Identification and Molecular Cloning of p75/AIRM1, A Novel Member of the Sialoadhesin Family That Functions as an Inhibitory Receptor in Human Natural Killer Cells

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Summary

In this study, by the generation of a specific monoclonal antibody, we identified p75/AIRM1 (for adhesion inhibitory receptor molecule 1), a novel inhibitory receptor that is mostly confined to human natural killer cells. p75/AIRM1 is a 75-kD glycoprotein that, upon sodium pervanadate treatment, becomes tyrosine phosphorylated and associates to src homology 2 domain–bearing protein tyrosine phosphatase (SHP)-1. The p75/AIRM1 gene is located on human chromosome 19 and encodes a novel member of the sialoadhesin family characterized by three immunoglobulin-like extracellular domains (one NH2-terminal V-type and two C2-type) and a classical immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic portion. The highest amino acid sequence similarity has been found with the myeloid-specific CD33 molecule and the placental CD33L1 protein. Similar to other sialoadhesin molecules, p75/AIRM1 appears to mediate sialic acid–dependent ligand recognition.

Key words: molecular cloning • natural killer cells • sialoadhesin • inhibitory receptor • immunoglobulin superfamily

Relevant progress has been made in recent years towards a better understanding of the molecular interactions responsible for the regulation of NK cell functions. In particular, the finding that NK cells are capable of killing target cells lacking MHC class I proteins on their surface (1) has been the basis for the identification of a series of inhibitory receptors present on NK cells that bind MHC molecules expressed on target cells. In humans, these receptors are expressed on subsets of NK cells and recognize either discrete HLA class I alleles or nonclassical HLA class I molecules (1–3). The first group includes members of the immunoglobulin superfamily (Ig-SF)1 that are referred to as killer inhibitory receptors (KIRs). KIRs are characterized by the ability to specifically recognize distinct groups of HLA-A (4), -B (5), or -C (6) alleles. The second group is represented by members of the C-type lectin superfamily that covalently associate to form the CD94/NKG2 receptors (7–9); the inhibitory CD94/NKG2A receptor has been shown to be specific for the nonclassical HLA class I molecule, HLA-E (3, 10). More recently, several additional inhibitory receptors, either HLA class I specific or not, have been identified that may exert a regulatory activity on human NK cell function; these include, for example, the leukocyte Ig-like receptor 1/Ig-like transcript 2 (LIR-1/ILT-2 [11, 12]) and the p40/leukocyte-associated Ig-like receptor 1 (LAIR-1) (13, 14) molecules. All of the inhibitory receptors identified so far are characterized by one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail. Upon tyrosine phosphorylation, ITIM binds the src homology (SH)2 domains of phosphatases such as SHP-1 and SHP-2 that, in turn, cause downregulation of NK cell triggering and cytotoxicity (15).

In an attempt to identify novel receptors possibly involved in the negative regulation of NK cell function, we immunized mice with human NK cell clones and screened for mAbs capable of modulating the NK-mediated cytoly-sis. Using this approach, we selected an mAb (Z176) specific for a novel inhibitory receptor molecule of ~75 kD (p75) termed adhesion inhibitory receptor molecule 1 (AIR M1). p75/AIR M1 is expressed on the majority of human NK cells. Molecular cloning revealed a novel member of the Ig-SF characterized by three Ig-like domains in the extracellular portion and an ITIM in the cytoplasmic tail.

*Abbreviations used in this paper: aa, amino acid(s); AIR M1, adhesion inhibitory receptor molecule 1; Ig-SF, immunoglobulin superfamily; ILT, Ig-like transcript; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer inhibitory receptor; LIR, leukocyte Ig-like receptor; MAG, myelin-associated glycoprotein; RT, reverse transcriptase; SH, src homology domain; SHP, SH2 domain–bearing protein tyrosine phosphatase.

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Although p75/AIR1 M does not recognize HLA class I molecules, its amino acid (aa) sequence, together with functional data, suggests that it represents a novel member of the sialoadhesin family. In conclusion, p75/AIR1 M is a novel, HLA-independent, inhibitory receptor that may play a relevant role in the negative regulation of human NK cell functions.

