A recessive mutant cell line with a constitutive IκB kinase activity

Netnaphis Chinanonwait, Hideyasu Miura, Naoki Yamamoto, Shoji Yamaoka*

Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

Received 15 July 2002; revised 9 September 2002; accepted 4 October 2002

First published online 1 November 2002

Edited by Richard Marais

Abstract To search for negative regulatory components of the NF-κB activation pathways, we mutagenized Rat-1 fibroblasts and established a stable mutant cell line with a constitutive NF-κB activity. This mutant cell line, designated as TK26, showed permanently elevated I kappa B kinase (IKK) activity and a genetically recessive phenotype revealed by somatic cell hybridization between TK26 and Rat-1. Our results suggested that lack of a negative regulation of IKK could lead to permanent NF-κB activation. The TK26 cell line will be useful to genetically identify a component necessary for keeping the IKK complex under an inactive form in resting cells.

Key words: Recessive mutation; NF-κB; IKK activation

1. Introduction

The nuclear factor-kappa B (NF-κB) family of transcription factors controls the expression of various genes involved in inflammatory and immune responses [1–4]. Under physiological conditions, NF-κB is sequestered in the cytoplasm by the inhibitor of I kappa B (IκB) proteins. Exposure of cells to a variety of extracellular stimuli leads to rapid phosphorylation, ubiquitination and proteolytic degradation of IκB, which free NF-κB to translocate to the nucleus, where it regulates gene transcription [5–8]. The multisubunit I kappa B kinase (IKK) complex lies at a converging regulatory step leading to IκB phosphorylation. The core of the IKK complex consists of two catalytic subunits, IKK1/α and IKK2/β and a non-catalytic regulatory subunit, NF-κB essential modulator (NEMO/IKKγ) [9–15]. Activation of IKKs is supposed to depend on phosphorylation of two serine residues in their activation loop and kinases responsible for this phosphorylation are under intense investigation. NF-κB is known to be critically involved in a variety of pathological conditions characterized by its aberrant activation [16] or caused by its defective signaling [17]. Thus, it has become increasingly important to elucidate the molecular mechanisms of NF-κB dysregulation. It should be noted that many of the disease states are characterized by dysregulation of the IKK activity, resulting in permanent or defective NF-κB activity [18]. This may reflect a central role that the IKK complex plays in the NF-κB signaling pathways. Normally, the IKK complex should be kept quiescent without stimulation, and following its activation, should be down-regulated at an appropriate time point. The mechanisms of inducible IKK activation in response to a wide variety of stimuli through cell surface receptors have been studied extensively, but little is known as to constitutive IKK activation or negative regulation of the IKK complex [19].

Given that a number of cellular regulatory systems such as the mitogen-activated protein kinase cascades, cell cycle regulation or apoptosis require both positive and negative regulators, it is reasonable to assume that the NF-κB signaling is also regulated negatively at multiple steps, loss of which may lead to its permanent activation. However, negative regulators are more difficult to identify in general unless they are inactivated by certain viral proteins [20]. One successful approach to this issue would be forward genetics, which has resulted in many gene discoveries based on their function [21]. To gain new insight into negative regulation of NF-κB, we mutagenized a rat fibroblastic cell line, Rat-1, which otherwise exhibits a very low NF-κB activity and is known to be able to grow under a high NF-κB activity, and report here a genetically recessive mutant cell line with a constitutive IKK activity.

2. Materials and methods

2.1. Reagents

Hygromycin B and cycloheximide were purchased from Wako. Blasticidin S was purchased from ICN. Lipopolysaccharide (LPS), poly d (I-C), neomycin (G418), puromycin and ganciclovir were purchased from Sigma.

2.2. Recombinant plasmids

Plasmids Igκ2bsrH, Igκ-ConA luciferase and EF1-lacZ were described previously [13,22]. Plasmid Igκ2tkH was constructed by replacing the bsr gene of Igκ2bsrH with the Herpes Simplex virus thymidine kinase (HSV-TK) gene, so that this gene can be expressed under the NF-κB-dependent promoter. Plasmid pPUR was purchased from Clontech. A dominant negative form of NEMO (aa 97–412) was described previously [23].

2.3. Antisera

Anti-p50, anti-RelA, anti-RelB and anti-IκBβ sera were kind gifts from Dr. Alain Israël (Institute Pasteur, Paris, France). Anti-IκBα, anti-p52, anti-cRel and anti-actin antibodies were purchased from Santa Cruz. Anti-IKK1 antibody was purchased from Imgenex. Anti-NEMO rabbit polyclonal antiserum (serum 44106) was described previously [24].

2.4. Cell culture, DNA transfection and mutagenesis

All cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum and antibiotics. To establish Rat-1 fibroblasts conditionally resistant to blasticidin S, Rat-1 cells were transfected with pIgκ2bsrH by the calcium phosphate

*Corresponding author. Fax: (81)-3-5803 0124.
E-mail address: shojmmb@tmd.ac.jp (S. Yamaoka).

Abbreviations: EMSAs, electrophoretic mobility shift assays; IκBα, I kappa B alpha; IκBβ, I kappa B beta; IKK, I kappa B kinase; LPS, lipopolysaccharide; NEMO, nuclear factor-kappa B essential modulator; NF-κB, nuclear factor-kappa B

0014-5793/02/$22.00
PII: S0014-5793(02)03618-9

coprecipitate method. Stable transfectants were selected with 500 μg/ml of hygromycin B. Individual hygromycin-resistant clones were isolated and tested for their ability to survive a high dose of blasticidin S selection after hybridization by polyethylene glycol with 5R cells that express the Tax protein of human T cell lymphotropic virus type I and lack NEMO. One of the established clones named B5 was used for further studies.

