IREM-1 is a novel inhibitory receptor expressed by myeloid cells

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Using a three-hybrid strategy, we have identified a novel cell surface molecule which interacts with the Src homology 2 (SH2) domains of SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1), termed “immune receptor expressed on myeloid cells 1” (IREM-1). The full-length cDNA coding for a polypeptide of 290 amino acids presents an extracellular single V-type Ig domain, a transmembrane region and a cytoplasmic tail with five tyrosine residues, two of which are in the context of an immunoreceptor tyrosine-based inhibitory motif. Moreover, cDNA encoding for three other splicing forms of IREM-1, named IREM-1 splice variant (Sv)1, Sv2 and Sv3 were cloned by reverse transcription (RT)-PCR. The gene encoding for IREM-1 contains nine exons, is located on human chromosome 17 (17q25.1) and is homologous to previously identified molecules termed CMRF-35 and IRp60. RT-PCR, northern blot and FACS analysis with specific monoclonal antibodies indicated that IREM-1 is expressed on monocytes, granulocytes, and myeloid leukemia cell lines. Western blot analysis confirmed the recruitment of SHP-1 to IREM-1 and demonstrated that phosphotyrosine residue 205 is the main docking site for this interaction. Finally, cross-linking of IREM-1 results in the inhibition of FcR-induced activation. Our results indicate that IREM-1 is a novel inhibitory receptor of the Ig superfamily in myeloid cells.

Key words: Inhibitory receptor / Myeloid cell / SHP-1 / Ig superfamily

1 Introduction

Leukocyte functions are regulated by a balance between positive and negative signals delivered by activating and inhibitory receptors. Inhibitory receptors play an essential role maintaining a threshold of activation in the immune system, which is overcome by triggering signals in response to pathological stimuli. These mechanisms allow leukocytes to discriminate between normal cells and pathogenic agents, tumors and grafts. There is experimental evidence supporting the notion that a dysfunction of inhibitory receptors may contribute to the development of chronic autoimmune diseases [1–3].

A group of inhibitory receptors belongs to the Ig superfamily, whereas others are lectin-like molecules. The first group includes several multigenic families of receptors (CD33-like subgroup of Siglecs, KIR, ILT, FCAR, and LAIR) that are encoded within the leukocyte receptor complex on chromosome 19 (19q13.4), as well as other gene families (i.e. SIRP, PILR, and TREM) located on different chromosomes [3, 4]. Lectin-like inhibitory receptors comprise CD72, NKG2A, and members of the Ly49 family [5, 6]. Some inhibitory receptors recognize well-defined endogenous molecules (i.e. MHC class I, IgG Fc, sialic acid, CD47), whereas for others, the nature of their ligands remains unknown.

The molecular basis for the functional activity of immune inhibitory receptors is the presence of cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM) [3]. Upon receptor-ligand interaction, ITIM become tyrosine phosphorylated, thereafter recruiting and activating Src homology 2 (SH2) domain-containing phosphatases like SHP-1 and SHIP, which mediate the inhibitory signal. The SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) is mainly expressed in the cytoplasm of hematopoietic cells [7]. It contains two SH2 domains, one catalytic phosphatase domain, and
two sites for tyrosine phosphorylation in the C-terminal region. Upon recruitment by the inhibitory receptor, SHP-1 becomes activated and dephosphorylates key elements of the activation pathway in lymphoid and myeloid cells, thus inhibiting cell activation. SHP-1 controls the activating signaling cascade at its proximal steps, shutting down subsequent events [8].

By using a three-hybrid strategy in yeast, we cloned a novel member of the Ig superfamily preferentially expressed by myeloid cells that contains cytoplasmic ITIM and is capable of delivering negative signals. This "immune receptor expressed on myeloid cells-1" (IREM-1) is a member of a multigenic family located on human chromosome 17 (17q25.1), which includes previously identified molecules termed CMRF-35 and CMRF35-H/IRp60 [9–11].

