

**Identification and characterization of a transmembrane isoform of CD160 (CD160-TM),
an unique activating receptor selectively expressed upon human NK cell activation¹**

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CD160 has been initially identified as a GPI-anchored MHC-class I activating receptor mainly expressed on peripheral blood NK cells. Here, we report the identification of three additional CD160-related mRNA generated through alternative splicings of *CD160* gene, among which one encoded a putative CD160 transmembrane isoform (CD160-TM). We first establish that CD160-TM surface expression is highly restricted to NK cells and is activation-dependent. In addition, we provide evidences that CD160-TM represent a novel activating receptor, as assessed by the increased CD107a NK cell surface mobilization observed upon its engagement. Finally, we demonstrate that CD160-TM cytoplasmic tail is by itself sufficient to mediate the recruitment of Erk1/2 signaling pathway, and that the initiation of this activation process is dependent on the Src-family kinase p56^{lck}. The identification of CD160-TM therefore provides new possibilities regarding the role of CD160 isoforms in the regulation of NK cell functions.

NK lymphocytes recognize abnormal or aberrant cells through multiple receptors that detect normal host molecules, as well as stress-induced or pathogen-expressed motifs (1, 2). Individual NK cells express both activating and inhibitory receptors, which together drive the specificity towards target cells (3).

The NK cell inhibitory receptors have been classified into three groups, namely the heterodimeric CD94/NKG2A, the Ig-like transcript (ILT) receptors, and the members of the killer cell Ig-like receptors (KIRs) (4, 5). All of them bind to classical or non-classical MHC-class I molecules. A common characteristic of the inhibitory receptors is the presence of immunoreceptor tyrosine-based inhibition motif(s) (ITIM) within their intracellular tail (6). Following engagement by their ligands, the inhibitory receptors become phosphorylated on the tyrosine residue(s) present in the ITIM(s), creating docking sites for the SH2-domains of the cytoplasmic protein tyrosine phosphatases SHP1 and SHP2. Their recruitment further results in the down-regulation of the intracellular activation cascade (7). In contrast, activating receptors recognize a large variety of ligands, mostly distinct from MHC-class I molecules, and exhibit more complex but well characterized signaling pathways. Natural cytotoxicity receptors (NCRs)⁴ and NKG2D are the major receptors involved in NK cytotoxicity (8). The NCRs (namely NKp46, NKp44 and NKp30) belong to the Ig-superfamily, and represent non-MHC-class I specific receptors whose cellular ligands still have to be confirmed (9). In contrast to NKp46 and NKp30, expressed on circulating NK lymphocytes, NKp44 expression is activation-dependent (10). The NCRs transduce signals through their association with ITAM-containing molecules such as CD3 ζ , Fc ϵ RI γ and DAP12 (11, 12). Besides the NCRs, NKG2D is a C-type lectin-like receptor shown to recognize the MHC-class I homologues MICA and MICB, and the family of UL16-binding proteins (ULBPs) (13). NKG2D uses the transmembrane polypeptide DAP10 for signaling, which interacts with the PI3-kinase once phosphorylated (14). Interestingly NKp80, an additional C-type lectin-like activating receptor exclusively expressed by human NK cells, has been identified (15). A search for NKp80 ligands led to the identification of activation-induced C-type lectin (AICL) (16). However,

NKp80 signaling pathway remains enigmatic as this receptor does not contain a transmembrane charged residue (a feature allowing association with ITAM-containing adaptor proteins) or any intracellular consensus activation motifs. Finally, beside these MHC- class Ia/Ib molecule-independent activating receptors, it is important to mention the well-characterized DAP12-associated CD94/NKG2C, and KIRs activating isoforms (17, 18), although the precise events leading to their specific recruitment still have to be better defined.

Using the original BY55 mAb, we previously reported the identification of BY55/CD160 receptor on functional cytotoxic peripheral blood (PB)-NK lymphocytes, and initially found that its expression was rapidly down-modulated after cell activation (19). CD160 shows a broad specificity with weak affinity for the MHC-class Ia/Ib molecules (20). An additional ligand for CD160 has been recently identified as the herpesvirus entry mediator protein (21). CD160 behaves as an activating receptor on NK lymphocytes as demonstrated by the induction of their cytotoxic potential upon engagement (22). Furthermore, CD160 triggering with its physiological ligand MHC-class I results in a unique profile of cytokine secretion by cytotoxic CD56^{dim}CD16⁺ PB-NK cells, with the release of TNF α , IFN γ and IL-6 (23). CD160 appears to be unique among the activating receptors since *CD160* gene was found to be located on human chromosome 1, and the corresponding protein was characterized as a glycosylphosphatidylinositol (GPI)-anchored cell surface molecule (24). In addition, CD160 is expressed by intestinal intraepithelial T lymphocytes and by a minor subset of circulating T lymphocytes including TCR $\gamma\delta$ and CD8^{bright}CD28⁻ T lymphocytes (24-26), and exerts a co-receptor function on CD8^{bright}CD28⁻ cytotoxic T lymphocytes (25). Thus CD160 might be involved in mechanisms regulating both adaptive and innate immunity. Interestingly, we reported that a down-modulation of CD160 surface expression occurs as a consequence of its proteolytic cleavage upon NK cell activation (27). The released soluble form of CD160 was found to impair the MHC-class I-specific cytotoxicity of CD8⁺ T lymphocytes and NK cells.