To generate mutant cells, approximately 40 million B5 cells were subjected to four rounds of chemical mutagenesis with 10 μg/ml of ICR191 (Sigma), which killed about 80–90% of cells in each round of mutagenesis. After the last round of mutagenesis, cells were subjected to selection with 3 μg/ml of blasticidin S. After 2 to 3 weeks of selection, individual colonies were ring-cloned and expanded. A blasticidin S-resistant clone 2-7 was transfected with the pIgκ2tkH and pPUR plasmids and selected with 2 μg/ml of puromycin. One isolated clone TK26, which exhibited the phenotype indistinguishable from that of 2-7 cells, was chosen for further experiments. TK26 cells were hybridized with Rat-1 cells resistant to neomycin (G418) and selected in media containing 500 μg/ml of G418 and 2 μg/ml of puromycin for 7–10 days before pooled hybrid cells were examined for NF-κB-DNA binding and IKK activity.

For measurement of luciferase activity in transiently transfected cells, approximately 1.5×10^5 cells were plated in 6-well plates. The next day, cells were co-transfected with 0.25 μg of Igκ-luciferase reporter plasmid, 0.25 μg of EF1-lacZ plasmid, and 2 μg of either vector or effector plasmid using the calcium phosphate coprecipitate method. Cells were harvested 48 h after transfection and assayed for luciferase activity. Luciferase values were normalized based on β-galactosidase activity. Experiments were repeated three times in duplicate.

2.5. Preparation of cell extracts

Cells were washed twice with cold phosphate-buffered saline and resuspended in hypotonic buffer (20 mM HEPES (pH 7.8), 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA), supplemented with protease inhibitors (0.1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin). After 15 min of incubation at 4°C, NP40 was added to 1% and the cells were vortexed for 20 s. After centrifugation, supernatants were recovered and used as cytoplasmic fraction. Nuclear pellets were briefly washed with isotonic buffer (20 mM HEPES (pH 7.8), 100 mM NaCl, 0.1 mM EDTA and 25% glycerol) and resuspended in extraction buffer (20 mM HEPES (pH 7.8), 400 mM NaCl, 0.1 mM EDTA and 25% glycerol, 1 mM DTT, 0.1 mM PMSF). After 30 min of incubation at 4°C with occasional agitation, supernatants were recovered by centrifugation at 14 000 rpm for 2 min and stored at −80°C until use as nuclear extracts. Whole cell lysate was prepared by lysis of cells with RIPA buffer (20 mM Tris–HCl (pH. 8.0), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP40, 0.5% deoxycholate, 0.1% SDS and 0.1 mM PMSF). Protein concentrations of cytoplasmic fraction, nuclear fraction and whole cell lysate were determined by the Bradford method.

2.6. Electrophoretic mobility shift assays (EMSAs)

Five micrograms of nuclear extracts were added to binding buffer for NF-κB probe (10 mM HEPES (pH 7.8), 100 mM NaCl, 1 mM EDTA, 25% glycerol final), or to binding buffer for activator protein-1 (AP-1) probe (12 mM HEPES (pH 7.2), 60 mM KCl, 0.6 mM EDTA, 0.6 mM DTT), 1 μg poly d (I-C) and γ-^{32}P-labeled κB probe derived from the H-2K^b promoter [25], or γ-^{32}P-labeled AP-1 probe (5′-GATCGTGATGACTCAGGTT-3′) and incubated for 30 min at room temperature. Samples were run on a 5% polyacrylamide gel in 0.5×Tris–borate EDTA (TBE). For competition studies, excess unlabeled probe was included in the binding reaction.

2.7. Western blot analysis

Proteins in cytoplasmic extracts were fractionated on 8 or 10% SDS–polyacrylamide gels, transferred onto immobilized membranes (Millipore), and blots were revealed with an enhanced chemiluminescence detection system (NEN™ Life Science products). When necessary, membranes were stripped by incubation in stripping buffer (62.5 mM Tris–HCl (pH. 6.8), 100 mM 2-mercaptoethanol, 2% SDS) for 30 min at 50°C with constant agitation, washed and then reprobed with other antibodies as indicated.

2.8. Immunoprecipitation and kinase assay

Approximately 4×10^6 cells were washed twice with cold phosphate-buffered saline and resuspended in hypotonic buffer (20 mM HEPES (pH7.8), 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA), supplemented with phosphatase inhibitors (0.1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μM Na_3VO_4 and 20 mM β-glycerophosphate). After 15 min of incubation at 4°C, NP40 was added to 1% and the cells were vortexed for 20 s. The supernatants (400 μg) were subjected to immunoprecipitation with NEMO antiserum (anti-NEMO) in TNT buffer (20 mM Tris–HCl (pH 7.5), 200 mM NaCl and 1% Triton X-100). Immunoprecipitates were collected on protein G-Sepharose beads, which were washed three times with TNT buffer and then divided into two, 50% for detection of IKK1 in the immunoprecipitates and 50% for in vitro kinase assay. Samples for kinase assay were washed three times with kinase reaction buffer (20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl). Kinase reaction was conducted in the kinase reaction buffer supplemented with 100 μM Na_3VO_4, 20 mM β-glycerophosphate, 2 mM DTT and 20 μM ATP at 30°C for 30 min in the presence of 5 μCi of (γ?-^{32}P)-ATP and GST-IκBα (1-72) wild type or GST-IκBα (1-72) S32A/S36A mutant protein as substrates [13]. Phosphorylated proteins were resolved on 12% SDS–poly-

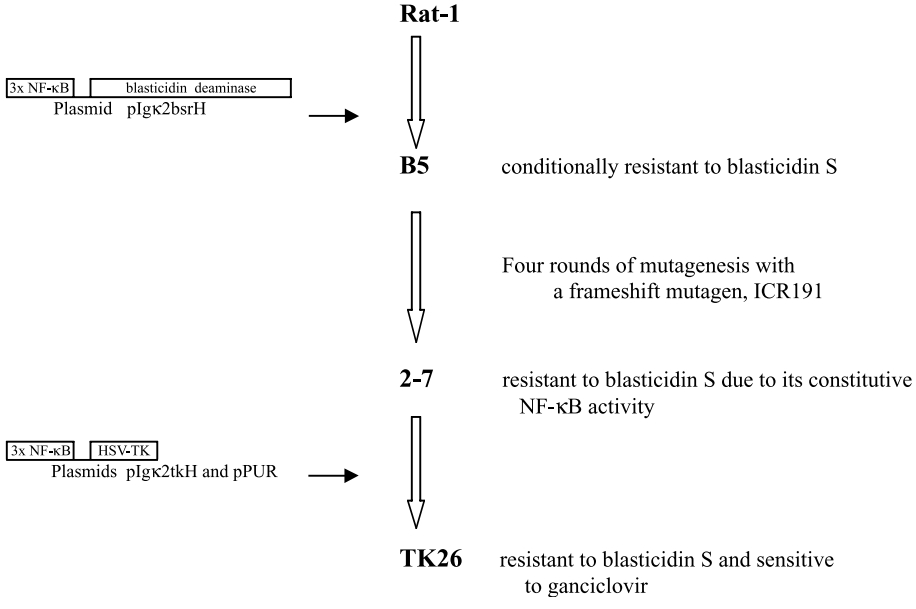

Fig. 1. Isolation of a mutant cell line, TK26.

