Molecular and Functional Characterization of CD300b, a New Activating Immunoglobulin Receptor Able to Transduce Signals through Two Different Pathways

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Molecular and Functional Characterization of CD300b, a New Activating Immunoglobulin Receptor Able to Transduce Signals through Two Different Pathways

Águeda Martínez-Barriocanal and Joan Sayós

In this study, we describe the characterization of human CD300b, a novel member of the CMRF-35/immune receptor expressed by myeloid cell (IREM) multigene family of immune receptors. Immune receptor expressed by myeloid cell-3 cDNA was cloned from a PHA-activated PBMC library and RT-PCR revealed the gene to be expressed preferentially in cells of myeloid origin. The CD300b cDNA open reading frame encodes a 201-aa type I protein composed of a single extracellular Ig V-type domain followed by a transmembrane region containing a positively charged residue (lysine) which is a common feature among receptors that associate with activating adaptor proteins. Indeed, CD300b was able to associate with DNAX-activating protein of 12 kDa (DAP-12) and deliver different activating signals through this ITAM-based adaptor. Unusually for an activating receptor, the 29-aa cytoplasmic tail of CD300b contains a tyrosine-based motif that, upon c-Fyn phosphorylation, became a docking site for the intracellular signaling mediator growth factor receptor-bound protein 2. Moreover, in the absence of DAP-12, CD300b was able to activate NFAT/AP-1-dependent transcriptional activity in RBL-2H3 cells. This activity could be abolished only by mutating both the cytoplasmic tyrosine and the transmembrane lysine. Our data suggest the existence of an unidentified molecule capable of interacting with CD300b through a charged residue of the transmembrane region and allowing receptor signaling independent of DAP-12. Therefore, CD300b defines a nonclassical Ig receptor able to trigger signals by coupling distinct mediators and thus initiating different signaling pathways. The Journal of Immunology, 2006, 177: 2819–2830.

Immune function is regulated by a balance between positive and negative signals delivered by activating and inhibitory receptors on the surface of leukocytes. These receptors are responsible for the correct transduction of information from the exterior to the interior of the cell and elicit a response that can range from reactivity to quiescence depending on the stimulus (1). Inhibitory receptors display long cytoplasmic tails presenting a variable number of ITIMs. Upon ligand binding, ITIMs are tyrosine phosphorylated by Src kinases and become docking sites for Src homology 2 (SH2)-containing phosphatases, such as SHIP and SH2 domain-containing phosphatase 1 (SHP-1). Dephosphorylation of key signaling molecules will lead to inhibition of cellular activation (2, 3). In contrast, classical activating receptors display both a short cytoplasmic tail having no known signaling motifs, and a positively charged residue in the transmembrane region that allows the receptor to associate with transmembrane adaptor proteins. To date, two types of transmembrane adaptor proteins have been described: the ITAM-bearing adaptors, including DNAX-activating protein of 12 kDa (DAP-12)/killer cell-activating receptor-associated protein (KARAP), FceRIy, and CD3ζ; and the PI3K-associated adaptor DAP-10. Engagement of activating receptors promotes tyrosine phosphorylation of transmembrane adaptors and subsequent recruitment of either protein tyrosine kinases containing SH2 domains such as Syk or Zap70, or PI3K that would contribute to activation of Akt and phospholipase C-γ pathways. Activating signaling events will lead to secretion of cell mediators, migration, proliferation, and/or differentiation, among other processes (4–8).

We recently identified immune receptor expressed by myeloid cell (IREM)-1 and IREM-2, members of the CMRF-35/IREM family (CD300) of Ig receptors (9, 10). The genes of this family are clustered in a region on chromosome 17 (17q25.1) spanning ~250 kb, and code for both activating and inhibitory receptors. IREM-1 and Irm60 have been shown to recruit SHP-1/2 phosphatases and deliver inhibitory signals (9, 11). Oppositely, IREM-2 has been found to associate with the DAP-12 adaptor and therefore deliver activating signals (10). The first identified member, CMRF-35, has a short cytoplasmic tail and a transmembrane region containing a negatively charged residue, structural features that make its classification uncertain (12).

In the present study, we report the identification and functional characterization of a novel Ig-like receptor belonging to the CMRF-35/IREM family. CD300b, expressed mainly on myeloid cells, is a nonclassical activating receptor able to deliver signals not only by associating with the transmembrane adaptor protein DAP-12, but also by recruiting growth factor receptor-bound protein 2 (Grb-2) through a tyrosine-based motif present in the receptor’s cytoplasmic tail.
**Materials and Methods**

**Cells and Abs**

Human T (Jurkat, Molt-4, T-All 103/102), B (Ramos, Daudi, RPMI 8866), NK (YT, NKL), and myelomonocytic cell lines (U937, THP-1, HL60, MonoMa6), and KU812, P815, and Rav26.7 cell lines were maintained in RPMI 1640-glutamine medium supplemented with 10% heat-inactivated FBS, 2 mM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin. T-All 103/102 and NKL cell culture media were additionally supplemented with 100 µ/ml human IL-2 (BD Pharmingen).

