Structural and functional analysis of the related transcriptional enhancer factor-1 and NF-κB interaction

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1College of Life Science, Liaoning University, Shenyang, China; 2Department of Biological Sciences, The University of Toledo, Toledo, Ohio; 3Cardiovascular Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; 4Division of Molecular Cardiology, Cardiovascular Research Institute, College of Medicine, Texas A & M University Health Science Center; 5Scott & White, Central Texas Veterans Health Care System, Temple, Texas

Submitted 31 January 2013; accepted in final form 1 October 2013

Ma J, Zhang L, Tipton AR, Wu J, Messmer-Blust AF, Philbrick MJ, Qi Y, Liu S-T, Liu H, Li J, Guo S. Structural and functional analysis of the related transcriptional enhancer factor-1 and NF-κB interaction. Am J Physiol Heart Circ Physiol 306: H233–H242, 2014. First published November 8, 2013; doi:10.1152/ajpheart.00069.2013.—The related transcriptional enhancer factor-1 (RTF-1) increases gene transcription of hypoxia-inducible factor 1α (HIF-1α) and enhances angiogenesis in endothelium. Both hypoxia and inflammatory factor TNF-α regulate gene expression of HIF-1α, but how RTF-1 and TNF-α coordinate regulate HIF-1α gene transcription is unclear. Here, we found that RTF-1 interacts with p65 subunit of NF-κB, a primary mediator of TNF-α. RTF-1 increased HIF-1α promoter activity, whereas expression of p65 subunit inhibited the stimulatory effect. By contrast, knockdown of p65 markedly enhanced RTF-1 stimulation on the HIF-1α promoter activity (7-fold). A physical interaction between RTF-1 and p65 was confirmed by coimmunoprecipitation experiments in cells and glutathione S-transferase (GST)-pull-down assays. A computational analysis of RTF-1 crystal structures revealed that a conserved surface of RTF-1 potentially interacts with p65 via four amino acid residues located at T347, Y349, R351, and Y352. We performed site-directed mutagenesis and GST-pull-down assays and demonstrated that Tyr352 (Y352) in RTF-1 is a key site for the formation of RTF-1 and p65-NF-κB complex. An alanine mutation at Y352 of RTF-1 disrupted the interaction of RTF-1 with p65. Moreover, expression of RTF-1 decreased TNF-α-induced HIF-1α promoter activity, IL-1β, and IL-6 mRNA levels in cells; however, the effect of RTF-1 was largely lost when Y352 was mutated to alanine. These results indicate that RTF-1 interacts with p65-NF-κB through Y352 and that they antagonize each other for HIF-1α transcriptional activation, suggesting a novel mechanism by which RTF-1 regulates gene expression, linking hypoxia to inflammation.

related transcriptional enhancer factor-1; nuclear factor κB; hypoxia-inducible factor-1α; tumor necrosis factor-α

RELATED TRANSCRIPTIONAL ENHANCER FACTOR-1 (RTF-1), also known as transcriptional enhancer activator (TEA) domain family member 4 (TEAD4), is a member of the transcriptional enhancer factor (TEF) family (21). The TEF-1 gene family, comprised of TEF-1, RTF-1, divergent TEF-1, and epidermal growth factor receptor-specific TF, is responsible for the regulation of expression of multiple genes in cardiac, skeletal, smooth muscle, and endothelial cells (15, 32, 33, 38, 41). All four members of the TEF-1 family share an evolutionarily conserved DNA binding domain, the so-called TEA or ATTS domain that specifically recognizes MCAT element CATN(T/C)(T/C) found in the promoter region of many genes in cardiac muscles (14, 37). Recently, several cofactors for TEF-1 family members have been identified, including p160 family of nuclear receptor coactivators (steroid receptor coactivator 1, transcriptional intermediary factor 2, and Ras-related C3 botulinum toxin substrate 3) (3), Src/Yes-associated protein (YAP)65 (40), the transcriptional coactivator with postsynaptic density 95/Drosophila disk large/zonula occludens-1-binding motif (29), and the vestigial-like proteins 2 and 4 (Vgl-2, -4) (5, 28). By interacting with other transcription factors, such as serum response factor (SRF), myocyte enhancer factor 1 (MEF2), and Max (2, 18, 19, 28), TEF-1 family members increase the activity of cardiac troponin T in myocardium and α-actin gene expression in skeletal muscle. Thus RTF-1 transcriptional networks for regulation of the target gene expression under different cellular context and environmental factors are complex and incompletely understood.

