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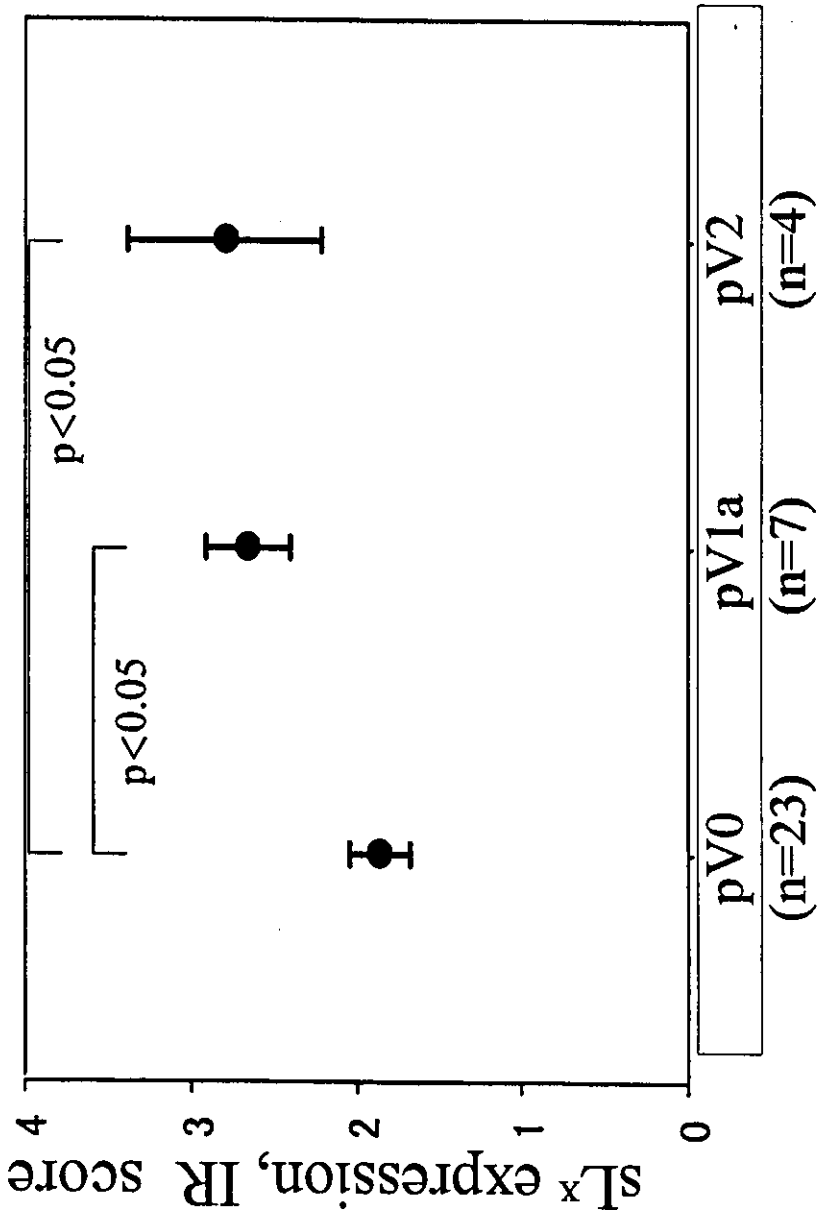

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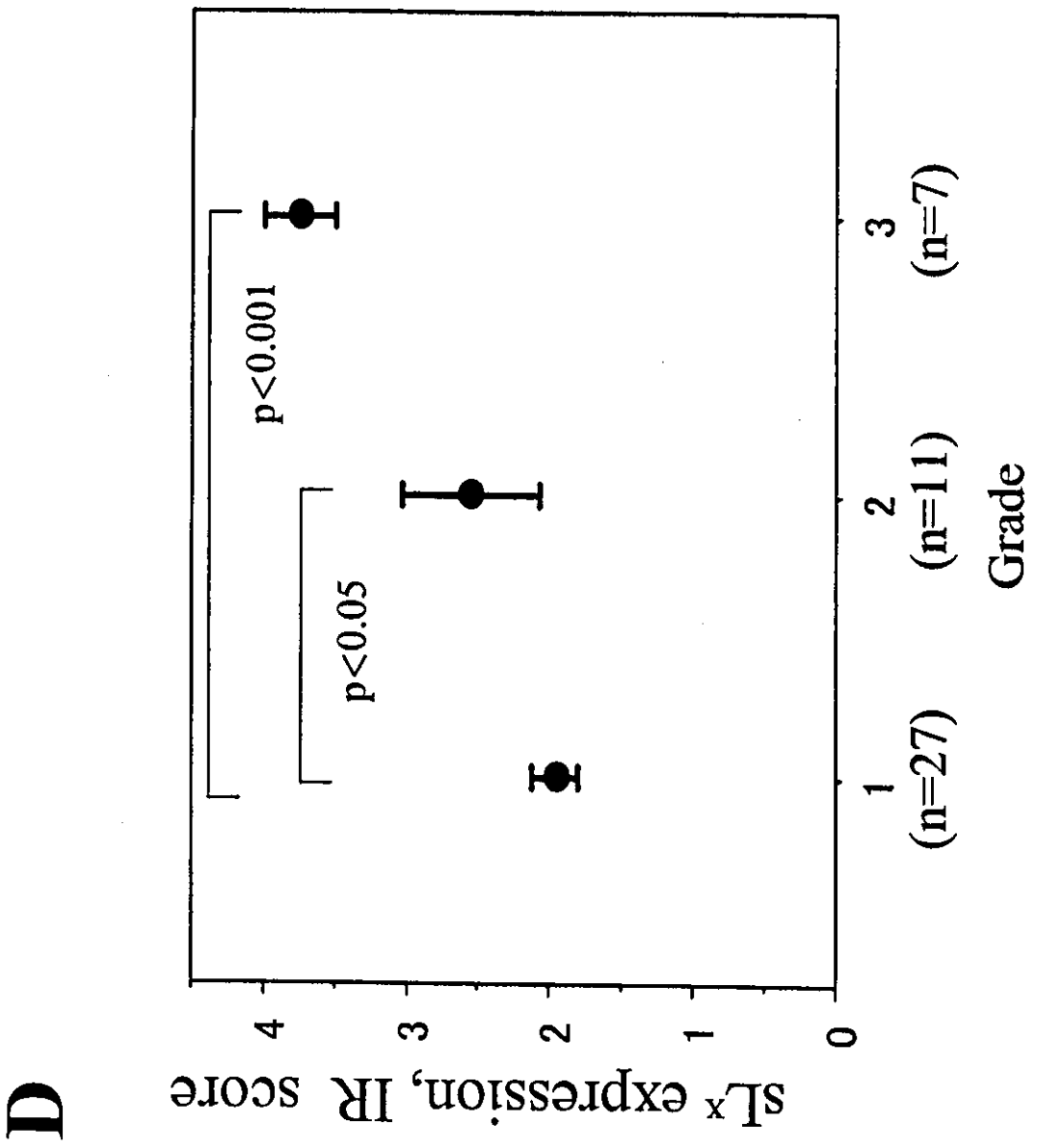

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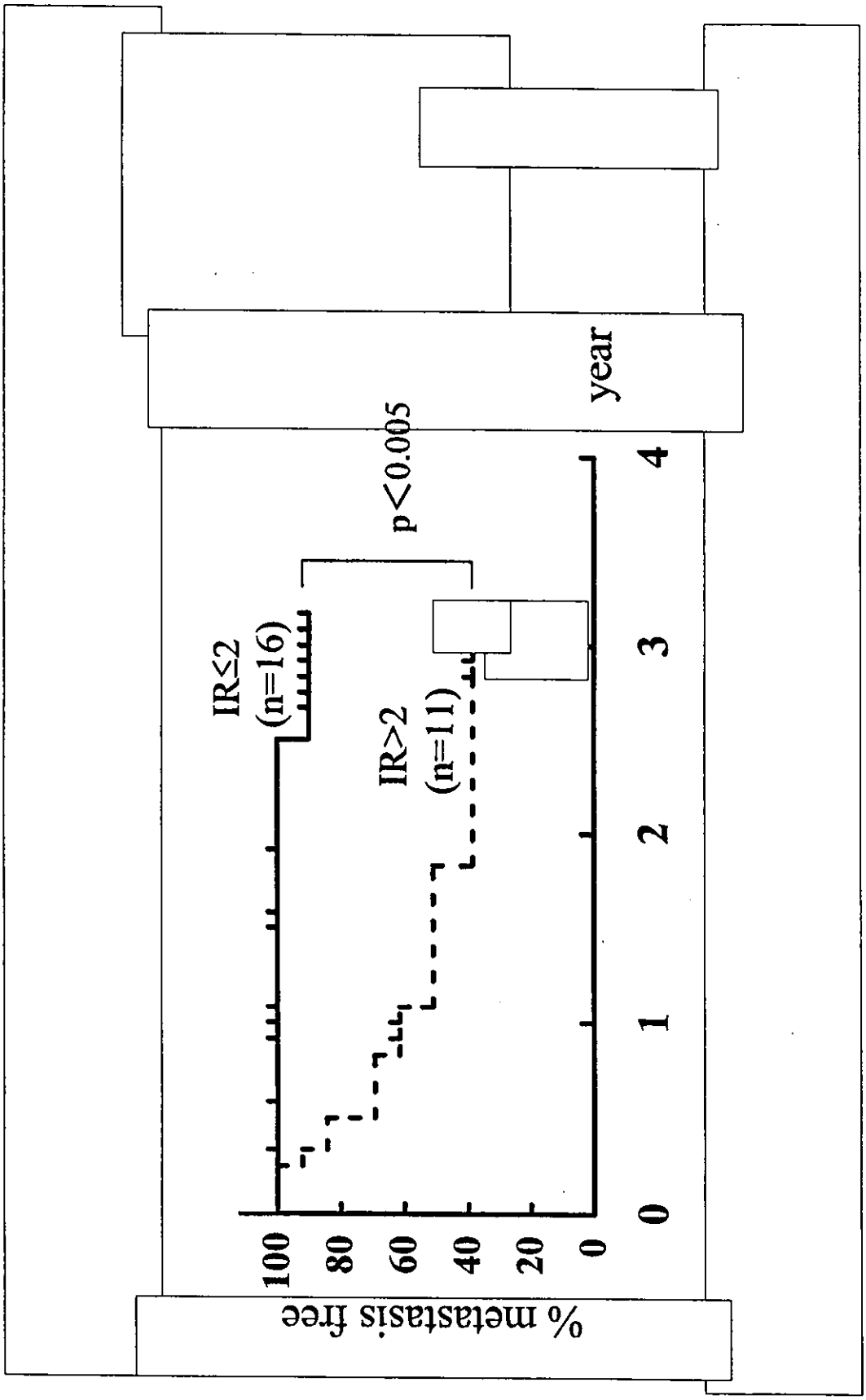

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REVIEW

2 Takashi Okamoto

The epigenetic alteration of synovial cell gene expression in rheumatoid arthritis and the roles of nuclear factor κB and Notch signaling pathways 1

Abstract Rheumatoid arthritis (RA) is a complex process of chronic and progressive inflammation associated with activation of numerous signaling molecules and transcription factors and hyperproliferation of synoviocytes of the affected joints, although the greater part of its pathophysiological process is explained by activation of nuclear factor κB (NF-κB). For example, the self-perpetuating nature of the rheumatoid inflammation is ascribable to overexpression of the proinflammatory cytokines tumor necrosis factor α and interleukin-1β, known to elicit the activation cascade for NF-κB and activator protein-1 that are responsible for transcriptional induction of these cytokines among other target genes, which conform a positive feedback loop for continuation and expansion of the inflammatory responses. In addition, comparative gene expression profile analyses have revealed activation of a number of genes that explain the "transformed-like" phenotype of synoviocytes. Among the genes expressed in rheumatoid synoviocytes upon inflammatory stimuli, induction of gene expression of Notch proteins and its ligand have been found. Possible roles of Notch signaling in RA synoviocytes are discussed.

