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Promoter paper

An Ets element regulates the transcription of the human 2B4 gene in natural killer cells

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Abstract

2B4 (CD244) acts as an activation receptor on human NK cells, whereas it sends inhibitory signals in murine NK cells. A previous study indicated a prominent role for AP-1 in the transcription of 2B4 gene. To further understand the transcriptional regulation we analyzed the upstream positive regulatory region (−1151 to −704) of the 2B4 promoter. We have identified an Ets element that regulates the 2B4 gene transcription in an AP1 dependent manner.

Keywords: 2B4 promoter; Transcription regulation; Natural killer cell

Natural killer (NK) cells mediate non-MHC restricted lysis of tumor and virally infected cells and play an important role in immune surveillance [1,2]. NK cells also secrete IFN-γ that regulates other immune cells. The natural cytotoxicity and cytokine secretion is mediated through a delicate balance of signals received through activating and inhibitory receptors expressed by the NK cells [3–5]. Previously we identified 2B4 (CD244) as an activating receptor on murine NK cells and a subset of T cells that mediate non-MHC restricted cytotoxicity and later cloned the human homologue of mouse 2B4 [6–8]. It belongs to the CD2 subset of the immunoglobulin superfamily. The ligation of surface 2B4 enhances the cytotoxicity of NK cells against tumor cell lines and enhances IFN-γ production [7]. 2B4 also augments MHC-restricted killing by CD8 T cells [9]. A mAb against 2B4 enhances the cytotoxicity of murine NK cells against various cancer cell lines [7]. The ligation of surface 2B4 activates murine DETCs leading to the secretion of IFN-γ and IL-2, to the proliferation of cells and the up-regulation of egr-1 and c-fos mRNA expression [10]. These activated DETCs have an enhanced capacity to kill YAC-1 cells and disrupt transformed keratinocyte monolayers, indicating a role of 2B4 in the killing of skin derived tumors [11]. Recently we have generated 2B4-deficient mice and studies on these mice reveal an in vivo role of 2B4 in the killing of cancer cells [12]. We have also shown that murine 2B4 has an inhibitory role as opposed to the activating function assigned from previous studies [12,13]. Unlike in murine NK cells, 2B4 acts as an activation receptor on human NK cells [14]. The activation of human NK cells through surface 2B4 increases their invasiveness and cytotoxicity against human cancer cell lines [15,16].

2B4 also plays a role in the immune response against viral infections. The importance of 2B4 in the clearance of Epstein–Barr virus (EBV) is evident from the studies on XLPD [17–21]. In patients with HIV infection the percentage of $2B4^+$ CD8 T cells in the periphery increases and this population increases as the infection progresses [22]. CD8 T cells raised in response to HSV infection have up-regulated expression of 2B4 that plays a role in MHC-independent lysis of the HSV infected blastoid cell line LCL [23]. The

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up-regulation of 2B4 by CD8 αβ-T cells correlates with the acquisition of effector properties by these cells [24]. 2B4 expression is up-regulated on IL-2 activated DETC [10], on PMA activated NK cells [25] and on CD8 T cells in response to HSV infection [23]. Thus, understanding how the 2B4 gene is expressed and regulated is of much interest. Earlier we cloned a 1.1 kb 5′ regulatory region of the 2B4 gene and mapped regulatory regions within this promoter fragment [26]. AP1 was shown to play an important role in 2B4 gene transcription.

In this study we decided to characterize the upstream positive regulatory region (−1151 to −704). Towards this end we performed DNase I protection assay on a promoter fragment corresponding to nucleotides −1151 to −900 of the promoter. Probe for the footprint was prepared by PCR using end-labeled primers. Briefly, the reverse primer was end-labeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The PCR reaction contained the labeled reverse and unlabeled forward primer. A full length construct of the human 2B4 promoter in the vector pGL2 (Promega, Madison, WI) was used as the template. For the DNA footprint, radiolabeled probe (40,000 cpm per reaction) was incubated on ice without or with 100 μg YT cell nuclear protein for 30 min and then digested with DNase I (Promega, Madison, WI). DNA was phenol-chloroform extracted and electrophoresed on a 7.5 M urea/6% acrylamide gel adjacent to a Maxam–Gilbert G+A sequencing ladder of the DNA fragment. The gel

