Characterization of KIR intermediate promoters reveals four promoter types associated with distinct expression patterns of KIR subtypes

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The human killer cell immunoglobulin-like receptor (KIR) genes contain multiple promoters that control the process of gene activation and variegated expression of KIR on natural killer (NK) and T cells. Specific subfamilies of KIR genes have differences in the timing and tissue specificity of expression: however, previous studies of the proximal KIR promoters have not shown significant differences in activity between differentially expressed KIR gene subsets. The recent identification of an intermediate KIR promoter (ProI) associated with KIR2DL1 expression suggested a central role for this element in KIR expression. The current study identifies ProI elements in all of the KIR genes, revealing four classes of ProI that correspond with four distinct expression phenotypes of KIR subgroups: KIR2DL2/S2/L3 that are expressed early in reconstituting NK after transplant; KIR2DL4 that is expressed by CD56-bright NK in a non-variegated manner; KIR3DL3 that is not expressed by circulating NK cells; and the remaining KIR that are expressed by subsets of CD56-dim NK. The four classes of ProI are structurally diverse and display distinct functional properties. Altogether, these results indicate that KIR ProI elements contribute to the tissue/cell-type specificity of KIR transcription and cooperate with the probabilistic proximal promoter to control KIR expression.

INTRODUCTION

The receptors for class I major histocompatibility complex expressed by human and mouse natural killer (NK) cells have evolved from distinct gene families to perform identical functions.¹ The murine Ly49 class I receptors are related to c-type lectins,² whereas the human killer cell immunoglobulin (Ig)-like receptors (KIRs) are members of the Ig-superfamily.³ In addition to their convergent functional evolution, the human and mouse gene families share a specialized pattern of variegated expression resulting in the majority (80%) of NK cells expressing from one to three receptors out of a repertoire of > 10 receptors in any given genotype.⁴ ⁵ ⁶ ⁷ This generates subsets of NK cells that are tuned to recognize altered expression of specific class I alleles. The KIR and Ly49 gene families make use of a similar molecular mechanism to generate stochastic expression on NK cells.⁹ Bidirectional promoters are found upstream of each gene, and the relative affinity of binding sites for transcription factors (TFs) involved in sense versus antisense promoter activity determines the probability of generating the sense transcript required for gene activation. In the Ly49 genes, the bidirectional promoter is only active in immature NK cells, and it is located several kb upstream of the promoter associated with Ly49 expression in mature NK cells. There is a clear separation of probabilistic gene activation from the onset of protein expression in the mouse genes. In contrast, the bidirectional KIR promoter is adjacent to the first coding exon of the gene and it produces sense transcripts in mature, KIR-expressing NK cells, suggesting a role for this element in both gene activation and protein expression. A recent study of a weakly expressed KIR2DL1 allele demonstrated that transcription from an additional promoter (ProI) located ~300 bp upstream was required for KIR2DL1 expression.⁹ ProI transcripts correlated with protein expression, whereas proximal transcripts did not. In addition, non-translatable splice variants of the proximal transcript were detected in cells with decreased ProI activity. These results suggested that the principal role of the proximal bidirectional promoter was to control variegated expression, similar to the distal Ly49 element, and that a separate upstream promoter was required for protein expression. A previous study of KIR2DL4 transcripts had revealed the presence of distal and intermediate promoter transcripts in this non-variegated KIR.¹⁰ A distal promoter is located 11 kb upstream and an intermediate promoter is present approximately 900 bp upstream of the KIR2DL4 translation-initiation site. Transcripts from the distal and intermediate KIR2DL4 promoters are spliced to a site 190 bp upstream of the KIR2DL4 start codon, allowing translation of these messages, thus bypassing the variegated expression associated with the bidirectional proximal promoter.