2 Results

2.1 Cloning of a novel IREM-1

Our goal was to identify molecules that could interact with the SH2 domains of the tyrosine phosphatase SHP-1 in a phosphotyrosine-dependent manner. To this end, we used a three-hybrid system in yeast to screen a library of PHA-activated PBMC (Clontech), using the SHP-1 SH2 domains as bait in the presence of the Src kinase c-fyn420, 531Y-F, 176R-Q mutant. The three-hybrid SH2 domains of the tyrosine phosphatase SHP-1 in a phosphotyrosine-dependent manner. To this end, we used a three-hybrid system in yeast to screen a library of PHA-activated PBMC (Clontech), using the SHP-1 SH2 domains as bait in the presence of the Src kinase c-fyn420, 531Y-F, 176R-Q mutant. The three-hybrid screening (8 × 10^6 independent clones) produced more than 200 positive clones [12]. Five of these clones coded for a polypeptide of 93 amino acids that contained five tyrosines, two of them in the context of ITIM. By searching human cDNA databases, we found partial homology of our sequence with a putative inhibitory receptor called CMRF-35H (database accession number AF176991) (Fig. 1B). A Blast search was carried out on the Ensembl genome database (Ensembl BLAST Server at www.ensembl.org), and a predicted cDNA containing this sequence at the 3' region was found. We next designed primers and cloned two cDNA fragments, one [IREM-1 splice variant (Sv) 1] with an open reading frame of 882 bp (AF375480) and a second (IREM-1 Sv2) with an open reading frame of 474 bp (AF375481). The IREM-1 Sv1 sequence is predicted to be translated into a type I glycoprotein with 293 amino acids (Fig. 1). This molecule displays a single V-Ig domain at the N terminus, a transmembrane fragment, and a cytoplasmic tail including five tyrosine residues (Fig. 1A). IREM-1 Sv2 is a splice variant that lacks exons 6 and 7, thus lacking the transmembrane domain (Fig. 2). By screening the human expressed sequence tag (EST) database with the complete IREM-1 Sv1 cDNA (www.ncbi.nlm.nih.gov/BLAST/) we found a number of EST clones containing both isoforms, Sv1 and Sv2, and a new isoform (AAH28199) that we called Sv3, with the insertion of an additional exon (exon 5). By reverse transcription (RT)-PCR, we cloned a cDNA fragment of 502 bp that contains exon 5 and a subsequent frame shift. Interestingly, we found sequences (AF251706, BQ073166) in which the 5' untranslated and the first translated nucleotides were different to those found in IREM Sv1 and Sv2. Through alignment of cDNA and EST sequences with the genomic sequence, we determined the genomic organization of the IREM gene (Fig. 2A). We realized that there were two different transcriptional starts at exon 1 and exon 2. With new primers, we amplified another IREM-1 isoform from activated PBMC-derived cDNA (accession number AY303545). This isoform has an open reading frame of 873 bp that encodes for a 290-amino acid polypeptide almost identical to IREM-1 Sv1, but with a different leader peptide. In fact, when we analyzed both putative signal peptides using the program SignalP V2.0 (www.cbs.dtu.dk), only that present in IREM-1 fitted with a valid signal peptide sequence. In summary, we here describe a putative inhibitory receptor with two different transcriptional starts (IREM-1 and IREM-1 Sv1) and two truncated forms that present a V-type Ig domain but do not display any transmembrane region (IREM-1 Sv2 and Sv3) (Fig. 2B).

