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ORIGINAL ARTICLE

Identification and analysis of the human CD160 promoter: implication of a potential AML-1 binding site in promoter activation

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CD160 is a glycosylphosphatidylinositol-anchored multimer expressed at the cell surface of subsets of cytotoxic T-lymphocytes and natural killer cells. Although CD160 is an important molecule for the control of cell-mediated cytotoxic responses, the mechanisms of regulation of its expression is unknown. We investigated the regulation of CD160 transcription by localizing and analyzing its promoter. The CD160 gene is encoded on chromosome 1, contains 6 exons with the translation initiation codon in exon 3. Bioinformatics analysis pointed to three potential promoter regions, two upstream of exon 1 and one in front of exon 3. RACE-PCR analysis identified a single transcription start site (TSS) and reporter gene transfections localized the active region immediately upstream of exon 1. Sequential deletion analysis led to the identification of a 371 bp sequence, located between −314 and +57 relative to the TSS, as the core promoter sequence driving CD160 gene transcription. Sequence alignment of the mouse and human CD160 genomic promoter region revealed a strong homology in the 371 bp sequence identified and pointed out to three conserved transcription factor binding sites for acute myelogenous leukemia-1 (AML-1), FREAC-4 and Sox17. Site-directed mutagenesis showed that the predicted AML-1 site is essential for the regulation of CD160 gene expression.
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Introduction

Natural killer (NK) cells are lymphocytes of the innate immunity that constitute an important component of immune surveillance against transformed and virus-infected cells.[1] NK cell activation and engagement to effector function depend on the resultant signal delivered through activating and inhibitory cell surface receptors. Most inhibitory NK receptors are surveying molecules for MHC class I level expression on target cells. They correspond to the CD94/NKG2A heterodimer,[2] the leukocyte immunoglobulin (Ig)-like transcript 2[3] and the long form of killer-cell Ig-like receptors (KIR-L) characterized by an ITIM-bearing intracytoplasmic tail.[4] In contrast, the KIR-S have a short intracellular tail and can mediate an activation signal through their association with an adaptor molecule bearing an ITAM motif. However, most of the activating NK receptors are not MHC class I specific. They include the NKG2D homodimer specific for several stress-inducible surface glycoproteins,[5] and the natural cytotoxicity receptors (NCRs). NCR are composed of NKp30, NKp44 and NKp46 and are uniquely expressed on NK lymphocytes. Their cellular ligands are still not well defined but several viral proteins have been identified as recognized molecules.[6–8]

CD160 has been described as an MHC class Ia/Ib-specific Ig-like type receptor.[9–11] It is mainly expressed as a glycosylphosphatidylinositol-anchored multimer at the surface of circulating cytotoxic T $CD8^+$ cells and NK cells.[12,13] The engagement of CD160 at the surface of these cells leads to the production of cytokines and the induction of cytotoxic functions. Importantly, CD160 is also expressed after activation of $CD4^+$ T-lymphocytes[14,15] and endothelial cells[16] where the physiological ligands HVEM and HLA-G molecules induce inhibition of the lymphocyte proliferation and endothelial cell apoptosis, respectively.

In humans, NK cells can be divided into cytotoxic and immunoregulatory subsets. The main circulating NK lymphocyte pool is represented by the effector cytotoxic subset, characterized by a $CD56^{dim}CD16^+$ phenotype and the expression of perforin and CD160. In contrast, the $CD56^{bright}CD16^-$ exerts immunoregulatory functions, poor cytotoxic activity and lacks the expression of CD160. Interestingly, CD160 mRNA synthesis can be induced in $CD56^{bright}$ NK lymphocytes following exposure to IL-15.[17] Also during activation, NK cells release a soluble form of CD160 through a proteolytic cleavage of the membrane-bound CD160 that functionally impairs MHC-I-specific cytotoxic $CD8^+$ T-lymphocytes.[17]

Although CD160 is an important molecule for the control of cytotoxic T $CD8^+$ and NK lymphocyte effector

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responses, the mechanisms of regulation of its expression are unknown. In this study, we investigated in NK lymphocytes the regulation of CD160 transcription by localizing and analyzing its promoter. Using *in silico* prediction based on human–mouse comparison and site-directed mutagenesis, we show that acute myelogenous leukemia-1 (AML-1), a transcriptional regulator of the Runx family (for review see[18]), may be an essential factor in the regulation of CD160 gene expression.

