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Activation of RelA homodimers by tumour necrosis factor α: a possible transcriptional activator in human vascular endothelial cells

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In vascular endothelial cells, cytokines induce genes that are expressed in inflammatory lesions partly through the activation of transcription factor NF-κB (nuclear factor-κB). Among the members of the NF-κB/rel protein family, homodimers of the RelA subunit of NF-κB can also function as strong transactivators when expressed in cells. However, the functional role of endogenous RelA homodimers has not been clearly elucidated. We investigated whether RelA homodimers are induced in cytokine-treated vascular endothelial cells. Gel mobility-shift and supershift assays revealed that a cytokine TNFα (tumour necrosis factor α) activated both NF-κB1/RelA heterodimers and RelA homodimers that bound to a canonical κB sequence, IgκB (immunoglobulin κB), in SV40 (simian virus 40) immortalized HMEC-1 (human dermal microvascular endothelial cell line 1). In HMEC-1 and HUVEC (human umbilical-vein endothelial cells), TNFα also induced RelA homodimers that bound to the sequence 65-2κB, which specifically binds to RelA homodimers but not to NF-κB1/RelA heterodimers *in vitro*. Deoxycholic acid, a detergent that can dissociate the NF-κB–IκB complex (where IκB stands for inhibitory κB), induced the binding of the RelA homodimers to 65-2κB from the cytosolic fraction of resting HMEC-1. Furthermore, TNFα induced the transcriptional activity of a reporter gene that was driven by 65-2κB in HMEC-1. These results suggest that in addition to NF-κB1/RelA heterodimers, TNFα also induces RelA homodimers that are functionally active. Thus RelA homodimers may actively participate in cytokine regulation of gene expression in human vascular endothelial cells.

Key words: cytokine, endothelial cell, microvascular, nuclear factor-κB1 (NF-κB1), RelA homodimer, tumour necrosis factor α (TNFα).

INTRODUCTION

In a variety of cell types, cytokines regulate the expression of many viral and cellular genes through the transcriptional factor NF-κB (nuclear factor κB) (reviewed in [1–3]). NF-κB was first described as a heterodimer that consists of NF-κB1 and RelA subunits [4–6]. As an inactive form, NF-κB is present in complex with its inhibitory protein IκB (inhibitory κB) in the cytosol of cells. Cytokines and other inducers activate NF-κB by releasing it from IκB [3,7]. This process depends on the degradation of IκB and is inhibited by the antioxidant PDTC (pyrrolidine dithiocarbamate) [8–11]. The inhibition of NF-κB activation by the antioxidant suggests that reactive oxygen species play an essential role in the immediate early response of cells [12].

In vascular endothelial cells, NF-κB activates the expression of genes involved in immune and inflammatory diseases, such as cytokines, IL-6 (interleukin-6) and IL-8 [13–15]. It also activates genes coding for adhesion molecules, VCAM-1 (vascular cell adhesion molecule-1) [16,17], E-selectin [18–20] and ICAM-1 (intercellular adhesion molecule-1) [21,22]. These genes are regulated by binding of NF-κB to the consensus κB motifs, albeit with some differences in the nucleotide sequences present in their promoters. We reported previously that PDTC selectively suppresses the induction of VCAM-1 expression by cytokines, but not that of E-selectin or ICAM-1, in endothelial cells [23]. These results suggest that oxidative stress differentially regulates these genes and raises questions of whether and to what degree vascular endothelial cells utilize NF-κB (NF-κB1/RelA heterodimer) or other NF-κB-like molecules to regulate these genes selectively.

NF-κB is a member of NF-κB/rel family of transcriptional proteins [1–3]. Members of this family share the homology of 300 amino acids at N-terminal, which is essential for dimerization and DNA binding, with the proto-oncogene product c-rel and *Drosophila* morphogen dorsal [1–3]. Five cDNA of NF-κB/rel family have been cloned, including NF-κB1/p110 [24–27], NF-κB2/p100 [27,28], RelA [29,30], c-rel [31] and RelB [32]. Among these, NF-κB (NF-κB1/RelA heterodimer) has been extensively studied as a potent transcriptional factor. In addition, recent *in vitro* studies suggest that most of these members can homo- or heterodimerize with each other and positively or negatively affect transactivation of κB-controlled genes [2,29,33–36]. There is increasing evidence that the RelA homodimer is a potent transactivator for κB-controlled reporter genes by transient transfection assays, although the activation of RelA homodimer has not been extensively studied *in vivo* [29,33–36].

Recently, Narayanan et al. [37] reported that the antisense oligonucleotide against mRNA of RelA subunit, but not that against NF-κB1 subunit, inhibits adhesion of a variety of cells including primary cultured endothelial cells. These results suggest that RelA plays an essential role in endothelial cell adhesion. In T-cell line Jurkart, the cytokine IL-8, which is also produced in endothelial cells, was shown to utilize the RelA-homodimer-like protein to drive the promoter activity through the κB consensus site [38]. Based on these studies, one can speculate that in endothelial cells

Abbreviations used: CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; DOC, deoxycholic acid; HMEC-1, human dermal microvascular endothelial cell line 1; HUVEC, human umbilical-vein endothelial cells; IκB, inhibitory κB; IgκB, immunoglobulin κB; IL, interleukin; NF-κB, nuclear factor-κB; NP40, Nonidet P40; TNFα, tumour necrosis factor α; TRE, PMA ('TPA')-responsive element.

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the RelA homodimer or homodimer-like protein may actively participate in gene regulation.

Recently, Kunsch et al. [39] characterized κB sequences that specifically bind to RelA homodimer but not to NF-κB1 homodimer or to NF-κB (NF-κB1/RelA heterodimer). These sequences were isolated from a pool of randomly prepared κB sequences using purified RelA protein as a probe [39]. This *in vitro* result prompted us to examine the possible involvement of RelA homodimer in cytokine-treated vascular endothelial cells with the RelA homodimer-specific sequence.

In the present study, we examined whether RelA homodimers were induced in cytokine-treated endothelial cells, HMEC-1 (human dermal microvascular endothelial cell line 1) and HUVEC (human umbilical-vein endothelial cells). With a canonical κB sequence, Igκ B (immunoglobulin κB), and a RelA homodimer-specific sequence, 65-2κB, as probes, we performed gel mobility and supershift assays using antibodies raised against members of the NF-κB/rel protein family. The results showed that in addition to NF-κB (NF-κB1/RelA heterodimers), TNFα (tumour necrosis factor α) also induced RelA homodimers in the vascular endothelial cells. Dissociation of cytosolic protein complexes with DOC (deoxycholic acid) suggested that, like NF-κB1/RelA heterodimers, the nuclear translocation of RelA homodimers is also regulated. Importantly, in HMEC-1, TNFα induced the transcriptional activity of a reporter gene that was driven by the RelA homodimer specific enhancer element. Thus homodimers of RelA may also participate in TNFα regulation of NF-κB-dependent gene expression in endothelial cells.

