The Human Leukocyte Antigen (HLA)-C-specific "Activatory" or "Inhibitory" Natural Killer Cell Receptors Display Highly Homologous Extracellular Domains but Differ in their Transmembrane and Intracytoplasmic Portions

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Summary

Natural killer cells express clonally distributed receptors specific for major histocompatibility complex class I molecules. The human leukocyte antigen (HLA)-C-specific receptors have been molecularly identified and cloned. They exist not only as inhibitory (p58) but also as activatory (p50) receptors. Here we show that p50 and p58 are highly homologous in their extracellular regions formed by two Ig-like domains. In contrast, major differences exist in their transmembrane and cytoplasmic portions. Whereas p58 displays a 76–84–amino acid cytoplasmic tail containing an unusual antigen receptor activation motif, p50 is characterized by a shorter 39–amino acid tail. In addition, whereas p58 has a nonpolar transmembrane portion, p50 contains the charged amino acid Lys. These data strongly suggest that receptors with identical HLA-C allele specificity can mediate functions of opposite sign owing to their different transmembrane/cytoplasmic portions.

The cytolytic activity of NK cells is regulated by several clonally distributed inhibitory receptors specific for different groups of HLA class I alleles. Interaction between these NK receptors and HLA class I molecules leads to inhibition of the NK-mediated target cell lysis (1–4). The receptors specific for two distinct groups of HLA-C alleles have been molecularly identified as p58 molecules, which react with either GL183 (5) or EB6 mAb (6). p58-encoding genes have recently been isolated and cloned (7). They encode for type 1 transmembrane proteins, which belong to the Ig superfamily. They are characterized by two extracellular Ig-like domains and display a high level of sequence homology. Recently, additional 50-kD molecular forms of EB6 or GL183 mAb–reactive receptors have been identified. They display the same HLA-C allele specificity of the corresponding p58 receptors but mediate NK cell triggering rather than inhibition (8). p50 receptors were found to be expressed only in some donors and to display a clonal distribution. Importantly, single NK cells did not co-express p58 and p50 receptors with an identical HLA-C

specificity (8). The protein backbone of p58 or p50 molecules has been shown to be 42 and 36 kD, respectively, after deglycosylation. These data, together with identical HLA-C specificity and mAb reactivity, suggested that p58 and p50 could be homologous in their extracellular domains but different in their intracellular portions. Cloning of several p58-homologous genes revealed the existence of molecules displaying cytoplasmic tails of different length. Here we show that, different from p58, the activatory p50 receptors are characterized by a short intracytoplasmic tail associated with a transmembrane portion containing a polar residue.

Materials and Methods

Isolation of EB6⁺ or GL183⁺ NK Clones and Definition of the Function of their Receptors. EB6⁺ or GL183⁺ NK cell clones were isolated as previously described (4). Screening of clones for the expression of triggering (p50) or inhibitory (p58) receptors was performed by cytolytic tests using P815 target cells in a redirected

killing assay. The HLA class I–negative 221 cells were transfected with Cw3 or Cw4 alleles as described in detail elsewhere (8).

Amplification of Specific cDNAs from NK Cell Clones. Total RNA was extracted using RNAzol (Cinna/Biotecx, Friendswood, TX) by standard procedure. cDNA was obtained by reverse transcriptase (RT) reaction using oligo dT priming. Primers used for cDNA amplification were 5′-ACCTACAGATGCTTCGG (common up, nucleotides (nt) 583–599), 5′-AAAACACAGT-GATCCAATTA (C, nt. 913–932), and 5′-GTTCCG(CT)GTA-CACGATGA (E, nt. 986–1003). Each RT-PCR reaction contained 0.1 μM of control primers specific for the HLA-C locus [C1, 5′-GACG(AC)CG(AC)GAGTCC(AG)AGAGG; and C2, 5′-CG(GT)CCTCGCTCTGGTTGTAG] and 0.5 μM of group-specific primers. The set of primers common up/C was used in a 30-cycle PCR, including 15 cycles at 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C; and 15 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C, followed by a final extension of 7 min at 72°C. PCR using the primer pair common up/E was carried out for 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by a final extension of 7 min at 72°C. cDNA fragments were subcloned into pCRII vector (Invitrogen, San Diego, CA) and sequenced.

