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PAPER

Involvement of DNA binding domain in the cellular stability and importin affinity of NF-κB component RelB

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NF-κB is a transcription factor for the immune activation and tissue stability, but excess activation of NF-κB often causes inflammation and cancer. An NF-κB component RelB is involved in B-cell maturation and autoimmunity. In the present research we studied the role of the RelB DNA binding domain on cellular stability and importin affinity. We prepared a RelB protein mutated at Arg141 to Ala and Tyr142 to Ala (AA mutant) having no DNA binding activity. The stability of this mutant protein was greatly reduced compared with that of the wild-type protein. We also constructed a nuclear localization signal-inactivated mutant of RelB, and found that this mutant was also unstable in the cells. Thus, RelB destabilization was caused by the loss of DNA binding possibly because of the change in cellular localization. The mutation also decreased the affinity to importin-α5 decreasing the nuclear localization. Our newly discovered NF-κB inhibitor (−)-DHMEQ binds to a specific Cys residue in RelB to inhibit DNA binding and also decreased the stability and importin affinity. These findings would indicate that the DNA binding activity of this transcription factor is a crucial for its stability and intracellular localization.

Introduction

NF-κB is a transcription factor that promotes expressions of many inflammatory cytokines, adhesion molecules, and anti-apoptosis proteins. It is important for immune activation and tissue stability. However, its excess activation often induces inflammation and cancer progression. NF-κB is a heterodimer of Rel family proteins including p65, RelB, cRel, p50 and p52. There are canonical NF-κB mainly consisting of p65/p50 heterodimers and noncanonical NF-κB mainly consisting of RelB/p52. Canonical NF-κB is important for natural immunity and general inflammation, and its mechanism of activation has been extensively studied. On the other hand, noncanonical NF-κB is more important in B-cell maturation and the etiology of autoimmune diseases.[1] Moreover, recent reports have suggested that the noncanonical pathway is also important in cancer progression.[2] However, the mechanism of noncanonical NF-κB activation has not been fully understood.

Nuclear import of NF-κB is necessary for its activation, and is regulated by importin-α/β pathways.[3] Importin-α proteins bind to the arginine/lysine-rich nuclear localization signal (NLS) of target proteins. Importin-β becomes associated with importin-α, whose complexes then induce the docking of importin-cargo proteins to the cytoplasmic side of nuclear pore complexes.[4] Recent studies have shown that each member of the importin-α family has a different affinity for Rel family proteins and that nuclear import of NF-κB dimers is regulated by the component-dependent NLS.[3,4] For instance, RelB/p52 mainly binds to importin-α5, and nuclear localization of the RelB/p52 dimers is regulated by the 2 NLSs present in RelB.[4,5]

We previously designed and synthesized (−)-DHMEQ as a specific inhibitor of NF-κB. It covalently binds to a specific Cys residue of Rel family proteins to inhibit their DNA binding.[6]

In the present research we have studied the role of the DNA binding domain in RelB on its stability and nuclear localization by preparing various function-deleted mutants. We found that mutations of RelB that inhibited its DNA binding induced cellular instability of RelB and also weakened the affinity of RelB for importin-α5. (−)-DHMEQ also reduced the stability and importin-α affinity in RelB. These observations suggest the importance of the DNA binding property of noncanonical NF-κB for their localization and stability.

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Results

Loss of DNA binding activity affects noncanonical NF-κB stability

As shown in Fig. 1A, Cys144 of RelB exists nearby Arg141 and Tyr142, which are essential for DNA binding by forming hydrogen bonds with DNA. It is likely that when (−)-DHMEQ binds to Cys144 of RelB, Arg141 and Tyr142 would have difficulty in accessing the DNA due to steric hindrance. Thus, (−)-DHMEQ inhibited RelB/p52 DNA binding both *in vitro* and *in situ* by inhibiting the formation of hydrogen bonds with DNA.

We prepared a RelB mutant that could not bind to DNA. It was earlier reported that p52 with its Arg54 mutated to Ala and its Tyr55 to Ala can not bind to DNA,[7] and these 2 residues are conserved in all the Rel family proteins (Fig. 1B). So we prepared the R141A and Y142A double-mutant RelB, referred to as RelB(AA), and over-expressed this protein in HeLa cells together with wild-type p52. Disappearance of NF-κB binding was observed in RelB(AA) mutant-expressing cells, whereas the binding was clearly detected in the case of the wild-type or RelB (C144S)-expressing cells, as expected (Fig. 1C). RelB(C144S) is known to possess higher DNA binding activity than the wild-type.[8] Interestingly, the protein levels of RelB and p52 were greatly lowered in the RelB(AA)-over-expressing cells, although their mRNA levels measured by semi-quantitative RT-PCR did not change (Fig. 1D). To analyze the stability of the RelB/p52 protein, we employed the cycloheximide (CHX)-chase assay. When protein synthesis was blocked by CHX, the decreases in RelB and p52 in RelB(AA)-expressing cells were faster than in the case of wild-type or C144S (Fig. 1E). The stability of p52 was also lowered in the RelB(AA)-expressing cells (Fig. 1E). Thus, the RelB(AA)/p52 dimers, which can not bind to DNA, are likely to have been unstable in the cells. These results indicate that the DNA binding ability of noncanonical NF-κB regulated the stability of its components. p65 has the conserved amino acid sequence of 141Tyr and 142Arg of RelB (Fig. 1B) and these 2 residues have similar 3D structure to RelB (Fig. 1A and 2A). Unexpectedly, p65(R35A, Y36A) known to be a DNA binding deficient mutant, referred to as p65(AA), was not unstable (Fig. 2B).

