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Identification and Characterization of a Transmembrane Isoform of CD160 (CD160-TM), a Unique Activating Receptor Selectively Expressed upon Human NK Cell Activation

Jérôme Giustiniani, Armand Bensussan,2,3 and Anne Marie-Cardine2

CD160 has been initially identified as a GPI-anchored MHC-class I activating receptor mainly expressed on peripheral blood NK cells. Herein, we report the identification of three additional CD160-related mRNAs generated through alternative splicings of the 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. Additionally, we provide evidence that CD160-TM represents a novel activating receptor, as assessed by the increased CD107a NK cell surface mobilization observed upon its engagement. Finally, we demonstrate that the 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 p56lck. The identification of CD160-TM therefore provides new possibilities regarding the role of CD160 isoforms in the regulation of NK cell functions. The Journal of Immunology, 2009, 182: 63–71.

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 toward target cells (3).

The NK cell inhibitory receptors have been classified into three groups, namely, the heterodimeric CD94/NKG2A, the Ig-like transcript receptors, and the members of the killer cell Ig-like receptors (4, 5). All of them bind to classical or nonclassical MHC class I molecules. A common characteristic of the inhibitory receptors is the presence of ITIMs 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 Src homology region 2 domain-containing phosphatase (SHP) 1 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 (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, which are expressed on circulating NK lymphocytes, NKp44 expression is activation-dependent (10). The NCRs transduce signals through their association with ITAM-containing molecules such as CD3ζ, FcεRIγ, and DAP12 (11, 12). Besides the NCRs, NKG2D is a C-type lectin-like receptor shown to recognize the MHC class I homologs MICA and MICB, as well as the family of UL16-binding proteins (ULBPs) (13). NKG2D uses the transmembrane polypeptide DAP10 for signaling, which interacts with the PI3K 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, the 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, besides these MHC class Ia/ib molecule-independent activating receptors, it is important to mention the well-characterized DAP12-associated CD94/NKG2C and killer cell Ig-like receptor-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 we 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 CD56dimCD16+ 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 the CD160 gene was found to

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

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be located on human chromosome 1, and the corresponding protein was characterized as a GPI-anchored cell surface molecule (24). Additionally, CD160 is expressed by intestinal intraepithelial T lymphocytes and by a minor subset of circulating T lymphocytes including TCRγδ and CD8α/CD28 T lymphocytes (24–26), and it exerts a co-receptor function on CD8α/CD28 CTL (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 is activation-dependent. We established that CD160-TM fulfills an activating function once expressed on activated PB-NK lymphocytes. Thus, the CD160 receptor represents a 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 SL (PAA Laboratories). PB-NK, CD4+ and CD8+ lymphocytes were purified using MACS and a specific cell subset isolation kit according to the manufacturer’s recommendations (Miltenyi Biotec). NKT cells were sorted and activated as previously described (27). The specific primers for the amplification of the cDNA corresponding to the GPI-anchored CD160 molecules were 5'-GGGTTGCTACGCTTTTGTAGTT CAGTAAGGTTGATAACCCG-3' (as well as the coding sequence corresponding to CD160-TM intracellular domain with an overlapping 5’ primer (5’-CGTGTATCTCCTTTCAGTCACCTCCAAAGGCGC TAAGACCCCCC-3') and CD160-TM-specific 3’ primer. The two overlapping fragments were used as templates for a PCR with CD8α-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 μg of the desired expression vector using a Gene Pulser II (Bio-Rad) with settings at 250 V and 950 μF. Forty-eight hours 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 Abs

Two rabbits were immunized with peptides located within the Ig-like domain (peptide 1, SSASQEGRNLIC; amino acids 31–44) and the membrane-proximal region (peptide 2, KQRQHLFSNHNMG, amino acids 144–158) of both CD160 and CD160-TM extracellular parts. Each rabbit antisera was further affinity purified on peptide 1 or peptide 2 (referred to as anti-CD160-TMppV or anti-CD160-TMppV), and the specificity of the purified Abs was 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 (VSTPSNEGAI IFLPP, amino acids 186–200, and SRRRLERMSRGRK, amino acids 204–218). The corresponding Abs are referred to as anti-CD160-TMppV.

Flow cytometry

For generation of Abs, PBMC were incubated with anti-CD160 mAb BY55 (IgM; 1 μg/test) or affinity-purified anti-CD160-TMppV Abs (3 μg/test) for 30 min at 4°C. An anti-CD34 mAb (IgM) or rabbit preimmune serum was used as negative control, respectively. PE-conjugated goat anti-mouse or goat anti-rabbit Iggs were added as secondary reagents. 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 preimmune or polyclonal anti-CD160-TMppV serum and protein G-Sepharose beads. Following proteins separation and transfer onto a nitrocellulose membrane, immunoblot analysis was conducted using the anti-CD160-TMppV Ab (dilution 1/5000) and HRP-conjugated anti-rabbit Ig Ab (Jackson ImmunoResearch Laboratories). Detection was realized using an ECL detection system (Amersham Biosciences).

