Functional redundancy of transcription factor-binding sites in the killer cell Ig-like receptor (KIR) gene promoter

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Abstract

Variegated expression of inhibitory killer cell Ig-like receptors (KIRs) for MHC class I molecules helps NK cells distinguish normal from aberrant self and avoid autoreactivity. Prior studies of KIR promoters have produced conflicting results and no cis-acting sites have been independently confirmed. We took a comprehensive linker-scanning mutagenesis approach and substituted 24 consecutive 10-bp segments in the human KIR3DL1 promoter. Our analysis revealed eight segments that activated and three segments that repressed KIR transcription. Site-directed mutagenesis and electrophoretic mobility shift assays indicated that optimal KIR transcription requires a proximal Ets site that binds several Ets family members, a cAMP response element (CRE), a Runx site and a site that mediates complex interactions between Ets family members, signal transducer and activator of transcription 5 (STAT5) and YY1; Sp1 also contributes to KIR transcription. KIR transcription was greatly reduced by several compound mutations and was abrogated by a combination of mutations that affected the proximal Ets site, and the CRE, Runx, Sp1 and Ets/STAT sites. The many transcription factors that contribute to KIR transcription are partially redundant in the setting of transient transfection assays, helping to explain why only 0–2 activating sites had been reported in each of three prior studies. We propose that the multiplicity of transcription factors enables NK cells to sustain continuous KIR expression in diverse cellular and cytokine milieus, thus preventing NK autoreactivity.

Introduction

NK cells are critical elements of innate immune defense and kill infected cells, cancer cells and other aberrant cells (1). NK cells also secrete large amounts of IFN-γ, tumor necrosis factor-α, granulocyte macrophage colony-stimulating factor and other cytokines, which have direct anti-tumor and anti-pathogen effects and which orchestrate subsequent T cell immune responses (2). Aberrant cells activate NK cells when NK stimulatory receptor signals overbalance inhibitory receptor signals (3). Because killer cell Ig-like receptors (KIRs) recognize human MHC class I molecules and because MHC class I molecules frequently are down-regulated by virus infection and tumor transformation, KIR molecules allow human NK cells to distinguish normal from aberrant self. The KIR gene complex is large and individual NK cells express a variable number of the available KIR genes and alleles (4, 5). Stochastic KIR expression patterns are maintained by epigenetic events, including DNA methylation and histone acetylation/methylation (6–8). Epigenetic modifications regulate access of transcription factors to critical cis-acting elements and, in turn, epigenetic modifications are regulated by transcription factors and associated protein complexes. These interactions are likely to be important both in the maintenance of KIR transcription in mature NK cells and in the initiation of KIR transcription during NK cell development. As a first step to understanding the interaction between transcription factors and epigenetic modifications, we must characterize the cis- and trans-acting elements that control KIR transcription.

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Three groups have characterized the KIR promoter (9–11). However, each purported cis-acting element either was not independently confirmed or was contradicted in another study. Therefore, the findings have been contradictory and there is no clear picture of KIR regulation has emerged. The major consistent finding by all three groups is that most mutations in the KIR promoter failed to reduce transcriptional activity. All point mutations tested thus far have been predicted factor-binding sites or at sites of KIR sequence divergence. Herein, we report a comprehensive approach to investigating the KIR promoter. We characterize the effect of mutating multiple cis-acting sites alone and in combination.

Methods

Materials

Unless otherwise indicated, all materials were from Sigma (St Louis, MO, USA).

Cells and constructs

YT-HY cells [hereafter referred to as YT, a gift of W. Leonard, National Institutes of Health (NIH), with permission of J. Yodoi (12)] were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% iron-supplemented bovine calf serum (Hyclone Laboratories, Logan, UT, USA). HeLa cells were cultured in DMEM supplemented with 10% iron-supplemented bovine calf serum, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 0.15% bicarbonate (Invitrogen). The 249-bp KIR3DL1 promoter pGL3 reporter plasmid was similar to that described previously (6), except that the KIR ATG start site was fused with the luciferase ATG start site and polylinker sequences were eliminated. Human Ets-1, Ets-2 and Fli-1 expression constructs and the pSG5 parent vector were generous gifts of Dennis Watson (Medical University of South Carolina, Charleston, SC, USA).