In order to determine whether the presence of intermediate promoters was a general feature of KIR gene regulation, we searched for the presence of ProI elements in all members of the KIR gene family. The current study reveals that all KIR genes possess intermediate promoters, and the characteristics of ProI elements define KIR subgroups that have distinct expression patterns. ProI therefore appears to represent the key element controlling KIR expression patterns, and the primary function of the proximal promoter is the control of variegated expression.
RESULTS

Identification of KIR2DL1-related ProI elements

A comparison of the region upstream of the KIR2DL1 Prol transcription start site (TSS) revealed a high degree of sequence conservation in many KIR genes, with the exception of KIR2DL4, KIR2DL2/L3/S2, KIR3DL2 and KIR3DL3 (Figure 1). Interestingly, the genes with low homology represent KIR with distinct expression characteristics. KIR2DL4 is expressed by CD56-bright and -dim NK cells in a non-variegated manner.11,12 KIR2DL2/L3/S2 are the first receptors to be expressed by NK cells after transplant.13 KIR2DL4 is more highly expressed by T cells than other KIR,14 and KIR3DL3 is not expressed by circulating CD56-dim NK cells but has been found in decidual CD56-bright NK cells.15 A luciferase reporter assay was used to test the promoter activity of the predicted promoter regions shown in Figure 1a. The genes with a high degree of homology to KIR2DL1 demonstrated varying levels of activity, but no Prol activity was observed for this region of the KIR2DL4, KIR2DL2/L3/S2 and KIR3DL2 genes (Figure 1b). The KIR3DL3 sequence present in this region had very little homology to KIR2DL1, but it was found to have a high level of promoter activity in uterine cancer lines. Allele- and gene-specific polymorphisms in activator protein-1 (AP-1)-, Oct-1- and Ets-1-binding sites affect TF binding

The KIR genes with a KIR2DL1-related Prol element were analyzed for the presence of putative TF-binding sites. Potential CCAAT/ enhancer-binding protein (C/EBP)-, AP-1-, Oct-1- and Ets-binding sites were identified. The C/EBP-binding site is conserved in all

Figure 1. Comparison of the KIR2DL1 ProI region with other KIR genes. (a) The 240 bp ProI region of KIR2DL1 is shown, with only nucleotide differences displayed for other KIR genes. The location of the KIR2DL1 sequence relative to the start codon of the gene is indicated in bold at the right end of each line of sequence. Dashes indicate deletions relative to the KIR2DL1 sequence. Underlined bold sequence represents putative TF-binding elements. The single bold A residue denoted by an asterisk indicates the TSS determined for KIR2DL1. KIR2DL1*003 represents the unique sequence found in the KIR2DL1*003 allele, and KIR3DL1*005 represent the sequence found in the KIR3DL1*001, *004, and *005 alleles. KIR listed in red print are genes with low homology to the KIR2DL1 Prol region. The complete sequence of the KIR3DL3 gene upstream of the Ets-1 site is shown owing to a very low level of homology with KIR2DL1 in this region. (b) Luciferase activity of pGL3 constructs containing the KIR promoter regions depicted in panel (a). Constructs were transfected into the cell lines listed, and relative luciferase activity was determined 48 h posttransfection. Values represent the mean, and error bars indicate the s.d. of at least three independent experiments. KIR genes listed in red correspond to the divergent KIR gene sequences identified in panel (a).
members of this class of Prol. However, gene-specific nucleotide changes are present in the AP-1 and Oct-1/Ets sites that are predicted to change the affinity for their respective TFs. In addition, the pair of adjacent AP-1-binding sites possess allele-specific polymorphisms in the KIR2DL1*003 and KIR3DL1*005 genes (Figure 1a). Gel-shift analysis was conducted with AP-1 or Oct-1/Ets site probes corresponding to individual KIR genes and alleles (Figure 2). The nucleotide changes present in the KIR2DL1*003 and KIR3DL1*005 probes resulted in a loss of AP-1 binding. Comparison of the tandem Oct-1- and Ets-1-binding sites revealed a loss of Ets-1 binding with probes from the KIR2DL2 and KIR2DS3 genes that have a G to T substitution in the core Ets-binding motif (Figures 1 and 2b). The KIR2DL2 gene has an additional T to C substitution in the Oct-1 site (Figure 1) that decreased observed binding to Oct-1 (Figure 2b).

