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Dual role of the nuclear factor of activated T cells insert region in DNA recognition and cooperative contacts to activator protein 1

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ABSTRACT **The transcription factors nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1) coordinately regulate cytokine gene expression in activated T-cells by binding to closely juxtaposed sites in cytokine promoters. The structural basis for cooperative binding of NFAT and AP-1 to these sites, and indeed for the cooperative binding of transcription factors to composite regulatory elements in general, is not well understood. Mutagenesis studies have identified a segment of AP-1, which lies at the junction of its DNA-binding and dimerization domains (basic region and leucine zipper, respectively), as being essential for protein–protein interactions with NFAT in the ternary NFAT/AP-1/DNA complex. In a model of the ternary complex, the segment of NFAT nearest AP-1 is the Rel insert region (RIR), a feature that is notable for its hypervariability in size and in sequence amongst members of the Rel transcription factor family. Here we have used mutational analysis to study the role of the NFAT RIR in binding to DNA and AP-1. Parallel yeast one-hybrid screening assays in combination with alanine-scanning mutagenesis led to the identification of four amino acid residues in the RIR of NFAT2 (also known as NFATC1 or NFATc) that are essential for cooperativity with AP-1 (Ile-544, Glu-545, Thr-551, and Ile-553), and three residues that are involved in interactions with DNA (Lys-538, Arg-540, and Asn-541). These results were confirmed and extended through *in vitro* binding assays. We thus conclude that the NFAT RIR plays an essential dual role in DNA recognition and cooperative binding to AP-1 family transcription factors.**

The recognition of antigens by the T-cell receptor results in the activation of mitogenic signaling pathways and the induced expression of cytokines such as interleukin 2 (1, 2). The expression of interleukin 2 is controlled by an enhancer region located within 300-bp upstream of the transcription start point (3). A sequence in the interleukin 2 enhancer termed the upstream antigen receptor response element, or ARRE2 (4), serves as a composite recognition site for the nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1) (5–9). Whereas AP-1 transcription is induced upon activation of the Ras/Map kinase and protein kinase C pathways, NFAT is sequestered in the cytoplasm of resting T cells and translocates to the nucleus in response to a sustained rise in Ca^{2+} concentration (1, 10). The nuclear translocation of NFAT is blocked by the clinically important immunosuppressants FK506 and cyclosporin A, which function by inhibiting calcineurin, a Ca^{2+}-activated serine/threonine phosphatase (11, 12). Calcineurin is thought to dephosphorylate NFAT directly (13, 14), thereby unmasking a nuclear localization sequence and passivating a nuclear export sequence in the N-terminal domain (10, 15, 16).

The NFAT family of transcription factors comprises at least four distinct genes (8, 17–20). NFAT1 and NFAT2 represent two of the best characterized isoforms (NFAT1 and NFAT2 are also known as NFATp and NFATc, respectively, and have been designated by the Genome Data Base Nomenclature Committee as NFATC2 and NFATC1). These proteins show characteristic patterns of tissue-specific expression and encode functionally nonredundant products, as evidenced by the distinctive phenotype of NFAT1 knockout mice (21, 22). NFAT proteins share a highly conserved region of ≈300 amino acids, which is responsible for DNA binding and cooperation with AP-1. Despite the structural similarity between the DNA-binding domains of NFAT proteins and that of prototypical Rel transcription factors such as NF-κB (23), the two subfamilies of Rel proteins recognize DNA in rather distinct ways; for example, NFAT proteins bind DNA as monomers whereas NF-κB binds DNA as a dimer (20, 24).

NFAT1 and NFAT2 bind with $\approx 10^{-8}$ M affinity to the consensus sequence 5′-GGAAAA-3′ (25). The NFAT binding site in ARRE2 closely abuts a nonconsensus AP-1 site, 5′-TGTTTCA-3′ (refer to Fig. 1), to which AP-1 binds very weakly. The presence of NFAT on ARRE2 increases the apparent affinity of AP-1 for the nonconsensus site roughly 10-fold (25). By the same token, the dissociation half-life of the ternary NFAT/AP-1/ARRE2 complex is 10 times longer than that of the binary NFAT/ARRE2 complex (9). Thus, NFAT and AP-1 mutually stabilize each other's interaction with ARRE2, this being a hallmark of bona fide thermodynamic cooperativity. The cooperative interaction between NFAT and AP-1 on ARRE2 is essential for activation of the interleukin 2 gene (17, 26).