Dot Blot Hybridization. Amplified fragments were denaturated in 0.45 N NaOH, spotted into nylon membrane (Gene-Screen Plus; Dupont, NEN, Boston, MA) and hybridized with the following ³²P-labeled oligonucleotides: 5′-CAGTGGT-CAAA(CA)TCCCTTTCA (K-Lys nt. 752–772), 5′-CAGACA-CCTGCATGTTCTG (K2 nt. 723–741), and 5′-TGACTCTC-CATACGAGTGG (K4 nt. 609–627). Hybridization was carried out for 4 h at 56°C in 0.1% SDS, 5 × Denhardt's solution, and 5 × SSPE (probes Lys and K4), or 3 × SSPE (probe K2). Membranes were washed in 0.1% SDS and 0.1 × SSPE for 10 min at room temperature and for 10 min at 42°C.

Isolation of Full-Length cDNAs. The sequences of the forward primers used to obtain complete open reading frame (ORF) amplification products were 5′-ATGTCGCTCATGGTCG (SP1), 5′-ATGTCGCTCATGGTCATCAT (SP2), and 5′-ATGTCG-CTCTTGTTCG (SP3). The PCR cycling condition used for the sets of primers SP1/C, SP2/C, and SP3/C was 15 cycles of 20 s at 94°C, 30 s at 60°C, 30 s at 72°C followed by 15 cycles of 20 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a final extension of 7 min at 72°C. 5′ end nucleotide sequences of EB6-ActI and 183-ActI cDNAs were checked by rapid amplification of cDNA ends (RACE) (9).

Transient Transfectants. The monkey COS cell line (5 × 10⁵/plate) was transfected by the DEAE–dextran method with different cDNA constructs (10): RSV.5gpt/cl47.11, RSV.5gpt/EB6-ActI, RSV.5gpt/cl6.11, and RSV.5gpt/183-ActI. After 48 h, transfectants were trypsinized, stained with EB6 or GL183 mAb, followed by a PE-conjugated goat antibody to mouse IgG1, and analyzed by a FACSort® (Becton Dickinson & Co., Mountain View, CA).

Stable Transfectants. Jurkat cells (2 × 10⁷) were transfected by electroporation (960 μF/250 V) with the cDNA construct RSV.5gpt/cl42 or RSV.5gpt/EB6-ActI. 48 h after electroporation, cells were selected in mycophenolic acid and xantine-containing medium (10 μg/ml each). 3–4 wk later, cells were analyzed for cell surface expression of p58/p50 molecules.

Immunoprecipitation and Deglycosylation Experiments. Jurkat transfectants were lysed in 1% NP-40 lysis buffer and immunoprecipitated with EB6 mAb coupled to CNBr-Sepharose. Samples were fractionated in a 8.5% SDS-PAGE, blotted on a polyvinylidene difluoride membrane, and analyzed by Western blot with ¹²⁵I-

EB6 mAb (8). Surface ¹²⁵I-labeled (lactoperoxidase) Jurkat transfectants were lysed in 1% NP-40 and immunoprecipitated with EB6 mAb coupled with CNBr-Sepharose. Undigested or N-glycanase–digested molecules were analyzed on SDS-PAGE (8).