2.2 IREM-1 is expressed by myeloid cells

We analyzed by RT-PCR the expression pattern of IREM-1 mRNA. The band of the expected size (985 bp) was amplified from non-activated PBMC as well as from PHA-activated PBMC from different donors (data not shown). To more accurately address the distribution of IREM-1, we evaluated by RT-PCR purified T lymphocytes, NK cells and monocytes. Transcripts were detected in isolated peripheral blood monocytes, whereas polyclonal NK and T cell populations were negative for the presence of IREM-1 mRNA (Fig. 3A). Northern blot analysis with total RNA extracted from several hemopoietic cell lines was also performed, in order to assess the expression pattern of IREM-1. Specific hybridization with a single band of 1.5 kb was detected in the U937 human monocyctic cells as well as in the HL60 promyelocytic leukemia cell line, but not in T or B lineage cell lines (Fig. 3B). We developed specific anti-IREM-1 mAb, UP-D1 and UP-D2, to accurately study this molecule. These antibodies did not cross-react with IREM-2 (accession number AF395839), another member of the family (Aguilar et al., submitted) expressed only by monocytic cells (Fig. 4A). In accordance with the mRNA detection results, double-color FACS analysis of different PBMC populations revealed that IREM-1 was detectable on the surface of all CD14+ monocytes and CD15+ granulocytes.
Fig. 1. (A) Nucleotide and predicted amino acid sequences of IREM-1 (AY303545) and IREM-1 Sv1 (AF375480). The nucleotide sequences of IREM-1 and IREM-1 Sv1 containing an open reading frame of 873 bp and 882 bp are shown in upper case. The 5' UTR of both isoforms are shown in lower case. The putative signal peptides are underlined (IREM-1, dotted line; IREM-1 Sv1, double line). The Ig-like domain is in bold type, the transmembrane domain is underlined (single line), and the consensus ITIM-like sequences are in bold type and underlined (single line). The N-glycosylation site is boxed, and cysteines involved in the Ig-like fold are circled. (B) Protein sequence alignment of IREM-1 with CMRF-35 and CMRF-35H/IRp60. Identical amino acids are shown on black background, and similar residues are in gray. Alignment was done for the three proteins using IREM-1 amino acids 1–142 (extracellular domain) and only for IREM-1 and CMRF-35H/IRp60 using IREM-1 amino acids 143–290 (transmembrane domain and cytoplasmic tail).

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Expression of IREM-1 was maintained after culturing monocytes alone, with LPS for 72 h (Fig. 5), or with IL-10 (10 ng/ml) for 48 h (data not shown). By contrast, surface expression of the molecule was markedly down-regulated in monocyte-derived immature dendritic cells (Fig. 5), and the molecule was not re-expressed after inducing the maturation of these cells by LPS treatment, which was monitored by the induction of CD83 expression (data not shown).

To further study IREM-1, immunoprecipitation of a biotin-labeled U937 monocyctic cell line using the anti-IREM-1 UP-D1 mAb was carried out. When resolved by SDS-PAGE, IREM-1 showed two discrete bands of approximately 53 and 59 kDa, both under reducing or non-reducing conditions, showing that this molecule does not oligomerize at the cell surface through disulfide bonds (Fig. 6).

(Fig. 4B, C), whereas no expression was detected on CD19+ B lymphocytes and the CD56+ population including NK cells and CD3+ T lymphocytes (Fig. 4B). Expression of IREM-1 was maintained after culturing monocytes alone, with LPS for 72 h (Fig. 5), or with IL-10 (10 ng/ml) for 48 h (data not shown). By contrast, surface expression of the molecule was markedly down-regulated in monocyte-derived immature dendritic cells (Fig. 5), and the molecule was not re-expressed after inducing the maturation of these cells by LPS treatment, which was monitored by the induction of CD83 expression (data not shown).
2.3 IREM-1 interaction with SHP-1 is dependent on tyrosine phosphorylation of the receptor

Next, we cotransfected the CG1945 yeast strain with pGAD10/IREM-1 and pBridge, pBridge/SHP-1, pBridge/c-fyn and pBridge/SHP-1/c-fyn, to perform a three-hybrid system assay. Association between IREM-1 and SHP-1 was only detected as β-galactosidase activity when the kinase c-fyn was present (Fig. 7A). These data suggested that tyrosine phosphorylation within the cytoplasmic tail of IREM-1 was required for the interaction.