X-ray crystallographic studies have revealed that the basic region-leucine zipper (bZIP) domains of the AP-1 family proteins c-Fos and c-Jun pair to form continuous α-helices that splay to present a Y-shaped fork into which the major groove of DNA binds (27). Whereas c-Fos and c-Jun alone bind DNA in two orientations related by the interchange of two protein subunits (27, 28), NFAT forms a cooperative complex with only one of the two AP-1 orientational isomers (28). Although the mechanism of orientational specificity in this system is not well understood, biochemical experiments have suggested that the most important contacts contributed by AP-1 are located at the junction between the basic region and Jun leucine zipper (25, 29). A conserved arginine residue in this so-called "spacer

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Abbreviations: NFAT, nuclear factor of activated T cells; AP-1, activator protein 1; RIR, Rel insert region; DBD, DNA biding domain; β-gal, β-galactosidase; ARRE, antigen receptor response element; EMSA, electrophoretic mobility-shift assay; AD, activator domain; bZip, basic region-leucine zipper.

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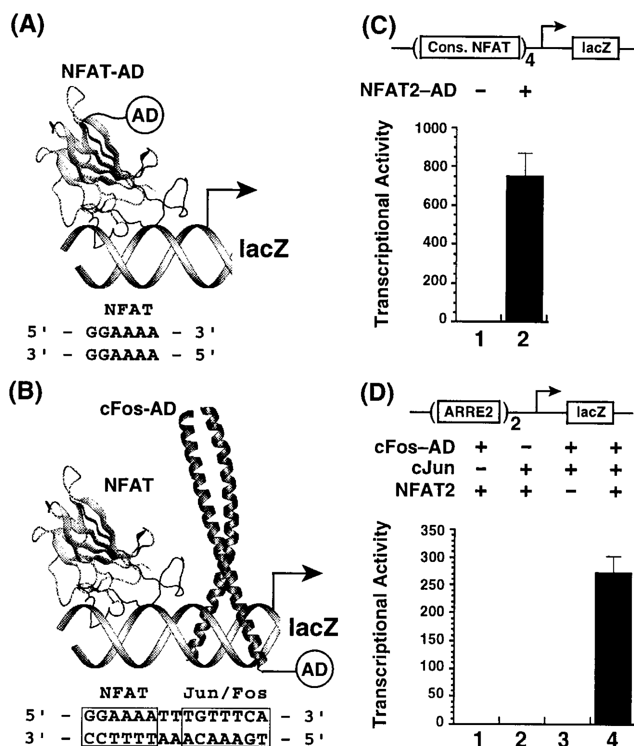

FIG. 1. Activation of *lacZ* reporter gene transcription by NFAT acting alone and in cooperation with AP-1. (*A*) A model of the NFAT2-DBD (23) bound to a consensus NFAT site. AD denotes the B42 activation domain, which is fused to the N terminus of the NFAT2-DBD. (*B*) A composite model of the NFAT2-DBD, bound to a polypurine tract in ARRE2, adjacent to AP-1 bound to the non-consensus AP-1 site. Cooperative recruitment of c-Jun/c-Fos-AD to DNA by NFAT activates reporter gene expression (25). In the cooperativity assay the B42 activation domain is fused to the N terminus of c-Fos. (*C*) Reporter gene expression driven by NFAT-AD binding alone to a consensus NFAT site in yeast. (*D*) ARRE2-linked reporter assay detects assembly of the cooperative AP-1/NFAT/ARRE2 complex in yeast (25). Transcriptional activity refers to units of β-gal activity.

region" of c-Jun, c-Jun-R285, is especially critical for cooperative NFAT/AP-1 complex assembly on DNA; mutation of c-Jun-R285 to Ala resulted in virtually complete loss of cooperativity with NFAT, but had no effect on DNA recognition by AP-1 alone (25).

Solution structural studies of the DNA binding domain of NFAT2 (NFAT2-DBD) have revealed that NFAT and NF-κB p50 share significant structural homology, but have important differences in their DNA recognition strategies (23). The greatest structural divergence between the NFAT2-DBD and NF-κB p50 is found in a long insert connecting the G′ and H β-strands. Known as the Rel insert region (RIR), this structural feature varies widely in size and in sequence amongst members of the Rel transcription factor family. For example, whereas the RIR in DNA-bound NF-κB p50 comprises 67 amino acids that form a tight α-helical bundle packed against the immunoglobulin-like β-barrel (30, 31), the RIR of DNA-unbound NFAT2 is a $\approx$20-amino acid loop devoid of stable secondary structure (23). In a computer-generated model of the NFAT2/AP-1/ARRE2 complex, the portion of NFAT closest to AP-1 is the RIR (23), leading to the proposal that the RIR is an important site for cooperative contacts to AP-1. Interestingly, the N terminus of the RIR in this model is located directly over the 3′-end of the NFAT recognition sequence, suggesting that the RIR might also contact DNA directly (23).

