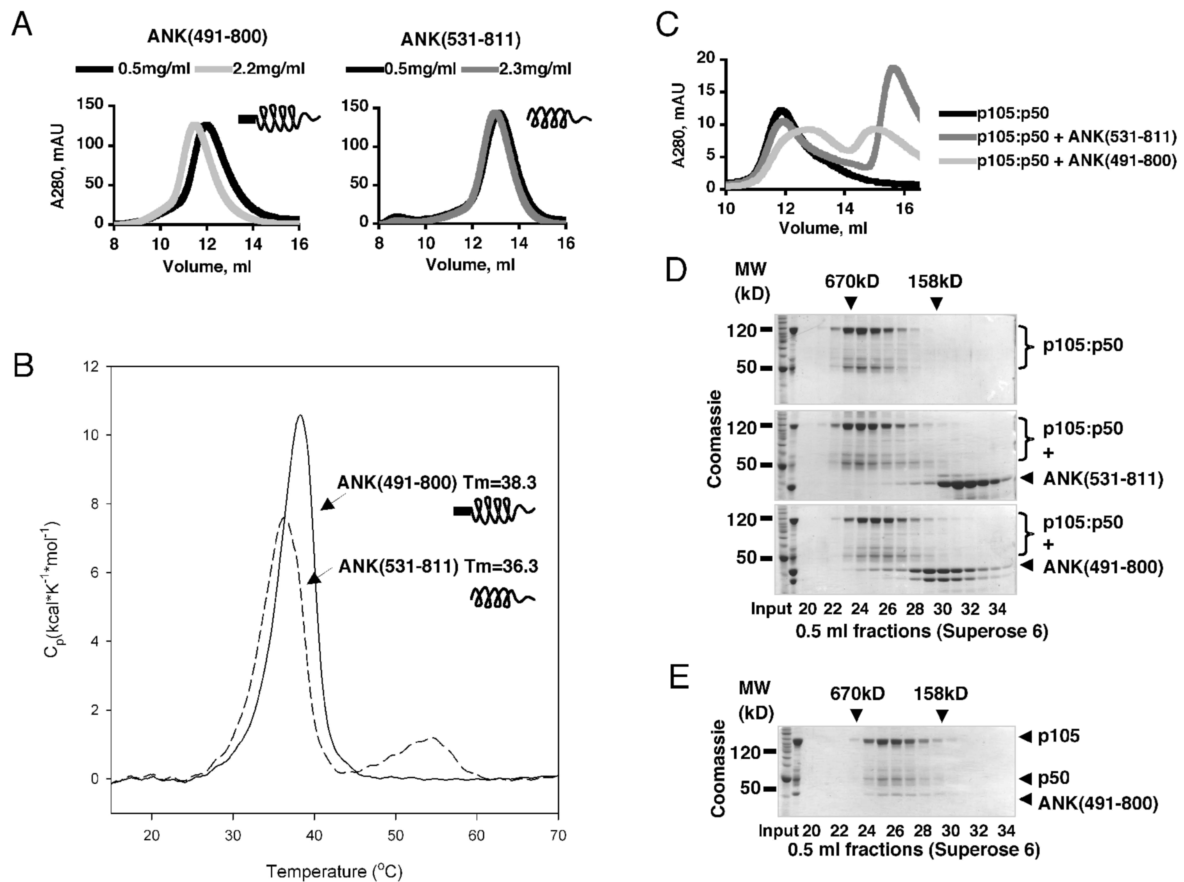

Figure S5. Identification of the second conserved dimerization domain in p105

(A) Purified p105 fragments, ANK(491-800) (left panel) and ANK(531-811) (right panel) were analyzed by gel filtration chromatography (GF) at two concentrations. Normalized absorbance at λ280nm (A280) was plotted against retention volume.
(B) Melting temperatures, Tm, for ANK(491-800) and ANK(531-811) were determined from the results of Differential Scanning Calorimetry (DSC). Heat capacities, C_p (kcal*K^{-1}*mol^{-1}) were plotted against temperature, ^{0}C.
(C) Purified p105 fragments, ANK(531-811) and ANK(491-800), were incubated with the recombinant p105:p50 complexes *in vitro* and analyzed by GF. A280 was plotted against retention volume.
(D) The resulting GF fractions (as in C) were analyzed by SDS-PAGE followed by Coomassie staining.
(E) ANK(491-800) complex with p105:p50 (detected in D, bottom panel) was re-purified by GF. The resulting GF fractions were analyzed SDS-PAGE followed by Coomassie staining.