Importantly, murine CD160 tissue expression, specificity and molecular structure show similarities to what has been described in human (28, 29).

In this study we further identified and characterized a transmembrane isoform of CD160 (CD160-TM). In contrast to the GPI-anchored isoform, its expression is restricted to NK cells and activation-dependent. We established that CD160-TM fulfills an activating function once expressed on activated PB-NK lymphocytes. Thus, CD160 receptor represents an unique receptor that might recruit alternative activating signaling pathways through the differential expression of its isoforms on resting or activated NK lymphocytes.

Materials and Methods

Cells

PBMC were isolated from heparinized venous blood from healthy volunteers by density gradient centrifugation over MSL (PAA Laboratories, Les Mureaux, France). PB-NK, CD4⁺ and CD8⁺ lymphocytes were purified using a magnetic-activated cell sorter (MACS) and specific cell subset isolation kit according to the manufacturers' recommendations (Miltenyi Biotec, Bergish-Gladbach, Germany). NKT cells were sorted and activated as previously reported (30, 31). Individual cell purity was >95%. For activation, cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated human serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), L- glutamine (2 mM) (Invitrogen, Cergy Pontoise, France) and IL-2 (200 UI/ml, a gift from Sanofi-Aventis, Labège France), IL-12 (50 ng/ml; R&D Systems), IL-15 (10 ng/ml; Peprotech, Princeton, USA) or IL-18 (80 ng/ml, MBL, Woburn, MA). Cord blood mononuclear cells and CD34⁺ cells were isolated as previously described (32). IL-2-activated NK cells were expanded up to 5 weeks from sorted NK cells in culture medium supplemented with 200UI/ml IL2, and mixed allogenic PBMC plus 2 µg/ml PHA (Sigma-Aldrich, St Quentin Fallavier, France) were added each two weeks as feeder cells.

Wild type and Lck-deficient (JCam) Jurkat cells and derived transfectants, 221 target cells, Daudi, YTindi and Molt4 cell lines, were cultured in RPMI 1640 medium supplemented with penicillin/streptomycin, L-glutamine, and 10% heat-inactivated FCS (Perbio Science, Brebières, France). For the NK malignant cell lines NKL and NK92, 100UI/ml of IL-2 were added to the medium. The TCRαβ-expressing clones B12g, JF1 and C1, and the TCRγδ-expressing clone LSO, were developed and expanded as reported elsewhere (33, 34).

Reverse transcription and cDNA amplification (RT-PCR)

Total RNA extraction and reverse-transcription were performed as previously described.(27) The specific primers for the amplification of the cDNA corresponding to the GPI-anchored CD160 molecules were 5'-ATGCTGTTGGAACCCGGCAGAG-3' (forward) and 5'-

TTACAAAGCTTGAAGGGCCAC-3' (reverse). The same forward primer in combination with the reverse primer 5'-TCAGTGAAACTGGTTTGAACCTTCCTG-3' was used for the detection of the cDNA encoding the transmembrane isoforms. PCR were performed according to standard procedures, and amplified products were separated by electrophoresis on 1% agarose gel.

Expression vectors and transfection

CD160 and CD160-TM full-length cDNA were generated by PCR using the pair of primers corresponding to each type of isoforms, with the exception that a Flag-tag coding sequence was added at the 5' end of CD160-TM reverse oligonucleotide.

The chimeric CD8-CD160TM construct consists of the extracellular and transmembrane region of CD8 α fused to the intracellular part of CD160-TM. It was generated by separately amplifying the cDNA encoding the extracellular and transmembrane domains of CD8 α using a CD8 α -specific 5'-primer (5'-ATGGCCTTACCAGTGACCGCCTTG-3') and a chimeric 3'-primer (5'-GGGGTGCTTACGGCTCTTTTGGAGTTGCAGTAAAGGGTGATAACCAG-3'), and the coding sequence corresponding to CD160-TM intracellular domain with an overlapping 5'-primer (5'-CTGGTTATCACCTTTACTGCAACTCCAAAAGAGCCGTAA GCACCCC-3') and CD160-TM-specific 3'-primer. The two overlapping fragments were used as templates for a PCR with CD8 α -5' and CD160-TM-3' primers.

Each purified cDNA was ligated into the pcDNA3.1 or pEF6 expression vector according to the manufacturer's recommendation (Invitrogen). Mutants of the chimeric CD8-CD160-TM construct were produced by site-directed mutagenesis. The following mutants were generated: mutF²²⁰SS (Y²²⁰ to F) and mutF²²⁵PQ (Y²²⁵ to F). The integrity of all sequences was finally assessed by double-strand sequencing.

Cells were transfected by electroporation with 30 μ g of the desired expression vector using a Gene Pulser II (Biorad) with settings at 250 V and 950 μ F. 48h later, cells were subjected to

antibiotic-selection. The synthesis of CD160, CD160-TM or CD8-CD160-TM chimera by the growing clones was assessed by flow cytometry.