Results

Genomic organization of the human CD160 locus

Human CD160 gene is located on position q21.1 of chromosome 1. It is encoded on the minus DNA strand by 6 exons, of which the first two are untranslated (Figure 1). Two main messenger RNA of 1.5 and 1.6 kb have been identified encoding, a 181 amino-acid polypeptide containing a C2 Ig-like extracellular domain.[10] Several isoforms of CD160 have been identified, resulting from alternative splicing of different exons, including the untranslated exon 2 and the Ig-like domain encoding exon 4.[19] Alternative splicing in exon 5 is also responsible for the generation of a transmembrane form of CD160 observed after NK cell activation.[19] To determine the potential existence of several promoter regions active in the transcriptional regulation of these CD160 transcripts, we first analyzed *in silico* for such suitable regions. Analysis of the CD160 locus was performed using the PromoterInspector software of the Genomatix informatics suite.[20] As shown in Figure 1, three potential promoter regions were identified and designated P1, P2 and P3. P1 and P2 were located about 3 Kb and immediately upstream of exon 1, whereas region P3 was found upstream to exon 3 in which the translation initiation start is encoded. A classical TATA box element was also found in region P2 and P3.

Determination of the transcription start site

The localization of the transcription start site(s) (TSS) was performed in human NK cell line YT by 5′-RACE PCR. As shown in Figure 2, three main bands were obtained from the cDNA amplified using a gene-specific primer located in the exon 5 of the *CD160* gene. After cloning and sequencing, these three products corresponded to the three main alternative transcripts of CD160 mRNA described earlier:[19] the full-length transcript (665 bp band), the spliced exon 2 (590 bp) and the spliced exon 4 (410 bp band). The DNA sequence performed on 11 clones from these different bands revealed the 5′-ends, each indicated by a star '*' in Figure 2. They were located at 22, 49, 73, 76 and 83 nucleotides from the TATA box. The extremity indicated with an arrow in Figure 2 (73 bp from the TATA box), and located at position 904 892 of the genomic sequence NT_004434 of human chromosome 1, was chosen as the TSS, as corresponding to the highest frequency of clone 5′-ends. The absence of sequence found with a 5′-extremity corresponding to a region close to exon 3 argue against an active role of region P3.

Localization of the promoter region of the CD160 gene

To functionally identify the CD160 promoter, the three potential regions of about 2 kb P1, P2 and P3, were cloned by genomic PCR into a luciferase reporter vector (pGL4.10). Transfection of these plasmids in the NK cell line YT, identified the P2 region as the only sequence presenting a promoter activity, as compared with the basal activity observed with the empty vector (Figure 3).

To further characterize the CD160 promoter and identify potential regulatory regions, a 3.2 kb genomic

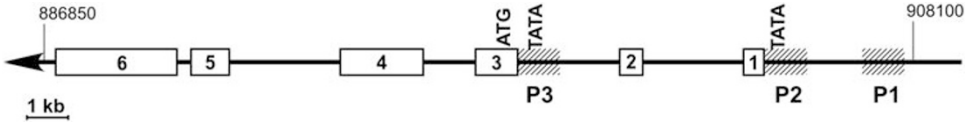

Figure 1 Schematic representation of CD160 genomic region on chromosome 1. Human *CD160* gene (Hs.488237) is located in human chromosome 1 and encompasses 6 exons, with the exons 1 and 2 being untranslated. Bioinformatics analysis identified three potential promoter regions denoted P1, P2 and P3, located as represented.

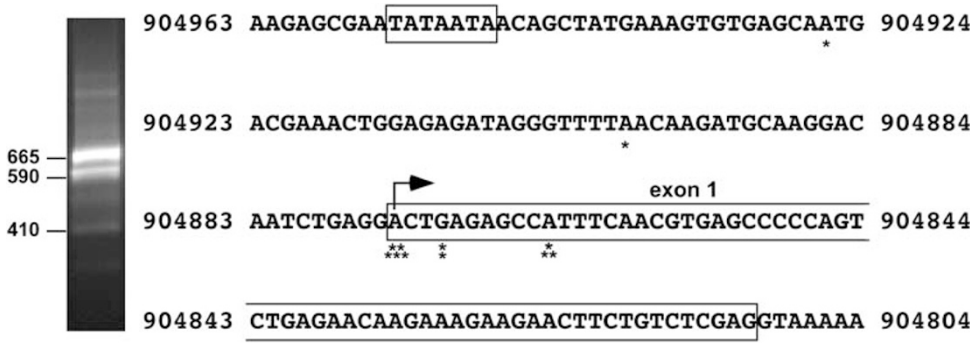

Figure 2 Localization of TSS of human *CD160* gene. Using RACE-PCR method, we amplified the 5′ extremity of CD160 transcripts. Three major bands of 665, 590 and 410 bp, corresponding to the alternative splice variants of CD160 transcripts, were cloned in a TA vector and sequenced. All clones presented a common extremity located upstream of exon 1 and identified here by an asterisk under the genomic sequence.

