targets for anti-NF-κB therapy.

Table1 Alterations of *nf-kb/ikb* genes in hematological malignancies

Genes	Locus	Alteration type	Disease
c-rel	2p14-15	Amplification	Diffuse large cell lymphoma
		Amplification	Mediastinal B-cell lymphoma
		Amplification	Follicular large cell lymphoma
		Rearrangement/overexpression	Follicular lymphoma
		Rearrangement/overexpression	Diffuse large cell lymphoma
relA	11q13	Chromosomal rearrangement	B-cell non-Hodgkin's lymphoma
		Chromosomal rearrangement	Multiple myeloma
		Amplification	Diffuse large cell lymphoma
		Amino acid substitution	Multiple myeloma
nfkb1 (p105)	4q24	Chromosomal rearrangement	Acute lymphoblastic leukemia
nfkb2 (p100)	10q24	Rearrangement/overexpression	Cutaneous T-cell lymphoma
		Chromosomal rearrangement	B-cell non-Hodgkin's lymphoma
		Chromosomal rearrangement	B-cell chronic lymphocytic leukemia
		Chromosomal rearrangement	Multiple myeloma
bcl-3	19q13.1	Rearrangement/overexpression	B-cell chronic lymphocytic leukemia
		Rearrangement/overexpression	B-cell non-Hodgkin's lymphoma
ikba	14q13	Mutation/truncation	Hodgkin's disease
ikbb	?	Mutation/truncation	Hodgkin's disease

(Rayet B. and Gelinas C., 1999 Oncogene)

Table2 Neoplasms in which constitutive activation of NF-κB are reported

Hematological malignancies	Solid tumors
Hodgkin's disease	Hepatocellular carcinoma
Diffuse large B-cell lymphoma	Colorectal cancer
Cutaneous T-cell lymphoma	Non-small cell lung cancer
Anaplastic large T-cell lymphoma	Prostate cancer
MALT lymphoma	Breast cancer
Multiple myeloma	Ovarian cancer
Chronic lymphocytic leukemia	Pancreatic cancer
Acute lymphocytic leukemia	Pharengeal carcinoma
Adult T-cell leukemia/lymphoma	
NK/T-cell leukemia/lymphoma	
Chronic myeloid leukemia	

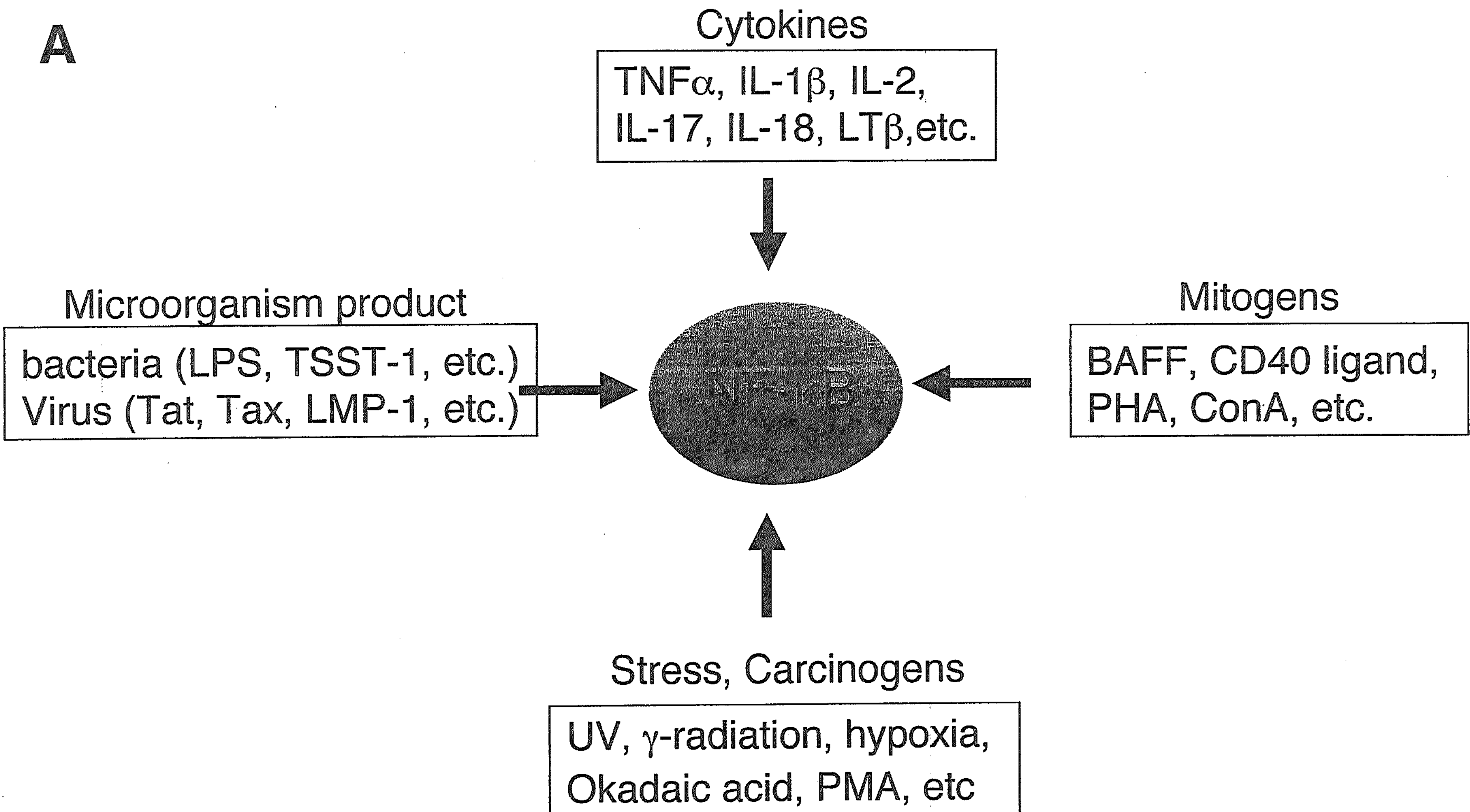


Fig.1A (Okamoto et. al.)

