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Three Structurally and Functionally Divergent Kinds of Promoters Regulate Expression of Clonally Distributed Killer Cell Ig-Like Receptors (KIR), of KIR2DL4, and of KIR3DL3

Hans-Ingo Trompeter,* Natalia Gómez-Lozano,† Simeon Santourlidis,* Britta Eisermann,* Peter Wernet,* Carlos Vilches,† and Markus Uhrberg*†

The generation of killer cell Ig-like receptor (KIR) expression patterns in NK cells involves variegated silencing of KIR genes by DNA methylation. To identify regulatory elements involved in KIR gene activation, upstream regions of KIR genes were functionally characterized in NK3.3 cells as well as in primary NK cells. Three kinds of KIR promoters were defined, controlling clonally expressed KIR genes, the constitutively active KIR2DL4, and the weakly expressed KIR3DL3. Upstream of a short core promoter common to all KIR genes, a region containing functionally divergent elements was characterized. Although this region had no impact on the activity of the KIR2DL3 promoter, an inhibitory element was identified in the KIR2DL4 promoter and an activating element was found in the KIR3DL3 promoter. Upon treatment with a methyltransferase inhibitor, KIR3DL3 expression could be readily induced showing that the low levels of KIR3DL3 expression in peripheral blood are due to sustained DNA methylation of an otherwise fully functional promoter. Analysis of transcription factor binding sites identified a functional acute myeloid leukemia (AML) site common to all three KIR promoters. Mutation of this site led to a substantial increase in activity of all KIR promoters. Among the different members of the AML family, AML-2 was identified as the predominant KIR binding factor. The present study suggests that AML-2 acts as a repressor of KIR expression in mature NK cells and opens the possibility that AML factors and associated cofactors are involved in regulation of KIR expression during NK cell development. The Journal of Immunology, 2005, 174: 4135–4143.

Natural killer cells constitute an early line of defense against tumorigenic cells and viruses (1–3). NK cells are able to recognize target cells either lacking self-MHC class I molecules or expressing reduced levels on their surface by impaired engagement of inhibitory NK cell receptors (4, 5). Three major kinds of MHC class I receptors are present in humans: the lectin-like CD94:NKG2 (6), the killer cell Ig-like receptors (KIR), and the leukocyte Ig-like receptors (7–11). KIR can be further subdivided into inhibitory receptors carrying an inhibitory signal motif within their cytoplasmic domain (KIR2DL and KIR3DL) and stimulatory receptors (KIR2DS and KIR3DS) lacking this motif (12). The extracellular domains of KIR consist of either two (KIR2D) or three (KIR3D) Ig-like domains called D0, D1, and D2. Most of the inhibitory KIR are specific for the products of HLA class I genes like KIR3DL2 (HLA-A), KIR3DL1 (HLA-B), and KIR2DL1, KIR2DL3 and KIR2DL7 (HLA-C) (13–16). KIR2DL4 combines structural and functional features of both stimulatory and inhibitory KIR and was reported to bind to the nonclassical class I protein HLA-G (17, 18). The ligand specificities of KIR2DL5, KIR3DL3, as well as the six stimulatory KIR (KIR2DS1-KIR2DS5 and KIR3DS1) are uncertain and might include non-HLA class I ligands (19).

The KIR genes are densely clustered within the leukocyte receptor complex on chromosome 19q13.4 (20). Remarkably, the number and combination of KIR genes present in the genome varies within the human population (21). Family segregation studies in the caucasoid population have shown the presence of >20 different KIR haplotypes, which contain variable numbers of inhibitory and stimulatory KIR genes. Although all haplotypes contain several inhibitory KIR, the number of stimulatory KIR varies from zero (in case of group A haplotypes containing the mutant 2DS4*003 allele) up to five in the highly diverse group B haplotypes (22).

Like the members of the Ly-49 family in mice, KIR genes are expressed in a clonally distributed fashion in human NK cells (23, 24). One to eight different receptors from the variety of inhibitory and stimulatory KIR present on a given KIR genotype are expressed on individual NK cell clones and individual expression patterns are stably maintained through cell divisions (25). Two KIR genes do not fit into this KIR group: firstly, KIR2DL4, which is constitutively expressed in all NK cells on the transcriptional level, although cell surface expression appears to be variable (18, 25, 26), and secondly, KIR3DL3, which is expressed at low or undetectable levels in peripheral blood NK cells (27, 28). The distribution patterns of KIR on NK cells appear to be largely stochastic. Although each NK cell expresses at least one inhibitory receptor for a self class I allotype (either a KIR or a CD94:NKG2A...
KIR repertoires are generally not biased for expression of certain combinations of KIR genes. Thus the expression of KIR appears to be largely independent of each other and the impact of the requirement for inhibition by self class I molecules on the shape of the KIR repertoire is rather subtle. The influence of HLA class I is best seen on the identical genetic background of sibling pairs, where the relative frequencies of cells expressing a certain KIR correlate with HLA class I type (29). Additional evidence for class I is best seen on the identical genetic background of sibling cells, which are largely similar to that found on NK cells of the same individual (30). Thus, despite the apparent differences in ontogeny and selection of NK and T cells, KIR repertoires are similar, which again points to a rather subtle effect of selection on KIR expression.