**Heparinized venous blood of healthy donors by Ficoll-Hypaque gradient**

COS-7 and RBL-2H3 cells were grown in DMEM containing 10% heat-inactivated FBS, 25 mM HEPES, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Human PBMC were obtained from heparinized venous blood of healthy donors by Ficol-Hyapaque gradient centrifugation. Human PBMC were isolated and cultured as described (13).

**Anti-HA.11 mAb was obtained from Covance. Mouse anti-phosphotyrosine mixture coupled to HRP was obtained from Zymed Laboratories. Anti-FLAG M2 mAb and anti-DNP SPE7 mAb were obtained from Sigma-Aldrich. Anti-Grb2 mAb was obtained from BD Transduction Laboratories, and anti-Grb2 (C-23) polyclonal Ab was from Santa Cruz Biotechnology. Anti-Grb2 mAb was obtained from BD Transduction Laboratories, and anti-Grb2 (C-23) polyclonal Ab was from Santa Cruz Biotechnology.**

**DNA reagents**

Mammalian and yeast expression vectors pMES/hFyn (15), pFLAG-CMV2/Fc/ErFy (10), pSresa/CD3δ (10), and pACT2/Grb2-2 (16) were described previously. Sequence encoding CD300b lacking the peptide signal was amplified by PCR using oligos 1 and 2 (Table I) and cloned into the BglII/SalI sites of the pDisplay vector. pDisplay/CD300b substitution mutants were generated with sets of mutagenic oligos according to the instructions of the QuickChange Site-Directed Mutagenesis kit (Stratagene).

**pDisplay/CD300b**

The pDisplay constructs were amplified by PCR using oligos 1–2 and cloned into the BglII/SalI sites of pDisplay vector. CD300b amplification was performed using primers 1–2 (Table I) and cloned into the BglII/SalI sites of pDisplay vector.

**RT-PCR**

RNA extracted with TRizol reagent (Invitrogen Life Technologies) from human polyclonal NK cells and hemopoietic cell lines was retrotranscribed using a ProtoScript First Strand cDNA Synthesis kit (New England Biolabs) according to the manufacturer’s instructions. CD300b amplification from human cell lines with oligos 15–16 was performed using 2 µl of cDNA as template. The conditions used were: 94°C 10 min; 40 cycles of 94°C 30 s, 60°C 30 s, 72°C 1 min; and 72°C 10 min. CD300b amplification from human isolated blood populations was performed using 10 ng of commercial cDNA (poly(A)⁺ RNA origin) (BD Clontech) or 2.5 µg of cDNA from polyclonal NK cells (total RNA origin). The conditions used were: 94°C 10 min; 40 cycles of 94°C 30 s, 60°C 30 s, 72°C 1 min; and 72°C 10 min. PCR products were cloned into the pCR2.1-TOPO vector and sequenced for confirmation using the universal T7 primer as described above.

**Cloning strategy of CD300b and murine IREM-3 (mIREM-3)**

A PCR strategy was used to amplify the full-length CD300b sequence from a PHA-activated PBMC cDNA library. Oligos 15–16 in Table I, mapping to the 5’ and 3’ untranslated regions of CD300b, were used for this purpose. PCR conditions were as follows: 94°C 5 min; 25 cycles of 94°C 1 min, 60°C 30 s, 72°C 1 min; and 72°C 10 min. mIREM-3 full-length sequence was amplified from Raw264.7 cDNA using oligos 17–18, which also map to the 5’ and 3’ untranslated regions of mIREM-3. PCR conditions were as follows: 94°C 5 min; 35 cycles of 94°C 1 min, 55°C 30 s, 72°C 1 min; and 72°C 10 min. DNA products were resolved in a 1% agarose gel and visualized by ethidium bromide staining. Expected size fragments were cloned into the pCR2.1-TOPO cloning vector (Invitrogen Life Technologies) and sequenced for confirmation using the universal T7 primer as described above.

**Table I. Oligos used in this study**

<table>
<thead>
<tr>
<th>Sequences</th>
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<tbody>
<tr>
<td>1 Sense S'-CCTGACTCAGGCAAGCCGATCAGTGAGA-3'</td>
</tr>
<tr>
<td>2 Antisense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
</tr>
<tr>
<td>3 Sense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
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<tr>
<td>4 Antisense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
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<td>5 Sense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
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<td>6 Antisense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
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<td>7 Antisense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
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<td>19 Sense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
</tr>
<tr>
<td>20 Antisense S'-GCCGTCGACCTAAGTGGCCATGTCTTTAGT-3'</td>
</tr>
</tbody>
</table>

* Nucleotide changes introduced in the sequence for changing the desired amino acid are shown in bold letters. Restriction sites used for cloning are underlined.
and fetal tissues was performed using a Human Rapid-Scan Gene Expression Panel (Origene Technologies) following the manufacturer’s recommendations. β-actin amplification was used as an internal control. FastStart TaqDNA Polymerase (Roche) was used for all PCRs.