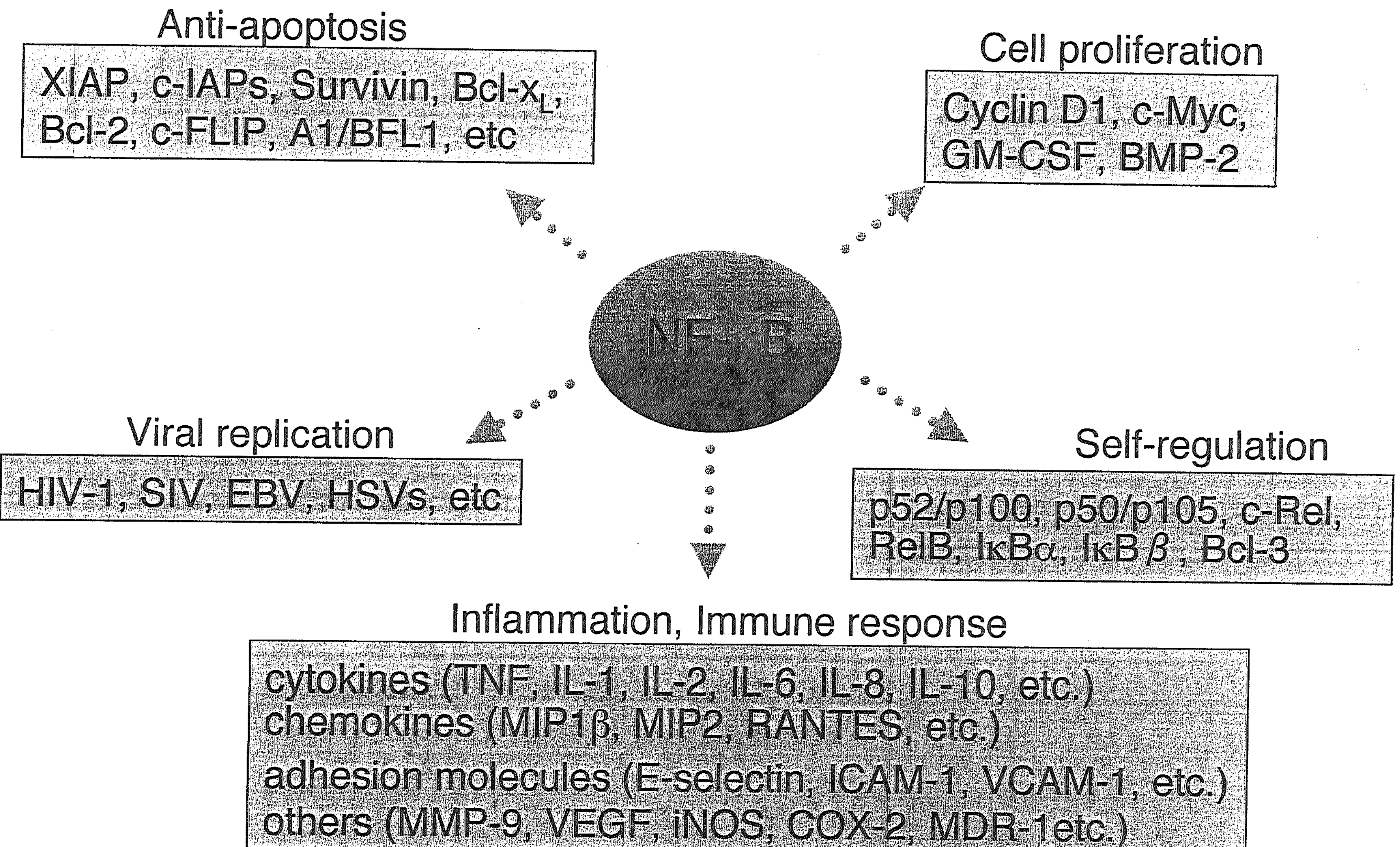


Fig.1B (Okamoto et. al.)

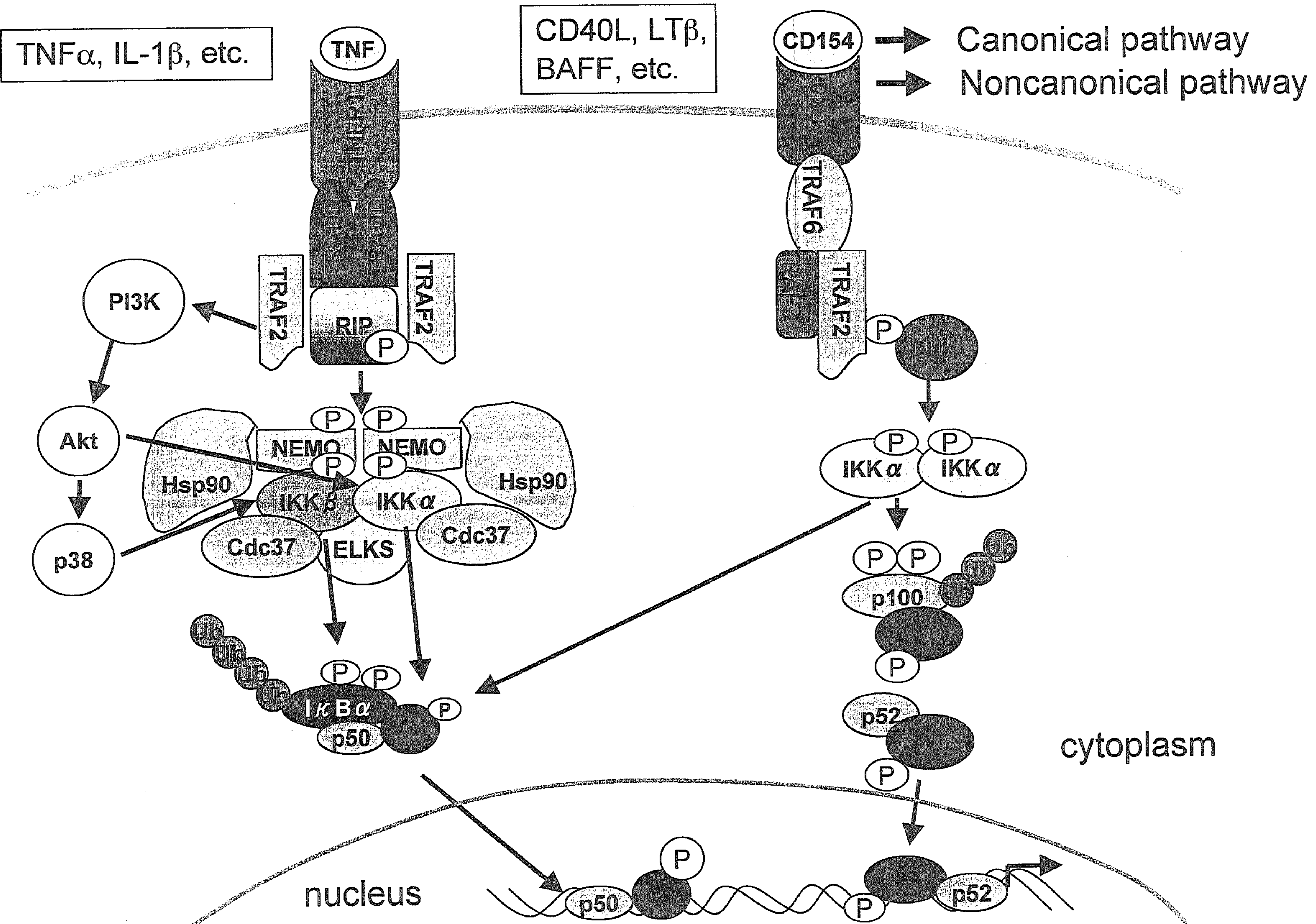


Fig.2 (Okamoto et. al.)

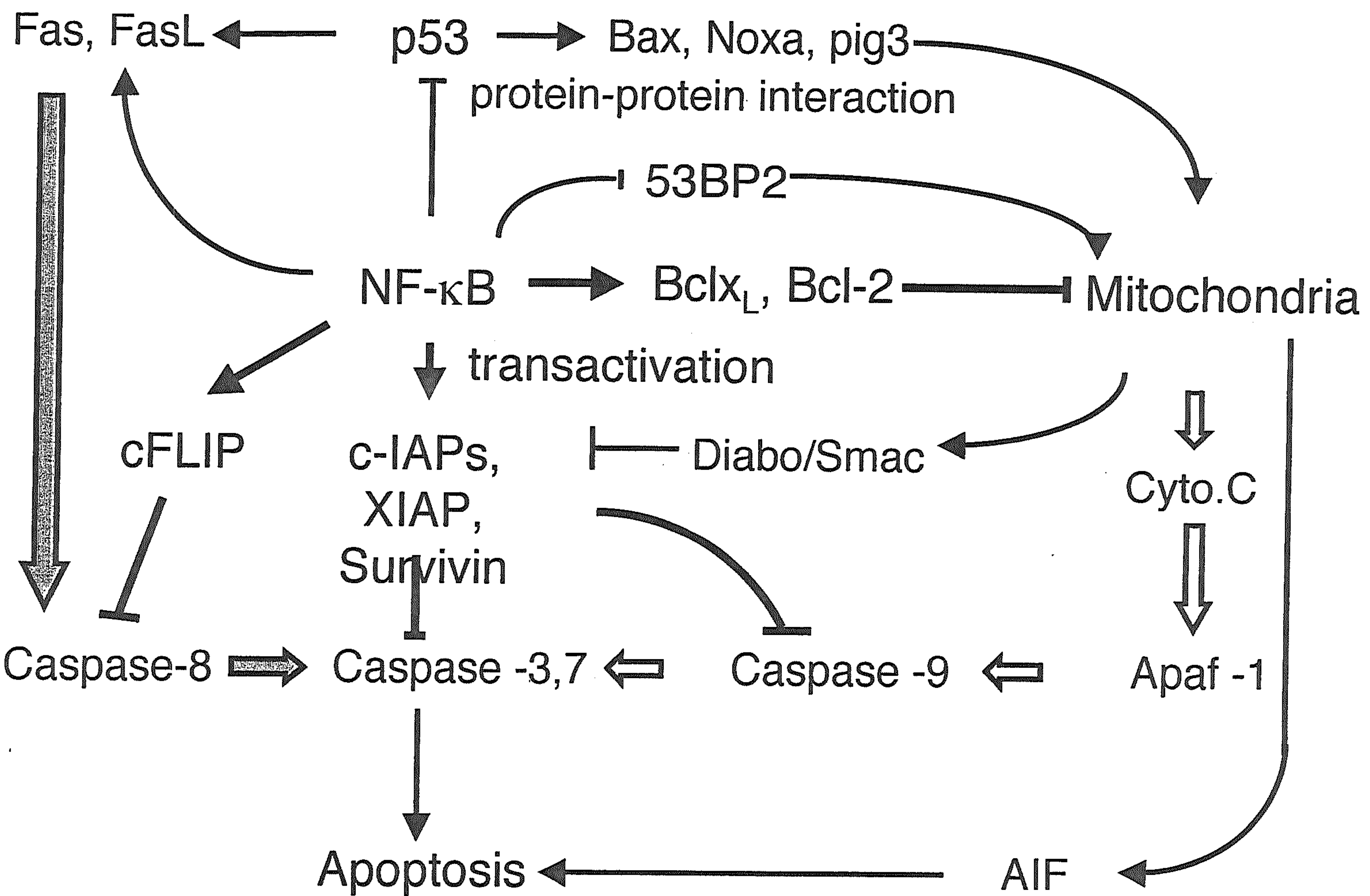


Fig.3 (Okamoto et. al.)