EXPERIMENTAL

Cell culture

HMEC-1 was immortalized with SV40 (simian virus 40) large T-antigen [40]. The established cell line, immortalized human dermal microvascular endothelial cells, termed CDC/EC, was generously provided by E.W. Ades (Centers for Disease Control, Atlanta, GA, U.S.A.) and maintained in EBM medium obtained from Clonetics (San Diego, CA, U.S.A.) supplemented with 15 % (v/v) FBS (fetal bovine serum; Atlanta Biological, Norcross, GA, U.S.A.), 10 ng/ml epidermal growth factor (Intergen, Purchase, NY, U.S.A.), and 1 μg/ml cortisol (Sigma, St. Louis, MO, U.S.A.). HUVEC were purchased from Clonetics and were cultured in M199 medium (Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 20 % FBS, 16 units/ml heparin (ESI Pharmaceuticals, Cherry Hill, NJ, U.S.A.), 50 mg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA, U.S.A.) and 25 mM Hepes buffer. Both cell types were cultured in a humidified atmosphere of 5 % CO_2 in air. The medium for both cell types contained 2 mM L-glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. HUVEC were grown on tissue culture plates coated with 0.1 % gelatin and were used within the first six passages. Human recombinant TNFα was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). All other chemicals were of reagent grade.

Nuclear extract and gel mobility-shift assays

Confluent HMEC-1 was exposed to TNFα (100 units/ml) for 1 h, and nuclear proteins were purified by a modification of the method of Dignam et al. [41]. Briefly, after washing with PBS, cells were centrifuged and the cell pellet was suspended in 500 μl of buffer A [10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM DTT (dithiothreitol)]. After re-centrifugation, the cells were resuspended in 80 μl of buffer A containing 0.1 % NP40 (Nonidet P40) by gentle pipetting. After incubating for 10 min at 4 °C, the homogenate was centrifuged and the supernatant was stored at − 70 °C as a cytosolic extract. Then the nuclear pellet was washed once with buffer A and resuspended in 50 μl of buffer C (20 mM Hepes, pH 7.9, 25 %, v/v, glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 1 mM DTT). This suspension was incubated for 30 min at 4 °C followed by centrifugation at 20000 ***g*** for 10 min. The resulting supernatant was stored at −70 °C as a nuclear extract. Protein concentrations were determined by the Bradford method [42]. To minimize proteolysis, all buffers contained 1.0 mM PMSF, 10 μg/ml antipain, 10 μg/ml leupeptin and 0.023 TIU/ml aprotinin.

The sequences of oligonucleotides used for probes are as follows (κB sequences are underlined) [39]: 65-2κB: 5′-GTACC-GGAAATTCCGGGCTCGAGATCCTATG-3′; and Igκ B: 5′-GT-AGGGGACTTTCCGAGCTCGAGATCCTATG-3′. The dotted underlined sequences represent a tail sequence added to serve as a template for synthesis of the double-stranded DNA. Other oligonucleotide sequences used for unlabelled competition are as follows: mutated Igκ B (mut Igκ B): 5′-GTAG**CTC**AAT**C**TCCG-AGCTCGAGATCCTATG-3′ (boldface letters show the mutated sequences); AP-1 (consensus sequence is underlined): 5′-AGC-TTAAAAAAGCATGAGTCAGACACCTGGCTCGAGATCCT-ATG-3′. Radiolabelled double-stranded DNA was made by annealing an oligonucleotide complementary to the 3′-end of the sequences listed above (dotted underlined) (5′-CATAGG-ATCTCGAGC-3′) and extending with DNA polymerase and 50 μCi of ^{32}P-dCTP and unlabelled dATP, dGTP and dTTP followed by the addition of 500 μM unlabelled dCTP to complete the reaction. Unincorporated nucleotides were removed by column chromatography over a Sephadex G-50 column. Unlabelled double-stranded DNA was prepared in the same way, by replacing radiolabelled dCTP with unlabelled dCTP.

The DNA binding reaction was performed at 30 °C for 15 min in a volume of 20 μl, which contained 2 μg of nuclear extract, 225 μg/ml BSA, 100000 c.p.m. ^{32}P-labelled probe (∼1 ng), 0.1 mg/ml poly(dI-dC) and 15 μl of binding buffer (12 mM Hepes, pH 7.9, 4 mM Tris, 60 mM KCl, 1 mM EDTA, 12 % glycerol, 1 mM DTT and 1 mM PMSF) with or without 100-fold molar excess of unlabelled competitor. Samples were subjected to electrophoresis on native 4 % (w/v) polyacrylamide gels in 1 × Tris/glycine buffer.

For dissociation experiments, 5 μg of cytosolic extract or 2 μg of nuclear extract in binding buffer was treated with 1.0 % DOC followed by 1.2 % (v/v) NP40, and incubated with a radiolabelled probe.

For the supershift assay, the antibody was added to each reaction mixture before adding the radiolabelled probe. The mixture was then incubated for 15 min at room temperature (26 °C). Anti-RelA, c-rel and RelB antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-NF-κB1 antibody and *in vitro* translated NF-κB1, NF-κB1/RelA and RelA were gifts from C. Kunsch and C. Rosen (Human Genome Sciences, Rockville, MD, U.S.A.).

CAT (chloramphenicol acetyltransferase) assays

At 1 day before transfection, HMEC-1 was split at a ratio to yield 80 % confluence. Transfection was performed by the calcium phosphate co-precipitation technique using 10 μg of reporter plasmids for 18 h. After a 2 h recovery period, cells were either left untreated or treated with TNFα for 20 h. For co-transfection with expression vectors, each expression vector was co-transfected with reporter genes for 40 h. The amount of expression vector is indicated in Figure legends. Promoterless plasmid poLUC [43] was used for adjusting the amount of transfected

DNA. Following transfection, cells were harvested and extracts prepared using three rapid freeze–thaw cycles in 0.25 M Tris (pH 8.0). Protein content was determined using the Bradford technique [42]. Same amounts of proteins were assayed for CAT activity according to the method described in [44,45]. Acetylated and unacetylated forms of chloramphenicol were separated by TLC. Each assay was performed in duplicate and in at least two separate experiments.

