A p105-based Inhibitor Broadly Represses NF-κB Activities*

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An IκBα-based NF-κB super repressor (sr) has been used widely for studying genes regulated by NF-κB transcription factors. Repression of NF-κB by IκBα(sr) also facilitates tumor necrosis factor α-induced apoptosis in the cell. However, IκB primarily targets RelA and c-Rel-containing complexes, leaving other NF-κB/Rel protein complexes, such as p50 and p52 homodimers, and RelB heterodimers uninhibited. Because these atypical NF-κB complexes also contribute to gene regulation and are activated in pathological conditions, broad inhibition of all NF-κB species is of significant pharmacological and clinical interests. We have designed, generated, and tested a p105-based NF-κB super repressor. We showed that p105(sr), which no longer generates p50 and undergoes signal-induced degradation, effectively inhibits all NF-κB activities. In addition, we also demonstrated that p105(sr) significantly enhances tumor necrosis factor α-mediated killing of MT1/2 skin papilloma cells where p50 homodimer activity is elevated. Our results suggest that p105(sr) is a broader range and effective NF-κB super repressor and can potentially be used in cells where a noncanonical NF-κB activity is dominant or multiple NF-κB activities are activated.

Nuclear factor κB (NF-κB) is a latent dimeric complex sequestered in the cytoplasm by its inhibitor IκB and is activated to engage transcription in the nucleus by various stimuli. In normal physiological conditions such activation is transient, because of autoregulatory mechanisms. However, this family of transcription factors is constitutively activated in many types of cancer cell and is thought to regulate anti-apoptosis factors that aid survival of the cancer cells. Chemotherapy reagents induce death of cancer cells but also activate NF-κB pathways. Therefore, activation of NF-κB is a contributing factor of chemoresistance. Introduction of an IκBα-based NF-κB super repressor (sr) into cancer cells has not only enhanced stimuli-induced apoptosis but also facilitated systematic identification of genes regulated by NF-κB that may contribute to the malignancy and progression of the tumor.

Activation of prototypic NF-κB requires degradation of IκBs, and the prerequisite of the process is stimuli-induced phosphorylation of IκBs by the IκB kinase-constituted signalsome. IκBα(sr) was generated by either mutating serine 32 and 36, which are the targets of the IκB kinases or deleting the N-terminal portion of IκBα that harbors these targets. IκBα(sr) suppresses stimuli-induced NF-κB activation, because the inhibitor now cannot be phosphorylated and therefore will not be subjected to immediate degradation, and the bound NF-κB subunits will not be released into the nucleus.

Although in most normal and cancer cells, the NF-κB activity detected is that of prototype p50/RelA heterodimer, atypical/noncanonical NF-κB species also play significant roles in gene regulations. For example, the RelB/p52 complex plays a key role in B cell development. It has also been observed that NF-κB p50 homodimer activity is significantly elevated in certain types of cancers such as murine B cell leukemia and chemical-promoted mouse skin carcinomas, and such elevation has been linked to the survival of the cancer cells.

Furthermore, more than one species of NF-κB can be elevated in cancer cells, and broad inhibition of this family of proteins is pharmacologically and clinically significant.

p105 has been regarded as an atypical NF-κB/Rel protein inhibitor. It binds other Rel proteins and retains them in the cytoplasm. However, wild-type p105 cannot be employed as an NF-κB super repressor, because expression of the nfkbia gene always produces both p105 and p50 in the cell, and the latter is a component of the NF-κB transcription complex. In addition, wild-type p105, like IκBα, also undergoes signal-induced complete degradation, releasing the bound NF-κB species into the nucleus. Compared with IκBα, however, p105 possesses important features that make it potentially a broader range NF-κB super repressor. First, p105 interacts with all Rel proteins, including p50, p52, and RelB, with high affinity through the Rel homology domain (RHD) and IκB-like ankyrin repeats. Second, although both require the proteasome activity, p50 biogenesis and p105 degradation are separate processes. The middle region of p105, which contains a glycine-rich region and a putative degracion signal for the proteasome, is likely to be responsible for p50 generation. The C terminus of p105, which harbors the consensus sequences for the IκB kinases, the ubiquitin ligase β-transducin repeat-containing protein, and a death domain (DD), is required for signal-induced p105 degradation.