Preparation of YT nuclear extracts

YT cells (2 × 10^6) were pre-incubated with or without 200 U ml^−1 of recombinant IL-2 for ~1 h before harvest. All the following procedures were carried out at 0–4°C: cells were centrifuged and re-suspended in PBS with NaF (5 mM) and Na2VO4 (1 mM), and then re-suspended in 400 μl lysis buffer (20 mM HEPES, 10 mM KCl, 20 mM NaF, 2 mM MgCl2, 0.1 mM EDTA, 0.2% NP-40, 1 mM Na2VO4) and protease inhibitors [2 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM Nα-tosyl-L-lysine chloromethyl ketone, 1 mM Nα-tosyl-L-phenylalanine chloromethyl ketone, 100 μg ml^−1 N-(trans-epoxysuccinyl)-L-leucine 4 guanidinobutylamide, 100 μg ml^−1 pepstatin A, 50 μg ml^−1 aprotonin and 30 μg ml^−1 chymostatin]. The cells were lysed with 10–20 strokes of a Dounce glass homogenizer. After incubating for 10 min, dithiothreitol (DTT), ovomucoid trypsin inhibitor and soybean trypsin inhibitor were added to final concentrations of 2 mM, 1 mg ml^−1 and 1 mg ml^−1, respectively. Lysate was centrifuged at 2400 × g for 10 min, and the pellet was re-suspended in 400 μl lysis buffer, protease inhibitors and 2 mM DTT. This extract was centrifuged at 220 × g for 10 min, and the pellet was re-suspended in 400 μl nuclear extraction buffer (20 mM HEPES, 600 mM KCl, 20 mM NaF, 2 mM MgCl2, 0.1 mM EDTA, 25% glycerol, 1 mM Na2VO4) and protease inhibitors. The extract was rotated for 30 min, and then centrifuged at 11,000 × g for 10 min. DTT was added to a final concentration of 2 mM, the extract was aliquoted, flash frozen in liquid N2 and stored at −80°C.

Electrophoretic mobility shift assay

Probe sense (Table 1) and anti-sense oligonucleotides (Integrated DNA Technologies Coralville, IA, USA) were hybridized and labeled with [α-32P]dCTP (Perkin Elmer, 3000 Ci mmol) using Klenow DNA polymerase I (New England Biolabs, Ipswich, MA, USA); unincorporated label was removed using a protein desalting column (Pierce, Rockford, IL, USA). All procedures were carried out at 0–4°C: nuclear extract (8–10 μg) was incubated in a total volume of 10 μl in binding buffer (10 mM HEPES, 60 mM KCl, 10% glycerol, 1 mM EDTA, 10 mM Na2PO4, 1 mM DTT, 10 mM NaF, pH 7.8), including 1 mM Na2VO4, 1 mM PMSF, 1× Roche protease inhibitor cocktail (Roche), 200 ng sonicated salmon sperm dsDNA, 100 ng poly (dI–dC), competitor DNA and labeled probe (~2 ng) for 30 min. For supershift experiments, 2 μg of antibody was added and incubated for an additional 30 min. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA): signal transducer and activator of transcription 5 (STAT5) (385X), YY1 (1703X), cAMP response element binding protein (CREB) (186X), activating transcription factor-1 (ATF-1) (270X), Ets-1/ Ets-2 (112X), GA binding protein α (GABPα) (22810X), Elf-1 (28682X), c-myc (7874X) and Sp1 (59X). Samples were electrophoresed on a non-denaturing 4–6% PAGE gel (29:1 (m:m)

Table 1. EMSA oligonucleotides

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP4 WT</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>AP4-23 mutant</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>AP4-4 mutant</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>CRE</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>Proximal Ets WT</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>Proximal Ets mutant</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>S1 WT</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>S1 mutant</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>Sp1</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>YY1/Ets/STAT</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>YY1 mutant</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
<tr>
<td>Ets/STAT mutant</td>
<td>GGTTGCCTGCTGAGTGGGCGCA</td>
</tr>
</tbody>
</table>
acrylamide:N,N'-methylenebisacrylamide) at 10 V/cm at 4°C for 3–4 h in 0.25× Tris–Borate EDTA (13). Gels were removed, washed with 5% glycerol for 15 min, vacuum dried onto filter paper at 65°C and exposed to film at –80°C. Each electrophoretic mobility shift assay (EMSA) figure shows a single gel; in some cases lanes were rearranged for clarity.

**Transient transfection**

Samples of plasmids were purified from overnight growths of transformed DH5α bacteria using a plasmid mini-prep kit (Bio-Rad Quantum Prep or Qiagen miniprep). YT cells were transfected using diethylaminoethyl dextran as previously described (14), but using 1 μg of the test firefly luciferase plasmid and 50 ng of control renella luciferase plasmid (pRL-CMV, Promega, Madison, WI, USA). After 40 h incubation (37°C, 5% CO2), cells were harvested, washed with PBS, centrifuged and vortexed in 50 μl of ice-cold 1× Passive Lysis buffer (Promega) including 1 mM PMSF and 1× protease inhibitor cocktail (Roche); this lysate was flash frozen in liquid N2 and stored at –80°C. Promoter activity was determined using a luminometer (Lumat L9507; EG & G Berthold) and a Dual-Luciferase Reporter Assay kit (Promega), following manufacturer’s instructions. Firefly luciferase activity was divided by renella activity and this value was normalized to the value for the wild-type KIR3DL1 promoter vector.