Cell transfections

COS-7 cells (10⁶) were transiently transfected using the DEAE-dextran method (18) and lysed for the immunoprecipitation assay after 48 h. For the generation of RBL-2H3 stable transfectants, 20 × 10⁶ cells were electroporated in the presence of 20 μg of linearized construct at 280 V and 950 μF in a Gene Pulser Electroporator (Bio-Rad). Transfectants were selected and maintained in culture with 1 mg/ml G418 (Invitrogen Life Technologies). RBL-2H3 double transfectants were generated sequentially and maintained in 1 μg/ml Puroromycin (Sigma-Aldrich) in addition to G418. COS-7 cells (10⁶) for flow cytometry analysis were transfected with Fugu gene 6 reagent (Roche) according to the manufacturer’s instructions.

Flow cytometry

Cell surface expression of the desired molecules was checked by flow cytometry with the corresponding Abs following standard techniques (19). Analysis was performed using a FACScan instrument and CellQuest software (BD Pharmingen).

Immunoprecipitation and Western blotting

Cells were lysed at 4°C for 15 min using 1% Triton X-100 (Tx-100)-containing buffer described previously (15). Cell lysates were clarified by centrifugation at 14,000 × g for 15 min at 4°C and the crude lysates were preclaried for 1 h at 4°C using 20 μg of protein G-Sepharose beads (Amersham Biosciences) and 5 μg of mouse or rabbit IgG (Sigma-Aldrich). Two additional preclarings were conducted for 30 min at 4°C with 20 μl of protein G-Sepharose beads. For immunoprecipitations, preclaried lysates were incubated with 30 μl of protein G-Sepharose beads and 1 μg of Ab for 3 h at 4°C. Proteins in the crude lysates and immunoprecipitates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) filters (Millipore). Filters were blocked for 1 h with 5% skim-milk or 3% BSA (Sigma-Aldrich) and then probed with the indicated Abs. Bound Abs were detected using ECL reagent (Pierce).

Luciferase assays

RBL-2H3 transfectants were transiently electroporated with a luciferase reporter plasmid (pT81Luc) containing three tandem copies of the distal NFAT/AP-1 site of the murine IL-2 promoter (20) (0.5 μg/10⁶ cells) and a TK Renilla construct (Promega) (0.1 μg/10⁶ cells). Twenty-four hours posttransfection, 1.5 × 10⁶ cells were stimulated for 1 h with 5 μg/ml anti-HA12CA5 or a negative isotypic Ab using the murine mastocytoma cell line H9262 (Promega) as a substrate as described (15). Supernatants (20 μl) were added 0.2 M glycine buffer (pH 10.7) (150 μl/H9262) and incubated for an additional hour at 37°C with 1 mM 4-nitrophenyl-D-glucosaminide in 0.05 M citrate buffer (pH 4.5) (50 μl; Sigma-Aldrich). Reactions were stopped by adding 0.2 M glycine buffer (pH 10.7) (150 μl), and the OD was measured at 405 nm. For determining the maximum degree of hexosaminidase release, cells were lysed with Tx-100 (1%) before incubation with the substrate.

Three-hybrid system assay

To characterize the interaction between the cytoplasmic tail of CD300b and Grb-2, a three-hybrid system assay was conducted by co-transforming COS-7 cells with pBridge/c-Fyn420, 531Y-F, 176R-Q or pBridge/c-Fyn catalytic mutant containing in supplemented RPMI 1640/L-glutamine was used as negative control.

Results

Cloning and sequence analysis of CD300b and its marine ortholog mIREM-3

Following a three-hybrid system strategy in yeast, we recently described a novel cell surface inhibitory receptor termed IREM-1/CD300f (AY303545) (9). This immune receptor is a member of a multigene family of activating/inhibitory receptors located on human chromosome region 17q25.1 which includes the previously identified molecules CMRF-35/CD300c and CMRF35-H/IRp60/CD300a (11, 12, 22-24). With the aim of identifying new members of the same family, we searched for sequences having homology to IREM-1 and CMRF-35 in the Ensembl genome database (Ensembl BLAST server at (www.ensembl.org)). This yielded a cDNA coding for the activating receptor IREM-2/CD300e (AF395839) (10). In the process, we identified a second cDNA sequence that shared homology not only with IREM-1 and CMRF-35 but also with IREM-2, that we termed IREM-3. The sequence was found in the database as a another cclone expressing receptor expressed on myeloid cell (TREM)-5 (AF427618, AY359025, BC028091) and recently named CD300b (25). We designed primers for amplification of the predicted cDNA from a PHA-activated PBMC cDNA library. The obtained 732-bp fragment contains an open reading frame of 606 bp that encodes a protein of 201 aa with a predicted molecular mass of 22.7 kDa (AY646929) (Fig. 1A). Sequence analysis revealed CD300b to be a type I transmembrane protein driven by a signal peptide 17 aa in length (SignalP 3.0 server at (www.cbs.dtu.dk/services/SignalP)). The extracellular region of CD300b displays a single Ig V-type domain followed by a 29-aa membrane-proximal region containing a high proportion of proline, serine, and threonine residues that would confer on the molecule an extended open conformation (26). Additionally, the Ig domain stem presents many potential O-glycosylation sites. The transmembrane domain, which has a positively charged residue (lysine) in a central position, is followed by a short cytoplasmic tail of 29 residues in which a tyrosine-based motif can be distinguished (Fig. 1A).

