The nuclear factor κB (NF-κB) transcription factor family regulates genes involved in cell proliferation and inflammation. The promoters of these genes often contain NF-κB-binding sites (κB sites) arranged in tandem. How NF-κB activates transcription through these multiple sites is incompletely understood. We report here an X-ray crystal structure of homodimers comprising the RelA DNA-binding domain containing the Rel homology region (RHR) in NF-κB bound to an E-selectin promoter fragment with tandem κB sites. This structure revealed that two dimers bind asymmetrically to the symmetrically arranged κB sites at which multiple cognate contacts between one dimer to the corresponding DNA are broken. Because simultaneous RelA-RHR dimer binding to tandem sites in solution was anti-cooperative, we inferred that asymmetric RelA-RHR binding with fewer contacts likely indicates a dissociative binding mode. We found that both κB sites are essential for reporter gene activation by full-length RelA homodimer, suggesting that dimers facilitate DNA binding to each other even though their stable co-occupation is not promoted. Promoter variants with altered spacing and orientation of tandem κB sites displayed unexpected reporter activities that were not explained by the solution-binding pattern of RelA-RHR. Remarkably, full-length RelA bound all DNAs with a weaker affinity and specificity. Moreover, the transactivation domain played a negative role in DNA binding. These observations suggest that other nuclear factors influence full-length RelA binding to DNA by neutralizing the transactivation domain negative effect. We propose that DNA binding by NF-κB dimers is highly complex and modulated by facilitated association–dissociation processes.

The NF-κB family of dimeric transcription factors regulates the expression of a multitude of genes involved in cell proliferation, survival, and inflammation. NF-κB dimers are combinatorially formed from five subunits: p50, p52, RelA/p65, cRel, and RelB. The dimers regulate transcription by sequence-specific binding to DNA that is collectively known as the κB site. The 3D X-ray structures of several NF-κB–DNA complexes identified a common DNA recognition mode by the dimers. The G:C-rich flanking sequences of κB sites contact NF-κB with sequence specificity, whereas the central sequences are recognized with less sequence specificity (1, 2). General rules for the DNA-binding strategy adopted by these dimers are depicted in Fig. 1A. The broad conservation in DNA-binding chemistry by the dimers arises from high sequence homology in the N-terminal DNA-binding domain (DBD) of NF-κB proteins. This homologous DBD is commonly referred to as the Rel homology region (RHR). The RHR contains two folded immunoglobulin-like domains connected by a short linker, the N-terminal domain (NTD), and the dimerization domain (DD) (Fig. 1B). The flexible connection between of the two domains confers a unique DNA-binding property to the NF-κB dimers where one of the two NTDs in a dimer may not contact DNA with sequence specificity (2). This NTD still can make nonspecific DNA backbone contacts; thus most of the DNA-binding affinity is retained. This results in much broader DNA sequence spectrum for recognition with only half-site binding specificity than that would be possible if both half-sites were absolutely required for recognition (3–5).

Sequence variations in the central region often provide differential transcriptional specificity by the NF-κB dimers. Even one nucleotide change can switch transcriptional program from gene activation to repression without altering DNA-binding specificity by a dimer (6, 7). The precise mechanism of how one nucleotide change confers such a dramatic change in transcriptional output is still unclear. Taken together, these observations suggest that κB sites play at least two critical roles in transcriptional regulation: to select one or a subgroup of NF-κB dimers over other dimers at the level of binding specificity and to dictate different transcriptional outcome without changing binding specificity by a dimer.
Most of the NF-κB target genes contain multiple κB sites in their promoters (8, 9). These κB sites are arranged randomly with respect to orientation and spacing between them (Fig. 1C). Differences in their sequences at few positions guide them to recruit specific but overlapping set of dimers at a specific site (5, 10). Three well-characterized promoters containing multiple κB sites are E-selectin, HIV, and IP-10. E-selectin promoter contains three κB sites; two of them are inverted repeats with no spacing between these two sites, and the third κB site is located 12 bp downstream of the tandem sites (11). The HIV promoter contains two κB sites direct repeat sequences separated by 43 bp (6). It is unclear, however, whether and how the two sites cooperate. The X-ray crystal structure of p50–RelA heterodimer bound to the tandem HIV κB sites is known (13). Both dimers interact with each κB site nearly identically. Although the structure predicts probable cooperation between the dimers to stably interact with DNA, biochemical experiments suggest that the p50–RelA heterodimers binds HIV κB sites anti-cooperatively. Thus the relationship between transcriptional activation by NF-κB dimers and its DNA binding to multiple sites on a promoter remains unresolved. It is thought that these sites act independently (14).