Based on these assumptions, we have designed and generated a p105(sr) by deletion of residues 356–498 in the middle region of murine p105 and the C-terminal residues 800–971. This p105 deletion mutant no longer generates p50 and responds to stimuli to undergo degradation. We showed that p105(sr) retains its ability to interact with all five NF-κB spe-
cies and inhibits tumor necrosis factor (TNF) α-induced NF-κB activities effectively. Unlike IκBζ(sr), which does not interact with p50 and p52 and hence does not inhibit the homodimer activities, p105(sr) interacts with both proteins, and we have shown that it effectively inhibits p50 homodimer activity. In addition, p105(sr) can serve as a potent inhibitor for RelB, which is an important player in B cell development (14). To apply p105(sr) in a specific biological context, we also tested p105(sr) in mouse skin papilloma MT1/2 cell line (31). p50 homodimer activity is significantly elevated in MT1/2 cells, and the cells are resistant to TNFα-mediated apoptosis. We demonstrated repression of p50 homodimer activity and TNFα-mediated killing of these tumorigenic cells by p105(sr). Our results indicate that p105(sr) is an effective NF-κB super repressor and can be used as a broader range alternative to IκBζ(sr).

**EXPERIMENTAL PROCEDURES**

Construction of p105(sr)—PCR was used to generate two deletions within murine nfκb1 gene (32). Two sets of primers were used to generate deletion in the middle region first: 5'-CGGAGATTCATGCCAG-CAGGATG, and 5'-CCTACTGGTTCTGCCCTGTTGATTTC to amplify the first half of nfκb1 and 5'-GACTTGAAGGCTGCTGACG-CGCC and 5'-GCTCTAGACTAAAAATCTCCCTTAAAGY to amplify the second half of the gene. The DNA fragments were cloned into the expression vector pEVP2 (33), with a blunted HindIII site from the first fragment directly ligated to the uncut 5'-end of the second fragment to preserve the correct reading frame. The above plasmid was then used as the template, and the primers 5'-GGGAGATTCATGCCAGGATG and 5'-GGATCCATGCCAGGATG and 5'-GGAATTCGAGTCTCCAGATATCATCAG were used for PCR. The PCR fragment was subsequently cloned into a modified pEVRF expression vector pEVRF (33), with a blunted HindIII site from the first half of the gene. The DNA fragments were cloned into the uncut 5'-end of the second fragment.

**Cell Culture and Transfection—**Human embryonic kidney 293 cells, human cervical carcinoma HeLa cells, Chinese hamster ovary cells with a stably transfected human CD14 surface marker (CHO-CD14) (35), and several mouse skin cell lines were used in the studies. HeLa and 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics and CHO-CD14 cells in RPMI1640 medium with the same supplements. The cells were seeded in either 24- or 6-well plates and transfected with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Skin papilloma cell line MT1/2, carcinoma line CH1 (spindle cell carcinoma), and the control keratinocyte line C50 were cultured in minimum essential Eagle’s medium with Earle’s balanced salt solutions supplemented with 4% (for MT1/2) or 1% (for C50 and CH7T24) fetal bovine serum, l-glutamine, antibiotics, and other growth supplements (36). The skin cells were transfected with FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions.

**Measurement of Protein Concentration—**A Bio-Rad protein concentration kit was used according to the manufacturer’s instructions, with bovine serum albumin as the standard.

**Immunoblotting and Co-immunoprecipitation—**Transfected CHO-CD14 cells were harvested 12–24 h post-transfection, and lysed with ELB buffer (300 mM NaCl, 9% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). For direct immunoblotting, 2–5 μg of lysates were resolved with SDS-PAGE (10%) and transferred to Immobilon-P membrane (Millipore). The membrane was blotted with antibodies, and the reactive bands were detected with ECL reagents (Amersham Biosciences). For co-immunoprecipitations followed with immunoblotting, expression vectors carrying gp10-tagged p105(sr) or IκBζ(sr) and the testing NF-κB species were co-transfected into CHO-CD14 cells, and the lattes were first immunoprecipitated with anti-gp10 monoclonal antibodies (Novagen) and protein A-Sepharose CL-4B beads (Sigma) at 4 °C for 3 h to overnight (rotating). The protein A beads were then washed several times with ELB buffer containing 1 mM NaCl and suspended in SDS-PAGE loading buffer. After boiling, the supernatants were resolved with SDS-PAGE, transferred to Immobilon-P membrane, and blotted with antibodies either to gp10 or to the NF-κB species (rabbit polyclonal; Santa Cruz Biotechnology).