The CD300b gene is located at chromosomal region 17q25.1. Through alignment of cDNA and genomic sequences, we determined the organization of the CD300b gene (Fig. 1B). The GT-AC rule was used to design intron-exon boundaries (27). The CD300b gene spans ~10 kb and contains four exons: exon 1 encodes the 5′ untranslated region and the protein’s signal peptide; exon 2 encodes the Ig domain; exon 3 encodes the membrane-proximal and transmembrane regions; and exon 4 encodes the entire cytoplasmic tail.

Alignment of residues of the extracellular Ig domain of CD300b with sequences of the CMRF-35/IREM family and other closely related immune receptors showed high levels of homology among the V-type Ig folds: 77% with IREM-1, 57% with IREM-2, 58% with IRp60, 60% with CMRF-35 and 44% with NKp44 (Fig. 2A). A lower, although significant, degree of protein sequence homology was detected between the Ig domains of CD300b and TREM-1 and TREM-2 (41 and 42%, respectively, alignments not shown). The conservation of not only the pair of cysteine residues describing the Ig-V-type fold (Cys36 and Cys51) but also the second pair of cysteines also in the Ig domain (Cys80 and Cys82) is noteworthy. It has been proposed that the additional disulfide bond created by the second cysteine pair could define a discrete evolutionary group of receptors stemming from duplication of a common ancestral V-type Ig domain gene (28).

To identify mouse orthologs of CD300b, we used the human CD300b cDNA sequence to search the Ensembl mouse genome database. We identified a cDNA sequence highly homologous but...
not identical with CML-7, a member of a recently described family of murine myeloid receptors (29). Amplification of the mIREM-3 full-length transcript was performed using cDNA from the murine myelomonocytic cell line Raw264.7 as template. From a complex pattern of bands we cloned a cDNA fragment 684-bp long containing an open reading frame of 630 bp. The cDNA codes for a 209-aa protein with a predicted molecular mass of 23.7 kDa that we termed mIREM-3/CD300b (AY996128). Alignment of the cDNA to the mouse genome sequence located the mIREM-3 gene to chromosome 11, which is considered syntenic to human chromosome 17. We cloned four transcript variants of the gene, but, with the exception of mIREM-3, they all specified incomplete mRNAs (data not shown). The human and murine forms of CD300b share 73% amino acid identity (Fig. 2B). It is striking that the tyrosine-based motif of the cytoplasmic tail is lost from the murine molecule while the charged amino acid of the transmembrane domain is retained.

CD300b is expressed mainly in myelomonocytic cells

To determine the distribution of CD300b transcript, we performed RT-PCR on human hemopoietic cell lines, PBMC, and purified blood populations. CD300b transcript was found abundantly in all human myelomonocytic cell lines tested (U937, THP-1, MM6, and HL60), and weakly in both the NK cell line NKL and the B cell line RPMI 8866 (Fig. 3A). However, when assessing the presence of the transcript in human hemopoietic isolated populations, CD300b was found exclusively in peripheral blood monocytes (Fig. 3B). No specific cDNA amplification was observed in activated NK and B cells. To ensure that the detected transcripts did indeed correspond to CD300b, the PCR products were cloned and sequenced. Based on these data, it seems that expression of CD300b is restricted to myeloid lineages, as we observed previously for IREM-1 and IREM-2 (9, 10).

Next, we determined the distribution of CD300b transcript in human tissues. A wide set of tissues was positive for the presence of CD300b (Fig. 3C). We were able to amplify CD300b from colon and lung, where mature differentiated myeloid cells can be found, and from placenta, bone marrow, and fetal liver that constitute the main reservoirs for myeloid precursors (30). A small amount of transcript was amplified from spleen. In the future, CD300b protein distribution will be assessed with mAbs to correlate transcript detection with cell surface expression of the molecule.