This is in contrast with stable binding assembly of multiple transcription factors on promoters containing multiple binding sites. The current study investigates how RelA homodimer recognizes tandem κB sequences and how its binding to these DNA sequences affect transcription.
null
mutate invariant residues, Arg^{41} and Ser^{42} of loop L1 and Arg^{187} of the linker, often display DNA sequence-sensitive variations in binding (supplemental Fig. S2). In our structure, only Arg^{41} of subunits A and C directly contact the inner 5-bp κB half-sites. This residue donates two hydrogen bonds to O6 and N7 of the guanine at position 5. Arg^{187} on these subunits does not make direct contact with DNA. In the case of Arg^{187}, it forms a hydrogen bond with main chain oxygen of Phe^{34}. The other two subunits, B and D, bind to the outer 4-bp κB half-sites displaying different binding fashion. In subunit B, Arg^{11} donates hydrogen bonds to O6 and N7 of a non-consensus guanine at position 5 in the opposite DNA strand. Arg^{11} of subunit D contacts backbone phosphate of C+4. Arg^{187} forms a hydrogen bond with O4 of T+2 of both half-sites (Fig. 2A). Ser^{42} on any of the four RelA subunits, however, does not interact with the DNA sequence. A schematic representation of the interactions described above is shown in Fig. 2B.

In general, these specific contacts confirm, as predicted previously, that RelA can bind both 5- and 4-bp κB half-sites. A single base pair switch at the first position from a G:C to non-G:C is responsible for this. In the case of the 5-bp GGGAN κB half-site, three Gs are contacted by Arg^{41}, Arg^{35}, and Arg^{187}, and in the case of the 4-bp GGAN κB half-site, GGA is contacted by Arg^{35}, Arg^{187}, and Arg^{187} (T from the reverse strand). Our results reveal that if a G:C bp is present at the ±5 position, that will be specifically recognized by Arg^{41}, and this contact alters the structure of the DNA in such a way that Arg^{187} cannot contact A:T bp at ±2 position. The result of their differential binding might explain the alteration of the overall conformation of the two half-complexes. The consequence of these conformational variations in gene expression has yet to be determined.

Tandemly arranged κB sites cooperate in activating transcription

To test whether the tandem arrangement of κB sites and protein–protein interaction between the two dimers observed in the current structure have any consequence in gene expression, we used luciferase reporter assay where the luciferase gene expression is driven by E-selectin promoter. As we mentioned above, the natural E-selectin promoter has three κB sites (site I, site II, and site III), where sites I and II are tandemly arranged. Initially, we tested the natural E-selectin promoter and a mutant version where the site III was deleted leaving behind the tandem sites (E-Sel I+II). Luciferase activity was measured 48 h after co-transfecting RelA and luciferase reporter into HeLa cells. Although the natural E-Sel I+II+III promoter showed the greatest activity, the E-Sel I+II promoter was transcriptionally active (Fig. 3A, see black bar corresponding to E-Sel I+II promoter). We next tested whether sites I and II synergize in activating RelA-mediated transcription. Mutation of either site, which are both weak-binding κB sites, reduced the reporter activity to near background levels, suggesting their functional cooperativity in cells (Fig. 3A, see gray and striped bars, respectively). These results also suggest that functional cooperativity might arise from direct interactions between the two dimers bound to two closely spaced κB sites.

Two other NF-κB classical target promoters with tandemly arranged κB sites, HIV and IP-10, were analyzed in a similar manner. The HIV promoter has two near consensus identical κB sites for RelA homodimer separated by 4 bp. Mutation of one κB site decreased reporter activity more than 2-fold, suggesting that both κB sites functionally cooperate to activate transcription (Fig. 3B). Similar results were obtained when the IP-10 promoter was tested. This promoter contains two κB sites separated by 46 bp; one of them is a strong site for RelA homodimer binding, whereas the other is a weaker binding site. When both sites were individually mutated, reporter activity was greatly affected, and the effect was greater when the strong binding site was mutated. The non-additive nature of reporter activity suggests functional cooperation between the two sites physically separated by nearly 150 Å (Fig. 3C). This result suggests that two κB sites placed in tandem or separated by tens of bp can synergistically activate transcription but that synergy
direct dimer-dimer interactions cannot be responsible for this

According to our structural results shown in Fig. 1, E showed significantly enhanced transcriptional activity (Fig. 3).

homodimers is expected when the sites are separated by 5 bp (5bp).