Information on the molecular regulation of KIR gene expression is limited. The thirteen clonally distributed KIR genes share a remarkably high homology (>91%) in their 5′-untranslated regions (5′UTR), which lack typical promoter features like TATA and CAAT boxes(31, 32). Certain transcription factor binding sites have been predicted in the upstream regions of KIR on the basis of the regions of sequence inspections including sites for transcription factors CREB, SP1, ETS, AP-1, AP-4, and acute myeloid leukemia (AML) factor. More recently, it was demonstrated that DNA methylation plays an important role in regulation of KIR gene expression (33, 34). CpG islands located upstream of the translation initiation codon of each KIR gene are either densely methylated in case of repressed KIR genes or demethylated in expressed KIR genes. Expression of formerly silenced KIR genes can be readily induced using a demethylating agent. Reporter gene experiments in vitro methylated 5′UTR constructs indicated that KIR CpG islands largely overlap with regions having promoter activity (33).

The purpose of the present study was to analyze structure and function of different KIR promoters and correlate these results with the expression characteristics of each KIR gene. We show that expression of the KIR gene family is regulated by three different types of KIR promoters corresponding to the three different expression modes of KIR: one unique for KIR2DL4, which is expressed in all NK cells, one for KIR3DL3, which is generally not expressed in peripheral blood, and a third kind common to all clonally distributed KIR genes. Both functional mutation analysis and protein binding studies show that the transcription factor AML-2 is involved in negative regulation of all three types of KIR promoters.

Materials and Methods

Construction of reporter plasmids

All reporter plasmids were constructed using the luciferase reporter gene vector pGL3Basic (Promega) backbone. All primers were purchased HPLC-purified from Thermo Hybaid. All restriction sites within primers were underlined. The 494-bp fragment containing the human GAPDH promoter region was isolated from pGL3Basic via SfiI and HincIII, respectively, to obtain constructs KIR2DL3Intron and KIR2DL3 65Intron as well as KIR2DL3 225Intron. Constructs containing mutated AML sites were generated by PCR using primers with mutated AML sites (wild type, TGTGGT, mutated site, TGTAGT).

PCR and cloning procedures

All PCRs were performed using 10/1 mixtures of Taq polymerase (Qiagen) and the proofreading enzyme Pwo polymerase (Roche) with a hot-start procedure. In general, PCR was performed using 35 cycles at 50–58°C annealing temperature. Vector-insert ligations were purified using the Qiagen PCR purification kit and transformed into Escherichia coliSURE strain (Strategene) by electroporation using 2500 V, 201 Ohm, and 25 μF. Following sequencing, DNA preparations used for nucleofections were done at least twice using the Qiagen QIAfilter Plasmid Maxi kit. DNA pellets were resuspended in 500 μl of TE buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA; pH 8.1).

NK cell isolation and culture

The cloned NK cell line NK3.3 (36) was kindly provided by Dr. J. Kornbluth (St. Louis University, St. Louis, MO) and cultured in RPMI 1640 (BioWhittaker), 10% FCS (Biochrom), 15% Lymphocult (Biotest Diagnostics), 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C and 5% CO2. Optionally, Lymphocult was replaced by 10% IL-2-containing PBMC supernatant as described (37). CD34+ hemopoietic progenitor cells were isolated from cord blood by enrichment of PBMC using the Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec) and subsequent flow cytometric cell sorting. Primary NK cells were enriched from PBMC using the NK cell isolation kit II (Miltenyi Biotec). A total of 5 × 10⁶ NK cells were cultivated in the presence of 1 × 10⁵ irradiated autologous PBMC and 1 × 10⁵ irradiated 721.221 cells (38) in RPMI 1640, 5% human serum, 10% FCS (Biochrom), 1000 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C and 5% CO2. For KIR3DL3 induction experiments, NK cells were incubated for 72 h in the presence of 2.5 μM 5-aza-2′-deoxycytidine (Aza) and/or 50 nM trichostatin A (TSA). Bisulfite conversion of genomic DNA and subsequent sequencing was performed as described (33).