Expression vectors and CAT reporter genes

The eukaryotic expression vectors CMV-NF-κB1 (also called p50), CMV-RelA (also called p65) and CMV-NF-κB1/RelA (also called p50/65) contain the respective cDNAs cloned between a CMV (cytomegalovirus) promoter β-globin intron and SV40 poly(A) signal [35]. CMV-NF-κB1/RelA encodes chimaeric protein consisting of a DNA-binding domain of NF-κB1 (amino acids 1–370) and transactivation domain of RelA (amino acids 309–550). The reporter plasmids p(IgκB)4CAT and p(65-2κB)-4CAT contain four tandem copies of the respective κB DNA sequences cloned upstream of the HIV-1 LTR (long terminal repeat) and fused to the coding region of the bacterial CAT gene [39]. These vectors were gifts from C. Kunsch and C. Rosen.

RESULTS

TNF-activated HMEC-1 NF-κB proteins have differential affinity for IgκB and 65-2κB

The canonical NF-κB motif, IgκB (5′-GGGACTTTCC-3′), present in the enhancer region of immunoglobulin light chain gene and in LTR of HIV has been well characterized to bind to NF-κB (NF-κB1/RelA heterodimer) [46,47]. By gel mobility-shift assays, IgκB made two complexes with nuclear proteins, fast mobility (F) and slow mobility (S), of TNF-activated HMEC-1 (Figure 1A, lane 2). The intensity of complex F was severalfold greater intensity than that of complex S. The nuclear proteins from unstimulated cells formed a minor complex that had mobility similar to that of the major complex F (Figure 1A, lane 1). The competition studies with unlabelled IgκB (100-fold excess) suggested that the complexes were specific (Figure 1A, lane 3). Neither the mutated IgκB (Figure 1A, lane 4) nor the unrelated sequence TRE [PMA ('TPA')-responsive element; Figure 1A, lane 6] competed for the NF-κB complexes. The unlabelled 65-2κB, which specifically binds to RelA homodimers, completely competed for proteins in the complex S and partially for the major complex F. This suggests that 65-2κB has greater affinity for proteins associated with the S complex than those associated with F complex.

We next examined whether TNFα could activate 65-2κB-binding nuclear proteins in HMEC-1. 65-2κB, core sequence 5′-CGGAATTTCC-3′, was isolated from a randomly prepared pool of sequences and was characterized to bind to RelA homodimers and c-rel protein but not to NF-κB1 homodimer or to NF-κB1/RelA heterodimer *in vitro* [39]. As shown in Figure 1(B), 65-2κB also formed two complexes, F and S, with the TNFα-treated HMEC-1 nuclear proteins (Figure 1B, lane 2). The mobility of these complexes was similar to that formed by IgκB. However, in this situation, the intensity of complex S was severalfold greater than that of complex F, which is the reverse of that observed with IgκB. The control nuclear extract formed a minor complex that had mobility similar to that of complex S, opposite to that observed with IgκB (Figure 1B, lane 1). The competition studies with unlabelled 65-2κB (100-fold excess) suggested that both complexes were specific (Figure 1B, lane 3). The unlabelled IgκB (100-fold excess) also competed for both

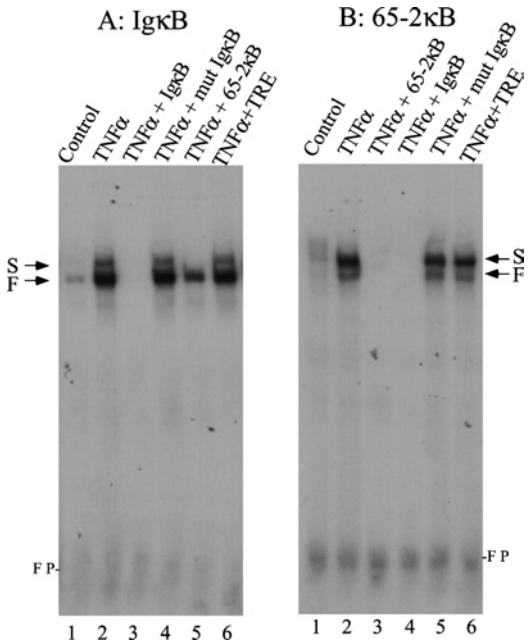

Figure 1 TNFα-mediated activation of DNA-binding proteins to IgκB and 65-2κB sequences in HMEC-1

Nuclear extract was prepared from HMEC-1 either untreated (lane 1) or treated with 100 units/ml TNFα for 1 h (lanes 2–6). Unlabelled IgκB (100-fold molar excess; **A**, lane 3; **B**, lane 4), mutated IgκB (**A**, lane 4; **B**, lane 5), 65-2κB (**A**, lane 5; **B**, lane 3) and TRE (lanes 6) probes were added to the respective lanes just before incubating with a ^{32}P-labelled probe (**A**, IgκB; **B**, 65-2κB). Then the reaction mixture was incubated at 30 °C for 15 min. The samples were subjected to electrophoresis on native 4 % polyacrylamide gels in 1 × Tris/glycine buffer. Slow (S) and fast (F) moving complexes are shown by arrows. FP stands for the free probe. Data are representative of two experiments.

S and F complexes (Figure 1B, lane 4). This suggests that the IgκB can also bind to the 65-2κB-binding proteins. Neither the mutated IgκB (Figure 1B, lane 5) nor the unrelated sequence TRE (Figure 1B, lane 6) competed for the binding. These studies suggest that the NF-κB complexes F and S bind to both IgκB and 65-2κB but with different affinities.

TNFα activates RelA homodimers in addition to NF-κB1/RelA heterodimers in HMEC-1

Both F and S complexes were further characterized by supershift assays using specific polyclonal antibodies raised against NF-κB1, RelA, c-rel and RelB proteins. The complex F formed by IgκB (Figure 2A, lane 1) was completely shifted by Ab-NF-κB1 (where Ab stands for antibody), while the S complex was not affected (Figure 2A, lane 2). When Ab-RelA was used alone (Figure 2A, lane 3) or in combination with Ab-NF-κB1 (Figure 2A, lane 4) both F and S complexes were shifted. Neither Ab-c-rel (Figure 2A, lane 5) nor Ab-RelB (lane 6) shifted the complex. These results suggest that IgκB mainly associates with NF-κB1/RelA heterodimers (complex F), although it can also associate with RelA- or RelA-homodimer-like protein(s) (complex S).