SWAP promoter showed less transcriptional activity both the

We also analyzed the effect of swapping both κB sites on the E-selectin promoter was increased by 2 or 5 bp (2bp, 5bp). These observations led us to propose that other proteins present endogenously might play a role that should be further addressed. Overall, our structural data, together with reporter assays, suggest that tandemly arranged κB sites in different RelA-dependent promoters cooperate to activate transcription. However, the cooperation is not unconditional but depends on their specific sequence, orientation, and spacing.

In vitro DNA-binding affinities of recombinant full-length or RelA-RHR fail to explain differential transcriptional outcomes

We wanted to test whether differential transcriptional outcomes of WT and mutant E-selectin promoters could be explained by different binding strengths of RelA homodimers for these promoters. We performed EMSA using RelA-RHR to determine their relative binding efficiency (Fig. 4). Our results show that RelA-RHR binds the tandemly arranged κB sites (E-Sel II+I, E-Sel I+II (2bp), E-Sel II+I SWAP (2bp)) sequentially where binding of the first RelA dimer was detected at a concentration range between 0.12 and 3 nM to all the probes except for E-Sel II+I SWAP probe, in which the second κB site occupancy was detected at concentration around 500 nM. This observation suggests that the second RelA dimer binds the second site anti-cooperatively, while binding of the first dimer is inhibitory to the binding of the second dimer. More profound
inhibition in the case of E-Sel II SWAP probe is perhaps due to strong steric repulsion (Fig. 4A). We also tested E-Sel I+II (5bp), E-Sel II+I SWAP (5bp), E-Sel I+II (mut), E-Sel I(mut)+II, and E-Sel I(mut)+II(mut) (supplemental Fig. S3A). No binding difference was observed between E-Sel I+II (5bp) or E-Sel II+I SWAP (5bp) probes. The last three probes tested showed the expected results: one site occupied when one κB single site was mutated and no binding when both κB sites were simultaneously mutated. These results suggest no correlation between in vitro DNA-binding affinity of RelA–RHR for κB sites and the cell-based reporter activity that used full-length RelA (Fig. 3). To examine whether full-length RelA differentially
binds to the E-Sel promoters under study, we prepared full-length recombinant RelA from baculovirus-infected Sf9 cells (supplemental Fig. S3B). We tested the binding efficiency of full-length RelA for all probes discussed above using EMSA (Fig. 4B and supplemental Fig. S3C). Results of EMSA experiments led us to make two conclusions: first, full-length RelA binds poorly to all probes tested compared with the RelA-RHR (supplemental Fig. S3D), and second, in contrast to the sequential occupancy of both κB sites observed with RelA-RHR, two shifted complexes appeared simultaneously for full-length RelA (Fig. 4B and supplemental Fig. S3C).

To determine the specificity of the complexes, we performed antibody supershifts using anti-RelA and anti-His antibodies. The faster mobility complex was shifted in lesser degree than the slower mobility complex. It is possible that AD of one of the RelA subunits is degraded in the faster mobility complex (because the anti-RelA antibody used recognizes the RelA AD). However, this is unlikely because the protein appeared minimally degraded (supplemental Fig. S3B). Alternatively, the epitope recognized by the antibodies might be partly masked in the faster mobility complex. Strikingly, both shifted complexes appeared in probes where one site was mutated (supplemental Fig. S3C, see probes E-Sel I(mut)+II and E-Sel 1+II(mut), respectively). It is likely that somehow recruitment of the homodimer at one site facilitates the recruitment of a second homodimer even when the second site is not a κB site. Mutation of both sites mostly abolished dimer binding (supplemental Fig. S3C, see probe E-Sel I(mut)+II(mut)), suggesting that the binding observed in probes containing only one consensus κB site is specific. In summary, our results suggest that recombinant full-length RelA poorly and indiscriminately binds tandem κB sites. We speculated whether that poor binding by full-length RelA might be due to the inhibitory effect of the AD (Fig. 1B). To test this, we assayed RelA-RHR binding to tandem E-selectin κB sites in the presence of increasing concentrations of RelA AD-TA (Fig. 4C). Our results showed that RelA-RHR was progressively released from the probe as the concentration of AD increased. Although it is not clear whether RelA AD competes with the DNA by directly binding to RelA-RHR or indirectly modulate DNA binding by RHR, the inhibitory effect of the AD in RelA DNA binding is clear in the purified in vitro system.