A recent study of expression patterns of KIR alleles confirmed the low expression intensity of KIR3DL1*005 relative to other KIR3DL1 alleles and revealed that the average intensity of KIR2DL1*003 expression is lower than the KIR2DL1*002 allele. Therefore, reduced intensity of KIR2DL1 expression may be associated with a reduced activity of the KIR2DL1*003 Prol element. Interestingly, the average frequency of expression of KIR2DL1*003 was slightly higher than KIR2DL1*002, suggesting that the intermediate promoter polymorphism does not affect the frequency of KIR-positive cells.

Analysis of the enhanced activity of the KIR3DL3 intermediate promoter in cervical cancer lines

Although the sequence of the KIR3DL3 Prol fragment 5’ of the Ets-1 site had no significant homology to KIR2DL1, it possessed a high level of activity in two cervical cancer cell lines, Hela and CaSkI (Figure 1b), suggesting that this gene might be expressed in uterine tissue. However, reverse transcriptase-PCR (RT-PCR) analysis of these cell lines did not detect any KIR3DL3 mRNA (data not shown), indicating that the increased transcriptional activity in Hela and CaSkI may not be due to tissue specificity of the KIR3DL3 promoter per se, but instead it may be associated with changes in transcription activity owing to transformation of these cervical cancer cell lines by papilloma virus, with the KIR3DL3 Prol being responsive to the virus-induced alterations.

The KIR3DL3 Prol region contains a putative CREB/AP-1 element. Electrophoretic mobility shift assay revealed that the CREB/AP-1 site produced a strong complex in Hela cells, with only faint bands appearing in the other nuclear lysates tested, suggesting that this region has a role in the enhanced activity of the KIR3DL3 Prol element in these cells (Figure 3a). Antibody inhibition of complex formation on the putative CREB-binding site revealed that numerous members of the Fos, Jun and CREB family of TFs were capable of binding to this element (Figure 3b).

A comparison of the factors bound to the KIR3DL1 AP-1 site in Hela nuclear lysate showed a similar pattern of Fos/Jun binding but no detectable ATF/CREB binding to the KIR3DL1 AP-1 site (Figure 3c). This indicates that the higher activity of the KIR3DL3 Prol element in Hela cells relative to other cell types may be due to higher expression of CREB family members in these cells or perhaps a result of papilloma virus transformation and production of the viral E7 protein that is known to associate with the AP-1 family of TFs.17
The **KIR2DL2/L3/S2** intermediate promoter is located further upstream

A previous examination of **KIR2DL4** distal transcripts revealed the presence of an intermediate promoter approximately 900 bp upstream of the translation initiation site, indicating that a ProI-like element might exist elsewhere for the other non-homologous genes. Because of the lack of detectable promoter activity in the **KIR2DL2/L3/S2**, and **KIR3DL2** region homologous to **KIR2DL1** ProI, a search for a distinct **KIR2DL2** ProI was initiated. 5′ RACE (Rapid Amplification of cDNA Ends) was performed for the **KIR2DL2** gene using primers upstream of the proximal promoter TSS, and a series of upstream gene fragments were tested for promoter activity. Figure 4a shows the **KIR2DL2** region found to contain promoter activity. Mapping of promoter activity through the analysis of a series of PCR fragments localized optimal transcription activity to the region from −864 to −438 relative to the translation start site (Figure 4b). This region contains putative binding sites for YY1, Oct-1, IRF-1, C/EBP and TBP. The transcripational start site mapped by 5′ RACE was located within the nucleotide sequence of the 3′ **KIR2DL2** primer associated with the highest promoter activity (−438; Figure 4b). There is a TATA-like element located 24 bp upstream of this start site, and a C/EBP site is located 59 bp upstream, indicating the structure of a typical TATA-based promoter. A construct lacking the transcription initiation site (−953 to −566 fragment; Figure 4c) has reduced promoter activity, and a construct lacking the TATA element had no detectable activity (−953 to −589 fragment; Figure 4c). The 5′ boundary of the effective promoter region is defined by a repetitive element insertion containing distal promoter elements that is present only in the **KIR2DL2/L3/S2** and **KIR3DL2** genes. The decrease in promoter activity seen in constructs containing additional 5′ sequence (−1320, −1240, −1141) may be due to the generation of transcripts from the distal region that inhibit Prol function.

