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In vitro selection of optimal RelB/p52 DNA-binding motifs

Liudmila V. Britanova [a], Vsevolod J. Makeev [a,b], Dmitry V. Kuprash [a,*]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia*
[b] *Institute of Genetics and Selection of Industrial Microorganisms, State Research Centre GosNIIgenetika, Moscow, Russia*

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Abstract

Rel/NF-kB dimers of different subunit composition can activate distinct subsets of target genes *in vivo*, however, the role of DNA recognition in this specificity is not well understood. We set out to study the DNA-binding ability of RelB/p52, the least studied of all NF-kB proteins and the main transcriptionally active product of the alternative NF-kB signaling pathway. We searched for optimal binding sites for RelB/p52 by random site selection method, using full-length proteins expressed in eukaryotic cells. The subset of RelB/p52-binding sequences defines a consensus which is very similar to the classical RelA/p50 consensus. Importantly, each of these binding sites is also recognized by RelA/p50 heterodimer with comparable affinity, questioning the existence of RelB/p52-specific kappa B sites.
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The family of mammalian Rel/NF-kB transcription factors consists of five members: RelA (p65), c-Rel, and RelB (in humans called I-Rel, [1]), NF-kB1 (p105/p50), and NF-kB2 (p100/p52). All of them have an N-terminal RHD (Rel Homology Domain) which contains NLS (Nuclear Localization Signal) and is responsible for dimerization and sequence-specific DNA binding. RelA, RelB, and c-Rel (but not p50 and p52) proteins contain a C-terminal transactivation domain. Active Rel/NF-kB proteins function as homo or heterodimers, except for RelB which is only able to form heterodimers with NF-kB1 or NF-kB2.

Despite significant structural and functional similarities, different NF-kB proteins show remarkably different biological activities, as seen by phenotypes of mice deficient in single Rel/NF-kB family members (reviewed in [2]). Phenotypes of double-knockout animals reveal additional important activities, indicating some functional redundancy between Rel/NF-kB family members.

The complex role of Rel/NF-kB transcriptional factors in immunity is often described in terms of two major signaling pathways, so-called "classical" and "alternative" pathways, which involve different Rel/NF-kB dimers (RelA/p50 and RelB/p52, respectively) and activate distinct subsets of target genes (for review, see [3]).

The recruitment of specific NF-kB dimers to particular kB DNA-binding sites is controlled at multiple levels (for review, see [4]), including temporal control of subunit expression [5,6] and chromatin structure [7]. Covalent modifications [8], such as phosphorylation, also affect NF-kB activity [9,10] by modifying its ability to bind DNA [11,12] and to interact with co-activators [13].

To which extent the biological effect of a given NF-kB protein is defined by the specific nucleotide sequences of its binding site has been an intriguing question for a number of years. The known binding sites for the most intensively studied RelA/p50 heterodimer define a remarkably loose consensus sequence, often cited as 5′-GGGRNYYYCC-3′ [14,15]. Reported X-ray structures of several NF-kB: DNA complexes also include a wide variety of different kB site sequences [14,16–18].

Some NF-kB sites demonstrate preference for specific subsets of NF-kB dimers and this preference may also contribute to regulation: for example, kB sites in *Il2* enhancer (AGAAATTCC) and mouse urokinase plasminogen

* Corresponding author. Fax: +7 495 1351405.
E-mail address: kuprash@online.ru (D.V. Kuprash).

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activator gene promoter (AGGAAAGTAC) deviate from the classical kB consensus and preferentially bind c-Rel homodimer and c-Rel/p65 heterodimer, respectively [19–21]. The effect of the specific DNA sequence of the binding site can be intensified by NF-kB binding partners that do not contact DNA directly [22,23].

Interaction of active RelB/p52 with a specific DNA sequence was reported to directly contribute to specificity of the alternative NF-kB pathway towards promoters of its target genes [24]. A potential unique kB site recognized by RelB/p52, but not by RelA/p50, was identified in the promoter of CXCL13 (BLC) gene using high concentrations of truncated proteins expressed in bacteria.

In this work, we used the random site selection (RSS) approach to address the question of whether the recruitment of RelB/p52 heterodimers to certain promoters can be explained based on their DNA-binding preferences *in vitro*. Our results indicate that high affinity kB sites that would selectively bind RelB/p52 *in vitro* do not exist.