Here we report the identification of residues in the NFAT RIR that mediate interactions with AP-1 and DNA. Mutation of four residues in the NFAT RIR (I544, E545, T551, or I553) to alanine affects cooperative interactions of NFAT with AP-1 but leaves unchanged the ability of NFAT to bind DNA. Two different mutations in the NFAT RIR (K538A and R540A) strongly decrease the affinity of NFAT for DNA, and another (N541A) exerts weaker effects. These and other data presented here reveal that the NFAT RIR plays a key role in cooperative contacts to AP-1 and in DNA recognition by NFAT.

MATERIALS AND METHODS

The yeast strains, β-galactosidase (β-gal) reporter gene assays, and oligonucleotides used have been described (25).

Plasmid Construction. Plasmids used to reconstitute the core cooperative NFAT/c-Fos/c-Jun/DNA complex in yeast have been previously described (25). For the NFAT2 DNA binding assay, NFAT2 and alanine mutants were expressed under the control of the *GAL1* promoter as B42 activation domain fusion proteins from the *LEU2*-selectable plasmid pBC103-AD. The pBC103-AD plasmid was constructed by subcloning the small fragment from pJG4–5 (32) into the large fragment of pBC103 after digestion with *Kpn*I and *Xho*I. All mutants of the NFAT2-RHR were prepared by PCR megaprimer mutagenesis using *Pfu* polymerase (Stratagene) (33). All new constructs and those mutants affecting DNA binding or cooperative interactions with AP-1 were confirmed by dideoxy DNA sequencing.

Recombinant Proteins. The recombinant human NFAT2-RHR (residues 415–710) was expressed in *Escherichia coli* BL21(DE3) from the vector pLM1 (34) as a C-terminal $(His)_6$ fusion protein with a heart muscle kinase recognition site (RRASV) spacer between the NFAT2-RHR and $(His)_6$ tag. Protein expression was induced with isopropyl β-D-thiogalactoside and the cells were harvested after a 4 hr induction at 30°C. The proteins were purified to homogeneity under native conditions by chromatography on Ni^{2+}-nitrilotriacetic acid agarose (Qiagen, Chatsworth, CA) and eluted with a step gradient of 10–500 mM imidazole in phosphate buffer (50 mM NaH_2PO_4·NaOH/250 mM NaCl/10 mM 2-mercaptoethanol, pH 8). All NFAT2-RHR wild-type and mutant proteins were quantified by bicinchoninic acid assay (Pierce). Recombinant c-Jun (247–340) and c-Fos (118–211) bZip fragments were expressed and quantified as described (25).

Gel Electrophoretic Mobility-Shift Assays (EMSAs). protein–DNA complexes studied by EMSA [6% native polyacrylamide (Protogel, National Diagnostics)] were formed by incubation at room temperature for 1 hr with <100 pM end-labeled DNA probe in 20 μl of binding buffer [16 mM Hepes·KOH, pH 7.5/60 mM KCl/30 mM NaCl/10% glycerol/1 mM dithiothreitol/BSA (10 μg/ml^{-1})/poly-(dI·dC) (5 μg/ml^{-1})].

RESULTS

Tandem Yeast Genetic Assays Allowed Dissection of Mutations Affecting NFAT/AP-1 Cooperativity and NFAT/DNA Recognition. We recently reported (25) the development of parallel modified yeast one-hybrid assays (35) based on the interaction trap system (32) and their use in identifying mutations in AP-1 that affect cooperative DNA binding with NFAT, and also in distinguishing these mutants from those that affect DNA-binding by AP-1. The present, complementary study uses parallel modified yeast one-hybrid assays to distinguish mutations in NFAT that affect cooperativity from those that impair the ability of NFAT to bind DNA (Fig. 1). As described earlier (25), the primary yeast screen specifically

detects formation of the cooperative NFAT/AP-1/ARRE2 complex (Fig. 1 *B* and *D*). For the present study, it was necessary to develop a secondary screen, which detects DNA binding by NFAT alone in the absence of AP-1 (Fig. 1*A*). Thus, NFAT2 containing a fused B42 activation domain (NFAT-AD) was used to drive expression of a *lacZ* reporter linked to four tandemly repeated consensus NFAT sites. As shown in Fig. 1*C*, NFAT-AD gave rise to a substantial (200-fold) induction of β-gal activity (compare lanes 1 and 2); these levels were sufficient to monitor the DNA-binding activity of NFAT in yeast cells.