As observed previously, 65-2κB formed a major complex (S) and a minor complex (F) with the nuclear proteins of TNFα-treated HMEC-1 (Figure 2B, lane 1). The Ab-NF-κB1 did not shift the S complex (Figure 2B, lane 2), while this complex was completely shifted by Ab-RelA (Figure 2B, lane 3). When Ab-NF-κB1 and Ab-RelA were used in combination, both S and F complexes were completely shifted (Figure 2B, lane 4). Neither Ab-c-rel nor Ab-RelB shifted the complexes F and S (Figure 2B, lanes 5 and 6). These results suggest that 65-2κB

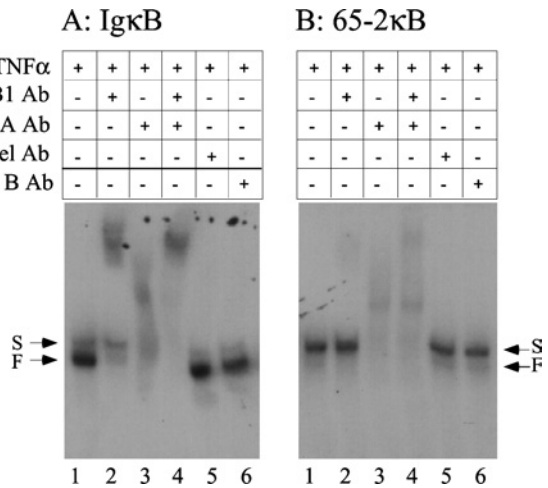

Figure 2 Supershift assay of TNFα-treated HMEC-1 nuclear extract

Nuclear extract from TNFα-treated HMEC-1 was preincubated without (lane 1) or with anti-NF-κB1 (lane 2), anti-RelA (lane 3), both anti-NF-κB1 and RelA (lane 4), anti-c-rel (lane 5) and anti-RelB (lane 6) antibodies for 15 min at room temperature and then incubated with the ^{32}P-labelled probe (A, IgκB; B, 65-2κB) for 15 min at 30 °C. The samples were subjected to electrophoresis on native 4 % polyacrylamide gels in 1 × Tris/glycine buffer. Slow (S) and fast (F) moving complexes are shown by arrows. Data are representative of two experiments.

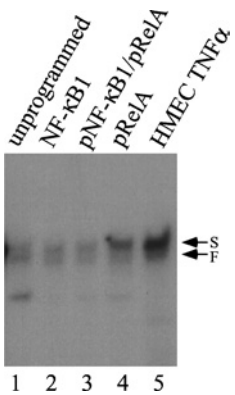

Figure 3 Both *in vitro* translated RelA and the nuclear extracts of TNF-activated HMEC-1 made complexes with 65-2κB that had the same mobility

To compare the mobility in gel shift assay between *in vitro* translated proteins of NF-κB1 homodimer (lane 2), NF-κB1/RelA heterodimer (lane 3) and RelA homodimer (lane 4) with the unprogrammed protein as control (lane 1) and the binding complex of TNFα-treated HMEC-1 nuclear extract (lane 5), samples were incubated with ^{32}P-labelled 65-2κB probe at 30 °C for 15 min and subjected to electrophoresis on native 4 % polyacrylamide gels in 1 × Tris/glycine buffer. Data are representative of two experiments.

predominantly associates with RelA- or RelA-homodimer-like proteins (S complex), although it can also associate with NF-κB1/RelA heterodimers with low affinity.

65-2κB made complexes with *in vitro* translated RelA and nuclear proteins of TNF-activated HMEC-1 that had the same mobility

As shown in Figure 3, lane 4, the *in vitro* translated RelA made a complex with 65-2κB that had mobility similar to that of the complex S formed by the nuclear proteins of TNFα-activated HMEC-1 (lane 5). The *in vitro* translated NF-κB1 (lane 2) or NF-κB1 + RelA (lane 3) or unprogrammed control (lane 1) did not make any specific complex with 65-2κB. Consistent with previous studies [39], these results suggest that 65-2κB binds to RelA homodimers and that the homodimers are present in the nuclear extracts of TNF-activated HMEC-1.

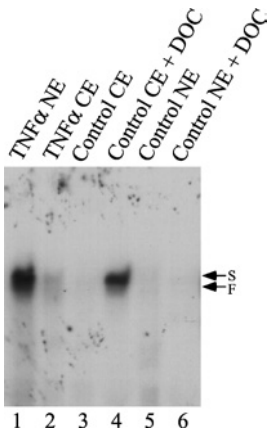

Figure 4 A dissociating agent induces the binding of RelA homodimers from the cytosolic extract of untreated HMEC-1

Cytosolic (lanes 2–4) or nuclear (lanes 1, 5 and 6) extract of TNFα-treated (lanes 1 and 2) or untreated (lanes 3–6) HMEC-1 was either untreated (lanes 1–3 and 5) or treated (lanes 4 and 6) with 1.2 % (v/v) DOC followed by 1 % NP40, then incubated with ^{32}P-labelled 65-2κB probe at 30 °C for 15 min and subjected to electrophoresis on native 4 % polyacrylamide gel in 1 × Tris/glycine buffer. Data are representative of three experiments.

DOC activates RelA homodimers from the cytosolic extract of untreated HMEC-1

NF-κB (NF-κB1/RelA heterodimers) is retained in the cytosol of cells by its inhibitory protein IκB. Cytokines or other activators translocate NF-κB into the nucleus through a mechanism that involves phosphorylation and proteolytic degradation of IκB. DOC can also dissociate NF-κB from IκB that can efficiently bind to κB consensus sequences [1–3]. Using 65-2κB as a probe, we demonstrate that DOC can also dissociate RelA homodimers from the cytosol of unstimulated HMEC-1 (compare lanes 3 and 4 of Figure 4). The mobility of these homodimers was comparable with that obtained through stimulation of HMEC-1 with TNF (compare lanes 1 and 4). Both cytosolic extracts of TNFα-treated (Figure 4, lane 2) and control (Figure 4, lane 3) cells did not show any significant amount of the binding complex. DOC treatment of nuclear extracts from unstimulated cells also did not show any 65-2κB-binding proteins (compare lanes 5 and 6 of Figure 4). These results suggest that like NF-κB1/RelA heterodimers, RelA homodimers are also retained in the cytosol by IκB or IκB-like proteins and that TNFα activates RelA homodimers through a mechanism similar to that used for NF-κB (NF-κB1/RelA heterodimers).

