Research Square

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Chemical shift assignments of the dimerization domain of NF-KappaB c-Rel subunit

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Abstract

The NF-κB family of transcription factors serve as master regulator of a broad range of cellular processes in vertebrates ranging from immune activation and inflammation to cell survival, proliferation, and development. The five NF-κB family members can potentially assemble into 15 different dimers, each capable of recognizing a 9–11 base pair κB DNA sequence on its target-gene promoter/enhancer region. Here, we report the NMR backbone chemical shift assignment for the 23.2 kDa homodimer of NF-κB c-Rel subunit. In contrast to the ubiquitously expressed NF-κB subunits such as RelA and p50, c-Rel expression is confined largely to hematopoietic lineages. Dysregulated c-Rel activity drives immune dysfunction, chronic inflammation, and blood cancer. Chemical shift-derived secondary structure of the dimer is broadly consistent with existing DNA-bound crystal structures of the protein.

Biological context

The Nuclear Factor-KappaB (NF-κB) family of transcription factors is universally recognised as a critical regulator of innate and adaptive immune response in higher vertebrates. The pathway is complex and composed of five structurally related members, including NF-κB1 (*aka* p50), NF-κB2 (*aka* p52), RelA (*aka* p65), c-Rel (*aka* Rel) and RelB. All of these members share a highly conserved DNA binding domain called the rel homology region (RHR). The RHR is composed of two subdomains, namely, the N-terminal domain (NTD) that recognises the cognate κB sequence on the promoter/ enhancers of its target genes, and the dimerization domain (DD) which is responsible for the formation of dimers among NF-κB subunits. c-Rel is the cellular homolog of v-Rel, the oncogenic viral protein first identified in the avian retrovirus REV-T, the first NF-κB family member ever isolated (Hrdlicková et al. 1994). High levels of c-Rel expression are largely restricted to the hematopoietic cell, particularly B and T lymphocytes (Carrasco et al. 1994). Furthermore, aberrant c-Rel expression contributes to lymphomas and autoimmune disorders (Leeman et al. 2008). c-Rel exists as a homodimer or as heterodimers with other NF-κB subunits like p50 across a variety of cell types, playing a major role in activating canonical NF-κB target genes (Hoffmann et al. 2003). The mechanism driving specific NF-κB dimer formation remains poorly understood, even with existing crystal structures. Assignment of the resonances of the DD of c-Rel-homodimer (c-Rel DD) is a first step towards understanding the mechanism of subunit exchange during heterodimer formation between c-Rel and other NF-κB partners. Here, we report the backbone resonance assignments of c-Rel DD (23.2 kDa excluding the first methionine).

Methods and experiments

Cloning, expression, and purification

The murine c-Rel DD (182–282 aa residues) was sub-cloned into the pET-28c vector from the full-length c-DNA sequence, obtained from Addgene (RRID: Addgene_20013, (Sanjabi et al. 2005)). The construct was over-expressed in *Escherichia coli* BL-21(DE3) cells in untagged form in M9 minimal media (Anderson 1946; Sambrook et al. 1989) supplemented with 2 g/L of ^{13}C-labeled glucose, 1 g/L ^{15}N-

labeled ammonium chloride (Cambridge Isotope Laboratories, Andover, MA, USA) and 30 µg/ml kanamycin. The cells were cultured at 37°C until the optical density at 600 nm reached 0.6, at which point protein expression was induced with 0.5 mM isopropyl-beta-thiogalactopyranoside (IPTG) followed by overnight incubation with agitation at 20°C. The cells were centrifuged and harvested at about 3200 g for 10 min and stored at − 80°C. For protein purification, the cells were thawed and resuspended in buffer containing 20 mM Tris (pH 7.2), 50 mM NaCl, 20 mM β-mercaptoethanol (β-ME), 1 mM EDTA, 10 % v/v glycerol and 0.5 mM PMSF. Cell lysis was performed b sonication (45 % power; 1 s pulse/9 s rest duty-cycle, for 40 min) followd by centrifugation at 14,000 g for 40 minutes. The supernatant containing the soluble fraction of the protein was collected following centrifugation and subjected to anion-exchange chromatography using Q-Sepharose Fast-Flow beads (GE Healthcare). The protein bound to the column was washed with the wash buffer (lysis buffer without PMSF) and then batch eluted using elution buffer (wash buffer containing 75 mM NaCl). The eluted fractions containing c-Rel DD was concentrated to a total volume of ~ 2 mL using 10 kDa cut-off centrifugal filters (MERCK Millipore) and subjected to size-exclusion chromatography using Superdex-75 column (GE-Healthcare) equilibrated with degassed 20 mM Tris-HCl (pH 7.2), 50 mM NaCl, 20 mM β-ME. The eluted fractions were checked on 15 % SDS-PAGE and the protein containing fractions were poole together followed by further concentration and buffer exchange to NMR buffer containing 20 mM Bis-Tris (pH 6.8), 50 mM NaCl and 20 mM β-ME.