**Discussion**

The X-ray crystal structures of the NF-κB–DNA complexes known to date are mostly DNA sequences containing a single κB site bound to one dimer (2). Although these studies provided fundamental information on how different NF-κB dimers recognize κB sites, they failed to provide information on the relationship between the two dimers bound to tandem sites. This is an important question because a large number of NF-κB targets contain multiple κB sites (8, 9). Only the structure of tandem HIV-κB site bound to two p50–RelA heterodimers provide insights into the cooperativity between two dimers bound to nearby sites (13). Because differences in spacing, orientation, and sequence can result in numerous arrangements of dimers even with two κB sites, structural information from other multidimer–DNA complexes are required for better understanding of different assemblies that occur and their mechanism of formation. We chose E-selectin tandem κB sites as the model DNA for further study. The work presented here sheds new light on binding by two RelA homodimers to a specifically arranged tandem κB site and its effect on transcription. We show that two weak κB sites, which independently fail to induce transcription because they each bind RelA homodimer inefficiently, can drive transcription synergistically if placed in proper context. However, a random arrangement of the tandem sites is not optimal for transcription; their specific arrangement is important. Our study provides the first demonstration that not only the presence of multiple κB sites but also their arrangement could impact transcription, and these positional factors need to be considered while interpreting NF-κB–dependent transcriptional outcome.

Our effort to thoroughly explain differential RelA-driven transcription from differently arranged tandem sites using in vitro binding data and structural insights remained incomplete. Nonetheless, we made some intriguing observations; first, despite protein–protein interactions between the two dimers visualized in the structure, in vitro experiments indicate that the dimers do not cooperate for simultaneous stable interactions with DNA in solution. Second, the dimers could bind asymmetrically to symmetrically arranged sites in which one subunit of one dimer does not satisfy all DNA contacts. These broken DNA contacts suggest unstable DNA binding. This mode of binding possibly represents dissociating state of one dimer. Consistently, in vitro data for binding by the RelA-RHR (DBD) also shows the anti-cooperative mode of DNA binding by the two dimers. Finally, the full-length recombinant RelA binds DNA with poor specificity and weaker affinity as compared with RelA-RHR, suggesting an unexpected role for RelA AD. The AD domain being highly negatively charged might be involved in repulsive interactions with the negatively charged phosphates in the flanking exposed DNA. These observations are tallies with observations of earlier reports suggesting that RelA does not bind κB sites and functions as a non-DNA binding partner of p50 to activate transcription (18).

An important question now is how full-length RelA recognizes κB sites in vitro to activate transcription overcoming the negative effect of the AD. Multiple factors have been shown to influence transcriptional activity of RelA, including RPS3, OGG1, E2F1, and p53 (19–22). These later factors are DNA binding transcription factors, but their DNA binding activity is not essential to activate the ability of RelA to activate transcription (21, 22). We suggest that these factors, as shown in the cases of RPS3, OGG1, and p53, augment the ability of RelA to bind DNA at least in part by neutralizing the negative effect of the AD. However differential transcriptional activity observed with wild-type and various mutants of E-selectin promoters cannot be explained simply by neutralization of a negative effect of AD on RelA-RHR by these regulatory factors. We observe that multiple κB sites present in native promoters functionally cooperate among themselves to activate transcription by RelA, although artificially altering the arrangement of these κB sites resulted in transcriptional repression. At this stage, we are not clear about how RelA differentially selects these promoters. Perhaps NF-κB dimers in association with other regulatory factors facilitate each other in binding DNA through
physiological conditions, both dimers cannot co-occupy the two sequence, positional arrangement of multiple the binding kinetics of RelA and other NF-effective of other associated nuclear factors together determine (3). E-selectin, HIV, and IP-10 luciferase reporters containing 18828 and GST-RelA AD(429–551) have been previously described Plasmids and antibodies Experimental procedures Figure 5. A schematic representation of transcription activation domain (TAD)–mediated inhibition of RHR binding to κB sites. A, the TAD engages in a highly dynamic interaction to inhibit DNA binding rather than forming a stable interaction with RHR. Other factor(s) must neutralize the negative effect of TAD to allow RHR to bind DNA. B, RelA homodimers facilitate each other for stable interaction of one homodimer to one κB site. However, under physiological conditions, both dimers cannot co-occupy the two κB sites. The model depicts cooperation between the two dimers at a long range but anti-cooperation on the DNA. The resultant effect is more profound when κB sites of tandem arrangements are weak. Therefore, when both sites are weak and one site is abolished, the other site cannot efficiently engage a dimer because of the absence of a long-range support from the other dimer.