TNFα induces RelA homodimers in the primary cultures of endothelial cells

As shown in Figure 5, TNFα also activated 65-2κB-binding proteins in HUVEC (compare lanes 1 and 2). The binding proteins made a major complex S and a minor complex F. The Ab-RelA shifted both complexes F and S (lane 4), while the Ab-NF-κB1 shifted only the minor complex F without affecting the major complex S. These results suggest that, in addition to NF-κB1/RelA heterodimers, TNFα also activates RelA or RelA-homodimer-like factors in primary cultured endothelial cells.

TNFα activates the RelA homodimer specific CAT reporter gene in HMEC-1

To determine whether RelA homodimers were transcriptionally active, we performed CAT assays. We used p(65-2κB)4CAT, a CAT reporter gene attached to four tandem repeats of the 65-2κB,

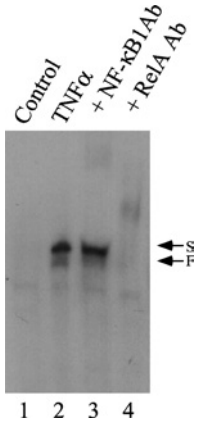

Figure 5 TNFα activates RelA homodimer in primary cultures of endothelial cells

The nuclear extract was prepared from HUVEC either untreated (lane 1) or treated with 100 units/ml TNFα for 1 h (lane 2). Then the nuclear extract of TNFα-treated HUVEC was preincubated with anti-NF-κB1 (lane 3) or anti-RelA (lane 4) antibody for 15 min at room temperature. The samples were then incubated with ^{32}P-labelled 65-2κB probe for 15 min at 30 °C and subjected to electrophoresis on native 4 % polyacrylamide gels in 1 × Tris/glycine buffer. Slow (S) and fast (F) moving complexes are shown by arrows. Data are representative of three experiments.

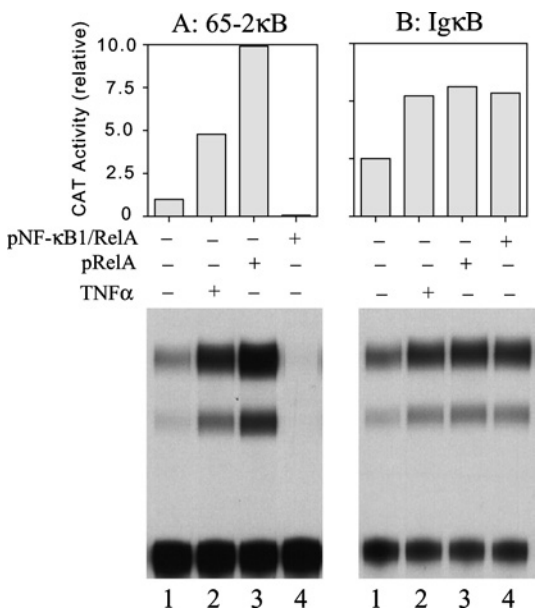

Figure 6 TNFα activates the RelA homodimer-driven CAT reporter gene in HMEC-1

HMEC-1 was transfected either with 10 μg of p(65-2κB)4CAT (**A**) or p(IgκB)4CAT (**B**). The transfected cells were either untreated (lane 1) or treated (lane 2) with 200 units/ml TNFα for 16 h. The cells were also co-transfected either with 2 μg of expression vectors encoding RelA (CMV-RelA) (lane 3) or chimaeric protein NF-κB1/RelA (CMV-NF-κB1/RelA) (lane 4). Data are representative of two experiments performed in duplicate with similar results.

to assess the activity of RelA homodimers. As a control, we used p(IgκB)4CAT, a CAT reporter gene attached to four tandem repeats of IgκB. In transfection studies, TNFα increased the activity of p(65-2κB)4CAT severalfold (Figure 6A, lane 2) above the basal level (Figure 6A, lane 1) in HMEC-1. Similarly, co-transfection of CMV-RelA, an expression vector encoding the RelA subunit of NF-κB, transactivated the activity of p(65-2κB)4CAT several-fold above the basal level (Figure 6A, lane 3).

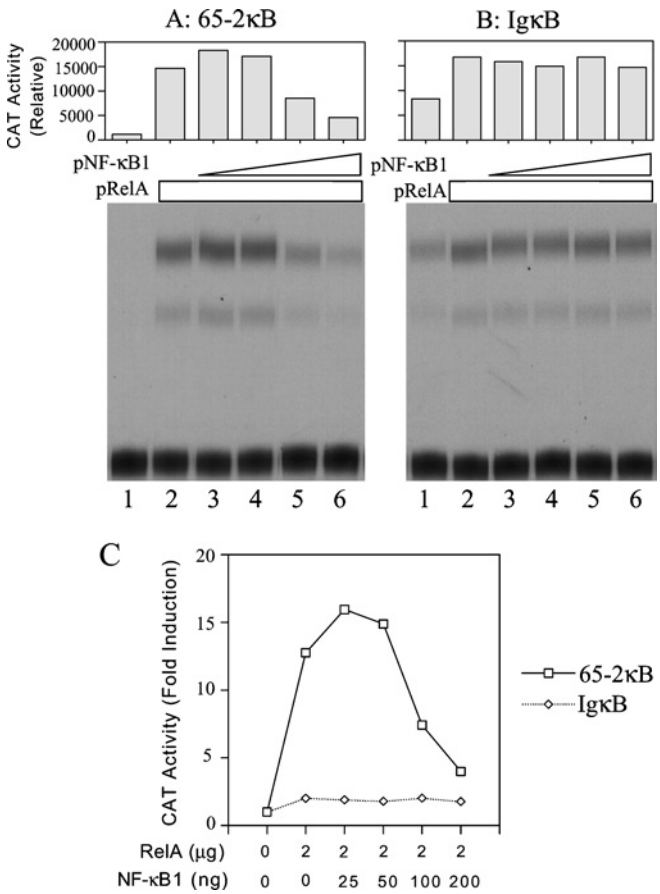

Figure 7 RelA homodimers but not the reconstituted NF-κB1/RelA heterodimer activates the p(65-2κB)4CAT

HMEC-1 was transfected either with 10 μg of p(65-2κB)4CAT (**A**) or p(IgκB)4CAT (**B**) along with a constant amount of CMV-RelA (2 μg) and increasing amount of CMV-NF-κB1. In both the Figures, lane 1 shows the basal levels of each CAT reporter gene. Lanes 2–6 had 2 μg of CMV-RelA and 0, 25, 50, 100 and 200 ng of CMV-NF-κB1. The total amount of transfected DNA was kept constant by using promoterless plasmid poLUC. (**C**) The data from (**A**, **B**) are expressed graphically and values are presented as the means for two experiments performed in duplicate with similar results.