Results and Discussion

p58 and p50 Receptors Correlate with Different Types of Transmembrane and Intracytoplasmic Portions. NK cell clones expressing either the activatory (p50) or the inhibitory (p58) form of EB6 or GL183 mAb-reactive receptor were used to analyze whether a correlation exists between the receptor function and the type of transmembrane/cytoplasmic portion. To this end, a panel of PCR primers specific for unique sequences of genes encoding for either short or long cytoplasmic tails has been designed. The PCR primers used included (*a*) a common forward primer annealing at the 5′ end of the sequences encoding for the transmembrane portions (common up); (*b*) a backward primer specific for the short (39–amino acid) tail (primer C); and (*c*) a backward primer specific for the long (76–84–amino acid) tail (primer E). The common up/C primer combination was found to amplify a 350-bp fragment, whereas the common up/E combination amplified a 421-bp fragment. In NK clones expressing p50 receptors (either EB6 or GL183), a 350-bp fragment was consistently amplified by the common up/C primer combination. In contrast, in clones expressing p58 receptors, the 421-bp fragment was always amplified by the common up/E primer combination (Fig.
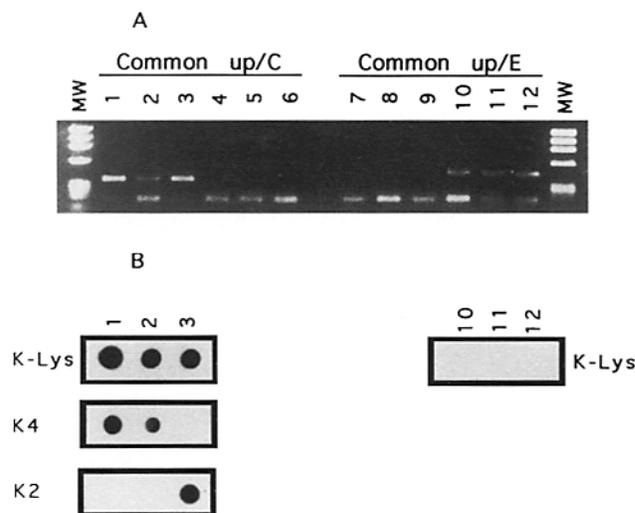
Figure 1. Amplification of specific cDNAs from NK cell clones expressing p50 or p58 receptors. (*A*) Common up/C and common up/E amplified fragments are shown. (Lanes *1* and *7*) Clone 82G16 (p50 EB6); (lanes *2* and *8*) clone PA4 (p50 EB6); (lanes *3* and *9*) clone DF (p50 GL183); (lanes *4* and *10*) clone 1F32 (p58 EB6); (lanes *5* and *11*) clone A4.12 (p58 GL183); (lanes *6* and *12*) clone A1.20 (p58 GL183). As control, a 160-bp HLA-C fragment was coamplified. (*B*) The common up/C amplified fragments from the NK clones 1:82G16, 2:PA4, and 3:DF were hybridized with K-Lys, K4, and K2 probes, whereas the common up/E cDNAs amplified from the NK clones 10:1 F32, 11:A4.12, and 12:A1.20 were hybridized only with K-Lys.
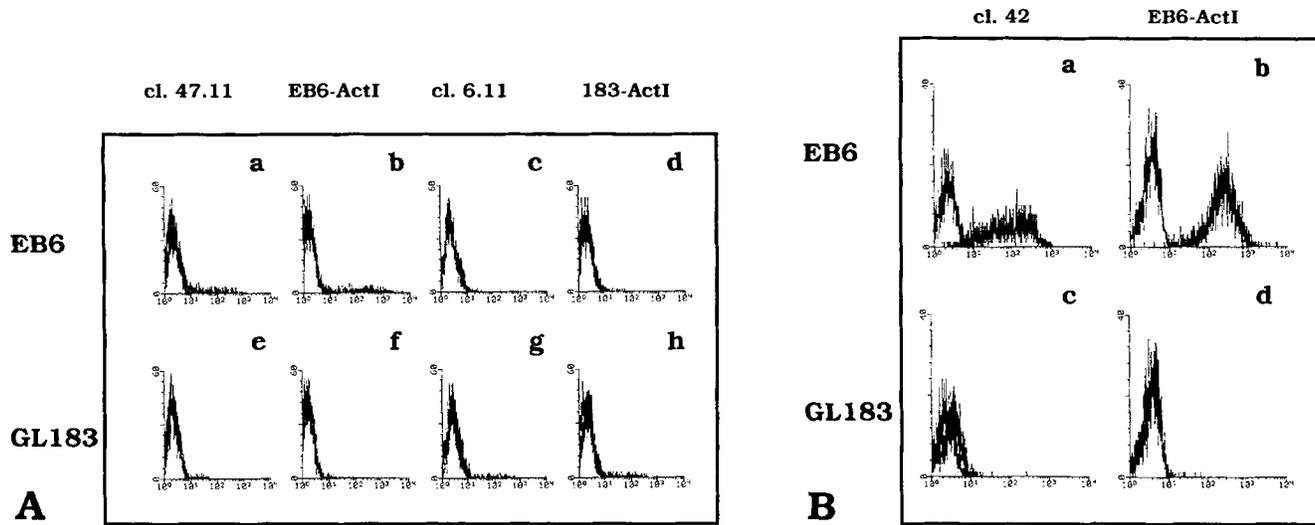
Figure 2. Cell surface expression of EB6– or GL183 mAb–reactive proteins in cell transfectants. (A) COS cell transfectants. COS cells were trypsinized, stained with EB6 or GL183 mAb, followed by a PE-conjugated goat antibody to mouse IgG1, and analyzed by FACSort®. Isotype-matched mouse IgG were used as controls. Cells were transfected with the following cDNA constructs: RSV.5gpt/cl47.11 (inhibitory EB6) (*a* and *e*), RSV.5gpt/EB6-ActI (activatory EB6) (*b* and *f*), RSV.5gpt/cl6.11 (inhibitory EB6) (*c* and *g*), and RSV.5gpt/183-ActI (activatory GL183) (*d* and *h*). (*B*) Jurkat cell transfectants. Jurkat cells were transfected with the cDNA construct RSV.5gpt/cl42 (inhibitory EB6) or RSV.5gpt/EB6-ActI (activatory EB6) and analyzed for cell surface expression of p58/p50 molecules by using EB6 (*a* and *b*) or GL183 (*c* and *d*) mAb as above. In each panel, IgG1 control has been overlayed, and the percentage of positive cells is indicated.