**Pulse-Chase Metabolic Labeling—**Transfected HeLa cells were pre-incubated with Dulbecco’s modified Eagle’s medium without methio-nine and cysteine for 1 h and pulse-labeled with 10 μCi/ml [35S]methionine/cysteine (PerkinElmer Life Sciences) for 30 min following immunoprecipitation as described previously (23).

**Luciferase Reporter Assay—**293 or HeLa cells (105 in 24-well plate) were co-transfected with either p105(sr) or IκBζ(sr) with two luciferase reporter constructs: B-luciferase (firefly) construct and thymidine kinase-Renilla luciferase construct (Promega). The latter reporter driven by thymidine kinase promoter provides a low level, constitutive expression of Renilla luciferase in the cell and serves as the internal control. 36 h post-transfection, the transfected cells were treated with TNFa (20 μg/ml) for 30 min and lysed. The lysates were then assayed with luciferase substrates provided by the Promega kit. For each experiment, lysates from three independently transfected cells were assayed, and all of the data were normalized with the readout of Renilla luciferase activity.

**Electrophoretic Mobility Shift Assay (EMSA)—**100 ng of double-stranded κB probe (Santa Cruz Biotechnology) was labeled with 15 μCi of [γ-32P]ATP (Amer sham Biosciences) and T4 polynucleotide kinase and purified with MicroSpin G-50 mini columns (Amer sham Biosciences). 8 μg of TNFa-treated or 2.5 μg of p50-transfected HeLa cell nuclear extracts were incubated with 1 μg of poly(dI-dC), 1 μl of labeled κB probe, and the binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 12.5% glycerol, and 0.2 mM dithiothreitol) at room temperature for 30 min before loading to 5% non-denaturing polyacrylamide gel. For antibody-mediated supershift, the reaction mixture was pre-incubated with 1 μl of either anti-p50 or anti-RelB antibodies (Santa Cruz Biotechnology) at room temperature for 20 min before loading of the samples. For cold κB nucleotide competition, 20 ng of the wild-type or mutant κB nucleotides were preincubated with the reaction mixture.

**Cell Fractionation—**Transfected CHO-CD14 cells (2 × 105) were lysed with sucrose buffer (0.32 mM sucrose, 10 mM Tris-HCl pH 8.0, 3 mM CaCl2, 2 mM MgOAc, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 500 × g for 5 min. The supernatants were saved as the cytosolic fraction. The pellets were washed once with sucrose buffer without Nonidet P-40 and suspended in low salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). An equal volume of high salt buffer (same composition as the low salt buffer except with 800 mM KCl) was added slowly. The lysates were incubated for 30–45 min at 4 °C on a rotator and centrifuged at 14,000 × g for 15 min. The supernatants were saved as nuclear fractions.

**Apoptosis Assays—**MT1/2 and C50 cells were treated with TNFa (20 μg/ml) for 16 h. The cells were washed with phosphate-buffered saline buffer and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then stained with 5 μg/ml of Hoechst dye (32324, Sigma) for 10 min and examined with fluorescence microscopy. The percentage of cells undergoing apoptosis was calculated by counting apoptotic cells in eight random areas (each area contains approximate 500 cells) of each treated sample as previously reported (37, 38). The data represent three sets of separated experiments. Similar analyses were applied to transfected MT1/2 cells.