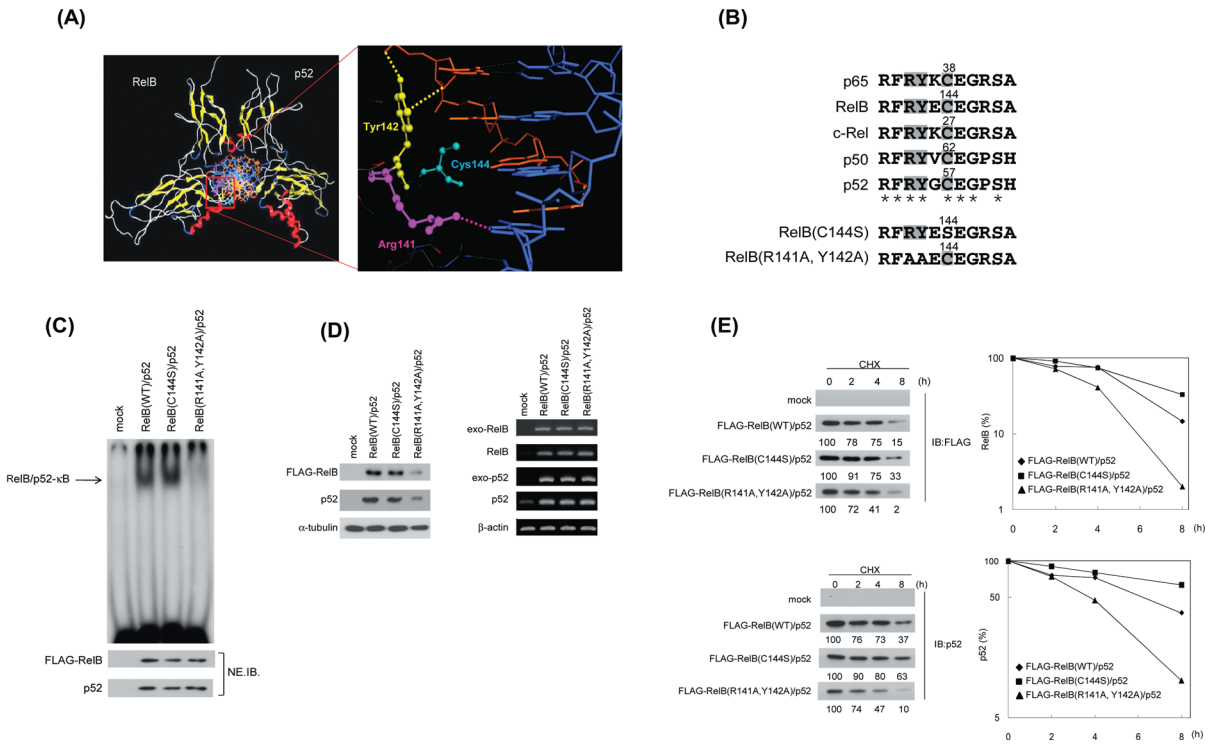

Fig. 1 Involvement of DNA binding activity for RelB/p52 stabilization. (A) Three-dimensional structure drawn by MOE of RelB/p52/DNA complex (PDB: 3DO7, left) and hydrogen-bond interaction of Arg141 and Tyr142 with DNA (right). Hydrogen-bonds are shown as dotted-lines. (B) Sequence alignment of Rel family proteins, RelB(C144S), and RelB(AA). Sequence homology was analyzed by ClustalW. (C) RelB(AA) does not bind to DNA. HeLa cells were made to transiently over-express FLAG-tagged RelB(WT), RelB(C144S) or RelB(AA) together with wild-type p52. Mock is for empty vector transfection. Nuclear proteins of the transfected cells were extracted and used for EMSA (top). The expression of each protein was detected by nuclear extracts (NE) Western blotting (bottom). (D) Decreased RelB(AA) stability in cultured cells. HeLa cells transiently over-expressed FLAG-tagged forms of RelB(WT), RelB(C144S) or RelB(AA) together with wild-type p52. The proteins (left) and mRNA (right) levels were detected by Western blotting and semi-quantitative RT-PCR, respectively. The exo-RelB and exo-p52 mRNA were detected by using specific primers which only recognized exogenous mRNA. (E) Cycloheximide-chase analysis. HeLa cells transiently over-expressed FLAG-tagged versions of RelB (WT, closed diamonds), RelB(C144S, closed squares) or RelB(AA, closed triangles) together with wild-type p52. The cells were treated with 20 μg ml^{-1} CHX for the indicated times, and the total cell extracts were thereafter subjected to Western blotting. The protein amount of RelB (top) and p52 (bottom) was determined by ImageJ.