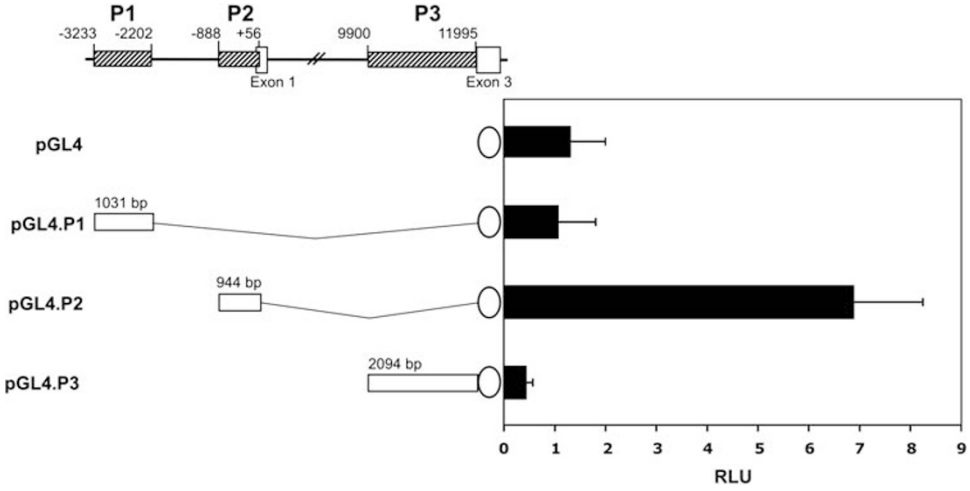

Figure 3 Luciferase reporter assay for promoter activity of region P1, P2 and P3. PCR generated fragments corresponding to region P1 (1031 bp), P2 (944 bp) and P3 (2094 bp) were cloned in the pGL4.10 luciferase reporter vector, cotransfected in YT cells with the pTK-renillase internal control (pRluc). After 6 h of culture, cell lysates were prepared and assayed for luciferase activity. RLU, relative luciferase units.

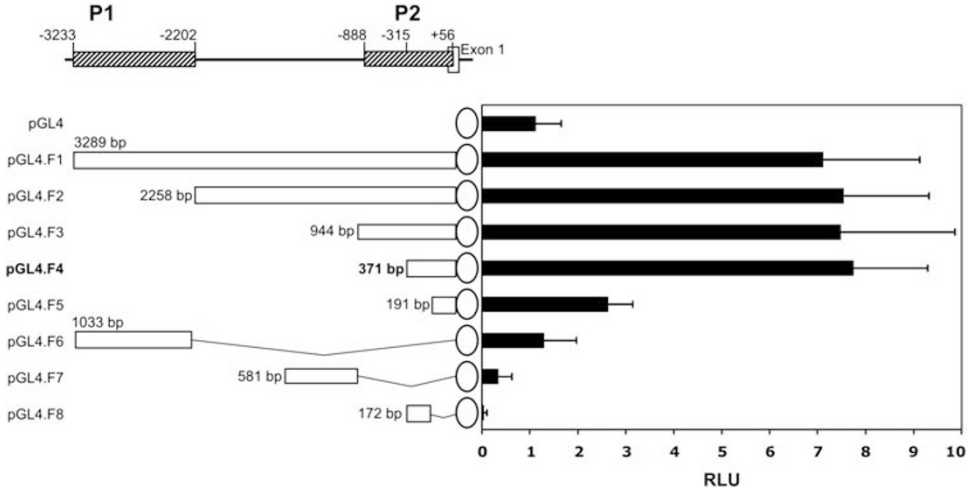

Figure 4 Identification of the minimal promoter region of *CD160* gene. A sequential deletion analysis of the 3.5 kb region upstream of exon 1 (fragment F1) was performed to identify the 371 bp of fragment F4, representing the minimal promoter region. All fragments were generated by PCR and cloned in the pGL4.10 reporter vector, cotransfected in YT cells with the pRluc internal control. After 6 h of culture, cell lysates were prepared and assayed for luciferase activity. RLU, relative luciferase units.

region (fragment F1), covering the P1 and P2 region, was cloned in the pGL4.10 vector. Sequential deletion analysis of fragment F1, as presented in Figure 4, shows that region upstream to P2 is devoid of transcriptional activity (see pGL4.F6 and F7). Progressive deletion of fragment F1 did not increase the promoter activity, suggesting the absence of negative regulatory element in these regions. The minimal promoter sequence is included in the 371 bp F4 fragment extending from positions +56 to −315 relative to the TSS. All other deletion constructs in this region showed a reduced promoter activity. These results suggest that the CD160 promoter is located in a 300 bp sequence upstream of exon 1.