HeLa cells (~1 × 10⁶) in individual 35-mm wells were transfected with 1.5 μg of KIR3DL1 promoter plasmid, 1 ng of control SV40-renella plasmid (Promega), 0.015 and 0.4 μg of the desired Ets construct and enough parent plasmid so that equivalent amounts of DNA were present in each condition tested, using Lipofectamine and Plus reagent (Invitrogen). Manufacturer’s instructions were followed, except that media was replaced with fresh media 3 h after transfection. Cells were harvested and promoter activity was determined as described above.

**Mutagenesis**

We performed PCR-based mutagenesis essentially as described (15), except that we used Deep Vent DNA polymerase (New England Biolabs). Linker-scanning mutagenesis was performed as described (16), beginning 7 bp upstream of the KIR3DL1 translational start site (Fig. 1A and Table 2). All constructs were verified by sequencing (Northwestern University Biotech Lab, Chicago, IL, USA).

**Results**

**Systematic dissection of the KIR3DL1 promoter reveals several important cis-acting elements**

Previous studies that tested potential KIR promoter cis-acting elements focused on potential transcription factor-binding sites that were implicated by computer algorithms or KIR sequence divergence (9–11). The paucity of positive cis-acting elements that were identified by these approaches suggested that important transcription factors have yet to be recognized. Therefore, we systematically replaced 24 segments upstream of the KIR3DL1 ATG translation start site showing the 24 contiguous 10-bp segments (denoted S1–S24 under the corresponding segment) that were replaced with the linker sequence, GCAGAGCTGTC. The ATG translational start site at +1 is noted by a bold letter A. Putative cis-acting elements identified by TESS are noted by a single line above or below the corresponding segment) that were replaced with the linker sequence, GCAGAGCTGTC. The ATG translational start site at +1 is noted by a bold letter A. Putative cis-acting elements identified by TESS are noted by a single line above or below the corresponding segment (Fig. 1B). YT cells were transfected as described in Methods. Values represent averages from tests of at least three different plasmid preparations (each measured in duplicate), with error bars representing 95% confidence limits. WT, wild type. B, background level produced by the promoterless pGL3-basic vector.
Table 2. KIR3DL1 mutations

<table>
<thead>
<tr>
<th>Site</th>
<th>Motif</th>
<th>KIR sequence</th>
<th>Mutated sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segments 1–24</td>
<td></td>
<td>Various</td>
<td>GCAGATCCGC</td>
</tr>
<tr>
<td>AP4-2</td>
<td>RYCACTGGYĜ</td>
<td>GCAGCTGCTGACTGĜ</td>
<td>C&gt;T, –40</td>
</tr>
<tr>
<td>AP4-3</td>
<td>RYCACTGGYĜ</td>
<td>GCAGCTGCTGACTGĜ</td>
<td>C&gt;T, –45</td>
</tr>
<tr>
<td>AP4-3</td>
<td>GCGTGGTĜ</td>
<td>GCGTGGTĜ</td>
<td>G&gt;T, –49; C&gt;A, –40; C&gt;T, –45, –35</td>
</tr>
<tr>
<td>AP4-1234</td>
<td>GCGTGGTĜ</td>
<td>GCGTGGTĜ</td>
<td>C&gt;C, –56 –55</td>
</tr>
<tr>
<td>Sp1</td>
<td>GGGMGĜ</td>
<td>GGGĜ</td>
<td>G&gt;A, –68, –71</td>
</tr>
<tr>
<td>E2F</td>
<td>TAGCCĜ</td>
<td>TAGCCĜ</td>
<td>AAA&gt;A, –65 to –63</td>
</tr>
<tr>
<td>CRE</td>
<td>TGACGTĈ</td>
<td>TGACGTĈ</td>
<td>C&gt;A, –116; C&gt;T, –113</td>
</tr>
<tr>
<td>Distal segment 12</td>
<td></td>
<td>GAGCTĈ</td>
<td>GAGCCT&gt;AGATCT, –127 to –122</td>
</tr>
<tr>
<td>Proximal segment 12</td>
<td></td>
<td>GAGCTĈ</td>
<td>GCCGAC&gt;AGATCT, –121 to –116</td>
</tr>
<tr>
<td>Runx (GA)</td>
<td>TGTGGT</td>
<td>TGTGGT</td>
<td>G&gt;A, –97</td>
</tr>
<tr>
<td>Runx (GT)</td>
<td>TGTGGT</td>
<td>TGTGGT</td>
<td>G&gt;T, –97</td>
</tr>
<tr>
<td>Runx (Triplic)</td>
<td>TGTGGT</td>
<td>TGTGGT</td>
<td>TGTTG&gt;ACTA, –100 to –97</td>
</tr>
<tr>
<td>YY1 (3)</td>
<td>VDCATNWYĜ</td>
<td>AGCATTĜ</td>
<td>CAT&gt;TGG, –177 to –175</td>
</tr>
<tr>
<td>YY1 (1)</td>
<td>VDCATNWYĜ</td>
<td>AGCATTĜ</td>
<td>CAT&gt;TGG, –177 to –175</td>
</tr>
<tr>
<td>STAT (1)</td>
<td>TCCTNGGÂ</td>
<td>TCCTNGGÂ</td>
<td>A&gt;G, –176</td>
</tr>
<tr>
<td>STAT (2)</td>
<td>TCCTNGGÂ</td>
<td>TCCTNGGÂ</td>
<td>T&gt;G, –188</td>
</tr>
<tr>
<td>STAT (3)</td>
<td>TCCTNGGÂ</td>
<td>TCCTNGGÂ</td>
<td>T&gt;A, –188; A&gt;T, –180</td>
</tr>
<tr>
<td>Distal Ets</td>
<td>CMGGAWGŶ</td>
<td>TMGGAWGŶ</td>
<td>T&gt;TCTGCTA, –188 to –186</td>
</tr>
<tr>
<td>Ets/STAT</td>
<td></td>
<td>TCCCCGÂ</td>
<td>G&gt;C, –183</td>
</tr>
</tbody>
</table>