To confirm the interaction between SHP-1 and IREM-1, we used the U937 monocytic cell line that expresses both IREM-1 and SHP-1. Cells were treated or not with sodium pervanadate to induce tyrosine phosphorylation, lysed and immunoprecipitated with UP-D1 mAb or an isotypic control Ig, followed by incubation with the indicated PE-conjugated mAb. Western blot analysis of the precipitates with anti-phosphotyrosine antibodies showed that IREM-1 was phosphorylated only after

Fig. 3. IREM-1 mRNA is expressed by human monocytes and myeloid cell lines. (A) RT-PCR using RNA from human fresh and PHA-activated PBMC, isolated blood monocytes, and polyclonal NK and T cells was performed to amplify full-length IREM-1. cDNA products were resolved in 1% agarose. Molecular weight markers are shown on the left. (B) Northern blot analysis of total RNA from several hematopoietic cell lines was carried out with a 32P-radiolabeled probe. GAPDH 32P-radiolabeled probe was used as loading control. The position of 18S ribosomal subunit is indicated.

Fig. 4. IREM-1 is expressed on the surface of human myeloid cells. (A) COS-7 cells were transfected with HA-IREM-1 or HA-IREM-2 and stained by indirect immunofluorescence with the UP-D1 anti-IREM-1 mAb, an anti-HA mAb or an isotypic control Ig, and a FITC-labeled goat anti-mouse Ig. (B) PBMC were sequentially stained with UP-D1 anti-IREM-1 mAb and FITC-labeled goat anti-mouse Ig, followed by incubation with the indicated PE-conjugated mAb. (C) Dextran-enriched granulocytes were sequentially stained by indirect immunofluorescence with anti-IREM-1, anti-TREM-1 mAb or an isotype control Ig and a PE-conjugated goat anti-mouse Ig, followed by incubation with a FITC-conjugated anti-CD15 mAb. Cells were gated according to FLS/90° LS.
pervanadate treatment. Re-blotting of the membrane with anti-SHP-1 antibodies revealed that this phosphatase was coprecipitated only when IREM-1 was tyrosine phosphorylated (Fig. 7B). These results confirmed the interaction between IREM-1 and SHP-1 and showed the capability of IREM-1 to recruit SHP-1.

2.4 IREM-1 tyrosine residue 205 is required for SHP-1 recruitment

In order to verify that phosphorylation of putative ITIM within IREM-1 is required for and mediates the recruitment of SHP-1 via its SH2 domains, we generated a number of IREM-1 tyrosine-to-phenylalanine substitution mutants. Two tyrosine residues, Tyr 205 and Tyr 249, match with the strict ITIM consensus sequence, whereas a third residue, Tyr 284, is in the context of an ITIM-like motif. COS-7 cells were transfected with wild-type IREM-1 or the different mutants, together with SHP-1 and the c-fyn kinase. Cell lysates were immunoprecipitated with anti-HA antibodies, and samples were analyzed by Western blot with anti-phosphotyrosine, biotinylated anti-HA and anti-SHP-1 antibodies. As shown in Fig. 8, SHP-1 failed to interact with IREM-1 when Tyr 205 was mutated, suggesting that this tyrosine is essential for the recruitment of the phosphatase. On the other hand, mutation of both Tyr 249 and Tyr 284 did not interfere with SHP-1 recruitment, indicating that these residues are not relevant for this interaction. Taken together, our results support that SHP-1 binding to IREM-1 is dependent on the phosphorylation of tyrosine residue 205.
2.5 IREM-1 engagement delivers an inhibitory signal

Taking into account the association between IREM-1 and SHP-1, we addressed the putative inhibitory role of this molecule. To this end, we used the rat basophilic leukemia (RBL) cell line stably transfected with IREM-1. RBL cells can be activated through their FcεR with IgE, and inhibitory receptors have been shown to down-regulate this activating pathway by recruiting SHP-1 [3, 13]. Cells were transfected with plasmids encoding the luciferase reporter gene under the control of NFAT- or NF-κB-dependent promoters. Cells were stimulated through the FcεR by cross-linking with an anti-DNP IgE mAb, in the presence of the UP-D2 anti-IREM-1 mAb or an isotypic Ig control. Cross-linking of the FcεR induced cell activation as revealed by the increase of luciferase activity (Fig. 9A, B); the activities of NFAT and NF-κB transcription factors were dramatically reduced when both FcεR and IREM-1 were simultaneously engaged. These data confirmed that IREM-1 is able to function as an inhibitory receptor in myeloid cells.

