LIR9, an Ig-superfamily activating receptor, is expressed as a transmembrane and a secreted molecule

Short title:
LIR9, an activating receptor in monocytes

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Abstract

LIRs are immunoglobulin-like receptors that have both activating and inhibitory functions in leukocytes. Here we report the identification of the first LIR family member, LIR9, that is expressed both as a membrane-bound receptor and a secreted molecule. We identified 4 different forms of LIR9, two of which encode transmembrane molecules and two that encode secreted molecules. The transmembrane forms of LIR9 contain a short cytoplasmic domain and a charged arginine residue within the transmembrane region, which is likely to mediate its association with another co-receptor. LIR9 is mostly expressed in myeloid cells including monocytes and neutrophils. Crosslinking of LIR9 on the surface of monocytes induces calcium flux and secretion of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, indicating that LIR9 could play a role in triggering innate immune responses.

Introduction

The LIR family includes 11 members 1 that are mostly expressed in monocytes, macrophages, and dendritic cells, but which can also be present in T, B, and NK cells (for review see 2-4). These receptors are also known as ILTs 5, MIRs 6, or HM and HL clones 7. The LIR family can be subdivided into three groups: transmembrane molecules with two to four immunoreceptor tyrosine-based inhibitory motifs (ITIM) (LIR1, -2, -3, -5, and -8); transmembrane molecules with short cytoplasmic domains, a positively charged arginine residue within the transmembrane domain, and no ITIMs (LIR6 and -7); and a single soluble molecule with no transmembrane domain (LIR4). ITIM-containing LIRs interact with the SH2-domain containing phosphatase SHP-1 8-10 and send inhibitory
signals. LIR6 and LIR7 do not signal by themselves and require association with another cell surface receptor. ILT1/LIR7 associates with the FcR common γ-chain to send an activating signal. Most of the LIRs have unknown ligands; the only exceptions are LIR1, LIR2, and LIR6 which bind to MHC class I molecules.

LIR-4 is the only member in this family that is expressed as a soluble molecule, but its function is still unknown. LIR4 could potentially act as an antagonist of other LIRs by preventing binding of a common ligand to the membrane-bound LIRs. However, we have not been able to detect any binding of LIR4 to MHC class I or any other ligands (our unpublished data) despite the fact that the extracellular domain of LIR4 is 84% identical to LIR1. This has led us to ask whether there is a membrane-bound form of LIR4 or whether other membrane-bound LIRs have soluble versions. To address this question, we performed RACE on leukocyte RNA derived from a large pool of human donors and identified the first LIR family member, LIR9, that is expressed both as a membrane-bound and secreted molecule.

Material and methods

RACE cloning and cDNA constructs. For the RACE template, we used Marathon-Ready Leukocyte cDNA prepared from a pool of 550 human donors (ClonTech, CA). To clone LIR9, we used the following primers to perform 5’ RACE:

5’tcaaggtccaggagcctggctcc3’, 5’gatatgcctgcgagagccatagcatctgagc 3’.

The following primers were used to perform 3’ RACE: 5’gagaccacaagctctggacctggactc3’, 5’gtggatgctagatctagctcgcagg3’.
**Calcium mobilization assay.** Primary human monocytes were labeled with Fluo-4 (Molecular Probes, OR), followed by incubation on ice for 15 min with no antibody or 5 μg/ml of anti-LIR9, anti-LIR7, or isotype-matched control antibody. The unbound antibodies were then washed off and the bound primary antibody was crosslinked by the addition of donkey anti-mouse F(ab’)_2 fragments at 5 μg/ml (Jackson Laboratories, PA). Calcium mobilization was measured at room temperature on the FLIPR-384 Fluorometric Imaging Plate Reader system (Molecular Devices, CA). The calcium measurements were done in “real time” before, during, and for 4 minutes after the addition of the secondary antibody. The assay buffer was Hanks Salt Solution with calcium and magnesium, but no phenol red, 20mM HEPES, 1% FBS, and 2.5mM Probenecid.