CD160 promoter activity is restricted to CD160-expressing cells

The tissue specificity of F4 fragment promoter activity was tested by transfecting the pGL4-F4 construct into various cells and cell lines, differentially expressing CD160. As shown in Figure 5, whereas YT and NK3.3 cell lines expressing CD160 displayed a good F4 promoter activity, other cell line lacking CD160 expression were unable to show any F4 promoter activity. We also tried to confirm these results on freshly isolated non-transformed cells. To this aim, we purified $CD160^+$ and $CD160^-$ cells from peripheral blood lymphocytes by fluorescence cell sorting. Twenty-four hours after transfection with the pGL4-F4 or the empty vector, relative luciferase activity was measured. As shown in Figure 5 (lower panel), $CD160^+$ cells transfected with the F4-containing plasmid, presented a much higher F4 promoter activity than the $CD160^-$ fraction, the latter being less that twice the basal transcriptional level of the empty vector. We think that the residual F4 promoter activity observed in $CD160^-$ cells may result from some low membrane-CD160-expressing cells or from cells in transition toward the acquisition of this receptor. Altogether, the results

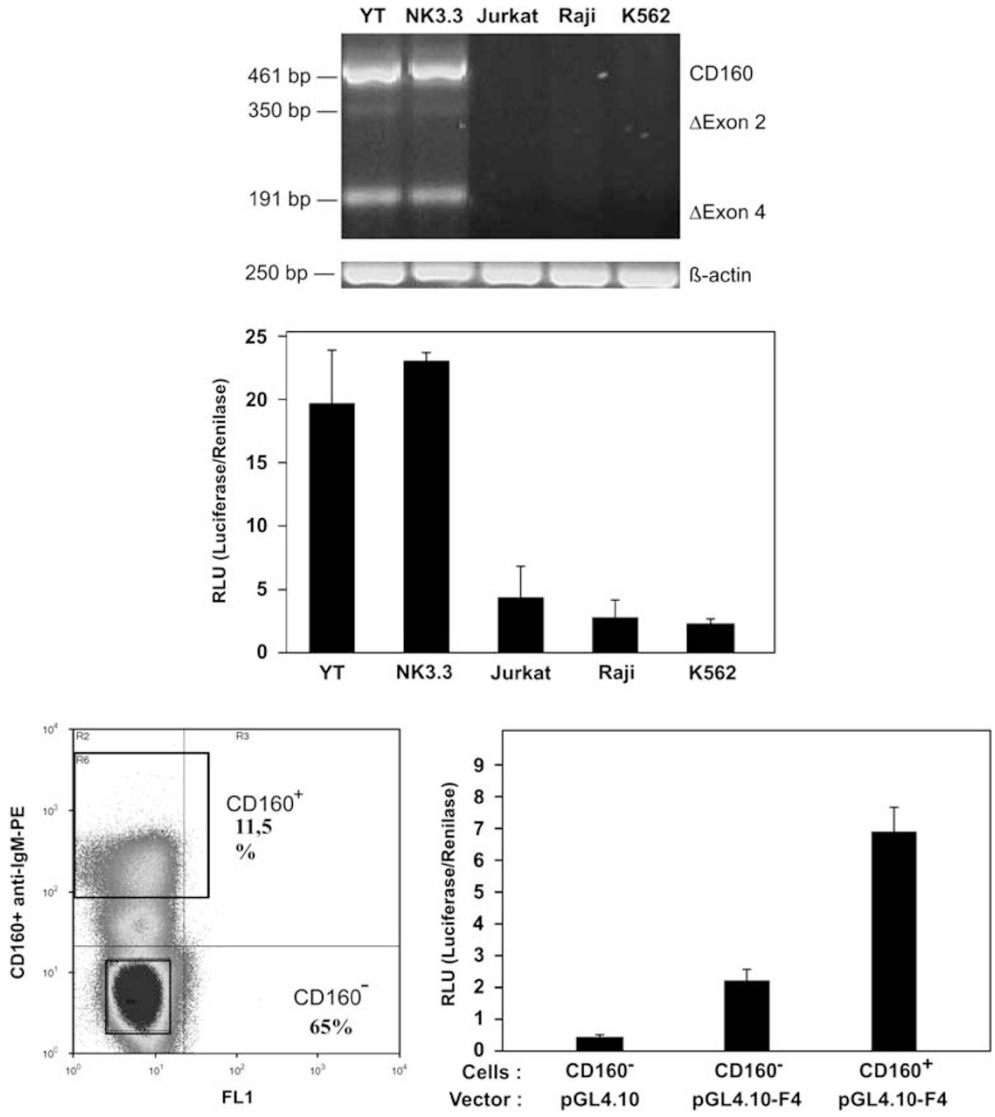

Figure 5 Tissue specificity of fragment F4 promoter activity. To demonstrate that *CD160* promoter activity is restricted to CD160-expressing cells, we transfected the pGL4.10-F4 plasmid in various cells expressing or not CD160. The upper panel shows the expression of CD160 transcripts, detected by RT-PCR in various cell lines. In the middle panel, pGL4.10-F4 were transfected in these cell lines and the relative luciferase activity (RLU) was measured 24 h after transfection and normalized for the background activity generated by the empty vector. The lower panel represents the luciferase activity measured in $CD160^+$ and $CD160^-$ cells. The two cell populations were isolated by flow cytometry cell sorting of CD160 labeled human peripheral blood lymphocytes (see lower left for gating). Intermediate CD160-labeled population corresponded essentially to cytotoxic αβ or γδ T-cells. RLU was measured 24 h after transfection with pGL4.10-F4 as compared with the empty vector.