**RESULTS**

**Design and Generation of p105(sr)—**Although the C-terminal portion of p105, IκBζ, has been shown to co-immunoprecipitate p50 (20, 39, 40), its affinity to other NF-κB species and even to p50 is not as high as the N-terminal RHD. We hence chose to design a super repressor based on p105, which contains both the RHD and the ankyrin repeat. For designing a p105-based NF-κB super repressor, two conditions must first be satisfied. First, expression of the modified p105 should no longer generate p50, a component of the NF-κB transcription complex. Second, the modified p105 should not be degraded immediately upon extracellular signals, because such a process will release bound NF-κB species. Based on studies of regulation of p50/p105 homeostasis (25–30), we deleted residues 356–498 in the middle region that covers from the nuclear localization signal (NLS) to the putative degradation signal in the middle of p105 to include p50 generation (Fig. 1A). To abolish signal-induced p105 degradation, we deleted residues 800–971 of p105 that include the degradation signal in the C terminus and the DD (28) (Fig. 1A).
p105(sr) no longer generates p50 and does not respond to TNFα-induced degradation. A, anatomy of wild-type murine p105. Regions corresponding to p50 and IκBα are shown, and residue 453 marks the C terminus of p50. GRR, the glycine-rich region; DSM, degradation signal in the middle of p105; DSC, degradation signal in the C terminus of p105 that includes the DD. B, p105(sr) does not generate p50. gp10-tagged p105(sr) and p105(wt) were expressed in CHO-CD14, HeLa, and 293 cells. Twice the volume of HeLa cell lysates was loaded on the gel, because expression in HeLa cells is lower. The lysates were resolved with SDS-PAGE (10%) and immunoblotted with anti-gp10 antibodies. C, p105(sr) is stable upon TNFα treatment. p105(sr) is stable upon TNFα treatment. gp10-tagged p105(sr) and p105Δ356–498 were expressed in HeLa cells. The transfected cells were treated, labeled with [35S]methionine/cysteine for 30 min, and chased with normal growth medium supplemented with TNFα (20 μg/ml) for the time indicated. At each time point, the cells were lysed and immunoprecipitated with anti-gp10 antibodies. The precipitants were then resolved with SDS-PAGE and visualized with fluorography.

To test whether expression of this p105 mutant still generates p50, we expressed the p105(sr) construct in three different cell lines: 293, HeLa, and CHO-CD14. As demonstrated by immunoblotting with antibodies to the epitope tag gp10 attached at the N terminus of p105(sr), expression of this mutant gene no longer generates p50 in any of the tested cells, whereas under the same condition, the wild-type p105 generates p50 normally (Fig. 1B).

We next tested whether p105(sr) responds to TNFα-induced degradation. HeLa cells were transfected with either p105(sr) or a p105 mutant, p105Δ356–498, that carries the same middle region deletion but maintains an intact C terminus. The cells were metabolically labeled with [35S]methionine/cysteine and chased with normal growth medium supplemented with TNFα. As shown in Fig. 1C, p105(sr) is more stable than p105Δ356–498 upon TNFα treatment. Together, these results suggested that the designed p105(sr) can potentially function as an effective NF-κB super repressor. In addition, the results in Fig. 1C also showed clearly that the degradation signal in the C-terminal portion of p105 is critical for degradation of p105, whereas the degradation signal in the middle of p105 protein regulates p50 production.

p105(sr) Maintains the Ability to Interact with Rel/NF-κB Proteins—Because p105(sr) harbors two areas of deletion, it was not clear whether it still maintains the ability to interact with Rel/NF-κB proteins. To test this aspect, we co-transfected p105(sr) with five different NF-κB species, p50, p52, RelA, c-Rel, and RelB, into CHO-CD14 cells. The transfected cells were lysed and immunoprecipitated with anti-gp10 antibodies first (co-immunoprecipitation), and the immunoprecipitants were then resolved with SDS-PAGE and immunoblotted with antibodies either to the gp10 tag or to the corresponding NF-κB protein. p105 maintains the ability to interact with all five NF-κB proteins (Fig. 2, left lanes). As reported previously, IκBα interacts with RelA and c-Rel only (41, 42), and its interactions with p50, p52, and RelB are rather weak; IκBβ(sr), which carries the S32A,S36A mutation (a kind gift from Dr. Warner Greene’s lab) with a similar gp10 epitope tag, co-immunoprecipitates RelA and c-Rel only and does not co-immunoprecipitate p50, p52, or RelB (Fig. 2, middle lanes). Thus, p105(sr) maintains its ability to interact with all NF-κB species and hence may have a broader inhibitory range that includes p50, p52, and RelB proteins.