Alanine-Scanning Mutagenesis Reveals a Bifunctional Role of the NFAT2 RIR. To identify regions of NFAT that play an important role in formation of the cooperative NFAT/AP-1/DNA complex, we initially converted blocks of residues in the NFAT2 RIR to alanines, then determined the effect of these block mutations on cooperative binding in the yeast ARRE2-driven reporter assay. Alanine substitution was chosen to eliminate interactions beyond the side-chain β-carbon, while minimizing the perturbation of secondary structure (36). Of the six NFAT2 block-alanine mutants tested in the cooperative binding assay, four (residues 536–539, 540–542, 543–545, and 550–553) virtually abolished cooperative interactions with AP-1 (Fig. 2). To localize further the positions within the four deleterious blocks that had the greatest effect on cooperative binding, we analyzed mutant proteins containing double and single alanine substitutions throughout the region of interest. Five alanine point mutants (K538A, R540A, N541A, E545A, and T551A) resulted in almost complete loss (>50-fold reduction) of ARRE2-driven β-gal activity (Fig. 2), and two alanine point mutants (I544A and I553A) caused significant but less pronounced ($\approx$6-fold) reductions in ARRE2 reporter gene expression.

To distinguish mutations that affect cooperativity from those that affect DNA-binding, the NFAT2 point mutants that strongly diminished cooperativity were evaluated *in vivo* as NFAT2-AD fusion proteins. In this secondary screen, four of the mutant NFAT-AD proteins (I544A, E545A, T551A, and I553A) activated transcription from the consensus NFAT-driven reporter as well as wild-type (Fig. 2), thus indicating that the mutations in these proteins did not affect the NFAT/DNA interaction. On the other hand, two mutants (K538A and R540A) were essentially devoid of the ability to activate the NFAT-driven reporter gene ($\approx$350-fold reduction in activity), and one (N541A) had a significant but less substantial effect ($\approx$17-fold reduction in transcriptional activity) (Fig. 2); we conclude that these mutations affect NFAT–DNA interactions.

***In Vitro* EMSA Analysis of Mutant NFAT2 Proteins Confirmed the Importance of Residues Identified *in Vivo*.** To characterize further the effects of NFAT2 mutations on its interactions with AP-1 and DNA, we carried out EMSA on mutant and wild-type NFAT2, in the presence and absence of the AP-1 bZip domain (Figs. 3 and 4). To quantify cooperative binding by the wild-type and mutant NFAT proteins, we measured the effective K_d for binding of AP-1 to the preformed NFAT2/DNA complex (Fig. 3 *A–C*). In these experiments, the NFAT2 concentration was held fixed at 20 nM to afford >80% binary NFAT2/DNA complex in the absence of AP-1 (lane 1). Whereas $\approx$50 nM wild-type AP-1 resulted in 50% formation of the ternary AP-1/NFAT2/DNA complex (Fig. 3*A*, lane 4), $\approx$160 nM of AP-1 was required to produce the same amount of ternary complex with the T551A-NFAT2 mutant (Fig. 3*C*, lane 6). Thus, mutation of Thr-551 to alanine reduces the ability of NFAT to cooperate with AP-1 by $\approx$3.4-fold. Similarly, $\approx$130 nM of AP-1 was required to supershift by half the E545A-NFAT2/DNA mutant complex (Fig. 3*B*, lane 6), corresponding to a $\approx$2.8-fold reduction in cooperativity. The I544A- or I553A-NFAT mutations had less dramatic effects on cooperativity, $\approx$2.1-fold and $\approx$1.3-fold, respectively (apparent AP-1 K_d values of 100 and 60 nM). Significantly, the DNA affinities of all four NFAT2 mutants (I544A, E545A, T551A, and I553A) were experimentally indistinguishable from wild-type (K_d $\approx$16 nM, compare Fig. 3