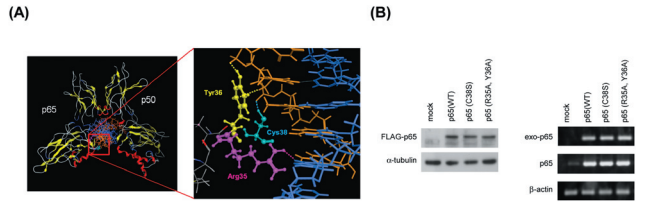

Fig. 2 Effect of AA mutation on cellular p65 amount. (A) Three-dimensional structure drawn by MOE of p65/p50/DNA complex (PDB: 1VKX, left) and hydrogen-bond interaction of Arg35 and Tyr36 with DNA (right). (B) HeLa cells transiently over-expressed FLAG-tagged forms of p65(WT), p65(C38S) or p65(AA). The proteins (left) and mRNA (right) levels were detected by Western blotting and semi-quantitative RT-PCR, respectively. The exo-p65 mRNA was detected by using a specific primer which only recognized exogenous p65 mRNA.

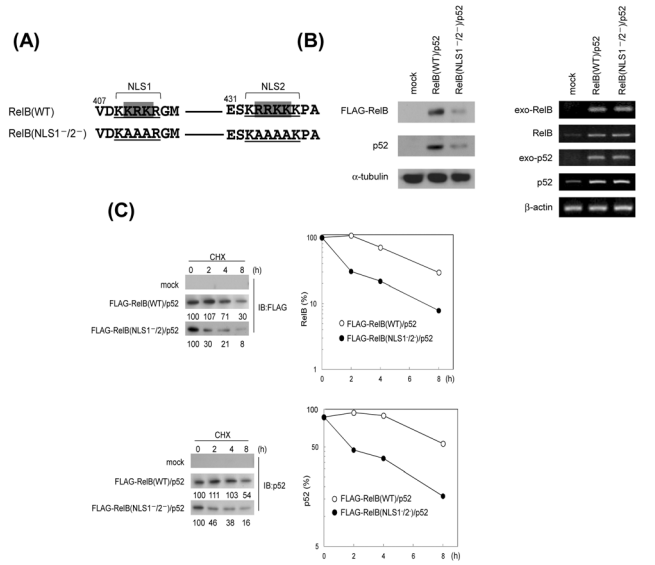

Fig. 3 Effect of NLS inactivation of RelB on RelB and p52 stabilities. (A) Sequence alignment of RelB(WT) and RelB(NLS1⁻/2⁻). (B) Decreased stability of RelB NLS mutant. HeLa cells were made to transiently over-express FLAG-tagged forms of either RelB(WT) or RelB (NLS1⁻/2⁻) together with wild-type p52. The proteins (left) and mRNA (right) levels were detected by Western blotting and semi-quantitative RT-PCR, respectively. The exo-RelB and exo-p52 mRNA were detected by using specific primers which only recognized exogenous mRNA. (C) Cycloheximide-chase analysis. HeLa cells were made to transiently over-express FLAG-tagged versions of RelB(WT) (open circles) or RelB (NLS1⁻/2⁻, closed circles) together with wild-type p52. The cells were then treated with CHX for the indicated periods, after which total cell extracts were prepared and then subjected to Western blotting. The protein amount of RelB (top) and p52 (bottom) was determined by ImageJ.

Involvement of nuclear localization in the regulation of RelB/p52 stability

To examine whether the intracellular localization between nucleus and cytoplasm may cause instability of proteins, we prepared a RelB mutant in which its NLS activity had been lost. As shown in Fig. 3A, RelB has 2 NLS sequences, 409–413 (NLS1) and 433–438 (NLS2), and both of them contribute to the nuclear localization.[5] Furthermore, nuclear import of RelB/p52 heterodimers is determined by the NLSs of RelB.[5] We transiently over-expressed wild-type (WT) or NLS1⁻/NLS2⁻ mutant (K410A, R411A, and K412A/R434A, R435A, K436A, and K437A) in HeLa cells together with wild-type p52. We found that the protein amounts of RelB and p52 were greatly decreased in the RelB(NLS1⁻/NLS2⁻)-over-expressing cells, although their mRNA levels did not change (Fig. 3B). In the presence of CHX, the decrease in the level of RelB(NLS1⁻/NLS2⁻) or p52 was faster than in RelB(WT, Fig. 3C). Moreover, the degree of RelB and p52 instability in the RelB(NLS1⁻/NLS2⁻)-expressing cells was similar to that in the RelB(AA)-expressing ones (*cf*. Fig. 1E and 3C). Thus, the change in intracellular localization from the nucleus to the cytoplasm, caused by the loss of the NLSs, induced the instability of RelB and p52.

(−)-DHMEQ induced destabilization of RelB and p52 by inhibition of DNA binding activity

We previously reported that (−)-DHMEQ (Fig. 4A) covalently bound to Cys144 of RelB.[6] As shown in Fig. 4B, when we over-expressed either the wild-type (WT) or Cys144Ser (C144S) mutant RelB together with wild-type p52 in HeLa cells, (−)-DHMEQ completely inhibited the NF-κB activity in RelB (WT)-expressing cells, whereas it did not inhibit the activity in the RelB(C144S)-expressing ones. Thus, (−)-DHMEQ would target the Cys144 residue of RelB even in cultured cells.

