A new human gene complex encoding the killer cell inhibitory receptors and related monocyte/macrophage receptors

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Activation of various immune cell types can be prevented by negative signaling receptors. Natural killer (NK) cells, which can lyse tumor or virus-infected cells, express inhibitory receptors that recognise distinct 'self' class I molecules of the major histocompatibility complex [1]. Recognition of self class I molecules results in a negative signal to prevent NK-mediated killing of healthy cells [2]. Human and mouse NK cells express both immunoglobulin-like type I inhibitory receptors – such as the human killer cell inhibitory receptor (KIR) and the mouse gp49B glycoprotein – and lectin-like type II inhibitory receptors – such as the human CD94/NKG2 heterodimer and the mouse Ly-49 receptor family [1]. These receptors use tyrosine phosphorylation motifs in their cytoplasmic tails to deliver a dominant-negative signal by recruiting the tyrosine phosphatase SHP-1 [3–5]. We have identified a new family of monocyte/macrophage immunoglobulin-like receptors (MIRs) related to KIR. Two cDNA clones with sequence similarity to each other and to the gp49B gene were isolated from human lymphocytes; both encode proteins containing four immunoglobulin domains and the conserved cytoplasmic inhibitory motifs, and transcription of both was detected primarily in monocytes/macrophages, rather than T, NK, or mast cells. The MIR genes are closely linked to the KIR gene family and the gene for FcαR on chromosome 19, at cytogenetic band q13.4. A mouse sequence related to MIR was mapped to a region on mouse chromosome 7 syntenic with human 19q13.4. Our findings should facilitate studies of the evolution and function of the MIR and KIR families.

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Results and discussion

We investigated whether gp49B-related sequences are expressed in human lymphocytes. A search of human expressed sequence tags (ESTs) revealed a 215 bp EST (GenBank accession number T73907) which encodes a stretch of 52 amino acids with 46% sequence identity to gp49B [6]. A larger fragment (clone-4) extending this EST was amplified by 5′ rapid amplification of cDNA ends (RACE) using the polymerase chain reaction (PCR), and used to screen a human lymphocyte cDNA library. Two clones, cl-7 and cl-10, were sequenced completely (GenBank accession numbers AF004230 and AF004231). The nucleotide sequences of the three cDNAs are 74–83% identical to each other; cl-7 and cl-10 encode type I transmembrane proteins with four immunoglobulin domains of the C-2 type, transmembrane regions of 23 amino acids, and cytoplasmic tails of 168 and 115 amino acids, respectively. The amino-terminal immunoglobulin domains of the cl-7 and cl-10 proteins are most closely related to regions of mouse gp49B (41–43% identity). GenBank searches with the extracellular portion of the mature proteins indicated, however, that the newly described p91 or PIR (paired immunoglobulin-like receptor) mouse protein, which contains six immunoglobulin domains [7,8], is the closest relative (63% identity with the third immunoglobulin domain of the cl-7 protein). The next closest relatives of the proteins encoded by cl-7 and cl-10 are bovine Fcγ2R and human KIR.

The cytoplasmic tails of the cl-7 and cl-10 proteins contain tyrosine phosphorylation motifs similar to those in several

Figure 1

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  KIR    HRWCSNKKKNAAV............14.............DSDEQDPQEVTYAQLDHCVF..14..PPTDTIVYTELPNA..10..
gp49B    GYQYGHKKKANAS..19..EDPQGIVYAQVKPSR..3...DTACKETQDVTYAQLCIRTQ..5..

  p91    .44.QEESLYASV..19..EDPQGETYAQVKPSR..33..AAESGESQDVTYAQLCSRTL..14..APEEPSVYATLAAA..11..
 cl-7    .44.QEENLYAAV..18..EDPQAVTYAEVKHSR..33..AAASEAPQDVTYAQLHSLTL..14..SPAVPSIYATLAIH
cl-10    .44.QEENLYAAV............14.............AAASEAPQDVTYAQLHSLTL..14..PPAEPSIYATLAIH
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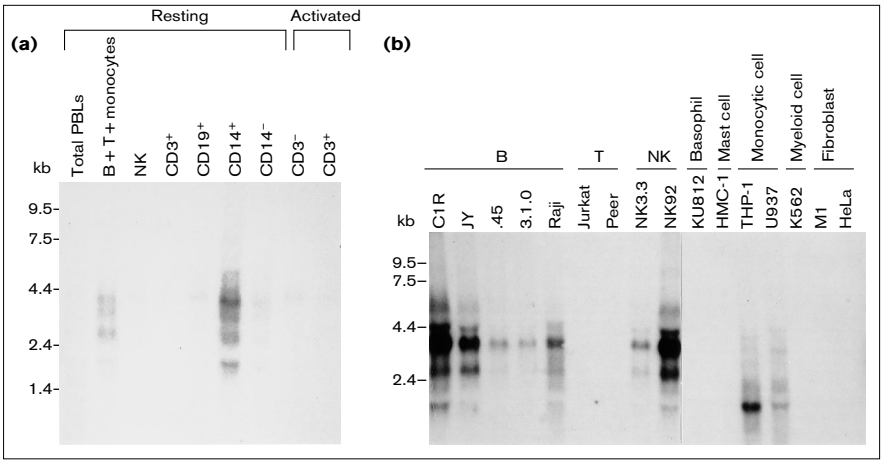
The cytoplasmic tails of the proteins encoding cl-7 and cl-10 share conserved sequence motifs with inhibitory receptors. Amino-acid sequences of the cl-7 and cl-10 cytoplasmic tails were aligned with those of mouse p91/PIR, human KIR, and mouse gp49B inhibitory receptors. Dark and light shading indicate identity between and within the two groups of receptors, respectively. The complete cytoplasmic tails are shown, with the spaces between conserved regions given as numbers of amino acids.