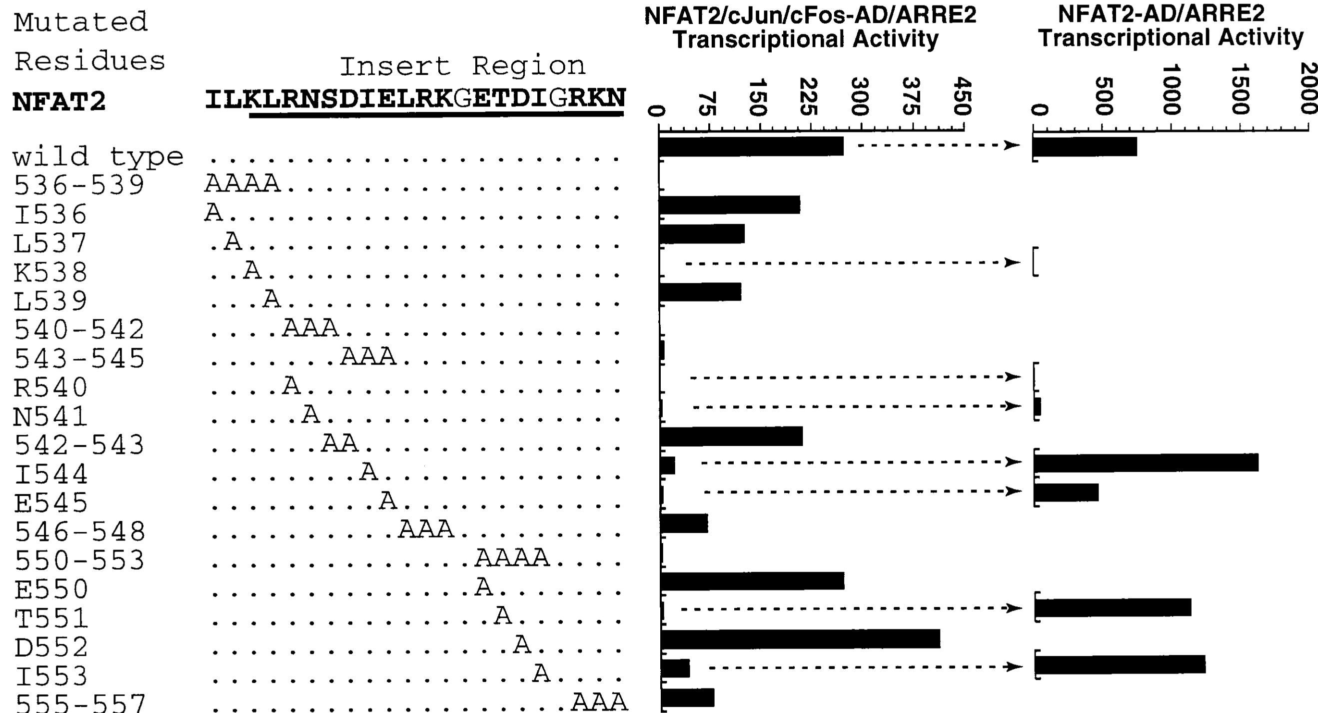

FIG. 2. The effects of alanine-scanning mutations on NFAT2 activity *in vivo*. Sequence of the NFAT2 RIR, with positions mutated to alanine denoted below. To the right of the sequences are shown the transcriptional activity of wild-type and mutant NFAT2-RHR proteins in the cooperative ARRE2 reporter assay, and (for selected examples) in the NFAT-AD reporter assay. The standard error in the measurements of transcriptional activity assays are estimated at ± 25%.

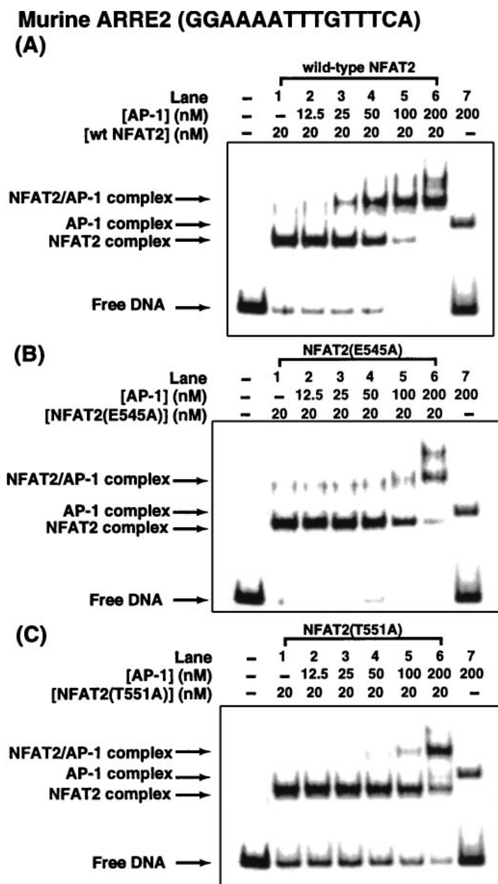

FIG. 3. EMSA analysis of NFAT and AP-1 DNA-binding activity *in vitro*. (*A–C*) Wild-type, E545A, and T551A mutant NFAT2 proteins bind with essentially identical affinities to the ARRE2 ($K_d \approx 16$ nM). (*B* and *C*) The E545A and T551A mutants show a substantially diminished ability to cooperate with NFAT2. From the data in *A*, the effective K_d of AP-1 for the wild-type NFAT2-RHR/DNA complex is estimated at 50 nM. From the data in *B* and *C*, the effective K_d of AP-1 for the NFAT2(E545A)/ARRE2 complex is estimated at 130 nM, and for the NFAT2(T551A)/ARRE2 complex is 160 nM. Nonspecific binding of proteins to DNA was observed at high protein concentrations (lane 6 of both *A* and *B*).