Key words Notch · Nuclear factor κB (NF-κB) · Rheumatoid arthritis · Signal transduction · Synoviocyte

Introduction

Rheumatoid arthritis (RA) is a common human autoimmune disease with a prevalence of about 1%.[1] While there has been progress in defining its etiology and pathogenesis, these are still incompletely understood.[1–3] Proposed causes for RA include (i) genetic predisposition, (ii) pathogenetic immunoinflammatory responses triggered by environmental agents, particularly microbes, (iii) autoimmunity directed against components of synovium and cartilage, (iv) dysregulated production of cytokines (usually upregulation of proinflammatory and inflammatory cytokines and chemokines), (v) recruitment of immunoinflammatory cells through induction of inflammatory cell adhesion molecules (such as E-selectin, intracellular adhesion molecule-1, and vascular cell adhesion molecule-1), and, last but not least, (vi) transformation of synovial cells into autonomously proliferating cells with highly invasive nature (often referred to as "transformed-like" phenotype[4–6]).

Rheumatoid arthritis is characterized by a chronic inflammation of the synovial joints associated with proliferation of synovial cells and infiltration of activated immunoinflammatory cells including memory T cells, macrophages, and plasma cells,[1,2,7] which eventually leads to progressive destruction of cartilage and bone. This process is considered to be mediated by a number of cytokines including tumor necrosis factor α(TNFα), interleukin (IL)-1, IL-6, IL-8, IL-12, IL-16, IL-18, and interferon γ(IFNγ) (reviewed in Refs. 1–3). Most of these pathophysiological features of RA can be explained by activation of limited number of transcription factor and its activation signals such as nuclear factor κB (NF-κB) and activator protein (AP)-1.[3,8] In fact, some effective anti-RA drugs are now known to inhibit NF-κB and its activation cascade (reviewed in Ref. 8). However, the mechanism by which rheumatoid synoviocytes exhibit the tumor-like nature has been yet to be clarified.

Involvement of NF-κB in RA as a primary pathogenic determinant

Among the various signaling and transcription regulation pathways, NF-κB and AP-1 are known to be the target of inflammatory responses. In fact, most of the factors involved in RA pathophysiology are under the control of these transcription factors.[3,8] Particularly, various cytokines

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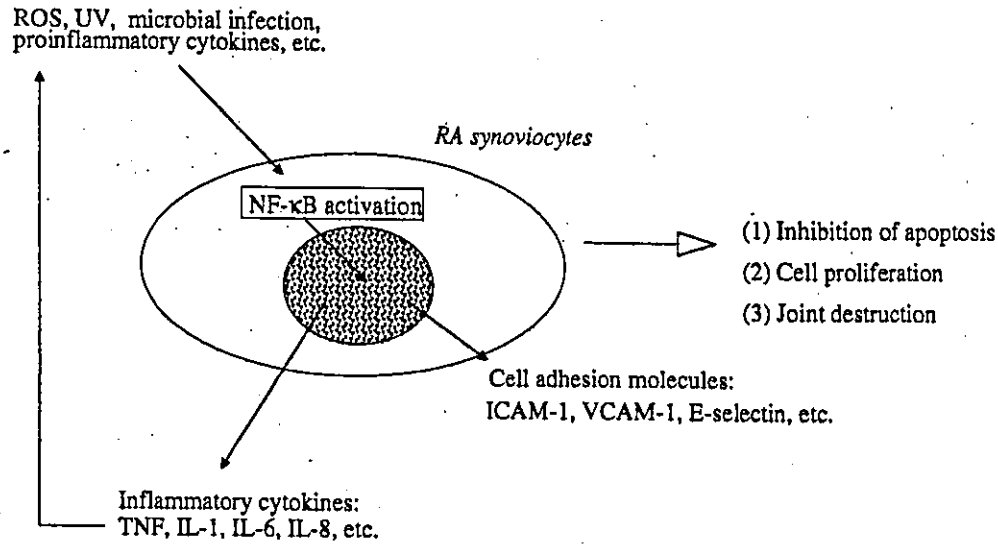

Fig. 1. Involvement of nuclear factor κB (*NF-κB*) in rheumatoid arthritis (*RA*) pathophysiology. NF-κB induces gene expression of inflammatory mediators such as cytokines and cell adhesion molecules. Since proinflammatory cytokines, tumor necrosis factor α (*TNF*), and interleukin (*IL*)-1β stimulate the NF-κB activation cascade that induces expression of these cytokines, there will be a positive feedback loop that perpetuates and expands the inflammatory responses even systemically. NF-κB also stimulates synovial proliferation by inhibiting apoptosis. See also Fig. 2. *ROS*, reactive oxygen species; *ICAM*, intracellular adhesion molecule; *VCAM*, vascular cellular adhesion molecule

and cell adhesion molecules activated in the rheumatoid joints are under the transcriptional control of NF-κB. The self-perpetuating nature of rheumatoid inflammation is ascribable to TNFα and IL-1β, known to elicit the activation cascade for NF-κB and AP-1, as they constitute another positive feedback loop in the logic of the inflammatory responses associated with RA (Fig. 1).

In addition, besides its action in upregulating inflammatory cytokines and cell adhesion molecules, NF-κB also induces gene expression of cell growth-promoting factors such as cyclin D1 and c-Myc, and physiological inhibitors of apoptosis such as cIAPs, Bcl-X_L, and cFLIP.[9,10] (Fig. 2). Moreover, it has been shown that NF-κB blocks apoptosis in the absence of de novo protein synthesis[11] through protein–protein interaction with p53 and proapoptotic protein 53BP2.[12,13] These actions of NF-κB explain not only the inflammatory responses but also the hyperproliferation of synovial tissues in RA, indicating that NF-κB acts as a major determinant for RA pathophysiology. Nuclear factor κB induces TNFα and IL-1β gene expression, and both TNFα and IL-1β stimulate NF-κB signaling, a vicious cycle formed to perpetuate and even expand the inflammatory responses.[8] The intervention therapy against using anti-TNF antibody and IL-1β receptor antagonist has been thus developed.[14,15] In addition, some of the drugs for RA have been shown to block NF-κB-activation cascade or its actions (Table 1).[16–18]

delete "against"

The signal transduction cascade for NF-κB activation

The members of the NF-κB family in mammalian cells include the proto-oncogene c-Rel, Rel A (p65), Rel B, NFkB1 (p50/105), and NFkB2 (p52/p100). These proteins share a conserved 300-amino-acid region known as the Rel homology domain, which is responsible for DNA binding, dimerization, and nuclear translocation of NF-κB. In most cells, Rel family members form hetero- and homodimers with distinct specificities in various combinations.[8,19,20] A common feature of the regulation of NF-κB family is their sequestration in the cytoplasm as inactive complexes with a class of inhibitory molecules known as IκBs.[20,21] Upon stimulation of the cells such as by proinflammatory cytokines, IL-1β and TNFα, IκBs are degraded, and NF-κB is translocated to the nucleus and activates expression of target genes (Fig. 2).

The IκB kinase complex capable of specifically phosphorylating Ser32 and Ser36 of IκBα was originally identified as a ~700kDa of high molecular complex.[21,22] Subsequently, two catalytic subunits (IKKα and IKKβ) and a scaffold subunit of this complex (IKKγ/NEMO/IKKAP) were identified and cloned (for review see Refs. 20–22). The IKK complex, consisting of IKKα, β, and γ, can be activated by a variety of stimuli, including TNFα, IL-1β, and LPS. Activation of the complex involves the phosphorylation of two serine residues located in the "activation loop" within the kinase domain of IKKα and IKKβ. IKK complex is stimulated by upstream kinases that belong to MAP kinase kinase kinases (MAP3Ks), including MEKK1, MEKK2, MEKK3, and NIK, capable of phosphorylating these serines in vitro, and activating NF-κB.[23,24] Phosphorylation on specific serine residues of IκBs leads to ubiquitination of IκBs and subsequent degradation by the proteasome complex.

There is accumulating evidence suggesting the involvement of additional kinases that phosphorylate the p65 (RelA) subunit of NF-κB and regulate its transcriptional

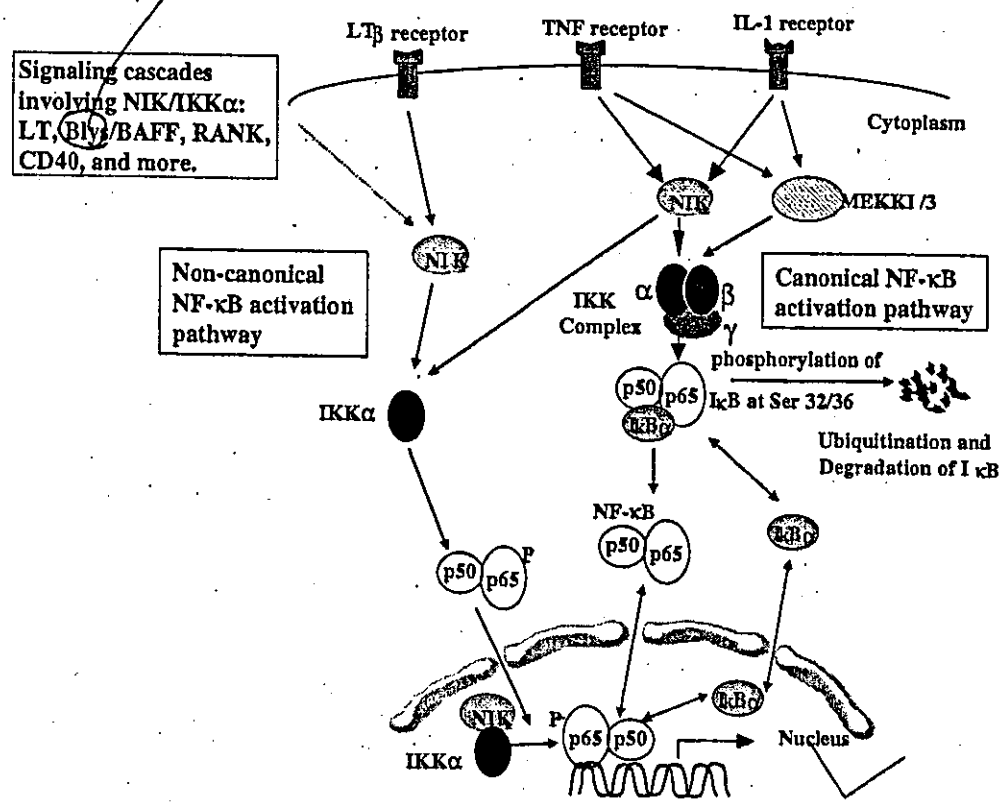

Fig. 2. NF-κB activation cascades. In addition to the canonical pathway involving IκB phosphorylation and ubiquitination followed by its proteolytic degradation in 26S proteasome within the cytoplasm, there appears to be another cascade not involving IκB phosphorylation. Lymphotoxin (*LT*) β-receptor signaling, CD40, RANK, and Blys/BAFF stimulate the NIK–IKKα cascade that leads to p100/p52 processing and p65 phosphorylation at its C-terminal transactivation (Ser536). IKKα also phosphorylates histone H3 in the nucleus and derepresses the otherwise silent nucleosome, thus reactivating the dormant genes. The effect of p65 (Ser536) phosphorylation is considered to activate the transcriptional competence of NF-κB