Biochemical characterization of CD300b

COS-7 cells were transiently transfected with CD300b tagged with an HA epitope. Forty-eight hours posttransfection, the cells were lysed and subjected to anti-HA immunoprecipitation and SDS-PAGE analysis under both reducing and nonreducing conditions. Despite CD300b having a predicted molecular mass of 22.7 kDa, the molecule appeared as three discrete bands ranging in size from 26 to 32 kDa (Fig. 4A), suggesting the occurrence of posttranslational modifications to the mature protein. The equivalent electrophoretic mobilities observed under reducing and nonreducing conditions indicates that the receptor was not forming dimers/multimers through disulfide bridges (Fig. 4A). The lack of asparagine residues in an N-glycosylation context suggests the possibility that the different bands may correspond to O-glycosylated and/or Ser/Thr-phosphorylated forms of the molecule. Treatment of the immunoprecipitates with a mixture containing the most common O-glycosidases or with alkaline phosphatase had no effect on the number and size of bands (data not shown). Expression of the molecule without its cytoplasmic tail led to a reduction in molecular mass of the bands but did not alter the triplet pattern (Fig. 4B), indicating that whatever the modification may be, it affects the extracellular portion of the molecule. Similar results

FIGURE 1. A, Predicted amino acid sequence of CD300b (AY646929). The putative signal peptide is underlined (dotted line). The Ig-like domain is marked in bold and the transmembrane domain is underlined (single line). Cysteine residues involved in the Ig-like domain fold are circled and the transmembrane-charged lysine residue is boxed. The tyrosine-based motif located in the cytoplasmic tail is in bold and underlined (double line). B, CD300b gene organization. Schematic organization of the CMRF-35/IREM locus at chromosomal region 17q25.1. CD300b genomic organization is shown below. Exons are represented by boxes (respective lengths in coding amino acids and domain architecture are shown); introns are represented by connecting lines.
were obtained when the molecule was expressed in RBL-2H3 cells (data not shown).

CD300b associates with DAP-12/KARAP and becomes tyrosine phosphorylated in transfected COS-7 cells

The presence of a positively charged amino acid in the transmembrane region of an immune receptor strongly suggests binding to a signaling transmembrane adaptor molecule (5, 6). Considering that CD300b has a lysine residue in its transmembrane portion, we wanted to test whether the molecule was able to associate with any ITAM-bearing adapters such as DAP-12/KARAP, FceRIγ, and CD3ζ. For this purpose, COS-7 cells were transiently transfected with CD300b-HA and the different ITAM-bearing adaptor molecules in both the presence and absence of c-Fyn kinase. CD300b was able to interact with DAP-12 only (Fig. 5A). Furthermore, Western blot analysis with anti-phosphotyrosine mAb revealed phosphorylation of the CD300b receptor. A low level of phosphorylation of CD300b was observed in the presence of the c-Fyn kinase alone and none when the kinase was absent, but it increased dramatically when c-Fyn was coexpressed with DAP-12 (Fig. 5A). Some receptors such as signal regulatory protein-β1 and murine NKG2D are able to couple not only DAP-12 but also the PI3K-associated adaptor DAP-10 (31, 32). We tested CD300b for its ability to recruit FLAG-tagged DAP-10. CD300b did not associate with DAP-10 nor did it become efficiently tyrosine phosphorylated (data not shown).

It has been suggested that DAP-12 acts not only as a signaling molecule but also as a chaperone for certain immune receptors, preventing their retention in the endoplasmic reticulum and intracellular degradation by forming stable receptor-DAP-12 complexes (33, 34). To determine whether the presence of DAP-12 was necessary for CD300b surface expression, COS-7 cells were transfected with CD300b-HA in presence or absence of DAP12-FLAG. Surface expression of CD300b was evaluated after 48 h by flow cytometry with anti-HA mAb. CD300b was detected on the cell surface independently of DAP-12 expression, indicating that CD300b does not require its adaptor counterpart for membrane localization (Fig. 5B). Nevertheless, CD300b expression was enhanced in the presence of DAP-12 (25.87 ± 7.64% mean of expression = 39.22 ± 2.34 vs 41.08 ± 6.66% mean of expression = 76.60 ± 4.37) (Fig. 5B). Oppositely, DAP-12 expression was not modified by coexpression of the receptor (data not shown). Increased expression of CD300b was not detected when the receptor was cotransfected with any other transmembrane adaptor protein and it was abolished when a CD300b mutant affecting transmembrane lysine 158 (K158L) was used (Fig. 5B and data not shown).