p105(sr) Effectively Represses TNFα-induced NF-κB Activity—To test whether p105(sr) represses TNFα-induced NF-κB activity, we co-transfected p105(sr) with luciferase reporter constructs into 293 cells. The cell lysates were prepared and assayed for firefly luciferase activity, and the readouts were normalized with that of Renilla luciferase activity, which serves as the internal control. TNFα potently induced NF-κB-dependent firefly luciferase activity in cells co-transfected with

![Fig. 1. p105(sr) no longer generates p50 and does not respond to TNFα-induced degradation. A, anatomy of wild-type murine p105. Regions corresponding to p50 and IκBα are shown, and residue 453 marks the C terminus of p50. GRR, the glycine-rich region; DSM, degradation signal in the middle of p105; DSC, degradation signal in the C terminus of p105 that includes the DD. B, p105(sr) does not generate p50. gp10-tagged p105(sr) and p105(wt) were expressed in CHO-CD14, HeLa, and 293 cells. Twice the volume of HeLa cell lysates was loaded on the gel, because expression in HeLa cells is lower. The lysates were resolved with SDS-PAGE (10%) and immunoblotted with anti-gp10 antibodies. C, p105(sr) is stable upon TNFα treatment. p105(sr) is stable upon TNFα treatment. gp10-tagged p105(sr) and p105Δ356–498 were expressed in HeLa cells. The transfected cells were treated, labeled with [35S]methionine/cysteine for 30 min, and chased with normal growth medium supplemented with TNFα (20 μg/ml) for the time indicated. At each time point, the cells were lysed and immunoprecipitated with anti-gp10 antibodies. The precipitants were then resolved with SDS-PAGE and visualized with fluorography.](image)

![Fig. 2. Co-immunoprecipitation of p105(sr) with different NF-κB species. N-terminally tagged (with a gp10 epitope tag) p105(sr) or IκBα(sr) were co-transfected with untagged NF-κB species to CHO-CD14 cells. An empty vector was also co-transfected with these species as the negative control. The transfected cells were lysed, and the lysates were divided into two parts. One part was immunoprecipitated with anti-gp10 antibodies, and the precipitants were resolved with SDS-PAGE (10%) and blotted with antibodies specific to each NF-κB species (middle panels). To examine the expression of each specific NF-κB species, a portion of the second part of lysates (about 2.5 μg) was directly resolved with SDS-PAGE (10%) and blotted with antibodies specific to each NF-κB species (bottom panels). Because of in vivo homologous recombination, p50 co-transfected with p105(sr) (both were cloned into and expressed from the same vector) resulted in a small portion of gp10-tagged p50 that migrated slightly faster than the IgG heavy chain (marked as an asterisk in A). A, co-transfection with p50. B, co-transfection with p52. C, co-transfection with RelA. D, co-transfection with c-Rel. E, co-transfection with RelB. IP, immunoprecipitation; IB, immunoblot.](image)
two luciferase vectors and assayed as described. C was repressed by the increased input of p105(sr) (Fig. 4, NF-κB antibodies to either p50 or RelA supershifted the detected tracts of p105(sr)-transfected cells (Fig. 3). Induced NF-κB activity from vector-transfected cell lysates without treatment of TNFα was used as the background reading and had been excluded from column 1. B, p105(sr) repression is dose-dependent. 293 cells (10⁶/well) were transfected with 0.05, 0.1, 0.2, and 0.4 μg of p105(sr) (columns 2–5, respectively) and the two luciferase vectors and assayed as described. C, EMSA of TNFα-induced NF-κB activity. HeLa cells (10⁶/well) were transfected with p105(sr) or IκBα, and nuclear extracts were prepared from the cells 36 h post-transfection after treatment of with 20 ng/ml TNFα for 30 min. Lanes 1 and 8, vector-transfected; lanes 2–4, p105(sr)-transfected (0.1, 0.2, and 0.4 μg of DNA); lanes 5–7, IκBα-transfected (0.1, 0.2, and 0.4 μg of DNA); lane 9, supershift with anti-p50 antibodies; lane 10, supershift with anti-RelA antibodies; lane 11, cold wild-type κB nucleotides competition; lane 12, cold mutant κB nucleotides competition. The lower panel shows binding of Oct-1 by the same lysates as the internal control (lanes 1–7) and cold wild-type and mutant Oct-1 nucleotides competition (lanes 11 and 12).