**Figure 3.** Enhanced formation of CREB/AP-1 complexes in Hela cell nuclear lysates. (a) Comparison of complexes formed by a probe containing the **KIR3DL3** CREB/AP-1 site in nuclear extracts from various cell lines. Probe sequence is shown underneath the panel with the CREB site underlined. The position of the CREB/AP-1 complex is indicated by a labeled arrow. (b) The ability of a panel of antibodies reacting with AP-1, CREB or control TFs to inhibit the complex formed in Hela cells is shown. Probe and CREB/AP-1 complex are indicated as in panel (a). The numbers in parentheses after each of the CREB1 antibodies indicate different commercial antibodies tested. (c) Similarity of AP-1 binding but not CREB binding to the **KIR3DL1** tandem AP-1 sites. The sequence of the probe used is shown below, with the AP-1 sites underlined. The position of the CREB/AP-1 complex is indicated by a labeled arrow.

**DISCUSSION**

The initial characterization of the **KIR** promoter elements immediately upstream of the coding region revealed a high degree of sequence conservation (91.1–99.6%), with only **KIR2DL4** possessing a significantly divergent promoter structure (67% homology to **KIR3DL1**), consistent with its non-variegated expression at the earlier CD56-bright stage of NK cell development. The **KIR** promoters were divided into four groups based on polymorphisms found in YY1 and Sp1 TF-binding sites: group 1 = most variegated **KIR** genes; group 2 = **KIR2DL1/S1** and **KIR2DL5/S5**; and group 3 = **KIR3DL3**; group 4 = **KIR2DL4**. Although these groupings provided a potential explanation for the distinct expression patterns of **KIR3DL3** and **KIR2DL4**, they could not account for the observation that **KIR2DL2/S2** and **KIR2DL3** were the first **KIR** to be expressed by NK cells developing in vitro or after hematopoietic stem cell transplantation. The subsequent discovery of the bidirectional nature of the **KIR** proximal promoter revealed that the polymorphisms found in the YY1 and Sp1 sites...
Identification of the KIR2DL2/L3/S2 and KIR3DL2 Promoter Region

(a) The sequence of the 793 bp KIR2DL2 region tested for ProI activity is shown with only nucleotide differences shown for the other KIR genes. The vertical lines through the sequence indicate the ends of PCR fragments tested for promoter activity as shown in panel (b), and the numbers shown indicate the position relative to the start codon of the KIR2DL2 gene. Predicted TF-binding sites are underlined in bold. The non-conserved KIR2DL1 and KIR3DL1 sequences are shown in red. The dashed lines show the location of a region that is deleted in the KIR2DL1 and KIR3DL1 genes. The single bold A residue denoted by an asterisk indicates the TSS determined for KIR2DL2 ProI.

(b) Mapping of the 5′ boundary of KIR2DL2 ProI activity. PCR fragments were generated with a series of 5′ primers together with a common 3′ primer containing the TSS and cloned into the pGL3 reporter vector. Constructs were transfected into the cell lines listed, and relative luciferase activity was determined 48 h posttransfection. Values represent the mean, and error bars indicate the s.d. of at least three independent experiments.

(c) Mapping of the 3′ boundary of KIR2DL2 ProI. Two additional pGL3 constructs were generated by PCR using the −864 primer found to mark the 5′ end of the active promoter region and 3′ primers deleting either the transcription initiation site (−477) or the putative TATA element (−500). The activity of the −864 to −438 construct is shown again for comparison.