Analysis of CD300b/DAP-12 complex formation and CD300b phosphorylation

The association between an activating immune receptor and its adaptor counterpart relies on the formation of a membrane-embedded salt bridge between a basic residue on the receptor (lysine or arginine) and an acidic residue on the adaptor protein (aspartate) (5, 6). As CD300b phosphorylation was occurring only in the presence of both c-Fyn and DAP-12, we addressed whether tyrosine phosphorylation was required for association of CD300b and DAP-12. For this purpose, we generated a set of HA-tagged CD300b mutants affecting the transmembrane lysine (K158L), the cytoplasmic tyrosine (Y188F) or both residues. The CD300b Lys mutant affecting the transmembrane lysine (K158L) was used (Fig. 5C). Therefore, in this system, tyrosine phosphorylation of CD300b depends on the expression of DAP-12 but does not require its binding. The CD300b Tyr mutant cotransfected into COS-7 cells with c-Fyn kinase and DAP-12 was unable to recruit the adaptor molecule but, unexpectedly, it retained the ability to become tyrosine phosphorylated (Fig. 5C). Therefore, in this system, tyrosine phosphorylation of CD300b depends on the expression of DAP-12 but does not require its binding. The CD300b Tyr mutant recruited DAP-12 as efficiently as the wild-type form (WT), indicating that CD300b tyrosine phosphorylation is not needed for recruitment of the adaptor (Fig. 5C). As expected, the CD300b double mutant could neither recruit DAP-12 nor became phosphorylated in the presence of c-Fyn kinase (Fig. 5C). Association between CD300b and DAP-12 was maintained in absence of the c-Fyn kinase, indicating that phosphorylation of the adaptor was not essential for the formation of the complex (data not shown). Immunoprecipitation of DAP-12 resulted in coimmunoprecipitation of the three bands corresponding to CD300b, indicating that modification to the extracellular portion of the immune receptor does not affect its ability to recruit adaptor (data not shown).

CD300b engagement is able to activate transcriptional activity in the absence of DAP-12

Signal propagation to the intracellular compartment by activating receptors requires their association with transmembrane adaptor
proteins (5–7). CD300b, unlike other activating immune receptors described to date, has a tyrosine-based motif in its cytoplasmic tail that could potentially deliver positive signals independently of DAP-12. To evaluate the activating capacity of the receptor, we stably expressed CD300b-HA on the surface of the RBL-2H3 cell line, in which DAP-12 is absent (31) (Fig. 6A). The cell line was transiently transfected with a vector containing three copies of the NFAT/AP-1 response element fused to the firefly luciferase gene. Transfected cells were stimulated with anti-HA mAb that would mimic CD300b natural ligand, as shown previously for IREM-2 (10). An increase in promoter activity, comparable to the one delivered by FcεRI stimulation, was achieved when the cells were stimulated with anti-HA mAb (Fig. 6B). To determine the importance of the tyrosine-based motif in activating the CD300b reporter gene, we generated an RBL-2H3 stable transfectant expressing the CD300b Tyr mutant form of the molecule. Substitution of the tyrosine residue caused a marked decrease in the activating capacity of the receptor but did not abolish it (Fig. 6C). To address the possibility that other residues of CD300b were important for recruitment of signaling molecules, we generated a wider set of RBL-2H3 CD300b-HA stably transfected mutants (Fig. 6A). The set included the previously described substitution mutants CD300b K158L and CD300b K158L+Y188F, and two deletion forms affecting the cytoplasmic tail: CD300b Δ1 (del 178–201) in which the whole cytoplasmic tail was removed, and CD300b Δ2 (del 188–201) in which the amino acids preceding the tyrosine-based motif were maintained. The polyproline motif in the truncated cytoplasmic tail of CD300b Δ2 resembled a potential docking site for Src homology 3 (SH3) domain-containing molecules. However, both deletion mutants showed an activating capacity similar to the CD300b Y188F mutant, suggesting there are no other motifs in the cytoplasmic region able to contribute to reporter activation. Surprisingly, the CD300b K158L mutant caused a dramatic reduction in reporter activity, thus also establishing a role for the receptor’s transmembrane region in signaling in the absence of DAP-12 (Fig. 6C). Complete abrogation of reporter activity was achieved only when both residues, K158 and Y188, were mutated simultaneously (Fig. 6C). No differences were observed among transfectants in the activating capacity and cell surface levels of FcεRI (≥99% expression, data not shown).

**CD300b tyrosine does not mediate transcriptional activity in the presence of DAP-12**

To explore the stimulatory function of CD300b in the presence of DAP-12, we stably expressed both molecules at the cell surface of...
the RBL-2H3 cells, which do not express either protein endogenously (Fig. 7A). NFAT/AP-1-dependent transcriptional activity was enhanced more efficiently in the presence of DAP-12 (Fig. 7B). To test whether the observed increase could be due to the convergence of two pathways initiated by CD300b, we performed a comparative analysis among RBL-2H3 cells expressing on the cell surface DAP-12 and mutants of CD300b altered at K158, Y188, or at both residues (Fig. 7C). Mutation of the cytoplasmic tyrosine did not reduce transcriptional activity, indicating that the signal observed was generated solely from the association of CD300b with DAP-12 (Fig. 7C). Additionally, we can conclude that the signal induced by the receptor-adaptor complex was stronger than that produced by the receptor alone (Fig. 7B). Consistent with these interpretations, mutation of the transmembrane lysine of CD300b, alone or in combination with mutation of the tyrosine residue, completely eliminated NFAT/AP-1-mediated transcriptional activity (Fig. 7C).