3 Discussion

The aim of our work was to develop a comprehensive search for molecules that interact with SHP-1 and thus could mediate an inhibitory role in the immune response. Herein, we describe a novel inhibitory receptor that belongs to the Ig superfamily, termed IREM-1. Upon engagement by specific ligands, ITIM-containing im-
munoreceptors act by recruiting phosphatases (e.g. SHP-1) after phosphorylation of cytoplasmic tyrosine residues. The inhibitory signal down-regulates cytokine release, cytotoxic activity, and cell proliferation [3]. Most of these regulatory molecules belong to families of proteins that also include activating members; moreover, their genes are often organized in clusters (e.g. ILT, KIR) [4, 14, 15]. Unlike inhibitory molecules, the activating counterparts lack signaling motifs and present a charged residue in the transmembrane region that allows their association with ITAM-bearing adapters delivering the activating signal.

Sequence analysis of IREM-1 revealed this molecule as a member of the CMRF-35 family of receptors described elsewhere [9, 11]. This family is encoded in a region of human chromosome 17 (17q24–q25), and as in other inhibitory receptor families, the CMRF-35 cluster includes ITIM-bearing molecules as well as short-tailed proteins [16, 17]. Members of this gene family have been shown to be expressed on cells of different hematopoietic lineages, such as T cell subsets, B lymphocytes, NK cells, monocytes, macrophages, and granulocytes [18]. An inhibitory member of the family (CMRF-35H or IRp60) is expressed by several human hematopoietic cell types, including myelomonocytic cells, NK cells, and T lymphocytes [10, 11]. This receptor delivers a negative signal after engagement by mAb, inhibiting human NK cell cytolysis. In the present report, we show that IREM-1 mRNA was detected in both fresh and PHA-activated PBMC, as well as in monocytes and a number of myeloid cell lines (i.e. HL60 or U937). Double-color flow cytometry analysis by using the UP-D1 and UP-D2 anti-IREM-1 mAb confirmed that IREM-1 is expressed by all blood CD14+ monocytes, as well as by CD15+ granulocytes. Remarkably, IREM-1 expression was maintained after treatment of monocytes with LPS or IL-10 (data not shown), whereas it was down-regulated after in vitro differentiation to dendritic cells. These results support the notion that IREM-1 is mainly expressed by cells of the myeloid lineage. A putative IREM-1 murine orthologue, CLM-1, has been recently shown to be also expressed mainly by myeloid cells [19]. A myeloid-restricted expression pattern has been observed for a number of immunoreceptors, such as TREM-1 [4] and PILRα [20], some ILT [21, 22], and IREM-2, another member of the same family (Aguilar et al., submitted), indicating a possible role for these molecules in the regulation of the innate immune response. IREM-1 and IREM-2 are the only known members of the CMRF-35 family with a myeloid-restricted expression pattern; it is of note that UP-D1 and UP-D2 mAb do not cross-react with IREM-2.