Figure 2



The cl-7 and cl-10 genes are preferentially expressed in monocytes. **(a)** Northern blot of RNA isolated from the indicated subsets of resting or activated cells (3 weeks in 100 units per ml rIL-2), purified by incubation with antibodies conjugated to magnetic beads and separation on MACS columns (Miltenyi Biotec). The probe was a fragment containing most of the coding region of cl-7. The final wash was in 0.2× SSC at 68°C. **(b)** Northern-blot analysis of cl-7 expression in the indicated human cell lines using the same probe as in (a). The final wash was in 2× SSC at 68°C.

receptors known to have inhibitory functions, including KIR, gp49B, Ly-49, and NKG2 [3,5,6,9,10]. Notably, the cytoplasmic tails of the cl-7 and cl-10 proteins contain the amino-acid sequence QDVTYAQL (in single letter code) found in the mouse NK inhibitory receptor gp49B and in the p91/PIR molecule (Figure 1). Members of the KIR family contain the sequence QEVTYAQL and a second, related, tyrosine phosphorylation motif with the consensus sequence V/IxYxxL, in which x is any amino acid (Figure 1). Phosphotyrosine residues in these motifs recruit and activate the tyrosine phosphatase SHP-1 [3,9,11], which is crucial to the inhibition of NK-mediated lysis [3]. The specificity of the binding of SHP-1 to phosphorylated KIR is determined by residues around the tyrosine, with the amino acid at position –2 being particularly important [12]. The presence of the conserved QDVTYAQL motif in the cl-7 and cl-10 proteins, and of an additional VxYxxV motif in the cl-7 protein, suggests that these proteins are inhibitory receptors. Additional sequence conservation in the cytoplasmic tails of these receptors is evident (Figure 1). Recently, a related molecule (ILT3) that has two immunoglobulin domains was shown to inhibit macrophage activation when co-ligated with stimulatory receptors [13].

The cl-7 and cl-10 proteins have unusually long stems, of about 30 amino acids, connecting the membrane-proximal immunoglobulin domain to the transmembrane region. KIR, p91, and gp49B have stem regions of 17, 16, and 13 amino acids, respectively. Remarkably, about 50% of the stem of KIR, p91, cl-7 and cl-10 is composed of prolines or serines. These stems, which are absent from the related but non-inhibitory FcαR and Fcγ2R receptors, contribute to the definition of a new subfamily of inhibitory receptors within the immunoglobulin superfamily.