Materials and Methods

mAbs. Z176 (IgG2b) mAb was obtained by immunizing a 5-wk-old BALB/c mouse with the NK clone SA260 (surface phenotype: CD3+CD16+CD56-, Nkp46+, Nkp44+, p70/ NKB1+, CD94/NKG2A+) as described previously (16). The following mAbs were produced in our laboratory: JT3A (IgG2a, anti-CD3), BAB281 (IgG1, anti-NKp46), Z231 and KS38 (IgG1 and IgM, respectively, anti-NKp44), Z199 (IgG2b, anti-NKG2A), Z27 (IgG1, anti-p70/NKB1), K1D and c127 (IgG2a and IgG1, respectively, anti-NKp44), Z28 (IgG2b, anti-NKG2A), Z199 (IgG2b, anti-NKp46), Z231 (IgG1, anti-NKp44), and Z199 (IgG2b, anti-NKG2A). The MCA531 mAb (IgM, anti-CD20) was purchased from Serotec. The D1.12 mAb (IgG2a, anti-HLA-DR) was provided by Dr. R.S. Accolla (Università di Pavia, Pavia, Italy). The HP2.6 mAb (IgG2a, anti-CD16) and c218 and A6-90 (IgG1 and IgM, respectively, anti-NKp44), Z27 (IgG2b, anti-NKG2A), Z28 (IgG1, anti-NKp44) and Z231 (IgG1, anti-NKp46) were produced in our group.

Purification of Polyclonal or Clonal NK and T Cell Populations.

To obtain PBLs, PBMCs derived from healthy donors were isolated on Ficoll-Hypaque gradients and depleted of plastic-adherent cells. PBLs were either used directly or were incubated with anti-CD3 (JT3A), anti-CD4 (HP2.6), and anti-HLA-DR (D1.12) mAbs for 30 min at 4°C, followed by immunomagnetic depletion with goat anti–mouse coated Dynabeads (Dynal, 30 min, 4°C; reference 17). NK or T cell clones were obtained by limiting dilution in the presence of irradiated feeder cells, 1.5 ng/ml PHA (GIBCO BRL), and 100 U/ml IL-2 (I-2; Immunotech). The set of primers AIRM1-up (5'-GTC AGT TTG TAT GCT G) and AIRM1-down (designed in the 3'-untranslated region; 5'-ACA AGC CCG AGC CTC TGC) were used to amplify the AIRM1-transfected COS-7 cells by DEAE-dextran method and immunofluorescence staining as described previously (2). The amplification products obtained were separated in 10 pools of 200,000 different inserts, was transfected into COS-7 cells by DEAE-dextran method and immunocytochemical staining using the Z176-specific mAb and sb selection (21).

DNA Sequencing. DNA sequencing was performed using d-R-hodamine Terminator Cycle Sequencing kit and a 377 ABI automatic sequencer (Perkin Elmer-Applied Biosystems).

Identification and Cellular Distribution of a Novel Surface Molecule with Inhibitory Function.

Cytotoxicity Assays. NK cell cytotoxicity was determined by standard 4-h 51Cr-release assay as described previously (2). The E/T ratio used was 8:1 in all instances. The amplification products obtained were separated in 10 pools of 200,000 different inserts, was transfected into COS-7 cells by DEAE-dextran method and immunocytochemical staining using the Z176-specific mAb and sb selection (21).

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CD3⁻CD16⁺, CD56⁺, NKP46⁺, NKP44⁺, p70/NKB1⁺, CD54/NKG2A⁺), characterized by a strong cytolytic activity against the P815 murine mastocytoma cell line. After cell fusion, mAbs were analyzed for their ability to inhibit the cytotoxicity mediated by NK cell clones in a classical redirected killing assay against the FcγR⁺ P815 cell line. By using this screening procedure, we isolated the Z176 mAb (IgG2b) that inhibited the cytolytic activity of the majority of the NK cell clones analyzed. Fig. 1 shows four representatives of such clones, including the immunizing SA260 clone. In three of these clones, the addition of Z176 mAb (but not of an isotype-matched anti-CD56 mAb) resulted in inhibition of the spontaneous cytolytic activity of the majority of the NK cell clones analyzed (Fig. 1). Clone D414 is representative of the infrequent NK cell clones in which no inhibitory effect could be detected. Immunofluorescence and FACS analysis of the same clones revealed that Z176 mAb reacted with clones SA260, LM15, and LM8 but not with clone D414. Similar data were obtained in a large panel of NK cell clones, thus suggesting that the Z176 mAb-reactive molecule is expressed and delivers inhibitory signal in the majority of, but not all, human NK cells.