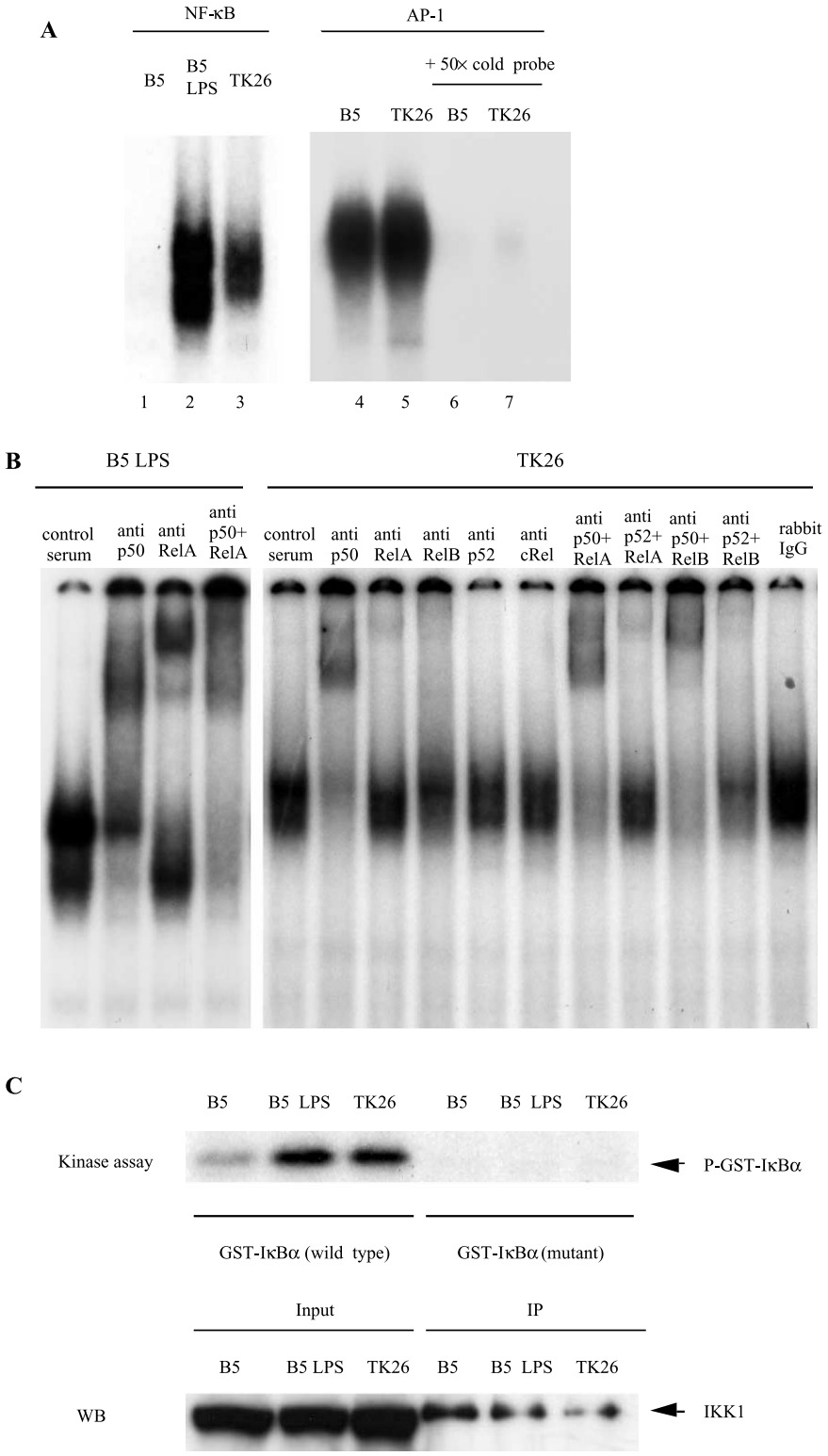

Fig. 2. Elevated NF-κB DNA binding and IKK activity in a mutant cell line, TK26. A: Five micrograms of nuclear extracts prepared from B5 (lane 1, 4 and 6), B5 stimulated with LPS for 1 h (lane 2) and TK26 (lane 3, 5 and 7) were analyzed by EMSAs, using the NF-κB-binding sequence derived from the H-2 K^b promoter and AP-1-binding sequence as probes. For competition studies, 50-fold excess of unlabeled probe was included in the binding reaction. B: The protein composition was determined by pre-incubating 5 μg of nuclear extracts with antibodies specifically directed against p50, RelA, RelB, p52 or cRel for 30 min at room temperature. C: Cytoplasmic extracts (400 μg) were immunoprecipitated with anti-NEMO and specific IκB kinase activity was determined by in vitro immune complex kinase assay, using GST-IκBα (1-72) wild type or GST-IκBα (1-72) S32A/S36A mutant proteins as substrates. To control for amounts of input and immunoprecipitated IKK1, part of the immunoprecipitates or cell extracts used for the kinase assay was subjected to Western blot analysis.