presented in Figure 5 confirm that the F4 promoter activity is restricted to CD160-expressing cells.

CD160 promoter activity is dependent of AML-1 transcription factor binding site

To gain insight into the putative transcription binding sites, we performed a comparative sequence analysis between the human and mouse CD160 promoter regions. Using the program ConSite (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite), with a conservation cutoff of 60%, we identified a conserved non-coding sequence of about 400 bp, on a 3 kb sequence upstream of exon 1. The conserved sequence corresponded almost perfectly with the fragment F4 identified experimentally (Figure 6). When a search for conserved transcription binding sequences, three putative binding sites were identified with the highest score: FREAC-4, SOX17 and AML-1 binding sites. Two other TF site, Hunchback and bZip910, with lower (although significant) scores were not studied. When analyzed by RT-PCR, for their expression in YT cells, the FREAC-4 transcription factor was found not to be expressed in the YT cells and only a faint band is observed with Jurkat cells, quite in accordance to data of the literature showing that FREAC-4 (FOXD1) is not expressed in the hematopoietic system[21] (Figure 7). To investigate the implication of the other TF, mutagenesis experiments were performed on the SOX17 and AML-1 binding sites. As shown in Figure 7, a mutation in the core-binding sequence of AML-1 completely inhibited the promoter activity of fragment F4 when transfected in YT cells. Although some inhibition was also observed with the mutated SOX17

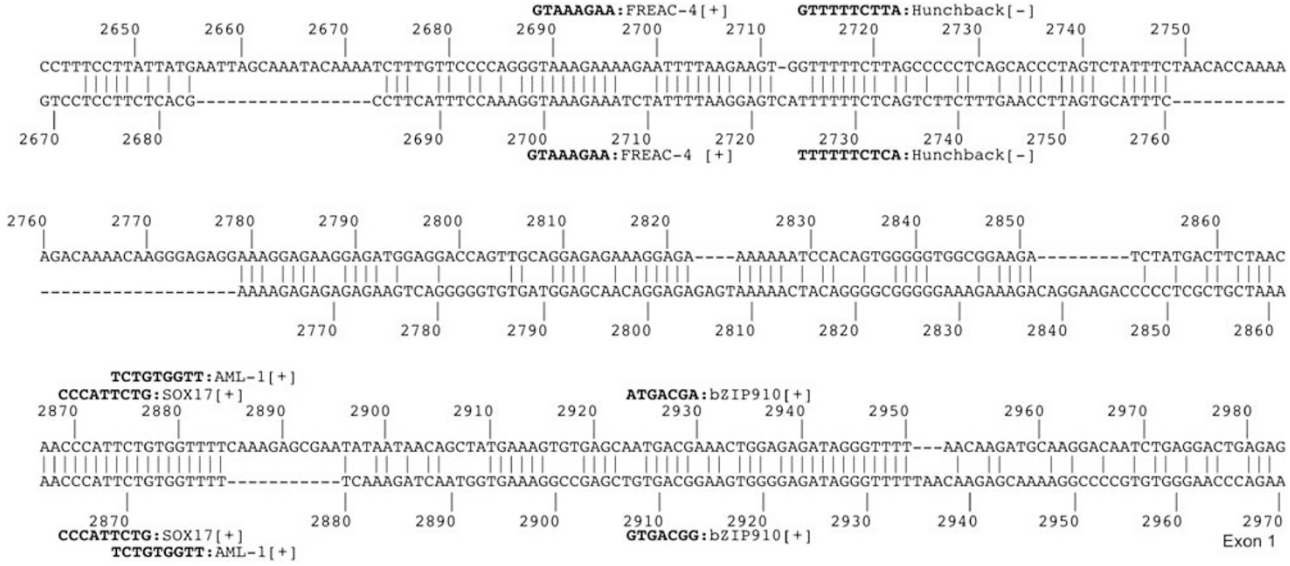

Figure 6 Genomic sequence alignment of human and mouse *CD160* promoter regions. Human and mouse sequences 3 kb upstream of exon 1 were compared. Significant homologies were observed only in the 400 bp closed to exon 1, using a conservation cutoff of 70%. The putative transcription binding sites conserved in the two species with the highest scores is represented (TF score cutoff of 80%).