Hypoxia, a common pathophysiological phenomenon with a profound impact on the cellular responses and properties during many cardiovascular disease processes, induces gene expression of RTF-1 (22). In response to hypoxia, cells produce hypoxia-inducible factor (HIF)-1α, a transcription factor that is usually rapidly degraded via ubiquitination in normoxic conditions (hypoxic conditions promote HIF-1α protein stability) (12). Moreover, HIF-1α also can be activated under normoxic conditions, initiating inflammatory responses; in turn, inflamed lesions often become severely hypoxic (12, 31). NF-κB, a key mediator of inflammatory responses, links innate immunity to the hypoxic responses through transcriptional regulation of HIF-1α (23). We recently demonstrated that RTF-1 increases HIF-1α gene expression that promotes angiogenesis (36) and accelerates myocardial recovery from ischemia (22). We also demonstrated that RTF-1 in endothelial cells modulates blood glucose levels in vivo (30). However, whether RTF-1 regulates HIF-1α and hypoxia-related gene expression involving inflammatory factors and NF-κB is unknown.

In this study, we report that RTF-1 directly interacts with p65 subunit of NF-κB. By building up a molecular model of p65-NF-κB binding domain with the TEF-1 family members, we identified a conservative surface-exposed area on both RTF-1 and TEF-1 protein crucial for p65-NF-κB binding. Further analyses indicated that p65-NF-κB binding to RTF-1 resulted in a competitive and negative effect on RTF-1-mediated HIF-1α transcriptional regulation. These studies reveal an important combinatorial interaction between these two
classes of transcription factors and enhance our understanding of the relation between hypoxia and inflammatory pathways. Targeting this interactive pathway may provide an effective strategy to disrupt RTEF-1 and NF-\(\kappa\)B interactions in controlling hypoxia and inflammation-related gene expression.

**MATERIALS AND METHODS**

**Cell culture and siRNA transfection.** Human embryonic kidney (HEK)293 cells were maintained in DMEM containing 10% fetal bovine serum. Human microvascular endothelial cells (HMEC-1, Center of Disease Control) were cultured in the MCDB-131 medium containing 10% fetal bovine serum, 1% glutamine, 0.1% endothelial growth factor, and 0.1% hydrocortisone (Invitrogen). HMEC-1 cells were treated with 20 ng/ml TNF-\(\alpha\) (Sigma) or 20 \(\mu\)M wendelolacnone (Sigma) for 5 h in the culture medium before harvest and measurement. The primers for p65 and p50 siRNA were synthesized by GenePharma, and the sequences are as follows: siRNA1: 5'-GGGACAUUGAGACCUCUAAATT-3'; siRNA2: 5'-GCUGAUUGUCCACCGAAGG-3'; p50: siRNA1: 5'-AAGGGGCAUAUACCUGCUAC-3'; siRNA2: 5'-AUAGUUACUGAAUACUG-3'. An siRNA targeting none of the genes in human/mouse genome was used as a control.

**Gene transfection and reporter gene analysis.** HEK293 cells were seeded in 24-well plates and transfected using polyethylenimine (Polysciences). Human pHIF-1\(\alpha\) (557/300)-Luc reporter construct was kindly provided by Drs. Scot Ebbinghaus and Daekyu Sun, University of Arizona. The PGL2-TK-HRE-Luc plasmid containing the three copies of the hypoxia response element (HRE) (5'-GTGTCACGTGCTGCTAG-3') on the luciferase reporter promoter region was provided by Giovanni Melii (Developmental Therapeutics Program, National Cancer Institute). Transfections with 300 ng of either reporter plasmid pHIF-1\(\alpha\) (557/300)-Luc or PGL2-TK-HRE-Luc, or with or without 300 ng of pXJ40-RTEF-1, pXJ40-TEF-1, pXJ40-p65, or pXJ40-p50 expression plasmids, were performed in HEK293 cells. Empty vector pXJ40 was used as a control. Luciferase activity was determined by the dual luciferase assay system (Promega). For the endothelial cell transfection, we used the retrovirus-expressing control medium before harvest and measurement. The primers for p65 and p50

**In vitro GST-pull-down binding assay.** Transfected cells with the PGL2-TK-HRE-Luc reporter construct were incubated for 5 h in a hypoxia chamber before harvest and measurement of luciferase activity.