Generation of anti-CD160-TM specific antibodies

Two rabbits were immunized with peptides located within the Ig-like domain (peptide 1, SSASQEGTRLNLIC, aminoacids 31-44) and the membrane-proximal region (peptide 2, KQRQHLEFSHNNEGTL, aminoacids 144-158) of both CD160 and CD160-TM extracellular part. Each rabbit antiserum was further affinity-purified on peptide 1 or peptide 2 (referred to as anti-CD160-TM^{pep1} or -CD160-TM^{pep2}), and the specificity of the purified Ab assessed by immunostaining on CD160-TM-expressing cells.

A second immunization protocol was similarly performed using peptides belonging to the intracellular domain of CD160-TM (VSTPSNEGAIIFLPP, aminoacids 186-200 and SRRRRLERMSRGREK, aminoacids 204-218). The corresponding Abs are referred to as anti-CD160-TM^{IC}.

Flow cytometry

For detection of CD160 or CD160-TM, cells were incubated with anti-CD160 mAb BY55 (IgM; 1 µg/test) or affinity purified anti-CD160-TM^{pep2} Abs (3 µg/test) for 30 min at 4°C. An anti-CD34 mAb (IgM) or rabbit pre-immune serum was used as negative control, respectively. PE-conjugated goat anti-mouse or goat anti-rabbit Ig were added as secondary reagent. Expression of CD8-CD160-TM chimera was established using a PE-conjugated anti-CD8 mAb (clone B9.11; Beckman Coulter). Cells were analyzed using an EPICS XL apparatus (Beckman Coulter).

Immunoprecipitation and immunoblotting

Immunoprecipitations were performed as described elsewhere (27) using rabbit pre-immune or polyclonal anti-CD160-TM^{IC} serum and protein G-sepharose beads. Following proteins

separation and transfer onto a nitrocellulose membrane, immunoblot analysis was conducted using the anti-CD160-TM^{IC} Ab (dilution 1/5000) and HRP-conjugated anti-rabbit Ig Ab (Jackson Immunoresearch). Detection was realized using an ECL detection system (Amersham Biosciences, Orsay, France).

To detect Erk1/2 activation, cells were incubated with mouse IgG1 or anti-CD8 mAb (1µg/ml) and cross-linked with rabbit anti-mouse IgG Ab for 20min at 37°C. Cells were harvested immediately after stimulation and lysed. Cellular lysates were analyzed by immunoblotting with a specific anti-phospho-Erk1/2 mAb (Sigma-Aldrich) and purified polyclonal anti-Erk1/2 Abs (Cell Signaling Technology).

Proliferation assays

Stably transfected Jurkat cells (10⁴/well) were activated with immobilized anti-CD8 mAb or mouse IgG1 (Beckman-Coulter) for 6h at 37°C. Cells were then pulsed with 1 µCi of ³H-thymidine for the next 6h, and ³H-thymidine incorporation was measured. All conditions were done in triplicate.

NK cell activation and CD107a analysis

The NK cell line NK92 was cultured for 4h in wells that had been pre-coated with anti-CD8 mAb, anti-CD160TM^{IC} or -CD160TM^{pep2} purified Abs. Following extensive washes, cells were collected and analyzed by flow cytometry for CD107a cell surface expression using a PE-Cy5-conjugated anti-CD107a mAb (BD Biosciences). Statistical analyses were performed using a Student *t* test. Values of $P < 0.005$ were considered significant.

Accession codes

The GenBank accession numbers for CD160-TM and CD160ΔIg-GPI cDNA are EU016100 and EU016101, respectively.

Results

Identification of new CD160 mRNAs

We previously reported the characterization of the GPI-anchored CD160 molecule as a multimeric activating receptor capable to trigger PB-NK cell lysis and cytokine production following engagement by its physiological ligand HLA-C (22, 23). To investigate CD160 functions and potential molecular associations, experiments were conducted to generate a full-length cDNA construct for subsequent cloning into an eukaryotic expression vector. Unexpectedly, RT-PCR experiments performed on total RNA isolated from the NK cell line NK92 constantly led to the detection of two amplified products (Fig. 1, lane 1). Thus a 550 bp cDNA corresponding to CD160 full coding sequence was observed, as well as a second product of approximately 220 bp. Because additional verifications confirmed the specific amplification of the shorter product, its purification and sequence analysis were undertaken. The results obtained revealed that this cDNA encoded a putative truncated form of CD160, as it corresponds to a CD160 mRNA that lacks the coding region for the Ig domain (Fig. 2A). This deletion would result in a GPI-anchored protein devoid of extracellular Ig domain (Fig. 2A). We will refer to this protein to as CD160 Δ Ig-GPI.

We further realized a computer assisted sequence comparison of the newly identified mRNA against available databases, and found an additional mRNA (Gene Bank accession number AK128370) presenting a high level of identity with the CD160 Δ Ig-GPI transcript (Fig. 2A). Upon translation, this transcript would lead to the synthesis of a CD160 protein presenting no extracellular Ig domain, but exhibiting a transmembrane and an intracellular domain (Fig. 2B). This putative variant of CD160 was therefore called CD160 Δ Ig-TM.