core-binding site, it was likely due to the vicinity of this binding sequence with the flanking sequence of the AML-1 binding site. From these data, we conclude that the 371 bp fragment upstream of the first exon contains the promoter of human *CD160* and that its potential AML-1 binding site is essential in the control of the expression of this gene.

Discussion

In this study, we present the first characterization of the human *CD160* gene promoter. We localized the TSS upstream of the untranslated exons 1 and 2, and common to the different alternative transcripts present in the YT NK cell line. Using PromoterInspector (www.genomatix.de), the search for potential promoter regions led to localize three regions, two upstream of the exon 1 (P1 and P2) and a third sequence upstream of the exon 3 (P3) that contains the translation initiation site. Although the search for CpG islands was not informative for these regions, the regions P2 and P3 contained a consensus TATA box for transcription initiation. To better understand the molecular mechanisms involved in the expression of *CD160*, the putative promoter regions were cloned upstream to a luciferase reporter gene to test for promoter activity in the CD160-expressing YT cell line. Transfection experiments showed that the about 1 kb region P2, upstream of exon 1, was the only active region. Deletion mapping performed from a 3.3 kb region upstream of exon 1, identified a 371 bp sequence as the minimal promoter sequence and excluded the presence of other negative regulatory element potentially present in this region. A human/mouse comparison of the upstream genomic regions of *CD160* gene confirmed this result and pointed toward three highly conserved transcription factor binding sites: FREAC-4, SOX17 and AML-1. Whereas FREAC-4 is normally not expressed in the hematopoietic cells,[21] SOX17 and AML-1 are expressed in human NK cells (Figure 7) and belong to families of transcription factors essential for the development of the hematopoietic system. SOX17 is a member of the SRY-related HGM box family, which includes SOX13, recently shown to orient early thymocyte differentiation toward the Tγδ lineage.[22] SOX17 is an endodermal transcription factor and is required for the maintenance of fetal and neonatal, but not adult, hematopoietic stem cells.[23] Here, we showed that mutation of its specific binding site in the promoter of CD160 only induced a marginal loss of the reporter gene expression in YT cells, suggesting that if existing, its role in the transcriptional control of *CD160* gene is not essential. Strikingly, mutating the potential AML-1 binding site totally abolished the promoter activity of fragment F4, revealing that it is essential to express CD160 in YT NK cells. AML-1, also called RUNX1 or CBFα, is a member of a gene family of heterodimeric transcriptional regulators.[24] AML-1 plays a crucial role in hematopoiesis and is one of the most frequent targets for leukemia-associated translocations. It is widely expressed in all hematopoietic lineages and regulates the expression of a variety of myeloid and lymphoid genes.[18] RUNX family proteins play an important role in NK cell differentiation.[25] In humans, AML-2 was identified as the predominant KIR binding factor controlling clonally expressed KIR gene during NK cell development.[26] AML-1 participates to the transcriptional control of other important genes implicated in the cytotoxicity such as IL2, IFN-γ and granzyme B.[27,28] The ubiquitous expression of AML-1 in hematopoietic cells versus the more restricted CD160 expression on cytotoxic T and NK cells clearly exclude a correlated expression of the two genes. CD160 promoter activity as well as accessibility of its AML-1 binding site is restricted to CD160-expressing cells. AML-1/Runx1 transcriptional activity is influenced by its association with co-activators, such as histone acetyltransferases, or co-repressors such as Sin3A, and further work will be required to