We next analyzed the cell surface distribution of the Z176 mAb-reacting molecule in PBLs by two-color immunofluorescence and FACS analysis. As shown in Fig. 2, all CD56⁺ cells were stained by the Z176 mAb. In contrast, Z176 mAb did not stain CD3⁺ T cells or CD20⁺ B cells. Only in some individuals was a minor fraction (<5%) of T cells found to be Z176⁺ (see donor B). Although not shown, further analysis of the Z176 surface expression in NK cell–enriched fractions of PBLs (upon depletion of CD3⁺HLA-DR⁺ cells) confirmed that most NK cells reacted with Z176 mAb while Z176⁺ NK cells were rather infrequent. Analysis of a large panel (>100) of T cell clones showed that Z176-reactive molecules are not expressed by T lymphocytes after cell activation or clonal expansion (not shown). Finally, Z176 mAb did not stain EBV-transformed B cell lines (including Raji, Daudi, and C1R) or T cell lines (such as JA3, HSB-2, CEM, MOLT-4, H9, and Jurkat). Remarkably, it also failed to stain NK cell lines, including NK3.3, NKL, and YT (not shown).

Biochemical Characterization of the Surface Molecule Recognized by Z176 mAb. A polyclonal NK cell population was surface labeled with ¹²⁵I and immunoprecipitated with Z176 mAb. This antibody immunoprecipitated a molecule with a molecular mass of ~75 KD (p75) both under nonreducing (not shown) and reducing conditions (Fig. 3). To analyze their glycosylation pattern, p75 molecules were treated with different enzymes. The molecular mass was...
not modified by treatment with O-glycosidase (not shown). In contrast, digestion with N-glycosidase revealed a protein backbone of ~48 kD (Fig. 3 a), thus suggesting a relatively high N-glycosylation pattern. Finally, in two-dimensional peptide mapping (2DPM) analysis, p75 displayed a digestion pattern that was different from those of known inhibitory receptors, including p70/NKB1 and NKG2A (not shown). Altogether, these data support the notion that p75 is a novel surface molecule with inhibitory function expressed by the majority of human NK cells.

It is well known that inhibitory receptors, including p58 (CD158), p70/NKB1, CD94/NKG2A, and LIR-1/ILT-2, are characterized by the presence of ITIM sequences in their cytoplasmic tail. ITIMs, upon tyrosine phosphorylation, recruit SH2-containing phosphatases such as SHP-1 and SHP-2. To assess whether the p75 molecule could also belong to the ITIM-bearing receptor family, a polyclonal NK cell population, treated or not with sodium pervanadate, was immunoprecipitated with Z176 mAb. Samples were probed with antiphosphotyrosine, anti–SHP-1, or anti–SHP-2 mAbs. Fig. 3 b shows that treatment with sodium pervanadate leads to p75 tyrosine phosphorylation (left panel) and association with SHP-1 (right panel). It is of note that, under the same conditions, no association with SHP-2 could be detected (not shown). These data strongly suggest that p75 contains at least one typical ITIM in the cytoplasmic tail.

Molecular Cloning and Characterization of the cDNA Encoding the p75 Molecule. A cDNA library, prepared from RNA derived from 2 polyclonal NK cell populations, fractionated in 10 different pools, was transiently transfected into COS-7 cells. After 48 h, cells were tested for reactivity with Z176 mAb by immunocytochemical staining. The plasmidic DNA of the positive pool was amplified in Escherichia coli, fractionated in smaller subpools, and transfected into COS-7 cells. Six rounds of transfections and screening allowed the isolation of an individual cDNA termed AIRM1. As shown in Fig. 4 a, COS-7 cells transfected with VR1012–AIRM1 construct were brightly stained with Z176 mAb in cytofluorimetric analysis. Cell transfectants were then surface labeled with 125I, and cell lysates were immunoprecipitated with Z176 mAb. As shown in Fig. 4 b, the immunoprecipitated molecule displayed a molecular mass slightly lower than that immunoprecipitated from NK cells. This difference in molecular mass could reflect a non-
complete N-glycosylation in COS-7 cell transfectants. Indeed, upon treatment with N-glycosidase, the molecule immunoprecipitated from AIR M1 transfectants displayed a 48-kD protein backbone identical to that of the p75 molecule isolated from N K cells.

Fig. 5 shows the nucleotide sequence (1766 bp) of AIR M1 and its predicted amino acid translation (467 aa). The 5’ noncoding region consists of 64 nucleotides, and in the 3’ noncoding sequence a possible polyadenylation signal (AAUAUA) preceded the poly A tail. The putative protein appears as a type I transmembrane molecule belonging to the IgSF. An 18 aa leader peptide precedes a 335 aa extracellular portion characterized by an NH₂-terminal V-type domain followed by two C2-type domains. Six putative O-glycosylation sites and eight putative N-glycosylation sites are present in the extracellular portion. The predicted polypeptide mass is ~48 kD, thus corresponding to the protein backbone of the p75 molecule immunoprecipitated by Z176 mAb from both NK cells and p75/AIRM1 COS-7 transfectants.