To assess if CD160 Δ Ig-TM mRNA was synthesized by the NK92 cell line, specific primers allowing the amplification of CD160 Δ Ig-TM cDNA were designed (see Fig. 2A). As previously observed for the synthesis of CD160 cDNA, two specific products of roughly 400 and 700 bp were detected (Fig. 1, lane 2). The sequence analysis of these two cDNA showed

that the smaller one corresponded to CD160 Δ Ig-TM cDNA, while the larger one coded for a fourth possible isoform of CD160. Indeed, it presents an open reading frame coding for the complete extracellular domain of CD160, but then enters into a nucleotide sequence coding for a transmembrane and an intracellular domain (Fig. 2A). The corresponding polypeptide would therefore be a transmembrane protein sharing the same extracellular domain that the original GPI-anchored molecule (Fig. 2B and C). We will refer to this isoform to as CD160-TM. Thus, we demonstrated that four CD160 transcripts were synthesized by NK92 cells, two coding for GPI-anchored molecules, and two corresponding to their transmembrane (TM) counterparts. In addition, each pair of GPI- and TM-proteins can be distinguish from the other according to the presence or absence of the Ig domain within their extracellular moiety. Note that complementary sequence comparisons of the four cDNA sequences with genomic DNA showed that all transcripts were produced by alternative splicing of *CD160* gene.

Expression of CD160 transcripts in peripheral blood NK cells

To investigate the physiological relevance of the identification of four CD160 transcripts, RT-PCR experiments were realized on freshly isolated PB-NK cells. Total RNA were extracted from purified PB-NK cells, reverse-transcribed and amplified with the primer pair specific for either the GPI-anchored or the TM-molecules (see Fig. 2A for primers sequences). As shown in Fig. 3, the transcripts corresponding to the two GPI-bound proteins (CD160 and CD160 Δ Ig-GPI) were present in resting PB-NK cells (Fig. 3, top panel, day 0), while the ones encoding the TM-isoforms remained undetectable (Fig. 3, middle panel, day 0). Interestingly, incubation of the cells with either IL-2, IL-12, IL-15 or IL-18 resulted in the neo-synthesis of CD160-TM and CD160 Δ Ig-TM mRNA (Fig. 3, middle panel). Notably, while the latter transcripts were no longer detectable at day 6 of IL-18 treatment, they remained present up to 14 days in all other activation conditions. The transcription of the GPI-isoforms mRNA did not seem modified along the activation process (Fig. 3, top panel) in agreement with our previous observation (27). Thus, unlike the NK92 cell line that

constitutively expressed all four CD160 transcripts (Fig. 1), PB-NK cells show an activation-dependent synthesis of CD160 mRNAs, as the potential expression of the transmembrane molecules can only be achieved following activation.

The synthesis of CD160 Δ Ig-TM and CD160-TM transcripts is restricted to activated NK cells

To further evaluate the cellular specificity of CD160 TM-isoforms transcripts synthesis, RT-PCR was conducted on total RNA extracted from PB-sorted cells, tissue-isolated cells, or from various established cell lines. The results reported in Table I showed that among the PB cell types tested, the transcripts encoding CD160 TM-isoforms were only detected in IL-15-treated NK cells. These mRNA were also not found in thymocytes, cord blood mononuclear cells or CD34⁺ cells. In agreement with previous studies (21, 27, 35), the GPI-isoforms transcripts showed a broader distribution pattern and were detected in resting and activated PB-NK and PB-CD8⁺ lymphocytes, and in activated PB-CD4⁺ T lymphocytes of all donors. Additionally, CD160 Δ Ig-TM and CD160-TM transcripts were successfully amplified from NK tumoral cell lines, but not from established normal and malignant T or B cell lines (Table I). These data strongly suggested that, in contrast to the GPI-anchored CD160, CD160 TM-isoforms are exclusively expressed by activated NK cells and by their transformed counterparts.

CD160-TM isoform expression upon activation of PB-NK cells

To establish whether the activation-dependent synthesis of the TM-isoforms mRNA in PB-NK cells was correlated to the expression of the corresponding proteins, the generation of Abs specific for the TM-isoforms was required. Polyclonal Abs were obtained by immunization of rabbits with a mix of two peptides, one belonging to the Ig-like domain (peptide 1) and one located within the membrane-proximal domain (peptide 2) of the molecules (see the Material and Methods section, and Fig. 2C, for sequence details). Each

serum was further affinity-purified on either peptide 1 or 2, and the reactivity of the purified Abs assessed by flow cytometry analysis. Initial experiments showed a better reactivity of the Abs affinity-purified on peptide 2 (anti-CD160-TM^{pep2}) on NK92 cell line (data not shown). To further determine whether these Abs were specific for CD160-TM, immunolabelings were realized on transfected Jurkat cells stably expressing CD160 or CD160-TM receptor. We clearly observed that the anti-CD160-TM^{pep2} Abs efficiently labeled CD160-TM transfectants while no signal was obtained using the anti-CD160 mAb BY55 (Fig. 4A, left panel). Thus, despite the presence of the membrane-proximal domain in both the GPI-anchored CD160 and CD160-TM, these affinity-purified Abs allow to discriminate between the two isoforms. Conversely, the anti-CD160 mAb BY55 positively stained CD160-expressing cells but gave no signal on CD160-TM transfectants (Fig. 4A, middle panel). Note that the anti-CD160 mAb CL1-R2, that presents a reactivity different from the one of BY55 mAb (20, 22, 23, 27), also failed to stain CD160-TM transfectants (data not shown). To definitely assess the specificity of CD160-TM recognition, long term IL-2-activated NK cells were tested for their reactivity with the anti-CD160-TM^{pep2} Abs or BY55 mAb. A positive staining was obtained on IL-2-activated cells labeled with the anti-CD160-TM^{pep2} Abs while no labeling was observed using BY55 mAb (Fig. 4A, right panel), although both CD160 and CD160-TM transcripts were detected (data not shown). Thus, we obtained purified polyclonal Abs that, according to their peptide recognition ability, allow the detection of CD160-TM molecule, but not of its GPI-anchored counterpart.