**Immunoblot analysis and immunoprecipitation.** Transfected cells were washed with cold PBS twice and lysed in cold RIPA buffer (Boston Bio-Products), which contains 50 mM Tris-HCl, \(pH\) 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Roche). Protein concentrations were determined with the DC Protein Standard Assay (Bio-Rad). Samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Whatman), and subsequently blocked in TBS-Tween 20 containing 5% non-fat milk for 1 h. The membranes were incubated with the indicated primary antibodies: polyclonal anti-RTEF-1 antibody (1:10,000 dilution, Genemed Synthesis), monoclonal anti-vinculin antibody (1:100,000, Sigma), polyclonal anti-p50 antibody (1:1,000), polyclonal anti-p65 antibody (1:1,000, Santa Cruz Biotechnology), and monoclonal anti-glutathione S-transferase (GST) (1:10,000) followed by incubation with horseradish peroxidase-conjugated secondary antibodies anti-mouse IgG (1:3,000), anti-goat IgG (1:2,000, Calbiochem), or anti-mouse IgG (1:5,000 dilution, Vector Laboratories). Blots were developed using the chemiluminescence detection system (Thermo Fisher). Densitometric analysis was performed using the NIH software Image J. For immunoprecipitation, nuclear extracts (50 \(\mu\)g protein) were prepared from HEK293 cells overexpressing RTEF-1 or NF-\(\kappa\)B. After hypoxic stimulation for 5 h, 500 \(\mu\)g of the cellular protein extracts were incubated with 0.5 \(\mu\)g normal goat serum, 0.8 \(\mu\)g anti-p65, or 0.8 \(\mu\)g anti-p50 antibodies at 4\(^\circ\)C for 3 h. Alternatively, 50 \(\mu\)g of nuclear extracts was incubated with 1 \(\mu\)g anti-HA antibody or 0.8 \(\mu\)g anti-RTEF-1 antibody at 4\(^\circ\)C for 3 h. Protein A/G-agarose was then added and incubated for 1.5 h at 4\(^\circ\)C. Immunocomplexes were washed with NP-40 lysis buffer five times, resolved by SDS-PAGE, and then analyzed by immunoblotting using antibodies against RTEF-1, p50, or p65.

**Gene cloning and site-directed mutagenesis.** TEF-1 cDNA and RTEF-1 cDNA were generated by PCR amplification using oligonucleotides as follows and cloned into pENTR/D-TOPO (Invitrogen). The PCR primer sequences used are as follows: TEF-1: 5'-CAGCGAGAGGTGATGACTCGG-3' and 5'-TCAGTCCTTTACAGCCCTCTAG-3'; RTEF-1: 5'-GAGCTGACGTGCTGCTACT-3'. The underlined sequences denote the site that was mutated, and all constructs were carried out DNA sequencing from Genewiz.

**Recombinant protein expression and purification.** The p65 gene was cloned into pGEX-4T expression vector (Pharmacia) and induced with 0.1 mM isopropyl-\(\beta\)-thiogalactopyranoside for expression in Escherichia coli BL21 at 28\(^\circ\)C, in which p65-GST recombinant protein was purified using glutathione-agarose columns (Pharmacia). The concentration of recombinant proteins was assessed by measuring the absorbance at 280 nm. For each reaction, the amount of recombinant protein used was determined with the DC Protein Standard Assay (Bio-Rad).

**Quantitative real-time PCR analysis.** Total RNA was extracted from cells overexpressing RTEF-1 using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. A total of 2 \(\mu\)g of RNA was reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems) with random primers according to the manufacturer's protocol. Quantitative real-time PCR amplification was performed using the SYBR Green Master Mix kit (Applied Biosystems). The PCR primer sequences used are as follows: IL-8: 5'-TCCCAAGCCCTTCTTTTGCAG-3' and 5'-TAAGAACAATAAGTGCCGCGG-3'; IL-6: 5'-GGTACATCCTGACGGGCAATCT-3' and 5'-GTCCTTTGTGTGAGACCACTTCTG-3'; and IL-1\(\beta\): 5'-GACGGCATCT-3' and 5'-AAAGTAAGTGCTTCTTCTTGAGACCACTTCTG-3'. The underlined sequences denote the sites that were mutated, and all constructs were carried out DNA sequencing from Genewiz.

**In vitro GST-pull-down binding assay.** The troponin T-coupled rabbit reticulocyte lysate system (Promega) was used to translate wild-type and mutant RTEF-1 in vitro. The integrity of translated proteins was analyzed by immunoblotting. For binding assays, in vitro translated wild-type or mutant RTEF-1 was incubated with either 700 \(\mu\)g of GST alone or GST-tagged p65 bound to glutathione-agarose beads in 1 \(\times\) protein interaction buffer (PIB) [40 mM Tris-HCl \(pH\) 7.5, 150 mM NaCl, 5 mM MgCl\(_2\), 0.5% NP-40, 5% glycerol, 1 mM tris(2-carboxyethyl) phosphine, 200 \(\mu\)g/ml BSA] for 4 h at 4\(^\circ\)C with continuous shaking. Proteins on the bead were washed with 1 \(\times\) PIB three times, resolved by SDS-PAGE, and analyzed by immunoblotting.

**AJP-Heart Circ Physiol · doi:10.1152/ajpheart.00069.2013 · www.ajpheart.org**
Sequence alignments. The protein sequences of TEAD1/TEF-1(NP_068780.2), TEAD2/TEF-4(NP_003589.1), TEAD3/TEF-S(NP_003205.2), and TEAD4/RTF-1(NP_003204.2) were obtained from the National Center for Biotechnology Information (NCBI). These sequences were subjected to multiple sequence alignment using Clustal W2.0.12 under default parameters (8).