To assess the inhibitory function of p105(sr) further, we also transfected HeLa cells with p105(sr) and monitored TNFα-induced NF-κB activities by EEMAs. Consistent with luciferase assay, NF-κB activity is clearly inhibited in the nuclear extracts of p105(sr)-transfected cells (Fig. 3C, lanes 2–4). Because antibodies to either p50 or RelA supershifted the detected NF-κB complex in EMSA (Fig. 3C, lanes 9 and 10), and the same complex diminished with increased input of p105(sr) or IκBα (Fig. 3C, lanes 5–7), we conclude that p105(sr) can effectively repress the prototypic κB (p50/RelA) activity.

p105(sr) Is a Potent Repressor to p50 Homodimer Activity—Although IκBα is a potent repressor to RelA and c-Rel containing NF-κB complexes, it does not inhibit activities of p50 or p52 homodimers and RelB containing complex because of the weak interactions between IκBα and these proteins. We next tested whether p105(sr) represses p50 homodimer activity by EEMAs. p50 and p105(sr) were co-transfected into HeLa cells, and nuclear extracts from the transfected cells were prepared. Nuclear extracts from p50-transfected cells exhibited high p50 homodimer activity (Fig. 4, lane 1), and such activity was repressed by the increased input of p105(sr) (Fig. 4, lanes 2–4). In contrast, the increased input of IκBα did not affect p50 homodimer activity. (Fig. 4, lanes 6–8).

p105(sr) Inhibits NF-κB Activity by Forming p105(sr)/Rel Protein Heterodimeric Complex in the Cytoplasm—Wild-type p105 is located exclusively in the cytoplasm (21). It is not clear whether the deletions change the subcellular localization of p105(sr). To assess the mechanism of p105(sr)-mediated inhibition upon NF-κB activity, we co-transfected gp10 Tagged p50 and p105(sr) into CHO-CD14 cells and fractionated the cell lysates into nuclear and cytoplasmic portions. The two portions were resolved with SDS-PAGE and immunoblotted with anti-gp10 antibodies. Without p105(sr), p50 is largely located in the nucleus (Fig. 5, lane 3). However, when co-transfected with extra amounts of p105(sr), majorities of p50 were found in the cytoplasmic portion (Fig. 5, lane 6). Similar results were obtained in transfected 293 and HeLa cells (data not shown). These results suggest that p105(sr) inhibits NF-κB activity by formation of the p105(sr)/Rel protein heterodimer in the cytoplasm.

p105(sr) Inhibits p50 Homodimer Activity in Skin Papilloma Cells and Facilitates TNFα-mediated Killing—Multi-stage car-
cinogen treatment of mouse skin results in papillomas, squamous cell carcinomas, and spindle cell carcinomas (a metastatic form of squamous cell carcinoma) (43). In papilloma and squamous cell tumor tissues, the activity of p50 homodimer, rather than p50/RelA heterodimer, was found to be constitutively elevated (15). We therefore chose mouse skin tumor cells as a model system to test whether p105(sr) effectively represses endogenous p50 homodimer activity and whether such repression leads to effective killing of the tumor cells by TNFα.

To verify that in MT1/2 papilloma cells and CH72T4 carcinoma cells p50 homodimer activity is indeed constitutively elevated as observed previously in tissues (15), we first performed EMSAs on nuclear extracts of these cells. As shown in Fig. 6A (lanes 2 and 3), CH72T4 and MT1/2 cells both exhibit elevated p50 homodimer activity. In contrast, p50 homodimer activity is not significantly elevated in nontumorigenic mouse skin cell line C50 (Fig. 6A, lane 1). Two closely migrated κB complexes were detected in the EMSAs. Anti-p50 antibodies shifted both complexes (Fig. 6A, lane 4), whereas anti-c-Rel antibodies shifted the slowly migrating complex only (Fig. 6A, lanes 5 and 7). Because the rest of antibodies did not shift these κB complexes (Fig. 6A, lanes 5, 6, and 8), we conclude that these NF-κB complexes contain p50/c-Rel and p50/p50, respectively. Compared with p50 homodimer complex, p50/c-Rel heterodimer complex appears to the minor species. These results are consistent with the previous observation in tissues (15).