We further investigated the expression pattern of the GPI-anchored molecules *versus* the full length TM-protein on PB-NK cells by performing immunolabeling on resting or IL-15-activated PB-NK cells using either BY55 mAb or the anti-CD160-TM^{pep2} Abs. As previously reported (27), CD160 is expressed by circulating NK lymphocytes and becomes almost undetectable after 3 days of activation (Fig. 4B, upper panel). Note that the disappearance of CD160 from the cell surface resulted from an activation-dependent

proteolytic process involving a metalloprotease (27). This down-modulation step was then followed by a re-acquisition phase, as assessed by the recovery of a positive signal with BY55 mAb at later time points of activation (Fig. 4B, top panel). Moreover, in agreement with CD160 mRNA analysis (Fig. 3), no CD160-TM was detected on resting PB-NK cells, while a low level of membrane expression was found after 3 days of IL-15-treatment (Fig. 4B, lower panel). Interestingly, we constantly observed that this initial induction of CD160-TM expression was followed by a down-modulation step, the TM-receptor becoming undetectable at day 5 and 7 of activation. Remarkably, after 10 days of stimulation, two cell populations were detected, one expressing high levels of CD160-TM, and one being barely CD160-TM-positive cells. Depending on the donors, both cell types remained present up to 2 to 3 weeks after the beginning of the activation process (data not shown). Importantly, we evidenced a complete loss of CD160 and an exclusive expression of CD160-TM on *in vitro* activated cells, as exemplified in Fig. 4A, suggesting that upon longer activation time, CD160 can be totally replaced by CD160-TM at the NK cell surface. Thus, it seems that during the activation process, NK cells went through a time-dependent regulation of CD160 and CD160-TM membrane expression, the receptors being available at the cell surface depending on the level of proteolytic activity (for CD160) or translational activity (for CD160-TM) existing within the cells during the time course of activation.

Molecular characterization of CD160-TM

To better characterize CD160-TM, additional protein analyses were performed on CD160-TM-expressing Jurkat cells or IL2-activated NK cells. Immunoprecipitates were prepared using either anti-CD160-TM^{IC} Abs, directed against the intracellular domain of the protein (see Fig. 2C and the Material and Methods section for peptides sequence), or the corresponding rabbit pre-immune serum. Immunoblot analysis conducted with the anti-CD160-TM^{IC} Abs showed the presence of a 100 kDa protein in the immunoprecipitates prepared from both cell types (Fig. 5). No protein was recovered when using rabbit pre-

immune serum for precipitation, inferring the specificity of signal detected in the anti-CD160-TM immunoprecipitates (Fig. 5). Importantly, Western blot analysis using the anti-CD160 mAb CL1-R2 similarly allowed the detection of a 100 kDa protein (data not shown). As CD160-TM aminoacids sequence corresponds to a polypeptide with an estimated molecular weight of 25.6 kDa, it is likely that CD160-TM is expressed in activated NK cells as a multimeric molecule which appears to be quite resistant to reducing agent, as already observed for CD160 (19, 24) and soluble CD160 (27).

CD160-TM represents an activating receptor on PB-NK cells

Our observation that the clearing of GPI-anchored CD160 from the NK cell surface parallels the appearance of the transmembrane molecule prompted us to further investigate the potential function of CD160-TM. To determine whether CD160-TM might trigger activating or inhibitory signals following engagement, NK92 cells were activated with either a control mAb, or affinity-purified anti-CD160-TM^{IC} or -CD160-TM^{pep2} Abs, and the corresponding cellular degranulation response was analyzed through the detection of CD107a cell surface mobilization. We observed that a fraction of NK92 cells (representing 12% of the overall cell population) exhibited a spontaneous level of degranulation, and that this amount of CD107a-expressing cells was not modified in the presence of CD160-TM^{IC} Abs (Fig. 6A). In contrast, a 1.5 fold increase in the percentage of CD107a positive cells was detected upon stimulation with CD160-TM^{pep2} Abs, thus demonstrating that CD160-TM triggering can lead to the generation of positive signals involved in the process of degranulation.