**Cytokine secretion.** Primary human monocytes were incubated in a 96-well tray coated with the following antibodies: anti-LIR9 m660, anti-LIR1 m402, anti-LIR2 m421, anti-LIR7 m473, and the isotype-matched control anti-leucine-zipper m15 antibody. The cells were cultured for 2 days and the conditioned media were harvested. All the reagents used in this assay were free of endotoxin contamination. Ten different cytokines were analyzed in a multiplex assay system containing fluorescently labeled microsphere beads (Beadlyte Human Multi-Cytokine Detection System 3, Upstate Biotechnology, NY) and cytokine levels were quantitated using the Luminex 100 system (Luminex, TX).

**Results and discussion**

We performed RACE cloning on a large pool of leukocyte RNA and identified a new member of the LIR family, which we named LIR9. Four different forms of LIR9 were identified: two membrane-bound molecules, LIR9m1 (also known as ILT11) and
LIR9m2, and two molecules, LIR9s1 and LIR9s2, which lack a transmembrane domain (Figure 1A). The difference between LIR9m1 and LIR9m2, as well as LIR9s1 and LIR9s2, resides in the presence or absence of a small exon encoding 12 amino acids. This exon is present near the amino-terminus of LIR9m1 and LIR9s1 and is partially contained within the signal peptide of these molecules. After cleavage of the signal peptide, LIR9m1 and LIR9s1 are predicted to have different amino-termini from LIR9m2 and LIR9s2. The insertion of this exon also creates a new N-linked glycosylation site in LIR9m1 and LIR9s1. It is not clear whether these differences will impose any changes on LIR9 function, expression, or stability.

LIR9 transcripts are present mostly in tissues of the hematopoietic system including bone marrow, spleen, lymph node and peripheral leukocytes (data not shown). Among leukocytes, monocytes and neutrophils express the highest levels of LIR9 transcripts (Figure 1B). At the protein level, LIR9m is expressed in CD14+ monocytes (Figure 1C), but not in T cells, B cells, and NK cells.

The LIR9s1 and LIR9s2 splice variants contain a signal peptide and lack a transmembrane domain, suggesting that these molecules might be secreted. To determine whether LIR9s1 could be secreted, we cloned the LIR9s1 cDNA including the native signal peptide into a mammalian expression vector and transfected this plasmid into COS cells. Supernatants from LIR9s1-transfected cells, but not control cells, contain a protein of ~35kDa, which is the predicted size for glycosylated LIR9s1 (Figure 1D). Based on these results and the cell surface staining, we concluded that LIR9 is expressed both as a membrane-bound and secreted protein. The function of LIR9 in vivo is expected to be modulated by the ratio of LIR9s/LIR9m in the environment around the cells.
Crosslinking LIR9 on the surface of monocytes induces mobilization of calcium (Figure 2A), a known signaling mediator that is released during cell activation. In addition, triggering of LIR9 on monocytes induces secretion of IL-1β, TNF-α, and IL-6 (Figure 2B). Secretion of these cytokines was detected as early as 12 hours after crosslinking of LIR9 on the surface of monocytes (our unpublished data). IL-1β, TNF-α, and IL-6 are normally released in the early stages of inflammatory responses suggesting that LIR9 might play a role in modulating monocyte function in inflammatory settings. Other LIRs, LIR2 and LIR7, have been shown to be up-regulated in monocytes and neutrophils infiltrating the synovium of patients with early rheumatoid arthritis. Evaluation of LIR9 expression in diseased tissues, particularly in conditions that have an inflammatory component, might help to further delineate LIR9 function and define its potential as a therapeutic target.

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Figure legends

Figure 1. LIR9 amino acid sequences and LIR9 expression in leukocytes and transfected cells. A) Alignment of the amino acid sequences of the four forms of LIR9 and LIR6b, another activating LIR with two Ig-like domains. The two arrows indicate the predicted cleavage sites for the signal peptides; the first arrow indicates the cleavage site for LIR9m1 and LIR9s1 and the second arrow is for LIR9m2 and LIR9s2. The box highlights the 12 amino acid exon that is present in LIR9m1 and LIR9s1. The underlined amino acids are N-linked glycosylation sites. The arginine residue within the transmembrane domain is shown in bold face. B) Expression of LIR9m(1 and 2) and LIR9s(1 and 2) transcripts in peripheral blood leukocytes and monocyte-derived dendritic cells as determined by TaqMan quantitative RT-PCR. The data is expressed on a relative scale using the LIR9s expression in monocytes as the reference value. C) LIR9m expression in CD14⁺ monocytes as determined by 2-color FACS analysis. D) LIR9s1 is detected in the conditioned media of COS cells transfected with the LIR9s1 cDNA, but not with the LIR9m1 cDNA or empty vector. The cells were transfected with the indicated plasmids and two days later were metabolically labeled with ³⁵S-[Cysteine-methionine] for 5 h. Ten microliters of conditioned media were loaded per lane and the gel was exposed to film for 49 h (COS). The arrow indicates the LIR9s1 protein.