The 23 aa hydrophobic transmembrane portion is followed by a cytoplasmic tail (91 aa) containing two tyrosine residues. Remarkably, Tyr 437 is part of a typical ITIM motif (435–440 IQYAPL). Finally, other consensus sequences for putative phosphorylation sites are present in the cytoplasmic tail: a 3’–5’ adenosine monophosphate (cAMP)-dependent protein kinase phosphorylation site, three protein kinase phosphorylation sites, and five casein kinase 2 (CK2) phosphorylation sites.

Comparison of the aa sequence of p75/AIRM1 with those of known proteins in the EMBL/GenBank/DDBJ database revealed significant similarity with the placenta antigen CD33L1 (24), as well as with the myeloid lineage molecule CD33 (25) (Fig. 6). In particular, both the IgV domain and the transmembrane region of p75/AIRM1 display a high degree of aa identity (55 and 61%, respectively) with CD33 molecule. On the other hand, the IgC2a and IgC2b domains display a remarkable similarity with those of CD33L1. In this context, the highest degree of aa identity (73%) was found between the IgC2a domain of p75/AIRM1 and that of CD33L1. The IgV and IgC2a domains of p75/AIRM1 contain two Cys residues at position 41 and 174. Cys residues located at the same relative positions are also present in different sialoadhesins (26, 27) and are likely to be involved in the formation of interdomain bridges. Altogether, these results suggest that the p75/AIRM1 molecule may represent a novel member of the sialoadhesin family.

Figure 4. Surface expression and biochemical analysis of Z176-reactive molecules in COS-7 cells transfected with VR1012–AIRM1 construct. (a) AIRM1-transfected COS-7 cells were analyzed for surface expression using 2176 mAb followed by PE-conjugated isotype-specific goat anti-mouse second reagent. The open profile represents cells stained with the second reagent alone. (b) A polyclonal NK cell population (lanes A and B) and AIRM1-transfected COS-7 cells (lanes C and D) were surface labeled with °¹²⁵I and immunoprecipitated with 2176 mAb. Samples were analyzed in an 8% SDS-PAGE under reducing conditions either undigested (−) or digested (+) with N-glycosidase. Molecular weight markers (in kD) are indicated on the right.

Figure 5. AIR M1 cDNA nucleotide sequence and its predicted aa translation. aa included in the signal peptide are indicated in small letters. Cysteine residues are circled. The predicted transmembrane portion is underlined. Potential N- and O-glycosylation sites are boxed. The ITIM sequence is double underlined. The AIR M1 nucleotide sequence is available from EMBL/GenBank/DDBJ under accession no. AJ007395.
Cloning of a Novel Sialoadhesin Member Expressed by NK Cells

analyzed with a 1203-bp AIR M1-specific probe. We could localize the AIR M1 gene on human chromosome 19, since only the somatic cell hybrids containing this chromosome were positive in Southern blot analysis (Fig. 7). Moreover, the simple pattern of hybridization observed suggests that AIR M1 is a single gene or a few copy genes. It is of note that other members of the sialoadhesin family, including CD33 (28) and CD33L1 (24), myelin-associated glycoprotein (MAG [29]), CD22 (30), and Siglec-5 (31), have been mapped on human chromosome 19.

Reverse transcriptase (RT)-PCR analysis was performed on polyclonal NK cell populations and clones, derived from five different donors. Using the set of primers AIRM1-up and AIRM1-down, we could detect, not only in polyclonal populations but also in NK cell clones, three amplified products of 1.4, 1.1, and 0.7 kb, respectively (not shown). These cDNA fragments were subcloned and sequenced. The 1.4-kb product was found to correspond to the AIRM1 cloned cDNA. Moreover, the sequence analysis, performed in five different donors, revealed only an allelic variant of AIRM1 cDNA (termed AIRM1b; sequence data available from EMBL under accession no. AJ130710) characterized by two silent substitutions (codon 105, ACC instead of AAT; and codon 452, GGT instead of GGA).