Nucleofection of NK cells

NK3.3 cells were transfected by nucleofection as described (39). Luciferase activity was analyzed 4 h after transfection by lysis of cells in 70 μl of reporter lysis buffer followed immediately by luciferase activity measurement using the Luciferase Reporter Gene Assay (high sensitivity; Roche). Samples were measured in a Beckman LS6000 IC scintillation counter (Beckman Coulter) or in a Berthold MiniLumat LB9506 (Berthold). Luciferase activities in all transfection samples were normalized for protein content, which was determined by absorption at 750 nm with a DC Protein Assay (Bio-Rad) according to the manufacturer’s instructions. Protein-normalized luciferase activities were subsequently normalized to the activity of water-transfected cells (specific luciferase activity). Normalization to the empty vector pGL3Basic was avoided due to significant background activities measured in all tested cell types. This activity (approximately one-third of the 65-bp core promoter elements in NK3.3 cells) could, however, be completely blocked by transcriptionally inactive inserts (e.g., KIR2DL3 225b4bp). Thus, to avoid “negative” values of these fragments, luciferase activities were normalized to water-transfected cells. All transfection assays were performed at least three times. A total of 5 × 10⁵ cultivated primary NK cells were transfected with 10 μg of DNA using method U-01 and lysed in 30 μl of reporter lysis buffer after 4 h of incubation. A total of 20 μl were used for luciferase measurement.

EMSAs

Nuclear extracts of NK cells were prepared by collection of 0.5–5 × 10⁶ cells followed by a PBS wash. Cell pellets were lysed in 400 μl of ice-cold buffer A (10 mM HEPES, pH 7.6, 1.5 mM MgCl2, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 2 μg/ml aprotinin, leupeptin, bestatin, 0.5 mM PMSF, and 10 mM Na3MoO4) and incubated on ice for 15 min followed by short vortexing. Following lysis by addition of 25 μl of 10% Nonidet P-40, nuclei were spun down at 16,000 × g for 0.5 min at 4°C and the clean pellet was resuspended in 70 μl of ice-cold buffer C (20 mM

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HEPES, pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 4 µg/ml aprotinin, leupeptin, bestatin, 0.5 mM PMSF, and 10 mM Na₂MoO₄) and mixed on ice for 30 min. Residual debris was spun down and protein content of the supernatant was determined by a Bradford assay (Bio-Rad). Two hundred nanograms of double-stranded oligonucleotides was end-labeled with [³²P]ATP and T4 kinase and purified in 100 l of TE buffer using Microspin G-25 columns (Amersham Biosciences). EMSA reactions were set up as follows: 7 l of EMSA buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 2 mM DTT, 20% (v/v) glycerol); 1 l of poly(dI:dC) (2 g/ml); 1 l of 100 mM MgCl₂; 1 l of labeled oligonucleotides (plus 100-fold molar excess of unlabeled competitor oligonucleotides in certain samples); and 5 g of nuclear extract in a total volume of 20 l. Supershifts included each 2 g of AML-specific Abs (AML-1, sc-8563 and AML-3, sc-12488; both Santa Cruz Biotechnology; AML-2, 39301; Active Motif). All AML Abs used in supershift analyses were pretested with an AML consensus oligonucleotide and AML protein-expressing extracts (a kind gift of Dr. A. Puig-Kroger and Dr. Á. L. Corbi, Centro de Investigaciones Biológicas, Madrid, Spain). NF-κB competitor oligonucleotides were purchased from Promega. Reactions were incubated on ice for 45 min and separated on 6% polyacrylamide acid gels. Gels were dried and autoradiographed at 70°C for 2–16 h.

Sequence analysis software
Putative transcription factor binding sites were analyzed by MatInspector software using the Transfac 4.0 database (40).

nek04137fig1.jpg

FIGURE 1. Sequence variability of putative promoter regions of KIR2DL3, KIR3DL3, and KIR2DL4. The region from −225 up to the translation initiation codon is depicted. Putative transcription factor binding sites predicted by MatInspector software are shown in boxes. Sequence identity to KIR2DL3 is indicated by periods, and nucleotide deletions by dashes.