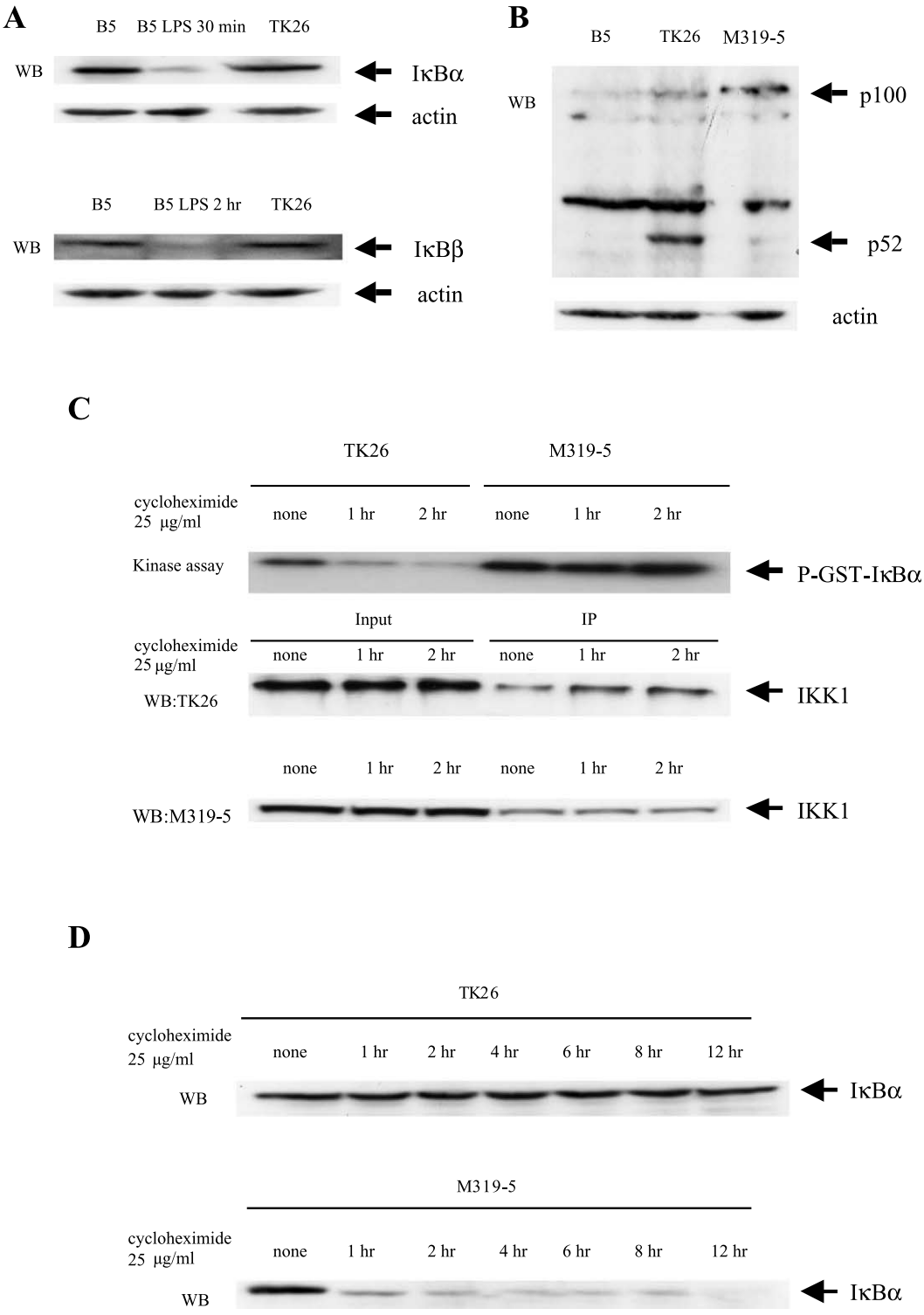

Fig. 3. Distinct IKK and IκB kinetics in TK26 and Rat-1 cells expressing Tax. A: Approximately 50 μg of cytoplasmic extracts were subjected to Western blot analysis with IκBα or IκBβ antisera. The same blot was then probed with anti-actin serum, showing equivalent sample loading in each lane. B: Approximately 50 μg of whole cell lysates were subjected to Western blot analysis with a p52-antibody. The positions for p100 and p52 are indicated by the arrows. The same blot was then probed with anti-actin serum. C: Cytoplasmic extracts (400 μg) from TK26 and M319-5 untreated or treated with 25 μg/ml of cycloheximide for 1 or 2 h were subjected to immunoprecipitation with anti-NEMO, and specific IκBα kinase activity was determined by in vitro immune complex kinase assay with GST-IκBα (1-72) wild type as substrates. Western blotting was performed to control for amounts of IKK1 in each cell lysate or immune complexes. D: Tk26 and Tax-expressing M319-5 cells were treated with 25 μg/ml of cycloheximide for the indicated periods, and the steady-state levels of IκBα were determined by Western blot analysis.

acrylamide gels and revealed by autoradiography. Amounts of IKK1 in each lysate or immunoprecipitates were verified by Western blot analysis.

3. Results

3.1. Generation of a mutant cell line

We attempted to generate a mutant cell line with a constitutive NF-κB activity through chemical mutagenesis of Rat-1 fibroblasts, aiming at identification of a negative regulator of the NF-κB signaling. Rat-1 cells had been used successfully for genetic studies in the NF-κB signaling pathway [13]. We first established sublines of Rat-1 cells capable of expressing an inducible drug resistance gene under elevated NF-κB activity (Fig. 1). A conditional drug resistance gene, Igκ2bsrH, contains the blasticidin deaminase gene [22] linked to a minimal IL-2 promoter following three repeats of the NF-κB-binding element derived from the immunoglobulin κ gene. Rat-1 cell clones obtained by stable transfection with this construct were tested for survival in the presence of blasticidin S following hybridization with 5R cells, which express the Tax protein of human T cell leukemia virus type 1 but lack NEMO [13]. Hybrid cells express Tax that persistently activates NF-κB, and should eventually survive the drug selection. One of the transfected Rat-1 cell clones, B5, was chosen for further experiments, as hybrids between B5 and 5R cells were able to survive selection with a high dose of blasticidin S and showed elevated NF-κB DNA-binding activity (data not shown). There could be seen no survival of B5 cells cultured in the presence of a low concentration of the drug without the hybridization step, demonstrating that B5 is amenable to mutagenesis and the following lethal selection. B5 cells were then subjected to four rounds of mutagenesis with a frameshift mutagen ICR191 followed by selection in medium containing blasticidin S. Of 27 cell clones that survived blasticidin S selection, we found one clone, 2-7, that stably showed an elevated NF-κB DNA-binding activity in EMSAs. Subsequently, sublines of 2-7 cells were established which could express the thymidine kinase gene derived from Herpes Simplex virus (HSV-TK) under the control of the same NF-κB-dependent promoter as that for the blasticidin deaminase gene. Since HSV-TK is known to kill cells efficiently in the presence of ganciclovir, 2-7 cells carrying this HSV-TK construct were supposed to be killed in the presence of ganciclovir because of their high NF-κB activity, but to be able to survive if they restore the normal NF-κB activity. TK26 cells showed strong NF-κB DNA-binding activity similar to that of 2-7 cells, but no significant change in the AP-1 DNA-binding activity, when compared to B5 cells. (Fig. 2A).