Table 1. List of rheumatoid arthritis drugs that inhibit nuclear factor κB

Acetylsalicylic acid
Aurothioglucose
Aurothiomalate
Auranofin
Dexamethasone
Ibuprofen
Sodium salicylate
Sulfasalazine

competence.[25–27] We recently found that IKKα is responsible for the p65 phosphorylation at Ser536 upon the lymphotoxin β receptor signaling mediated by NIK, and induces NF-κB activation independently of the IκB phosphorylation and its degradation.[28–30] Interestingly, this NIK–IKKα cascade is also involved in Blys/BAFF, RANK, and most likely CD40 signaling.[31,32] In contrast to the classical (or canonical) pathway involving IKKβ and the phosphorylation of IκB, this cascade ("non-canonical pathway") does not necessarily involve IKKβ and IκB phosphorylation but involves p100 (NFkB2) processing and p65 phosphorylation (Fig. 2). Since Blys/BAFF and CD40 signaling cascades induce B-cell activation and RANK signaling is involved in osteoclast differentiation, the NIK–IKKα cascade is considered to play important roles in disease progression of RA. The TNFα-dependent phosphorylation of Ser529 has also been demonstrated to increase the transcriptional activity of p65. For example, casein kinase II was implicated in the TNFα-dependent phosphorylation of p65 on Ser529.[33] It was shown that Ser529 and Ser536 of p65 were required for transcriptional activation of p65 by AKT and the IL-1β signaling.[30,34]

Inducible phosphorylation of p65 appears to function at many different levels, including conformational changes in the transcriptional activation domain and promoting association with coactivator proteins CBP/p300.[20] It is possible that the phosphorylation of p65 may lead to dissociation from corepressor proteins such as histone deacetylases and Groucho proteins (TLE/AES) and selective interaction with FUS/TLS coactivator protein.[35–37] Regarding the cross-talk with the camp–PKA cascade, although my group and others found that it downregulates the NF-κB-dependent gene expression presumably mediated by C/EBPβ,[38–40] it has also been reported that the catalytic subunit of PKA (PKAc), associated with the NF-κB/IκBα complex, upregulates the NF-κB-dependent gene expression by directly phosphorylating p65 on serine 276,[27] thus pending the physiological relevance.

Cytological characteristics of rheumatoid synoviocytes

Although it appears that NF-κB plays a major role in the pathophysiology of RA, there is no evidence to support the possibility that NF-κB or its signaling cascade is impaired in RA. To clarify the transformed-like nature of rheumatoid synoviocytes, my group have performed gene expression

profile analyses of synoviocytes.[41] When compared with control synoviocytes obtained from healthy individuals (upon injury) or osteoarthritis patients, we found that both platelet-derived growth factor (PDGF) receptor α and a chemokine, SDF-1, genes are activated in RA synoviocytes without any external stimulus. Gene knockout studies showed that PDGF receptor α is required for the development of limb joints. During the early developmental stages, PDGF and SDF-1 are known to act as chemotactic factors for fibroblasts[42] and macrophages,[43,44] respectively. It is possible that synovial fibroblasts (type B synoviocytes) and synovial-lining macrophage-like cells (type A) communicate with each other by producing SDF1A and PDGF, respectively, in order to form the primordial joint tissue during the early embryonic development (reviewed in Ref. 41). Thus, it is likely that rheumatoid synoviocytes may have reacquired the "revertant" phenotype of the primordial synoviocytes, like cancer cells, although the underlining mechanism is yet to be clarified.

As mentioned above, rheumatoid synovial tissues are usually under inflammatory stimuli as synovial fluid contains high concentrations of TNFα, IL-1, and oxidants (Ref. 46 and references therein). Thus, we extended the gene expression profile analysis with synoviocytes in the presence of physiological concentration (200pg/ml) of TNFα and compared the genes induced by TNFα in RA and control synoviocytes. Although well-known genes under the control of NF-κB were similarly stimulated by TNFα, we found that the genes encoding Notch-1, Notch-4, and Jagged-2, a ligand for Notch proteins, were activated only in RA synoviocytes.[49] (Fig. 3). We also found that genes encoding MMP-11 and -17, and Wee1 and cyclin B1, were induced by TNFα only in rheumatoid synoviocytes. These findings indicate that one of the effects of phenotypic reversion of rheumatoid synoviocytes, as described above, could be attributable to the induction of Notch signaling and that the activation of Notch signaling, known to be involved in cell-fate determination, may directly or indirectly cause induction of genes responsible for cell proliferation (such as induction of Wee1 and cyclin B1 genes) and tissue invasion (such as induction of MMP-11 and -17). These findings support an idea that RA synoviocytes may have reacquired the "revertant" phenotype mimicking the primordial synoviocytes, by presumably involving Notch signaling, and exhibit the hyperproliferative and invasive nature of cells.

Activation of Notch signal in RA

As TNFα induced Notch-1 and its ligand Jagged-2 in RSF, we examined if the Notch signaling is elicited by the TNFα stimulation. Rheumatoid synovial fibroblasts (RSF) and normal synovial fibroblasts (NSF) were stimulated with TNFα and the intracellular localization of Notch intracellular domain (NICD) of Notch-1 was examined by immunostaining. We found the nuclear translocation of Notch-1 NICD, a hallmark of the Notch signaling,[48,49] only in TNFα-stimulated RSF[47] (Fig. 4). These results suggested that in response to TNFα stimulation RSF expressed both Notch-1 and Jagged-2 proteins, which then interacted with each other between adjacent cells and elicited the signaling. In RA tissues we found that hyperproliferative synovial tissues were clearly stained by Notch-1, Notch-4, and Jagged-2 antibodies, and that some of the RA synovial cells showed the nuclear staining of Notch-1 and Notch-4.

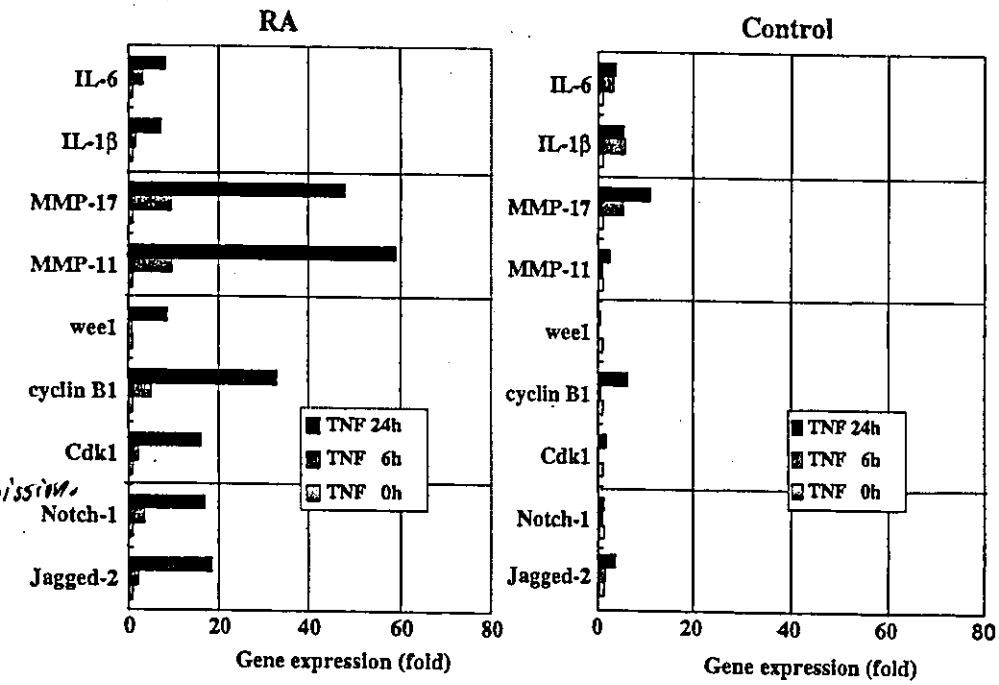

Fig. 3. Comparative gene expression profile analysis of rheumatoid and control synoviocytes upon stimulation with tumor necrosis factor α (*TNF*). Synoviocytes were cultured with or without TNF (200pg/ml). The mRNA was purified from each cell culture harvested at 0, 6, and 24h after TNF stimulation, cDNA probe was synthesized, then hybridized with a cDNA array membrane. The quantitation of gene expression level was performed and standardized based on the average levels of housekeeping genes. Based on the observation by Ando et al.[47]

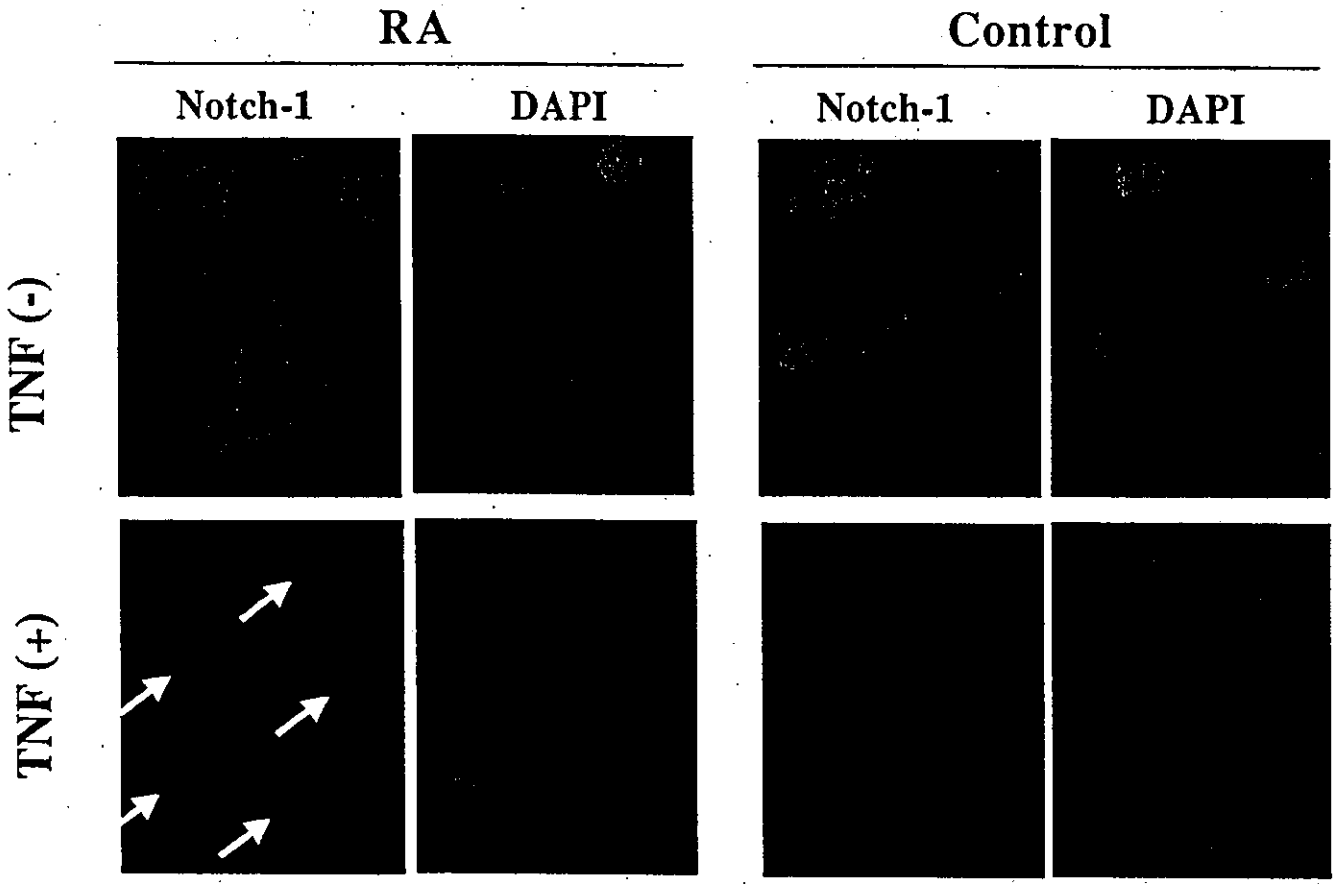

Fig. 4. Nuclear translocation of Notch-1 intracellular domain (NICD) in rheumatoid synoviocytes after tumor necrosis factor α (*TNF*) stimulation. Cells were immunostained with anti-Notch-1 C-terminus polyclonal antibody (C-20) before and after 12 h of TNF stimulation and examined by fluorescent microscopy. *Green*, Notch-1 intracellular domain (detected by fluorescein isothiocyanate-conjugated rabbit antigoat IgG as secondary antibody); *Blue*, nuclear staining with 4',6'-diamidino-2-phenylindole hydrochloride (*DAPI*). Based on the observation by Ando et al.[47]

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We also detected expression of these proteins in the developing synovial and cartilage tissues of embryonic mice.[47] In more developed joints of newborn mice, expression of these proteins was restricted in the synovium, raising a possibility that the Notch signaling pathway might control the differentiation and development of joints.