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**Figure 4.** Identification of the KIR2DL2/L3/S2 and KIR3DL2 Pro region. (a) The sequence of the 793 bp KIR2DL2 region tested for ProI activity is shown with only nucleotide differences shown for the other KIR genes. The vertical lines through the sequence indicate the ends of PCR fragments tested for promoter activity as shown in panel (b), and the numbers shown indicate the position relative to the start codon of the KIR2DL2 gene. Predicted TF-binding sites are underlined in bold. The non-conserved KIR2DL1 and KIR3DL1 sequences are shown in red. The dashed lines show the location of a region that is deleted in the KIR2DL1 and KIR3DL1 genes. The single bold A residue denoted by an asterisk indicates the TSS determined for KIR2DL2 ProI. (b) Mapping of the 5′ boundary of KIR2DL2 ProI activity. PCR fragments were generated with a series of 5′ primers together with a common 3′ primer containing the TSS and cloned into the pGL3 reporter vector. Constructs were transfected into the cell lines listed, and relative luciferase activity was determined 48 h posttransfection. Values represent the mean, and error bars indicate the s.d. of at least three independent experiments. (c) Mapping of the 3′ boundary of KIR2DL2 ProI. Two additional pGL3 constructs were generated by PCR using the −864 primer found to mark the 5′ end of the active promoter region and 3′ primers deleting either the transcription initiation site (−477) or the putative TATA element (−500). The activity of the −864 to −438 construct is shown again for comparison.
influenced the relative strength of competing sense and antisense promoters, and the loss of Sp1 binding correlated with increased sense promoter activity and a higher frequency of expression by NK cells.\textsuperscript{21,22}

The KIR gene groupings generated as a result of identifying the Prol elements upstream of the bidirectional proximal KIR promoter may provide an explanation for the more rapid expression of the KIR2DL2/S2/L3 genes in reconstituting NK. The inclusion of the framework KIR3DL2 gene in this group indicates that it should also be expressed early; however, previous studies of reconstituting NK have not tested KIR3DL2 expression. The higher frequency of T cells expressing KIR3DL2 than other KIR may reflect an expanded window of opportunity for KIR3DL2 expression owing to Prol activation earlier in T-cell development.

A comparison of the intergenic regions of KIR family members (Figure 6) reveals that the distinct Prol elements have arisen as a result of differential insertion/deletion of repetitive elements in the region upstream of the conserved bidirectional proximal promoter. The KIR2DL1-related Prol elements are in a region of non-repetitive DNA; however, the

\begin{figure}
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\caption{Identification of TFs binding to the KIR2DL2 Prol region. (a) Probes containing predicted binding sites are listed with the putative binding region underlined. (b) Electrophoretic mobility shift assay of the probes listed in panel (a) performed with either Jurkat (left panel) or YT-Indy (right panel) nuclear extracts. (c) Antibody inhibition of complexes formed in Jurkat nuclear lysates with Oct (left panel), YY1 (center panel) or C/EBP (right panel) probes. Antibodies used are listed above each lane, and those shown in red produced complex inhibition and/or supershift.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Generation of distinct Prol elements as a result of repetitive element insertion and deletion. A schematic of the four classes of KIR genes based on Prol analysis is shown. The start codon of each gene is indicated by (ATG) over a vertical line. Yellow rectangles labeled ‘Prol’ indicate the intermediate promoters characterized. The boxes labeled (1) and (4) represent the promoter regions depicted in Figures 1a and 4a, respectively. The white rectangle labeled ‘Prol-D’ represents the previously characterized distal KIR3DL1 promoter. Insertions and deletions in KIR2DL2 relative to KIR2DL1 are indicated by heavy black lines marking locations of the events. Repetitive elements of different classes are indicated by different colored rectangles labeled with the name of the element. For the KIR2DL4 gene, (SD) and (SA) indicate the location of the splice donor and splice acceptors, respectively, used to produce a spliced intermediate transcript.}
\end{figure}
three other classes of Prol are all associated with repetitive elements. The KIR2DL2 and KIR2DL4 intermediate promoters are contained within LINE elements, whereas KIR3DL3 Prol contains a unique Alu element insertion. It is of interest to note that the previously characterized KIR3DL1 distal promoter (Pro-D)\(^{8,10}\) is located within the same L1M5 LINE element that contains KIR2DL2 Prol. It appears that the KIR2DL2-related genes have replaced the KIR3DL1-associated Prol with a distal promoter element owing to a 406-bp deletion of the Prol region. This event may account for the expression of KIR2DL2/L3/S2 earlier in NK development than other variegated KIR, as transcription from the distal promoter was detected at the earliest stages of NK cell differentiation.\(^{25}\)