long-range electrostatics. Only when two sites are in close proximity does this facilitation rely on specific arrangement of the two sites. In contrast, when the sites are far apart as in IP-10, orientation of the sites may not be critical. A schematic representation of these multiple options is depicted in Fig. 5. Binding data reflecting anti-cooperative nature suggest that at the concentrations of RelA observed in the nucleus, both dimers could occupy the sites simultaneously. These results are consistent with hyperdynamicity of DNA binding by NF-κB as shown previously and opposed to a stable enhanceosome formation by multiple transcription factors bound to tandem sites (23, 24). Thus the dynamic engagement of RelA rather than a stable complex in equilibrium state is a critical determinant of transcriptional output. Combined effects of specificity of κB sequence, positional arrangement of multiple κB sites, and effect of other associated nuclear factors together determine the binding kinetics of RelA and other NF-κB factors. Future studies will address these intriguing possibilities.

Experimental procedures Plasmids and antibodies Untagged mouse RelA(19–304), pRC-HA-hRelA(1–551), and GST-RelA AD(429–551) have been previously described (3). E-selectin, HIV, and IP-10 luciferase reporters containing specific κB DNA promoter were cloned in CMXTK-Luciferase reporter (a kindly gift from Dr. D. Chakravarti, Northwestern University Feinberg School of Medicine) using Sall and BamHI restriction enzymes (New England Biolabs). Sequences of oligonucleotides containing the N-terminal Sall restriction site, the C-terminal BamHI restriction site, and the specific κB site used for reporter cloning are listed in supplemental Table S1. The antibody recognizing RelA (sc–372) was purchased from Santa Cruz. Anti-His antibody was a gift from Biobharati Life Science, Kolkata, India.

Protein expression Recombinant untagged mouse RelA(19–304) was expressed and purified with modifications of a previously published protocol (25). Briefly, cells were lysed with lysis buffer (25 mM MES, pH 6.5, 50 mM NaCl, 0.5 mM EDTA, pH 8.0, 0.5 mM PMSF, and 10 mM 2-mercaptoethanol (BME)) and sonicated. Lysate was cleared by centrifugation at 13,000 rpm for 30 min at 4 °C. Supernatant was decanted into an ice-cold beaker, and 1/27 of streptomycin (10%) was added to a 0.3% final concentration while gently stirring. The sample was left stirring 20 min more at 4 °C and cleared by centrifugation at 13,000 rpm for 30 min at 4 °C. Supernatant was loaded onto an SP-Sepharose Fast flow column pre-equilibrated (Amersham Biosciences) with lysis buffer. Column was washed with 20 column volumes of Lysis buffer and then eluted at 4 °C with elution buffer (25 mM MES, pH 6.5, 300 mM NaCl, 0.5 mM EDTA pH 8.0, 0.5 mM PMSF, and 10 mM BME). Peak fractions were pooled and concentrated in an Amicon concentrator and further purified by size exclusion chromatography on a Superdex 75 column in 25 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 1 mM DTT. GST-tagged RelA-AD was expressed in Escherichia coli Rosetta cells by growing cells harboring the expression plasmid (pGEX-4T containing RelA 429–551) to A660 0.4 followed by induction with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at room temperature. The fusion protein was purified in a single step using a glutathione-Sepharose column (gift from Biobharati Life Science, Kolkata, India) from the crude cell lysate (150 mM NaCl, 25 mM Tris–HCl, pH 7.5, glycerol 10% (v/v)) followed by elution with 10 mM glutathione. Recombinant His-hRelA full-length baculovirus was kindly provided by Dr. James Kadonaga. SF9 suspension cultures were infected with His-hRelA full-length baculovirus at a cell density of 1 × 10⁶/ml and allowed to grow for 60 h postinfection. The cells were harvested and lysed in lysis buffer (10 mM Tris–HCl, pH 7.5, 500 mM NaCl, Nonidet P-40 0.1% (v/v), glycerol 10% (v/v), 15 mM imidazol, 10 mM BME, 2 mM PMSE, and protease inhibitor mixture (Sigma)) by sonication. The lysate was clarified by filtering with 0.22 μm and mixed with slurry of nickel-nitritolriacetic acid resin (Qiagen) in batch in the cold room for 3 h. The resin was thoroughly washed with lysis buffer containing 30 mM imidazole and 300 mM NaCl prior to elution using the same buffer with 400 mM imidazole and 200 mM NaCl. Elution was done twice in the cold room for 30 and 10 min, respectively. Both elutions were pooled and dialyzed three times 1 h each. First and second dialysis were done against dialysis buffer 1 (10 mM Tris–HCl, pH 7.5, 200 mM NaCl, 10 mM BME, 10% glycerol (v/v)), and the third dialysis was against
dialysis buffer 2 (10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM BME, 5% glycerol (v/v)). Protein was concentrated by centrifugation using Centriprep 30-kDa cutoff membrane concentrator unit (Millipore) and loaded onto preparative Superdex 200 size exclusion column connected to an AKTA purifier (GE Healthcare) equilibrated with buffer containing 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM DTT, 5% glycerol (v/v) at room temperature. Peak fractions were concentrated again with Centriprep 30-kDa. Protein concentration was determined using Bradford reagent and was snap frozen in liquid N$_2$ for long-term storage at −80 °C.