NMR spectroscopy

Protein samples used in this study were in NMR buffer with 7% $^{2}H_2O$, containing 0.7 mM of the protein. NMR experiments were recorded on Bruker Avance 700 MHz NMR spectrometer equipped with a 5 mm triple-resonance cryogenic probe. The standard set of NMR experiments, namely, HNCA, HNcoCA, HNCACB, CBCAcoNH, HNCO, HNcaCO for the backbone resonance assignments along with a sensitivity-enhanced 2D [^{15}N, ^{1}H]-HSQC experiment as described by(Schleucher et al. 1994) was acquired. The [^{15}N, ^{1}H]-HSQC spectrum was acquired with 2048 and 256 complex points along ^{1}H and ^{15}N dimensions, respectively. Additionally, [^{15}N-^{1}H]-NOESY-HSQC (with a 100 ms mixing time) was acquired (Davis et al. 1992). All the NMR experiments were performed at 30 ^{o}C. The NMR data were processed in NMRPipe(Delaglio et al. 1995) and analyzed in CARA (Keller 2005). The chemical shifts for ^{15}N and ^{13}C were calibrated indirectly, whereas ^{1}H chemical shifts were directly referenced using DSS.

Extent of assignments and data depositions

c-Rel DD construct comprises 101 amino acids as a monomer, including an additional Methionine and Glycine appended at the N-terminus to maintain reading frame during subcloning into the pET-28c vector. Resonance assignments for the c-Rel DD homodimer (23.2 kDa) are mapped onto its [^{15}N, ^{1}H]-HSQC spectrum in Fig. 1, shown here. Residue numbering scheme used in this paper corresponds to that of the full-length c-Rel subunit. Following the classical assignment strategy, an overall 99% completeness of resonance assignments was achieved ($^{1}H^{N}$ 99%, ^{15}N 99%, $^{13}C_{\alpha}$ 99%, $^{1}H_{\alpha}$ 98%, $^{13}C_{\beta}$ 99% and ^{13}CO 99%).

Amide resonances from the N-terminal methionine and the five proline residues, that lack an amide proton, were not observed. The unusually up-field shifted amide proton chemical shift of C250 residue in the [^{15}N, ^{1}H]-HSQC spectrum can be attributed to its close spatial proximity to the aromatic ring of the conserved W225 residue, as found in the c-Rel homodimer crystal structure from chicken (PDB ID: 1GJI).

Backbone dihedral angles were estimated from assigned chemical shifts using TALOS-N web server (Shen and Bax 2013), which additionally predicted secondary structure elements and RCI order parameters (S^2). Secondary structure analysis (Fig. 2A) identified nine β-strands within the c-Rel DD; eight of these (β1 (187–192), β2 (204–209), β3 (217–223), β4 (226–232), β5 (237–238), β6 (242–246), β7 (258–266), and β8 (276–280)) are consistent with the previously reported X-ray structure of the same domain from chicken (Huang et al. 2001). Most RCI-S^2 values are close to 1, a sign of a rigid structure, except for the N- and C-termini and the loops connecting the β-strands in which the values are lower (S^2 < 0.8), a sign of more flexible regions. In contrast to the X-ray structure, the NMR-derived secondary structure lacks the second β-strand (195–197) and first helix (212–216), suggesting it is dynamic in solution and stabilized primarily by crystal-packing interactions in the crystalline state (Fig. 2B).

Declarations

Data Availability

^{1}H, ^{15}N and ^{13}C sequential backbone (^{1}H, ^{15}N, $^{13}C_{\alpha}$, $^{1}H_{\alpha}$, $^{13}C_{\beta}$ and ^{13}CO) resonance assignments for c-Rel DD have been deposited in the BioMagResBank under the accession number 53211. Additional data supporting the findings are available upon request.

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Contributions

SP and MN prepared the samples for the experiments and recorded the NMR experiments. SP processed and analyzed the data. SP and SPM wrote the main manuscript text and figures. All authors reviewed the manuscript.

Ethics declarations

Ethical approval

The authors declare that the experiments described in this publication were done in compliance with the ethical standards of the countries in which they were performed.

Competing interests

The authors declare no competing interests.