MT1/2 cells are also more resistant to TNFα-mediated killing than C50 cells. C50 and MT1/2 cells were treated with 20 μg/ml TNFα for 16 h, fixed with paraformaldehyde, and stained with Hoechst dye. As shown in Fig. 6B (B and C), MT1/2 papilloma cells exhibit marked resistance to TNFα-induced apoptosis in comparison to the nontumorigenic C50 cells. A similar level of resistance was also found in CH72T4 cells (data not shown).

We next transfected p105(sr) or IκBα(sr) into MT1/2 papilloma cells and performed EMSAs. Similar to the co-transfection results shown in Fig. 4, p105(sr) effectively inhibits endogenous p50 homodimer and p50/c-Rel heterodimer activities in MT1/2 cells (Fig. 7, lane 2). In contrast, IκBα(sr) inhibits p50/c-Rel activity only (Fig. 7, lane 3).

We then tested whether introducing p105(sr) into MT1/2 cells would enhance TNFα-mediated apoptosis. As shown in Fig. 8 (A, columns 2 and 3, and B), p105(sr) and IκBα(sr) both facilitate TNFα-mediated apoptosis in MT1/2 cells, with p105(sr) consistently exhibiting a more effective enhancement than IκBα(sr). Such a difference is statistically significant (Student’s t test, p < 0.05). The enhancement of TNFα-mediated killing by the super repressors in MT1/2 appears to be specific.
rather than the nonspecific toxicity caused by expression of an exogenous protein, because expression of a full-length gp10 only slightly affects the cell death (Fig. 8, A and B). Because p105(sr) inhibits all NF-κB activities, whereas IκBα(sr) represses RelA and c-Rel containing NF-κB activities only, our results suggest that p50 homodimer activity, although it may not be the sole anti-apoptotic resource, is at least partially responsible for the survival of skin tumor cells.

**DISCUSSION**

The NF-κB family consists with five different members, all of which can form various homo- and heterodimers. This family of transcription factors regulates cell growth and developmental processes and is a component of innate immunity network (44, 45). Persistent activation of NF-κB often occurs during inflammation and other pathological conditions such as cancers and pathogen infections (5, 7). Inhibition of NF-κB therefore helps to circumvent or ease these conditions. Inhibition of NF-κB also facilitates drug- and chemotherapy reagent-mediated cell killing (11). Although an IκBα-based super repressor and various small chemical-based inhibitors repress NF-κB activities, they mainly target RelA and c-Rel complexes, leaving other NF-κB complexes, such as p50 and p52 homodimers, and RelB heterodimer uninhibited. In most cells, the p50/RelA heterodimer is the prototypical and dominant complex. However, other atypical NF-κB complexes also participate in cellular regulations, and dysregulation of these atypical species under pathological conditions has been reported (15, 16, 46).

In this report, we designed, generated, and tested a p105-based super repressor on the basis of the current knowledge of the mechanism of p50/p105 homeostasis. This p105-based super repressor broadly and effectively may inhibit all NF-κB activities in the cell. Potentially, p105(sr) can be used as an adjuvant for anti-inflammatory, anti-cancer drugs and chemotherapy reagents to increase the killing of inflamed and cancerous cells. This application may be particularly useful for tissues where the atypical NF-κB activities are dominant or in a situation multiple NF-κB complexes are activated. Because many of the downstream products of atypical NF-κB transcription complexes are unknown, introducing p105(sr) into a cell in combination with DNA microarray analyses will allow systematically dissection of these gene targets, leading to a clearer understanding of the role of various NF-κB species in cell development and pathogenesis. Revealing the identity of these target genes will also help future development and design of drugs against inflammatory diseases and cancers.