Co-transfection of CMV-NF-κB1/RelA, an expression vector encoding chimaeric protein containing DNA-binding domain of NF-κB1 and the transactivation domain of RelA, a functional mimic of NF-κB1/RelA heterodimers, did not transactivate p(65-2κB)4CAT (Figure 6A, lane 4). Under the same conditions, co-transfection of CMV-NF-κB1/RelA into HMEC-1 transactivated p(IgκB)4CAT (Figure 6B, lane 4). As expected, both TNFα treatment (Figure 6B, lane 2) and CMV-RelA co-transfection (Figure 6B, lane 3) also increased the activity of p(IgκB)4CAT in HMEC-1 (Figure 6B, lane 1). These results suggest that the endogenous RelA homodimers present in TNFα-stimulated HMEC-1 is functionally active.

To confirm the functional specificity of the p(65-2κB)4CAT for RelA homodimers, we performed the reconstitution experiment by co-transfecting the reporter gene with a constant amount of CMV-RelA and increasing amounts of CMV-NF-κB1. This method was used to form the NF-κB1/RelA heterodimers in cells and was shown to transactivate p(IgκB)4CAT [33,36,48]. As shown in Figure 7, co-transfection of 2 μg of CMV-RelA transactivated both p(65-2κB)4CAT and p(IgκB)4CAT (Figures 7A and 7B, lane 2, and Figure 7C) above the basal levels (Figures 7A and 7B, lane 1). Increasing amounts of co-transfected

CMV-NF-κB1 suppressed the RelA transactivation of p(65-2κB)4CAT (Figure 7A, lanes 3–6, and Figure 7C). Under the same conditions, consistent with previous studies [38,39], the RelA transactivation of p(Igκ B)4CAT could not be inhibited (Figure 7B, lanes 3–6). These results suggest that p(65-2κB)4CAT is specifically transactivated by RelA homodimers but not NF-κB1/RelA heterodimers and hence strongly suggest that TNFα activates p(65-2κB)4CAT by inducing RelA homodimers in HMEC-1.

DISCUSSION

We provide evidence that, in addition to NF-κB (NF-κB1/RelA heterodimers), RelA-homodimer-like factors are present in the cytosol of both immortalized and primary vascular endothelial cells. Like NF-κB1/RelA heterodimers, TNFα also stimulates the translocation of RelA-homodimer-like factors to the nucleus. These homodimers were transcriptionally active as judged by the activity of RelA homodimer-driven reporter gene, p(65-2κB)4CAT, in HMEC-1. Our results suggest that in addition to NF-κB1/RelA heterodimers, RelA-homodimer-like factors may also play a significant role in regulating the expression of several genes containing RelA homodimer-specific or canonical κB motif in HUVEC.

Previous studies have shown that the treatment of the cytosolic extract from unstimulated cells with dissociating agent DOC induces the binding of NF-κB (NF-κB1/RelA heterodimers) to Igκ B by dissociating the NF-κB–Iκ B complex [8,49]. We demonstrate that the treatment of the cytosolic extract from unstimulated endothelial cells with DOC induces the binding of the RelA homodimers to the 65-2κB sequence. This suggests that RelA homodimers are present in the cytosolic fraction of untreated endothelial cells possibly in complex with an inhibitory protein like Iκ B, which restricts them in the cytoplasm. Consistent with these results, an isoform of Iκ B, Iκ Bε, has been shown to function predominantly in the cytoplasm to sequester RelA homodimers [50]. Since Iκ Bepsilon is expressed in various cell types, it is possible that this inhibitory protein also retains RelA homodimers in the cytosol of resting endothelial cells.

Using gel mobility-shift assay, we showed both Igκ B (5′-GGGACTTTCC-3) and 65-2κB sequences make two complexes, slow migrating (S) and fast migrating (F), with nuclear proteins of TNFα-treated endothelial cells. The predominant form of the complex formed by Igκ B was ‘F’ and that formed by 65-2κB was ‘S’. The S complex was characterized to contain RelA homodimers or an RelA-homodimer-like protein since this complex was supershifted by anti-RelA antibody but not by antibodies against NF-κB1, c-rel or RelB. The involvement of p49/NF-κB2 in the S complex is unlikely because the mobility of NF-κB2/RelA heterodimer is similar to that of NF-κB1/RelA heterodimers, which was characterized to be an F complex [51,52]. That the complex S contained RelA homodimers was further supported by similar mobility of the complex made by 65-2κB with RelA homodimers prepared from *in vitro* translated RelA. Importantly, the 65-2κB probe also formed complex S with nuclear proteins of primary cultured HUVEC. This complex was also characterized to contain RelA homodimers. Like NF-κB1/RelA heterodimers, RelA homodimers appeared only after activation of the cells with TNFα. Thus TNFα activates both RelA homodimers as well as NF-κB1/RelA heterodimers in endothelial cells through processing subtypes of Iκ B that retain these dimers in the cytosol. Recent studies have suggested that RelA homodimers are activated through a mechanism that is distinct from that which activates NF-κB1/RelA heterodimers. ATP stimulation of the cells of macrophage lineage activated solely RelA homodimers, whereas lipopolysaccharide induced prototypical NF-κB/NF-κB1 homodimers and RelA/NF-κB1 heterodimers [53]. It is possible that various stimuli differentially activate RelA homodimers and NF-κB1/RelA heterodimers in endothelial cells, providing basis for the differential regulation of NF-κB-dependent genes. Further studies will determine which stimuli preferentially activate RelA homodimers.

Transient transfection studies revealed that the co-transfection of RelA expression vector that presumably forms RelA homodimers in cells potently activates the κB-controlled reporter gene activity [29,33–36]. Our studies suggest that endogenous RelA homodimers could also play a significant role in regulating inflammatory responses in endothelial cells. First, the amount of RelA homodimers activated by TNFα is not less than that of NF-κB1/RelA heterodimers. Secondly, the RelA homodimers were shown to activate transcription through both the canonical κB as well as the RelA homodimer-specific 65-2κB sites. These results are consistent with previous studies which suggest that RelA homodimers can recognize DNA targets consisting of only one cognate half-site containing four base-pairs (5′-GGAA-3′) and differences in the other half-site provide variations in confirmation and affinity of the complex [54,55]. Crystal structure of NF-κB1/RelA heterodimer bound to DNA has revealed a 5 bp 5′ subsite for NF-κB1, and a 4 bp 3′ subsite for RelA. These studies suggest that RelA homodimers could activate transcription through κB consensus sequences that may have weak affinity for NF-κB1/RelA heterodimers.