Discussion

In this study, we describe a novel inhibitory receptor, termed p75/AIRM1, which is expressed by resting and activated human NK cells and may play a role in the regulation of their function. Molecular and functional analysis revealed a novel member of the sialoadhesin family; thus, p75/AIRM1 differs from the other major inhibitory receptors expressed by NK cells that are characterized by their specificity for MHC class I molecules.

p75/AIRM1 is a transmembrane glycoprotein characterized by one IgV and two IgC2-type Ig-like domains in the extracellular portion that displays similarity with members of the sialoadhesin family. The only molecule reported to display inhibitory function (i.e., similar to p75/AIRM1) is CD22 that is expressed on B cells, in which it may downregulate the B cell receptor–mediated cell triggering. CD33 is selectively expressed by hemopoietic cells, in which it represents an important marker in normal dif...
differentiation as well as in leukemia typing. In view of the similarity with p75/AIRM1, which includes the presence of an ITIM sequence in the cytoplasmic tail, it is important to reinvestigate the role of CD33, especially with respect to its possible inhibitory effect on hemopoietic cell function and/or differentiation. Experiments along this line are in progress in our laboratory.

p75/AIRM1 is encoded by a gene localized on human chromosome 19. Remarkably, genes coding for other members of the sialoadhesin family map on chromosome 19 as well. This may suggest that all of these molecules, including p75/AIRM1, may have evolved through duplication of a common ancestral gene. It is of note that other inhibitory receptors involved in the regulation of NK-mediated cytotoxicity, including KIRs, LIR/ILT, and LAIR-1, have also been mapped on this chromosome. Although not shown, the AIRM1-specific probe, under low stringency conditions, hybridized with genomic DNA from Rhesus monkey, thus suggesting a cross-species conservation between humans and monkeys.

DNA sequencing of seven cDNA clones obtained by RT-PCR experiments, performed in five different donors, revealed only an allelic variant characterized by two silent substitutions. However, considering both the relatively low number of samples analyzed and the fact that all donors belonged to the Caucasian race, a polymorphism of the p75/AIRM1 gene cannot be ruled out. RT-PCR analysis, in addition to the 1.4-kb AIRM1 transcript, also allowed the identification of two alternatively spliced products of 1.1 and 0.7 kb, respectively. Sequence of these products revealed that the 1.1-kb fragment encoded a putative protein identical to the p75/AIRM1 but lacking the IgC2a domain (from codon 146 to codon 238) (termed AIRM2; sequence data available from EMBL under accession no. AJ130711; manuscript in preparation). The 0.7-kb amplified product contains two cDNA fragments that are both carrying an early stop codon at position 146 (EMBL accession nos. AJ130712 and AJ130713).

Consistent with the structural similarity between p75/AIRM1 and other sialoadhesins, p75/AIRM1 was also found to bind RBCs. This binding was specifically inhibited by Z176 mAb as well as by the neuraminidase treatment of RBCs. These data suggest that p75/AIRM1 may function as a receptor which recognizes its putative ligand(s) in a sialic acid-dependent manner. Carbohydrate-binding proteins are known to play a role in a wide variety of biological processes involving specific cell-cell interactions. As suggested for CD22 (41), it is possible that sialic acids may be required for correct orientation and presentation of the epitope (on the ligand) recognized by p75/AIRM1.

Cross-linking of p75/AIRM1 in human NK cells delivers an inhibitory signal resulting in downregulation of spontaneous NK cell-mediated cytotoxicity. An inhibitory effect could also be detected on the NK cell triggering mediated by activating receptors such as CD16, NKp46, and NKp44 (not shown). Coherent with its ability to mediate inhibition, the cytoplasmic tail of p75/AIRM1 was found to contain an ITIM that recruited the SHP-1 phosphatase upon tyrosine phosphorylation. This, in turn, is likely to...
inhibit downstream molecular events that are critical for the induction of NK cell-mediated cytotoxicity. One may ask the meaning of the inhibitory effect of p75/AIRM1 in NK-mediated function, and what could be the functional relationship with KIRs. KIRs appear to play a predominant role in the discrimination between HLA class I molecules and cells that do not express sufficient amounts of HLA class I, such as tumor- or virus-infected cells. Since p75/AIRM1 does not appear to recognize HLA class I molecules, it is possible to speculate that this receptor may play a role in recognition of still undefined sialylated proteins, possibly present in normal cells that physiologically express low amounts of HLA class I molecules. The expression of ligand(s) for p75/AIRM1 may protect these cells from the NK-mediated attack. It is evident that the identification of the p75/AIRM1 ligand(s) will greatly help to clarify this issue. In addition, it is possible that p75/AIRM1 may function during stages of NK cell differentiation from immature precursors in which cells have acquired cytolytic potential but have not yet expressed HLA class I-specific inhibitory receptors. Indeed, cells with these phenotypic characteristics have recently been identified in our laboratory (our unpublished data).

In conclusion, we have identified, characterized, and cloned a novel inhibitory receptor primarily confined to human NK cells which may play a role complementary to that of KIRs in the regulation of NK cell function.

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