3.2. Characterization of TK26 cells

To determine the composition of the NF-κB complexes bound to an NF-κB-specific DNA probe in EMSAs, we performed supershift analysis, using anti-p50, anti-RelA or anti-RelB antibodies. Anti-p50 and anti-RelA antibodies readily supershifted the two protein–DNA complexes that appeared following stimulation of B5 cells with LPS (Fig. 2B, lanes 2, 3 and 4), indicating that the faster migrating complexes but not RelA contain p50, and that the more slowly migrating complexes contain p50 and RelA. Regarding TK26, the faster migrating complexes contain p50, but not RelA, and the more slowly migrating complexes were supershifted by anti-p50 antibody to an extent similar to B5 cells. The anti-RelA antibody failed to supershift efficiently the slowly migrating complexes, but instead, anti-RelB antibody supershifted the faster migrating complexes and attenuated the binding as a whole. Neither anti-p52 nor anti-cRel affected the binding.

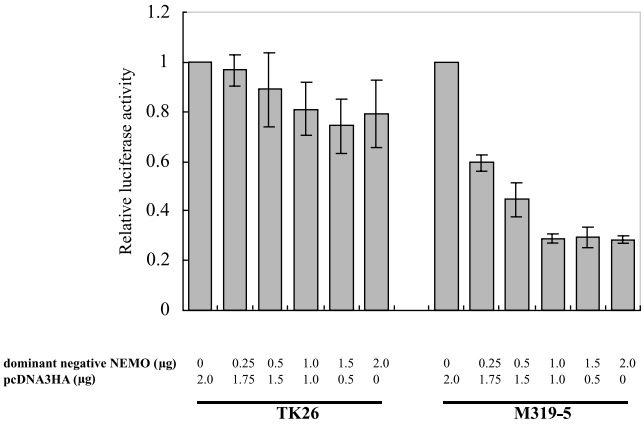

Fig. 4. A dominant negative form of NEMO fails to suppress NF-κB activity in TK26 cells. TK26 and M319-5 cells were transiently transfected with 0.25 μg of Igκ-ConA luciferase and EF1-lacZ along with 2 μg of effector plasmids, pcDNA3HA control vector or indicated amounts of expression vector for a dominant negative form of NEMO (dN97 NEMO). The total amount of the effector plasmids was adjusted to 2 μg. Results are shown as average luciferase values relative to that of vector-transfected cells obtained by three independent experiments in duplicate.

We next asked if the enhanced nuclear DNA-binding activity in TK26 cells resulted from constitutive IKK activation. In vitro kinase assays using anti-NEMO (NF-κB essential modulator) antibody detected a markedly elevated phosphorylating activity on recombinant IκBα substrates in immunoprecipitates from TK26 cells (Fig. 2C). This activity failed to phosphorylate a mutant form of the substrate with amino acid substitutions of alanine for serine at the residues 32 and 36 of IκBα, establishing the specificity of the activity. In each assay, equivalent amounts of the IKK1 protein were detected in the immune precipitates by Western blot analysis. Thus, IKK is constitutively activated in TK26 cells. Western blot studies revealed that the steady-state levels of IκBα and IκBβ in TK26 are similar to those in the parental cells (Fig. 3A), while NF-κB2/p52 is aberrantly expressed in TK26 (Fig. 3B), which was even more abundant than that in Rat-1 cells expressing Tax, suggesting active processing of its precursor p100 in TK26 cells. We also demonstrate a rapid down-regulation of the IKK activity in TK26 cells when treated with cycloheximide, a protein synthesis inhibitor, which contrasts with the sustained IKK activity in M319-5 cells expressing Tax (Fig. 3C) [13]. Consistently, the steady-state level of IκBα in TK26 cells was barely affected following protein synthesis inhibition, whereas this protein disappeared quickly in Tax-expressing cells (Fig. 3D). Moreover, a dominant negative form of NEMO, known to suppress TNFα-mediated NF-κB activation, potently counteracted Tax-mediated NF-κB activation, while its effects were only marginal in TK26 cells (Fig. 4). These results suggested distinct mechanisms of persistent IKK activation in TK26 and Tax-expressing cells.

We next examined if TK26 can respond to LPS and further activate NF-κB. As shown in Fig. 5A, both IκBα and IκBβ proteins disappeared shortly after LPS treatment in B5 cells,

