REL Domain of NFATc2 Binding to Five Types of DNA Using Protein Binding Microarrays

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ABSTRACT: NFATc2 is a DNA binding protein in the Rel family transcription factors, which binds a CGGAA motif better when both cytosines in the CG dinucleotide are methylated. Using protein binding microarrays (PBMs), we examined the DNA binding of NFATc2 to three additional types of DNA: single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) with either 5-methylcytosine (5mC, M) or 5-hydroxymethylcytosine (5hmC, H) in one strand and a cytosine in the second strand. ATTTCCAC, the complement of the core GGAA motif, is better bound as ssDNA compared to dsDNA. dsDNA containing the 5-mer CGGAA with either 5mC or 5hmC in one DNA strand is bound stronger than CGGAA. In contrast, the reverse complement TTCCG is bound weaker when it contains 5mC. Analysis of the available NFATc2:dsDNA complexes rationalizes these PBM data.

1. INTRODUCTION

Nuclear Factor of Activated T-cells cytoplasmic 2, or NFATc2 (also known as NFAT1, NFAT1a, or NFATP) is a member of the calcium-responsive NFAT family of transcription factors (TFs). NFATc2 is expressed in many somatic tissues including T-cell differentiation and activation, and development.2,6 NFAT TFs can bind cooperatively with other TFs including AP-1,13 GATA4,14 IRF4,15,16 FOXP3,17 and MEF2.18 For clarity, we present DNA sequences using the bold font.

Several structures of monomeric NFATc2 bound with double-stranded DNA (dsDNA) have been solved12,19,20 and amino acids that interact with both strands in the major groove of dsDNA have been highlighted. Two arginines in the loop regions of RHR-N (R421 and R430) form bidentate hydrogen bond interactions in the major groove with G1 and G2 of the NFAT DNA motif.21 The amide side chain of Q571 forms hydrogen bonds with A4 of the core motif.22 Several amino acids’ N-terminal of R421, R430, and Q571 interacts with the thymines in the complementary strand T6′, T5′, T4′, T3′, C2′, C1′ (nucleotide bases in the complementary strand are denoted with a ′”). Y424 interacts with T3′ and T4′, and R572 interacts with T5′ and T6′ via van der Waals contacts, and both R572 and Y424 form hydrogen bonds with the DNA backbone.11

It was shown that NFATc2 can bind strongly to sequences containing 5-mer C1′G′G′A′A′ when a cytosine in both strands of the C1′G′ dinucleotide is methylated (e.g., M′G′G′A′A′ in one strand and T4′C′ in the second strand),23 expanding the DNA sequences bound by NFATc2. To further explore the range of DNA binding of NFATc2, we performed protein binding microarray (PBM) experiments24 with microarrays containing three different types of DNA, single-stranded DNA (ssDNA), dsDNA with 5mC in one strand and a cytosine in the second strand (dsDNA(5mC C)), and dsDNA with 5hmC in one strand and a cytosine in the second strand (dsDNA(5hmC C)),25 and compared these data to previous data of NFATc2 binding to dsDNA with cytosine in both strands (dsDNA(5mC C)) and where both cytosines in all CG dinucleotides contain 5mC (dsDNA(5mCG)).23 Previously, we have examined several bZIP family TFs binding with these modified dsDNAs and identified the critical roles of conserved amino acids in their sequence-
specific DNA binding ability.\textsuperscript{26,27} Here, the contribution of 5mC and 5hmC in one strand of dsDNA to NFATc2 binding was examined.

2. RESULTS

2.1. Protein Binding Microarrays with Four Types of DNA. Previously, we examined NFATc2 binding to two types of dsDNA using PBMs, dsDNA where both strands contain a cytosine (dsDNA(C)) and dsDNA where both cytosines in all CG dinucleotides are methylated (dsDNA(5mC)).\textsuperscript{23} NFATc2 preferentially bound many 8-mers containing a methylated CG dinucleotide in 5-mer CGGAAA.\textsuperscript{23} To learn more about NFATc2 binding to different types of DNA, we examined NFATc2 binding to three additional forms of DNA using PBMs. The 16 grids of the Agilent HK design 40k DNA microarray\textsuperscript{28,29} were divided into four chambers containing four grids each using a gasket slide from Agilent (Figure S1). One chamber was left as ssDNA and the three other chambers were double-stranded using either a cytosine producing dsDNA(C), or 5mC producing dsDNA(5mC), or 5hmC producing dsDNA(5hmC).\textsuperscript{30–33} NFATc2 was bound to two grids in each of the four sectors. Examination of binding to all four types of DNA on a single microarray slide allows for comparisons between DNA types. These new data for NFATc2 binding dsDNA(C)\textsuperscript{32} agree with our previously published data ($R = 0.8$, Figures S2 and S3E).