Biological implications of Notch signal activation in RA

Notch signaling is involved in three different biological processes including (i) lateral specification, in which adjacent equipotent precursor cells coordinate each other's developmental fate, (ii) inductive signaling, in which one cell type determines the differentiation of another cell type, and (iii) cell-autonomous effects, in which a developing precursor (stem) cell regulates its own fate and maintains its status (reviewed in Refs. 50 and 51). Notch genes encode single-pass transmembrane receptors that transduce the extracellular signals responsible for cell fate determination during crucial steps of metazoan development.[52,53] The large transmembrane receptors encoded by Notch genes interact with membrane-bound ligands encoded by the Delta and Jagged (Serrate) genes at the extracellular surface of cells. The signal induced by this ligand binding leads to proteolysis of Notch, generation and nuclear translocation of NICD, and regulation of target gene expression (Fig. 5). Genes homologous to members of the Notch signaling pathway have been cloned from numerous vertebrate organisms and many have been shown to be essential for normal embryonic development. In humans, the importance of Notch signaling for growth and development is supported by the findings that T-lymphoblastic leukemia[54] and some inherited diseases involving affected organogenesis[55,56] can be ascribed to the mutations in Notch/Jagged (Delta) genes. The Notch signaling pathway is evolutionarily conserved, and mutations in its components disrupt cell fate specification and embryonic development in diverse organisms.[48,50]

Interestingly, a targeted mutation that removes a domain of the Jagged-2 protein required for the interaction with Notch-1 caused perinatal death associated with defects in craniofacial morphogenesis and syndactyly (digit fusions) of the fore- and hindlimbs, implicating that Jagged-2/Notch signaling is indispensable for the development of the joint.[57]

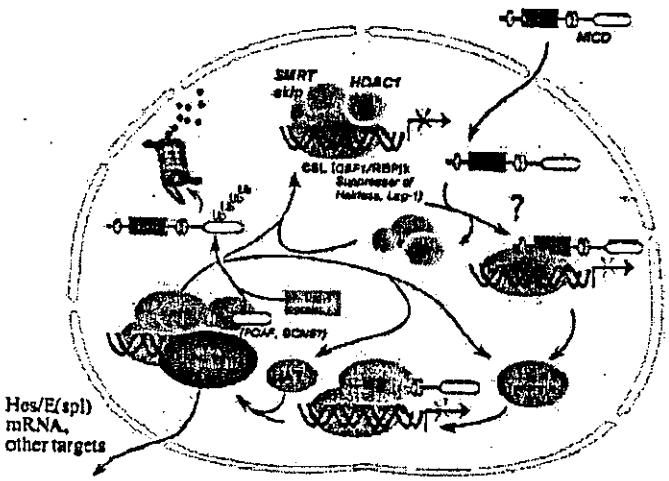

Fig. 5. Notch signaling and the transcriptional control by the Notch intracellular domain (*NICD*). Upon ligand binding, Notch protein is proteolytically cleaved by furin and presenilin to generate NICD, which is translocated to the nucleus. Once in the nucleus, NICD displaces the corepressor proteins such as *SKIP*, *SMRT*, and *HDAC-1* from the specific DNA-binding proteins *CSL* (CBP or RBP-Jκ in vertebrates, Su(H) in *Drosophila*, and Lag-1 in *Caenorhabditis elegans*; thus collectively called "CSL") and associates with the coactivator complex containing *Mastermind* (MAM) and *p300*;[62] MAM is considered to bridge the NICD/RBP-Jκ complex and p300.[63] The Notch intracellular domain is subjected to ubiquitination followed by proteasome-mediated degradation, thus terminating the transcriptional activation

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A similar phenotype has been observed in mice lacking the IKKα subunit of IκB kinase complex.[58] In embryonic day 16 (E16) mutant embryos, forelimbs (but not hindlimbs) were visible but were considerably shorter than those of normal ($Ikk\alpha^{+/+}$ and $Ikk\alpha^{+/-}$) littermates and lacked separated digits. At an earlier stage, E14.5, the fore- and hindlimbs of mutant embryos were not much shorter than those of normal counterparts, but were devoid of distinct digits. Therefore, it appears that the TNF-mediated NF-κB activation through IKKα is involved in expression of Jagged-2 in the developing joints. Thus, activation of the Notch signaling found in rheumatoid synoviocytes not only confirms the phenotypic reversion of synoviocytes but also indicates its active role in pathophysiological processes of RA, which presumably involve NF-κB cascade.

Cross-talk between NF-κB activation cascade and Notch signaling

In mammals, all four known Notch family members can physically interact with recombinant signal binding protein Jκ (RBP-Jκ), a DNA-binding repressor protein, and inhibit the activity of RBP-Jκ.[59,60] Oswald et al.[61] reported that NICD overcame the RBP-Jκ-mediated repression and strongly activated NF-κB2. In the absence of Notch signaling, RBP-Jκ interacts with SKIP and SMRT that recruit transcriptional corepressor complex[62] (Fig. 5). However, upon Notch signaling NICD induces changes in the DNA-bound protein assembly containing RBP-Jκ in the nucleus, thus displacing the corepressor complex and converting it to a transcriptionally active complex. It has been shown that a non-DNA-binding transcriptional coactivator Mastermind (MAM) is essential for the Notch/RBP-Jκ complex to recruit p300 coactivator to DNA.[63] Thus, activation of Notch signaling observed in rheumatoid synoviocytes appears to stimulate the noncanonical NF-κB pathway (Fig. 5).

It is conceivable that this noncanonical NF-κB activation pathway may be responsible for the altered response to the inflammatory environment involving IKKα. It is known that IKKα is translocated, together with NF-κB, to the nuclear chromatin compartments where target genes are present, and phosphorylates Ser10 of the histone H3 component of nucleosome.[64,65] (Fig. 5). Although the histone H3 with methylated lysine 9 of H3 renders the local nucleosome to be "repressive," the adjacent serine 10-phosphorylation of H3 histone reverses this effect and derepresses the transcriptional activity of the genes located in the "derepressed" nucleosome.[66] Thus, chronic and persistent NF-κB stimulation in synoviocytes of RA patients could also lead to the change in "histone code"[66] and eventually transform synoviocytes.

Conclusion

Rheumatoid arthritis is a complex process of chronic and progressive inflammation involving numerous transcription factors and signaling molecules. Based on the unexpected transcriptomic characteristics of rheumatoid synoviocytes, suggesting the phenotypic reversion, I have explored the mechanism by which chronic inflammatory stimuli could endow normal synoviocytes with "transformed-like" phenotype and could ascribe activation of the Notch signaling to this altered cellular status. This may explain the progressive and self-perpetuating nature of the rheumatoid inflammation, at least in part. Based on these considerations, future therapeutic strategy of RA should be developed based on the action of Notch signaling on its pathophysiology, which includes the action of the noncanonical NF-κB activation pathway, its therapeutic intervention, and elucidation of the Notch target genes, particularly in synoviocytes.

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RNA helicase A interacts with nuclear factor κB p65 and functions as a transcriptional coactivator

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RNA helicase A (RHA), a member of DNA and RNA helicase family containing ATPase activity, is involved in many steps of gene expression such as transcription and mRNA export. RHA has been reported to bind directly to the transcriptional coactivator, CREB-binding protein, and the tumor suppressor protein, BRCA1, and links them to RNA Polymerase II holoenzyme complex. Using yeast two-hybrid screening, we have identified RHA as an interacting molecule of the p65 subunit of nuclear factor κB (NF-κB). The interaction between p65 and RHA was confirmed by glutathione-*S* transferase pull-down assay *in vitro*, and by co-immunoprecipitation assay *in vivo*. In transient transfection assays, RHA enhanced NF-κB dependent reporter gene expression induced by p65, tumor necrosis factor-α, or NF-κB inducing kinase. The mutant form of RHA lacking ATP-binding activity inhibited NF-κB dependent reporter gene expression induced by these activators. Moreover, depletion of RHA using short interfering RNA reduced the NF-κB dependent transactivation. These data suggest that RHA is an essential component of the transactivation complex by mediating the transcriptional activity of NF-κB.

Keywords: coactivator; NF-κB; protein–protein interaction; RNA helicase A; transcription.

Nuclear factor κB (NF-κB) is an inducible cellular transcription factor that regulates a wide variety of cellular and viral genes including cytokines, cell adhesion molecules and HIV [1–3]. The members of the NF-κB family in mammalian cells include the proto-oncogene c-Rel, RelA (p65), RelB, NFkB1 (p50/105), and NFkB2 (p52/p100). In most cells, Rel family members form hetero- and homodimers with distinct specificities in various combinations. p65, RelB and c-Rel are transcriptionally active members of the NF-κB family, whereas p50 and p52 serve primarily as DNA binding subunits [1–3]. These proteins play fundamental roles in immune and inflammatory responses and in the control of cell proliferation [4,5]. A common feature of the regulation of NF-κB is their sequestration in the cytoplasm as an inactive complex with a class of inhibitory molecules known as IκBs. Treatment of cells with a variety of inducers such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) results in phosphorylation, ubiquitination and degradation of the IκB proteins [1–3].

The protein regions responsible for the transcriptional activation [called 'transactivation (TA) domain'] of p65, Rel B and c-Rel have been mapped in their unique C-terminal regions. p65 contains at least two independent TA domains within its C-terminal 120 amino acids (Fig. 1A). One of these TA domains, TA1, is confined to the C-terminal 30 amino acids of p65. The second TA domain, TA2, is localized in the N-terminally adjacent 90 amino acids and contains TA1-like motif. As the nuclear translocation and DNA binding of NF-κB were not sufficient for gene induction [6,7], it was suggested that interactions with other protein molecules through the TA domain [8–10] as well as its modification by phosphorylation [11–14] might play critical roles in the NF-κB-mediated gene expression.

It has been shown that NF-κB requires multiple coactivator proteins including CREB-binding protein (CBP)/p300 [8–10,15,16], CBP associated factor, and steroid receptor coactivator 1 [17]. These proteins have histone acetyl transferase activity that modifies chromatin structure and provides molecular bridges to the basal transcriptional machinery. p65 was also found to interact with a newly identified coactivator complex, activator-recruited cofactor/vitaminD receptor-interacting protein, which potentiated chromatin-dependent transcriptional activation by NF-κB *in vitro* [18]. Aside from coactivators, the transcriptional activity of gene-specific activators can also be mediated by general transcription factors.

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Abbreviations: AD, (transcriptional) activation domain; AES, amino-terminal enhancer of split; CREB, cAMP response element binding protein; CBP, CREB-binding protein; CMV, cytomegalovirus; DBD, DNA-binding domain; GIR, Groucho-interacting region; Grg, Groucho-related genes; GST, glutathione-*S* transferase; ICAM-1, intercellular adhesion molecule-1; IFN-β, interferon-β; IL-1, interleukin-1; MLE, maleless; MSL, male-specific lethal; NF-κB, nuclear factor κB; NIK, NF-κB inducing kinase; NLS, nuclear localization signal; RAI, RelA-associated inhibitor; RHA, RNA helicase A; RNA Pol II, RNA polymerase II; TLE1, transducin-like enhancer of split 1; TLS, translocated in liposarcoma; TNF-α, tumor necrosis factor-α.