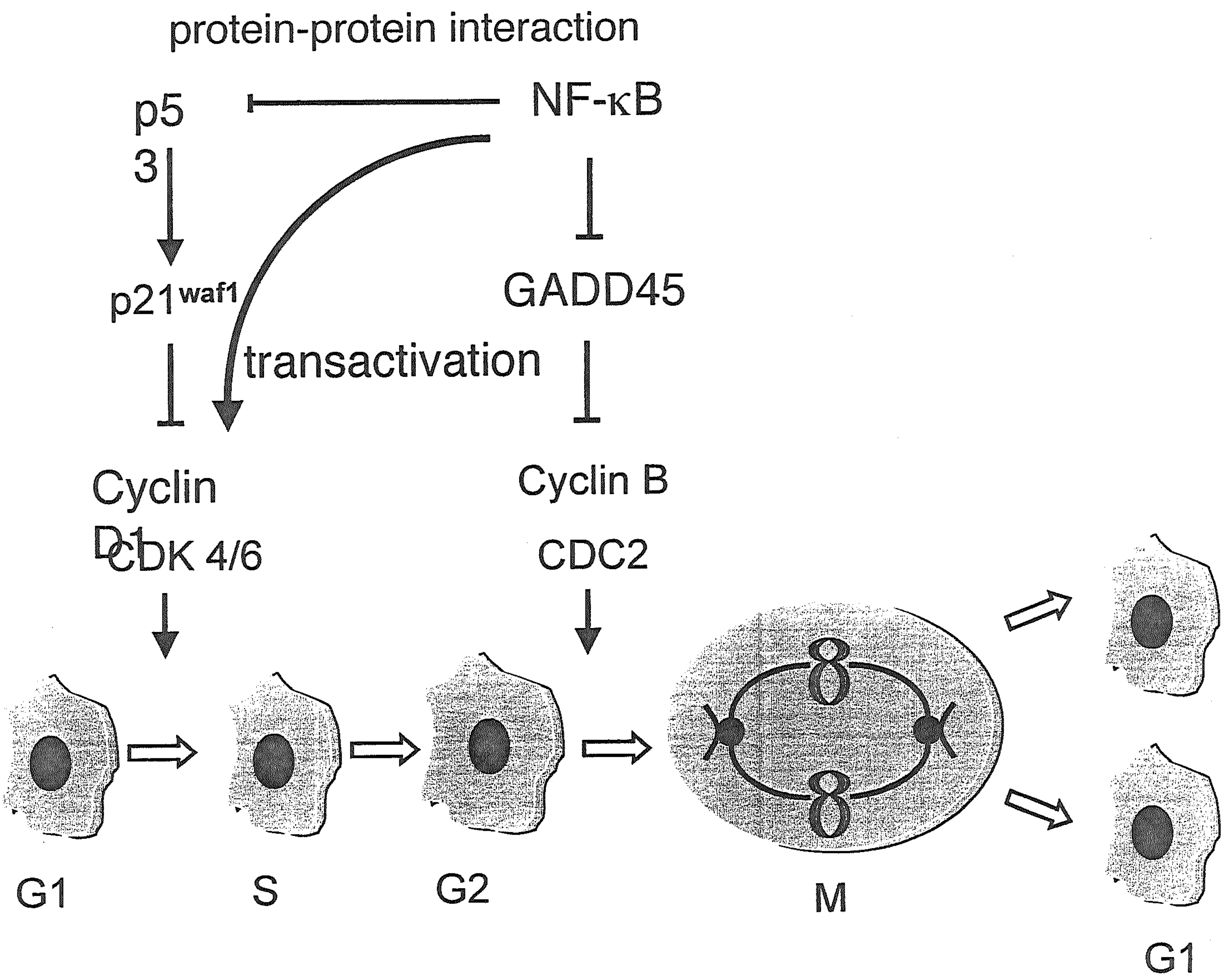


Fig.4 (Okamoto et. al.)

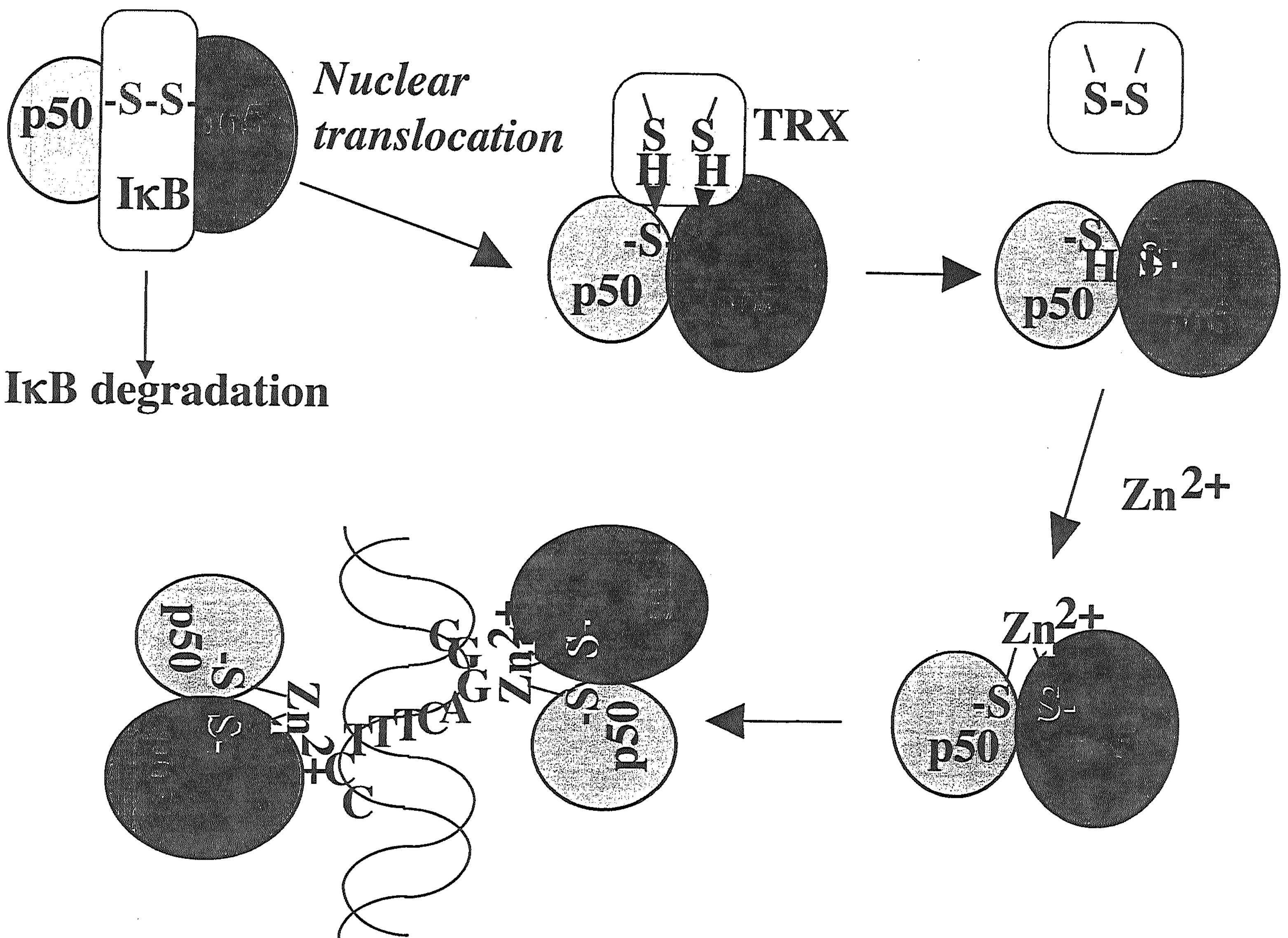
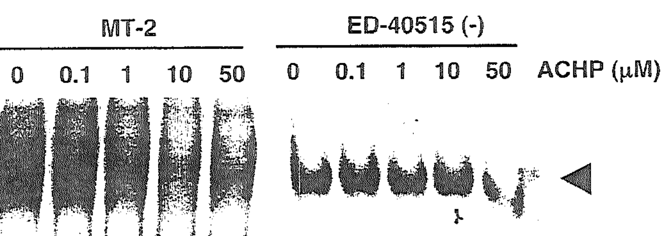
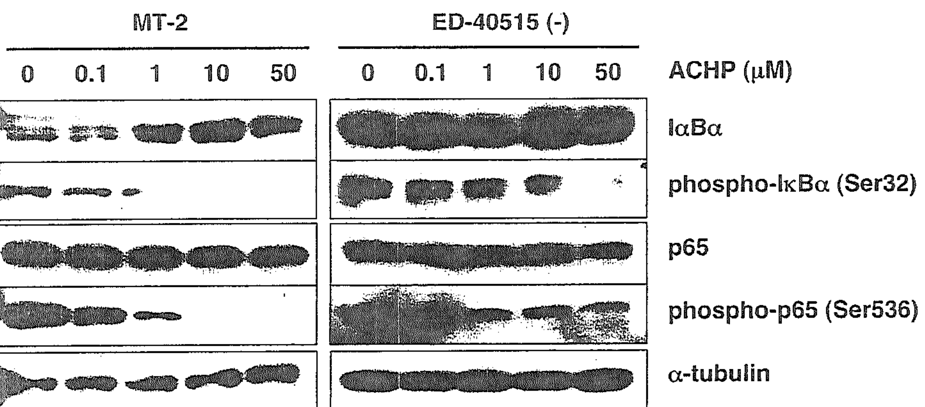
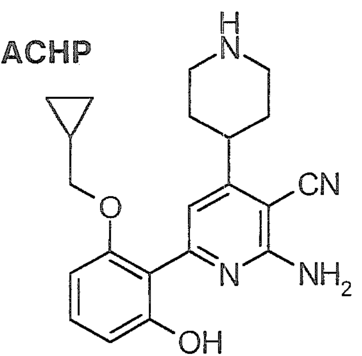
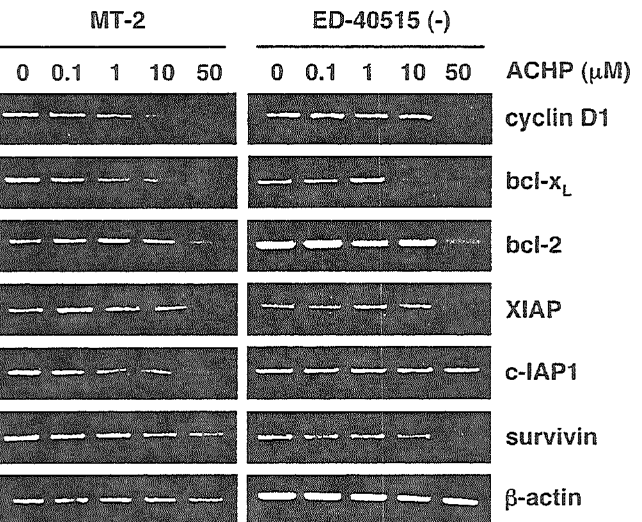