Construction of protein structural modeling. Available mouse RTF-1 crystal structure (PDB code: 3JUA) with 96% amino acid sequence identity to human RTF-1 was analyzed by Position-Specific Iterated BLAST search (1). Homology model of human RTEF-1 was generated using MODELLER 9v8 based on a template clustered using the Rosetta Cluster application with a cluster radius cutoff of 4 Å, and then a representative model is subsequently selected.

Molecular docking. Two widely accepted protein-protein docking programs, ZDOCK (7) and RosettaDock (16), were used to predict and assess the interactions between RTEF-1, TEF-1, and NF-κB (p50, p65, and NF-κB heterodimer, respectively). ZDOCK uses fast Fourier transform to globally search rigid-body transformations of two proteins and is amenable to large-scale decoy generation (7). The RosettaDock was run in full atom mode, allowing spin around the axis to connect the two proteins through the standard Monte Carlo movements. First, the initial stage-docking program ZDOCK was used as a rigid-body globally searching method to generate 2,000 poses for each docking. The top 100 poses were chosen by their score, which is determined by the scoring function of ZDOCK, which consists of a van der Waals term and an electrostatic term. Second, the 100 structures were clustered using the Rosetta Cluster application with a cluster radius cutoff of 4 Å, and then a representative model is subsequently selected.

Fig. 1. Related transcriptional enhancer factor (RTF)-1-stimulated hypoxia-inducible factor (HIF)-1α promoter activity is regulated by expression of p65-NF-κB subunit. A: human embryonic kidney (HEK)293 cells were cotransfected by pHIF-1α-Luc reporter gene together with the plasmid DNA expressing p65, p50, or RTF-1, as indicated, and luciferase activity was measured 24 h posttransfection and control vector pXJ40 used as a control. *P < 0.05 vs. control pXJ40. B: HEK293 cells were cotransfected by pHIF-1α-Luc together with p65, p50, and TEF-1 expression vectors, and luciferase activity was measured 24 h posttransfection. *P < 0.05 vs. control pXJ40. C: HEK293 cells were cotransfected by pHIF-1α-Luc together with RTF-1 or control plasmid pXJ40, treated with 20 μM wedelolactone (solid bars, IKK inhibitor) or with vehicle (0.01% DMSO) for 5 h, and luciferase activity was measured. *P < 0.01 vs. control. D: HEK293 cells were cotransfected by phospho-hypoxia response element (HRE)-Luc together with p50, p65, and RNK-1 expression vectors, and luciferase activity was measured. *P < 0.05 vs. p65. E: HEK293 cells were transfected with siRNA against p65, p50, or a control, and expression of endogenous p65, p50, and vinculin was determined by Western blot. HIF-1α promoter activity was measured after cotransfection with the RTF-1 expression vector for 18 h. Results from 3 independent experiments are shown as means ± SD. *P < 0.05 vs. control siRNA (c).
according to the best score from each obvious large cluster. Then each of the representative models was validated by using the local enrichment method of the RosettaDock program. The search procedure is repeated from different random starting orientations to create 10,000 decoys, whose energy scores were plotted against the root mean square distance from the starting input conformation. The models that achieved a “docking funnel” are considered to be the most robust complex structures (27).

Protein-protein interface analysis. The structures of the complex were submitted to protein-protein interface analysis server (PROTORS) (34) to analyze their Interface Accessible Surface Area.

Computational alanine scanning to predict the hotspots in the complex interface. The RTEF-1-p65 and TEF-1-p65 complex were submitted to the Robetta Alanine scanning server to predict the free energy changes brought about by alanine mutation at protein-protein interfaces by using a simple free-energy function (25). The alanine scan free-energy changes helped us identify the hotspot residues in the complex interface. This analysis served as an initial approach to identify the amino acid residues that are crucial for the interaction between the TEF family members and NF-κB.

Statistical analysis. Data presented are from at least three independent experiments. For statistical analysis, if differences were established, the values were compared using the Student’s t-test. Values were expressed as means ± SD. The results were considered significant at P < 0.05 or P < 0.01 as indicated.