nek04137fig2.jpg

FIGURE 2. Analysis of the KIR2DL3 promoter. A, Outline of the genomic region (top) consisting of 5'UTR, exon 1, and intron 1 of KIR2DL3. Promoter constructs 2447bp, 936bp, and 225bp as well as promoter/intron constructs 225Intron and Intron225 are indicated. The proximal promoter region (bottom) from −225 to +1 is shown and putative binding sites for transcription factors CREB, AML, SP-1, ETS, and AP-4, as well as a GC-rich region are indicated. Relative positions and structures of constructs 225d64bp, 155bp, 65bp, and 15bp (light gray arrows), and of deletion variants dCREB, dSP-1, dGC, dAP-4, and dETS (dark gray arrows) are given. In all constructs except 225d64bp, the 3’ end is immediately upstream of the ATG initiation codon. B, Promoter activities of constructs 2447bp, 936bp, 225bp, 225d64bp, and 65bp (light gray) as well as of GAPDH promoter control (dark gray) and water-transfected cells are given. Constructs cloned into luciferase + expression vector pGL3Basic were transiently transfected into NK3.3 cells using the nucleofection method and analyzed for specific luciferase activity as described in Materials and Methods. All constructs were analyzed in the same experimental series and each series was repeated at least three times with resulting mean values ± SD as indicated. C, Promoter activities of KIR2DL3 deletion variants. Activities of constructs 155bp and 65bp (light gray) were compared with transcription factor binding site deletion variants dCREB, dSP-1, dGC, dETS, and dAP-4 (dark gray) and analyzed as described in B. D, Contribution of Intron 1 to KIR2DL3 promoter activity. Constructs 225bp, Intron225, and 225Intron were analyzed as described in B.
Results

Expression of KIR2DL3 as well as other clonally distributed KIR is regulated by structurally and functionally similar promoters

A major obstacle for functional analysis of KIR promoters has so far been the lack of an efficient technique to transiently transfect NK cells. Our use of the recently developed nucleofection technology enabled transfection efficiencies of NK cells of >50% and thus provided a suitable methodologic basis for highly sensitive functional studies of KIR promoters (39). Analysis initially focused on KIR2DL3 as a representative of other clonally distributed KIR genes with highly similar upstream regions. To ensure that all regulatory factors necessary for KIR2DL3 expression are available in the cell, KIR2DL3 promoter analysis was performed in the NK3.3 cell line, which endogenously expresses KIR2DL3 and KIR2DL4.

A nucleotide sequence alignment of the upstream regions of KIR2DL3, KIR3DL3, and KIR2DL4 is given in Fig. 1. Because an initial computer-based search for conserved promoter elements had failed to identify classical features like TATA or CAAT boxes in the upstream regions of these KIR, fragments from the complete 2447-bp 5’UTR of KIR2DL3 extending from the 3’UTR of the preceding KIR3DL3 to the initiation codon of KIR2DL3 were included in the initial study (Fig. 2A). A construct containing the region from −225 to −1 (termed 225bp) possessed the strongest promoter activity but a very short segment representing only 65 bp (termed 65bp) upstream of the initiation codon showed almost equally strong activity (Fig. 2B). Inclusion of additional upstream regions did not increase promoter activity; in fact, the extended fragments 936bp and 2447bp exhibited significantly decreased promoter activity indicating that the essential promoter elements are located close to the transcription initiation site. The decrease in promoter activity found with longer fragments is most likely due to a loss of transfection efficiency of longer plasmids. Notably, promoter activity was almost completely lost when the 3’-terminal 64 bp were deleted from fragment 225bp (resulting in a fragment termed 225d64bp) (Fig. 2B). Within the promoter region represented by fragment 225bp of KIR2DL3, putative binding sites were found for transcription factors CREB, AML, SP-1, ETS, AP-4, and a GC box element, as determined by MatInspector software (40) using Transfac 4.0 database (Fig. 1). However, deletion of the consensus binding sites corresponding to variants dSP-1, dETS, dAP-4, and dGC did not show a significant change in promoter activity in NK3.3 cells (Fig. 2C). Only variant dCREB exhibited a slight but significant reduction in activity.

To assess whether the same functional characteristics of the KIR2DL3 promoter are also found in other clonally distributed KIR genes, we compared five different promoter fragments (936bp, 622bp, 451bp, 225bp, and 65bp) from KIR2DL3 with homologous fragments from KIR3DL2, an inhibitory KIR gene located at the opposite (telomeric) end of the KIR locus. Promoter activities of homologous fragments from both KIR genes were highly similar suggesting that clonally distributed KIR are regulated in similar ways (data not shown).

We next extended the promoter analysis to the first intron of KIR2DL3, which has a length of 909 bp and consists of 37 repeat units of a minisatellite sequence. As seen in Fig. 2D, insertion of the first intron downstream of the promoter (fragment 225Intron) led to a moderate increase in transcription. The inductive effect was position-dependent as insertion of the intron upstream of the promoter (fragment Intron225) led to decreased promoter activity (Fig. 2D).