2.2. Rel Domain of NFATc2 Binding to Four Types of DNA. We evaluated NFATc2 binding to four types of DNA by examining the median and average binding to the 40,000 DNA features on each sector of the Agilent microarray (Figure 1 (replicate data 1), Figure S4 (replicate data 2), and Table S1). The difference reflects sequence-specific DNA binding. The median binding of NFATc2 to ssDNA (2256, Figure 1) is higher than the three types of dsDNA, indicating that NFATc2 preferentially binds ssDNA. However, the average binding intensities for dsDNA(C) (2830) and dsDNA(5mC) (3404) are higher compared to ssDNA (2351), indicating more sequence-specific binding. This is observed in the longer right tail of all the three dsDNA distributions (Figure 1 and Table S1), indicating that NFATc2 is binding specifically to some dsDNA. Both average and median binding is highest for dsDNA with 5mC, followed by a cytosine, and finally 5hmC. A search for the most enriched DNA motifs in the best-bound 1% of the 40,000 PBM features for all the four DNA types finds the core motif 5-mer TTTCC as the best bound sequence in both ssDNA and dsDNA(C). The preference for binding ssDNA TTTCC suggests that these interactions are stronger than the interactions with the opposite strand GGAAA. The best bound features for dsDNA(5mC) and dsDNA(5hmC) contain the 5-mer MGGAA and HGGAA (Table S2).

2.2.1. NFATc2 Binding to ssDNA and dsDNA(C). We next calculated the relative binding affinities (Z-scores)\textsuperscript{29} for NFATc2 binding to all 8-mers. While other 8-mers measures have been developed such as the rank-based E-score,\textsuperscript{34,35} we prefer to use Z-scores as they are directly associated with binding affinity and give an idea of the specificity of the protein under investigation. Figure 2 (Figure S5A for replicate data) presents a scatter plot comparing NFATc2 Z-scores to all DNA 8-mers for dsDNA(C) (x-axis) or ssDNA (y-axis). The 8-mers are divided into two groups, those that contain 5-mer TTTCC, which is enriched in the top bound array features for both ssDNA and dsDNA(C), and all other 8-mers. For dsDNA(C), reverse complements are equivalent (i.e., TTTCC is equivalent to GGAAA), but with ssDNA, complements are not equivalent. We obtain lower Pearson correlation values for the 8-mer Z-scores for ssDNA replicate experiments (Figures S2B and S3) compared to those obtained for the raw intensity values (Figure S2A), highlighting the lower sequence specificity for ssDNA. Nevertheless, among the top bound 8-mers bound by NFAT as ssDNA are those containing TTTCC and GTTCC (Figures 2, SSA-C, Table S3).

2.2.2. NFATc2 Binding to dsDNA(5mC). Recently, it was shown that the Rel domain of NFATc2 binds 8-mers...
containing the 5-mer CGGAA stronger when both cytosines in the CG dinucleotide are 5mC. We examined binding when only one strand contains 5mC. Figure 3A shows the scatter plot comparing 8-mers bound by NFATc2 to dsDNA(C|C) (x-axis) and dsDNA(5mC|C) (y-axis). For this comparison, we divided 8-mers into four groups: (1) those that contain the NFATc2 canonical 5-mer CGGAA, (2) those that contain the reverse complementary 5-mer TTCCG, (3) all other 8-mers with a cytosine, and (4) 8-mers with no cytosine. NFATc2 binding to 8-mers without a cytosine is along the diagonal and acts as an internal control. The best bound 8-mer in both dsDNA(5mC|C) and dsDNA(C|C) in the non-cytosine 8-mer group is TGGAAAAT; thymine and 5mC both contain a methyl group at the same position of the pyrimidine ring. In general, 8-mers with CGGAA are better bound with dsDNA(5mC|C), while 8-mers containing the reverse complement TTCCG are better bound with dsDNA(C|C). Examination of NFATc2 binding to 8-mers containing a single cytosine or 5mC (Figure 3B) shows a similar pattern to Figure 3A. Here, all 8-mers containing the MGAAA 5-mer are well bound, suggesting that methylation of a single cytosine at position −1 in the M−1GGAA 5-mer is sufficient for the stronger binding of NFATc2 (Figure 3B).