Adult T-cell leukemia MT-1 cells were established by infection of T-cells with human T-cell leukemia virus type 1[9,10] and NF-κB is constitutively activated in them.[11–13] Among Rel family proteins, RelB and p52, which are noncanonical NF-κB components, were predominantly expressed in MT-1 cells and (−)-DHMEQ selectively inhibited *in vitro* the DNA binding of noncanonical NF-κB prepared from the nuclear extract of MT-1 cells, without inhibiting Oct-1 DNA binding (Fig. 4C and D). Furthermore, the amounts of RelB and p52 in total cell lysate were lower after treatment with (−)-DHMEQ for 4 h and these decreases in RelB and p52 proteins were not due to a change in their mRNA levels measured by semi-quantitative RT-PCR (Fig. 4E).When protein synthesis was blocked by CHX, the decreases in RelB and p52 were accelerated in the presence of (−)-DHMEQ (Fig. 4F). Thus, the inhibition of DNA binding activity of RelB/p52 caused by (−)-DHMEQ binding to Cys144 of RelB should have induced the instability of RelB and p52.

Loss of DNA binding activity of noncanonical NF-κB decreases its affinity with importin-α5

When WT, C144S or AA mutant RelB protein was over-expressed in HeLa cells together with wild-type p52, the nuclear/cytoplasmic ratio decreased in the RelB(AA)-expressing cells (Fig. 5A). Nuclear import of RelB/p52 heterodimers is mainly mediated by importin-α5 *via* the 2 NLSs of RelB, as reported before.[5] So we prepared recombinant GST-conjugated importin-α5 for use in a GST pull-down assay. As shown in Fig. 5B, the importin-α5 pull-down amount of RelB was lowered in the RelB(AA)-expressing cells. Thus, RelB(AA) showed lower affinity for importin-α5. In RelB and p52 transfection experiments, the amount of nuclear RelB(WT) in HeLa cells was lowered by incubation with (−)-DHMEQ for 1 h, whereas

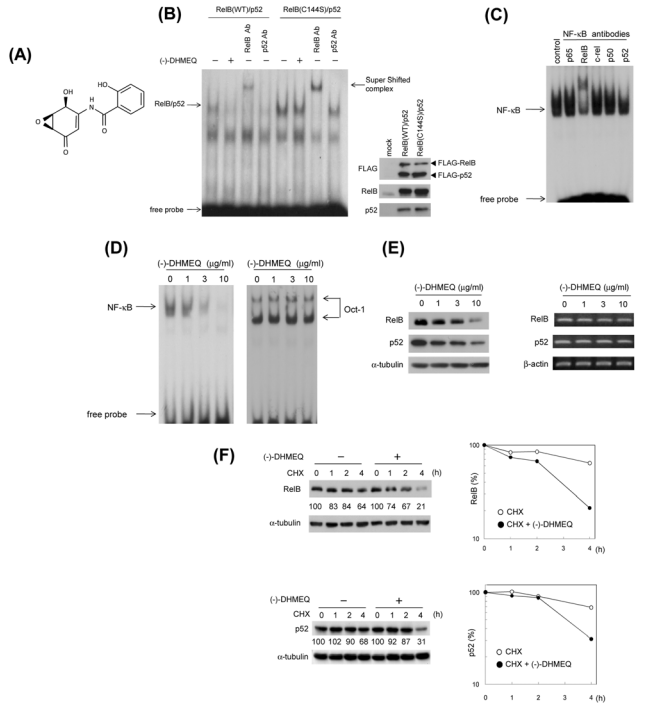

Fig. 4 Induction of RelB and p52 destabilization by (−)-DHMEQ. (A) Structure of (−)-DHMEQ. (B) (−)-DHMEQ inhibits DNA binding of RelB through the binding to Cys144. HeLa cells were made to transiently over-express either FLAG-tagged wild-type or C144S mutant RelB together with FLAG-tagged wild-type p52. Transfected cells were incubated with or without 10 μg ml^{-1} (−)-DHMEQ for 1 h, and nuclear proteins were then extracted and used for EMSA (left). The expression of each protein was detected by Western blotting (right). (C) Main NF-κB component in MT-1 cells is RelB. NF-κB components of MT-1 cells were analyzed by performing a supershift assay using the indicated NF-κB antibodies. (D) (−)-DHMEQ inhibits DNA binding of NF-κB in MT-1 cells. Nuclear extracts from MT-1 cells were treated with the indicated concentrations of (−)-DHMEQ for 1 h, and the *in vitro* DNA binding activity was then detected by using EMSA. The left panel shows inhibition of noncanonical NF-κB DNA binding activity by (−)-DHMEQ; and the right panel, the result when a control probe, Oct-1, was used. (E) Decreased stability of RelB and p52 in (−)-DHMEQ treated MT-1 cells. MT-1 cells were treated with various concentrations of (−)-DHMEQ for 4 h. The proteins (left) and mRNA (right) levels were detected by Western blotting and semi-quantitative RT-PCR, respectively. (F) Cycloheximide-chase analysis. The cells were treated with CHX for the indicated times with or without (−)-DHMEQ, and the total cell extracts were thereafter subjected to Western blotting. The protein amount of RelB (top) and p52 (bottom) was determined by ImageJ.

that of RelB(C144S) did not change much (Fig. 5C). The amount of p52 also decreased in the RelB(WT)-over-expressing cells. Inhibition of p52 nuclear accumulation by (−)-DHMEQ was also weakened in the RelB(C144S)-over-expressing cells (Fig. 5C). Furthermore, (−)-DHMEQ reduced the amount of importin-α5-bound RelB(WT), with no decrease in that of RelB (C144S, Fig. 5D).