1 *A*). Over 40 amplified 350-bp fragments, derived from 14 different p50+ NK clones, were further subcloned and sequenced. At least five different sequences could be identified, all containing the Lys codon AAA in the reading frame of cDNAs encoding for the transmembrane portion. To investigate further whether this codon was typical of all amplified 350-bp fragments, an oligonucleotide probe specific for the transmembrane encoding sequence was designed. This probe, termed K-Lys, was found to hybridize with all common up/C–amplified products but not with the common up/E ones (Fig. 1 *B*).

Isolation of Full-Length cDNAs Encoding for Activatory p50 Receptors. mRNA encoding for p50 receptors was reverse transcribed, amplified, and cloned by RT-PCR from

Figure 3. Alignment of amino acid sequences coding for GL183 mAb–reactive molecules (clone 6, clone 43, 183-ActI) or EB6 mAb–reactive molecules (clone 42, 47.11, EB6-ActI). Dashes were introduced to maximize homologies. Amino acids identical to the consensus are indicated by dots. Signal peptide and transmembrane regions are marked; cysteines predicted to form Ig domains are indicated by asterisks. cDNA sequences have been deposited in EMBL with the following accession numbers: EB6-ActI X89892 and 183-ActI X89893. Previously described sequences have the following GenBank accession numbers: clone 6, U24074; clone 43, U24075; clone 42, U24076; clone 47.11, U24078; clone 49, U24079.

selected activatory NK clones (either EB6⁺ or GL183⁺) to obtain the complete ORF of the corresponding genes. To this end, based on the known sequences of the genes coding for p58 molecules, three forward primers (SP1, SP2, and SP3) specific for different signal peptide sequences were designed and used in combination with the backward primer C. By using the primer combination SP1/C, an amplified product of 932 bp was obtained exclusively from NK clones expressing the p50 EB6 or GL183 activatory receptors. The products amplified from two representative clones (PA4 and DF), termed EB6-ActI and 183-ActI, respectively, were subcloned in the pCRII vector and sequenced. In Fig. 3, these sequences are compared with the several known p58 sequences.