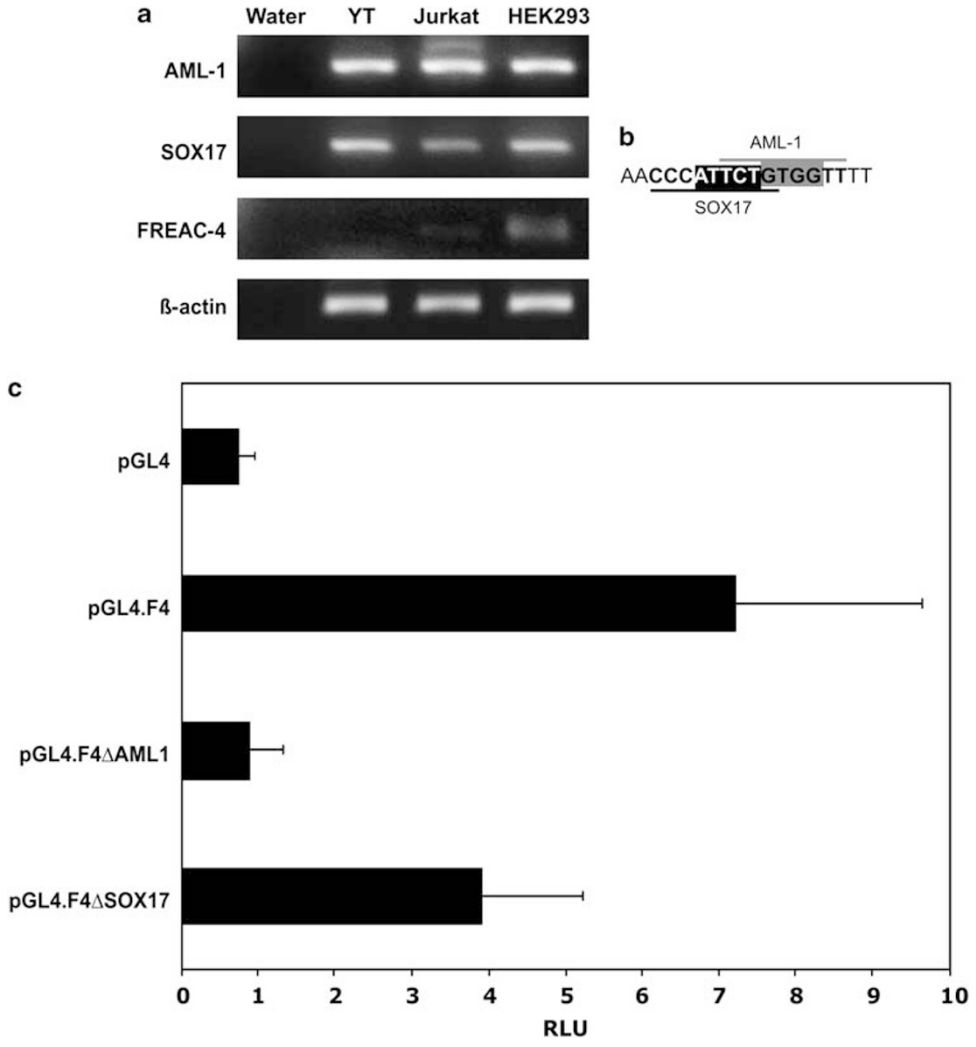

Figure 7 *CD160* promoter activity is dependent of a predicted AML-1 binding site. (**a**) RT-PCR analysis of AML-1, SOX17 and FREAC-4 transcription in YT, Jurkat and HEK293 cell lines. (**b**) The sequence of the overlapping AML-1 and Sox17 sites. The darken background nucleotides are the core TF sequences in which mutations were done (see Materials and methods section). (**c**) The promoter activity of site-directed mutagenesis of AML-1 or SOX17 binding site after transfection of the reporter plasmids in YT cells. Relative luciferase activity is measured 6 h after cell transfection.

demonstrate AML-1 binding and characterize the co-activator(s) participating in the regulation of the transcription of the human *CD160* gene. Our data clearly implicated a predicted AML-1 binding site in the regulation of the *CD160* promoter activity and offer new research perspectives to understand the way the CD160 receptor expression is controlled in NK and cytotoxic T-cells.

Materials and methods

Cell lines

Human cell lines were cultured in RPMI 1640 medium supplemented with penicillin (100 IU/ml, streptomycin 100 mg/ml), L-glutamine (2 mM) and 10% fetal calf serum (Perbio Science, Brebières, France) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The non-transformed NK-cell line NK3.3 was also supplemented with 100 U/ml of human IL2 (gifted from Sanofi-Adventis, Labège, France).

Preparation of peripheral blood NK cells

Peripheral blood NK cells were isolated from heparinized venous blood collected from human healthy donors. Briefly, mononuclear cells isolated by density gradient centrifugation over Ficoll-Isopaque (PAA Laboratories, Les Mureaux, France) were labeled by anti-CD160 (BY55) monoclonal antibody, revealed by phycoerythrin-conjugated anti-mouse IgM. Separation of $CD160^+$ and $CD160^-$ lymphocyte fractions were performed by fluorescence-activated cell sorting on a high speed MoFlo Dako Cytomation flow cytometer (Dako, Trappes, France).