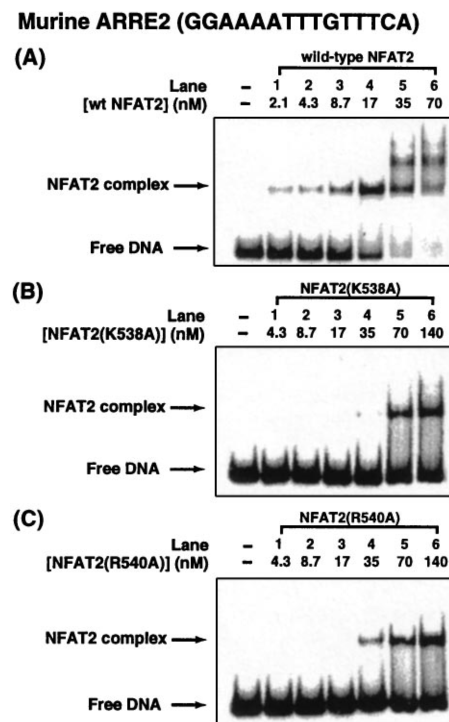

FIG. 4. EMSA analysis of NFAT DNA-binding activity *in vitro*. (*A*) The effective K_d of wild-type NFAT2-RHR for ARRE2 is estimated at 20 nM. (*B* and *C*) The K538A and R540 NFAT2 mutants bind ARRE2 with substantially lower affinity. From the data in *A–C*, the effective K_d of the wild-type, K538A and T551A mutants for ARRE2 is $\approx$20 nM, $\approx$165 nM, and $\approx$170 nM, respectively. Nonspecific binding of proteins to DNA was observed at high protein concentrations (lane 5 and 6 of *A*).

A–C, lane 1) (data not shown for I544A and I553A), thus indicating that the mutations cause specific defects in cooperative DNA binding only.

Three mutations in NFAT2 (K538A, R540A, and N541A) strongly diminish NFAT-driven reporter gene transcription in the absence of AP-1. EMSA analysis of the K538A and R540A NFAT2 point mutants revealed that either mutation lowers the affinity of NFAT2 for DNA by roughly 10-fold (compare Figs. 4 *A–C*). Mutation of Asn-541 to alanine resulted in only a 2-fold reduction in affinity (data not shown). These data indicate that three residues in the NFAT2 RIR serve a function in high-affinity DNA recognition.

DISCUSSION

Multiple Residues in the NFAT RIR Mediate Cooperativity Between NFAT and AP-1. The ability of NFAT and AP-1 to bind DNA cooperatively can be envisaged to arise from one or both of two limiting mechanisms: (*i*) directly, from energetically favorable contacts made by the two proteins to each other; or (*ii*) indirectly, from alteration of local DNA structure caused by binding of one protein to its site, so as to favor interaction of the other protein with its adjacent site. No information is available regarding the indirect readout mechanism; however, it has been observed that NFAT and AP-1 can be chemically crosslinked, but only when bound to ARRE2, suggesting that the two proteins do contact each other in the ternary NFAT/AP-1/ARRE2 complex (28). Consistent with this notion, the removal of a single arginine side-chain in the c-Jun subunit of AP-1 (R285A mutation) results in a nearly complete loss of cooperativity with NFAT, but has no effect on the binding of AP-1 alone to DNA (25). Together, these biochemical data have provided compelling evidence in favor of a model wherein AP-1 is recruited cooperatively to ARRE2 through direct contacts to NFAT. This being the case, then mutations in NFAT should also be capable of disrupting cooperative interactions with AP-1, without affecting NFAT/DNA interactions. Here we have investigated this issue through mutational analysis of the NFAT RIR, a segment suggested by modeling to be a probable contact interface with AP-1 (23).

The presence of NFAT on ARRE2 is known to stabilize the binding of AP-1 by $\approx$10-fold *in vitro* (25). This mutual stabilization encompasses multiple energetic contributions: the favorable energy of protein–protein contacts in the NFAT/AP-1 interface, balanced against potentially unfavorable costs of induced changes in DNA and protein structure required for formation of the cooperative complex. The present study demonstrates that the removal of side-chain functionality of multiple residues in the 20-amino acid NFAT RIR results in diminished cooperativity between NFAT and AP-1, without affecting the affinity of NFAT for DNA. Namely, at four positions in the NFAT RIR (I544, E545, T551, and I553) mutation to alanine substantially reduced cooperative transcriptional activation *in vivo*, but did not significantly alter transcriptional activity driven by NFAT-AD alone bound to DNA.