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

Proliferation assays

Stably transfected Jurkat cells (10^5/well) were activated with immobilized anti-CD8 mAb or mouse IgG1 (Beckman Coulter) for 6 h at 37°C. Cells were then pulsed with 1 μCi of [3H]thymidine for the next 6 h, 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 4 h in wells that had been pre-coated with anti-CD8 mAb or anti-CD160-TMppV, or anti-CD160-TMppV 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.
Results

Identification of new CD160 mRNAs

We previously reported the characterization of the GPI-anchored CD160 molecule as a multimeric activating receptor capable of triggering 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 a 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 an extracellular Ig domain (Fig. 2A). We refer to this protein as CD160\textsubscript{ΔIg-GPI}.

We further realized a computer-assisted sequence comparison of the newly identified mRNA against available databases, and found an additional mRNA (GenBank accession no. AK128370) presenting a high level of identity with the CD160\textsubscript{ΔIg-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\textsubscript{TM}.

To assess if CD160\textsubscript{ΔIg-TM} mRNA was synthesized by the NK92 cell line, specific primers allowing the amplification of CD160\textsubscript{ΔIg-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 cDNAs showed that the smaller one corresponded to CD160\textsubscript{ΔIg-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 as the original GPI-anchored molecule (Fig. 2, B and C). We refer to this isoform as CD160\textsubscript{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. Additionally, each pair of GPI and TM proteins can be distinguished 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 PB-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 was 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\textsubscript{ΔIg-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 neosynthesis of CD160-TM and CD160\textsubscript{ΔIg-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\textsubscript{ΔIg-TM} and CD160\textsubscript{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 mRNAs were also not found in thymocytes, cord blood mononuclear cells, or CD34\textsuperscript{+} 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\textsuperscript{+} lymphocytes, as well as in activated PB-CD4\textsuperscript{+} T lymphocytes of all donors. Additionally, CD160\textsubscript{ΔIg-TM} and CD160\textsubscript{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 Materials and Methods, Section 3). The Abs were affinity-purified, their specificity was verified by competition ELISA, and their content of IgG was determined by ELISA. They were used to identify a potential activation-dependent expression of the CD160 TM isoforms by NK cells.

CD160-TM expression upon activation of NK cells

To confirm whether the syntheses of CD160 TM isoforms were restricted to activated NK cells, the expression levels of the TM isoforms were determined following a 6-day culture of freshly isolated NK92 cells and PB-NK cells, performed in the presence of IL-2, IL-15, or IL-18. As shown in Fig. 4, the CD160 isoforms expression was detected in IL-15-treated NK cells, and was not observed in IL-2 or IL-18-treated ones. Moreover, the CD160 isoforms expression was not detectable in resting NK cells, as indicated by the small amount of CD160 produced by IL-2-activated NK cells. These data strongly suggested that the synthesis of CD160 isoforms was induced by IL-15, while it was not influenced by IL-2 or IL-18 treatment.
and Methods 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 was assessed by flow cytometry analysis. Initial experiments showed a better reactivity of the Abs affinity purified on peptide 2 (anti-CD160-TMpep2) 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-TMpep2 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 discrimination 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, which 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-TMpep2 Abs or BY55 mAb. A positive staining was obtained on IL-2-activated cells labeled with the anti-CD160-TMpep2 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 its GPI-anchored counterpart.

We further investigated the expression pattern of the GPI-anchored molecules vs 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\textsuperscript{exp2} 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 reacquisition phase, as assessed by the recovery of a positive signal with BY55 mAb at later time points of activation (Fig. 4B, upper 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, with the TM receptor becoming undetectable at days 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 2–3 wk after the beginning of the activation process (data not shown). Importantly, we found 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, with 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 IL-2-activated NK cells. Immunoprecipitates were prepared using either anti-CD160-TM\textsuperscript{IC} Abs, directed against the intracellular domain of the protein (see Fig. 2C and the Materials and Methods for peptide sequence), or the corresponding rabbit preimmune serum. Immunoblot analysis conducted with the anti-CD160-TM\textsuperscript{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 preimmune serum for precipitation, inferring the specificity of signal detected in the anti-CD160-TM\textsuperscript{IC} immunoprecipitates (Fig. 5). Importantly, Western blot analysis using the anti-CD160 mAb CL1-2 similarly allowed the detection of a 100-kDa protein (data not shown). As CD160-TM amino acids sequence corresponds to a polypeptide with an estimated molecular mass of 25.6 kDa, it is likely that CD160-TM is expressed in activated NK cells as a multimeric molecule that appears to be quite resistant to the 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\textsuperscript{IC} or anti-CD160-TM\textsuperscript{exp2} 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\textsuperscript{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\textsuperscript{exp2} 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

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**Table 1. Cellular distribution of D160 isoforms transcripts**