Fig.5 (Okamoto et. al.)









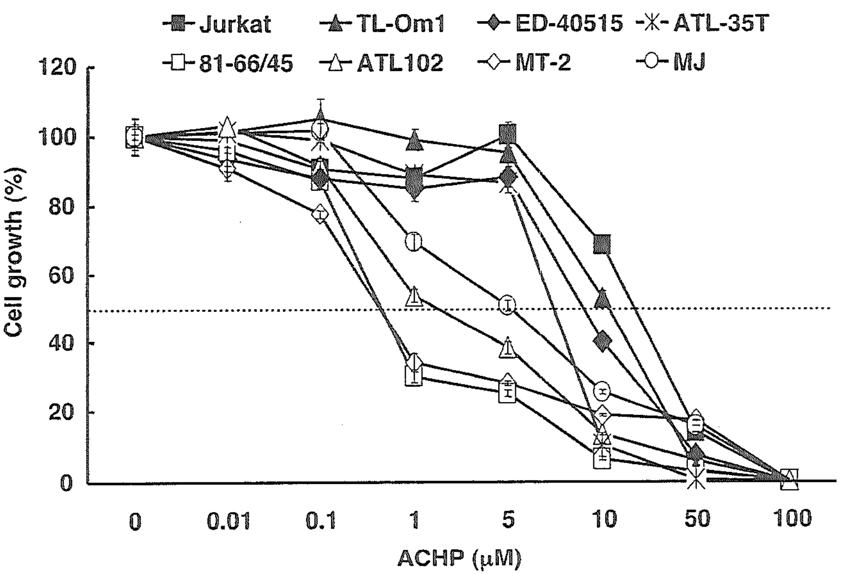


Fig.6 (Okamoto et. al.)

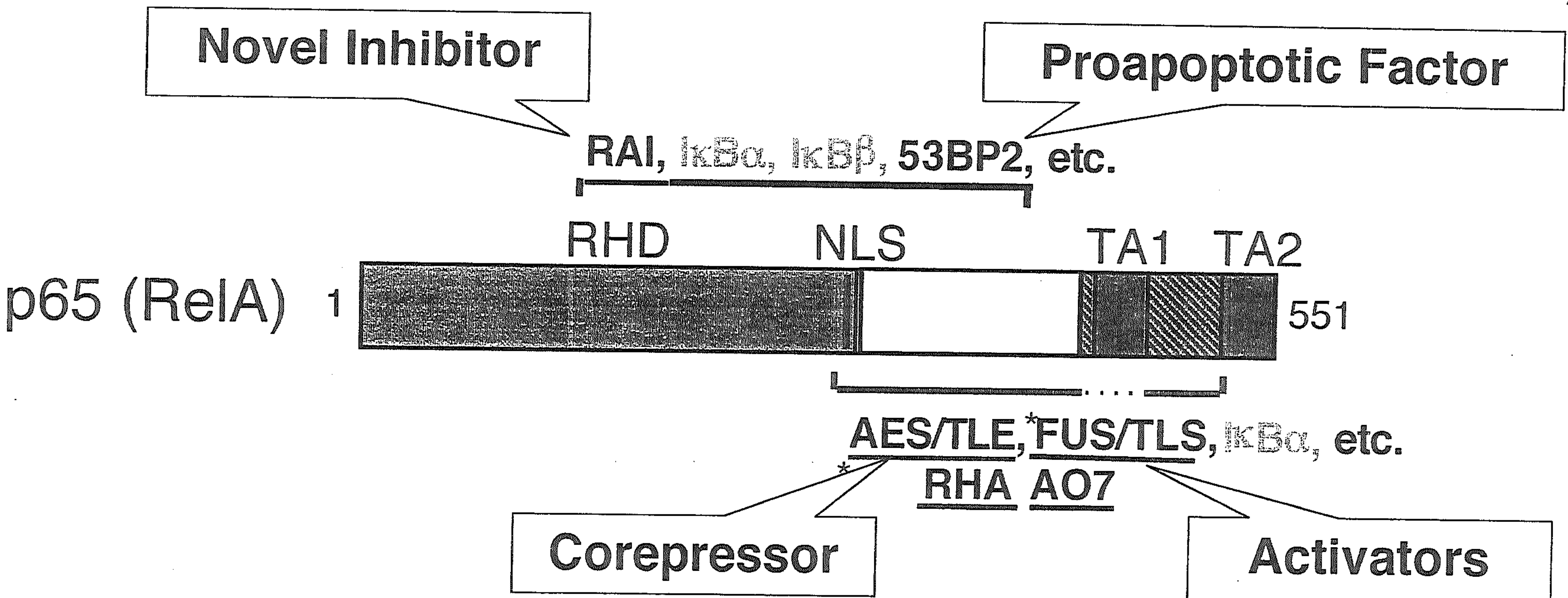


Fig.7 (Okamoto et. al.)