Discussion

RelB(AA)/p52 heterodimers, which could not bind to DNA (Fig. 1B–E) and RelB(NLS1^{-}/NLS2^{-})/p52, in which the NLSs were deleted (Fig. 3A–C), decreased the stability. It is likely that the transactivation dimers RelB/p52, exist transiently in the cytoplasm and are stabilized by nuclear accumulation and binding to DNA. Therefore, a change in the equilibrium of the nuclear/cytoplasm ratio caused by inhibition of DNA binding would result in RelB/p52 instability because of the difference in protease activity between the cytoplasm and nucleus. It was reported that a mutant p65 having no DNA binding activity, one in which the normal Arg35 was changed to Ala and Tyr36 to Ala.[8] We constructed these expressing vectors and unexpectedly this p65 mutant did not become unstable (Fig. 2B). This difference between RelB and p65 might be due to the protective proteins p52 and IκBα. IκBα masks the NLS of p65 and inhibits nuclear translocation, but it can not inhibit RelB nuclear translocation.[14]

The RelB monomer is known to be unstable, but it is protected from degradation by p100 and p52 in the cytoplasm.[6] We previously showed that a novel NF-κB inhibitor (−)-DHMEQ binds to a specific Cys residue of Rel family proteins except p52 and inhibits their DNA binding activity.[6] We showed that (−)-DHMEQ induced RelB destabilization by binding to the Cys144 of RelB (Fig. 4B–F). Since the major protein degradation pathway is the proteasome or lysosome, we examined whether a proteasome inhibitor, MG-132, or a lysosome inhibitor, pepstatin A, would inhibit the destabilization of RelB induced by (−)-DHMEQ. As a result, neither MG-132 nor pepstatin A inhibited (−)-DHMEQ-induced RelB destabilization (data not shown). Thus, the RelB instability was probably caused by other proteases. We also showed that (−)-DHMEQ induced p52 destabilization. It is likely that RelB regulates the p52 stability by regulating its localization as a dimer.

Activation of NF-κB is mainly regulated by nuclear accumulation of NF-κB components. (−)-DHMEQ inhibits not only DNA binding but also nuclear accumulation of NF-κB in many cell lines including lymphoma cells and solid tumor cells.[11,15] However, the mechanism of lower accumulation of NF-κB by the treatment with (−)-DHMEQ had not been elucidated until now. (−)-DHMEQ bound to Cys144 of RelB to inhibit its DNA binding activity (Fig. 4D); and, moreover, (−)-DHMEQ inhibited the nuclear accumulation of p52 (Fig. 5C), although it does not bind to p52. RelB does not form a homodimer, and the predominant RelB dimer is the RelB/p52 heterodimer.[7] Therefore, inhibition of p52 nuclear accumulation by (−)-DHMEQ was probably caused by the RelB intracellular localization, since p52 and RelB are always together.

(−)-DHMEQ weakly inhibited p52 nuclear accumulation in RelB(C144S)/p52-over-expressing HeLa cells (Fig. 5C). It would have occurred because p52 also forms a dimer with Rel family proteins other than RelB, such as p65, p50 and c-Rel. As shown in Fig. 5B and D, RelB(AA) and RelB with (−)-DHMEQ, both having no DNA binding activity, showed lower affinity for importin-α5. Thus, (−)-DHMEQ inhibited nuclear accumulation of RelB by loss of DNA binding activity and reducing the affinity of RelB with importin-α5.

These findings suggest that a change in the conformation of RelB by mutation or chemical binding may have reduced the amount of importin-bound RelB/p52. These effects should contribute to provide a more efficient effect of (−)-DHMEQ on nuclear accumulation and activity.