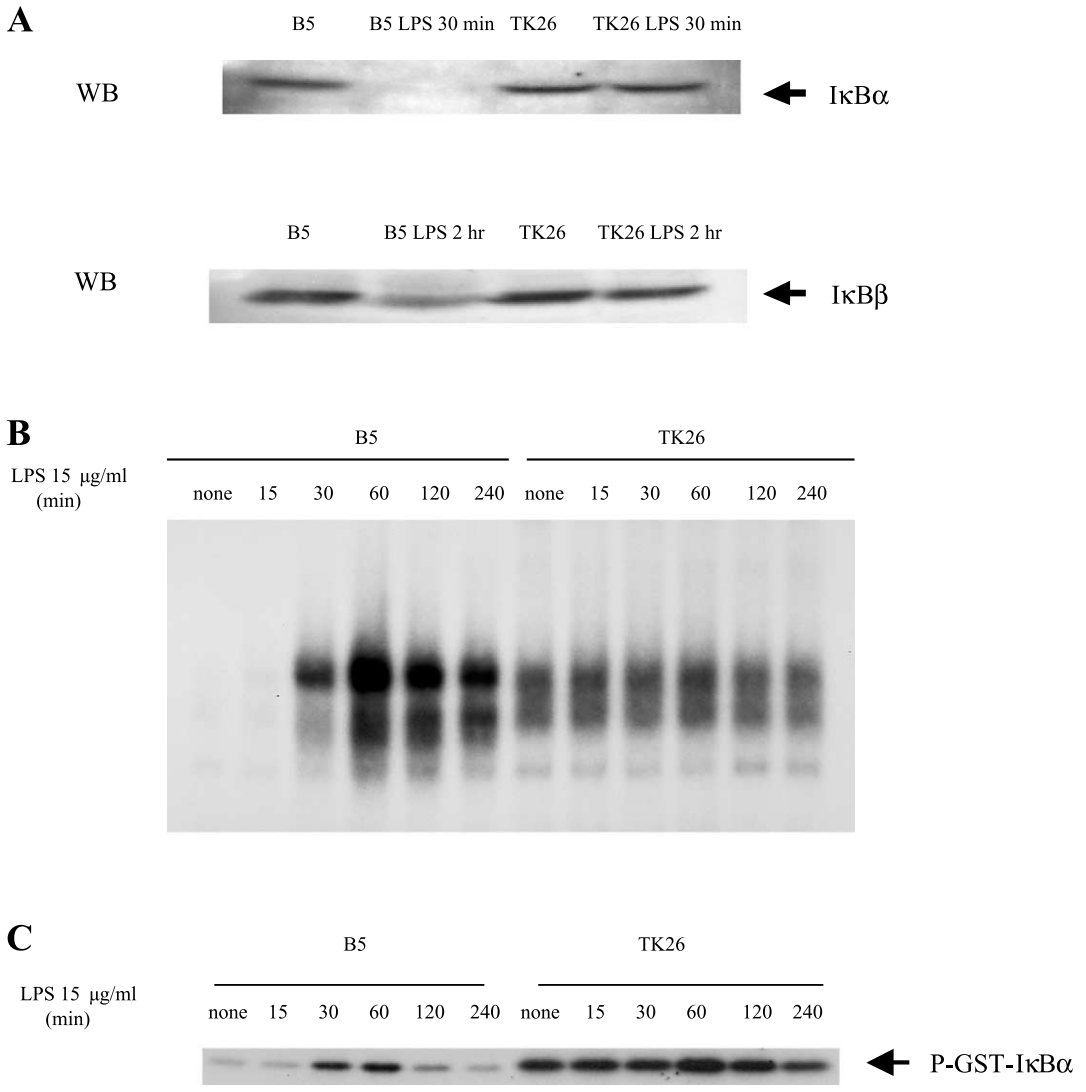

Fig. 5. Response of TK26 to LPS. A: Approximately 50 μg of cytoplasmic extracts was subjected to Western blot analysis with IκBα or IκBβ antisera. B: Five micrograms of nuclear extracts prepared from B5 and TK26 cells stimulated with 15 μg/ml of LPS for the indicated time were analyzed by EMSAs, using the NF-κB-specific probe. C: Cytoplasmic extracts prepared from the cells used for EMSAs were subjected to immunoprecipitation with anti-NEMO and IKK activity was determined by in vitro immune complex kinase assay with GST-IκBα (1-72) as a substrate.

while the steady-state levels of these proteins did not significantly change in TK26 cells. Fig. 5B shows only a marginal elevation of DNA-binding activity in TK26 cells treated with LPS, which contrasts to a sharp induction and subsequent down-regulation of the DNA-binding activity in B5 cells. We next examined the kinetics of IKK activation in LPS-stimulated B5 and TK26 cells (Fig. 5C). In B5 cells, IKK activity associated with NEMO rapidly increased and then quickly decreased following LPS treatment. TK26 cells showed a similar increase in IKK activity additive to its basally elevated one. Thus LPS treatment of TK26 cells further activated IKK, but did not result in the disappearance of the IκB proteins, so that the ultimate nuclear NF-κB-DNA-binding activity was not significantly altered.

3.3. The mutation in TK26 is recessive

We further analyzed the genetic phenotype of the mutation by somatic cell hybridization. The phenotype of hybrid cells between the mutant and wild type cells should provide an answer to the question as to whether TK26 cells carry a gain of function (dominant) mutation or loss of function (recessive) mutation. For this purpose, parental Rat-1 cells were fused to TK26 and stable hybrids were established. The obtained hybrids restored the DNA binding and IKK activity as low as those of B5 cells (Fig. 6A,B). This indicates that TK26 cells carry a recessive mutation that can be complemented by cell fusion with wild type cells.

4. Discussion

We hypothesized that NF-κB might become permanently activated without its negative regulation. To test this hypothesis and identify a component necessary for keeping NF-κB in a resting state, we tried to alter gene expression through chemical mutagenesis and generated a stable mutant cell line with a constitutive NF-κB activity. Both IκBα and IκBβ were shown to be present in TK26, therefore the elevated NF-κB activity could not be ascribed to a lack of these inhibitors.