Biochemical studies showed that IREM-1 is a monomeric type I transmembrane receptor. The IREM-1 polypeptide backbone has a predicted size of 32 kDa, whereas immunoprecipitated IREM-1 appeared as two discrete bands of 53 kDa and 59 kDa. The fact that a similar pattern was obtained in both transfectants and cells constitutively expressing IREM-1 suggests that its heterogeneous electrophoretic mobility is likely due to different levels of N- and/or O-linked glycosylation. IREM-1 was cloned for its ability to bind SHP-1, and consistent with the presence of intracellular ITIM, co-immunoprecipitation and Western blot analysis confirmed that IREM-1 can recruit the SHP-1 phosphatase in a phosphorylation-dependent manner in myeloid cells. The best candidates for phosphorylation by Src kinases and SHP-1 recruitment are Tyr 205 and Tyr 249, matching with the ITIM (I/L/VxYxxL/V) consensus sequence described for other inhibitory receptors [23]. Mutagenesis of ITIM tyrosine residues to phenylalanine supported the notion that tyrosine residue 205 was the main docking site for SHP-1. A model for SHP-1 activation has been proposed, in which the N-terminal SH2 domain of SHP-1...
serves both as a regulatory domain and as a high-affinity recruiting unit, whereas the C-terminal SH2 domain merely acts as a secondary recruiting unit [24]. According to this model, Tyr 205 would be responsible for recruiting the N-terminal SH2 domain of SHP-1, whilst the second tyrosine residue in the context of an ITIM (Tyr 249) might be able to recruit the C-terminal SH2 domain to fully activate SHP-1. This interaction has been described for other inhibitory receptors in which tyrosine phosphorylation by a Src kinase generates docking sites for phosphatases having SH2 domains (e.g. SHP-1).

It has been shown that SHP-1-mediated inhibition targets early stages of the activation response, shutting down subsequent events such as induction of gene transcription [3, 25]. The earliest events upon ligation of triggering receptors of the same [16] or other families i.e. FcR, TREM, ILT). In this regard, studies linking this gene family to psoriasis susceptibility [16] suggest that these receptors may have an important immunoregulatory role controlling inflammation. The identification of the IREM-1 physiological ligand becomes an essential step to establish the biological relevance of this molecule in the regulation of myeloid cell functions.

4 Materials and methods

4.1 Cells and antibodies

Human T (Jurkat, Molt-4), B (Ramos, Daudi and RPMI-8866) and myeloid cell lines (U937 and HL60) were grown in RPMI 1640/ Glutamax medium supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin (growing medium). COS-7 and RBL cells were grown in DMEM with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The γδ T cell line T-ALL 103/2 was kindly provided by Dr. D. Santoli (The Wistar Institute, Philadelphia, PA). PBMC were obtained from heparinized venous blood of healthy donors by Ficoll-Hypaque gradient centrifugation, washed with PBS, activated with 2 μg/ml PHA and further expanded in IL-2 (200 U/ml)-containing growing medium. Blood monocytes were isolated from PBMC by adhesion to plastic for 1 h at 37°C [21]; in some experiments, monocytes were cultured either alone or stimulated for 72 h with 1 μg/ml LPS (Sigma, St. Louis, MO) or 10 ng/ml IL-10 (PeproTech, UK). Immature dendritic cells were obtained by treatment of monocytes with 25 ng/ml IL-4 (R&D Systems, MN) + 100 ng/ml GM-CSF (Leucomax, Novartis) for 6 days and treated with LPS for an additional 48 h to achieve maturation. Polyclonal, activated NK and T cell populations were obtained from PBMC as described [29]. Granulocytes were separated from heparinized blood of healthy donors by 3%-dextran 500 (Amersham) sedimentation, followed by hypotonic lysis of erythrocytes. PE-conjugated anti-CD3, anti-CD19, anti-CD56, anti-CD14, anti-CD11a, anti-CD80 and anti-CD83 antibodies were from Becton Dickinson (San Jose, CA). FITC-labeled anti-CD15 antibody was purchased from Dako. Anti-HA monoclonal antibody 12CA5 was described before [12]. Anti-SHP-1 rabbit serum was obtained from Santa Cruz (Santa Cruz Biotechnology, Inc., CA). Anti-DNP IgE mouse antibody was purchased from Sigma. Phosphoantiregion-specific mAb was from Zymed Laboratories, Inc., CA. The anti-TREM1 mAb was kindly provided by Dr. M. Colonna (Washington University School of Medicine, St. Louis, MO).