Most KIR genes contain an approximately 2-kb intergenic region separating the start codon of one KIR gene from the polyA addition site of the previous gene. The majority of studies examining factors that control KIR transcription were concentrated on the 300 bp immediately upstream of the start codon. The current study of intermediate promoters together with previous analyses of distal promoter elements indicates that the majority of the intergenic region has a direct role in the regulation of transcription. The recent discovery of a direct correlation between intermediate promoter activity and protein expression of KIR2DL1\(^{9}\) raises the possibility that Prol elements control KIR expression, and the proximal promoter region exists solely to generate variegated expression of the KIR genes via the stochastic production of antisense RNA, leading to gene silencing.\(^{2,5}\) However, methylation of the proximal KIR promoter has been shown to control KIR expression,\(^{24,25}\) indicating that, at a minimum, the proximal promoter cooperates with the upstream promoter to enable expression, as no KIR transcripts are generated if the proximal promoter region is methylated. A role for methylation of Prol in gene silencing is excluded by the observation that there are no CpG sites in the KIR2DL1 intermediate promoter region, while 18 CpG sites are present in the proximal promoter region. In addition, a RUNX-binding site located in the proximal promoter has been shown to be required for KIR expression, as non-expressed variants of the KIR2DL5 gene lack RUNX binding to this region.\(^{26}\) Furthermore, demethylation of the silent KIR2DL5 allele gene leads to its expression, revealing a role for RUNX in gene activation. A possible collaboration between Prol and the proximal promoter in gene demethylation and activation is suggested by observations made on the KIR3DL3 gene. Trompeter et al.\(^{27}\) demonstrated that, although KIR3DL3 contains an intact RUNX-binding site, it is methylated and not expressed in peripheral blood NK cells. However, expression can be induced by demethylating the proximal promoter region, indicating the presence of a fully functional promoter. Perhaps the lack of detectable KIR3DL3 Prol activity in NK cells revealed by the current study represents the missing element required for KIR3DL3 proximal promoter demethylation. Previous studies of distal KIR promoters revealed that the KIR3DL3 distal element is inverted and transcribes only in the antisense direction.\(^{10}\) This observation, coupled with a lack of Prol transcription in NK cells, would result in a lack of distal transcripts traversing the proximal promoter region that may be required for access of RUNX to the DNA and subsequent demethylation of the region.

Whether or not the Prol elements of all KIR genes are absolutely necessary to drive protein expression is not known but can be tested by performing quantitative RT-PCR experiments comparing proximal and distal transcripts across all KIR genes. It will be important therefore to assay levels of Prol transcripts in future studies examining the relationship between levels of KIR transcription and the intensity of receptor expression on the surface of NK cells.

MATERIALS AND METHODS

Cell lines

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U ml\(^{-1}\) each of penicillin and streptomycin (P/S), sodium pyruvate and L-glutamine. YT-Indy and Jurkat cells were cultured in RPMI 1640 media containing 10% fetal bovine serum and penicillin and streptomycin. The NK cell line was cultured in RPMI 1640 containing beta-mercaptoethanol, nonessential amino acids, 10% fetal bovine serum, 100 U ml\(^{-1}\) each of penicillin and streptomycin, sodium pyruvate, L-glutamine, Hepes and 1000 U ml\(^{-1}\) of recombinant human IL-2. The cancer cell lines, Hela, CaSki and MCF7, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained according to the supplier’s instructions.