2.2.2.1. Contribution of Each 5mC in a Methylated CG Dinucleotide to Preferential NFATc2 Binding. We compared the dsDNA(5mC|C) data to previous data where both cytosines in all CG dinucleotides are 5mC (dsDNA-(5mCG)). We examined the 5096 8-mers that contain one cytosine that is in a CG dinucleotide (Figure 3C). 8-mers containing the CGGAA 5-mer are along the diagonal, indicating that methylation of the single cytosine in CGGAA is energetically similar to methylation of both cytosines in the CG dinucleotide. It also suggests that methylation of the cytosine on the other strand (e.g., TTCCMG) does not change NFATc2 binding. While our experiments do not allow for different combinations of unmethylated and methylated cytosines on the same strand (i.e., we cannot measure binding of TTCM14G), the reduction of NFATc2 binding to the sequence TTMMG observed in Figure 3A suggests that 5mC at position 2′ is responsible for the reduced binding to the complement motif.
2.2.3. NFATc2 Binding to dsDNA(5hmC/C).

5hmC is an oxidative product of 5mC that is associated with active demethylation of DNA in vivo.36 We compared NFATc2 binding to dsDNA(C/C) and dsDNA(5hmC/C) (Figure 4A). The effect of 5hmC on NFATc2 binding is not as dramatic as observed with 5mC. Similar to dsDNA(5mC/C), all 8-mers containing the reverse complement (TTCGG) are preferentially bound to dsDNA(C/C) compared to dsDNA(5hmC/C). Few 8-mers with CGGAA are moderately better bound with 5hmC (e.g., TGHGAAAAA). Examination of 8-mers with only one cytosine (Figure 4B) highlights a single 8-mer, with CGGAA (CGGAAAAA) being the best bound 8-mer with either 5hmC or C. 8-mers containing 5hmC outside of the core motif (e.g., AHTGGAAA) are also more strongly bound by NFATc2, indicating that 5hmC at positions outside of the core motif can influence NFATc2 binding.

Figure 4C,D compares NFATc2 binding to 8-mers for dsDNA(5mC/C) (x-axis) and dsDNA(5hmC/C) (y-axis). Most 8-mers containing MGGAA are better bound than HGGAA (e.g., TMGGAAAAA and AMGGAAAA), with the exception being 8-mer M/HGGAAAAA, which is similarly strongly bound by NFATc2 when it contains either 5mC or

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Figure 4. NFATc2 binding to dsDNA 8-mers with 5hmC in one strand. (A) 8-mer Z-score comparison of NFATc2 binding to dsDNA(C/C) (x-axis) and dsDNA(5hmC/C) (y-axis). (B) Same as in (A) but for 8-mers containing one cytosine. (C) 8-mer Z-score comparison of NFATc2 binding to dsDNA(5mC/C) x-axis and dsDNA(5hmC/C) (y-axis). 8-mers are colored as shown in Figure 3, with (D) same as in (C) but for 8-mers containing 1 cytosine. For all panels, 8-mers are colored as shown in Figure 3, with the sequences of several 8-mers indicated.
5hmC. NFATc2 modestly prefers 8-mers with TTMGG to TTHHGG. Some 8-mers containing cytosines outside the core CGGAA 5-mer are better bound when they contain 5hmC compared to 5mC or cytosine (e.g., the aforementioned AHTGAAA), highlighting that 5hmC at positions outside of the NFAT core motif can promote NFATc2 binding to dsDNA.