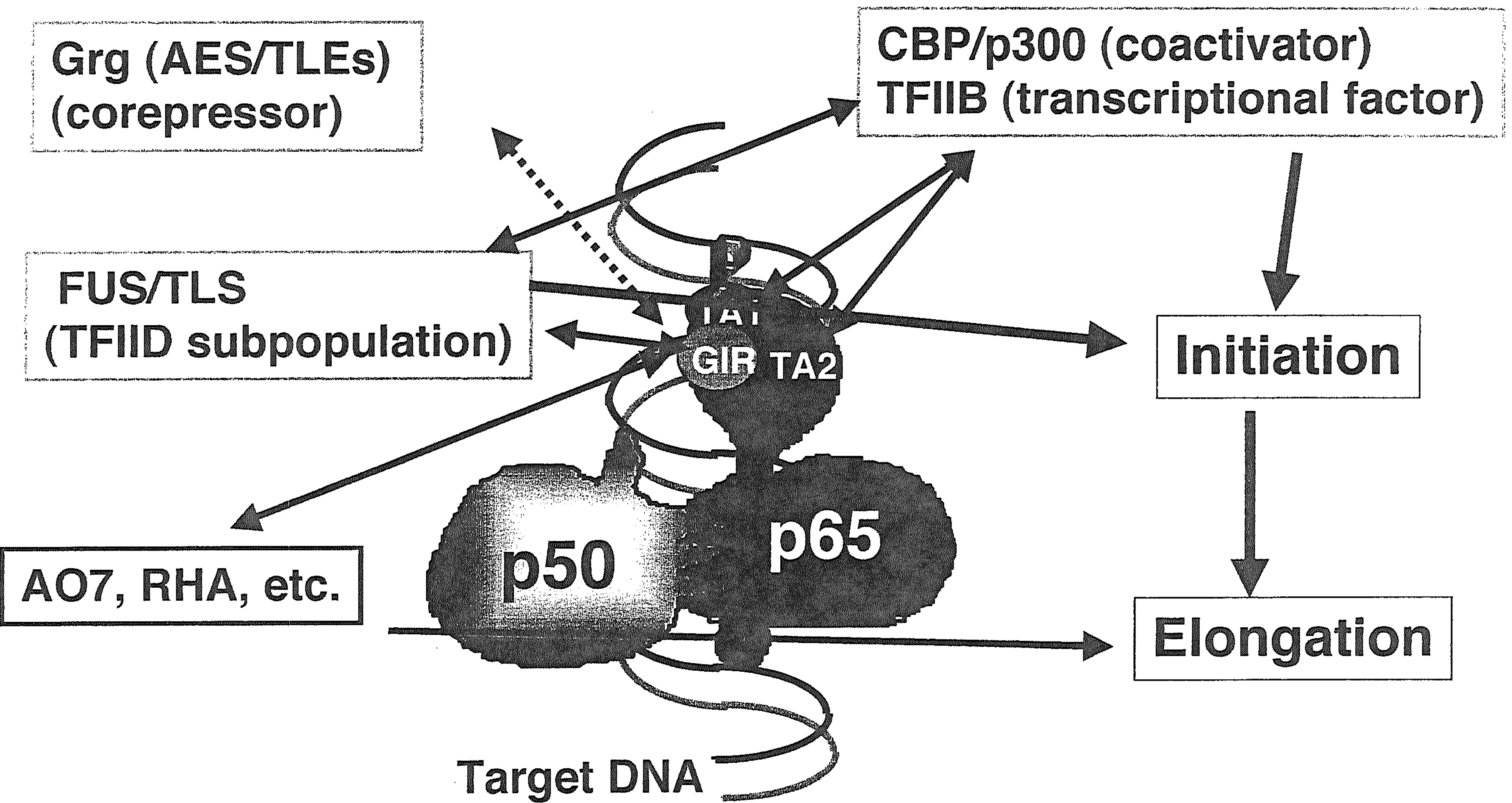


Fig.8 (Okamoto et. al.)

Table 1. Alterations of *nf-kb/ikb* genes in hematological malignancies

Genes	Locus	Alteration type	Disease
c-rel	2p14-15	Amplification	Diffuse large cell lymphoma
		Amplification	Mediastinal B-cell lymphoma
		Amplification	Follicular large cell lymphoma
		Rearrangement/overexpression	Follicular lymphoma
		Rearrangement/overexpression	Diffuse large cell lymphoma
relA	11q13	Chromosomal rearrangement	B-cell non-Hodgkin's lymphoma
		Chromosomal rearrangement	Multiple myeloma
		Amplification	Diffuse large cell lymphoma
		Amino acid substitution	Multiple myeloma
nfkb1 (p105)	4q24	Chromosomal rearrangement	Acute lymphoblastic leukemia
nfkb2 (p100)	10q24	Rearrangement/overexpression	Cutaneous T-cell lymphoma
		Chromosomal rearrangement	B-cell non-Hodgkin's lymphoma
		Chromosomal rearrangement	B-cell chronic lymphocytic leukemia
		Chromosomal rearrangement	Multiple myeloma
bcl-3	19q13.1	Rearrangement/overexpression	B-cell chronic lymphocytic leukemia
		Rearrangement/overexpression	B-cell non-Hodgkin's lymphoma
ikba	14q13	Mutation/truncation	Hodgkin's disease
ikbb	?	Mutation/truncation	Hodgkin's disease

(Rayet B. and Gelinas C., (1999))

Table 2. Neoplasms in which constitutive activation of NF-κB is reported

Hematological malignancies	Solid tumors
Hodgkin's disease	Hepatocellular carcinoma
Diffuse large B-cell lymphoma	Colorectal cancer
Cutaneous T-cell lymphoma	Non-small cell lung cancer
Anaplastic large T-cell lymphoma	Prostate cancer
MALT lymphoma	Breast cancer
Multiple myeloma	Ovarian cancer
Chronic lymphocytic leukemia	Pancreatic cancer
Acute lymphocytic leukemia	Pharengeal carcinoma
Adult T-cell leukemia/lymphoma	
NK/T-cell leukemia/lymphoma	
Chronic myeloid leukemia	

ELSEVIER

International Immunopharmacology 6 (2006) 170–181

International Immunopharmacology

www.elsevier.com/locate/intimp

Estrogen stimulates transcription of human immunodeficiency virus type 1 (HIV-1)

Daichi Katagiri [a], Hidetoshi Hayashi [a], Ann Florence B. Victoriano [b], Takashi Okamoto [b], Kikuo Onozaki [a,*]

[a] *Department of Molecular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan*

[b] *Department of Molecular and Cellular Biology, Graduate School of Medical Sciences, Nagoya City University, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601, Japan*

Received 14 June 2005; received in revised form 14 July 2005; accepted 27 July 2005

Abstract

Gene expression from human immunodeficiency virus (HIV) provirus is a crucial step for the viral replication. Here we examined a potential role of 17β-estradiol (E2) in HIV-1 transcription. Transient luciferase expression studies revealed that E2 activated HIV-LTR reporter gene in HEK293 cells when the cells were co-transfected with estrogen receptor α (ERα) but not ERβ expression plasmid. This E2 effect was abrogated by a specific antagonist to ER, ICI 182,780, indicating that it was mediated by ERα. Mutation analysis revealed that Sp1 binding site but not nuclear factor-kappa B (NF-κB) binding site of HIV-1 LTR is critical to the E2 effect. In addition, whereas E2 could not induce DNA-binding activity of NF-κB, E2 could augment both Sp1 DNA-binding and transcriptional activity. These findings suggest a contribution of estrogen for HIV-1 replication through ERα by augmenting Sp1 DNA-binding and transcriptional activity.
© 2005 Elsevier B.V. All rights reserved.

Keywords: HIV; Estrogen; Estrogen receptor; Sp1; NF-κB

1. Introduction

It is well documented that women are more vulnerable than men in acquiring HIV infection in heterosexual encounter, which has been ascribed to biological factors, such as the greater area of mucous membrane exposure during sexual intercourse in women than men, the greater quantity of fluids transferred from men to women, the higher viral content of male sexual fluids, and the micro mechanical tears that can occur in vaginal (or rectal) tissue from sexual penetration [1–3]. Social factors are also expected to contribute to the gender difference [4]. It is also reported that the rate of disease progression is greater

* Corresponding author. Tel.: +81 52 836 3419; fax: +81 52 836 3419.
E-mail address: konozaki@phar.nagoya-cu.ac.jp (K. Onozaki).

1567-5769/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2005.07.017

in women than men as shown by the rapid declines in $CD4^+$ cells [5] and higher rate of HIV-1 replication over time in women than in men [6].

Estrogen is a female sex hormone that has been recognized as a risk factor for breast and endometrial cancer, and autoimmune diseases [7]. There are two types of estrogen receptors, namely estrogen receptor α (ERα) and estrogen receptor β (ERβ) [8,9]. Following ligand binding, ERα and ERβ form homo- or heterodimmers that bind to specific DNA sequences (estrogen responsive element, ERE) of target genes through its DNA-binding domain (DBD), and activate the transcription of vitellogenin [10], progesterone receptor [11] and c-Myc [12] genes. Recent studies have revealed that ERα physically interacts with many transcription factors, including Sp1 [13–15], AP-1 [16], NF-κB [17], C/EBPβ and STAT5 [18], and modulates their transcriptional activity. Therefore, estrogen can modulate target gene activation through ER in both ERE-dependent and ERE-independent manners.