Similar transcriptional activity of a basic promoter common to all KIR genes

The region from −65 to −1, which contains basic promoter activity, is highly conserved among all clonally distributed members of the KIR gene family (92–100% nucleotide identity). Homologies of a lesser degree were found for KIR3DL3 (88%) and KIR2DL4 (61%). To elucidate whether sequence variations in this region might influence promoter activity, seven different 65bp fragments were made representing the putative basic promoters of all known KIR genes. As seen in Fig. 3A, all clonally distributed KIR genes, which were divided into five groups each sharing identical 65-bp fragments, exhibited similar activity. Moreover, also the ubiquitously expressed KIR2DL4 as well as the nonexpressed KIR3DL3 gene exhibited comparable promoter activities. To further characterize necessary core promoter elements, 35bp and 15bp fragments from KIR2DL3, KIR3DL3, and KIR2DL4 were analyzed (Fig. 3B). Because 35bp and in particular 15bp fragments from all three KIR types showed a substantial reduction in activity, the 65bp fragments can thus be viewed as KIR core promoter elements.

The divergent KIR2DL4 promoter contains an upstream inhibitor and an activating element in the first intron

Generally, the KIR2DL4 gene is constitutively active in primary NK cells as well as in cultured NK cell clones (18, 25). Because the core promoter activities of KIR appear to be similar (Fig. 3) we were interested to identify the features, which distinguish the KIR2DL4 promoter from the promoter type found in clonally distributed KIR. Deletion of a unique putative AP-1 site (fragment
dAP-1, (Fig. 4A) as well as of an ETS site (data not shown) did not lead to a significant reduction in promoter activity (Fig. 4B). In contrast to clonally distributed KIR, promoter activities were strongly reduced when an extended upstream segment spanning the region from −225 to −1 was used. Further deletion analysis demonstrated that the inhibitory element is located in the region between −132 and −66 (Fig. 4B). Notably, the silencing effect of the inhibitory element was overridden by insertion of the first intron downstream of fragment 225bp (Fig. 4C). An increase in promoter activity due to inclusion of the first intron was also seen with the shorter core promoter segment of KIR2DL4 (65Intron). The activating function of the intron was nearly lost when inserted upstream of the promoter, thus excluding a position-independent enhancer-like function.

KIR3DL3 contains a functional promoter with an upstream activating element and is epigenetically silenced in NK cells

Although KIR3DL3 is regarded as a nonexpressed KIR gene, the transcriptional activity of the 65bp fragment was comparable to 65bp fragments from other expressed KIR genes (Fig. 3). To identify promoter features, which could explain the lack of KIR3DL3 expression in NK cells, we tested several fragments ranging from 65bp to 936bp. In contrast to clonally expressed KIR as well as KIR2DL4, the 129bp fragment and in particular the 225bp fragment of KIR3DL3 exhibited substantially stronger promoter activity than the 65bp fragment (Fig. 5A) suggesting the presence of an activating element in this region. Extension of KIR3DL3 promoter fragments up to 936bp did not influence promoter activity any further (data not shown).

As described recently, silencing of clonally expressed KIR genes is maintained by DNA methylation (33). Because the promoter of KIR3DL3 appears to be fully functional we analyzed whether the general lack of expression could be explained by sustained epigenetic silencing of KIR3DL3. Treatment of NK3.3 as well as clonally expressed KIR (KIR2DL4) promoter constructs 225bp, 129bp, 15bp, and dAP-1 were cloned into pGL3Basic and transfected into NK3.3 cells followed by analysis for luciferase activity as described in Materials and Methods. Constructs 225Intron, Intron225, 65Intron as well as the wild-type fragments 225bp and 65bp were analyzed as described in B.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Functional analysis of the KIR2DL4 promoter. A. Overview of the genomic region (top) consisting of 5′UTR, exon 1, and intron 1 of KIR2DL4. Promoter constructs 1065bp, 225bp, 189bp, 132bp, 65bp, as well as promoter/intron constructs 225Intron, Intron225, and 65Intron are indicated. The proximal promoter region (bottom) from −65 to +1 is shown and constructs 35bp, 15bp, and dAP-1 are indicated. B. KIR2DL4 constructs 1065bp, 225bp, 189bp, 132bp, 65bp, and dAP-1 were cloned into pGL3Basic and transfected into NK3.3 cells followed by analysis for luciferase activity as described in Materials and Methods. C. Contribution of Intron 1 to KIR2DL4 promoter activity. Constructs 225Intron, Intron225, 65Intron as well as promoter/intron constructs 225bp, 132bp, 65bp were analyzed as described in B.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Functional analysis of KIR3DL3 promoter and DNA methylation status. A. Promoter activities of KIR3DL3 constructs 225bp, 129bp, and 65bp were determined as described in Materials and Methods. B. Induction of KIR3DL3 transcripts by Aza and/or TSA analyzed by RT-PCR in short-term cultivated primary NK cells as well as NK3.3 cells. C. Methylation of CpG dinucleotides of the KIR3DL3 gene was analyzed by sequencing, using the bisulfite conversion method as previously described (33). Positions of CpG dinucleotides are given together with the methylation rate averaged from 25 independent clones each from CD34⁺ NK cell precursors and NK3.3 cells. Note that due to a polymorphism the CpG dinucleotide position −165 is not present in KIR3DL3 in NK3.3 cells.
Disruption of the AML binding site leads to up-regulation of KIR expression