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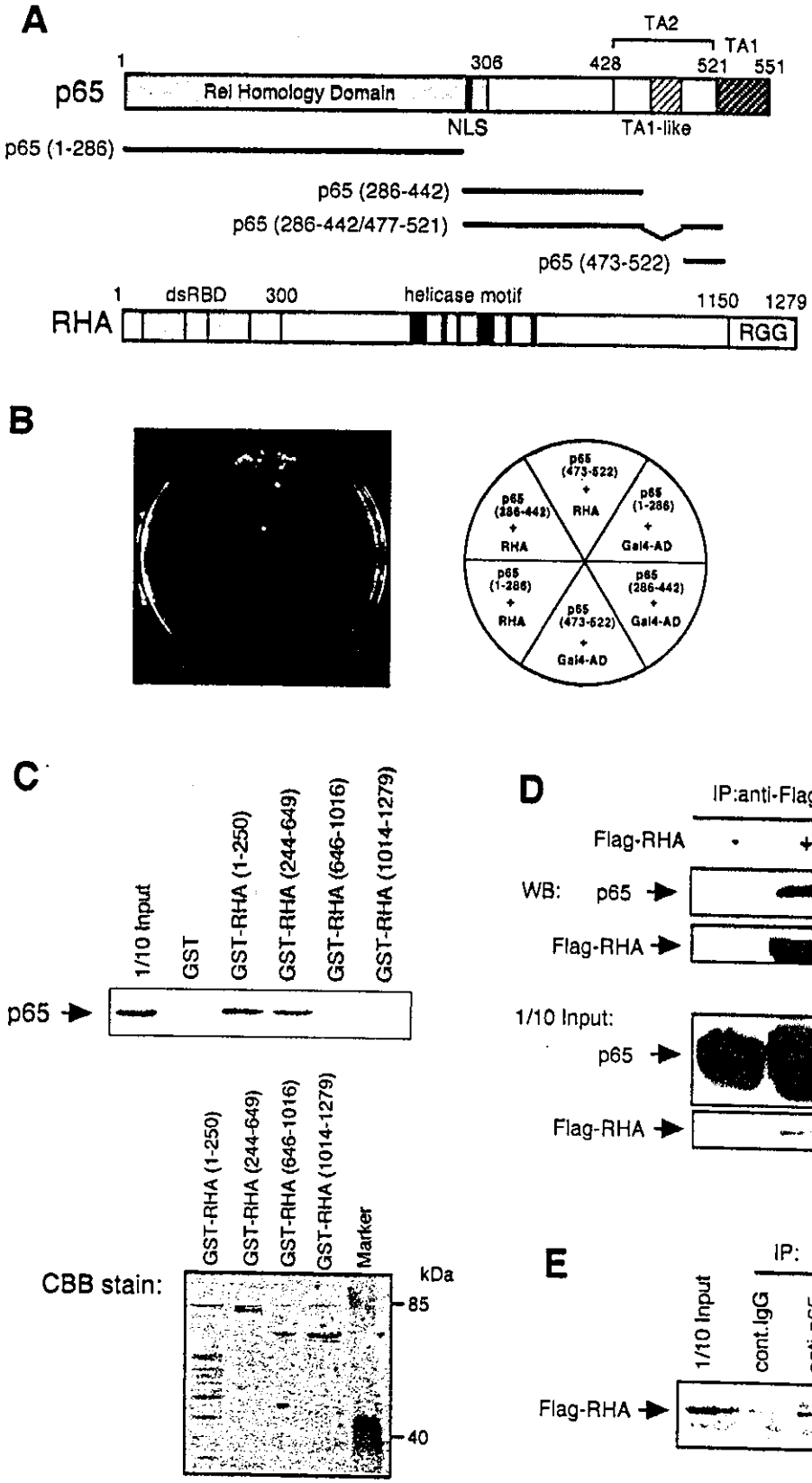


In the case of NF-κB, the association of p65 with general transcription factors such as TFIIB, $TAF_{II}105$, and TBP has been demonstrated [8,19–22]. It is thus postulated that specific protein–protein interactions with NF-κB determine its transcriptional competence. Up-regulation of the NF-κB transcriptional activity is mediated by interaction with basal factors and coactivators while its down-regulation is mediated by interaction with inhibitors and corepressors at

Fig. 1. Interaction between p65 and RHA. (A) Schematic illustrations of various functional domains of p65 and RHA. dsRBD, double stranded RNA-binding domain; NLS, nuclear localization signal; TA1, transactivation domain 1; TA2, transactivation domain 2 (containing TA1-like domain, Groucho-interacting region, and leucine-rich region); RGG, Arg-Gly-Gly rich region. (B) Growth of yeast transformants coexpressing p65 and RHA on the selective medium. The yeast Y190 was transformed with pACT2-RHA and pGBT plasmids expressing various portions of the p65 in fusion with Gal4-DBD. The yeast transformants grown on plates lacking Leu and Trp were streaked on plates lacking Leu, Trp and His, and containing 25 mM 3-aminotriazole. (C) p65 binds to RHA *in vitro*. p65 was labeled with [^{35}S]-methionine by *in vitro* transcription/translation. Radiolabeled p65 was incubated with GST, GST-RHA(1–250), GST-RHA(244–649), GST-RHA(646–1016) or GST-RHA(1014–1279) immobilized on glutathione-Sepharose beads. After incubation and further washing, the complexes were resolved by 10% SDS/PAGE and subjected to autoradiography. (D,E) p65 binds to RHA *in vivo*. HEK 293 cells were transfected with pCMV-p65 in combination with either pCMV-Flag-RHA or the empty vector. Whole cell extracts were harvested 48 h after transfection, and immunoprecipitated with 10 μL of anti-Flag M2 Affinity Gel, and the resulting precipitates were disrupted and immunoblotted with anti-p65 Ig and anti-Flag Ig (D, upper panel). Whole cell extracts (1/10 input) were also immunoblotted with anti-p65 Ig and anti-Flag Ig to show that the same amount of the immune complex containing p65 were loaded (D, lower panel). HEK 293 cells were transfected with pCMV-Flag-RHA and pCMV-p65 expression vectors. Whole cell extract was harvested 48 h after transfection, and RHA was immunoprecipitated with control rabbit IgG or anti-p65 rabbit polyclonal IgG. Ten microliters of protein G-agarose beads was added and the reaction was further incubated for 1 h. The immunoprecipitated proteins were resolved by 10% SDS/PAGE and immunoblotted with anti-Flag Ig (E).

multiple levels. In our previous studies, yeast two-hybrid screening yielded several novel regulators of NF-κB that interact with the p65 subunit: amino-terminal enhancer of split (AES) and transducin-like enhancer of split (TLE1) [23], both belonging to the Groucho-related genes (Grg) and acting as corepressors. The pro-oncoprotein TLS (translocated in liposarcoma), a homologue of $TAF_{II}68$, stimulates the transcriptional activity of p65 [24]. These proteins interact with a small intervening region between TA1 and TA1-like motifs, termed 'Groucho-interacting region' (GIR), within the C-terminal TA domain of p65 [23,24]. In addition, we also identified a novel nuclear protein RelA-associated inhibitor (RAI), containing ankyrin repeats and interacting with the central region of p65 that blocks the DNA binding activity of NF-κB [25,26], similar to the cytoplasmic inhibitors IκBs.

There is accumulating evidence indicating that RNA helicase A (RHA) acts as a transcriptional coactivator. RHA was found to interact with the CREB-binding protein (CBP) [27] and BRCA1 [28], and to be required for transcriptional activation. The ATP binding and/or ATP hydrolysis activities of RHA appear to be required for transcriptional activation as the RHA mutant, in which Lys417 within the conserved ATP-binding motif is substituted by Arg, resulted in the loss of RHA activity and a great reduction in transcriptional activity [27].

In this study, we demonstrate that RHA interacts directly with p65 and activates NF-κB-mediated transcription. We confirmed the interaction between p65 and RHA *in vitro* using the bacterially expressed fusion proteins and an *in vivo* co-immunoprecipitation assay. Depletion of endogenous RHA using siRNA reduced the NF-κB-mediated gene expression. These data indicate that RHA mediates the transcriptional activity of NF-κB.

Experimental procedures

Plasmids

Mammalian expression vector plasmids Gal4-Sp1, pCMV-NIK, ICAM-1-luc (−339 to −30) and E-selectin-luc, IFN-β-luc were generous gifts from S. T. Smale (UCLA School of Medicine, Los Angeles, CA, USA), D. Wallach (Weitzmann Institute of Science, Rehovot, Israel), L. A. Madge and J. S. Pober (Yale University School of Medicine, New Haven, CT, USA), and T. Taniguchi (Tokyo University, Tokyo, Japan), respectively. pCMV-RHA, pCMV-RHA-mATP, pCMV-p65, pGal4-p65, pGBT-p65(1–286), pGBT-p65(286–442), and pGBT-p65(473–522) had been described previously [23,29]. To create pACT2-RHA, the RHA cDNA was amplified by PCR using pCMV-RHA as a template with oligonucleotides containing *Bam*HI-*Xho*I site. These products were digested with *Bam*HI-*Xho*I, and subcloned in-frame into pACT2 vector at the *Bam*HI-*Sal*I site. Construction of a luciferase reporter plasmid, 4κB-luc, containing four tandem copies of the HIV-κB sequence upstream of minimal simian virus 40 (SV40) promoter had been described previously [30]. The other luciferase reporter plasmid, pGal4-luc (pFR-luc), containing five tandem copies of Gal4 binding site upstream of the TATA box, was purchased from Stratagene.

Yeast two-hybrid screening and protein–protein interaction assay

The yeast two-hybrid screening was performed as described previously [23,24,26]. The C-terminal regions of p65 corresponding to amino acids 286–442/477–521 was fused in-frame to Gal4 DNA binding domain (positions 1–147) using the pGBT9 vector (Clontech), and used as a bait for library screening. Yeast strain Y190 was transformed with pGBT-p65-(286–442/477–521) and the human placenta cDNA expression library fused to the Gal4 transactivation domain in the pACT2 vector (Clontech). Approximately one million transformants were screened for their ability to grow on the plates with medium lacking Trp, Leu, and His, and containing 25 mM 3-aminotriazole. Plasmids were rescued from clones that were positive for β-galactosidase activity and identified by nucleotide sequencing. cDNA sequences and their amino acid sequences were compared with GenBank™ and Swiss-Prot databases for identification of the interacting proteins.

Cell culture and transfection

Human embryonic kidney (HEK 293) cells were maintained in DMEM with 10% fetal bovine serum, 100 $U \cdot mL^{-1}$ of penicillin and 100 $\mu g \cdot mL^{-1}$ of streptomycin. Cells were transfected using Fugene-6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's

instruction. At 48 h post-transfection, the cells were harvested, and the extracts were prepared for luciferase assay. Luciferase activity was measured by the Luciferase Assay System (Promega, Madison, WI) as described previously [26]. Transfection efficiency was monitored by *Renilla* luciferase activity using the pRL-TK plasmid (Promega) as an internal control. The data are presented as the fold increase in luciferase activities (mean ± SD) relative to the control of three independent transfections. Human recombinant TNF-α was purchased from Roche.