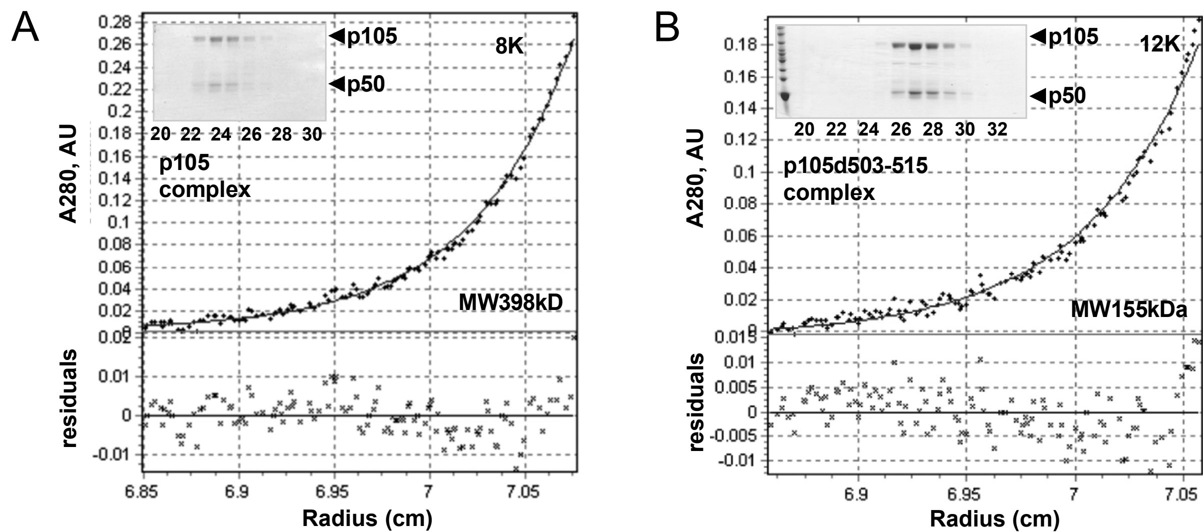

Figure S6. Molecular weight of p105 complexes

(A) His-tagged wild type p105 protein was expressed and purified from *E. coli* by Ni-affinity chromatography followed by ion exchange and gel filtration chromatography GF (insert shows the last step of purification) and analyzed by analytical ultracentrifugation (AUC). The representative result of the sedimentation equilibrium (SE) experiment performed at the rotor speed 8000rpm is shown.
(B) His-tagged mutant p105, p105d503-515, was purified from *E. coli* by Ni-affinity chromatography followed by GF (Fig. 5C). The lower MW peak was collected and re-purified by GF (insert shows the last step of purification) and analyzed by AUC. The representative result of the SE experiment performed at the rotor speed 12000rpm is shown.