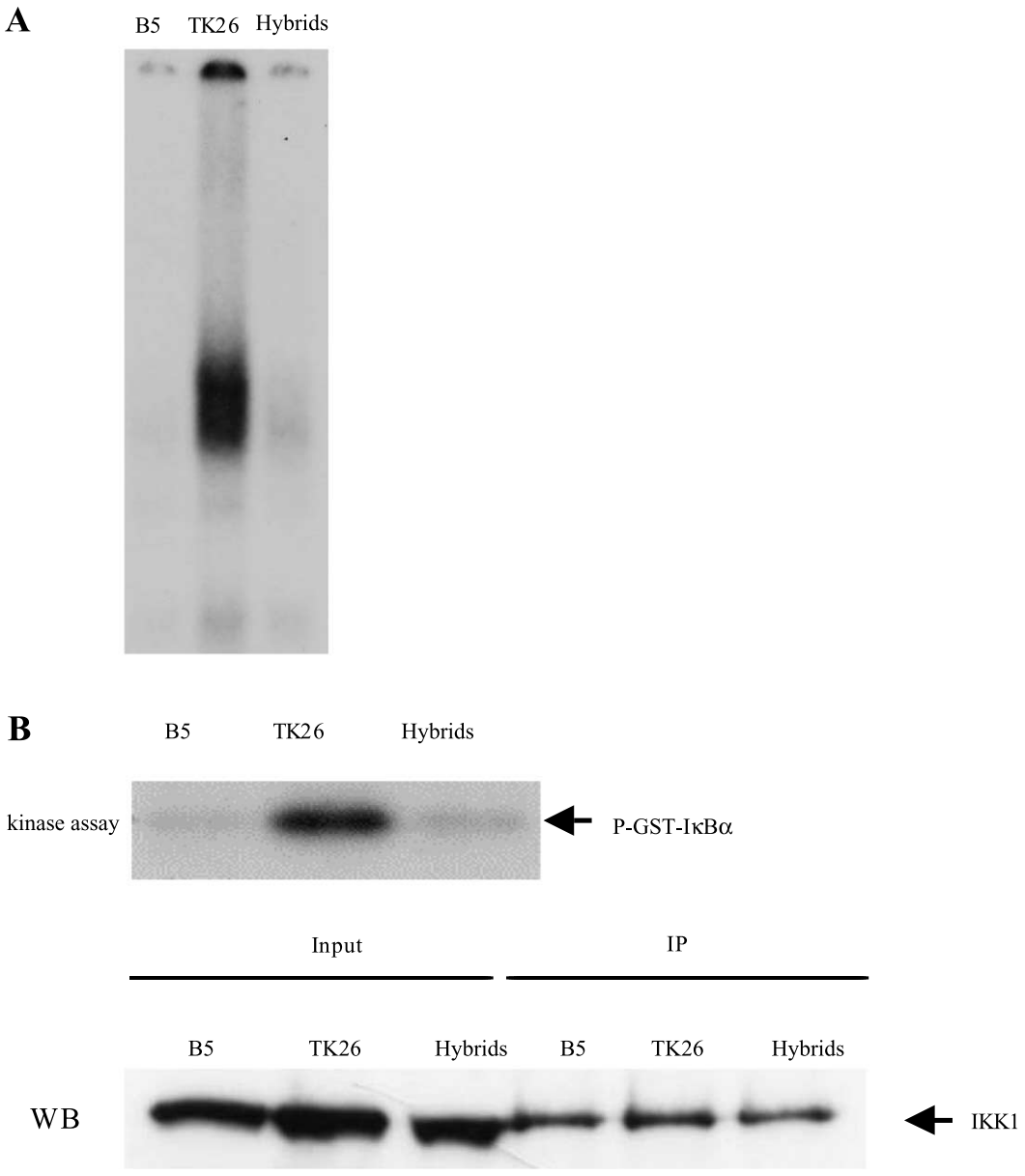

Fig. 6. Genetically recessive phenotype of TK26 cells. A: EMSAs of B5, TK26 and a pool of hybrid cells between TK26 and Rat-1. Five micrograms of nuclear extracts were analyzed using the H-2 K^b-derived κB probe. B: In vitro kinase assay was conducted, using cytoplasmic extracts derived from the indicated cell lines. Western blotting was performed to control for amounts of IKK1 as described in Fig. 2C.

The constitutive NF-κB activity resulted most likely from the elevated IKK activity revealed by the in vitro kinase assay following immunoprecipitation with NEMO, although we were unable to determine whether the elevated IKK activity in TK26 was caused by a lack of suppressor at the level of the IKK complex or by a deregulated upstream signaling molecule. However, IKK activation through an autocrine stimulation of cell surface receptor seemed unlikely, because exposure of Rat-1 cells to the supernatant of TK26 cells did not result in any NF-κB activation (data not shown). Interestingly, the supershift assays revealed that NF-κB complexes present in the nucleus of TK26 include substantial amounts of p50 and RelB as well as a tiny amount of RelA. This contrasts with the nuclear NF-κB complexes detected in Rat-1 cells either stimulated with LPS or stably expressing Tax, which are comprised of p50 and RelA [26]. The distinct nuclear NF-κB components may result from a qualitative difference in the active IKK complex. RelB differs in its regulation from the other two NF-κB activators, RelA and cRel, that are controlled by IκB proteins [27,28]. Previous studies revealed that p100 is associated with RelB and inhibits its nuclear localization, but IκBα, IκBβ, IκBε or p105 are not [29]. Indeed, nuclear translocation of RelB in TK26 cells is associated with aberrant expression of p52, which could result from enhanced p100 processing induced by constitutive IKK activity. P100 has a potent IκB function and its expression is induced by NF-κB activation. It is also known that processing of p100 is not triggered by the classical NF-κB activation that requires NEMO and IKK2 [30]. Thus, constitutive NF-κB activation may be achieved if the powerful IκB function of p100 is disrupted. LPS stimulation requires NEMO for IKK activation and causes rapid up-regulation and subsequent down-regula-

tion of the IKK activity within 4 h. The poor increase in the DNA-binding activity of TK26 cells in response to LPS despite the additive IKK activation may be explained by a possible rapid turnover of IκB proteins owing to the elevated IKK activity. The actual turnover of IκB proteins in TK26 cells is, however, unable to be evaluated in the presence of cycloheximide because the IKK activity in this cell line is sensitive to protein synthesis inhibition as is shown in Fig. 3C. This point remains to be addressed by a pulse chase analysis. It was recently reported that persistent NF-κB activation by Tax involved chronic phosphorylation of IKK2 and NEMO [31]. Indeed, a dominant negative form of NEMO efficiently inhibited Tax-mediated NF-κB activation while it suppressed the reporter gene activation only marginally in TK26 cells. Moreover, treatment of TK26 cells with cycloheximide rapidly abolished the IKK activity, whereas it barely affected LPS- (data not shown) or Tax-mediated IKK activation in Rat-1 cells, indicating that a labile factor mediates the permanent IKK activation in TK26 cells and that this IKK activation depends on a mechanism distinct from those for Tax or LPS. This particular feature of chronic IKK activation is also seen in cells established from patients with certain disease conditions (Miura and Yamaoka, unpublished observation), thus making it important to elucidate the mechanism of IKK activation in TK26 cells.