W = A, T; M = A, C; N = A, C, G, T; R = A, G; V = A, C, G; D = A, G, T. ‡ Motif on anti-sense strand. † Replacement of nucleotides –117 and –116 is predicted to affect the CRE.

(Fig. 1A) with a 10 bp sequence (Table 2) that is devoid of known transcription factor-binding motifs (16). Because cis-acting sites in the 5’ untranslated region can affect transcription (17), our comprehensive approach included segments downstream of the transcription start sites, usually mapped within 10–65 bp of the KIR3DL1 translation start site (6, 18, 19). Multiple deletion studies had shown that a fully active KIR promoter was contained within the 240-bp targeted region (6, 9–11, 18).

Replacement of several segments had minimal effect (Fig. 1B). Replacement of Segments 3, 8 and 9 increased KIR promoter activity by >1.5-fold. Replacement of Segments 4, 5, 6, 7, 14, 15 and 18 reduced KIR promoter activity by 22–40% and replacement of Segment 12 reduced activity by 50%. We systematically investigated the cis-acting elements and transcription factors that activate KIR transcription.

Segment 4 contributes to KIR3DL1 promoter activity

Segments 3–5 contain four overlapping sequences in the anti-sense strand that imperfectly match the AP4-recognition motif (Fig. 1A). Replacement of Segment 4 was expected to abrogate AP4 binding to the two central sites, which better match the AP4 motif. To test the importance of the putative AP4 sites, we performed site-directed mutagenesis (Table 2). Mutations predicted to damage or destroy the central two AP4 motifs in Segment 4 (AP4-1234, AP4-23 and AP4-2) significantly reduced KIR3DL1 promoter activity (Fig. 2). In contrast, the AP4-3 mutation, which was predicted to destroy the third most proximal AP4 site and damage the relatively weak fourth site, had no effect (Fig. 2). These results confirm that this region is required for optimal KIR transcription and suggest a cooperative effect of the central two AP4 sites. We further tested the role of AP4 using EMSA. The KIR wild-type probe and a probe containing the Segment 4 substitution produced the same number and intensity of shifted bands (data not shown). Furthermore, the intensity of bands bound by a standard AP4 probe was not diminished by wild-type KIR or AP4-1234 mutant competitor oligonucleotides (data not shown). Therefore, AP4 binding was not confirmed.

Ets family members activate KIR transcription

An Ets motif straddles Segments 5 and 6 on the anti-sense strand, hereafter referred to as the proximal Ets site, pEts. The role of the pEts site has been controversial (9, 11). Because previous studies replaced or eliminated 4–8 nucleotides, the third most proximal AP4 site and damage the relatively weak fourth site, had no effect (Fig. 2). These results confirm that this region is required for optimal KIR transcription and suggest a cooperative effect of the central two AP4 sites. We further tested the role of AP4 using EMSA. The KIR wild-type probe and a probe containing the Segment 4 substitution produced the same number and intensity of shifted bands (data not shown). Furthermore, the intensity of bands bound by a standard AP4 probe was not diminished by wild-type KIR or AP4-1234 mutant competitor oligonucleotides (data not shown). Therefore, AP4 binding was not confirmed.