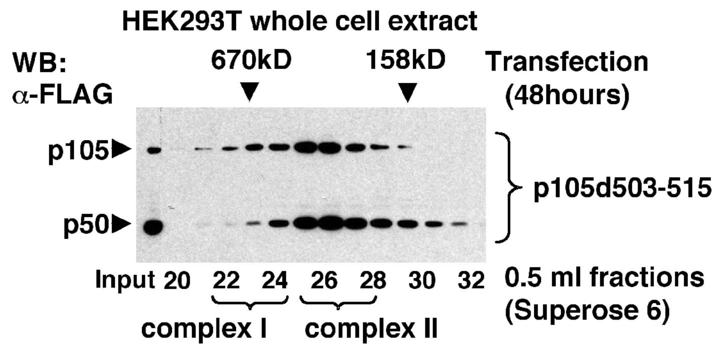

Figure S7. Complex II is the second intermediate of NF-κBsome assembly

The C-terminal helical dimerization domain mutant of p105, p105d503-515, was transiently expressed in HEK293T cells. Whole extract was prepared 48 hours after transfection and analyzed by gel filtration chromatography followed by Western blotting (WB). Complex II accumulated at 48 hours after transfection.

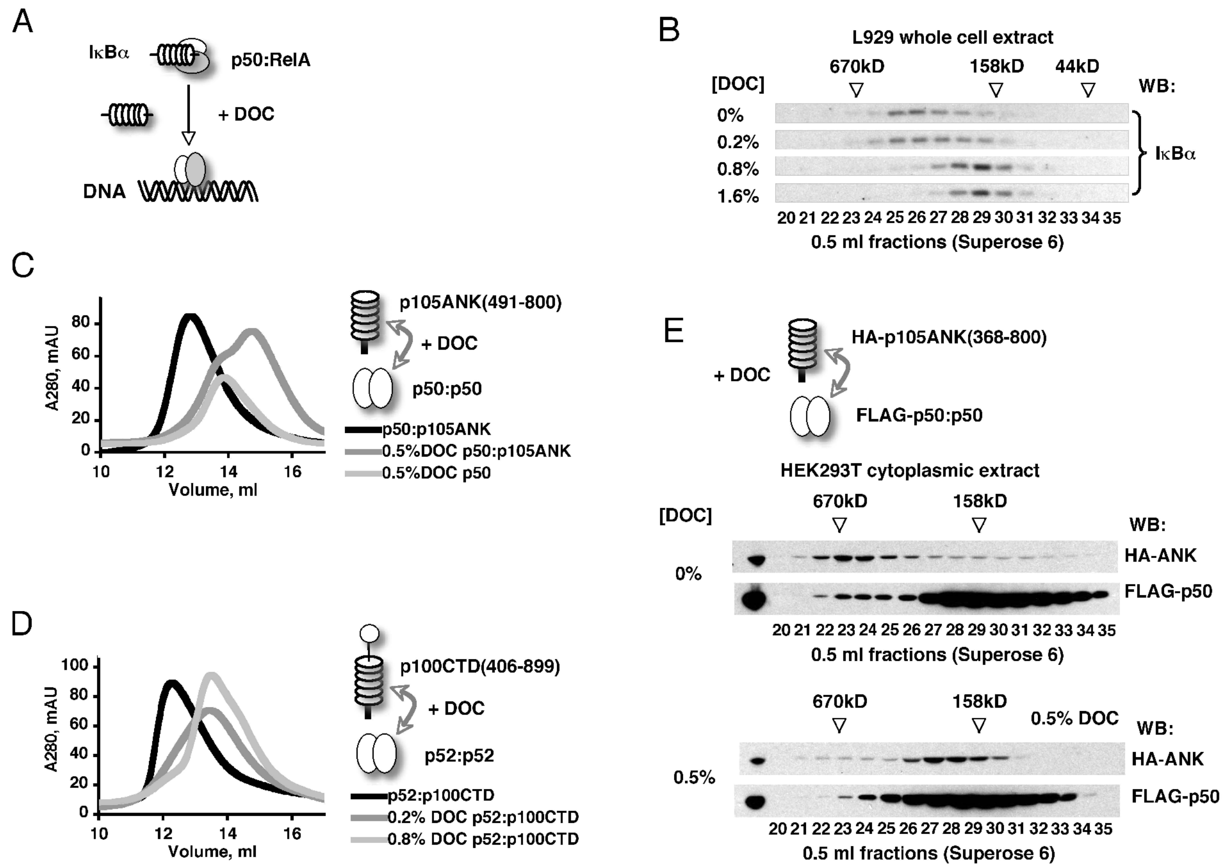

Figure S8. DOC sensitivity assay

(A) DOC treatment (Baeuerle and Baltimore, 1988) induces dissociation of latent cytoplasmic p50:RelA:IkBa complexes (Baeuerle and Baltimore, 1988)
(B) Using DOC treatment in combination with gel filtration chromatography (GF), we analyzed DOC sensitivity of endogenous IκBα complexes. IκBα was detected in gel filtration fractions by Western blotting (WB)
(C) Complete dissociation of p50:ANK(491-800) recombinant complexes in the presence of 0.5% DOC was detected by GF. A280 was plotted against retention volume.
(D) Dissociating effect of the increasing concentrations of DOC on p52:p100CTD(406-899) complex was determined by GF. A280 was plotted against retention volume.
(E) HA-tagged p105ANK(368-800) was co-expressed with FLAG-tagged p50 in HEK293T cell. Cytoplasmic fraction was prepared 24 hours after transfection and analyzed by GF in the absence or in the presence of 0.5% DOC. FLAG-tagged and HA-tagged proteins were detected by WB.

Supplemental Experimental Procedures

Cell Culture