RESULTS

RTEF-1-stimulated HIF-1α promoter activity is regulated by expression of p65-NF-κB subunit. Many of stimuli that induce HIF-1α gene expression are known to activate a number of transcription factors such as NF-κB (39), and RTEF-1 increases HIF-1α in endothelial cells, enhancing the vascular endothelial growth factor (VEGF) gene expression and promoting angiogenesis, as shown in our previous studies (22, 36). We next examined whether expression of NF-κB affects the RTEF-1-mediated transcriptional regulation of HIF-1α in cells. HEK293 cells were transfected with a HIF-1α promoter (−557/+300)-coupled luciferase reporter gene alone or cotransfected with plasmid DNA expressing RTEF-1, p50, or p65 of NF-κB subunit. As we previously demonstrated, expression of RTEF-1 increased the HIF-1α promoter activity ~2.5-fold (P < 0.05), whereas expression of p50 and p65-NF-κB individually or together had no such effect (Fig. 1A). By contrast, expression of p65, rather than p50, significantly reduced RTEF-1 and TEF-induced HIF-1α promoter activity (Fig. 1, A and B), suggesting that NF-κB negatively regulates the ability of RTEF-1 to increase the HIF-1α promoter activity. Conversely, treatment of an IKK-β inhibitor (20 μM wedelolactone) in cells increased RTEF-1-stimulated HIF-1α promoter activity threefold (P < 0.01, Fig. 1C). To further confirm the role of p65 in regulating RTEF-1-mediated HIF-1α promoter activity mediated by HRE, we transfected cells with a heterologous reporter gene that contains three consecutive copies of HRE-coupled luciferase (pHRE-luc), and expression of p65, rather than p50, reduced HRE-luc activity by 55% (P < 0.01, Fig. 1D). To determine the role of loss of p65 in RTEF-1-regulated HIF-1 promoter activity, we depleted endogenous expression of p65 by two different siRNA preparations, each of which increased RTEF-1-stimulated HIF-1α promoter activity by sevenfold (P < 0.01, Fig. 1E), whereas depletion of p50-NF-κB had no significant effort on the RTEF-1-stimulated HIF-1α promoter activity (Fig. 1E). Taken together, these results indicate that expression of p65-NF-κB regulates RTEF-1-mediated HIF-1α promoter activity.

p65-NF-κB interacts with RTEF-1. To test whether p65-NF-κB interacts with RTEF-1 in vitro, we extracted and purified recombinant p65-GST from bacteria and incubated with in vitro translated RTEF-1 for GST-pull-down assay. Incubation of p65-GST, rather than GST alone, pulled down RTEF-1, indicating that an interaction between p65 and RTEF-1 occurred (Fig. 2A). To further determine whether the interactions occur in vivo, HEK293 cells were transfected with plasmid DNA expressing RTEF-1 or control hemagglutinin-epitope, and immune complexes were isolated from nuclear extracts and examined for the coprecipitation of p50 and p65. In agreement with the observation from in vitro studies, p65 was coprecipitated with RTEF-1. Also, p50 was coprecipitated in the complex purified with antibody against RTEF-1 (Fig. 2B). Thus, the reciprocal immunoprecipitation of p65 and RTEF-1 from nuclear extracts confirmed a physical interaction of each other.

Computational structural analysis of RTEF-1 and NF-κB interaction surface. We next used two protein-protein docking programs (7, 16) to predict and assess the interactions between RTEF-1 and p65-NF-κB. Analysis of the crystal structures of RTEF-1 [protein data bank identification number (PDB ID):
we mutated each to alanine, a neutral amino acid, and trans-

muted them in vitro and examined their interaction for p65-GST in the GST-pull-down assay. Compared with RTEF-1-wild-
type (WT), all four alanine mutants of RTEF-1 displayed reduced binding affinity to p65-GST (Fig. 5A, A and B). More-
over, RTEF-1-Y352A mutation markedly reduced the interac-
tion between RTEF-1 and p65 (Fig. 5A), and p65 binding activity for RTEF-1-Y352A only reached <10% of RTEF-
1-WT (P < 0.01, Fig. 5B).

To examine the role of RTEF-1-Y352, which is required for p65-NF-κB interaction, in RTEF-1-stimulated HIF-1α promoter activity in cells, we examined the HIF-1α promoter activity in cells cotransfected with p65, RTEF-1-WT, or the RTEF-1 mutant. Coexpression of p65 together with RTEF-1-T347A, Y349A, or R351A decreased HIF-1α promoter activity by 50%, compared with the effect of RTEF-1-WT (Fig. 5C). By contrast, expression of RTEF-1-Y352A mutant completely disrupted the inhibitory effect of p65 expression on the pro-
moter activity (P < 0.01, Fig. 5C), suggesting that the amino
acid residue at Y352 of RTEF-1 interacts with p65 and mediates
the inhibitory effect of p65-NF-κB.