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![AP4 Mutations](https://academic.oup.com/intimm/article-abstract/18/8/1221/767843/121761863)
directed 2-bp substitution in the pEts motif (Table 2). The mutation reduced promoter activity by $\geq 60\%$ (Fig. 3A). To further test the role of the Ets family in KIR transcription, we performed EMSA. The KIR3DL1 probe bound several NK nuclear factors, and this binding was competed by the self-probe. Two complexes were not competed by the pEts mutant oligonucleotide, consistent with binding by an Ets family member (Fig. 3B). To identify protein in the EMSA complex, we tested antibodies to four Ets family members that are expressed in lymphocytes, Ets-1, Ets-2, Elf-1 and GABP (20). Antibody to GABP$\alpha$ supershifted a complex and antibody to Elf-1 reproducibly diminished Ets-specific band intensity (Fig. 3B). Antibody to Ets-1 and Ets-2 had no effect. These results implicated GABP and Elf-1, but did not rule out a role for Ets-1 and Ets-2, which have intramolecular interactions that inhibit efficient binding to probe in EMSA (20, 21).

To further test a role for Ets-1 and Ets-2, we co-transfected expression plasmids for these transcription factors together with mutant and wild-type KIR promoter reporter plasmids into HeLa cells, which does not express lymphocyte-specific Ets family members (20, 22, 23). The KIR reporter plasmid had little activity in HeLa cells, consistent with prior findings (6). Ets-2 increased transcription from the wild-type KIR promoter (Fig. 3C). Ets-2 also increased transcription from KIR promoters with mutations in the pEts site or with compound mutations affecting both the pEts site and a distal Ets site (see below). These results indicate that Ets-2 transcription activation was largely due to indirect effects and did not require Ets-2 binding to the KIR promoter. Ets-1 moderately increased transcription from the mutant KIR promoters (Fig. 3C). However, Ets-1-stimulated transcription from the wild-type promoter was significantly higher than from either mutant (Fig. 3C), indicating that the lymphocyte-specific Ets-1 interacted directly with the pEts site and possibly with both Ets sites in the KIR promoter.

**Sp1 has a weak effect on KIR promoter activity**

The Segment 7 substitution of KIR3DL1, nucleotides $-67$ to $-76$, consistently decreased KIR promoter activity by $\sim 20$–25$\%$ (Fig. 1B). Because previous findings appeared contradictory, we explored transcription factor binding to this region more thoroughly. The GC-rich Segment 7 contains potential overlapping binding sites for Sp1 and E2F, in addition to a site for binding by the RNA polymerase II complex general transcription factor, TFIIB. A 2-bp substitution that was predicted to eliminate Sp1 and TFIIB binding (Table 2) caused a small, but reproducible decrease in KIR promoter activity (Fig. 4A). A mutation directed at the putative E2F site caused a similar small decline in promoter activity that did not reach statistical significance (Fig. 4A). In EMSA, a probe based on the KIR3DL1 sequence (Table 1) bound YT nuclear factors and the complex was supershifted by anti-Sp1 antibody (Fig. 4B). These results indicate that Sp1 makes a small contribution to KIR promoter activity in NK cells.
shifted by individual antibodies to CREB and to ATF-1. KIR bound to the CRE-containing promoter site, we performed EMSA. YT nuclear complexes (9, 11), we systematically substituted the region around and 12. Due to the contradictory nature of previous findings response element (CRE) motif that straddles Segments 11 and 12. Substitution of Segment 12 caused the largest decline in KIR promoter activity (Fig. 1), implicating a potential cAMP response element site contributes to KIR promoter activity (Fig. 4).

A cAMP response element site contributes to KIR promoter activity

Substitution of Segment 12 caused the largest decline in KIR promoter activity (Fig. 1), implicating a potential cAMP response element (CRE) motif that straddles Segments 11 and 12. Due to the contradictory nature of previous findings (9, 11), we systematically substituted the region around Segment 12 (nucleotides –126 to –117) with a 6-bp linker (AGATCT). KIR promoter activity was reduced by substitution of proximal nucleotides –121 to –116, which involved the CRE, but not by substitution of distal nucleotides –127 to –122, upstream of the CRE (Fig. 5A). To more specifically focus on the CRE, we made a 2-bp substitution (Table 2). This mutation caused a ~50% decrease in KIR promoter activity (Fig. 5A). To investigate whether the CRE-binding factors, CREB and ATF-1, were present in YT cells and bound the KIR promoter site, we performed EMSA. YT nuclear complexes bound to the CRE-containing KIR probe and were supershifted by individual antibodies to CREB and to ATF-1 (Fig. 5B). These results suggest that the CRE site contributes to KIR transcription.