Co-transfection of NF-κB1 inhibited the RelA-mediated transactivation of 65-2κB-dependent reporter gene probably by making NF-κB1/RelA heterodimers. These studies imply that the presence of NF-κB1/RelA heterodimers could affect the RelA homodimer-mediated transcription.

The 65-2κB was originally characterized as an RelA homodimer-specific sequence [39]. In the present study, however, the 65-2κB sequence also bound to the NF-κB1/RelA heterodimer activated *in vivo* although with weak affinity. However, the NF-κB1/RelA heterodimer was unable to transactivate a promoter driven by 65-2κB. Although NF-κB1/RelA heterodimers can bind to 65-2κB with weak affinity, they cannot transactivate promoters through these sequences.

Using two different approaches, we demonstrate that a 65-2κB-dependent promoter construct, p(65-2κB)4 CAT, is specifically driven by RelA homodimers but not by NF-κB1/RelA heterodimers. In the first approach, co-transfection of the RelA expression vector, CMV-RelA, strongly transactivated p(65-2κB)4CAT, whereas under the same conditions the co-transfection of chimaeric protein NF-κB1/RelA expression vector, CMV-NF-κB1/RelA, did not. In the second approach, the reconstitution of NF-κB1/RelA heterodimers formed by co-transfection of constant amounts of CMV-RelA and increasing amounts of CMV-NF-κB1 also did not activate the p(65-2κB)4CAT. As observed previously, these results propose the functional specificity of the 65-2κB sequence for RelA homodimer [39]. So, by using p(65-2κB)4 CAT, an RelA homodimer-specific reporter gene, we could conclude that TNFα activates functional RelA homodimers in HMEC-1.

In conclusion, we have demonstrated the functional activation of the RelA homodimers by the cytokine TNFα in HUVEC. This proposes the fascinating possibility that RelA homodimers could participate in inflammatory responses by activating genes containing κB motifs. It would be interesting to find out whether RelA homodimers play any distinct role in endothelial cell apoptosis versus inflammatory response.