RAW264.7, THP-1 cells and mouse bone marrows (ALS strain) were provided by A. Savinov (BIMR, La Jolla, CA). BMDM were derived by L929 protocol (adapted for mouse tissues) as previously described (Boltz-Nitulescu et al., 1987). L929 cells were from ATCC. Immortalized wild type, *ikba-/-ikbb-/-ikbe-/-*, and *nfkb1-/-nfkb2-/-* mouse embryonic fibroblasts (MEF) were described previously (Hoffmann et al 2003, Basak et al 2007). All cell cultures, except THP-1, were pre-incubated with media containing 0.5% serum (low serum) for 36 hours before LPS stimulation; low-serum media was used for LPS treatment and chase periods. THP-1 monocytes were differentiated into macrophage-like cells with 50ng/ml (31nM) PMA for 48 hours (Tsuchiya et al., 1982), rested 48 hours with daily replenishment of media and stimulated with LPS in media containing 10% serum.

Antibody and other Reagents

Antibodies against RelA (sc372; sc372G), c-Rel (sc71; sc71G), RelB (sc226), p105/p50 (sc7178), IκBα (sc371), IκBβ (sc946), and IκBε (sc7176) were from Santa Cruz Biotechnology; p100/p52 (05-361) was from Upstate Cell Signaling Solutions. p105/p50 (#1157); p100/p52 (#1495), and p105-C (#1140) rabbit antisera were a kind gift from N. Rice (NIH, Bethesda, MD). Anti-FLAG M2 antibody (F3165) was from Sigma.
Protease Inhibitor Cocktail (P8849), LPS (L6529), FLAG peptide (F3290), Ni-affinity (P6611), and FLAG-affinity (A2220) resins were from Sigma. GST-affinity resin was from Amersham. TEV protease was purified in house. Gel filtration standards (151-1901) were from BioRad Laboratories. Gel Filtration media, Superdex 200 (17-1047-01), Superdex 75 (17-1044-01) and SP Sepharose FastFlow (17-0729-01); and pre-packed gel filtration column Superose 6 10/300 GL (17-5172-01) were from Pharmacia. All other reagents were from commercial suppliers.