It was previously observed, that a putative binding site for transcription factors of the AML family (also known as Runx, CBP, or PEBP2) is located around \(-100\) in all KIR genes (see Fig. 1) except certain nonexpressed KIR variants having a mutated AML site (41). We thus elucidated the influence of this site on KIR expression by destroying the AML binding sites of KIR2DL3, KIR2DL4, and KIR3DL3 promoters. For this purpose, a single base pair mutation, which was previously shown to effectively abrogate AML binding in AML-transfected COS cells (data not shown), was introduced into the AML site of the respective 225bp fragments. As shown in Fig. 6A, mutation of the AML binding site led to a substantial increase in transcriptional activity in all three types of KIR promoters. In case of KIR2DL4, the inhibiting influence of the silencing region from \(-225\) to \(-66\) was completely neutralized. In case of KIR3DL3, mutation of the AML binding site led to a substantial increase in promoter activity and the absolute values were the highest of all KIR constructs measured in this study. Similarly, a significantly increased promoter activity was observed for the mutated variant of the KIR2DL3 promoter.

Specific binding of transcription factor AML-2 to promoters of all three KIR types

Mutation analysis demonstrated inhibitory effects of the AML site found in all KIR but could not differentiate between the three members of the AML family. Mobility shift experiments using NK3.3 nuclear protein and oligonucleotides from KIR2DL3, KIR2DL4, and KIR3DL3 indeed indicated specific binding of AML proteins to all three KIR promoter types (Fig. 6B). An excess of unlabeled AML consensus competitor completely suppressed the appearance of bands in all three KIR types, whereas they were not affected by 100-fold excess of unlabeled NF-κB competitor. Similarly, a 100-fold excess of mutated AML competitor did not suppress an AML-specific complex (data not shown). Supershift assays using Abs specific for the three AML variants confirmed specific binding of AML-2, but not AML-1 and AML-3 to the AML sites of all three KIR promoter types by supershifting complex I in Fig. 6B. An additional band (complex II) of slower mobility was found predominantly with KIR3DL3 but in weaker amounts also with KIR2DL3 and KIR2DL4. Notably, the intensity of complex II increased upon supershifting complex I by AML-2 Abs.

Analysis of KIR gene regulation in primary NK cells

The experiments presented so far were mainly performed using NK3.3 cells, which represent a long-term cultivated monoclonal NK cell line. To exclude any cell line-specific artifacts and to elucidate whether the observed results were of physiologic relevance, primary polyclonal NK cells were isolated from peripheral blood and transfected with selected promoter constructs from KIR2DL3, KIR3DL3, and KIR2DL4. As seen in Fig. 7, in short-term cultivated primary NK cells, all three promoter types exhibited comparable activities to those observed in NK3.3 cells. The activating and inhibiting properties of the \(-225\) to \(-66\) region of KIR3DL3 and KIR2DL4 promoters, respectively, were confirmed.
as well as the activating effect of the KIR2DL4 intron. Most importantly, the inhibiting function of AML could be verified in all three KIR promoter types.

Discussion

Based on expression characteristics in NK cells, KIR genes can be divided into clonally distributed KIR (e.g., KIR2DL3), the constitutively active KIR2DL4, and the weakly expressed KIR3DL3. In the present study, we could identify structurally and functionally divergent promoters regulating each of these three KIR types in different ways.

Among the members of the large group of clonally distributed KIR, promoters appear to be structurally and functionally highly similar. They are characterized by the absence of classical promoter features like TATA and CAAT boxes as well as the lack of classical enhancer elements. Detailed analysis of KIR2DL3 and KIR3DL2 promoter structures exhibited a core promoter activity that could be assigned to a small 65bp fragment. Several putative transcription factor binding sites like AP-4, ETS, and a GC-rich element are located in the basic promoter region. However, deletion analyses showed that none of these elements alone was required for or did significantly increase promoter activity except a CREB site located outside of the core promoter region, which was shown to contain a weak activating function. No further cis-activating elements were found in the remaining upstream regions of >2 kb, which includes several Alu and long interspersed nucleotide repetitive elements. Thus, the basic KIR promoter appears to either require so far undefined transcription factors or alternatively does only interact with the basic transcription machinery of RNA polymerase II.