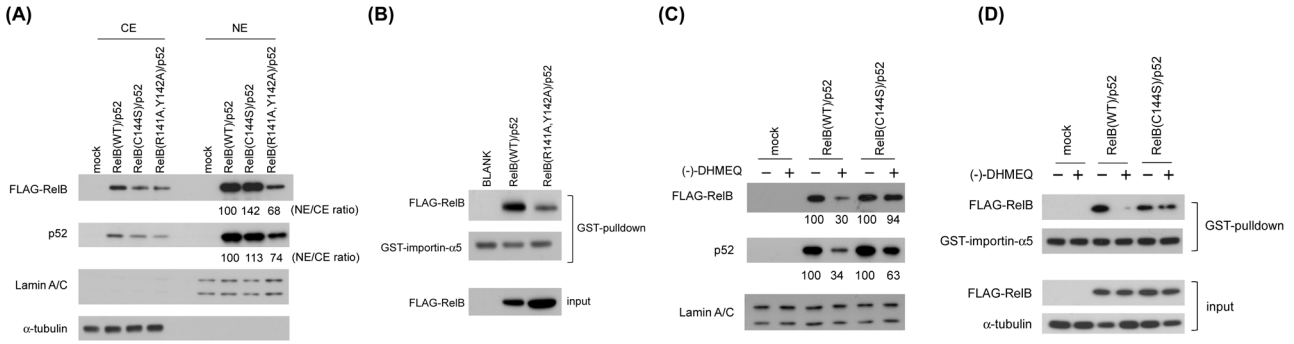

Fig. 5 RelB(AA) mutation or (−)-DHMEQ decreases the affinity to importin-α5. (A) Decrease of nuclear RelB and p52 in RelB(AA) mutant expressing cells. HeLa cells were made to transiently over-express FLAG-tagged forms of RelB(WT), RelB(C144S) or RelB(AA) together with wild-type p52, and thereafter fractionated into cytoplasmic (CE) and nuclear fractions (NE). The nuclear/cytoplasmic ratio was determined by ImageJ. (B) Decrease of GST-importin-α5 affinity in RelB(AA) mutant. Cell extracts of HeLa cells that transiently over-expressed the FLAG-tagged version of RelB(WT) or RelB(AA) together with wild-type p52 were prepared and then incubated with GSH-Sepharose-bound GST-importin-α5 for 2 h at 4 °C. GSH-Sepharose-bound proteins were dissolved and analyzed by Western blotting. (C) Treatment with (−)-DHMEQ decreased nuclear RelB. HeLa cells were made to transiently over-express FLAG-tagged forms of RelB(WT) or RelB(C144S) together with wild-type p52. The transfected cells were incubated with or without 30 μg ml^{-1} (−)-DHMEQ for 1 h, and the nuclear proteins were then extracted and used for Western blotting. (D) Decrease of importin-α5 affinity by (−)-DHMEQ. HeLa cells were treated as in "C" and the total cell extracts were incubated with GSH-Sepharose-bound GST-importin-α5 for 2 h at 4 °C. GSH-Sepharose-bound proteins were dissolved and analyzed by Western blotting.

Conclusions

Our results show that the DNA binding activity regulated the stability of noncanonical NF-κB *via* its localization. Therefore, inhibition of DNA binding causes the additional inhibitory activity of these transcription factors. A similar mechanism may be applicable to other DNA-binding proteins. These findings indicate that some means of inhibiting DNA binding should be an effective way to inhibit the function of DNA-binding proteins including transcription factors.

Experimental

Materials

(−)-DHMEQ was synthesized in our laboratory as described before.[16] Mouse monoclonal anti-p65 NF-κB (sc-8008), anti-p50 NF-κB (sc-8414), anti-p52 NF-κB (sc-7386), anti-c-Rel NF-κB (sc-6955), anti-GST (sc-138), and rabbit polyclonal anti-IκBα (sc-203) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal anti-RelB (#4922), anti-NF-κB2 p100/p52 (#3017), and polyclonal anti-Lamin A/C (#2032) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Mouse monoclonal anti-FLAG and anti-α-tubulin antibodies were obtained from Sigma (St. Louis, MO).

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's Medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% calf serum, 200 μg ml^{-1} kanamycin, 100 units per ml penicillin G, 600 μg ml^{-1} L-glutamine, and 2.25 g l^{-1} $NaHCO_3$. MT-1 cells were grown in RPMI1640 medium (Nissui) containing 10% heat-inactivated FBS, 100 μg ml^{-1} kanamycin, 100 units per ml penicillin G, 30 μg ml^{-1} L-glutamine, and 2.25 g l^{-1} $NaHCO_3$.

Cytoplasmic and nuclear protein extraction

Nuclear extracts were prepared according to the method of Andrews and Faller.[17,18] Cells were grown in 60 mm dishes and incubated with the desired chemicals. They were then harvested and washed with phosphate-buffered saline (PBS), suspended in 100 μl of buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM PMSF), and incubated on ice for 15 min. Nuclei were pelleted by centrifugation for 5 min at 500 × *g*, and the supernatant was used as the cytoplasmic fraction. Then, the pellets were resuspended in 400 μl of buffer A and incubated on ice for 15 min. After incubation the nuclei were pelleted by centrifugation for 5 min at 500 × *g*, resuspended in 40 μl of buffer C (50 mM HEPES [pH 7.9], 2.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 25% glycerol [v/v]), incubated on ice for 20 min, and centrifuged for 5 min at 14 000 rpm. The resulting supernatant was used as the nuclear extract.

Electrophoretic mobility shift assay (EMSA)

The binding reaction mixture contained nuclear extract (5 μg of protein), 1 μg poly(dI–dC), and 10 000 cpm ^{32}P-labeled probe (oligonucleotide containing NF-κB or Oct-1 binding site) in binding buffer (15 mM Tris-HCl [pH 7.0], 75 mM NaCl, 1.5 mM EDTA, 1 mM DTT, 7.5% glycerol, and 1.5% NP-40). Samples were incubated for 20 min at room temperature (RT) in this mixture. DNA–protein complexes were separated from free DNA on 4% native polyacrylamide gels in TBE buffer (22.5 mM Tris-HCl [pH 8.3] and 0.5 mM EDTA). The DNA probes used for NF-κB binding were purchased from Promega (Madison, WI, USA). The following sequences were used as NF-κB and Oct-1 binding probes: 5′-AGT TGA GGG GAC TTT CCC AGG C and 5′-GCC TGG GAA AGT CCC CTC AAC T for NF-κB binding, and 5′-TGT CGA ATG CAA ATC ACT AGA A and 5′-TTC TAG TGA TTT GCA TTC GAG A for

Oct-1 binding. These oligonucleotides were labeled with [γ-^{32}P]-ATP (3000 Ci mmol^{-1}; GE Healthcare, Little Chalfont, UK) by use of T4 polynucleotide kinase (Takara, Ohtsu, Japan), and purified by passage through a NICK column (GE Healthcare).