In vitro binding assay

Glutathione-*S* transferase (GST)-RHA(1–250), GST-RHA (244–649), GST-RHA(646–1016), and GST-RHA(1014–1279) were prepared as described previously [29]. These GST-RHA fusion proteins were expressed in *Escherichia coli* strain DH5α and purified. The *in vitro* protein–protein interaction assay ('pull-down' assay) was carried out as described previously [23,24,26]. The p65 protein was synthesized and labeled with [^{35}S]methionine by *in vitro* transcription/translation procedure using a TNT wheat germ extract coupled system (Promega) according to the manufacturer's protocol. Approximately 20 μg of GST fusion proteins was immobilized on 20 μL of glutathione-Sepharose beads and washed 2× with 1 mL of modified HEMNK buffer [20 mM HEPES/KOH (pH 7.5), 100 mM KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The beads were left in 0.6 mL of HEMNK and were incubated with radiolabeled proteins for 2 h at 4 °C with gentle mixing. The beads were then washed 3× with 1 mL of HEMNK buffer and 2× with 1 mL of HEMNK buffer containing 150 mM KCl. Bound radiolabeled proteins were eluted with 30 μL of Laemmli sample buffer, boiled for 3 min, and resolved by 10% SDS/PAGE.

Co-immunoprecipitation and Western blot assays

HEK 293 cells were transfected with pCMV-p65 in combination with either CMV-Flag-RHA or the empty vector. After transfection, cells were cultured for 48 h and harvested with lysis buffer [25 mM HEPES/NaOH (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% NP-40, 5% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride]. The lysates were incubated with 10 μL of anti-Flag M2 Affinity Gel (Sigma) at 4 °C for 1 h. The beads were washed 5× with 1 mL of lysis buffer. Antibody-bound complexes were eluted by boiling in Laemmli sample buffer, resolved by 10% SDS/PAGE, and transferred on nitrocellulose membrane (Hybond-C, Amersham). The membrane was incubated with anti-Flag Ig (Sigma) or anti-p65 Ig (Santa Cruz) and the immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce) as described previously [23,24,26]. To evaluate the level of exogenous p65 expressed from pCMV-p65 containing the His epitope-tag, rabbit polyclonal anti-$(His)_6$ Ig (Santa Cruz) was used for Western blotting.

RNA interference

The double-stranded RNA specific for RHA was synthesized by Takara Bio Inc. (Shiga, Japan). This RHA specific small interference RNA (siRNA) 5′-GCAUAAAACUUCUGC GUCU-3′ was targeted to the RHA portion from 2408 to 2426. Control siRNA 5′-AUUCUAUCACUAGCGU GAC-3′ was purchased from Dharmacon (Lafayette, CO, USA). siRNA transfections were performed using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction.

Results

Identification of RHA as a p65-binding protein

To identify proteins interacting with p65 subunit of NF-κB, we performed the yeast two-hybrid screen using pGBT-p65(286–442/477–521) as a bait for the screening. Yeast strain Y 190 was used for the screening of a human placenta cDNA library fused to the Gal4 transcriptional activation domain in the pACT2 vector (Clontech). Among $\approx 1.0 \times 10^6$ Y190 yeast transformants, 90 colonies grew on selective medium and turned blue when tested with a β-galactosidase assay. Each plasmid purified from the positive colony was cotransfected with the bait plasmid into the yeast to confirm the specific interaction. DNA sequencing and comparison with GenBank and SwissProt databases revealed the gene for RHA (one clone) in addition to IκBα/MAD3 (five clones) and Bcl3 (one clone) that are known to interact with p65.

In order to map the interaction domain of p65 with RHA, we performed the yeast two-hybrid protein–protein interaction assay (Table 1, Fig. 1B). Various regions of the p65 protein were fused to Gal4-DNA binding domain in the pGBT9 vector and cotransfected with pACT2-RHA, encoding RHA fused to Gal4–transactivation domain. Interactions were tested by β-galactosidase activity (Table 1) and by growth of yeast cells on plates with medium lacking His, Leu and Trp, and containing 25 mM 3-aminotriazole (Fig. 1B). pGBT-p65(1–286), pGBT-p65(286–442), and

Table 1. Yeast two-hybrid interaction assays between p65 and RHA. Yeast Y190 cells were cotransformed with expression vectors encoding various proteins fused to Gal4 DNA-binding domain (Gal4-DBD) and Gal4 transcriptional activation domain (Gal4-AD). pACT2-RHA is a rescued clone which encodes full length RHA fused to Gal4-AD. pACT2-IκBα encodes full length IκBα (amino acids 1–317) fused to Gal4-AD. Leu^+ Trp^+ transformants were streaked on selective medium lacking Leu and Trp, and allowed to grow for 2 days at 30 °C. At least three colonies of each transformant were tested for β-galactosidase activity using X-gal colony filter assay (Clontech). +, positive for β-galactosidase activity (blue colony) after 2–3 h; –, no β-galactosidase activity (white colony) after 24 h; ND, not determined.

	Gal4-AD hybrid		
Gal4-DBD hybrid	pACT2	pACT2-RHA	pACT2-IκBα
pGBT9	–	–	–
pGBT-p65(1–286)	–	–	–
pGBT-p65(286–551)	+	ND	ND
pGBT-p65(286–521)	+	ND	ND
pGBT-p65(286–470)	+	ND	ND
pGBT-p65(286–442)	–	–	+
pGBT-p65(473–522)	–	+	–

pGBT-p65(473–522) alone did not show any background in the prototrophic selection or in the β-galactosidase assay. Among these, pGBT-p65(473–522) was shown to interact with pACT2-RHA (Table 1, Fig. 1B). These results indicate that the minimal region of p65 responsible for the interaction with RHA resides within the amino acids 473–522.

Binding of RHA to p65

To confirm the interaction between RHA and p65, we performed an *in vitro* protein–protein interaction assay using various recombinant RHA proteins in fusion with GST. The radiolabeled p65 protein was synthesized by *in vitro* transcription/translation in the presence of [^{35}S]methionine using wheat germ extract. The radiolabeled p65 was incubated with GST-RHA fusion proteins immobilized on glutathione-Sepharose beads. As shown in Fig. 1C, p65 bound to GST-RHA(1–250) and GST-RHA(244–649) but not to GST-RHA(646–1016), or GST-RHA(1014–1279). No p65 binding was detected with beads containing GST alone (as a negative control).

To investigate the interaction between RHA and p65 *in vivo*, we expressed p65 and RHA containing the Flag-epitope in the N-terminus in HEK 293 cells. Lysates were prepared from the transfected HEK 293 cells and immunoprecipitated with anti-Flag M2 Affinity Gel (Sigma) and the resulting precipitate was disrupted and immunoblotted with anti-p65 and anti-Flag Igs. As shown in Fig. 1D, p65 was co-immunoprecipitated with Flag-RHA. To confirm this interaction, the cell lysates were immunoprecipitated with anti-p65 Ig or control IgG, followed by Western blotting using anti-Flag Ig. As shown in Fig. 1E, Flag-RHA was co-immunoprecipitated with p65. These data indicate the interaction between p65 and RHA *in vivo*.

RHA mediates NF-κB-dependent gene expression

We then investigated the effect of RHA on NF-κB-dependent gene expression. In Fig. 2A, the effect of RHA was examined on gene expression from the reporter plasmid 4κB-luc by transfection of pCMV-p65 with or without cotransfection of pCMV-RHA in HEK 293 cells. RHA augmented the NF-κB-mediated transactivation in a dose-dependent manner when the p65-expression plasmid was cotransfected. pCMV-p65 alone activated gene expression from 4κB-luc, but RHA further enhanced the p65-mediated gene expression. However, there was no detectable effect of RHA on the basal transcription level in the absence of pCMV-p65. These effects of RHA was not through increasing the level of p65, as Western blot analysis of the transfected cell lysate revealed no increase in the protein level of exogenously expressed p65 (Fig. 2A, lower panel). Similarly, RHA augmented NF-κB dependent gene expression induced by TNF-α or by NF-κB inducing kinase (NIK), the upstream kinase for NF-κB activation (Fig. 2B,C).

The catalytic activity is required for the effect of RHA

To determine whether endogenous RHA is involved in NF-κB mediated transcription, we used pCMV-RHAmATP,

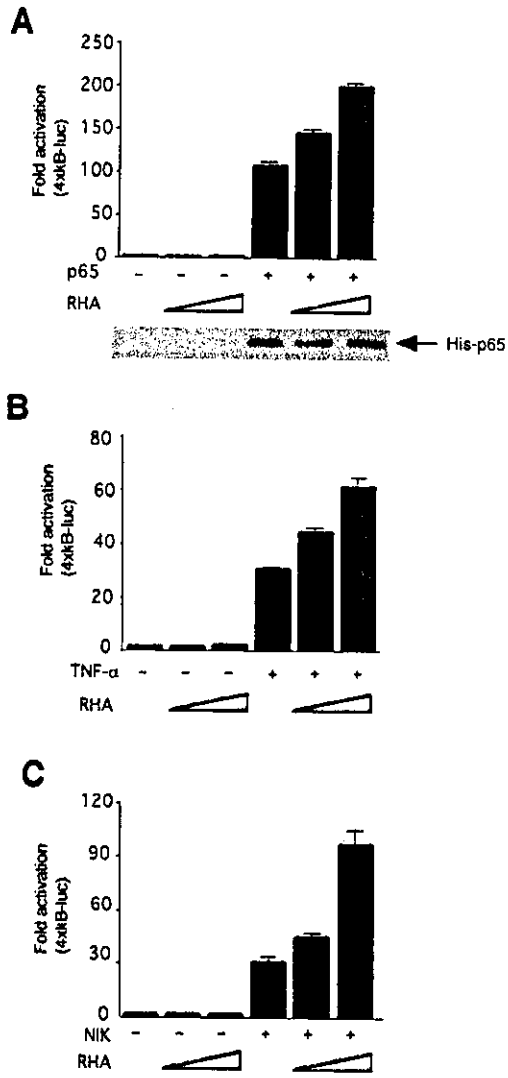
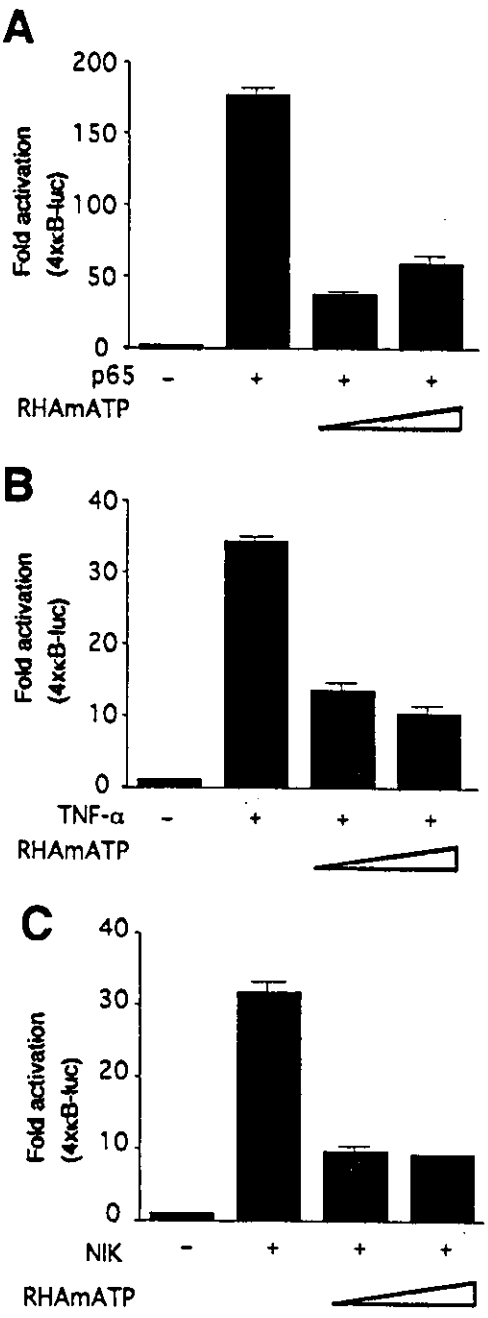


Fig. 2. RHA augments NF-κB-dependent gene expression. (A) HEK 293 cells were transfected with 20 ng of 4κB-luc in combination with pCMV-p65 [containing $(His)_6$ epitope] (10 ng) and pCMV-RHA expression plasmids (50 or 100 ng). Cells were harvested 24 h after transfection, and luciferase activity was measured. Western blot analysis of p65 levels in transfected cell extracts was done to confirm if equal amounts of the exogenous p65 are expressed irrespective of RHA overexpression (lower panel). A portion of each cell extract was separated by 10% SDS/PAGE and immunoblotted with anti-His Ig. (B) Effect of RHA on the NF-κB-dependent gene expression induced by TNF. HEK 293 cells were transfected with 4κB-luc (50 ng) and pCMV-RHA (50 or 100 ng). After 24 h of transfection, cells were stimulated with 1 ng·mL^{-1} of TNF and harvested after additional incubation for 24 h. (C) Effect of RHA on the NF-κB-dependent gene expression induced by NIK. HEK 293 cells were transfected with 4κBw-luc (50 ng) in the absence or presence of pCMV-NIK (10 ng) and pCMV-RHA (50 or 100 ng). Cells were harvested 24 h after transfection, and luciferase activity was measured. Extents of fold activation of luciferase gene expression as compared to the transfection with reporter plasmid alone are indicated. Values (fold activation) represent the mean ± SD of three independent transfections. Similar results were achieved repeatedly.

the expression plasmid for dominant negative mutant RHA, in which Lys417 of the conserved ATP-binding motif (Gly-Lys-Thr) of RHA catalytic domain was substituted by Arg, and the ATPase activity was abolished. NF-κB-dependent gene expression induced by p65, TNF-α and NIK was inhibited by the expression of RHAmATP (Fig. 3A–C), suggesting that the endogenous RHA mediates the transcriptional activity of NF-κB p65.