2.3. Structural Analysis of NFATc2 Binding Methylated dsDNA. We investigated the available NFAT/dsDNA structural complexes\(^\text{20,21}\) in order to understand the physical causes of the effects of cytosine modifications on NFATc2 dsDNA binding. Figure 5A examines the N-terminal segment of the dsDNA binding domain of NFAT1 (RHR-N) binding the beginning of the T\(^{1}\)GGAA consensus element. In particular, it is seen that the methyl group of the thymine at the 1 position (T\(^{1}\)) occupies a partially hydrophobic pocket formed by the side chain of R430 and the protein backbone. This is consistent with thymine being a preferred nucleotide at this position for complex formation, and why 5mC, for which the added methyl group is at the same location as in thymine, is preferred over cytosine. On the complementary strand, it is seen that the added methyl group on the cytosine at the 1 position (C\(^{1}\)) is distant from the protein, consistent with our suggestion that methylation at this position has no effect on the dsDNA binding affinity of the complex. Finally, it is seen that for the second cytosine on this strand (C\(^{2}\)), the added methyl group butts against the side chain of E427, which is not seen for the unmethylated version of the consensus (Figure 5B). This suggestion of a potential steric clash is consistent with the finding that methylation of this cytosine weakens binding. This is further supported by an NMR structure involving only the RHR-N domain of NFAT2 (PDB 1A66;\(^\text{27}\)). In this case, C\(^{2}\) and E427 are in close proximity, and methylation causes an obvious steric clash (Figure S6).

3. DISCUSSION

Previously, we showed that NFATc2 binds stronger to the 5-mer C\(^{1}\)G\(^{1}\)A\(^{1}\)A\(^{2}\) when both cytosines in the CG dinucleotide are methylated (i.e., M\(^{1}\)G\(^{1}\)A\(^{1}\)A\(^{2}\) in one strand and T\(^{4}\)T\(^{3}\)C\(^{2}\)M\(^{1}\)G\(^{1}\) in the second strand).\(^\text{23}\) Here, we examine how NFATc2 binds to three additional kinds of DNA: ssDNA, dsDNA(5mC), and dsDNA(5hmC). ssDNA 8-mers containing TTTCC or GTTCC are among the best bound 8-mers by NFAT, suggesting that interactions with the strand containing the TTTCC consensus may dominate interactions on the opposite strand. We evaluated how 5mC in either strand of dsDNA, one in the C\(^{1}\)GGAA and two in the complementary strand TTTCC, affects NFATc2 binding. 5mC in CGGAA and 5mC in both the cytosines of the CG dinucleotide in CGGAA are similarly bound by NFATc2, suggesting that 5mC in the opposite strand (TTCC\(^{2}\)C\(^{1}\)G) at C\(^{1}\) does not change binding. However, 5mC at position 2 in the complement TTCC\(^{2}\)C\(^{1}\)G inhibits DNA binding, which we attribute to a steric clash occurring between the methyl group of 5mC at position 2 of the core motif and Glu427 of the RHR-N domain of NFATc2. 5hmC is similar to cytosine for binding CGGAA and weakens binding to TTCCG more than 5mC.

The strong binding to certain ssDNA 8-mers may be biologically important. It is becoming increasingly clear that non-B-form DNA structures are bound in a sequence-specific manner and take part in gene regulation by selective binding of different TFs and small molecules.\(^\text{37-39}\) Sequence-specific binding to ssDNA has regulatory roles in eukaryotic transcription.\(^\text{40}\) The stronger binding to ssDNA may reflect the conformational flexibility of ssDNA\(^\text{40,41}\) and indicate that sequence-specific binding is primarily on one strand of DNA.

Our PBM data and structural analysis suggest a similarity between 5mC and thymine on NFATc2 binding (Figures 3
and 5). However, some 8-mers with MGAAA are better bound than those containing TGAAA (Figure S7A,B), suggesting that they are not equivalent. Many of these 8-mers differ from each other at locations outside of the core NFAT motif (e.g., AATMGGAA, \( p \)-val = 0.006, Figure S7B). In addition, several thymine to 5mC substitutions also reduce binding of NFATc2, including those outside of the core motif (e.g., TGGAAAT is better bound than TGGAAM, \( p \)-val = 0.04, Figure S7B). We currently cannot provide a physical explanation for NFATc2 binding these 8-mers. Our data highlight the effects of cytosine and modified cytosines inside and outside of the core motif on NFATc2 dsDNA binding, providing a richer description of the dsDNA binding specificity of NFATc2. The DNA binding domain of all NFAT members is highly conserved (64–72% sequence identity),\(^\text{42}\) suggesting that other NFAT family members may have similar changes in dsDNA binding with 5mC and 5hmC.