In vitro binding evaluated by EMSA

The binding mixture containing cell nuclear extract (5 μg) and 5% DMSO with or without (−)-DHMEQ in the binding buffer was incubated for 1 h at 4 °C. After incubation, 1 μg of poly(dI–dC) and 10 000 cpm ^{32}P-labeled probe were added to the reaction mixture. Then, electrophoresis was carried out as described above.

Total cell protein extraction

Cells were grown in 60 mm dishes and incubated with the desired chemicals. They were then harvested and washed with PBS, suspended in lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1mM EDTA, 0.1% SDS, 1% NP-40, 1 mM PMSF, and 0.3 μM aprotinin), sonicated, and centrifuged for 10 min at 14 000 rpm and the supernatant was used as the total cell extracts.

Western blotting

Total cell extracts, cytoplasmic extracts, and nuclear extracts were boiled in Laemmli loading buffer (58.3 mM Tris-HCl [pH 6.8], 1.7% SDS (w/v), 6% glycerol (v/v), and 0.83% 2-mercaptoethanol) and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred at 200 mA for 1 h onto Hybond-P membranes (GE Healthcare). Nonspecific binding sites were blocked for 30 min by immersing the membrane in TBST buffer (20 mM Tris-HCl [pH 7.6], 135 mM NaCl, and 0.1% Tween 20 [v/v]) containing 5% (w/v) nonfat milk. After a short wash in TBST, the membrane was incubated for 1 h at RT in a 1 : 1000 dilution of the desired primary antibody in TBST containing 5% (w/v) nonfat milk followed by 30 min of washing with TBST. The bound antibody was then detected with horseradish peroxidase-conjugated secondary antibody (diluted at 1 : 5000 in TBST containing 3% nonfat milk) by incubation for 1 h at RT. After having been washed for 30 min in TBST, the immunocomplexes were detected by using ECL reagent, Western Lightning (Parkin Elmer, Waltham, MA, USA) or Immobilon Western (Millipore, Billerica, MA, USA).

Semi-quantitative RT-PCR

Cells were grown in 60 mm dishes and incubated with the desired chemicals. Total cellular mRNAs were extracted from cells by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was converted to cDNA by using a High-Capacity cDNA Reverse Transcription Kit (Life Technology, Carlsbad, CA, USA). Two microliters of the cDNA mixture were used for enzymatic amplification with TaKaRa Taq™ (Takara Bio, Shiga, Japan). The primer sequences were 5′-AGATTGAGGCTGCCATTGAG and 5′-CGCAGCTCTGATGTGTTTGT (for total-RelB); 5′-TACAAGGATGACGACGATAAGGCC and 5′-AGGGTGACCGTGCTCAGGGA (for exo-RelB); 5′-CAGTCATCTCCCAGCCCATC and 5′-CTGGCTTGCGTTTCAGTTGC (for total-p52); 5′-GCTAGCCTCGAGAATTCATG and 5′-ATTGCTTGCCCACCAGACTG (for exo-p52); 5′-TACAAGGATGACGACGATAAGGCC and 5′-TAGAAGCCATCCCGGCAGTC (for exo-p65); 5′-CAGGCTCCTGTGCGTGTCTC and 5′-CTGGCTGATCTGCCCAGAAG (for total-p65); and 5′-CTTCGAGCAAGAGATGGCCA and 5′-CCAGACAGCACTGTGTTGGC (for β-actin). The number of PCR cycles for each product was determined after confirmation of the efficacy of amplification and after having defined the linear exponential portion of the amplification.

Plasmid construction

Human RelB cDNA containing an N-terminal BglII site and a C-terminal XhoI site was prepared from a Ramos cell cDNA pool by PCR. The primers used were 5′-TTTTTT **AGA TCT** ATG CTT CGG TCT GGG CCA GCC (forward-oligonucleotide, BglII recognition site in bold face and the initiation codon underlined) and 5′-TTTTTT **CTC GAG** TTA CTC GCT GCA GAC CCC ATC GG (reverse-oligonucleotide XhoI recognition sequence in bold face and the stop codon underlined). Then the cDNA was cloned into the BamHI–XhoI site of the pCMV-Tag2B vector (Stratagene, La Jolla, CA, USA). Similarly, human p52 corresponding to p100 (1–405 aa), human p65, and human importin-α5 cDNAs were prepared by PCR from the MT-1 cell cDNA pool. The primers used were 5′-TTTTTT **GGA TCC** ATG GAG AGT TGC TAC AAC CCA GG (for p52, forward-oligonucleotide, BamHI recognition site in bold face and the initiation codon underlined) and 5′-TTTTTT **CTC GAG** TTA CGC CCC GCC CCC GCC TCC CG (reverse-oligonucleotide, XhoI recognition site in bold face and the stop codon underlined); 5′-TTTT **GGA TCC** ATG GAC GAA CTG TTC CCC CTC ATC (for p65, forward-ologonucleotide, BamHI recognition site in bold face and the initiation codon underlined) and 5′- TTTT **CTC GAG** TTA GGA GCT GAT CTG ACT CAG CAG (reverse-oligonucleotide, XhoI recognition site in bold face and the stop codon underlined); 5′-TTTT **GGA TCC** ATG ACC ACC CCA GGA AAA GAG AAC (for importin-α5, forward-oligonucleotide, BamHI recognition site in bold face and the initiation codon underlined) and 5′-TTTT **CTC GAG** TCA AAG CTG GAA ACC TTC CAT AGG (reverse-oligonucleotide, XhoI recognition site in bold face and the stop codon underlined). Then p52 cDNA was cloned into the BamHI–XhoI site of the pCMV-Tag2B vector (Stratagene) or pCI-neo vector (Promega, Madison, WI, USA), and the importin-α5 cDNA was cloned into the BamHI–XhoI site of the pGEX-6P-1 vector (GE Healthcare).