CD160-TM cytoplasmic tail is sufficient to mediate activating signaling

The molecular basis underlying CD160-TM activating function was next analyzed. The protein sequence analysis of CD160-TM revealed the presence, within its transmembrane domain, of a positively charged lysine residue (see Fig. 2C). An association of the molecule with ITAM-bearing adaptors through the establishment of a stable salt bridge was therefore

considered. However, co-expression of CD160-TM together with DAP10, DAP12, ζ or Fc ϵ RI γ in COS cells did not evidence any association between these adaptor proteins and CD160-TM (data not shown). Another feature of CD160-TM is the presence, in its intracellular domain, of two tyrosine residues at positions 220 and 225, which might represent potential docking sites for signaling molecules upon phosphorylation. To investigate whether CD160-TM function was dependent on its intracellular domain, a chimeric construct coding for CD8 α extracellular and transmembrane domains fused to CD160-TM cytoplasmic tail was generated. CD8-negative wild type Jurkat cells stably expressing the chimeric protein (Jurkat/WT) were selected (see Fig. 6B, top panel, for expression of CD8-CD160TM molecules), and the activating potential of the chimera tested in proliferation assays. While the transfected cells did not show any proliferation increase in response to control mouse IgG1, a substantial enhancement of their growth rate, corresponding to a 30-35% increase in their proliferation level, was obtained following CD8 triggering (Fig. 6B, bottom panel). Furthermore we observed that point mutation of Y²²⁰ resulted in a chimeric protein still able to mediate an up-modulation of Jurkat cells growth following triggering (Fig. 6B, bottom panel, Jurkat/mutF²²⁰SS). In contrast, no more increase was detected upon ligation of the Y²²⁵-mutated chimera (Fig. 6B, bottom panel, Jurkat/mutF²²⁵PQ), demonstrating that this tyrosine residue is critically involved in the delivery of positive signals. A tyrosine-dependent activation signaling was confirmed by the observation that cross-linking of the WT chimera had no effect on the proliferation rate of Lck-deficient Jurkat cells (Fig. 6B, bottom panel, JCam/WT). These results establish that CD160-TM intracellular domain has the functional potential to transduce activating signals through a phosphotyrosine-dependent process that most likely involved p56^{lck}.

To further characterize the signaling pathways engaged upon CD160-TM triggering, Jurkat cells stably expressing WT or mutated CD8-CD160-TM chimera were either left unstimulated or activated with anti-CD8 mAb. The cellular lysates were then analyzed for the

activation of Erk by Western blot using an anti-phospho-Erk mAb (Fig. 6C). We observed that triggering of the CD8-CD160-TM chimera led to the efficient recruitment of Erk signaling cascade, as assessed by the detection of Erk phosphorylation (Fig. 6C, left panel). A similar activation of Erk was induced when targeting the Y²²⁰-mutated chimera, while Y²²⁵ mutation led to a complete loss of Erk activation. Finally, the triggering of WT chimera on Lck-negative cells did not result in any Erk phosphorylation (Fig. 6C, right panel). Thus, CD160-TM cytoplasmic tail exhibits structural features that allow the recruitment of Erk signaling pathway.

Discussion

CD160 has been initially identified as a MHC-class I specific activating receptor in PB-NK lymphocytes (22, 23), and was shown to act as a co-activating receptor in cytotoxic CD8⁺ T cells (25, 26). Here we report that, apart from CD160 mRNA, three additional transcripts generated through alternative splicings of *CD160* gene can be detected in NK cells. The predicted amino acid sequences for these newly identified mRNA correspond to: 1) a GPI-anchored CD160 molecule devoid of extracellular Ig-like domain (CD160ΔIg-GPI), 2) a transmembrane isoform of the original CD160 receptor (CD160-TM) and 3) a transmembrane version of CD160ΔIg-GPI (CD160ΔIg-TM). Notably, we observed a coupled synthesis of the transcripts, the mRNA encoding both GPI- or TM-isoforms being systematically produced in parallel (Fig. 1 and 3). By analyzing the synthesis pattern of the above mRNA, we confirmed the constitutive expression of CD160 by circulating NK and cytotoxic CD8⁺ T lymphocytes (19). In contrast, the TM-isoforms encoding mRNA were not amplified from these cells (Table I). More importantly, we demonstrated that the TM-isoforms transcripts became synthesized in PB-NK cells, but not in PB-CD8⁺ T cells, upon activation (Fig. 3 and Table I). This observation, added to the detection of all four transcripts in the IL-2-dependent NK tumoral cell lines tested (Fig. 1 and Table I), strongly suggested that the TM-isoforms are exclusively expressed by NK cells, and that their expression is activation-dependent. Furthermore, the concomitant synthesis of the GPI- and TM-isoforms transcripts by all NK cell lines tested suggested that the TM-isoforms expression may be detected in the whole activated NK cell population. Among the NK cell receptors, an activation-dependent expression has only been described for the NCR NKp44 (10). However, NKp44 mRNA synthesis was also evidenced in B-, T- and myeloid-cell lines indicating that its expression may not be specific to NK cells (36).