RTEF-1 decreases TNF-α-induced HIF-1α promoter activity through Y352. p65-NF-κB is a well-studied transcription
factor mediating inflammatory responses (31) and is rapidly
activated by cytokines, such as TNF-α or bacterial products.
Prolonged TNF-α exposure is present during inflammatory
conditions, and we investigated whether RTEF-1 regulates
TNF-α-induced HIF-1α promoter activity via Y352. Treatment
of TNF-α inhibited RTEF-1 stimulation on the HIF-1α pro-
moter activity by 40% (P < 0.05, Fig. 6A); however, the inhibitory effect of TNF-α was almost completely abolished
when RTEF-1-Y352 was changed to an alanine that blocks p65
interaction (P > 0.1, Fig. 6A). To further examine whether endogenous NF-κB has an effect on RTEF-1-stimulated HIF-1α
promoter, treatment of 20 μM wortmannin for IKK inhibi-
tion blocking p65 activity increased the ability of RTEF-1 to
promote HIF-1α promoter activity threefold (P < 0.01, Fig.

Identification of Y352 of RTEF-1 in NBD domain required
for interaction with NF-κB. We next used the computational
alanine scanning program to predict the hotspots in the complex
interface of RTEF-1, TEF-1, and p65, as shown in Table 1. With the
criteria that ΔΔG > 1.0 kcal/mol as a first round of selection
factor, two homologous residues in RTEF-1 (Y349, Y352) and
TEF-1 (Y341, F344) were picked out to be of significance. We
next used the second factor for selection, which is the percenti-
tile of Interface Accessible Surface Area as shown in Table 2.
The analysis indicated that another two more conserved resi-
dues are important within the RTEF-1 NBD located at T347
and R351 and the TEF-1 NBD located at T339 and R343, of
which percentile Interface Accessible Surface Area is greater
than 5%. To determine the role of four residues in RTEF-1
(T347, Y349, R351, and Y352) in interaction with p65-NF-κB,
we mutated each to alanine, a neutral amino acid, and trans-

3JUA; Fig. 3A) and p65-NF-κB subunits (PDB ID: 2RAM),
p50 (PDB ID: 1SVC), or the p50/p65 heterodimer (PDB ID:
1VKX; Fig. 3A) predicted an interaction between RTEF-1 and
p65, rather than p50-NF-κB (Fig. 3B). Similar results were
found between p65-NF-κB and TEF-1, another TEF family
member (PDB ID: 3KYS) (Fig. 3C).

A conserved domain of the TEF family interacts with
p65-NF-κB. Sequence alignment analysis indicated that the
TEF family members have two conserved regions, one is the
TEA domain located at the NH2-terminal end for DNA inter-
action, and the other is the NF-κB binding domain (NBD)
located at the COOH terminus (Fig. 4, A and B). Further
analysis of the RTEF-1 and p65-NF-κB interaction surface
revealed several key amino acid residues that are located in β7
and β8 of the IgG-like fold of RTEF-1, likely involving the
interaction with p65 (Fig. 4C). Furthermore, the NBD of
RTEF-1 appears to encompass the region between amino acids
231 and 434 (Fig. 4, B and C). Moreover, the NBD of RTEF-1
overlaps with the previously identified YAP binding domain
of TEAD2 (6).

Identification of Y352 of RTEF-1 in NBD domain required
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than 5%. To determine the role of four residues in RTEF-1
(T347, Y349, R351, and Y352) in interaction with p65-NF-κB,
we mutated each to alanine, a neutral amino acid, and trans-

6B); however, the stimulatory effect was abolished by RTEF-1-Y352A mutation (Fig. 6B). We further assessed the effect of RTEF-1-Y352A mutation on TNF-α-regulated HRE-heterologous reporter gene activity in cells; TNF-α treatment suppressed RTEF-1-stimulated HRE-promoter activity by 50% under either normoxia or hypoxia \((P < 0.05, \text{Fig. 6C})\), and TNF-α was unable to suppress RTEF-1-Y352A-stimulated HRE-promoter activities in cells (Fig. 6C).

RTEF-1 blocks TNF-α-induced genes in endothelial cells via Y352. Last, we examined whether RTEF-1-Y352 affects TNF-α-induced expression of genes, such as IL-1β and IL-6 in endothelial cells. Either RTEF-1 or RTEF-1-Y352A mutant was highly expressed in HMEC-1 cells after retrovirus infection of each gene, and both IL-1β and IL-6 mRNA levels were induced three- to fivefold by TNF-α \((P < 0.01)\), of which induction was decreased by RTEF-1 overexpression in cells.

Fig. 4. A conserved domain of TEF family members interacts with p65-NF-κB. A: sequence alignment of the TEF family members. The highly conserved domain in the NH2 terminus is transcriptional enhancer activator (TEA), and the other highly conserved domain in the COOH terminus is NF-κB binding domain (NBD). B: schematic domain organization of RTEF-1, TEF-1 and p65. The amino acid residue numbers for different domain boundaries are indicated. TEA, DNA-binding domain; DBD, p65 DNA-binding domain; TAD, transactivation domain. C: surface area of the RTEF-1/p65-NF-κB complex was analyzed by the protein-protein interface analysis server (PROTORP). p65 is shown in green, RTEF-1 in red, and TEF-1 in yellow. Diagrams of the interface between RTEF-1/p65 (left) and TEF-1/p65 (right) are shown. The putative key amino acid residues in RTEF-1 and TEF-1 are indicated for the interaction with p65-NF-κB.
However, expression of RTEF-1-Y352A mutant completely disrupted the TNF-α suppression on both IL-1β and IL-6 gene expression in the HMEC-1 cells (Fig. 7, B and C).