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Figures

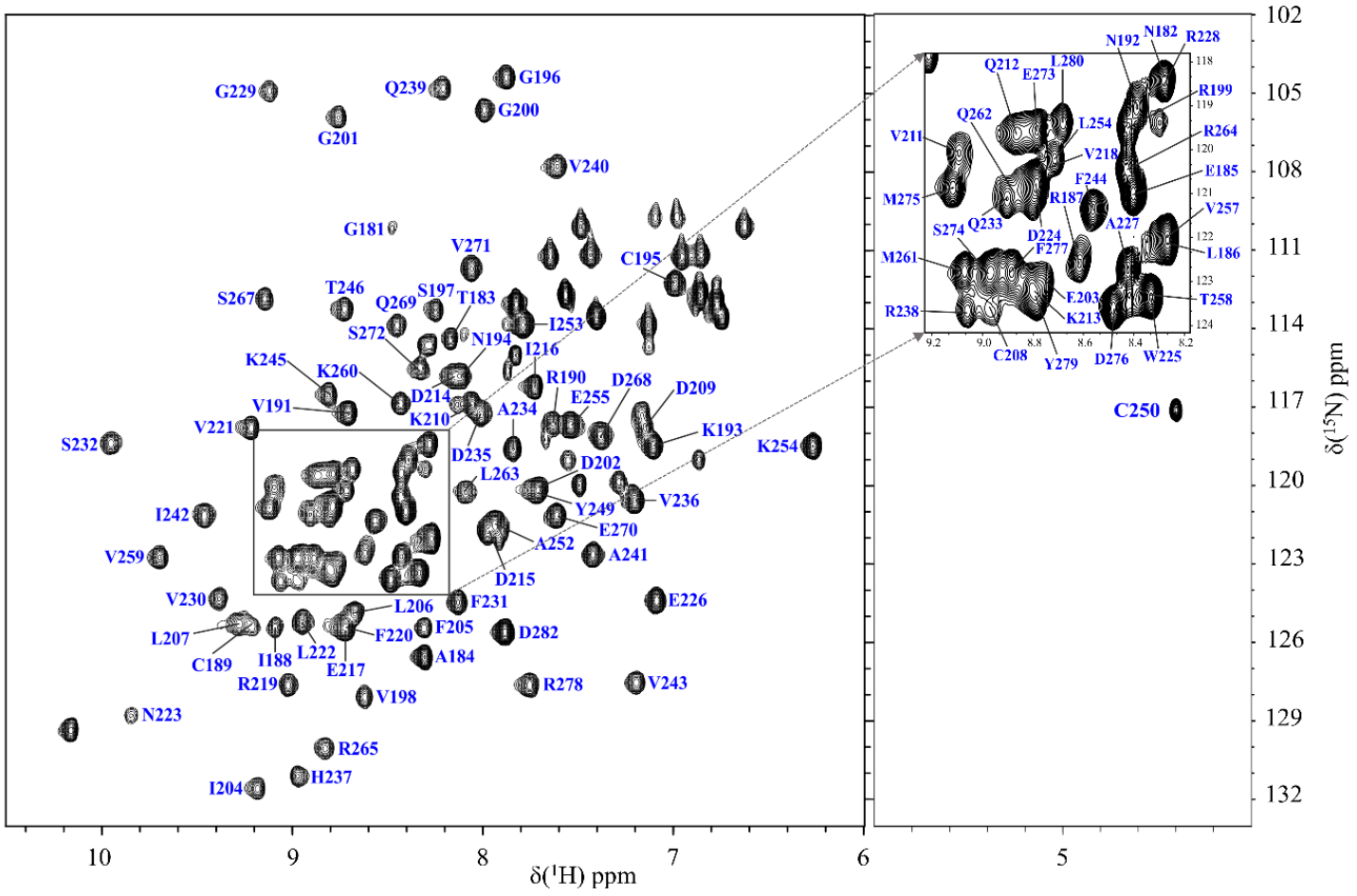


Figure 1

2D [^{15}N–^{1}H]–HSQC spectrum of the c-Rel DD at 303 K. The amide resonance assignments are shown in one letter amino acid code followed by the sequence number in the full-length protein. All the NMR experiments in this study were recorded on 700 MHz NMR spectrometer.