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REFERENCES

1 Grimm, S. and Baeuerle, P. A. (1993) The inducible transcription factor NF-kappa B: structure-function relationship of its protein subunits. Biochem. J. **290**, 297–308
2 Grilli, M., Chiu, J. J. and Lenardo, M. J. (1993) NF-kappa B and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143**, 1–62
3 Baeuerle, P. A. (1991) The inducible transcription activator NF-kappa B: regulation by distinct protein subunits. Biochim. Biophys. Acta **1072**, 63–80
4 Lenardo, M. J., Kuang, A., Gifford, A. and Baltimore, D. (1988) NF-kappa B protein purification from bovine spleen: nucleotide stimulation and binding site specificity. Proc. Natl. Acad. Sci. U.S.A. **85**, 8825–8829
5 Kawakami, K., Scheidereit, C. and Roeder, R. G. (1988) Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-kappa B) that activates transcription from a human immunodeficiency virus type 1 promoter *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **85**, 4700–4704
6 Baeuerle, P. A. and Baltimore, D. (1989) A 65-kappaD subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. Genes Dev. **3**, 1689–1698
7 Baeuerle, P. A. and Baltimore, D. (1988) I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science **242**, 540–546
8 Beg, A. A., Finco, T. S., Nantermet, P. V. and Baldwin, Jr, A. S. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol. Cell. Biol. **13**, 3301–3310
9 Brown, K., Park, S., Kanno, T., Franzoso, G. and Siebenlist, U. (1993) Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. Proc. Natl. Acad. Sci. U.S.A. **90**, 2532–2536
10 Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y. and Baeuerle, P. A. (1993) Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature (London) **365**, 182–185
11 Sun, S. C., Ganchi, P. A., Ballard, D. W. and Greene, W. C. (1993) NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science **259**, 1912–1915
12 Schreck, R., Meier, B., Mannel, D. N., Droge, W. and Baeuerle, P. A. (1992) Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J. Exp. Med. **175**, 1181–1194
13 Mantovani, A. and Dejana, E. (1989) Cytokines as communication signals between leukocytes and endothelial cells. Immunol. Today **10**, 370–375
14 Libermann, T. A. and Baltimore, D. (1990) Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol. Cell. Biol. **10**, 2327–2334
15 Mukaida, N., Mahe, Y. and Matsushima, K. (1990) Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. J. Biol. Chem. **265**, 21128–21133
16 Iademarco, M. F., McQuillan, J. J., Rosen, G. D. and Dean, D. C. (1992) Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). J. Biol. Chem. **267**, 16323–16329
17 Neish, A. S., Williams, A. J., Palmer, H. J., Whitley, M. Z. and Collins, T. (1992) Functional analysis of the human vascular cell adhesion molecule 1 promoter. J. Exp. Med. **176**, 1583–1593
18 Collins, T., Williams, A., Johnston, G. I., Kim, J., Eddy, R., Shows, T., Gimbrone, Jr, M. A. and Bevilacqua, M. P. (1991) Structure and chromosomal location of the gene for endothelial-leukocyte adhesion molecule 1. J. Biol. Chem. **266**, 2466–2473
19 Montgomery, K. F., Osborn, L., Hession, C., Tizard, R., Goff, D., Vassallo, C., Tarr, P. I., Bomsztyk, K., Lobb, R., Harlan, J. M. et al. (1991) Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Proc. Natl. Acad. Sci. U.S.A. **88**, 6523–6527
20 Whelan, J., Ghersa, P., Hooft van Huijsduijnen, R., Gray, J., Chandra, G., Talabot, F. and DeLamarter, J. F. (1991) An NF kappa B-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Nucleic Acids Res. **19**, 2645–2653
21 Voraberger, G., Schafer, R. and Stratowa, C. (1991) Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5′-regulatory region. Induction by cytokines and phorbol ester. J. Immunol. **147**, 2777–2786
22 Degitz, K., Li, L. J. and Caughman, S. W. (1991) Cloning and characterization of the 5′-transcriptional regulatory region of the human intercellular adhesion molecule 1 gene. J. Biol. Chem. **266**, 14024–14030
23 Marui, N., Offermann, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W. and Medford, R. M. (1993) Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant sensitive mechanism in human vascular endothelial cells. J. Clin. Invest. **92**, 1866–1874
24 Meyer, R., Hatada, E. N., Hohmann, H. P., Haiker, M., Bartsch, C., Rothlisberger, U., Lahm, H. W., Schlaeger, E. J., van Loon, A. P. and Scheidereit, C. (1991) Cloning of the DNA-binding subunit of human nuclear factor kappa B: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor alpha. Proc. Natl. Acad. Sci. U.S.A. **88**, 966–970
25 Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A. and Israel, A. (1990) The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product. Cell (Cambridge, Mass.) **62**, 1007–1018
26 Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P. and Baltimore, D. (1990) Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal. Cell (Cambridge, Mass.) **62**, 1019–1029
27 Schmid, R. M., Perkins, N. D., Duckett, C. S., Andrews, P. C. and Nabel, G. J. (1991) Cloning of an NF-kappa B subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352**, 733–736
28 Bours, V., Villalobos, J., Burd, P. R., Kelly, K. and Siebenlist, U. (1990) Cloning of a mitogen-inducible gene encoding a kappa B DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature (London) **348**, 76–80
29 Nolan, G. P., Ghosh, S., Liou, H. C., Tempst, P. and Baltimore, D. (1991) DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide. Cell (Cambridge, Mass.) **64**, 961–969
30 Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C. H., Maher, M., Baeuerle, P. A. and Rosen, C. A. (1991) Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-kappa B. Science **251**, 1490–1493
31 Brownell, E., Mittereder, N. and Rice, N. R. (1989) A human rel proto-oncogene cDNA containing an Alu fragment as a potential coding exon. Oncogene **4**, 935–942
32 Ryseck, R. P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P. and Bravo, R. (1992) RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B. Mol. Cell. Biol. **12**, 674–684
33 Ballard, D. W., Dixon, E. P., Peffer, N. J., Bogerd, H., Doerre, S., Stein, B. and Greene, W. C. (1992) The 65-kDa subunit of human NF-kappa B functions as a potent transcriptional activator and a target for v-Rel-mediated repression. Proc. Natl. Acad. Sci. U.S.A. **89**, 1875–1879
34 Fujita, T., Nolan, G. P., Ghosh, S. and Baltimore, D. (1992) Independent modes of transcriptional activation by the p50 and p65 subunits of NF-kappa B. Genes Dev. **6**, 775–787
35 Ruben, S. M., Narayanan, R., Klement, J. F., Chen, C. H. and Rosen, C. A. (1992) Functional characterization of the NF-kappa B p65 transcriptional activator and an alternatively spliced derivative. Mol. Cell. Biol. **12**, 444–454
36 Schmitz, M. L. and Baeuerle, P. A. (1991) The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. EMBO J. **10**, 3805–3817
37 Narayanan, R., Higgins, K. A., Perez, J. R., Coleman, T. A. and Rosen, C. A. (1993) Evidence for differential functions of the p50 and p65 subunits of NF-kappa B with a cell adhesion model. Mol. Cell. Biol. **13**, 3802–3810
38 Kunsch, C. and Rosen, C. A. (1993) NF-kappa B subunit-specific regulation of the interleukin-8 promoter. Mol. Cell. Biol. **13**, 6137–6146
39 Kunsch, C., Ruben, S. M. and Rosen, C. A. (1992) Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation. Mol. Cell. Biol. **12**, 4412–4421
40 Ades, E. W., Candal, F. J., Swerlick, R. A., George, V. G., Summers, S., Bosse, D. C. and Lawley, T. J. (1992) HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J. Invest. Dermatol. **99**, 683–690
41 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**, 1475–1489
42 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
43 Brasier, A. R., Tate, J. E. and Habener, J. F. (1989) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. Biotechniques **7**, 1116–1122
44 Ahmad, M., Marui, N., Alexander, R. W. and Medford, R. M. (1995) Cell-type specific regulation of vascular adhesion molecule-1 (VCAM-1) promoter through kB motifs enhancer. J. Biol. Chem. **270**, 8976–8983
45 Ahmad, M., Theofanidis, P. and Medford, R. M. (1998) Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-alpha. J. Biol. Chem. **273**, 4616–4621
46 Nabel, G. and Baltimore, D. (1987) An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**, 711–713
47 Sen, R. and Baltimore, D. (1986) Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell (Cambridge. Mass.) **47**, 921–928

48 Franzoso, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K. and Siebenlist, U. (1992) The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-kappa B-mediated inhibition. Nature (London) **359**, 339–342
49 Ganchi, P. A., Sun, S. C., Greene, W. C. and Ballard, D. W. (1993) A novel NF-kappa B complex containing p65 homodimers: implications for transcriptional control at the level of subunit dimerization. Mol. Cell. Biol. **13**, 7826–7835
50 Simeonidis, S., Liang, S., Chen, G. and Thanos, D. (1997) Cloning and functional characterization of mouse IkappaBepsilon. Proc. Natl. Acad. Sci. U.S.A. **94**, 14372–14377
51 Perkins, N. D., Schmid, R. M., Duckett, C. S., Leung, K., Rice, N. R. and Nabel, G. J. (1992) Distinct combinations of NF-kappa B subunits determine the specificity of transcriptional activation. Proc. Natl. Acad. Sci. U.S.A. **89**, 1529–1533
52 Duckett, C. S., Perkins, N. D., Kowalik, T. F., Schmid, R. M., Huang, E. S., Baldwin, Jr, A. S. and Nabel, G. J. (1993) Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3). Mol. Cell. Biol. **13**, 1315–1322
53 Ferrari, D., Wesselborg, S., Bauer, M. K. and Schulze-Osthoff, K. (1997) Extracellular ATP activates transcription factor NF-kappaB through the P2Z purinoreceptor by selectively targeting NF-kappaB p65. J. Cell Biol. **139**, 1635–1643
54 Chen, F. E., Huang, D. B., Chen, Y. Q. and Ghosh, G. (1998) Crystal structure of p50/p65 heterodimer of transcription factor NF-kappaB bound to DNA. Nature (London) **391**, 410–413
55 Chen, Y. Q., Ghosh, S. and Ghosh, G. (1998) A novel DNA recognition mode by the NF-kappa B p65 homodimer. Nat. Struct. Biol. **5**, 67–73

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