DISCUSSION

In this study, we present three important findings: 1) RTEF-1 interacts with p65-NF-κB subunit, and this interaction inhibits RTEF-1-stimulated HIF1 promoter activity; 2) we identified that Y352 in RTEF-1 has a key role in mediating the interaction between RTEF-1 and p65; and 3) RTEF-1 inhibits TNF-α and p65-stimulated HIF-1α promoter activity and expression of genes, such as IL-1β and IL6 in cells, whereas mutation of Y352A in RTEF-1 abolishes the effect of RTEF-1 inhibition. These results suggest that an interaction of RTEF-1 with p65-NF-κB via Y352 has important roles in control of gene expression, hypoxia, and inflammatory responses.

Table 1. Computational alanine scanning to predict hotspots in the complex interface

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>RTEF-1 DDG</th>
<th>TEF-1 DDG</th>
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<tbody>
<tr>
<td>GLU234</td>
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<td></td>
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<tr>
<td>ASP240</td>
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<td>GLU346</td>
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<tr>
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</tr>
<tr>
<td>ARG402</td>
<td>−0.06</td>
<td>ARG394</td>
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List of amino acids in related transcriptional enhancer factor (RTEF)-1 or transcriptional enhancer factor (TEF)-1 on the interface. DDG, changed free energy in response to the amino acids of TEF with a point mutation into Ala. DDG >1.0 kcal/mol implies a loss of binding affinity upon mutation, and DDG <−1.0 implies a reduction of binding affinity upon mutation.

($P < 0.01$, Fig. 7, B and C). However, expression of RTEF-1-Y352A mutant completely disrupted the TNF-α suppression on both IL-1β and IL-6 gene expression in the HMEC-1 cells (Fig. 7, B and C).

DISCUSSION

In this study, we present three important findings: 1) RTEF-1 interacts with p65-NF-κB subunit, and this interaction inhibits RTEF-1-stimulated HIF1 promoter activity; 2) we identified that Y352 in RTEF-1 has a key role in mediating the interaction between RTEF-1 and p65; and 3) RTEF-1 inhibits TNF-α and p65-stimulated HIF-1α promoter activity and expression of genes, such as IL-1β and IL6 in cells, whereas mutation of Y352A in RTEF-1 abolishes the effect of RTEF-1 inhibition. These results suggest that an interaction of RTEF-1 with p65-NF-κB via Y352 has important roles in control of gene expression, hypoxia, and inflammatory responses.

Table 2. Protein-Protein Interface Analysis

<table>
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<th>% Interface Accessible Surface Area</th>
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<tr>
<td>RTEF-1-p65</td>
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<td>PHE313 7.95</td>
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<td>ASN325 5.59</td>
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<td>THR347 5.09</td>
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<td>ARG351 6.71</td>
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<tr>
<td>HIS362 5.04</td>
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<tr>
<td>ARG363 9.69</td>
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</table>

List of the amino acids whose surface area on the interface is >5%.