Given that the CRE site contributes to KIR promoter activity and straddles Segments 11 and 12, we were puzzled why the Segment 11 substitution did not reduce promoter activity in five independent experiments (Fig. 1). Using the TESS computer algorithm (24), we searched the Segment 11 mutant KIR promoter and discovered a potential new Sp1 site at the junction of Segment 10 and the substituted Segment 11. To investigate this possibility, we performed EMSA using a probe centered on the Segment 11 linker (Table 1 and Fig. 5C). A major nuclear complex formed and was reproducibly supershifted with anti-Sp1 antibody (Fig. 5C). Furthermore, the mutated Segment 11 oligonucleotide competed with the Segment 7 wild-type KIR probe for Sp1 binding (Fig. 4B, lane 3). We propose that the Segment 11 substitution created an ectopic Sp1 site that compensated for the loss of the CRE. To our knowledge, this is the first published report of an inadvertent creation of a new activating site by linker-scanning mutagenesis.

Runx contributes to KIR promoter activity

The predicted Runx-binding motif (TGTGGT) straddles Segments 9 and 10 and substitution of these segments failed to reduce KIR promoter activity. However, we reasoned that a change of 7 or 8 out of 10 nucleotides in Segments 9 and 10, respectively, might have altered more than the Runx site alone. Because the effect of Runx mutations has been controversial, we generated three separate substitutions that were designed to disable the Runx site. All three of our Runx site substitutions diminished KIR promoter activity by ~40% (Fig. 6), including the same Runx substitution (GA; Table 2) that had been tested previously (9, 11). These data, along with demonstration by two groups that Runx family members bind to KIR sequences in EMSA (9, 10), support the conclusion that Runx proteins activate KIR transcription.

Overlapping Ets, STAT5 and YY1 sites affect KIR promoter activity

Substitution of Segment 18 decreased promoter activity by ~25% (Fig. 1B). We identified three putative overlapping cis-acting sites: Ets, STAT5 and YY1 (Fig. 1A). To test the importance of the putative Ets and STAT sites, we generated a 1-bp substitution that affected the Ets motif alone, 1-, 2- and 3-bp substitutions that affected the STAT motif alone and a 5-bp substitution that affected both motifs. All five mutations significantly inhibited promoter activity (Fig. 7A), consistent with the importance of Ets and STAT proteins in KIR transcription. To test the importance of the YY1 site, we made two substitutions that were not predicted to affect the other two sites. The 3-bp substitution had no effect and the 1-bp substitution slightly increased KIR promoter activity (Fig. 7A).

We performed EMSA to investigate which family members bound these sites. Several nuclear complexes bound the YY1/Ets/STAT probe (Table 1) and two complexes were not efficiently competed by an oligonucleotide with mutation affecting the Ets/STAT site (Fig. 6B). These bands were supershifted or significantly diminished by antibodies to GABP and Elf-1, respectively (Fig. 6B). As with the proximal Ets site, antibody to
Ets-1 and Ets-2 had no effect. In preliminary studies, we detected a faint STAT5-specific band in a minority of experiments. To enhance the level of activated STAT5, we stimulated YT cells with IL-2 (14, 25). One additional KIR probe-binding nuclear complex was induced by IL-2 stimulation (Fig. 7C). The IL-2-induced complex was competed by wild-type oligonucleotide, but not by the Ets/STAT mutant oligonucleotide, and was supershifted by antibody to STAT5A/B (Fig. 7C). Mutant YY1 oligonucleotide did not compete for binding to nuclear factors from IL-2-induced or -uninduced YT cells. Furthermore, anti-YY1 antibody significantly diminished the intensity of two complexes (Fig. 7C). These results suggest that YY1 binds to the KIR promoter, but does not greatly suppress transcription in YT cells under our transfection conditions. Collectively, these data indicate that Ets family members, STAT5 and YY1 bind overlapping sites in the KIR promoter.

Multiple factors cooperate to drive KIR transcription

A hallmark of published studies on the KIR promoter is the paucity of mutations that significantly inhibit transcription. Although we identified several activating sites, only substitution of the proximal Ets site and the CRE caused as much as a 50% reduction in promoter activity. This may be due to a redundancy of KIR promoter transcription factors in the setting of the transient transfection assay. To test this proposal, we tested mutations at multiple sites that had been implicated in both mutagenesis and EMSA experiments. Cis-acting site redundancy in this system was illustrated by the fact that no combination of two mutations abrogated KIR promoter activity (Fig. 8). However, we saw clear evidence of additive effects. The combination of the proximal Ets mutation and the distal Ets/Stat mutation and the Runx mutation decreased KIR promoter activity to that of the promoterless pGL3-basic control (Fig. 8). Addition of the CRE mutation or both the CRE and Sp1 mutations decreased promoter activity to levels significantly below that of the pGL3-basic control. Thus, KIR promoter activity was eliminated by a combination of mutations affecting six sites: proximal Ets, Sp1, CRE, Runx, STAT and distal Ets.