Figure 2. Crosslinking of LIR9m on the surface of monocytes induces calcium mobilization and cytokine secretion. A) Monocytes from two different donors were labeled with Fluo-4 and then incubated with anti-LIR9, anti-LIR7, an isotype-matched
control antibody), or no antibody. Calcium mobilization was measured on fluorometric plate reader as a response to crosslinking of the primary antibodies with donkey anti-mouse F(ab’)2 fragments. Data shown is representative for 2 out of 4 different donors. Among the 4 donors, the average fold changes and standard deviations in calcium flux relative to the non-antibody control were the following: control antibody: 0.91 ± 0.07; anti-LIR7: 3.10 ± 0.79; and anti-LIR9: 5.54 ± 1.57. **B** Monocytes were incubated on a 96-well tray coated with the antibodies against LIR1, LIR2, LIR7, LIR9, isotype-matched control antibody, or no antibody. Two days later, the conditioned media were harvested and cytokine levels were measured using the Luminex assay system as described in Material and Methods. Data shown is representative for 3 experiments on different donors.
Figure 1

A

1st Ig domain

LIR9m1  NSVTIRCQGTLEAQEYRLVKEGSPEPWDTQNPLEPKNKARFSIPSMTEHHAGRYRCYYYS
LIR9m2  NSVTIRCQGTLEAQEYRLVKEGSPEPWDTQNPLEPKNKARFSIPSMTEHHAGRYRCYYYS
LIR9s1  NSVTIRCQGTLEAQEYRLVKEGSPEPWDTQNPLEPKNKARFSIPSMTEHHAGRYRCYYYS
LIR9s2  NSVTIRCQGTLEAQEYRLVKEGSPEPWDTQNPLEPKNKARFSIPSMTEHHAGRYRCYYYS
LIR6b   SPVTLWCQGILETQEYRLYREKKTAPWITRIPQEIVKKGQFPIPSITWEHTGRYRCFYGS

2nd Ig domain

LIR9m1  -PAGWSEPSDPLELVVTGFYNKPTLSALPSPVVTSGENVTLQCGSRLRFDRFILTEEGDH
LIR9m2  -PAGWSEPSDPLELVVTGFYNKPTLSALPSPVVTSGENVTLQCGSRLRFDRFILTEEGDH
LIR9s1  -PAGWSEPSDPLELVVTGFYNKPTLSALPSPVVTSGENVTLQCGSRLRFDRFILTEEGDH
LIR9s2  -PAGWSEPSDPLELVVTGFYNKPTLSALPSPVVTSGENVTLQCGSRLRFDRFILTEEGDH
LIR6b   HTAGWSEPSDPLELVVTGAYIKPTLSALPSPVVTSGGNVTLHCVSQVAFGSFILCKEGED

Transmembrane region

LIR9m1  KLSWTLDSQLTPSGQFQALFPVGPVTPSHRWMLRCYGSRRHILQVWSEPSDLLEIPVSGA
LIR9m2  KLSWTLDSQLTPSGQFQALFPVGPVTPSHRWMLRCYGSRRHILQVWSEPSDLLEIPVSGA
LIR9s1  KLSWTLDSQLTPSGQFQALFPVGPVTPSHRWMLRCYGSRRHILQVWSEPSDLLEIPVSGE
LIR9s2  KLSWTLDSQLTPSGQFQALFPVGPVTPSHRWMLRCYGSRRHILQVWSEPSDLLEIPVSGE
LIR6b   EHPQCLNSQPRTHGWSRAIFSVGPVSPSRRWSYRCYAYDSNSPHVWSLPSDLLELLVPGA

B

Graph showing biological results.

C

CD14-PE

D

Western blot analysis.

LIR9-FITC

COS
Figure 2

A

B

C

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Figure 2

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