**CD300b induces hexosaminidase granule release only in the presence of DAP-12**

NFAT/AP-1-dependent transcription was activated in RBL-2H3 cells by engagement of CD300b in the presence and absence of DAP-12 adaptor protein. We decided to look for additional pathways activated by CD300b either on its own or in association with DAP-12. Mast cells, when encountering a proper stimulus, are able to secrete proteases stored in cytoplasmic secretory granules (35). For instance, RBL-2H3 cells accumulate β-hexosaminidase protease whose release can be induced upon FcεRI cross-linking (36). Stimulation of RBL-2H3 transfectants expressing CD300b alone failed to elicit a hexosaminidase exocytotic response, whereas co-expression of CD300b and DAP-12 resulted in a level of hexosaminidase release comparable to that induced by IgE cross-linking (Fig. 8A). Expression of a mutant form of CD300b in which the transmembrane lysine was mutated to a noncharged amino acid resulted in a reduction in protease release (Fig. 8B), confirming the dependence of this process on DAP-12. In contrast, CD300b conferring a substitution of the key cytoplasmic tyrosine elicited levels of hexosaminidase release comparable to WT CD300b (Fig. 8B), indicating that signaling through CD300b’s tyrosine-based motif is not involved in the exocytotic pathway.

**CD300b cytoplasmic tail recruits the Grb-2 adaptor molecule in the presence of the Src-kinase c-Fyn**

The cytoplasmic CD300b tyrosine-based motif matches the binding consensus sequence for the Grb-2 adaptor molecule (YXN) (37). To test for a phosphotyrosine-dependent interaction between CD300b and Grb-2, we performed a Gal4 three-hybrid system assay in the presence of either c-Fyn kinase or a c-Fyn kinase mutant lacking part of the catalytic domain. Interaction of CD300b and Grb-2 was detected only when c-Fyn kinase was fully active (Fig. 9A). No interaction was observed when CGI1945 yeast was co-transfected with CD300b and other molecules containing SH2/SH3 domains, such as Csk or PI3K (p85α subunit), in the presence of c-Fyn (data not shown). The interaction between both molecules was also tested in a mammalian system by transfecting COS-7 cells with the receptor in the presence and absence of the c-Fyn kinase. Endogenous Grb-2 was found to be bound to CD300b only when the kinase was coexpressed in the cells (Fig. 9B).

**Discussion**

With the identification and cloning of immune receptors, new forms of activating and inhibitory receptors that defy traditional classification are being recognized. In this study, we describe the molecular cloning and characterization of a novel-activating immune receptor belonging to the CMRF-35/IREM family. CD300b has a basic residue in its transmembrane domain and a functional tyrosine-based motif in its short cytoplasmic tail (Fig. 1A). This structural arrangement confers on the molecule the ability to signal through two independent pathways: one through formation of a complex with an ITAM-bearing adaptor, and the other through the tyrosine residue in its cytoplasmic tail. No precedents are found in which an activating Ig receptor displays motifs involved in delivering activating signals. The closest similarity can be established with NKp44, an activating receptor expressed exclusively on activated NK cells. One of the three known NKp44 isoforms produced by alternative splicing contains both a lysine residue in its transmembrane region and a tyrosine-based sequence in its short cytoplasmic tail (AI225109) that matches the ITIM consensus sequence (38, 39). Although able to be tyrosine phosphorylated, the ITIM on NKp44 does not recruit SHP-1/2 or SHIP phosphatases and, as a consequence, it has no inhibitory function (40).

CD300b by itself acts as a functional receptor in RBL-2H3 cells which endogenously lack DAP-12 (31). Stimulation of CD300b through the HA epitope promoted NFAT/AP-1-dependent transcriptional activity (Fig. 6B). The signal generated was transduced by the tyrosine-based motif on the receptor’s cytoplasmic tail but a cooperative effect involving the molecule’s transmembrane domain was needed (Fig. 7C). We hypothesize that in RBL-2H3 and in the absence of DAP-12, CD300b cytoplasmic phosphorylation requires the formation of a complex with an unknown molecule. This molecule would interact with CD300b through the positively charged residue in its transmembrane domain, similar to how ITAM-based adaptors or DAP-10 bind cell surface receptors. This could explain why substitution of the lysine residue of CD300b eliminated the transcriptional activity (Fig. 7C). The function of this unknown molecule, in the context of CD300b signaling, would be to recruit the kinase(s) responsible for the receptor’s tyrosine phosphorylation. It is noteworthy that the RBL-2H3 cell line expresses FcεRIy and DAP-10 adaptors, but neither was found to be associated with CD300b when assessed in COS-7 cells (31, 41).
The proposed complex, when coupled to the receptor, seems able to indirectly activate NFAT/AP-1-dependent transcription. This may explain why CD300b tyrosine or deletion mutants still exhibit a low level activation of the reporter gene (Fig. 7C). Future studies on this system will focus on phosphorylation status of the receptor under basal conditions and upon stimulation, and how the process is influenced by the integrity of its transmembrane region. In addition, efforts will move toward identifying the postulated coupling molecule.