Discussion

Three previously published studies reported very few activating cis-acting elements. Trompeter et al. (9) found only one cis-acting element, CRE, that was required for optimal KIR2DL3 transcription. Xu et al. (11) studied KIR2DL2, an allele of the
same KIR gene studied by Trompeter et al. (9), and reported two activating cis-acting elements in NK cells, Runx and the proximal Ets site. van Bergen et al. (10) did not identify any cis-acting sites that were required for optimal KIR3DL1 promoter activity. Each of the three putative activating KIR promoter sites, CRE, proximal Ets and Runx, was specifically tested and reported to be neutral or repressive by at least one other group (9–11). Multiple repressive cis-acting elements were...
Trompeter et al. (9) reported that deletion of a 10-bp segment, nucleotides −36 to −45, had little effect on KIR transcription. In contrast, we found that KIR promoter activity was significantly reduced by substitution of Segment 4 (nucleotides −37 to −46) and by targeted substitutions that included nucleotide −40. These mutations demonstrated the importance of this region and suggested a role for AP4. However, AP4 binding to this site was not confirmed in EMSA. An alternative hypothesis is that substitution of Segment 4 affects components of the RNA polymerase II complex rather than transcription factors. The KIR promoters do not contain a classic TATA box and KIR3DL1 start sites have been mapped to locations between nucleotides −65 and −10, with a ‘hot spot’ around nucleotide −30 (6, 18, 19). This hypothesis fails to explain why the Segment 3 substitution (nucleotides −27 to −36) did not reduce transcriptional activity or why substitution of nucleotide −40 did reduce transcriptional activity. The mechanism by which Segment 4 contributes to KIR transcription remains speculative.

The importance of the proximal Ets site is supported by the significant decline in promoter activity that was caused by a 2-bp mutation in the Ets motif and by Segments 5 and 6 substitutions. These findings are in agreement with Xu et al. (11), who reported that a 4-bp substitution in the putative Ets motif reduced KIR promoter activity, but stand in contrast to Trompeter et al. (9), who reported that an 8-bp deletion of the putative Ets-binding site did not affect KIR transcription. The importance of the proximal Ets site was further supported by our identification in EMSA of two KIR promoter-binding Ets family members, GABP and Elf-1. Neither we nor Xu et al. (11) demonstrated Ets-1 or Ets-2 binding to the KIR promoter sequences in EMSA, but these transcription factors are poorly demonstrated Ets-1 or Ets-2 binding to the KIR promoter activity. The level of KIR promoter activity stimulated by lymphocyte-specific co-transfected Ets-1 in HeLa epithelial cells approached that of YT NK cells. Although the effect of Ets-2 was largely indirect, Ets-1 required intact Ets sites in the KIR promoter for maximal activity. The level of KIR promoter activity stimulated by lymphocyte-specific co-transfected Ets-1 in HeLa epithelial cells approached that of YT NK cells. Although transfection results in YT cells and HeLa cells are not directly comparable, we speculate that the presence of appropriate Ets family member proteins is one reason why KIR expression is limited to NK and T cells.

Substitution of Segment 7 decreased promoter activity and at least some of that decrease could be attributed to Sp1. In agreement with earlier EMSA and DNase I footprinting experiments (10, 18), Sp1 bound to a KIR probe in EMSA. The effect of our Sp1 mutation was small and can be reconciled with the lack of effect of mutations in this region reported by Trompeter et al. (9) and Xu et al. (11). In contrast, van Bergen et al. (10) found that a replacement of nucleotides −63 to −85 prevented Sp1 binding and increased promoter activity. However, their combined 4-bp substitution and 5-bp deletion may have changed multiple cis-acting elements and the helical spacing between sites. We conclude that Sp1 makes a small contribution to KIR transcription.

We found that substitutions of Segments 3, 8 and 9 increased KIR promoter activity. Xu et al. (11) reported that a 6-bp substitution that straddled Segments 8 and 9 significantly

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**Fig. 8.** Multiple cis-acting sites contribute to KIR promoter activity. Promoter activity was measured as described in Fig. 1(B). Mutations were in the proximal Ets site (Ets or E), the Sp1 site (Sp1), the CRE (CRE or C), Runx site (Runx Triple mutation, designated Runx or R) and in the distal Ets/STAT site (Ets/STAT or E/S). The individual mutations are reproduced here to facilitate comparison with compound mutations. All refers to a compound mutation of the proximal Ets, Sp1, CRE, Runx and distal Ets/STAT sites. The dashed line indicates the level of luciferase activity produced by the promoterless pGL3-basic plasmid.
increased KIR promoter. Even though a potential c-myb site straddles Segments 8 and 9, a specific mutation of the putative c-myb site only slightly increased KIR promoter activity (1.2-fold ± 0.2, in five trials, data not shown), and we did not detect c-myb in YT nuclear extracts that bound KIR probe in EMSA (data not shown). We have not yet identified factors that repress transcription of the wild-type KIR promoter by binding Segments 3, 8 or 9.