On the other hand, it is well known that nuclear factor-κB (NF-κB) and Sp1 are potent cellular activators of HIV-1 gene expression [19–21], and activation of NF-κB together with constitutively active Sp1 could trigger the transcription of viral genes in cells chronically infected with HIV-1 [22–25]. Therefore, it is possible that estrogen activates HIV-1 transcription through interacting with NF-κB or Sp1. In this study we show that 17β-estradiol (E2) stimulates HIV-1 transcription via ERα but not ERβ by augmenting Sp1 DNA-binding and transcriptional activity.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) without phenol red, 17β-estradiol (E2) and anti-β-actin monoclonal antibody (AC-15) were purchased from SIGMA (St. Louis, MO). ICI 182,780 was from Torcris Cookson, Inc. (Ballwin, MO). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT) and was used after treatment with charcoal to remove serum-derived estrogen. Anti-human ERα rabbit antibody (HC-20), anti-human ERβ rabbit antibody (HC-150), anti-human p65 rabbit antibody (SC-372), anti-human Sp1 rabbit antibody (SC-59) and anti-human Sp3 rabbit antibody (SC-644) were purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) was from Jackson ImmunoResearch Lab. Inc. (West Grobe, PA). Horseradish peroxidase-conjugated sheep anti-mouse IgG was purchased from Amersham Bioscience.

2.2. Cell cultures

A human embryonic kidney cell line 293 was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and a human mammary tumor cell line MCF-7 was kindly provided by Dr. H. Utsumi (Kyushu Univ., Fukuoka, Japan). These two cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin [26].

2.3. Plasmids

The luciferase reporter plasmids containing the full length LTR (LTR-FF-Luc (κBw)) and with mutated two κB sites (MJW-37-Luc (κBm)) were generous gifts from Dr. J. Karn [27]. HIV-1 LTR-based luciferase expression plasmids including CD12-Luc (containing the full-size LTR U3 and R), CD23-Luc (containing positions −117 to +80 of HIV-1 LTR), CD52-Luc (containing positions −65 to +80 of HIV-1 LTR) and CD54-Luc (containing positions −48 to +80 of HIV-1 LTR) were constructed as described previously [24]. Expression vector pCIneo-Sp1 and GAL4-Sp1 were generous gifts from Dr. S. Kojima (RIKEN, Wako, Japan) [28]. Expression vectors pcDNA3-ERα and pcDNA3-ERβ, and a reporter plasmid $pERE_3tk$-Luc (containing three tandem repeats of ERE) were generous gifts from Dr. S. Kato (Univ. Tokyo, Japan) [29]. pFR-Luc (5× GAL4-Luc) and pFC2-dbd (GAL4-dbd) were purchased from Stratagene (La Jolla, CA).

2.4. Transfection conditions and luciferase assay

Transient transfection of 293 cells was performed by the Chen–Okayama method [30]. After 16 h of transfection, cells were incubated with the indicated concentrations of E2 for additional 24 h and then harvested. Gene expression was measured by luciferase activity as previously described [31]. Transfection efficiency was monitored by β-galactosidase activity with pCMV-β-gal plasmid as an internal control. All luciferase activities shown in transient transfection assays were corrected by the internal control activity of β-galactosidase activity by pCMV-β-gal.

2.5. Electrophoretic mobility shift assay (EMSA)

293 cells were transfected with or without pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated

with or without 10 nM E2 or TNFα (1 ng/ml) for 24 h. Preparation of nuclear extracts and EMSA was carried out as previously described [19,32,33]. The double stranded oligonucleotide probe for NF-κB or GC rich sequence was synthesized and end-labeled by γ-^{32}P-ATP. Both sequences were taken from the HIV-1 LTR. The κB sequence used was forward (5′-TTTCTAGGGACTTTCC-GCCTGGGGACTTTCCAG-3′) and complement (5′-TTT-CTGGAAAGTCCCCAGGCGGAAAGTCCCTAG-3′). The GC rich sequence used was forward (5′-CAGGGA-GGCGTGGCCTGGGCGGGACTGGGGAGTGGCGTC-3′) and complement (5′-G ACGCCACTCCCCAGTCCCGC-CCAGGCCACGCCTCCCTG-3′). Nuclear extracts were incubated in 10 μl EMSA buffer containing the radio-labeled oligonucleotide probe.

2.6. RNA preparation and reverse transcriptase (RT) reaction

Total RNA from cells was extracted according to the method of Chomczynski and Sacchi [34]. The amount of RNA was determined spectrophotometrically. The RT reaction was performed by incubating 2 μg of total RNA in 20 μl reaction volume containing 50 mM Tris–HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, 40 ng Random Primer $p(dN)_6$, 6 U ribonuclease inhibitor and 40 U M-MLV Reverse Transcriptase. The reaction mixtures were incubated at 37 °C for 60 min, then followed by 70 °C for 10 min in Gene Amp PCR System 2400 (Applied Biosystems). The reaction mixtures were diluted to five fold of volume with sterilized distilled H_2O and used as cDNA solutions. cDNA samples were stored at −20 °C until PCR analysis.

2.7. PCR analysis

Primers used for human ERα were 5′-GTCTGA-GGCTGCGGCGTTCGGCTCC-3′ and 5′-ATTCCATAGC-CATACTTCCCTTGTC-3′, for human ERβ were 5′-GGCAACTACTTCAAGGTTTCGAGAG-3′ and 5′-ACTC-GCATGCCTGACGTGGGACAGG-3′, and for human GAPDH were 5′-TGAAGGTCGGAGTCAACGGATTT-GGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′. PCR reactions contained 1× PCR buffer, 0.4 mM dNTPs, 2.5 ng forward and reverse primers, 0.005 U Ampli Taq Gold™ DNA Polymerase (Applied Biosystems), and 5 μl cDNA solution in a 10 μl volume. ERα and ERβ cDNAs were amplified for 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. GAPDH cDNA was amplified for 24 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. PCR products were analyzed on 1.5% agarose gels in the presence of ethidium bromide. Fragments of 281 bps (ERα), 265 bps (ERβ) and 983 bps (GAPDH) were generated and their identities were confirmed by restriction enzyme digestion and sequencing. Experiments were conducted three times.

2.8. Preparation of cell extracts and Western blot analysis

Preparation of cell extracts from cells seeded in 60-mm dishes and Western blot analysis were performed as described previously [35]. After the cells were treated for indicated times, the medium was removed. The cells were washed three times with ice-cold PBS, and 0.4 ml of ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 120 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml antipain, 100 μg/ml benzamidine hydrochloride, 50 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin A and 1 mM PMSF) was added into the plates. The cell lysates were collected and the cell debris was pelleted to collect the supernatants. For Western blots, equal amounts of proteins were suspended in SDS-sample buffer. The proteins were separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to an Immobilon™-P PVDF membrane (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST), membranes were incubated with appropriately diluted rabbit antiserum against human ERα, ERβ or anti-human β-actin mouse monoclonal antibody, and then with horseradish peroxidase-conjugated anti-rabbit IgG (1 : 10000) or anti-mouse Ig, horseradish peroxidase linked whole antibody (1 : 5000), respectively. The reactive proteins were detected with enhanced chemiluminescence reagents, ECL (Amersham Bioscience) and analyzed by a chemiluminescence image analyzer, LAS-1000 (Fuji Film, Japan).

3. Results

3.1. 293 cells express ERβ but not ERα

To examine the effect of E2 on HIV-LTR activation we used 293 cells because of their high transfection efficiency. First, we examined whether 293 cells express ERα or ERβ by determining mRNA and protein levels of ERα and ERβ by RT-PCR and Western blotting. As shown in Fig. 1, 293 cells express ERβ mRNA and low level of ERα mRNA. Western blot analysis confirmed the expression of ERβ but not ERα. In MCF-7 cells used as a positive control, ERα and ERβ were expressed at both mRNA and protein levels.

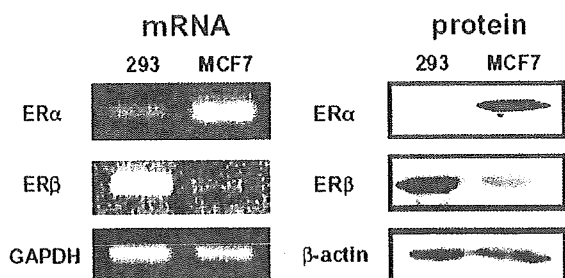


Fig. 1. Expression of ERα and ERβ in HEK293 cells and MCF-7 cells. Total RNA was extracted from each cell lines and then mRNA expression levels of ERα, ERβ and GAPDH were determined by RT-PCR. Cell lysates from each cell lines were separated by SDS-PAGE. Protein expression levels of ERα, ERβ and β-actin were determined by Western blotting. Representative data of three experiments are shown.