As might be expected from sequence homologies, 65bp fragments from all KIR genes including KIR2DL4 and KIR3DL3 exhibited comparable promoter strengths. Shorter fragments (35bp and 15bp) from KIR2DL3, KIR2DL4, and KIR3DL3 showed significantly reduced activities; the 65bp fragments might thus be viewed as KIR core promoters. Although for KIR2DL3 the reduced activities of the 35bp and 15bp fragments might point to a cooperative function of ETS, AP-4, and the GC box, this does not apply to transcription of KIR3DL3 and KIR2DL4, neither of which contain an AP-4 site or the GC box. Consistent with the presence of such small core promoters, the transcription start points of clonally expressed KIR and KIR2DL4 are clustered close to the translation start site (32).

KIR2DL4 is unique among KIR genes because it is constitutively active in all NK cells, though it is still debated whether expression is restricted to the transcriptional level or KIR2DL4 proteins are also expressed on the cell surface (18, 25, 26, 42). In contrast to clonally distributed KIR, the region from −225 to −66 was shown to provide a strong inhibitory effect on transcription of KIR2DL4. It was previously shown that KIR2DL4 binds to the nonclassical MHC class I gene HLA-G, which is strongly expressed on fetoplacental trophoblasts residing in the fetal-maternal interphase during pregnancy (18). Furthermore, NK cells infiltrating the maternal decidua are known to express higher levels of KIR2DL4 than peripheral blood NK cells (42). It might thus be speculated that the silencing element in this region of the KIR2DL4 promoter provides a regulatory switch for expression of KIR2DL4 in decidual NK cells. However, the inhibitory effect of this region could be overcome by inclusion of the first intron, which fully restored KIR2DL4 promoter activity. The first introns of KIR are generally located close to the promoter region and are only separated by a small exon of 34 bp. The first intron of KIR2DL4 consists of a 189-bp nonrepetitive sequence (31), which is a unique feature of KIR2DL4. In all other KIR genes, the first introns are almost completely taken up by a minisatellite sequence of 19–20 bp, which is repeated between 23 and 63 times. In contrast to KIR2DL4, these introns seem to have only limited influence on promoter activity as shown for the intron of KIR2DL3.

Significant differences between the promoters of KIR2DL4 and a clonally distributed KIR (KIR3DL1) were similarly reported recently (32). Notably, promoter activities of fragments comprising <100 bp upstream of the translation initiation codon were significantly reduced in those studies, contrasting with the smaller core promoter of 65 bp found in this study. The discrepancy in core promoter structure between both studies is probably attributable to the usage of different cell types: in agreement with the study by Stewart et al. (32), transfection of YT-Indy with the core promoter fragment 65bp led to similar reduction in transcriptional activity of all three promoter types, whereas the 225bp fragments exhibited strong promoter activity (data not shown). This suggests that divergent regulatory mechanisms might be operative in YT-Indy cells, which cannot easily be applied to other NK cell lines like NK3.3 or NKL. Most importantly, analysis of KIR promoters in primary NK cells is in good agreement with the results achieved in NK3.3 and NKL cells thus suggesting that the results of the present study closely reflect the in vivo situation found in peripheral blood NK cells.

The third kind of KIR promoter, KIR3DL3, exhibited the strongest activity of all KIR genes, which was not anticipated given the previously reported lack of expression of this gene in PBMC (27). The increased promoter activity is apparently due to a unique stimulatory element that could be identified in the region from −225 to −66. The weak expression levels of KIR3DL3 could be attributed to epigenetic silencing rather than a dysfunctional promoter. The KIR3DL3 promoter was densely methylated in NK3.3 cells as well as in CD34+ NK cell precursor populations. Moreover, upon treatment with the demethylating agent Aza, KIR3DL3 expression could be readily induced. Notably, it was recently reported that KIR3DL3 mRNA is present in human decidual NK cells (43). It would thus be interesting to analyze whether during pregnancy KIR3DL3 expression would be epigenetically up-regulated by demethylation of its promoter.

It was previously suggested that a binding site for the transcription factor AML/RUNX at −100 could be essential for KIR expression. This notion was based on the observation that this site is
present in all KIR genes except the pseudogene KIR3DP1 as well as the nonexpressed alleles KIR2DL5B*002 and KIR2DL5B*004 (41). The present results suggest the opposite: strongly increased promoter activities in all three different KIR types were found when the AML site was mutated; the activating effect of the region from −225 to −66 of KIR3DL3 was further accelerated, the inhibiting effect of the corresponding region from KIR2DL4 was reverted completely, and expression of KIR2DL3 promoter constructs was also significantly increased. These results strongly suggest that AML has a general inhibitory influence on KIR expression in mature NK cells and that AML exerts its repressor function by directly binding to the promoter of the different KIR genes.