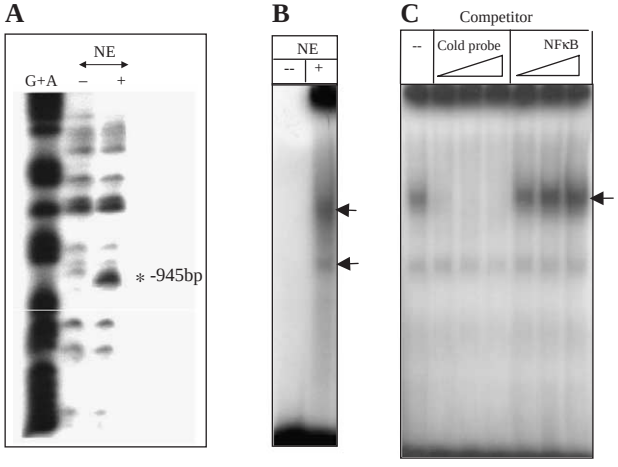

Fig. 1. DNase I protection and gel shift assays (A) DNase I protection assay was performed as described. First lane is the Maxam–Gilbert G+A sequencing lane. NE, YT cell nuclear extract. − and +, DNase I digestion in the absence and presence of nuclear protein, respectively. Asterisk indicates a site of DNase I hypersensitivity. −945 is the position of the hypersensitive nucleotide relative to the transcription start site (+1). (B) EMSA using the radioactive probe and YT cell nuclear extract (YT NE). − lane and + lane, binding reaction in the absence and presence of YT cell nuclear extract, respectively. Arrows indicate the positions of DNA–protein complexes. (C) Competition assay was performed with cold probe and the unrelated NK-κB oligonucleotide. Unlabeled competitor DNAs were added to the binding reaction at 5, 50 and 100 molar excess. − lane, binding reaction with no competitor DNA. Arrow indicates the sequence specific DNA–protein complex.

Table 1
Oligonucleotides used in the study

Name	Sequence
WT	5′-GCAGATCCAGAGCTGCCTGGCCTTC-3′
M1	5′-GCAGATCCAGAcaaGCCTGGCCTTC-3′
M2	5′-GCAGATatttAGCTGCCTGGCCTTC-3′

Bases in lower cases are mutated.

was fixed, dried and exposed to X-ray film at −70 °C between intensifying screens. As seen in Fig. 1A, the footprint failed to show any protection, however, a site of hypersensitivity was detected at the −945 nucleotide of the promoter region. Sites of hypersensitivity indicate binding of a protein close to the site. To determine whether the hypersensitivity in the 2B4 promoter was due to binding of a nuclear protein, we performed electrophoretic mobility shift assays. A 25 bp oligonucleotide (Table 1, WT) corresponding to nucleotides −955 to −931 (spanning the −945 hypersensitive site) and nuclear extract from YT cells were used in the EMSA. Labeled probes were incubated with YT cell nuclear extract on ice for 30 min in a 20 μl reaction containing 10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, 5% glycerol, 4 mM $MgCl_2$, 2 μg poly(dI-dC). Samples were electrophoresed on a 4% non-denaturing acrylamide gel at 200 V for 60–70 min. Gels were then dried and exposed to X-ray film. As seen in Fig. 1B, incubation of the labeled oligonucleotide with YT nuclear extract produced two DNA–protein complexes. Competition assay with cold probe and an unrelated cold NFκB probe revealed that the higher molecular weight DNA–protein complex is specific (Fig. 1C, shown by the arrow) while the lower complex is non-specific. This indicates that a YT nuclear protein binds specifically to the human 2B4 gene promoter close to the −945 nucleotide position.

To determine whether there is a functional cis-acting site at this region we performed luciferase reporter assays in YT cells. A full length, wild type human 2B4 promoter construct in the pGL2-Basic vector (Promega, Madison, WI) was made as described previously [26]. All mutant constructs were made from this construct by in vitro mutagenesis. Mutants 945M1 and 945M2 had mutations downstream and upstream of the hypersensitive site, respectively (Table 1). One million YT cells were transiently transfected with 4 μg of reporter construct and 0.4 μg of Renilla luciferase reporter plasmid using Fugene transfection reagent (Roche, Indianapolis, IN). Firefly and Renilla luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) as per the manufacturer's instructions, 48 h later. The reporter assay shows that mutation 945M1 does not change the activity of the promoter as compared to wild type (Fig. 2A). Mutation 945M2, however, reduces the activity of the promoter by about 30%. This indicates the presence of a functional element upstream of the hypersensitive −945 nucleotide. To identify the transcription factor binding at this region we analyzed the sequence using the transcription