These four cooperativity-specific mutations affect the energetics of ternary complex formation to different extents, as evidenced by the results of *in vitro* binding assays. The strongest effects are observed at the polar residues T551 and E545, which upon mutation to alanine each exhibits ≈3-fold reductions of cooperativity *in vitro*. On the other hand, either mutation that removes hydrophobic side-chain functionality, I544A or I553A, reduces cooperativity to a lesser extent (≈2-fold). Thus, in contrast to situation with AP-1, wherein alteration of one residue (c-Jun-R285) practically abolishes cooperativity, no single alanine mutation in the NFAT RIR abrogates cooperative interactions with AP-1 *in vitro* (25). This observation suggests that the cooperative binding energy is distributed over several residues of the NFAT RIR. Interestingly, whereas the driving force for protein–protein interactions is typically provided by hydrophobic contacts (36, 37), the NFAT/AP-1 interface appears to be dominated energetically by hydrogen-bonding and electrostatic interactions. Since such polar contacts tend to be relatively weak in aqueous solution, they may be uniquely suited to establish cooperative interfaces whose formation is dependent upon presentation by a DNA template.

Structural insights into the overall architecture of the NFAT/AP-1/DNA co-complex have been provided by a model constructed from the solution structure of NFAT2-DBD and the x-ray cocrystal structure of AP-1/DNA (23) (Fig. 5*A*). Even though the structure of the NFAT RIR in this model represents that of the free protein in solution, and hence is likely to be folded into a more compact structure in the actual case, it is nonetheless constrained by backbone connectivity to lie in close proximity to the junctional region of AP-1 and to the DNA backbone. Another surface projection on NFAT, the β-CD loop, also appears within reach of AP-1 (Fig. 5*A*), but its role in cooperative interactions was not investigated here. Notwithstanding the uncertainty in the structure of the RIR, we note that both residues that exhibit the greatest effects on cooperative binding, T551 and E545, are located in the middle of the RIR, where they can readily be envisioned to contact side-chain functionality of the AP-1 junctional region (Fig. 5*B*). Indeed, it is tempting to speculate that cooperative recruitment of AP-1 by NFAT could involve direct hydrogen-bonding or electrostatic interaction between NFAT-Thr-551 or NFAT-Glu-545 and the critical c-Jun-Arg-285 residue. Critical residues could also be provided by the backbone amide groups of the NFAT RIR, or be required to attain a proper folded conformation of NFAT RIR, such interactions would not be detected by the present methods of analysis. The RIRs of all NFAT isoforms identified thus far are highly conserved (Fig. 5*D*), suggesting a common mechanism by which NFAT family members interact with AP-1 family proteins at composite response elements such as ARRE2 (38).