Effect of RHA on the p65-mediated transactivation of ICAM-1, E-selectin, and IFN-β promoters

To confirm the effect of RHA on NF-κB in physiological promoters, we examined the effect of RHA on the promoters of ICAM-1, E-selectin, and IFN-β containing NF-κB binding sites. Various amounts of RHA expressing plasmid (pCMV-RHA) or RHAmATP plasmid (pCMV-RHAmATP) were transfected into HEK 293 cells along with ICAM-1-luc, E-selectin-luc or IFN-β-luc. As shown in Fig. 4, RHA enhanced the NF-κB dependent transcription for ICAM-1, E-selectin and IFN-β promoters (Fig. 4A–C, left panels). On the other hand, overexpression of RHAmATP inhibited the NF-κB dependent transcription from ICAM-1, E-selectin and IFN-β promoters (Fig. 4A–C, right panels). These data suggest that the enzymatic activity of RHA is involved in the NF-κB mediated gene expression in physiological promoters such as IFN-β, ICAM-1 and E-selectin.



RHA activates NF-κB through activation domain of p65

To further analyze the effect of RHA on p65, we used expression plasmids for fusion proteins of Gal4-p65, Gal4-CREB or Gal4-Sp1 in which the DNA-binding domain of Gal4 was fused with p65, CREB and Sp1. The extents of augmentation of transactivation of these Gal4-p65, Gal4-CREB and Gal4-Sp1 by RHA are shown in Fig. 5. RHA augmented the transactivation mediated by Gal4-p65(1–551) and Gal4-CREB, by 1.9-fold and 3.6-fold, respectively, whereas there was no significant effect on Gal4-Sp1 (Fig. 5A). The effect of RHA on the CREB-mediated transactivation was reported previously [27]. These observations indicated that the effects of RHA on transactivation appeared relatively specific for NF-κB and CREB. To further examine whether the effect of RHA depends on the transactivation domain of p65, we used plasmids expressing various portions of p65 in fusion with Gal4 DNA-binding domain including Gal4-p65(1–551), Gal4-p65(1–286) and Gal4-p65(286–551). As shown in Fig. 5B, RHA augmented the transactivation mediated by Gal4-p65(1–551) and Gal4-p65(286–551) whereas there was no significant effect

Fig. 3. RHAmATP inhibits NF-κB-mediated transcription. (A) Inhibition of p65-mediated transcription by RHA mutant (RHAmATP) containing a single amino acid substitution in the helicase domain that abolishes its ATP-binding and helicase activity. HEK 293 cells were transfected with 20 ng of 4κB-luc in combination with pCMV-p65 (10 ng) or pCMV-RHAmATP expression plasmids (50 or 100 ng). Cells were harvested 24 h after transfection, and the luciferase activity was measured. (B) RHAmATP inhibits NF-κB-dependent transcription induced by TNF-α. HEK 293 cells were transfected with 4κB-luc (50 ng) in combination with pCMV-RHAmATP (50 or 100 ng) or the empty vector. After 24 h of transfection, cells were stimulated with 1 ng·mL^{-1} of TNF and harvested after additional incubation for 24 h (C) RHAmATP inhibits NF-κB-dependent transcription induced by NIK. HEK 293 cells were transfected with 4κBw-luc (50 ng) in combination with pCMV-NIK (10 ng) and pCMV-RHAmATP (50 or 100 ng). Cells were harvested 24 h after transfection, and the luciferase activity was measured. pCMV control plasmids were included such that all transfections had equivalent amounts of expression plasmid. Total DNA was kept at 0.5 μg with pUC19 plasmid. Cells were harvested 48 h after transfection, and luciferase activity was measured. Extents of fold activation of luciferase gene expression as compared to the transfection with reporter plasmid alone are indicated. Values (fold activation) represent the mean ± SD of three independent transfections. Similar results were achieved repeatedly.

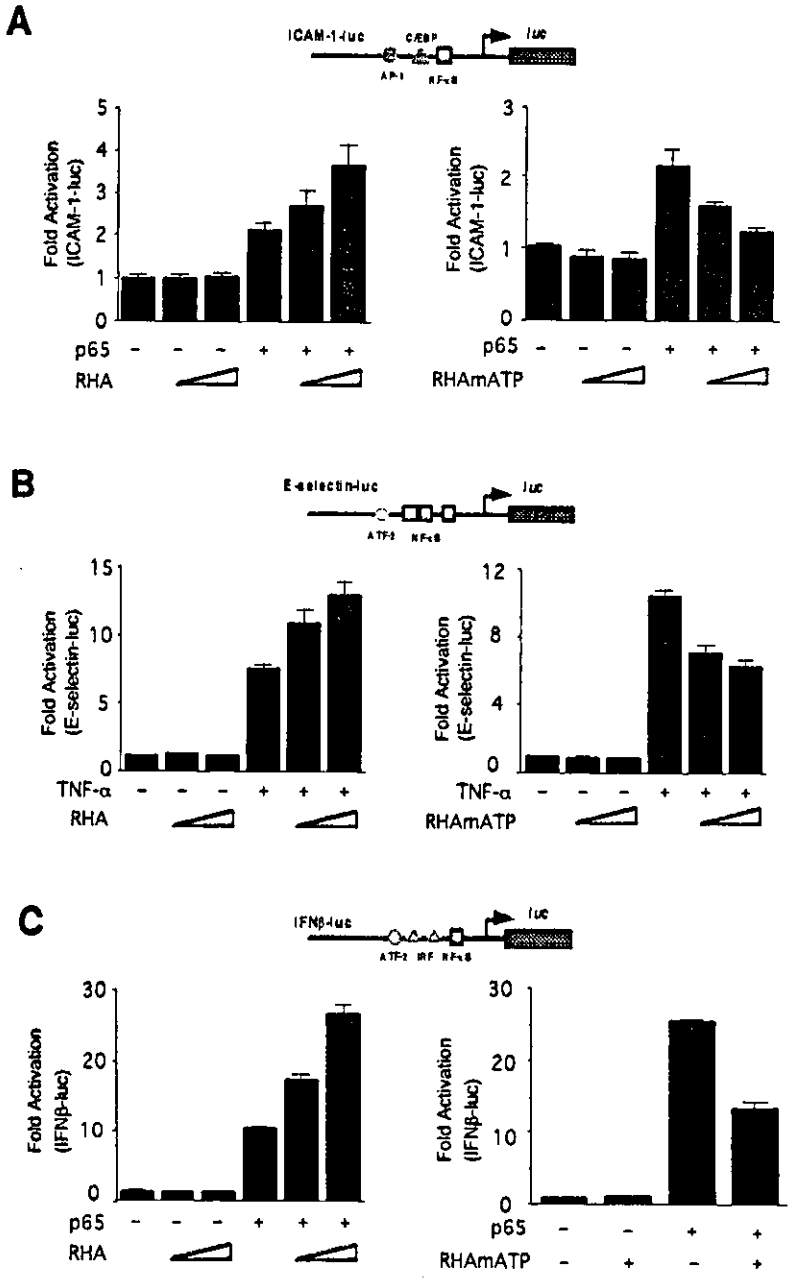


Fig. 4. RHA mediates NF-κB-dependent transcription in physiological promoters. (A) Effect of RHA on ICAM-1 promoter activity. HEK 293 cells were transfected with ICAM-1-luc (20 ng) in combination with pCMV-p65 (10 ng) and pCMV-RHA (50 or 100 ng) or pCMV-RHAmATP (50 or 100 ng). After 24 h of transfection, cells were harvested and luciferase activity was measured. (B) Effect of RHA on E-selectin promoter activity. HEK 293 cells were transfected with 20 ng of E-selectin-luc in combination with pCMV-RHA (50 or 100 ng) or pCMV-RHAmATP (50 or 100 ng). After 24 h of transfection, cells were stimulated with 1 ng·mL^{-1} of TNF-α and harvested after additional incubation for 24 h. (C) Effects of RHA on IFN-β promoter activity. HEK 293 cells were transfected with 20 ng of IFN-β-luc in combination with pCMV-p65 (10 ng) and pCMV-RHA (50 or 100 ng) or pCMV-RHAmATP (100 ng). After 24 h of transfection, cells were harvested and luciferase activity was measured. Values (fold activation) represent the mean ± SD of three independent transfections.

on Gal4-p65(1–286). These observations indicated that the C-terminal domain of p65 is required for the action of RHA.

Effect of RHA knockdown on the NF-κB-mediated transactivation

Finally, we investigated the physiological role of endogenous RHA with the use of RNA interference. We synthesized RNA duplex directed against the RHA-coding sequence (the nucleotide portion from 2408 to 2426). Transfection of HEK 293 cells with the RHA specific siRNA reduced the endogenous RHA protein level. The control siRNA had no effect (Fig. 6A). Neither RHA siRNA nor control siRNA had any effect on p65 and α-tubulin protein levels. We then examined the effect of RHA depletion on the NF-κB dependent reporter gene expression. As shown in Fig. 6B, the RHA siRNA reduced the NF-κB dependent gene expression from 4κB-luc induced by TNF-α. Similarly, we examined the effect of RHA siRNA on the TNF-mediated activation of E-selectin promoter. As shown in Fig. 6C, RHA siRNA significantly reduced the TNF-mediated induction of E-selectin gene expression. These data indicate that endogenous RHA is involved in the NF-κB-mediated gene expression.