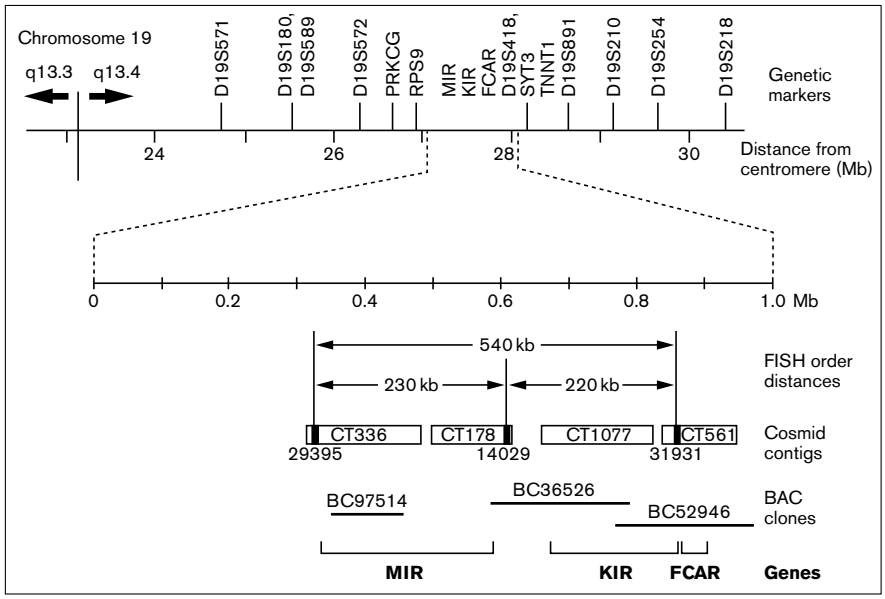
Hybridization of a cl-7 probe to a northern blot of human tissues revealed multiple mRNA species primarily in peripheral blood lymphocytes (PBLs) and lung (data not shown). Freshly isolated PBLs were sorted using specific monoclonal antibodies and magnetic separation, and RNA was extracted from each subset. Comparison of resting $CD19^+$ B cells, $CD3^+$ T cells, $CD3^-$ NK cells and $CD14^+$ monocytes revealed that the $CD14^+$ subset accounted for most of the hybridization signal (Figure 2a). $CD14^+$ cells in the periphery are primarily monocytes but this class also includes macrophages and some dendritic cells. Weak hybridization signals were detected in other subsets of cells on longer exposures; these signals may represent either low-level expression or contamination of the subsets by monocytes.

The cl-7 probe hybridized to mRNA from several monocytic and B cell lines, and from the NK cell lines NK3.3 and NK-92 (Figure 2b). Sequences (called ILT1 and ILT2) related to cl-7 and cl-10 were recently described that are expressed in several cell lines of the monocytic, NK, and B-cell lineages [14]. As shown here with normal cells isolated from peripheral blood, however, a strong hybridization signal with cl-7 was detected in the $CD14^+$ subset, but not in either resting or activated T cells or NK cells. Furthermore, using a PCR assay, cl-7 or cl-10 expression was detected in only one out of 23 randomly selected NK clones (data not shown), supporting the conclusion that $CD3^-$ NK cells generally do not express cl-7 or cl-10 sequences. A single-strand conformation polymorphism assay was used to show that sequences corresponding to cl-7 and cl-10 were expressed in the $CD14^+$ populations (data not shown). We conclude from our data that sequences corresponding to cl-7 and cl-10 are preferentially expressed in cells of the monocytic lineage; we therefore refer to them as monocyte/macrophage immunoglobulin-like receptors (MIRs).

On Southern blots, the cl-7 probe cross-hybridized at intermediate stringency (2× SSC, 68°C) with a few fragments in

Figure 3

Physical linkage of MIR and KIR genes. Diagram of the physical metric map of human chromosome 19q13.4 showing the location of the MIR, KIR, and FCAR genes. Indicated distances are from the centromere–19q euchromatin boundary (in Mb). FISH, fluorescent *in situ* hybridization; BAC, bacterial artificial chromosome.

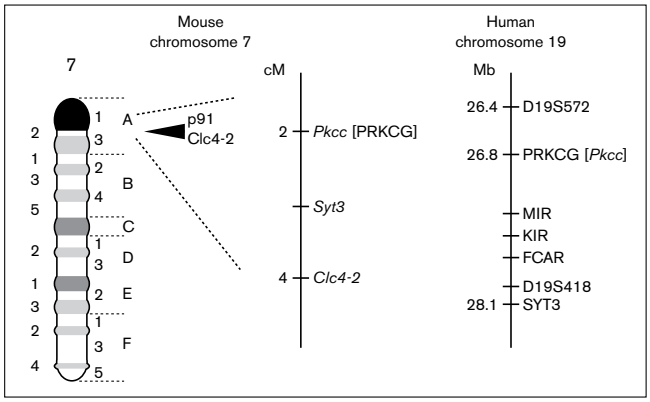


mouse genomic DNA (data not shown). This result contrasts with results using the mouse gp49B gene and human KIR genes, which do not cross-hybridize to sequences in the other species, even at low stringency. Human MIRs and mouse p91/PIR have 68% nucleotide identity over a 741 bp stretch. A mouse p91 probe hybridized to the same bands in mouse DNA and to a subset of the bands in human DNA as hybridized with cl-7 (data not shown). The cl-7 probe hybridized to several fragments in human DNA digested with various enzymes, indicating that there is a small family of genes related to cl-7 (data not shown), which would be consistent with the fact that a number of MIR cDNA clones — corresponding to five genes — have been described so far (this study and [13,14]).