The tyrosine-based motif of CD300b matches the consensus sequence for the Grb-2 docking site (YxN) (37). As predicted, Grb-2 was able to interact in yeast and mammalian cells with the cytoplasmic tail of CD300b in a phosphorytrosine-dependent manner (Fig. 9). Grb-2 is a ubiquitous adaptor protein consisting of an SH3 and two SH2 domains lacking catalytic activity of its own. However, Grb-2 is able to link cell surface receptors to downstream signaling molecules (42). Upon receptor cross-linking, Grb-2 translocates from the cytosol to the cell surface where it binds phosphorylated tyrosines on receptors via its SH2 domains. Simultaneously, Grb-2 is thought to use its SH3 domain to recruit other signaling molecules that would mediate a change in the activation status of the cell (43). For instance, Grb-2 contributes to the activation of the Ras/MAPK signal transduction pathway in response to extracellular signals (16). This could explain the CD300b-mediated transcriptional activation observed in our system of study. It will be necessary to map the signaling events conducted by Grb-2 upon CD300b cross-linking and determine their relevance to the function of myeloid cells.
Despite the presence of the tyrosine residue in the cytoplasmic tail, CD300b is able to act as a classical immune receptor and associate with an ITAM-bearing adaptor molecule (Fig. 5A). Interaction with DAP-12 occurred independently of the tyrosine phosphorylation status of both the receptor and the adaptor (Fig. 5C and data not shown). The association was confirmed to be functional in the RBL-2H3 cell expression system. Release of preformed intracellular mediators was promoted upon receptor
stimulation through its association with DAP-12 adaptor. No hexosaminidase release was observed in the absence of the adaptor molecule or when its recruitment was disrupted (Fig. 8). CD300b/DAP-12 complex was also able to activate NFAT/AP-1-dependent transcriptional activity (Fig. 7B). The observed signal was generated exclusively through DAP-12, and not as a combined effect of signals initiated by the ITAM-bearing adaptor and CD300b cytoplasmic tail (Fig. 7C). These data support the hypothesis of a complex recruited by the CD300b transmembrane region that is necessary for signaling through the receptor’s cytoplasmic tail. Even so, we cannot discount the possibility that both pathways could act simultaneously and in a complementary manner in other processes such as cytokine release, migration, cell proliferation, and/or differentiation (44).

For example, our biochemical experiments in COS-7 cells show how CD300b presents a curious dependence on DAP-12 in terms of cell surface expression and tyrosine phosphorylation (Fig. 5, B and C). In fact, the presence of DAP-12 enhanced more efficiently the phosphorylation of CD300b, even when no interaction with the adaptor through the transmembrane lysine took place (Fig. 5C). These data suggest that in COS-7 cells, DAP-12 is able to modulate the level of tyrosine phosphorylation of CD300b indirectly through its interaction with other molecules, such as a second set of kinases. Another possibility is that the interaction between CD300b and DAP-12 does not rely exclusively on the transmembrane lysine. For instance, it has been demonstrated that the SH2 domain of the SLAM-associated protein adaptor molecule binds the c-Fyn SH3 domain and directly couples this kinase to the cytoplasmic tail of the immune receptor CD150, promoting CD150 tyrosine phosphorylation (45). New experiments are needed to fully understand the regulation of CD300b phosphorylation.
To understand the function of CD300b it will be necessary to focus our studies on those cells in which both the receptor and DAP-12 are naturally expressed. DAP-12 has been shown to be expressed and functional in NK and myeloid cells (46, 47). CD300b expression at the mRNA level is restricted to monocytes and myelomonocytic cell lines, similar to what has been described for IREM-1 and IREM-2 (Fig. 3, A and B). In addition, data on the Gene Expression server (at http://symatlas.gnf.org/SymAtlas/) for TREM-5/CD300b revealed the presence of the transcript in dendritic cells, which could help to explain some of the transcripts detected in the tissue scan panel (Fig. 3C). Nevertheless, these data will need to be confirmed using specific mAbs. Several DAP12-associated activating myeloid Ig-like receptors, such as paired Ig-like type 2 receptor-β (48), signal regulatory protein-β (49), IREM-2 (10), and TREMs (50) have recently been described. Therefore, the existence of a DAP-12-free cell membrane in which CD300b would signal exclusively through its cytoplasmic tail seems unlikely. We think that CD300b signaling pathways are more likely to be controlled by engagement of the immune receptor with different ligands, compartmentalization of the molecule at the cell surface, and/or changes in CD300b affinity for extracellular, intracellular, and transmembrane coupling molecules.

Based on the results obtained, comparative functional analyses between human and murine CD300b molecules would be very interesting. Despite the high degree of homology between the two receptors, murine CD300b lacks the tyrosine-based motif of the cytoplasmic tail (Fig. 2B). The murine form will be essential for addressing the relevance of transmembrane associations in activating immune receptors by mechanisms distinct from the recruitment of classical adaptor proteins.

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Disclosures
The authors have no financial conflict of interest.