The existence of splice variants has been reported for numerous NK cell receptors. Thus, 2B4, CS1 (CRACC) or murine NKG2D present spliced isoforms that differed from the

full-length receptor in their cytoplasmic domain (37-39). A spliced isoform of CD33, devoid of extracellular Ig-like domain, has also been characterized (40). In all cases, the second isoform is thought to transduce different signals when compared to the full-length molecule as a result of its association with distinct signaling adaptor molecules, and/or of a cell-lineage specific expression pattern. One well-defined example was given by CD16 (FcγRIII), expressed on human NK cells as a transmembrane glycoprotein and on neutrophils as a GPI-anchored molecule (41-43). Notably, according to its membrane anchor, CD16 displayed distinct binding kinetic rates and affinity for a given ligand. In addition, anti-CD16 mAbs were found to exhibit a different reactivity towards the GPI- and the TM-isoform (44). In this regard, we similarly observed that while encompassing an identical extracellular domain, CD160 and CD160-TM were selectively recognized by BY55 mAb or anti-CD160-TM^{pep2} Abs, respectively (Fig. 4A). Furthermore, a significant reactivity was obtained with the anti-CD160 CL1-R2 mAb when CD160-TM-expressing cells were subjected to a fixation step prior to immunolabeling (data not shown). The most likely explanation will be that the replacement of the GPI-anchor motif by transmembrane and intracellular domains may result in some conformational modifications within the extracellular moiety, leading to differential Abs recognition of the molecules.

Although the complete identity between CD160 and CD160-TM extracellular part might suggest that both molecules could be specific for the same ligands (namely, the MHC-class I molecules or HVEM) (20-22), we cannot exclude the hypothesis of a different ligand specificity resulting from conformational differences. In any case, each isoform could be functionally characterized according to its related signal transduction pathway. Despite the presence of a charged lysine residue in CD160-TM transmembrane region, we did not evidence any association of the receptor with adaptor molecules usually involved in NK cell activating-receptor signal transduction, such as DAP12, FcεRIγ or CD3-ζ, in co-transfection experiments (data not shown). The generation of a CD8-CD160-TM chimera, encompassing

the intracellular domain of CD160-TM, suggested that CD160-TM is sufficient by itself to initiate intracellular signals leading to an increased cellular proliferation and to the recruitment of Erk activation pathway (Fig. 6). In addition, the lack of CD8-mediated response in CD8-CD160-TM JCam transfectants suggested a requirement for p56^{lck} in the initiation of CD160-TM-mediated activation process. We established that mutation of Y²²⁰ within CD160-TM intracellular domain did not affect the positive signals delivered through engagement of the CD8-CD160-TM chimera (Fig. 6). Furthermore, we observed that the F²²⁰SS mutated protein still underwent tyrosine-phosphorylation upon cell activation (data not shown). Accordingly, our data identified Y²²⁵ as the residue involved in the delivery of CD160-TM-dependent activation signals. We recently reported that CD160 activating function in NK cells depended on PI3-kinase recruitment (45). The sequence analysis of CD160-TM cytoplasmic tail showed no consensus tyrosine-based interaction motifs allowing a direct association with PI3-kinase. Similarly, CD160-TM does not contain any immunoreceptor tyrosine-based switch motif, thus not favoring the possibility of an interaction with the signaling lymphocyte activation molecule-associated protein (SAP) or its homolog EAT-2, such signaling pathways being described for the CD2 family receptors expressed in NK cells, namely 2B4, NTB-A and CRACC (46, 47). It is therefore possible that CD160-TM intracellular Y²²⁵ mediates an interaction with still undefined cytoplasmic signaling molecules involved in the transduction of activating signals. Work is in progress to clarify this issue.

Finally, we observed that the appearance of CD160-TM on activated-NK cells followed a two-steps expression process. Thus, low levels of CD160-TM were detected on IL-15-treated cells 3 days after activation, while CD160 became undetectable at the cell surface (Fig. 4B). We recently demonstrated that CD160 clearing from the NK cell surface resulted from a phospholipase-dependent proteolytic cleavage of the molecule (27). At longer activation times, a down-regulation of CD160-TM expression was observed, followed by a re-expression step leading to the detection of a NK cell population highly expressing the TM-receptor, the remaining cells exhibiting a lower cell surface expression. In contrast, no change

in the level of CD160-TM mRNA was detected during the time-course of activation (Fig. 3), suggesting that the level of CD160-TM expression results from a time-dependent regulation of its translation. CD160-TM re-expression parallels the progressive re-acquisition of GPI-anchored CD160, probably reflecting the shut-down of the previously induced GPI-phospholipase activity. One can therefore postulate that the tightly regulated expression of CD160 isoforms might be an important step in the cascade of events leading to a specific and efficient recruitment of CD160 molecules and of their respective signaling pathways. The relevance of the Δ Ig isoforms mRNA synthesis would also have to be addressed, in terms of protein expression, function, and specificity to determine whether these potential receptors may also play a role in the regulation of NK cell functions. In any case, the identification of CD160-TM opens new perspectives regarding the cellular events involved in the regulation of NK cell functions.

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Footnotes

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⁴ Abbreviations used: NCR, natural cytotoxicity receptor; TM, transmembrane; PB, peripheral blood.

Figure legends

Figure 1. Identification of novel CD160-related coding sequences. Total RNA were isolated from NK92 cells and subjected to reverse transcription. PCR were realized using a pair of primers corresponding to the 5' and 3' ends of CD160 (lane 1) or CD160 Δ Ig-TM (lane 2) reported coding sequence (NM007053 and AK128370, respectively). Amplification of β actin cDNA was performed in parallel as internal control. The position of each transcript is indicated by referring to the name of its putative translation product.