Discussion

In this study we found that the NF-κB p65 subunit interacts with RHA *in vitro* and *in vivo*. Transient transfection assays revealed that RHA is positively involved in the

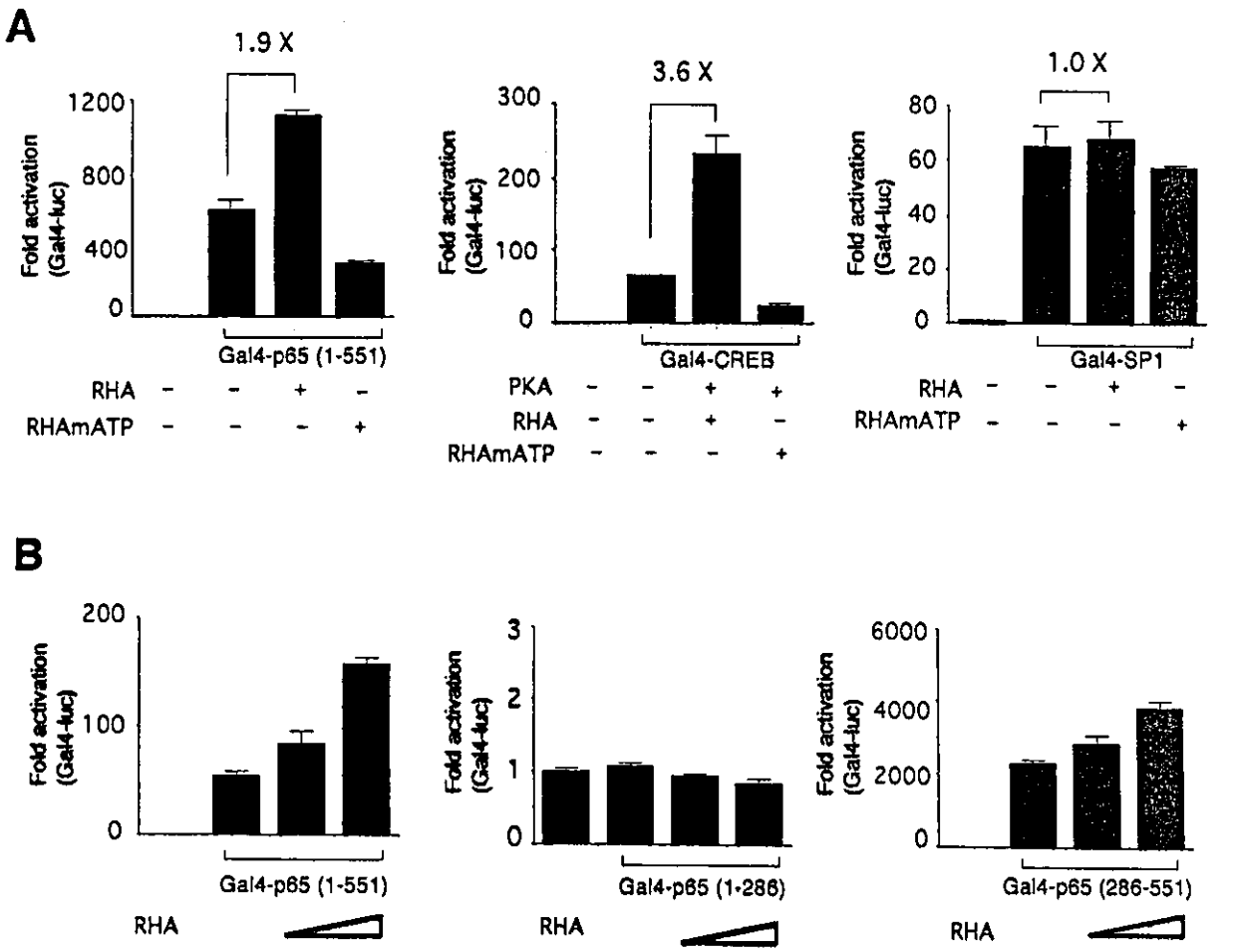


Fig. 5. Effects of RHA on Gal4-p65, Gal4-CREB and Gal4-Sp1-mediated transcription. (A) HEK 293 cells were transfected with 50 ng of 5x Gal4-luc reporter plasmid together with 10 ng of Gal4-p65 (left panel) or Gal4-CREB (10 ng) and PKA (10 ng) (middle panel) or Gal4-Sp1 (100 ng) (right panel) in combination with pCMV-RHA (100 ng) or pCMV-RHAmATP (100 ng). Cells were harvested 24 h after transfection and the luciferase activity was measured. Extents of fold activation of luciferase gene expression as compared to the transfection with reporter plasmid alone are indicated. (B) HEK 293 cells were transfected with 5x Gal4-luc reporter plasmid (50 ng) together with 10 ng of each of Gal4-p65 (1–551) (left panel), Gal4-p65 (1–286) (middle panel), Gal4-p65 (286–551) (right panel) and pCMV-RHA (100 or 200 ng). Cells were harvested 24 h after transfection, and luciferase activity was measured. Extents of fold activation of luciferase gene expression as compared to the transfection with reporter plasmid alone are indicated. Values (fold activation) represent the mean ± SD of three independent transfections.

NF-κB-dependent gene expression such as E-selectin, ICAM-1 and IFN-β. As NF-κB-dependent gene expression was inhibited by the dominant negative mutant form of RHA (RHAmATP) lacking the ATP-binding and helicase activity, the enzymatic activity of RHA is required for the transcriptional activation mediated by NF-κB.

RHA is a nucleic acid helicase that unwinds double-stranded DNA and RNA in ATP-dependent manner. It belongs to a large family of RNA helicases containing DEXD/H box that are known to be involved in various steps of gene expression including transcription, editing, splicing, RNA export, translation, and RNA turnover [31]. It is considered that RNA helicases prompt RNA molecules to initiate the interaction with other RNA molecules or proteins by catalyzing the folding and unfolding of these RNA molecules, just as proteins require chaperones to assist in folding and unfolding to form appropriate conformation [32,33].

RHA consists of two double-stranded RNA binding domains at the N-terminus, a helicase catalytic domain in the central part, and a Gly-rich single-stranded nucleic acid binding domain (RGG-box) at the C-terminus. Sequence analysis revealed that RHA contains seven helicase core motifs DEXD/H that are conserved among the helicase superfamily. It was shown previously that RHA stimulates transcription by interacting with CBP, BRCA1, and RNA Pol II [27,28]. Members of the ATPase/helicase family play important roles in many transcriptional processes including initiation, elongation, termination, and nuclear export [31]. For example, ATPase/helicase activity is found associated with TFIIH and chromatin remodeling complexes and plays crucial roles in transcriptional initiation and preinitiation. The ATPase/helicase activity of XPB/ERCC3 contained in TFIIH is required for promoter opening [34,35]. Similarly, the ATPase/helicase activity of SWI2/SNF2 in the chromatin remodeling complex SWI/SNF is involved

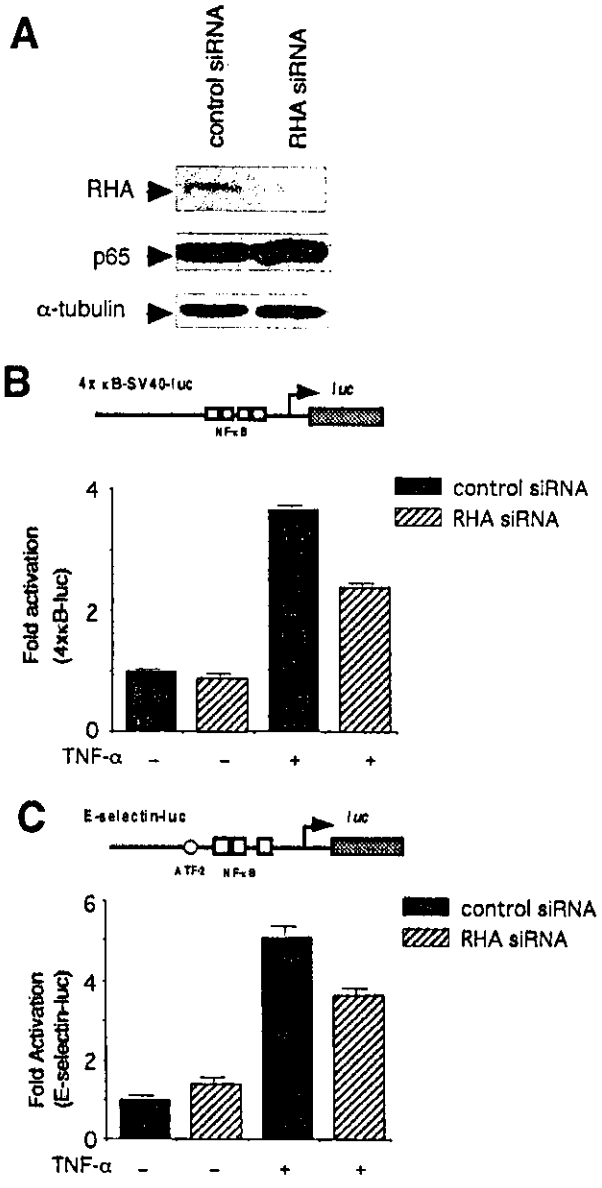


Fig. 6. Effect of RHA knockdown on NF-κB-mediated transactivation. (A) Knockdown of RHA by siRNA. HEK 293 cells (5×10^5) were transfected with 200 pmol of siRNA targeted to RHA. For the siRNA control, double-stranded RNA of unrelated sequences was used. The siRNA was transfected with lipofectamine 2000. After 48 h of transfection, cells were lysed and immunoblotted with antibodies to RHA, p65 and α-tubulin. (B) Inhibition of TNF-mediated NF-κB activation by RHA siRNA. HEK 293 cells (10^5) were transfected with 20 pmol of RHA siRNA or control siRNA together with 4κB-luc (20 ng). After 24 h of transfection, cells were stimulated with 10 ng·mL^{-1} of TNF-α and harvested after additional incubation for 24 h. (C) Inhibition of TNF-mediated E-selectin gene expression by RHA siRNA. HEK 293 cells (10^5) were transfected with 20 pmol of RHA siRNA or control siRNA together with E-selectin-luc (20 ng). After 24 h of transfection, cells were stimulated with 10 ng·mL^{-1} of TNF-α and harvested after additional incubation for 24 h. Extents of fold activation of luciferase gene expression as compared to the transfection with reporter plasmid alone are indicated. Values (fold activation) represent the means ± SD of three independent transfections. Similar results were obtained repeatedly.

in the relaxation of chromatin structure and promotes efficient transcription [36].

RHA was originally isolated as a human homologue of *Drosophila* maleless protein (MLE) [37]. MLE is involved in sex-specific gene dosage compensation and elevates the level of transcription derived from a single X-chromosome in male flies to a level equivalent to that derived from two X chromosomes in female flies [38]. MLE increases the transcriptional activity of X-linked genes through interaction with male-specific lethal (MSL) complexes [39,40]. In addition, the ATPase activity of RHA and that of MLE appeared to be essential for the CREB-dependent gene expression in mammals [27] and the gene dosage compensation in *Drosophila* [41], respectively. As MLE and its interaction with MSL are required for the specific histone H4 acetylation on X-chromosome [42,43], MLE may activate transcription of X-chromosome genes by promoting chromatin remodeling.

Another RNA helicase, p68 helicase belonging to the DEAD-box protein family, was shown to interact with human estrogen receptor α (ERα) and to act as a coactivator for ERα [44]. Although it was reported that RHA enhanced the CREB-dependent gene expression by bridging CBP and RNA Pol II, there has been no direct evidence that RHA interacts with CREB or any other gene-specific transactivators. In this study, we found that RHA binds to p65 through the interaction between the N-terminal region of RHA and the C-terminal GIR of p65. As the TA1-like and TA1 domains of p65 themselves recruit CBP/p300 coactivators, RHA appears to further facilitate the coactivator recruitment or assembly of transactivation complex by interaction with RNA Pol II.

Interestingly, we have reported previously that FUS/TLS activates the NF-κB-mediated transcription by interacting with the same region of p65 (amino acids 473–522) (GIR) [24]. There are some similarities between RHA and FUS/TLS. First, these proteins contain RGG domain that is capable of binding single-strand nucleic acids [45,46]. Second, they interact directly with the largest subunit of RNA Pol II and coactivator CBP/p300 [27,47]. Thus, NF-κB appears to form a functional transactivation complex ('enhanceosome') containing RHA, FUS/TLS, CBP/p300, RNA Pol II, and general transcription factors. Further studies are needed to clarify the action of RHA in transcriptional regulation.

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