Finally, the cell hybridization experiment indicated that TK26 cells carried a genetically recessive phenotype and hence were amenable to genetic complementation. Attempts at retroviral expression cloning are now underway to identify a gene product that is responsible for the constitutive IKK activation in TK26. Inhibition of aberrant NF-κB activity has received much attention to control certain malignancies, neurodegenerative and inflammatory diseases. Down-regulation of NF-κB activity can be achieved by expression of the IκBα protein or by a variety of natural and designed molecules, including antioxidants, protease inhibitors, salicylate, arsenite, proteasome inhibitors and peptides. [32]. Most of these reagents target molecules that work for activation at the level or downstream of the IKK complex, thereby inhibiting the general NF-κB activation pathway. A better understanding of the mechanisms of NF-κB activation in each pathological condition should result in the development of reagents that can specifically inhibit abnormal signalings and eventually contribute to establishing a new therapeutic strategy.

Acknowledgements: The authors thank Drs. Alain Israël, Nancy Rice, Robert Weil and Sylvie Memet for reagents. N.C. thanks Drs. Nurjahan Begum, Chang Myint Oo and Prateep Warnnissorn for critical reading of the manuscript. S.Y. thanks Dr. Gilles Courtois for helpful discussion. This work was partly supported by the Japan Human Science Foundation (grant SA14708 to S.Y.). N.C. was supported by grants from the Japanese Ministry of Education, Science, and Culture (Monbusho) scholarship.

References

[1] Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D. and Miyamoto, S. (1995) Genes Dev. 9, 2723–2735.
[2] Baeuerle, P.A. and Baltimore, D. (1996) Cell 87, 13–20.
[3] Baldwin Jr., A.S. (1996) Annu. Rev. Immunol. 14, 649–683.
[4] Karin, M. and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663.
[5] Henkel, T., Zabel, U., Van Zee, K., Muller, J.M., Fanning, E. and Baeuerle, P.A. (1992) Cell 68, 1121–1133.
[6] Traenckner, E.B., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S. and Baeuerle, P.A. (1995) EMBO J. 14, 2876–2883.
[7] DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S. and Karin, M. (1996) Mol. Cell. Biol. 16, 1295–1304.
[8] Gilmore, T.D. (1997) Semin Cancer Biol. 8, 61–62.
[9] Regnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z. and Rothe, M. (1997) Cell 90, 373–383.
[10] DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E. and Karin, M. (1997) Nature 388, 548–554.
[11] Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J., Young, D.B., Barbosa, M., Mann, M., Manning, A. and Rao, A. (1997) Science 278, 860–866.
[12] Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M. and Karin, M. (1997) Cell 91, 243–252.
[13] Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S.T., Weil, R., Agou, F., Kirk, H.E., Kay, R.J. and Israël, A. (1998) Cell 93, 1231–1240.
[14] Rothwarf, D.M., Zandi, E., Natoli, G. and Karin, M. (1998) Nature 395, 297–300.
[15] Mercurio, F., Murray, B.W., Shevchenko, A., Bennett, B.L., Young, D.B., Li, J.W., Pascual, G., Motiwala, A., Zhu, H., Mann, M. and Manning, A.M. (1999) Mol. Cell. Biol. 19, 1526–1538.
[16] Perkins, N.D. (2000) Trends Biochem. Sci. 25, 434–440.
[17] Courtois, G., Smahi, A. and Israël, A. (2001) Trends Mol. Med. 7, 427–430.
[18] Yamamoto, Y. and Gaynor, R.B. (2001) Curr. Mol. Med. 1, 287–296.
[19] Zetoune, F.S., Murthy, A.R., Shao, Z., Hlaing, T., Zeidler, M.G., Li, Y. and Vincenz, C. (2001) Cytokine 15, 282–298.
[20] Pahl, H.L. (1999) Oncogene 18, 6853–6866.
[21] Stark, G.R. and Gudkov, A.V. (1999) Hum. Mol. Genet. 8, 1925–1938.
[22] Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T. and Hanaoka, F. (1991) Exp. Cell Res. 197, 229–233.
[23] Doffinger, R., Smahi, A., Bessia, C., Geissmann, F., Feinberg, J., Durandy, A., Bodemer, C., Kenwrich, S., Dupuis-Girod, S., Blanche, S., Wood, P., Rabia, S.H., Headon, D.J., Overbeek, P.A., Deist, F.L., Holland, S.M., Belani, K., Kumararatne, D.S., Fischer, A., Shapiro, R., Conley, M.E., Reimund, E., Kalhoff, H., Abinun, M., Munnich, A., Israël, A., Courtois, G. and Casanova, J.L. (2001) Nat. Genet. 27, 277–285.
[24] Spindler, K.R., Rosser, D.S. and Berk, A.J. (1984) J. Virol. 49, 132–141.
[25] Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M.B., Kourilsky, P., Baeuerle, P.A. and Israël, A. (1990) Cell 62, 1007–1018.
[26] Yamaoka, S., Inoue, H., Sakurai, M., Sugiyama, T., Hazama, M., Yamada, T. and Hatanaka, M. (1996) EMBO J. 15, 873–887.
[27] Silverman, N. and Maniatis, T. (2001) Genes Dev. 15, 2321–2342.
[28] Jiang, H.Y., Petrovas, C. and Sonenshein, G.E. (2002) J. Virol. 76, 5737–5747.
[29] Solan, N.J., Miyoshi, H., Carmona, E.M., Bren, G.D. and Paya, C.V. (2002) J. Biol. Chem. 277, 1405–1418.
[30] Senftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.C. and Karin, M. (2001) Science 293, 1495–1499.
[31] Carter, R.S., Geyer, B.C., Xie, M., Acevedo-Suarez, C.A. and Ballard, D.W. (2001) J. Biol. Chem. 276, 24445–24448.
[32] Epinat, J.C. and Gilmore, T.D. (1999) Oncogene 18, 6896–6909.