3.2. E2 activates ERE reporter gene by expression with ERα or ERβ

Prior to examining the effect of E2 on HIV-1 LTR activation, we constructed E2 responding 293 cells. As 293 cells do not express ERα, the cells were transiently transfected with expression plasmid of ERα or ERβ, together with ERE_3tk-Luc, a reporter plasmid containing three tandem repeats of ERE, and stimulated with varying concentrations of E2. Up to 100 nM are physiological levels of E2 in circulation during the early and late pregnancy [36]. As shown in Fig. 2, E2-induced luciferase activity in a dose-dependent manner in the presence of ERα or ERβ, at up to 100 and 1000 pM, respectively. Without expression of either ERα or ERβ, E2 did not affect the level of luciferase activity, suggesting that endogenous ERβ is not functioning in 293 cells.

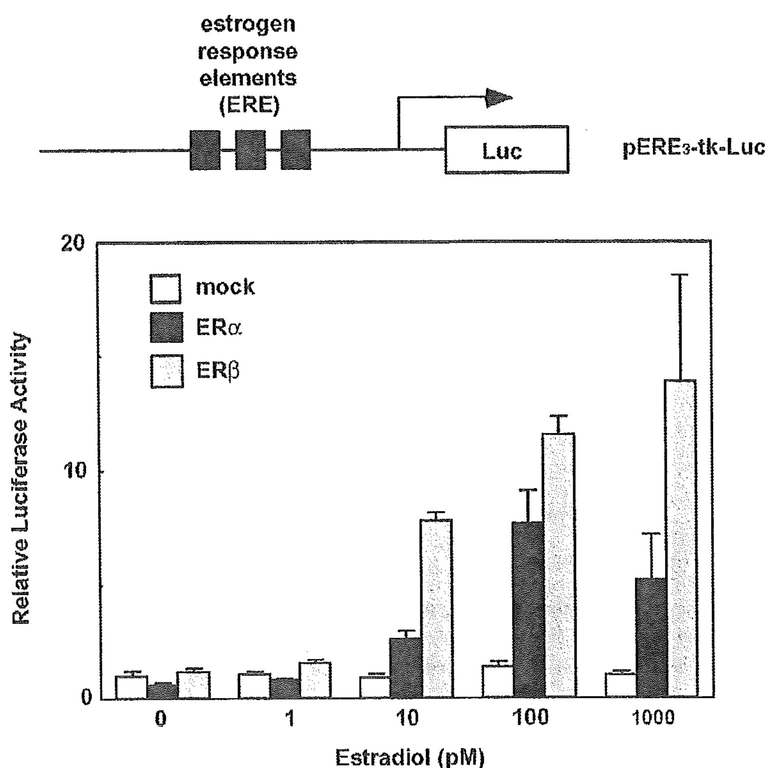


Fig. 2. The effect of 17β-estradiol (E2) on ER-dependent gene activation from estrogen response element (ERE) in HEK293 cell. 293 cells were co-transfected with ERE_3tk-Luc reporter gene with or without pcDNA3-ERα? or pcDNA3-ERβ plasmid. After 16 h of transfection, cells were incubated with varying concentrations of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured. The luciferase activity was normalized by the β-galactosidase activity and mean ± SD based triplicated cultures are shown. Representative data of three experiments are shown.

3.3. E2 activates HIV-1 LTR transcription via ERα

Using E2 responding 293 cells, we determined the effect of E2 on HIV-1 LTR activation. 293 cells were transiently transfected with expression plasmid of ERα or ERβ, together with the CD12-Luc reporter plasmid containing the full-length HIV-1 LTR, and stimulated with E2. As shown in Fig. 3B, the basal transcription level of CD12-Luc was not significantly stimulated by E2. In contrast, E2 activated transcription of CD12-Luc in a dose-dependent manner at up to 10 nM in the presence of ERα but not ERβ. The stimulating effect of E2 was also observed by increasing the expression level of ERα (Fig. 3C). In order to determine whether the stimulating effect of E2 is mediated

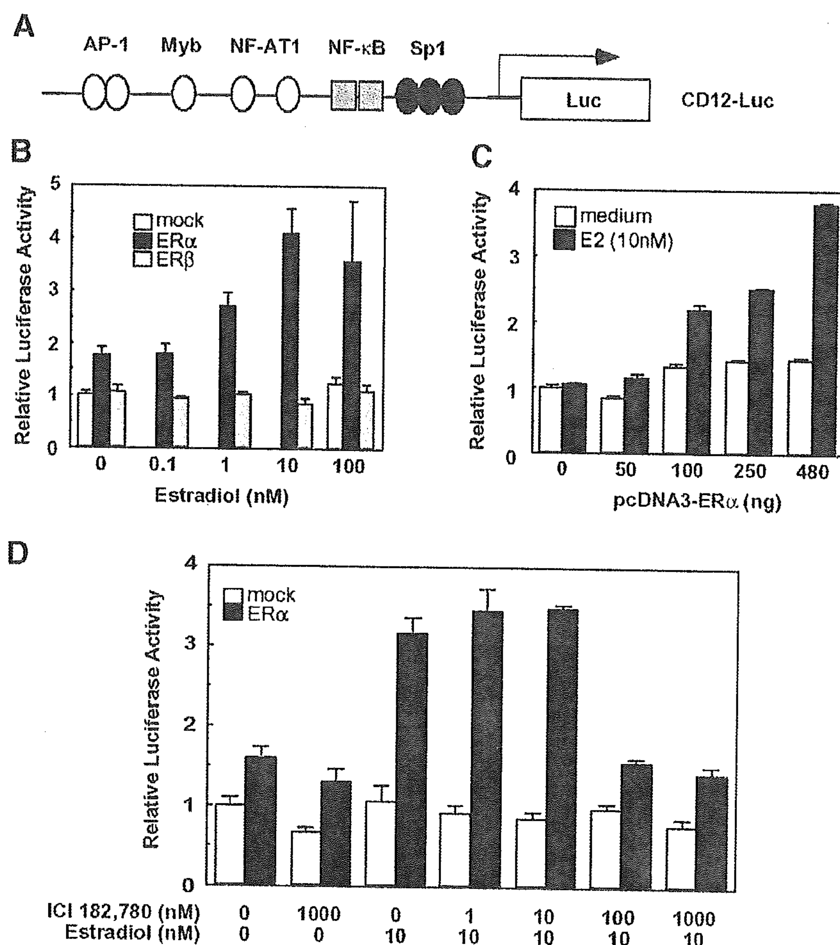


Fig. 3. The effect of E2 on gene expression from HIV-1 LTR. (A) Schematic diagram of HIV-1 LTR, position of various *cis*-elements for transcription factors, and reporter constructs. Binding sites for AP-1, Myb, NF-AT1, two tandem repeats of NF-κB binding sites, and three tandem repeats of Sp1 binding sites are indicated. CD12-Luc contains the full sequence of both U3 and R regions. (B) Dose-dependent effect of E2 on gene expression from HIV-1 LTR. 293 cells were co-transfected with CD12-Luc plasmid with or without pcDNA3-ERα or pcDNA3-ERβ plasmid.? After 16 h of transfection, cells were incubated with varying concentrations of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2. (C) Dose-dependent effect of ERα expression on the E2-induced activation of gene expression from HIV-1 LTR. 293 cells were co-transfected with CD12-Luc plasmid with pcDNA3 or without indicated amount of pcDNA3-ERα plasmid.? After 16 h of transfection, cells were incubated with 10 nM of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured. (D) Effect of ER antagonist on the E2-dependent activation of gene expression from HIV-1 LTR. 293 cells were co-transfected with HIV-1 LTR-Luc reporter plasmid (CD12-Luc) in the presence or absence of ERα plasmid. After 16 h of transfection, cells were incubated for 24 h with indicated concentrations of ICI 182,780, in the presence or absence of 10 nM E2, and luciferase activity was measured. Representative data of more than three experiments are shown.

by ERα, ICI 182,780, a specific antagonist to ER, was added to the reporter gene assay. As shown in Fig. 3D, the basal transcription level of CD12-Luc was not affected by ICI 182,780. However, the E2-induced CD12-Luc transcription was completely abrogated by ICI 182,780. These findings indicate that E2 activates transcription of HIV-1 LTR via ERα.