Human KIR family genes are located on chromosome 19, within the q13.4 region [15]. PCR analysis of DNA derived from a panel of hamster/human cell lines showed that the MIR sequences are also on chromosome 19 (data not shown). The exact location of KIR and MIR genes was determined by hybridization to arrayed cosmids derived from chromosome 19; both KIR and MIR genes mapped to cosmids in contigs which had previously been mapped to 19q13.4 [16]. The MIR genes were centromeric relative to the KIR genes, which in turn were centromeric relative to the gene for FcαR (FCAR; Figure 3). The genes encoding the MIRs, KIRs and FcαR map within approximately 600 kb and define a new gene complex encoding structurally related receptors.

The failure to identify mouse KIR gene homologs has long been an enigma. The linkage of the human MIR and KIR genes, and the existence of the MIR-related mouse p91/PIR sequences gave us an opportunity to test whether there is a mouse genomic region syntenic to the human genomic region containing the MIR genes. A genomic p91/PIR bacterial artificial chromosome (BAC) clone was isolated and used to determine the chromosomal location by fluorescent *in-situ* hybridization (FISH) mapping. The results revealed that p91/PIR sequences are located on band A2 of mouse chromosome 7 (Figure 4), in agreement with a recent report [8]. Another gene mapped to 7A2 using FISH is *Clc4-2*, which is 4 cM from the centromere (Mouse Genome Database at http://www.informatics.jax.org).

Figure 4



Mouse p91 genes map to a chromosomal region that is syntenic to the human region encoding MIRs and KIRs. The region of mouse chromosome 7 that contains *Pkcc* and *Syt3* is inverted relative to the rest of the synteny between mouse chromosome 7 and human chromosome 19. Distances from the centromere are indicated in cM (mouse) or Mb (human).

Interestingly, the PRKCG and SYT3 genes that flank the human MIR–KIR–FCAR gene complex have orthologs on mouse chromosome 7 [17], near *Clc4-2*. This region on mouse chromosome 7 is syntenic with the human 19q13.4 region [17], which contains the KIR–MIR gene complex. It is therefore likely that the mouse p91/PIR genes map between *Pkcc* and *Syt3*.

The family of type I transmembrane, immunoglobulin-like inhibitory receptors has been expanded by the identification of the MIR genes expressed in human monocytes. This receptor family now comprises the human KIR and MIR and the mouse gp49B and p91/PIR molecules. These receptors all have conserved tyrosine phosphorylation motifs in their cytoplasmic tails which have been implicated in negative signaling. Effector cells of the innate immune system, such as mast cells, monocytes and macrophages, and NK cells, may all be negatively regulated by such inhibitory receptors.

Materials and methods

Isolation of cDNA clones and hybridization

The oligonucleotide 5′-AGAATGAAGCCGCCAAATGCCAC, specific for nucleotides 116–138 of the EST yc54d09.r1 (GenBank accession number T73907), was used as reverse PCR primer in a 5′ RACE reaction containing human PBL cDNA and the forward adapter primer AP1 (Clontech). This resulted in a 576 bp PCR product called clone-4 (GenBank accession number U70665) [6]. Clone-4 was used to screen a cDNA library made from resting human PBLs (Life Technologies) at high stringency. Two clones (cl-7 and cl-10) with intact open reading frames were completely sequenced. For hybridization, total RNA (5 μg per lane) from human cell lines or from fresh, resting human PBLs or subsets of PBLs, was separated on a 0.8% agarose/formaldehyde gel, transferred to a Genescreen Plus filter using a pressure blotter (Stratagene), and probed with a ^{32}P-labeled *Bgl*I fragment (nucleotides 17–2112) of cl-7.

Chromosomal localizations

The MIR sequences were located to chromosome 19 by PCR analysis using DNA from human/hamster somatic cell hybrids (Bios). The MIR and KIR genes were assigned to cosmids by screening an 11× arrayed cosmid library specific for chromosome 19 as previously described [18]. The physical map of chromosome 19 was created by analyzing cosmids using a fingerprinting strategy [19,20]. Contigs were placed along the linear DNA molecule and estimates of genomic distances were obtained by 2-color, three-cosmid FISH analysis, as described [21,22]. In order to facilitate closure of the gaps among the contigs containing members of the gene family, long-range Alu PCR products [23] from cosmid templates were hybridized against a human BAC library [24]. The extent of overlap of the BAC clones with the previously identified cosmid contigs was confirmed by hybridization and also by construction of an *Eco*RI restriction digest map as described [16]. The overall strategy and a global view of the chromosome 19 metric physical map has been described [16]. An updated version of the map is available at http://www-bio.llnl.gov/bbrp/genome/genome.html. The chromosomal location of mouse p91/PIR was determined by FISH (Genome Systems) using a genomic p91/PIR clone labeled with digoxigenin-conjugated dUTP. Observations of specifically hybridized chromosomes 7 demonstrated that p91 is located immediately adjacent to the heterochromatic region in band 7A2. A total of 80 metaphase cells were analyzed, with 64 exhibiting specific labeling.

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