The AML/Runx transcription factor family consists of three homologous members (AML-1, AML-2, AML-3), which all bind to the same consensus sequence. The observation that the AML site in the KIR promoter is predominantly occupied by AML-2 proteins might thus simply reflect the fact that NK cells express larger amounts of AML-2 than AML-1 or AML-3. It is so far unknown how AML-2 interferes with KIR expression. It was recently reported that AML-2 is required for functional maturation of murine CD8 + T cells and is specifically involved in establishing epigenetic silencing of the CD4 gene through chromatin modification (44). For several reasons, repressive effects of AML-2 on KIR expression are probably not due to epigenetic silencing. First, it is unlikely that AML-2-induced chromatin remodeling and DNA methylation would play a significant role in plasmid-based reporter gene assays measured only 4 h post nucleofection. Second, analysis of the epigenetic status of KIR genes shows that active KIR genes, though binding to AML-2, are unmethylated (33) and also keep a highly accessible chromatin structure (data not shown). In contrast, it is well known that AML factors are involved in transcriptional repression through association with transcriptional corepressors like Groucho/transducin-like enhancer (TLE) of split (45, 46) or mSin3A (47). The interaction of the C-terminal VVWRPY motif with Groucho/TLE leads to tissue-specific inhibition by AML of genes like the multidrug resistance gene (48). Further analysis of the repressor function of AML-2 should thus involve a closer analysis of putative interacting partners like Groucho/TLE or mSin3A in NK cells.

AML might not only play a role in limiting KIR expression in mature NK cells but could also well be involved in regulation of KIR genes during NK cell development. Because all AML proteins bind to the same consensus sequence, they all could play regulatory roles in KIR expression. AML-1 is well known as a key regulatory factor in hemopoiesis and together with AML-2 is necessary for the development of CD8 T cells, which are in several regards functional relatives of NK cells (44). Through recruitment of coactivators like p300/CBP, which regulate transcription through acetylation of chromatin and recruitment of basal transcription factors, AML-1 could serve an activating function on KIR expression (49). AML-3 interacts in fibroblast and osteoblast cells with histone deacetylase 6, leading to transcriptional inaccessibility of DNA by higher order chromatin folding (50, 51). This implies that a putative functional role of AML on KIR might not only be direct transcriptional inhibition but could also lie in initial control of accessibility of KIR DNA during NK cell development. Changes in the expression of different AML isoforms during development, as already described in T cells, could thus alter or even reverse the effects of the AML site on KIR promoter function. To appreciate the significance of AML for KIR gene regulation it will thus be necessary to investigate expression and function of the different AML isoforms in NK cells as well as known interacting cofactors during NK cell development.

Based on the fact that the activating effect of the region from −225 to −66 was further increased in KIR3DL3 upon mutation of the AML site, it might be speculated that a yet unidentified activation factor would interfere with AML-2-mediated inhibition of transcription of the KIR3DL3 promoter. Mutation of the AML site would then increase transcriptional activity of the KIR3DL3 promoter. Similarly, a putative binding site for this activator might also be present in clonally expressed KIR because mutation of the AML site (225bp mAML) also led to an increased promoter activity of KIR2DL3. In contrast, KIR2DL4 promoter activity did not rise above that of the basic promoter upon mutation of the AML site, making the presence of an additional, competing activation protein unlikely. The regulation of KIR genes might thus be viewed as a result of transcriptional inhibition by AML-2 and transcriptional activation by a competing, yet unidentified, activation protein.

In summary, in the present study three different groups of KIR promoters were identified, which correlate with three distinct kinds of KIR expression patterns. The KIR promoter types share common elements within a core region of 65 bp and are subdivided on the basis of activating and inhibiting cis-regulatory elements further upstream. The promoters of the large group of clonally distributed KIR are structurally and functionally highly similar. It thus appears likely that in the absence of significant promoter variability, the variegated expression of these KIR is mainly regulated by a common process, involving epigenetic components like DNA methylation and chromatin remodelling. The identification of AML as negative regulator of KIR expression now not only allows detailed evaluation of the physiologic role of AML in mature NK cells but also analysis of its functional role in establishing epigenetic silencing of KIR during NK cell development.

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ciate with the nuclear matrix and repress RUNX (CBF1/AML/PEBP2b) depend-