The correlation of the significant elevation of p50 homodimer activity and resistance to apoptosis was previously reported in murine B cell lymphomas (16), and our studies found strong resistance to TNFα-induced apoptosis in MT1/2 (Fig. 6) as well as in CH72T4 (data not shown) skin cancer cells. Hence, development of p105(sr) may provide an effective means to curtail p50 homodimer activity and facilitate apoptosis in these cancer cells. As demonstrated in our studies, introducing p105(sr) into MT1/2 papilloma cells significantly enhances TNFα-mediated
Hence, p105(sr) is likely to target the newly synthesized NF-κB heterodimer (especially a heterodimer) in vivo activity only (Fig. 7, lanes 2 and 3). However, in apoptosis analyses, the difference of apoptotic enhancement by p105(sr) and IκBα(sr) is less dramatic (32.7% versus 22.9%; Fig. 5A). TNFα induces proteolytic RelA/p50 heterodimer activity, which is the primary inhibitory target of IκBα(sr). Our results suggest that, although p50 homodimer activity is constitutively elevated in MT1/2 cells and may contribute to sustained growth of the skin papilloma cells, it accounted for a portion of the anti-apoptotic repressors. IκBα will not result in transcription activation.

In this study, we consistently observed a slightly stronger inhibitory effect by IκBα(sr) than by p105(sr) in luciferase reporter assays and EMSAs (Fig. 3). Such difference may be due to different inhibitory mechanisms exerted by these two repressors. IκBα binds RelA in the NF-κB dimeric complex directly (47), forming a ternary complex, and is able to shuttle between the cytoplasm and the nucleus (48). Thus, introducing IκBα(sr) into the cell may render immediate repression of NF-κB activities. In contrast, p105 binds to individual NF-κB/Rel protein, forming a p105/Rel protein heterodimer in the cytoplasm. Because of the strong association of the Rel homology domains, the dissociation rate of a preformed NF-κB complex (especially a heterodimer) in vivo may be rather slow. Hence, p105(sr) is likely to target the newly synthesized NF-κB members (24) rather than the preformed NF-κB complexes. Indeed, repression of NF-κB by p105(sr) in 12 h post-transfection measured by both luciferase reporter assays and EMSAs was not as robust as that of 36–48 h post-transfection (data not shown), suggesting a slight leak of repression in the early phase. Because p105(sr) is overexpressed in the cell, and its natural turnover is slower than that of IκBα(sr) (data not shown), an effective repression by p105(sr) in the late phase (Figs. 3 and 4) can still be achieved.

Although the wild-type p105 resides exclusively in the cytoplasm, we found a fraction of p105(sr) in the nucleus, despite the lack of the NLS in this p105 mutant (Fig. 5, lanes 5 and 6). Because p50 was well fractionated in p50-transfected cells by the same method (Fig. 5, lanes 3 and 4), it is unlikely that p105(sr) detected in the nucleus was due to incomplete fractionation. In addition, nuclear localization of p105(sr) is not dependent upon binding to p50, which contains the NLS, because p105(sr) was also being detected in the nucleus when the cell was transfected by p105(sr) alone (data not shown). A recent report indicated that the DD in the C terminus of p105, in addition to the NLS, participates in regulation of the subcellular localization of p105 (22). Lack of DD in p105(sr) may therefore be responsible for its localization in the nucleus. Another version of p105(sr) generated by us, which includes the NLS and has similar inhibitory effect in the cell, exhibits increased nuclear localization (data not shown). Because p105 also interferes with NF-κB binding to the κB sequences (21), translocation of p105(sr)/Rel protein complexes into the nucleus will not result in transcription activation.

In this study, we have not extensively studied whether p105(sr) inhibits p52 homodimer or RelB heterodimer activity, although we found that both proteins co-immunoprecipitate with p105(sr) (Fig. 2, B and E), suggesting that similar repression of p52 and RelB activities can be achieved. Constitutive processing generates a low level of p52 in the cell (49, 50), and production of p52 is greatly enhanced by stimulation of a subset of cytokines (14). Derepression of nuclear genes also results in macrophage (51, 52), and inhibition of abnormal p52 homodimer activity may be of significant importance. Studies have shown that both constitutive and induced p52 generation require de novo protein synthesis, implying a co-translational mechanism during the process (49, 53, 54). Given that p105(sr) is likely to target nascent Rel proteins, it is possible to repress p52-related activity by formation of the p105(sr)/p52 complex. On the other hand, when the mechanism of p100/p52 regulation becomes clear, design and generation of a p100-based repressor that specifically targets p52 is also possible.

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