3.4. Sp1 binding site is critical to the E2-induced gene expression from HIV-1 LTR

To identify the *cis*-regulatory region responsible for the E2-induced activation of HIV-1 LTR transcription, we compared the effect of E2 on gene expression of CD12-Luc and various 5′-deletion mutants: CD23-Luc (deleting the upstream sequences from position-117 of HIV-1 LTR, including AP1-binding site but still retaining NF-κB and Sp1 sites), CD52-Luc (deleting the upstream from position-65 but retaining the promoter-proximal two tandem repeats of Sp1 sites), and CD54-Luc (deleting the upstream from −48 and retaining no Sp1 site) (Fig. 4A). Similar to the results with CD12-Luc, gene expressions of CD23-Luc and CD52-Luc were significantly activated by E2 in the presence of ERα (Fig. 4B). However, CD54-Luc lost its responsiveness to E2. These results indicate that E2 can activate HIV-1 gene expression even in the absence of NF-κB and that the Sp1 site is indispensable for the effect of E2.

3.5. NF-κB is not involved in the E2-mediated activation of HIV-1 gene expression

To further examine a role of NF-κB in the E2-induced HIV-1 gene activation, we examined the effect of E2 on the gene expression from HIV-1 LTR and its mutant containing mutations at two κB sites (MJW-37-Luc (κBm)). As demonstrated in Fig. 5B, E2 could activate the gene expression

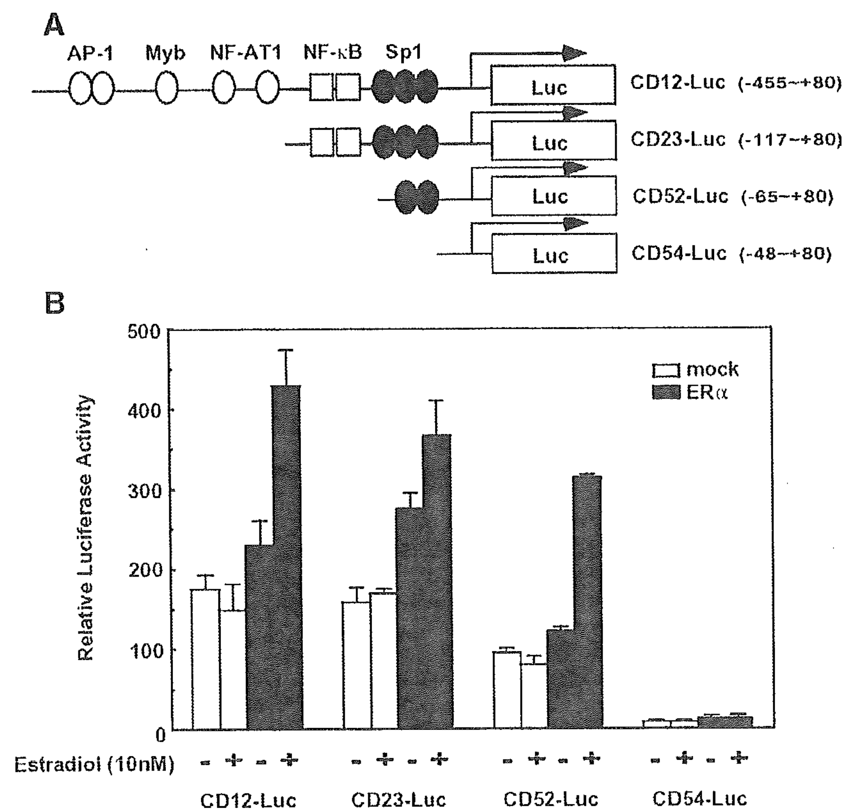


Fig. 4. Determination of crucial region for the E2-induced activation of gene expression from HIV-1 LTR. (A) Schematic diagram of HIV-1 LTR reporter genes, CD12-Luc (containing the full sequence of both U3 and R regions), CD23-Luc (containing both NF-κB and Sp1 sites), CD52-Luc (containing only the two Sp1 binding sites), and CD54-Luc (retaining no Sp1 site). (B) Each HIV-1 LTR reporter gene with or without pcDNA3-ERα plasmid was transfected into 293 cells. After 16 h of transfection, cells were incubated with or without 10 nM E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2.

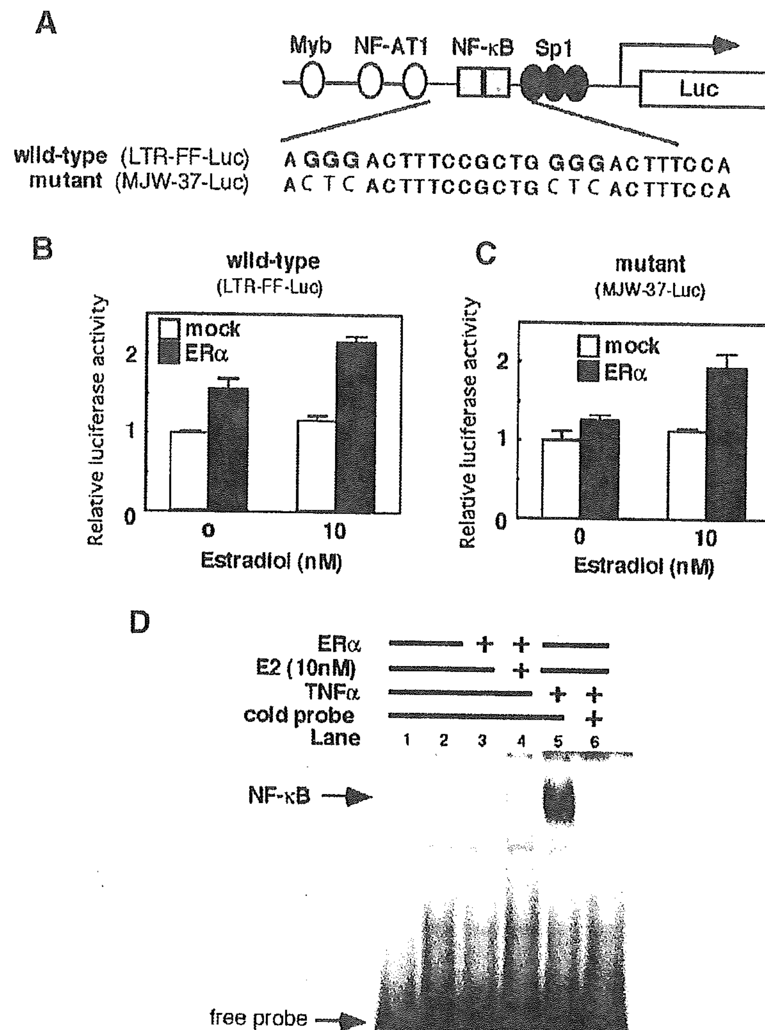


Fig. 5. NF-κB is not involved in the E2-induced activation of HIV-1 LTR. (A) Schematic diagram of LTR-FF-Luc (containing wild type NF-κB binding sites) and MJW-37-Luc (containing mutated NF-κB binding sites). (B, C) Mutation in the NF-κB site in HIV-1 LTR had no effect on the E2-induced activation of HIV-1 LTR-Luc in 293 cells. Cells were co-transfected with LTR-FF-Luc (wild type) (B) or MJW-37-Luc (mutant) (C) with pcDNA3-ERα plasmid. After 16 h of transfection, cells were incubated with or without 10 nM E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2. (D) DNA binding activity of nuclear extracts from the E2 treated 293 cells to the NF-κB site in HIV-1 LTR. Cells were transfected with or without pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated with or without 10 nM E2 or 100 U/ml TNFα. Twenty-four hours after cells were harvested and nuclear extracts were prepared and subjected to electrophoretic mobility shift assay (EMSA) analysis with ^{32}P-labeled NF-κB probe. Cells were treated with TNFα as a positive control (Lane 5). Lane 1; free labeled probe. Lane 6; 250-fold excess of cold NF-κB unlabeled was added to the labeled probe.

from the wild type HIV-1 LTR (LTR-FF-Luc (κBw)) in the presence but not in the absence of ERα, and the similar extents of stimulation by E2 were obtained even with the HIV-1 LTR promoter containing the mutated κB sites (Fig. 5C). We also assessed the effect of E2 on NF-κB DNA-binding by EMSA with κB DNA probe (Fig. 5D). Nuclear extracts obtained from 293 cells or from cells transfected with ERα stimulated with or without E2 or TNFα were allowed to bind with the end-labeled κB DNA probe. Although TNFα, used as a positive control, stimulated the NF-κB DNA-binding, E2 did not significantly augment the DNA-binding in the presence or absence of ERα. These observations indicate that NF-κB is not involved in the HIV-1 LTR activation induced by E2.