Figure 2. cDNA sequences and molecular organization of CD160 isoforms. **(A)** Sequence comparison of CD160 isoforms cDNA. The predicted coding region for the signal peptide (*italic*), the Ig-like domain (**bold**) and the hydrophobic region (underlined) are delineated. The sequences of the primers used for cDNA amplification are also indicated (dashed line). **(B)** Schematic representation of the predicted molecular organization of CD160 isoforms. Two types of proteins can be distinguished on the criteria of their mode of insertion into the membrane (GPI or TM), or of the presence or absence of the Ig-like domain within their extracellular part. The putative polypeptides share the same signal peptide. **(C)** Predicted amino acid sequence of CD160-TM protein. The amino acids predicted to form the signal sequence are underlined. The hydrophobic portion, deduced from a Chou and Fasman hydrophobicity analysis, and which contains a positively charged Lys (**K**) residue, is in **bold**. The intracellular KCYSSP motif, where is located a putative tyrosine phosphorylation site, is **boxed**. The sequence of the peptides used for immunization is in *italic*.

Figure 3. CD160 isoforms mRNA synthesis upon activation of PB-NK cells. PB-NK cells were purified from the peripheral blood of healthy donors, and either left unstimulated (day 0) or cultured in the presence of IL-2, IL-12, IL-15 or IL-18. Cells were collected at the indicated time points. Following total RNA extraction and reverse transcription, cDNA amplification was performed with a primer combination specific for the GPI-anchored (top panel) or the TM isoforms (middle panel) coding sequence (see Fig. 2A for primers sequence). The position and length of the cDNA corresponding to each isoform is as indicated. β actin cDNA synthesis was used as internal control (bottom panel). Data are representative of experiments performed on three independent healthy donors.

Figure 4. Expression CD160 isoforms following PB-NK cell activation. **(A)** Characterization of anti-CD160-TM polyclonal Abs. Jurkat cell stable transfectants expressing either CD160 or CD160-TM protein, or long term IL-2-activated NK cells, were immunolabeled with the affinity purified anti-CD160-TM^{pep2} Abs (top panel) or the anti-CD160 mAb BY55 (bottom panel). Rabbit pre-immune serum, or isotype-matched anti-CD34 mAb, was used for negative control. Following addition of the appropriate PE-coupled secondary Abs, cells were analyzed by flow cytometry. **(B)** CD160-TM surface expression upon PB-NK cells activation. Freshly isolated PB-NK cells were grown in medium alone (day 0) or supplemented with IL-15 for the indicated time. The presence of CD160 (top panel) or CD160-TM (bottom panel) isoform was detected by immunolabeling with BY55 mAb or the anti-CD160-TM^{pep2} purified Abs, respectively. Data are representative of experiments performed on three healthy donors.

Figure 5. Molecular characterization of CD160-TM. Transfected Jurkat cells stably expressing CD160-TM or long term IL-2-activated NK cells were lysed, and post-nuclear supernatants subjected to immunoprecipitation using the anti-CD160-TM^{IC} Abs (directed against the intracellular domain of CD160-TM) or the corresponding pre-immune serum (PI). After separation by SDS-PAGE under reducing conditions and transfer onto nitrocellulose,

protein revelation was performed by incubation of the blot with the anti-CD160-TM^{IC} Abs followed by a HRP-conjugated anti-rabbit Ig.

Figure 6. CD160-TM acts as an activating receptor **(A)** CD160-TM triggering enhances NK cell degranulation. NK92 cells were stimulated with either immobilized anti-CD8 mAb or purified anti-CD160-TM^{IC} or -CD160-TM^{pp2} Abs. CD107a cell surface mobilization was then analyzed by flow cytometry. A value of 1 was attributed to the % of cells spontaneously expressing CD107a. The increase in CD107⁺ cells observed upon CD160-TM triggering was significant ($P < 0.005$). Shown are results representative of three independent experiments. **(B)** Mapping of CD160-TM intracellular functional motif. Wild type (Jurkat) or Lck-deficient (JCam) cells were stably transfected with expression vector encoding either the wild-type (WT) or mutated (mut F²²⁰SS or mut F²²⁵PQ) CD8-CD160-TM chimeric receptor. *Top panel:* Expression of the chimeric protein was assessed for each transfectant by immunolabeling using a PE-conjugated anti-CD8 mAb vs. an isotype-matched control Ig. *Bottom panel:* For proliferation assays, transfectants were activated with increasing concentrations of either control mouse IgG1 (mIgG1) or anti-CD8 mAb, as indicated. Results were expressed as the percentage of proliferation \pm SD, with 100% corresponding to basal cell growth. Data are representative of four independent experiments. **(C)** CD8-CD160-TM cross-linking induces Erk1/2 activation. Jurkat cells expressing the WT or mutated CD8-CD160TM chimera were incubated with mouse IgG1 (control, C) or with an anti-CD8 mAb (left panel). Similar experiments were realized on JCam cells expressing the WT chimera (right panel). Immunoblot analysis was performed on total cell lysates using an anti-phospho-Erk1/2 (PErk) mAb. Equal protein loading was assessed by reprobing the membrane with anti-Erk1/2 Abs. Shown are data representative of five independent experiments.