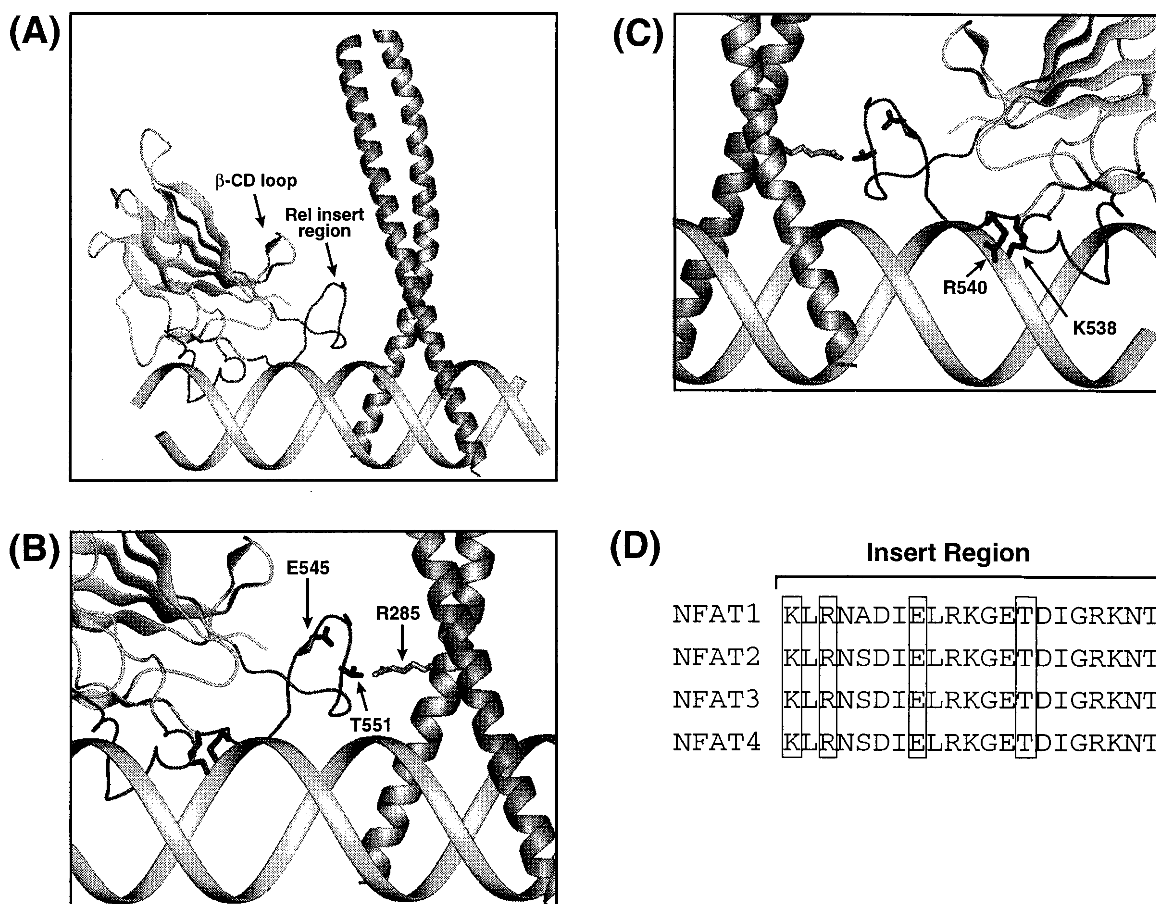

FIG. 5. (*A*) Model of the ternary NFAT/AP-1/DNA complex (23). The solution structure of the NFAT2-DBD (23) was superimposed with x-ray coordinates of DNA-bound p50 (30) by rigid body fitting of their respective β-barrels. The overall conformation of the NFAT RIR in the model was derived from a non DNA-bound structure and is likely to differ from that in the actual complex. The x-ray coordinates of the AP-1 bZip domain (27) were used to assemble the heterodimer over the nonconsensus AP-1 site in ARRE2, with positional and orientational information derived from affinity cleaving experiments (28). (*B*) An expanded view of the of the NFAT2 RIR and the junction of AP-1 between the basic and leucine-zipper domains. The side chains of three residues identified as being most critical for cooperativity (NFAT E545, NFAT T551, and c-Jun R285) are illustrated. (*C*) The view shown in *B* rotated by 180° about the vertical axis. The side chains of two NFAT residues critical for specific DNA recognition (K538 and R540) are shown. (*D*) Sequence alignment of the insert region of the four NFAT isoforms. Conserved residues identified here as being important for DNA recognition and cooperative binding with AP-1 are boxed.

The NFAT RIR Is Responsible for DNA Recognition by NFAT. Chemical crosslinking and methylation interference footprinting studies have shown that NFAT2 and p50 use identical side-chain functionality of a conserved DNA recognition loop to recognize the 5′-half site (5′-GGAAAA-3′) (Fig. 1*A*) (23). However the residues in NFAT responsible for recognition of the 3′-half site (5′-GGAAAA-3′) are not clearly understood. Template-directed interference footprinting analysis of NFAT1 has indicated strong major groove contacts only in the 5′-half site (C. Min and G.L.V., unpublished work). Switching of individual A·T base pairs to I·C in the NFAT site, which drastically alters the major groove functionality without affecting the minor groove (39), disrupted binding at the first A·T (GGA), but had little or no effect on the three A·T pairs in the 3′-half site (23). These data have suggested that recognition of the 3′-half site by NFAT is provided either via indirect readout of the 3′ poly(A) stretch, or by direct or water-mediated contacts to the DNA minor groove.

The present study describes the identification of three NFAT point mutations (K538A, R540A, and N541A) that significantly reduce β-gal gene expression driven by the *in vivo* assembly of the binary NFAT-AD/DNA complex. Two of these mutations, K538A and R540A, also strongly diminished ($\approx$10-fold) the affinity of NFAT for DNA *in vitro*. In our model (Fig. 5*C*), Lys-538 and Arg-540 are located at the N terminus of the RIR, directly over the 3′-half site. Based on these considerations, we propose that Lys-538 and Arg-540 make polar contacts to either the backbone or minor groove base-surface of the 3′-half-site, and that these interactions are likely to be play an important role in both the strength and specificity of NFAT-DNA interactions.

Although both NFAT-DBD and NF-κB p50 share a common structural fold, they differ substantially in their modes of sequence-specific DNA recognition. For example, whereas all known members of the NFAT branch of Rel proteins bind DNA as monomers, those of the NF-κB branch bind DNA as obligate dimers. The two branches show the greatest structural divergence in their RIRs, suggesting that this segment might play an important role in differentiating the functions of NFAT and NF-κB. Indeed, here we have shown that the NFAT RIR serves a key functional role in monomeric DNA-binding and in cooperative interactions with AP-1, operational features that are not shared by NF-κB. We thus conclude that the RIR is a key structural element that confers diversity on the range of biologic responses elicited by Rel proteins.

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