Fig. 5. Identification of the key amino acid residues in RTEF-1 for p65-NF-κB interaction. A: in vitro synthesized RTEF-1 and its mutant proteins were incubated with beads bound to recombinant protein GST or GST-p65. After being washed, proteins bound were immunoblotted with the antibody of RTEF-1. 10 % of each protein was used as input for comparison. B: binding of RTEF-1 and its mutant protein toward p65 examined in A were quantified and normalized against that of RTEF-1-wild-type (WT). Results are from 3 independent experiments, and means ± SD are shown. *P < 0.01 vs. WT. C: HEK293 cells were cotransfected by pHIF-1α-Luc together with control vector pXJ40 and expression vectors for p65-NF-κB, RTEF-1-WT, and RTEF-1 mutant T347A, Y349A, R351A, or Y352A. Luciferase activity was measured 24 h posttransfection, and results from 3 independent experiments are shown as means ± SD. *P < 0.05 vs. the control.
RTEF-1 has important roles in transcriptional regulation of genes in cardiovascular systems. Our previous reports indicate that RTEF-1 enhances hypoxia-related angiogenesis through the transcriptional regulation of VEGF and HIF-1α in endothelial cells (21, 22). Activation of gene transcription is a multistep process that is triggered by transcription factors and cofactors directing transcriptional initiation; the multiple cofactors for TEF family members include the p160 family of nuclear receptor coactivators (3), YAP65, Vgl-2, Vgl-4 (40), and the muscle-specific transcription factors SRF, MEF2, and Max (17). Here we have identified that the MCAT element, a consensus binding site for RTEF-1, and the NF-κB binding site are localized in a close proximity in the HIF-1α promoter. Based on the structural analysis, Thr374, Tyr349, Arg351, and Tyr352 in the COOH-terminal region of RTEF-1 might interact with p65 or other yet unidentified binding partners. Among those potential residues, Tyr352 was determined as a key residue in response to the interaction between RTEF-1 and NF-κB. This result suggests that the computer-based protein-protein interaction program is a powerful tool to identify a potential interaction between RTEF-1 and NF-κB, which was supported by in vitro cell-based experiments. Of note is that tyrosine is an amino acid that can be modified, such as by protein tyrosine kinases via phosphorylation-based mechanisms, and markedly contributes to conformational changes of the protein structure and function; thus whether there exists...
RTEF-1 potently increases the HIF-1α promoter activity, but p65-NF-κB has limited its capacity to induce the promoter activity. NF-κB is a pleiotropic protein complex formed by a family of subunits including RelA (p65), RelB, cRel, p52, and p50 (20). Recent studies have demonstrated that stimulation of HIF-1α gene by NF-κB provides an important, parallel level of regulation on HIF-1α expression (4, 35, 39). In the absence of p65-NF-κB, HIF-1α gene is not transcribed; hence no stabilization or activity is seen, even after prolonged hypoxia exposures (24, 39). There have been several studies demonstrating cross-talk between the NF-κB and HIF signaling pathways, but the mechanism is unclear. In our studies, we found that overexpression of p65 or treatment of IKK inhibitor in HEK293 cells had minimal effects on increasing or reducing the HIF-1α promoter activity, respectively (Fig. 1, A and C) and endogenous HIF-1α was also barely detectable (data not shown); however, p65 expression and the IKK inhibition had profound effects on RTEF-1 stimulation on the HIF-1α promoter activity. In particular, we demonstrated that NF-κB is a cofactor for RTEF-1 in control of both HIF-1α promoter activation and expression of cytokines, such as IL-1β and IL-6. We identified a region in RTEF-1 that directly interacts with p65-NF-κB in vitro by both coimmunoprecipitation and GST-pull-down assay. A loss of p65 enhanced the capacity of RTEF-1 to stimulate HIF-1α transcriptional regulation, whereas overexpression of p65 decreased TEF-dependent HIF-1α promoter activity. Moreover, activation of NF-κB following TNF-α treatments inhibited both RTEF-1-stimulated HIF-1α promoter activity and RTEF-1-activated HRE responses under normoxic and hypoxic conditions, supporting the notion that there is a competitive negative regulation on HIF1 by RTEF-1 and p65.

NF-κB transcription factor plays key roles in mediating inflammatory responses, and TNF-α is a proinflammatory cytokine with important roles in regulating inflammation, cell proliferation, and apoptosis. TNF-α activates p65-NF-κB and promotes its nuclear translocation in control of gene expression for cell survival. The endothelial cells from mice lacking IKK-β indicate that basal NF-κB activity is required for HIF-1 expression (35). p65-NF-κB binds a distinct element in the proximal promoter of HIF-1α gene and transcriptionally induces HIF-1α (39), suggesting that NF-κB promotes HIF-1α gene transcriptional expression. In this study, expression of RTEF-1 markedly inhibited the ability of TNF-α to stimulate HIF-1α promoter activities and endogenous gene expression of IL-1β and IL-6. However, these effects were largely blocked by RTEF-1-Y352A mutation, supporting the notion that RTEF-1 and its regulation via the Y352 affect expression of p65-NF-κB by interacting with the Y352 of RTEF-1. Arrows indicate stimulation, and T-bars stand for inhibition.

ACKNOWLEDGMENTS

We thank Dr. Lawrence M. Pfeffer (University of Tennessee) for the p65 construct, Dr. Melillo Giovanni (NIH-NCI) for the HRE construct, and Dr. Alexandre Stewart (University of Ottawa) for the pXJ40/RTEF-1 construct.

GRANTS

This work was supported in part by NIH Grant HLR01082837 (J. Li) and the American Heart Association BGIA7880040 (S. Guo). This material is the result of work supported with resources and the use of facilities at the Central Texas Veterans Health Care System, Temple, Texas.

DISCLOSURES

This work was prepared while Jian Li was employed at the Cardiovascular Institute, Beth Israel Deaconess Medical Center, Harvard Medical School. The opinions expressed in this article are the author’s own and do not reflect the
view of the National Institutes of Health, the Department of Health and Human Services, or the United States Government. No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