Figure 2

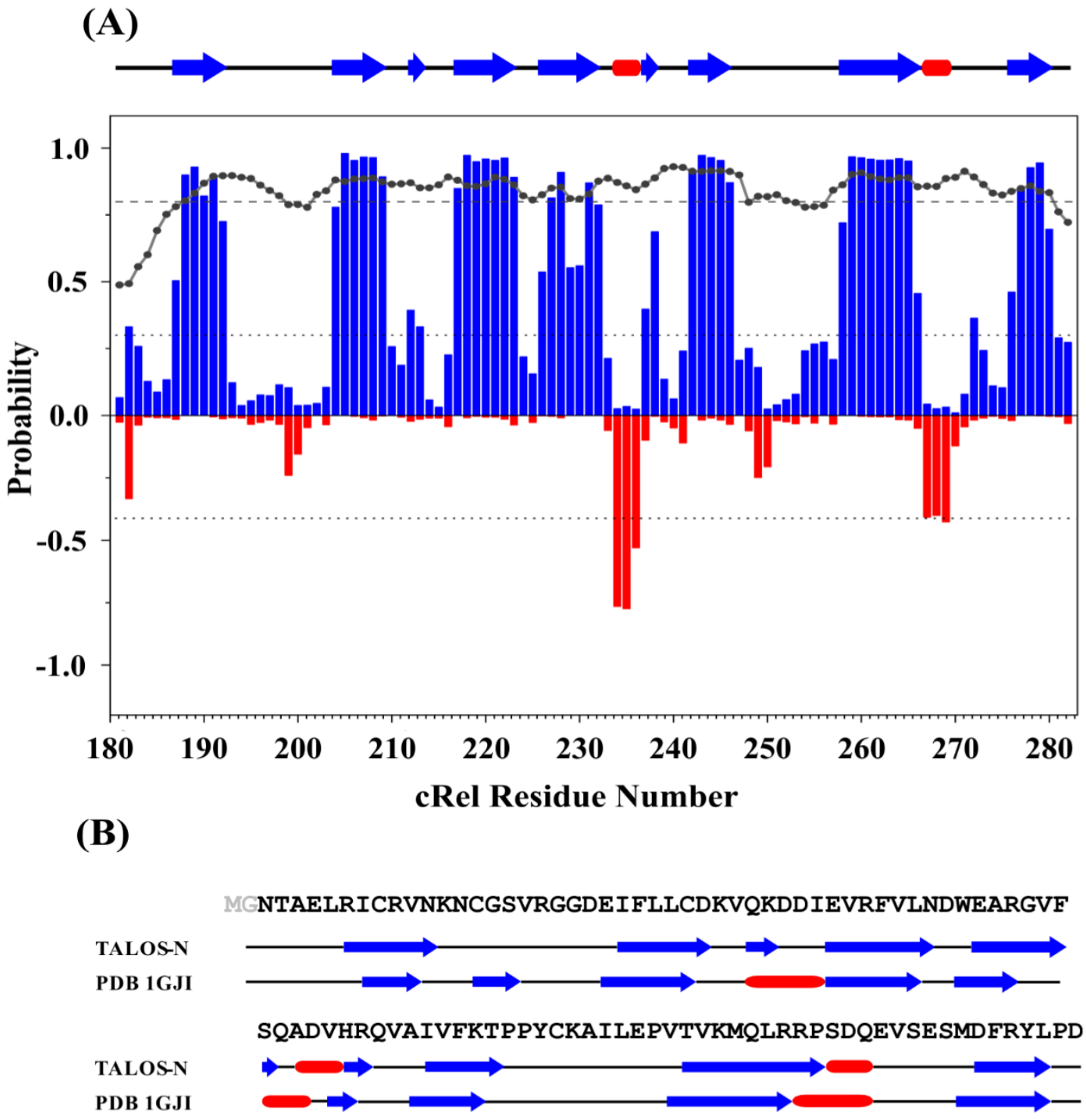


Figure 2

Secondary structure and flexibility analysis of the c-Rel DD. (A) Predicted secondary structure of c-Rel DD using TALOS-N program. The probabilities of β-strand and α-helix are given by positive (blue bars) and negative (red bars) values, respectively. Regions with probability values between − 0.3 and 0.3 are considered as loop. The secondary structure observed is depicted as a cartoon over the top of the plot with β-strand depicted as blue arrow and helical turn as red cylinder The flexibility of c-Rel DD is also

depicted as Random Coil Index (RCI) derived S^2 (RCI-S^2) values predicted by the TALOS-N server and shown with grey connected dots. A dashed line at 0.8 is plotted to facilitate visualization of relatively flexible regions. The reported amino acids for c-Rel DD are numbered in accordance with the native sequence of the full-length c-Rel. (B) Comparison of observed and predicted secondary structures for c-Rel DD. Shown are alignments of the predicted secondary structure based on backbone chemical shifts (using TALOS-N) in the present study (BMRB 53211) and observed secondary structure of c-Rel DD in the X-ray structure of the galline c-Rel RHR construct bound to kB DNA (PDB 1GJI).