The importance of the CRE site had been controversial, with Trompeter et al. (9) reporting that deletion of nucleotides –111 to –177 containing the CRE site decreased KIR promoter activity, while Xu et al. (11) reported that substitution of nucleotides –113 to –188 increased KIR promoter activity in NK cells by 2.7-fold. Consistent with an activating role, mutations affecting the CRE site diminished KIR promoter activity in our hands. The importance of the CRE is supported by ATF-1 and CREB binding in EMSA [this study and (10)] and by the location of the CRE in a DNase I footprint (18). TESS program analysis (24) of the CRE replacement mutation made by Xu et al. (11) suggests that potential new CRE and AP1 sites may account for their observed increased KIR promoter activity. Together, these considerations suggest that the CRE site contributes to KIR transcription.

There is general agreement that Runx family members bind to the KIR promoter (9, 10, 18). However, mutagenesis studies led Trompeter et al. (9) and Xu et al. (11) to opposite conclusions about whether Runx was activating or repressive. All three of our Runx site substitutions diminished KIR promoter activity, including the same G to A substitution that had been tested by both Trompeter et al. (9) and Xu et al. (11). In agreement with Xu et al. (11), we conclude that the Runx site is activating. Although Runx2 and Runx3 are often repressive proteins, several examples show that they activate transcription of some promoters (28).

Replacement of Segments 14 and 15 (nucleotides –137 to –156) diminished KIR promoter activity, but Sp1 did not bind a putative CACCC box in EMSA (data not shown) and few other high probability candidate transcription factor-binding sites were predicted for this region. Xu et al. (11) mutated a putative OctB2 site at nucleotides –137 to –142 and found significantly elevated KIR promoter activity. The transcription factors that bind to the KIR promoter in this region remain unknown.

In a region previously implicated by DNase I footprinting (18), we found evidence for transcription factor binding to three overlapping sites—Ets, STAT and YY1. van Bergen et al. (10) had tested a 1-bp YY1 mutation and they found it to increase KIR promoter activity by ~67%. Testing an identical mutation, we found a modest increase in KIR promoter activity. Although Runx2 and Runx3 are often repressive proteins, several examples show that they activate transcription of some promoters (28).

First, a second major lesson of this study is that large substitutions and deletions may produce misleading results because of the multiple effects of these changes. Our linker-scanning mutagenesis approach failed to reveal the importance of the Runx site, possibly because of an effect on neighboring sites. It is possible that Xu et al. (11) failed to identify the distal Ets site because their 6-bp mutation also affected the overlap- ing STAT and YY1 sites. As noted above, the increased KIR promoter activity caused by the CRE replacement mutation of Xu et al. (11) may have been due to inadvertent creation of new CRE and AP1 sites. Our Segment 11 substitution did not show the contribution of the CRE site because it created an ectopic Sp1 site. Because any mutation may have unanticipated consequences, it is important to search for potential transcription factor-binding motifs in both the original and the mutated constructs.

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It should be recognized that the cis-acting elements identified here may be limited to the context in which they
were tested. Plasmid DNA is not methylated and is not fully chromatinized in transfected cells, and the cis- and trans-acting elements identified may be important only for the maintenance of KIR expression. Despite the multiplicity of activating transcription factors, stochastic allele-specific KIR expression is maintained by powerful epigenetic controls that limit chromatin accessibility (6–8). The transcription factors identified here may or may not be involved in the initiation of KIR expression in developing NK cells. Initiation of KIR transcription likely requires DNA demethylation, recruitment of histone acetylases and chromatin opening. It is possible that factors which perform these functions do not associate with KIR promoters in mature NK cells. For example, some pioneer transcription factors that help open the chromatin of liver-specific genes in development do not contribute to ongoing transcription in mature hepatocytes (31). Other pioneer transcription factors continue to play a role in fully differentiated cells. We speculate that STAT5 is a pioneer transcription factor that helps initiate KIR transcription because many of the cytokines that induce NK cell differentiation also activate STAT5, including IL-3, IL-7 and IL-15 (32, 33). Finally, Davies et al. (unpublished results) have demonstrated that a KIR3DL1 promoter directed transcription in both the retrograde and antegrade directions (S. K. Anderson, personal communication). It will be important to investigate how antegrade and retrograde transcriptions are regulated during NK cell development.

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Abbreviations

ATF-1 activating transcription factor-1  
CRE cAMP response element  
CREB cAMP response element binding protein  
DTT dithiothreitol  
EMSA electrophoretic mobility shift assay  
GABPα GA binding protein α  
KIR killer cell Ig-like receptor  
NIH National Institutes of Health  
PMSF phenylmethylsulphonyl fluoride  
STAT5 signal transducer and activator of transcription 5

References