5′-RACE and RT-PCR

Total RNA was prepared from cells of the human NK cell line YT using the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). The TSS was determined using the 5′-rapid amplification of cDNA ends method (RACE), with a SMART RACE kit (Clontech, Saint Germain en Laye), using a gene-specific primer located in the exon 5 of the CD160 gene: 5′-TTT CAA TCC CGT CAC TGT GTA GTT

CCC T-3′. Expression of specific mRNA expression was performed by RT-PCR analysis. Total RNA extracted from the relevant cell lines was reverse transcribed using an oligo-dT primer and the Powerscript reverse transcriptase (Clontech). Amplification of the specific transcripts were done using the following pairs of primers: CD160 5′-TGC AGG ATG CTG TTG GAA CCC-3′ & 5′-TCA GCC TGA ACT GAG AGT GCC TTC-3′; AML-1 5′- CAC TCT GAC CAT CAC TGT CTT C-3′ & 5′-CTG CAT CTG ACT CTG AGG CTG A-3′; SOX17 5′-TGC AAC TAT CCT GAC GTG TGA C-3′ & 5′-TAG TGT GAC AGA GGT ACT AGT AG-3′; FREAC-4 5′-CCT GCA GCT GCC GCC TTA CG-3′ & 5′-TGG GCG CGC GAG GTC GAG A-3′. After an initial denaturation of 5 min at 94 °C, amplification consisted of 30 cycles of 94 °C for 30 s, annealing for 30 s at 62 °C (CD160), 60 °C (AML-1 and SOX17) or 68 °C (FREAC-4) and 72 °C for 90 s, followed by a final elongation at 72 °C for 10 min. The PCR products were analyzed on 1% agarose gel electrophoresis in the presence of 1/10 000 dilution of Syber safe (Invitrogen, Cergy-Pontoise, France) and photographed on a blue-light trans-illuminator (Invitrogen).

Luciferase reporter constructs

To generate human *CD160* promoter-luciferase reporter constructs, three fragments with potential promoter activity identified *in silico* (P1, P2 and P3) were amplified by PCR from genomic DNA purified from YT cells. The following primer pairs were used: P1 5′-GAT ATC CTC GAG ACG CCA CTG TAC TCC AGC CTG TG-3′ & 5′-AGG CCA AGC TTC ATA AAC TGC TAA ACT GGG CCT GGT-3′ ; P2 5′-GAT ATC CCT GAG ACG CCA CTG TAC TCC AGC CTG TG-3′ & 5′-AGG CCA AGC TTG AAG TTC TTC TTT CTT GTT CTC AGA CTG GG-3′; P3 5′-GAT ATC CTC GAG AAT TGC AAT TTG CCC TTT TTG ACC-3′ & AGG CCA GAT CTG CAG CCT CTG CCG GGT TCC A-3′. For sequential deletion analysis of region P2 a series of forward primers were used with the above common P2 reverse primer to generate the various fragments analyzed. The PCR-generated promoter fragments were digested with *XhoI* and *HindIII* (P1 and P2) or *XhoI* and *BglII* (P3) and cloned into the respective sites of the pGL4.10[luc2] plasmid vector (Promega, Charbonnière, France). For site-directed mutagenesis, the 'QuickChange' kit (Stratagene) was used to mutate the AML-1 and SOX17 binding site of the F4 fragment with the following primers: 5′-CTA ACA ACC CAT TCT CAA ATT T**CA AA**G AGC GAA TA-3′ (AML-1) and 5′-GAC TTC TAA CAA CCC **GCA TC**G TGG TTT TCA AAG AG-3′ (SOX17). Mutated core sequence nucleotides are in bold. All *CD160* promoter-luciferase construct clones were sequenced for validation before they were used in transfection experiments.

Cell transfection and luciferase assays

Transient transfections were performed by electroporation using the Amaxa technology (Amaxa Biosystems, Koeln, Germany). Briefly, 2×10^6 cells were resuspended in 100 µl of Nucleofactor solution V in the presence of 5 µg of pGL4.10 plasmid DNA and 0.5 µg of pGL4.74[hRluc/TK] (pRluc), used as internal control where the *Renillase* gene is expressed under the control of a HSV-thymidine kinase promoter. Electroporation were done using the programs O.017 for the NK cell lines YT and NK3.3; U.01 for fresh sorted-lymphocytes; A.017 for Jurkat, M.013 for Raji and T0.16 for K562 cells. Immediately after transfection, cells were resuspended in 2 ml of complete medium and cultured at 37 °C for 6 or 24 h. At the end of the culture period, cells were lysed and assayed for luciferase activity using the Dual-luciferase reporter assay system (Promega). Results were expressed as the ratio of luciferase versus renillase signals measured by luminometry (RLU), calculated from means of three independent experiments in which each condition is performed in triplicate.

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