4.2 DNA reagents

Yeast and mammalian expression constructs (SHP-1 and c-fyn) have been described [12]. IREM-1 (without signal peptide) was amplified by PCR and cloned into the Bgl II/Sal I sites of pDisplay ( Invitrogen Corp., CA) with primers 5’ CCT AGA TCT GGC TAC TCC ATT GCC ACT CAA 3’ and 5’ GCC GTC GAC CTA AGG CCT GCT GAT GTG GCT GTA 3’. IREM-1 Y205F was generated by amplification of two fragments with the two primer pairs 5’ CCT AGA TCT GGC TAC TCC ATT GCC ACT CAA 3’ and 5’ GGT CAG GTC TGC AAA GCA GAG GTC GCC CTC 3’, and 5’ GCC GAC CTC TGC TTT GCA GAC CTA ACC CTG 3’ and 5’ GCC GTC GAC CTA AGG CCT GCT GAT GTG GCT GTA 3’, and annealed at...
overlapping ends. After filling and amplification, DNA was cloned into the pDisplay vector. IREM Y249F was generated overlapping ends. After filling and amplification, DNA was cloned into the pDisplay vector. IREM Y249F was generated as described with the primer pairs 5' CCTAGATCT GGC TAC TCC ATT GCC ACT CAA 3' and 5' GGT CAG AGA TCG AAA GGA AAT GTC CTC CTT 3', and 5' GAG GAC ATT TCC TTT GCA TCT CTG ACC TTG 3' and 5' GCC GTC GAC CTA AGG CCT GCT GAT GGT GCT GTA 3'. IREM-1 Y284F was generated in a one-step PCR with the primers 5' CCT AGA TCT GAC TAC TCT ATT GCC ACT CAA 3' and 5' GCC GTC GAC CTA AGG CCT GCT GAT GGT GCT GTA 3'. The IREM-1 Y205F/Y249F double mutant was generated from the single mutant construct of IREM-1 Y249F by amplification of two DNA fragments with the primer pairs used previously to insert the Y205F mutation; all mutations were verified by sequencing.

4.3 Three-hybrid system assay

The three-hybrid screening was carried out as described [12]. To analyze IREM-1 interaction with SHP-1, three-hybrid assays were carried out by cotransferring the CG1945 yeast strain with pGAD10/IREM-1 and pBridge, or pBridge/SHP-1, or pBridge/c-fyn, as described before [30].

4.4 RT-PCR and cloning strategy

Retrotranscription of RNA from PBMC, T cells, monocytes and NK cells was performed using AMV retrotranscriptase enzyme (Promega Corp., Madison, WI) following the manufacturer's instructions. The PCR reaction to amplify IREM-1 was developed with 5 μg cDNA as template, using the primer pair 5' AAC GGG GAC CTG TCT GAA G 3' and 5' GTC GAT GAG GCA GGA GTG TGC TCA CAG 3'. PCR conditions used were 94°C 10 min, 94°C 30 s, 63°C 30 s, 72°C 1 min (40 cycles), 72°C 10 min, in the presence of FastStart Taq DNA Polymerase (Roche). Nested PCR to clone IREM-1 Ss1 and Ss2 and a cDNA fragment of IREM-1 Ss3 was done using 1 μg of PHA-activated PBMC cDNA as template. The first round of PCR was performed with the primers 5' CCA GAA CCA GTA ATC ATT AGG ACC 3' and 5' GTC GAT GAG GCA GGA GTG TGC TCA CAG 3', mapping in the 5' untranslated region (UTR) and the 3' UTR, respectively. Second-round PCR was done using the first PCR as template, with the primers 5' CCT AGA TCT ATG AGG GTC ATG GCT AGG AGT 3' and 5' GCC GTC GAC CTA AGG CCT GCT GAT GGT GTA 3'. A cDNA fragment of IREM-1 Ss3 was cloned in a second round of PCR with the primers 5' ACA ATC AGA AAA ACC GCA GTT 3' and 5' TGC TGA TGG TGC TGT ATT CGG 3'. In both rounds, PCR conditions were: 94°C 3 s min, and 94°C 1 min, 65°C 1.5 min, 72°C 3 min (30 cycles). PCR products were resolved in 1% agarose gels and visualized by ethidium bromide staining. Expected-size fragments were cloned into a pCR2.1 cloning vector (Invitrogen) and sequenced with ABI PRISM Big Dyes Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA) and the universal T7 primer.