S’ RACE analysis of KIR2DL2 prol transcripts

S’ RACE was performed on KIR2DL2-expressing NK92 cell RNA using the Invitrogen S’ RACE System (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s directions. Gene-specific cDNA was generated using a KIR2DL2/L3/S2-specific antisense primer located 147 bp upstream from the proximal promoter TSS: 5’-TTGACACCTGGGTCCTCCTACT-3’. A poly-dC tail was added to the cDNA to aid in the subsequent cDNA amplification, which was performed using a poly-dG-anchor primer 5’ and a KIR2DL2 antisense primer located 294 bp upstream of the proximal TSS: 5’-GTAAT GTGCAAAAGTGTCTAACGG-3’) at an annealing temperature of 59 °C for 35 cycles with Platinum PCR Supermix (Invitrogen). Excess primers from first-round PCR reactions were removed with the Charge-Switch PCR Clean-Up Kit (Invitrogen). Nested PCR was subsequently performed with the KIR2DL2 primer and the KIR2DL2 antisense primer located 366 bp upstream of the proximal TSS: 5’-CTCTGGTGAAGGTTCTTACTA-3’) at an annealing temperature of 60 °C for 30 cycles. PCR products were cloned into pCR2.1 Topo (Invitrogen) and sequences were analyzed to map the 5’ start sites.

RT-PCR of KIR transcripts

Total RNA was purified from 1 × 10\(^6\) cells with the RNeasy Kit (Qiagen, Valencia, CA, USA), and cDNA synthesis was carried out using Random Hexamer primer and Taqman Reverse Transcription Reagents Kit (Applied Biosystems Foster City, CA, USA) according to the manufacturer’s instructions. Reactions were carried out using the FastStart SYBR Green Master Kit (Roche Diagnostics, Indianapolis, IN, USA) on the 7300 Real-Time PCR System (Applied Biosystems). Quantitative RT-PCR was performed in duplicate and was repeated in at least three separate experiments. Melting curve analyses were performed to verify the amplification specificity. Relative quantification of gene expression was performed according to ΔΔCT method using the StepOne Software 2.0 (Applied Biosystems).

Electrophoretic mobility shift assays

Nuclear extracts were prepared from the various cell lines using the Celllytic NuCLEAR Extraction Kit (Sigma-Aldrich, St Louis, MO, USA). Protein concentration was measured with a Bio-Rad protein assay (Hercules, CA, USA), and samples were stored at –70 °C until use. Double-stranded DNA oligonucleotide probes corresponding to the predicted TF-binding sites of KIR Prol region were synthesized (Figures 2, 3 and 5). Labeling and DNA–protein binding reactions were performed as previously described.\(^{9}\) For antibody supershift experiments, nuclear extracts were incubated with 2 μl of antibody for 1 h on ice before addition of \(^{32}\)P-labeled DNA probe. After the addition of labeled DNA probe, the binding reaction was incubated for an additional 20 min at room temperature. The antibodies used were cFos (6-2H-2 F), FoxB (102), cJun (H-79), JunB (C-11), JunD (329), Fra-2 (Q-20), ATF-1 (F-11), Ets-1 (C-4), Erf-1 (C-20), Oct-1 (E-8, C-21 and 12F1), Oct-3/4 (C-10), Oct-2 (C-20), CREB-1 (2H4H8), C/EBPα (D-5), C/EBPβ (H-7), C/EBPδ (H-50), SP-1 (E-3), IRF-1 (C-20), IRF-3 (SL-12), IC3BP (C-19), PU.1 (A-7), E4BP4 (B-1) and MyoD (C-20) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) at gel shift grade (200 μg per 0.1 μl).