Materials and methods

Custom oligonucleotides synthesized by phosphoramidite method and purified by polyacrylamide gel electrophoresis were purchased from Syntol (Moscow, Russia). See Supplementary Material for sequences of the oligonucleotides used, plasmid vectors, cell culture and transfection, electophoretic mobility shift assay (EMSA) and random site selection (RSS) [25].

Results

Expression of full-length RelB/p52 heterodimers in eukaryotic cells

In order to investigate DNA-binding properties of RelB/p52 in comparison to RelA/p50, we looked for a source of recombinant proteins with an optimal combination of high concentration, native conformation and possible posttranslational modifications. For this purpose, we utilized total cell lysates from cell line 293 co-transfected with recombinant vectors expressing the desired heterodimer components. The optimal ratio of plasmids used for co-transfection was found to be 1:1 for both RelB/p52 (Fig. 1) and RelA/p50 (data not shown). The desired heterodimer composition was confirmed using EMSA and supershift with antibodies against individual heterodimer subunits. DNA-binding specificity was confirmed using several double-stranded synthetic oligonucleotides with known NF-kB-binding characteristics (Fig. 1, lanes 6–8, data not shown). With appropriate amounts of non-specific DNA competitors, both RelB/p52- and RelA/p50- containing lysates from cells transfected with 1:1 ratio of the corresponding vectors, demonstrated strong binding to classical kB sites and showed no detectable binding to a mutated kB oligo. Thus, our system is suitable to search for new kB sites that may preferentially bind one heterodimer or another.

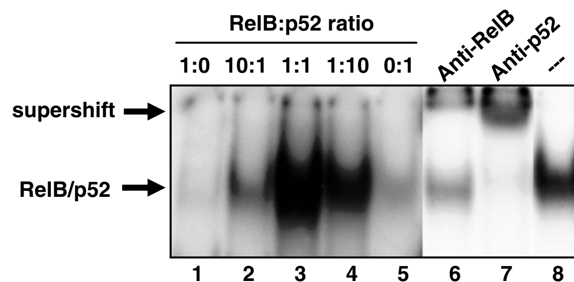

Fig. 1. Efficient forced expression of RelB/p52 heterodimers in 293 cells. Binding to positive control kB sequence (GGGACTTTCC from M-CSF gene) at different ratios of RelB to p52 expression vectors (lanes 1–5). Optimal binding (reflecting optimal heterodimer formation) is observed at 1:1 ratio (lane 3). Lanes 6–8: test of binding specificity using supershift with anti-p52 and anti-RelB antibodies.

BLC-kB site from mouse CXCL13 promoter does not bind full-length NF-kB heterodimers

As the RelB/p52 and RelA/p50 heterodimers play key roles in two different pathways of gene activation (alternative and classical, respectively), we decided to test their binding to the respective target promoters. The only DNA sequence to date that was reported to discriminate between two signaling pathways at the level of DNA binding is BLC-kB [24]. It demonstrated differential affinity towards truncated bacterially expressed RelA/p50 and RelB/p52 complexes and is located in CXCL13 (BLC) gene promoter [24]. We tested whether full-length Rel/NF-kB heterodimers expressed in eukaryotic cells recognize BLC-kB with different affinities. To our surprise, no detectable recognition of "alternative" kB site was observed with either RelB/p52 or RelA/p50 cell lysates (Fig. 2B). Therefore, our data indicate that RelB/p52 heterodimer formed *in vitro* using truncated proteins expressed in bacteria cannot reproduce DNA-binding properties of full-length factors from eukaryotic cells. It is possible that either domains that are absent in the truncated proteins and/or posttranslational modifications specific for eukaryotic cells are responsible for the formation of the heterodimer or the correct protein–DNA contacts. An additional biochemical and structural characterization of the RelB/p52 heterodimer used to identify the BLC-kB [24] would be useful to rectify these discrepancies.

All components of NF-kB signaling pathways are highly homologous between humans and mice. In particular, no functional difference between RelB and its human homologue I-Rel was found [1]. Many known functional kB sites are also fully conserved between men and mice [22]. However, this does not have to be the case for all target genes. Indeed, the sequence found in the human CXCL13 promoter in the position homologous to mouse BLC-kB site (hBLC-kB) is substantially different from its mouse counterpart (Fig. 2A). Unlike mouse BLC-kB probe which did not demonstrate any detectable Rel/NF-kB binding at all (Fig. 2B and data not shown), double-stranded oligonucleotide containing the hBLC-kB sequence was able to bind

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