4.5 Northern blotting

Total RNA was extracted from cells with Trizol reagent (GIBCO BRL) according to the manufacturer's instructions. Per lane, 10 μg of RNA were formamide/formaldehyde denatured, resolved in a 1% denaturing agarose gel and transferred onto nylon filters (Nytran Super Change, Schleider and Schuell), as detailed elsewhere [31]. The probe was obtained by PCR amplification of the cytoplasmic portion of IREM-1 (primers: 5' CCT CTG TCT TGC AGA CCT GAC CTC GTA AAG TGC TGT ATT CCG TAA ACC CCA ACT CCT AG 3', labeled with [a-32P]dCTP using the Random Primed DNA Labeling Kit (Roche)). Filters were prehybridized and hybridized under standard conditions. Filters were subjected to autoradiography for 10 days at –80°C (Kodak films, Rochester, NY).

4.6 Cell transfection

COS-7 cells (10^5) were transiently transfected with the DEAE-Dextran method and then lysed for immunoprecipitation assays as described [12]. RBL cells were stably transfected with 20 μg pDisplay/IREM-1 construct by electroporation at 280 V and 950 μF in a Gene Pulser Electroporator (Bio-Rad, CA) and selected with 1 mg/ml G 418 (Invitrogen).

4.7 Production of IREM-IgG2a fusion protein and anti-IREM-1-specific monoclonal antibodies

The IREM-1 extracellular domain was amplified by PCR with the primer pair 5' CAA AGC TTC GGC TAC TCC ATT GCC ACT CAA 3' and 5' CAT GAG AGG GTT CTT GAG TCG CAT AGC AGA CTT GCC CGC 5' and cloned into the cassette pSec/IgG2a [32] between BamHI and Hind III restriction sites. CHO cells were stably transfected with this construct; positive cells were selected with 300 μg/ml Zeocyn (Clontech). The chimeric protein was purified from the supernatant using a protein A column, and collected fractions were verified by Coomasie blue staining and Western blot. BALB/c mice were immunized with IREM-IgG2a fusion protein following standard methods [33]. Hybridoma supernatants were screened by ELISA using IREM-IgG2a fusion protein, and positive samples were further selected by FACS analysis of Jurkat/HA-IREM-1-transfected cells.
4.8 Flow cytometry analysis

IREM-1 expression on PBMC was tested with UP-D1 and UP-D2 mAb by immunofluorescence and flow cytometry analysis (FACScan; Becton Dickinson, Mountain View, CA) as described [13]. CellQuest software (Becton Dickinson) was used for data analysis.

4.9 Pervanadate treatment, immunoprecipitation, and Western blotting

U-937 cells were biotinylated using EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL), following manufacturer’s instructions. When indicated, cells were incubated with 1 mM sodium pervanadate for 15 min at 37°C, lysed and immunoprecipitated as described [12]. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham Pharmacia Biotech and streptavidin-HRP was from Roche. Blots were developed with West Pico SuperSignal kit (Pierce) and visualized on Hyperfilm (Amersham Pharmacia Biotech).

4.10 Luciferase assays

Luciferase reporter plasmids (0.5 μg/10⁶ cells) 3 × NFAT-Luc [34] and 2 × k-B-Luc [35], and TK renilla (0.1 μg/10⁶ cells) (Promega) plasmids were transiently transfected into RBL/IREM-1 cells by electroporation (10⁶ cells/point), and cells were cultured in growing medium for 24 h. Transfected cells were aliquoted in 12-well plates (Corning, NY) and stimulated (Promega) 6 h after transfection. Dual Luciferase reporter kit (Promega) 6 h after stimulation. Dual Luciferase reporter kit (Promega) 6 h after stimulation. Dual Luciferase reporter kit (Promega) 6 h after stimulation. Dual Luciferase reporter kit (Promega) 6 h after stimulation.

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References