Generation of luciferase reporter plasmids

Promoter fragments were generated by PCR using the primers listed in Table 1 and cloned into the TOPO-TA vector (Invitrogen). Inserts were excised with Xhol/HindIII and cloned into pGL3 (Promega, Madison, WI, USA) to generate constructs in forward orientation. All constructs were

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Table 1. Sequences and locations of primers used in this study

| Primer name and amplification | Sequence (′5′ to ′3′) | Construct length (bp) |
|------------------------------|-----------------------|----------------------|
| **Prol promoter assay**      |                       |                      |
| Forward primer               |                       |                      |
| 2DL1-For                     | AAACTCCAGAAATTTACAGGTGGGG |                      |
| 2DS1-For                     | AAAATCAGGAATTTAATGTTG   |                      |
| 3DL1-For                     | AATAATGAGAATTATAGGTG    |                      |
| 3DL5-For                     | GACAGAGGTGGAGCTGACAT    |                      |
| 2DL2/3DL2-For                | CTGAGACAGACCTTCCTCTCA   |                      |
| 2DL4-For                     | GACAGCTTCAATGACATATTG   |                      |
| 3DL3-For                     | CTGGGAGGTGGAGGCTGACAT   |                      |
| Reverse primer               |                       |                      |
| 2DL1/3DL1-Rev                | GAGATTCAAACTCTCTCTCTCT |                      |
| 2DL2/2DS2-Rev                | GAGATTCAAACTCTCTCATATG  |                      |
| 2DL4-Rev                     | GAGATTCAAACTCTCTCTGATAT|                      |
| 3DL2-Rev                     | GAGATTCAAACTCTCTCTGATAT|                      |
| 3DL3-Rev                     | GAGATTCAAACTCTCTCTGATAT|                      |
| **2DL2 5′-deletion assay**   |                       |                      |
| 2DL2-1527-For                | AACAGTTTTATGGAACCAAC    | 1001                 |
| 2DL2-1230-For                | GAGACGCTCTCCTACTCCTCA   | 794                  |
| 2DL2-1240-For                | AGCCAGTTCTCCCTCACGCTCT  | 714                  |
| 2DL2-1141-For                | GACCTGGGTTGACAGTTGGG    | 615                  |
| 2DL2-953-For                 | CACCCGATGGATAGGACGAC    | 427                  |
| 2DL2-815-For                 | CCTACGTTGAAACCCCTGTC    | 289                  |
| 2DL2-764-For                 | CACCATTTAATACACTGCGACC  | 234                  |
| 2DL2-527-Rev                 | GGTGACAGGAGGAAGATCTCTTC | 427                  |
| **2DL2 3′-deletion assay**   |                       |                      |
| 2DL2-953-For                 | CACCCGATGGATAGGACGAC    | 1001                 |
| 2DL2-589-Rev                 | CAAATCGAGGACCTAGTTCAG   | 365                  |
| 2DL2-566-Rev                 | GCAAACTAAAATACCTTAATACA | 388                  |
| 2DL2-527-Rev                 | GGTGACAGGAGGAAGATCTCTTC | 427                  |

verified by sequencing. Sequence analysis was carried out with the SeqWeb package at the NCI-Frederick supercomputing center and the Molecular Evolutionary Genetics Analysis (MEGA, The Biodisgn Institute, Tempe, AZ, USA) software version 4.5.

Cell transfection and luciferase assays

The cell lines, including breast cancer cell line MCF7, cervical cancer cell lines Hela and CaSkii, mouse NK cell line LNK and human embryonic kidney 293T cells, were plated at 1×10^5 cells per well in a 24-well plate the day before transfection and incubated overnight at 37 °C in 5% CO2. For each well, 1 μl of HilyMax transfection reagent (Dojindo, Rockville, MD, USA) was diluted in 30 μl of serum-free RPMI medium and incubated at room temperature for 30 min. YT-Indy cells were transfected by electroporation with 25 ng of the pRLSV40 control vector, and each construct was tested in triplicate in at least three independent experiments.

Statistical analysis

Mann–Whitney U and two-tailed t tests were performed using GraphPad Prism version 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA); P < 0.05 was regarded to be statistically significant.
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