Activatory EB6 and GL183 Molecules Differ in their Transmembrane Portions. Probes specific for the regions flanking the AAA Lys codon of either EB6-ActI (K4) or 183-ActI (K2) were further designed. K2 and K4 probes were hybridized with the common up/C PCR–amplified products obtained from a panel of NK clones expressing p50 EB6 or GL183 activatory receptors. A precise correlation was found between hybridization with K2 or K4 probes and the EB6 or GL183 phenotype. Fig. 1 *B* shows the results obtained in three representative NK cell clones. It is evident that the K4 probe only hybridized with the common up/C PCR products derived from the p50/EB6⁺ cell clones. Conversely, the K2 probe only hybridized with that derived from the p50/GL183⁺ cell clone.

MAb Reactivity with COS Transient Cell Transfectants. 183-ActI and EB6-ActI cDNAs were subcloned in the pRSV.5gpt expression vector (10) and transiently transfected in COS cells. EB6-ActI transfectants were found to react with EB6 but not with GL183 mAb, whereas 183-ActI transfectants were stained by GL183 but not by EB6 mAb (Fig. 2 *A*). In both transfectants, 50-kD molecules were revealed by immunoprecipitation experiments, followed by Western blot, using either EB6 or GL183 mAbs. Conversely, 58-kD molecules were immunoprecipitated from COS cells transfected with either 47.11 or 6.11 cDNAs (data not shown) (7), coding for p58 EB6 or GL183 inhibitory receptors, respectively (not shown).

Sequence Alignment of Different p50 and p58 Receptors. EB6-ActI and 183-ActI cDNAs were compared with the previously described GL183 and EB6 cDNA sequences (not shown). EB6-ActI did not correspond to any of the known sequences, whereas 183-ActI was highly homologous to the previously described clone 49 (7). However, the ORF of the 183-ActI cDNA encodes for a protein containing, at residue 20, Gly instead of Trp. In addition, it encodes for three additional amino acids (Met-Ser-Leu) at the NH₂ terminus (Fig. 3), which appear to be critical for an efficient expression of GL183 molecules (data not shown). Alignment of all known p58-related amino acid sequences also indicated that the extracellular regions of both EB6 and GL183 molecules are highly homologous. However, at least 11 amino acid residues in the extracellular domains were unique to EB6 or GL183 molecules. 8 of these residues were localized in the more external Ig-like

A

B
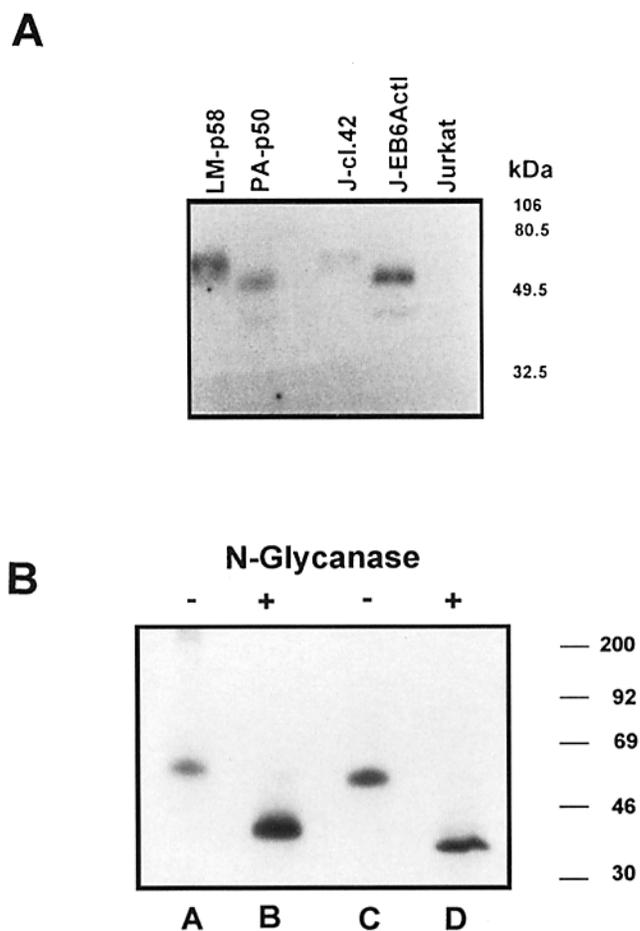
Figure 4. Biochemical analysis of p58/p50 molecules expressed by cell transfectants. (*A*) Immunoprecipitation of p58/p50 molecules from Jurkat cells. Jurkat cells untransfected or transfected with RSV.5gpt/cl.42 and RSV.5gpt/EB6-ActI were lysed in 1% NP-40 lysis buffer and immunoprecipitated with EB6 mAb coupled with CNBr-Sepharose. Samples were size fractionated in an 8.5% SDS-PAGE and analyzed by Western blot with ¹²⁵I-EB6 mAb. Cell lysates of a p58⁺ NK clones (*LM*) and a p50⁺ NK clone (*PA*) were used as controls. On the right vertical side of the figure, the position of molecular mass standard is reported. (*B*) N-glycanase digestion. EB6 mAb immunoprecipitates from surface ¹²⁵I-labeled transfectants. Lanes *A* and *B* are from RSV.5gpt/cl.42, and lanes *C* and *D* are from RSV.5gpt/EB6-Act-I transfectants, respectively. Samples undigested (lanes *A* and *C*) or digested with N-glycanase (lanes *B* and *D*) were analyzed in an 8% SDS-PAGE under nonreducing conditions. On the right vertical side of the figure, the position of molecular mass standard is reported.