**Crystallization, data collection, and structure solution**

Protein–DNA complex was formed by mixing 2:1 (RelA dimer–DNA) to a final concentration of ~10 mg/ml. The crystals were grown using hanging drop vapor diffusion method by mixing 1:1 ratio of the complex solution to reservoir solution (100 mM sodium citrate, pH 5.5, 10 mM CaCl$_2$, 1 mM spermine, 10 mM DTT, 15% PEG3350 (v/v), 0.1% n-octyl-β-D-glucopyranoside (BOG) (v/v)). Before data collection, the crystals were soaked for about 1 min in a cryoprotectant buffer containing the original reservoir solution plus 25% PEG400 and flash-cooled under liquid nitrogen. X-ray diffraction data were collected at APS 19ID synchrotron source. The diffraction patterns revealed that the crystals belong to the monoclinic space group P2$_1$, with unit cell: $a = 88.91$, $b = 117.80$, $c = 70.81$ Å, and $β = 91.20°$. X-ray diffraction data were integrated and scaled to 2.5 Å by using HKL2000. The data processing statistics are included in Table 1.

The structure was determined by molecular replacement using MolRep, with the structure of RelA homodimer and IL-8 DNA complex as the search model. Two copies of the search model were located in the asymmetrical unit with DNA end-to-end stacking at the junction. The orientation and position of this initial model were refined by rigid body refinement in CNS (cns_solve_1.3). The structure was further refined using minimization and simulated annealing with a maximum likelihood target function and a flat bulk-solvent correction using the CNS system. The model rebuilding was performed based on $2F_o - F_c$ maps using Xtalview. After the individual temperature factors were included in the refinement, the $R$ factor was 21.0%, and the free $R$ factor was 27.2% for the final model. The detailed results of the refinement are included in Table 1. The coordinates have been deposited into the Protein Data Bank with entry code 5U01.

**Luciferase reporter assays**

HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin–glutamine. The cells were transiently transfected with pRC-HA-hRelA(1–551) or empty HA vector and the luciferase reporter DNA with specific κB DNA promoters. The total amount of plasmid DNA was kept constant for all assays. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. *Renilla* luciferase expression plasmid was co-transfected as an internal control. The cells were collected 48 h after transfection. Luciferase activity assays were performed using a Dual-Luciferase reporter assay system (Promega) following the manufacturer’s protocol. The results from promoter fold activation of the reporter plasmids are given as the ratio of relative luciferase activity (luciferase units/ Renilla units) values from each sample relative to the corresponding control empty vector. The values represent the averages of three independent wells. The experiments were performed a minimum of three times with identical results. The data are represented as means ± S.D. κB sequence promoters are listed in supplemental Table S1.

**Electrophoretic mobility shift assay**

EMSA assays were performed as previously described (26). Briefly, E-selectin probes were radiolabeled and incubated with His-hRelA full-length or untagged mRelA-RHR for 20 min at room temperature. The amount of protein was quantified by Bradford assay (Bio-Rad). Protein complexes were analyzed by native electrophoresis on a 4% (w/v) non-denatured polyacrylamide gel. The probe sequences are listed in supplemental Table S2.

**Author contributions**—G. G. conceived the idea with help from M. C. M. and D.-B. H. determined the crystal structure. D.-B. H., G. G., and T. B. interpreted the structural results. M. C. M., H. T. N., V. Y. F. W., and Y. L. conducted biochemical experiments. G. G. and M. C. M. wrote the manuscript with help from T. B.

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