Western Blotting and Immunoprecipitation

Human p105/p50 and p100/52 were detected by Western blotting using commercial rabbit polyclonal p105/p50 (sc7178) and mouse monoclonal p100/p52 (05-361) antibodies, respectively. Mouse p105/p50 and p100/52 were detected using p105/p50 (#1157) and p100/p52 (#1495) rabbit antisera respectively. Other NF-κB subunits and IκB proteins were detected by a panel of commercially available rabbit polyclonal antibodies as listed in Antibody and other Reagents section. FLAG-tagged p105 was detected by anti-FLAG M2 antibody.
To immunoprecipitate endogenous p105 from gel filtration fractions we used p105-C (#1140) rabbit polyclonal antisera raised against C-terminal peptide of human p105 (0.1μl per reaction). To precipitate RelA and c-Rel, goat polyclonal antibodies RelA (sc372G) and c-Rel (sc71G) were used at 0.2μg per reaction. RelB and IκBα were immunoprecipitated with 0.2μg of rabbit polyclonal anti-RelB (sc226) and anti-IκBα (sc371) antibodies per reaction. Each immunoprecipitation reaction was supplemented with 50mg/ml of bovine serum albumin to normalize for total protein content and performed according to standard immunoprecipitation protocol.

Protein Expression and Purification

Mouse p105 cDNA was used as a template for all p105 cloning. p105 cDNA was cloned into two expression vectors. pEGFP-C1 (Clonetech), in which YFP sequence was replaced by FLAG sequence, was used to express p105 in HEK293T cells. The bacterial expression vector, pHis8, provided by J. Noel (Salk Research Institute, La Jolla, CA), was used to express p105 in *E. coli*. cDNA corresponding to C-terminal fragments of p105, ANK(491-800) and ANK(531-811), was amplified by PCR and cloned into pHis8 and pET15b (Invitrogen) vectors, respectively. pET3a-RelA(19-304) plasmid was previously described (Huxford et al., 1998). Human cDNA corresponding to p100CTD ("C-terminal domain", amino acids 406-899) was cloned into bacterial expression vector pGV67 provided by G. Van Duyne (U. Penn). Human cDNA corresponding to N-terminal p100 fragment, p52 (amino acids 1-341, referred to as "p52" to facilitate discussion), was cloned into pET11a vector (Invitrogen). pHis8-p105(1-531) plasmid was derived from pHis8-p105 by site-directed mutagenesis introducing stop codon. This plasmid was used to express p50. p50 was obtained as a product of bacterial proteolysis of p105(1-531). FLAG-p105 was purified by FLAG affinity chromatography from HEK293T cells. His-tagged p105, p105 ANK(491-800), p105 ANK(531-811), and p50 were expressed in BL21(DE3) *E. coli* cells and purified by Ni-affinity chromatography followed by gel filtration chromatography. To obtain p105 complex with RelA(19-304), His-tagged p105 was co-expressed with RelA(19-304) in BL21(DE3) *E. coli* cells from plasmids conferring kanamycin and ampicillin resistance, respectively. p105 complex with RelA was purified by Ni-affinity chromatography followed by gel filtration chromatography. Co-expression of p105 with RelA(19-304) in *E. coli* also yielded the lower molecular weight p50:RelA(19-304) complex that was separated from p105:p50:RelA(19-304) complexes at the second step of purification. p52 and GST-p100CTD were expressed in *E. coli* and purified by ion-exchange chromatography and GST affinity chromatography, respectively. p52:GST-p100CTD complex was formed *in vitro* and, after removal of GST by TEV cleavage, p52:p100CTD complex was re-purified by gel filtration chromatography. p50:(ANK491-800) and p50:(ANK531-811) complexes were formed from purified components *in vitro* and used directly. All cells expressing recombinant proteins were disrupted or lysed in buffer suitable for the first step of purification and supplemented with protease inhibitor cocktail. *E. coli* cells were disrupted by sonication. HEK293T cells were lysed in RIPA buffer. All proteins were purified from soluble fractions of cell lysates. Ni-affinity buffer contained: 25mM Tris-HCl, pH7.5; 140mM NaCl; 10mM Imidazole. Ion-exchange buffer contained: 25mM Tris-HCl, pH7.5; 50 mM NaCl; 1mM DTT. The standard gel filtration buffer contained: 25mM Tris-HCl, pH7.5; 140mM NaCl; 1mM DTT. GST-p100CTD purification was performed according to GST affinity resin manufacturer protocol.

References (Supplemental Data section)

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