domain. Both 183-ActI– and EB6-ActI–encoded proteins displayed a 39–amino acid cytoplasmic tail. Moreover, they shared four unique residues in their transmembrane portions (one represented by Lys) not present in molecules characterized by the 76/84–AA tails.

Biochemical Analysis of p50/p58 Molecules Expressed by Jurkat Cell Transfectants. To characterize further the p50 or p58 molecules encoded by EB6-ActI and EB6-cl42 cDNAs and reactive with EB6 mAb, stable transfectants were generated in Jurkat cells using RSV.5gpt constructs. Both transfectants were brightly stained by EB6 but not by GL183 mAb (Fig. 2 *B*). In addition, EB6 mAb immuno-

precipitated 50-kD molecules from EB6-ActI cell transfectants and 58-kD molecules from EB6-cl42 transfectants (Fig. 4 *A*). In addition, N-glycanase digestion resulted in molecules of 36 and 42 kD, respectively, in agreement with previous data on EB6 activatory or inhibitory molecules expressed by NK cell clones (8) (Fig. 4 *B*).

These data indicate that the HLA-C–specific NK receptors that mediate either cell triggering or inhibition display a high sequence homology in their extracellular domains, but not in their transmembrane or cytoplasmic portions. Therefore, receptors with identical specificity and mAb reactivity may transduce signals of opposite sign because of structural differences in the portions involved in signal transduction.

The finding that 11 amino acid residues in the extracellular domains are unique to EB6 or GL183 receptors may be related to the different HLA-C allele specificity and mAb reactivity. On the other hand, the activatory and inhibitory receptors reacting with the same anti-p58 mAb only differed for 2–7 residues in their extracellular domains. In contrast, major differences existed in their transmembrane and cytoplasmic portions. Thus, only the transmembrane portion of the activatory receptor contained a polar (Lys) residue, in association with a 39–amino acid intracytoplasmic tail. This residue in the transmembrane portion of p50 molecules represents a potential site of association with proteins involved in signal transduction. The inhibitory form is characterized by a 76–84–amino acid tail containing an antigen receptor activation motif (ARAM)-like motif characterized by tandem Tyr-X-X-Leu sequences spaced by 26 amino acids. Notably, in the typical ARAMs present in the TCR-associated CD3 chains, the Tyr-X-X-Leu sequences are spaced by 6–8 amino acid residues (11). In contrast, the 39–amino acid form did not contain the ARAM-like motif because of an in-frame stop codon within the first Tyr-X-X-Leu sequence. These data suggest that different molecules involved in signal transduction may be associated to the activatory or inhibitory HLA-C–specific receptors. It will be of interest to analyze whether NK receptors specific for other MHC class I molecules display similar structural characteristics. In this context, differing experimental evidence in rat and mouse suggests that MHC-class I–specific receptors may also deliver triggering signals (12, 13). In addition, signaling via CD94, the putative Bw6-specific human NK receptor, resulted in either NK cell triggering or inhibition (14).

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