Site-directed mutagenesis

To obtain the RelB Cys144Ser mutant, we employed inverse PCR using the pCMV-RelB plasmid as the template. The following primers were used for the construction of the mutant protein: 5′-**C**C GAG GGC CGC TCG GCC GGC AGC ATC (forward-oligonucleotide, mutation point in bold face) and 5′-A CTC GTA GCG GAA GCG CAT GCC GCG CTG (reverse-oligonucleotide). The PCR product was treated with DpnI for 120 min at

37 °C. The mutated pCMV-Tag2B-RelB was separated on 1% agarose gel in TBE buffer and extracted from the gel by using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). It was then mixed with 1 mM ATP and T4-PNK (Takara) at 37 °C for 1 h and then mixed with the components of a ligation kit, Ligation High (Toyobo, Osaka, Japan) at 16 °C for 1 h. The plasmid was amplified in *E. coli* HB101 cells. To obtain RelB AA and NLS1^{-}/2^{-} deficient mutants by using PCR, we used the followed sequence primers (with mutation point in bold type): 5′-GC ATG CGC TTC **GCC GCC** GAG TGC GAG GGC C (for AA mutant, forward-oligonucleotide) and 5′-GA GCG GCC CTC GCA CTC **GGC GGC** GAA GCG C (for AA mutant, reverse-oligonucleotide); 5′-GAC AAG **GCG GCG GCA** CGG GGG ATG CCC GAC (for NLS1^{-} mutant, forward-oligonucleotide) and 5′-CCC CCG **TGC CGC CGC** CTT GTC CAC GCC GTA (for NLS1^{-} mutant, reverse-oligonucleotide); 5′-AAA **GCA GCT GCG GCA** AAG CCG GCC ATC CTG (for NLS2^{-} mutant, forward-oligonucleotide) and 5′-CTT **TGC CGC AGC TGC** TTT GCT CTC GAT GCC (for NLS2^{-} mutant, reverse-oligonucleotide). To obtain p65 C38S and p65 AA mutants by using PCR, we used the followed sequence primers (with mutation point in bold type): 5′-C CGC TAC AAG T**C**C GAG GGG CGC TCC GCG G (for C38S mutant, forward-oligonucleotide) and 5′-C CGC GGA GCG CCC CTC G**G**A CTT GTA GCG G (for C38S mutant, reverse-oligonucleotide); 5′-CAG CGG GGC ATG CGC TTC **GCC GCC** AAG TGC GAG (for AA mutant, forward-oligonucleotide) and 5′-GGA GCG CCC CTC GCA CTT **GGC GGC** GAA GCG CAT (for AA mutant, reverse-oligonucleotide). The cDNA was cloned into the BamHI–XhoI site of the pCMV-Tag2B vector (Stratagene).

Transfection with plasmids

HeLa cells (1×10^5 cells per dish) were grown in 60 mm dishes. The cells were transfected with the desired DNA by using Lipofectamine LTX (Invitrogen, Grand Island, NY, USA) as described by the manufacturer. 24 h after transfection, the cells were treated with the desired chemical prior to EMSA and Western blotting.

Production of GST-fusion proteins in *E. coli* and preparation of cell lysates from cells

Recombinant proteins were expressed in *E. coli* BL21 cells (Promega) as GST fusion proteins by induction at 25 °C for 3 h with 100 μM isopropyl-1-thio-β-D-galactopyranoside (GE Healthcare). The bacteria were lysed in a sonication buffer (PBS containing 0.1% NP-40 [v/v], 1 mM DTT, and 1% protease inhibitors [Nacalai Tesque, Kyoto, Japan]), sonicated for 10 min on ice, and centrifuged for 10 min at 14 000 rpm at 4 °C. The supernatant was mixed with 500 μl of equilibrated glutathione-Sepharose 4B (GE Healthcare) at 4 °C for 1 h followed by washing 5 times with the sonication buffer. For preparation of cell lysates, MT-1 cells and HeLa cells transfected with plasmids were incubated with or without (−)-DHMEQ. The cells were then lysed in L-buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) on ice for 30 min and centrifuged for 10 min at 14 000 rpm at 4 °C.

GST pull-down assay

For GST pull-down experiments, GST fusion importin-α5 was immobilized on glutathione-Sepharose beads and then washed 3 times with L-buffer for equilibration. 200 μg of cell lysate was mixed with 25 μl of glutathione-Sepharose-immobilized GST fusion importin-α5 beads and rotated at 4 °C for 2 h followed by washing 5 times with L-buffer. The Sepharose beads were dissolved in 60 μl of 2× Laemmli sample buffer, and the proteins were separated by 10% SDS-PAGE as described above for Western blotting.

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