<table>
<thead>
<tr>
<th>Samples</th>
<th>GPI</th>
<th>TM</th>
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<tr>
<td>Fresh tissues</td>
<td>+</td>
<td>-</td>
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<tr>
<td>PBMCs</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Activated PBMCs</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cord blood mononuclear cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD3\textsuperscript{+} cells</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Thymocytes</td>
<td>-</td>
<td>-</td>
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<tr>
<td>NK cells</td>
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<tr>
<td>IL-15-activated NK cells</td>
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<tr>
<td>CD4\textsuperscript{+} T cells</td>
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<td>IL-15-activated CD4\textsuperscript{+} T cells</td>
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<td>CD8\textsuperscript{+} T cells</td>
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<tr>
<td>IL-15-activated CD8\textsuperscript{+} T cells</td>
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<td>Activated NKT cells</td>
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<tr>
<td>T cell clones</td>
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<tr>
<td>Thymocytes TCR\alpha\beta\textsuperscript{+} (B12g)</td>
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<tr>
<td>TCR\gamma\delta cells (LSO)</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CD4 TCR\alpha\beta\textsuperscript{+} cells (C1)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CD8 TCR\alpha\beta\textsuperscript{+} cells (JF1)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tumoral cell lines</td>
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<tr>
<td>B cells (Daudi)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>T cells (Jurkat, Molt4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NK cells (NK92, YTIndy, NKL)</td>
<td>-</td>
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\textsuperscript{a} RT-PCR using the primers pair specific for the amplification of the GPI-anchored (GPI) or the transmembrane (TM) transcripts was performed on total mRNA extracted from the indicated sorted cells or cell lines. Where indicated, cells were grown in the presence of IL-15 (10 ng/ml) for 3 days. Data are representative of three independent experiments. - , not detected; +, positive amplification; a, depending on the donor.
of a stable salt bridge was therefore considered. However, coexpression of CD160-TM together with DAP10, DAP12, CD3 \( \gamma \)/H9256, or Fc \( \varepsilon \)/H9255RI/H9253 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 extracellular and transmembrane domains fused to CD160-TM cytoplasmic tail was generated. CD8-negative wildtype Jurkat cells stably expressing the chimeric protein (Jurkat/WT) were selected (see Fig. 6B, top panel, for expression of CD8-CD160-TM molecules), and the activating potential of the chimera was 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 cell 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 crosslinking of the wild-type 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 p56lk.

To further characterize the signaling pathways engaged upon CD160-TM triggering, Jurkat cells stably expressing wild-type 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 antiphospho-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 wild-type chimera on Lck-negative cells did not result in any Erk phosphorylation (Fig. 6C, right panel). Thus, the CD160-TM cytoplasmic tail exhibits structural features that allow the recruitment of the Erk signaling pathway.

**Discussion**

CD160 has been initially identified as a MHC class I-specific activating receptor in PB-NK lymphocytes (22, 23), and it was shown to act as a coactivating receptor in cytotoxic CD8⁺ T cells (25, 26). Herein we report that, apart from CD160 mRNA, three additional transcripts generated through alternative splicings of the 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

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**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-TMIC or anti-CD160-TMpep2 Abs. CD107a cell surface mobilization was then analyzed by flow cytometry. A value of 1 was attributed to the percentage 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 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 are expressed as the percentage of proliferation ± 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 wild-type (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 wild-type 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.
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, with the mRNA encoding both GPI or TM isoforms being systematically produced in parallel (Figs. 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 encoding mRNA were not amplified from 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 presents 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 transude different signals when compared with 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 (Fc\(\gamma\)RIII), which was 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. Additionally, anti-CD16 mAbs were found to exhibit a different reactivity toward the GPI and the TM isoforms (44). In this regard, we similarly observed that while encompassing an identical extracellular domain, CD160 and CD160-TM were selectively recognized by BY55 mAbs or anti-CD160-TM\(^{gpl}\) 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 before 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 Ab recognition of the molecules.

Although the complete identity between CD160 and the 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 the CD160-TM transmembrane region, we did not find any association of the receptor with adaptor molecules usually involved in NK cell activating-receptor signal transduction, such as DAP12, Fc\(\gamma\)RIy, or CD3\(\zeta\), in cotransfection 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). Additionally, the lack of CD8-mediated response in CD8-CD160-TM JCam transfectants suggested a requirement for p56\(^{lck}\) in the initiation of the CD160-TM-mediated activation process. We established that mutation of Y220 within the 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 the CD160-activating function in NK cells depended on PI3K recruitment (45). The sequence analysis of the CD160-TM cytoplasmic tail showed no consensus tyrosine-based interaction motifs allowing a direct association with PI3K. 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, with such signaling pathways having been 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-step 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 reexpression step leading to the detection of a NK cell population highly expressing the TM-receptor, with 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 reexpression parallels the progressive reacquisition 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 ΔIg 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.

Disclosures

The authors have no financial conflicts of interest.

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