4. MATERIALS AND METHODS

4.1. Cloning and Expression of Human NFATc2 DNA Binding Domain. The construct containing human NFATc2 is an N-terminal GST construct cloned into a Gateway system pDEST15 vector.\(^\text{33}\) The protein was expressed using the PUREExpress In vitro protein synthesis kit (NEB) as per the manufacturer’s protocol\(^\text{33}\) in a 12.5 \( \mu L \) reaction volume containing 90 ng of plasmid. The amino acid sequence of NFATc2 (https://www.ncbi.nlm.nih.gov/protein/Q13469.2) with the Rel homology DNA binding domain (RHR-N) is shown below in bold:

LVPPTWPKLPVAPAICSPVTAAPLPLEWPLSSQSGSYELRIEVQPKPHRAHYETGESRGAV-KAPTGGHGVQVQVLMENKPLGQLIFITADERILK-PHYFQVRHITGKTTTSSYIEKIVGNTKVLEIPLEPKNNRAATIDCAGILKRNADIELRKGGET-DIGKRRNTRVLRVFVHPESSGRVLQTAASNIPIECQSRAHELPMVERQTDSCLYVYGGQMQILTGNQFTSESKVFTKTDTDQGQWEMATEVDKDSQPNMLFVEILPEYRNKHIRTVPVKNYFVINGKRRSQPQHFTYHPV-PAIKTEPTDEYDPTL.

4.2. Design of 40k Feature PBM and Double Stranding of the Microarray. The 40,000 array feature PBM design contains 16 sectors, each containing a DNA grid of 400 8-mers. These features are represented 32 times. T7 DNA polymerase was used to incorporate cytosine (NEB) or 5mC (NEB) or 5hmC (Zymo Research) into the DNA, respectively, in the probes during double-stranding reaction (see Supporting Information). All 8-mers (including complements) are once on the array, and all 8-mers (including complements) are represented 32 times. T7 DNA polymerase was used to incorporate cytosine (NEB) or 5mC (NEB) or 5hmC (Zymo Research) into the DNA, respectively, in the probes during double-stranding reaction, creating unmethylated, hemi-methylated, or fully methylated dsDNA.\(^\text{30,33,44}\) To monitor the double-stranding efficiency, the double-stranding reaction mixture was spiked with Cy3-dCTP (4%).\(^\text{30,33,44}\)

4.3. DNA-Protein Binding Reaction and Data Analysis. DNA-protein binding reactions were performed using these four types of DNA.\(^\text{35}\) The image generated from the Agilent Surescan microarray scanner was quantified using ImaGene 9 software (BioDiscovery Inc.), and the extracted probe intensity values were used for the calculation of Z-scores.\(^\text{44}\) In previous studies, complementary 8-mers were combined,\(^\text{33}\) but because of the asymmetric nature of the double-stranding protocol for 5mC and 5hmC on PBMs, complementary 8-mers are different. Therefore, the Z-scores of the reverse complement 8-mer were only considered and extracted from the array probe design as these represent the sequences containing the modified cytosine in dsDNA. Z-score is the measure of standard deviations from the 8-mer intensity from the global array median intensity.\(^\text{34}\) All proteins were assayed at least twice with good agreement (\( R > 0.85 \)) between replicates (Figures S2 and S3). For all visualizations in the main figures, we used data from representative experiments (indicated in Figure S2). Data (raw probe intensities and 8-mer Z-scores) have been deposited at the NCBI Gene Expression Omnibus (GEO) database under accession GSE10463.

4.4. Motif Enrichment. Motif enrichment analysis in the top 1% (403) of array probes ranked by intensity was performed using the MEME tool of the MEME software suite version 4.11.2.\(^\text{46}\) Motifs were searched on the forward strands for the ssDNA PBMs and the reverse strand for the dsDNA(5mC) and dsDNA(5hmC) experiments. Enriched motifs were searched for on both strands of the dsDNA(C) experiments.

4.5. Structural Modeling. Models of the NFATc2 protein binding the sequence TGGAAA and the methylated complement TTTTMA were developed from the crystal structure of the unmethylated complex (ref 20; PDB: 1pzu) with the UCSF Chimera software package.\(^\text{47}\) Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).
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Notes
The authors declare no competing financial interest.

ABBREVIATIONS
M, S-methylcytosine; H, 5-hydroxymethylcytosine; PBM, Protein Binding Microarray; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA

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