CD229 (Ly9) Lymphocyte Cell Surface Receptor Interacts Homophilically through Its N-Terminal Domain and Relocalizes to the Immunological Synapse

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CD229 (Ly9) Lymphocyte Cell Surface Receptor Interacts Homophilically through Its N-Terminal Domain and Relocalizes to the Immunological Synapse

Xavier Romero, Nuria Zapater,† María Calvo,‡ Susana G. Kalko,‡ Miguel Angel de la Fuente,* Victoria Tovar,* Charlotte Ockelen,§ Pilar Pizcueta,* and Pablo Engel*†

CD229 is a member of the CD150 family of the Ig superfamily expressed on T and B cells. Receptors of this family regulate cytokine production and cytotoxicity of lymphocytes and NK cells. The cytoplasmic tail of CD229 binds to SAP, a protein that is defective in X-linked lymphoproliferative syndrome. To identify the CD229 ligand, we generated a soluble Ig fusion protein containing the two N-terminal extracellular domains of human CD229 (CD229-Ig). CD229-Ig bound to CD229-transfected cells, whereas no binding was detected on cells expressing other CD150 family receptors, showing that CD229 binds homophilically. Both human and mouse CD229 interacted with itself. Domain deletion mutants showed that the N-terminal Ig-domain mediates homophilic adhesion. CD229-CD229 binding was severely compromised when the charged amino acids E27 and E29 on the predicted B-C loop and R89 on the F-G loop of the N-terminal domain were mutated to alanine. In contrast, one mutation, R44A, enhanced the homophilic interaction. Confocal microscopy image analysis revealed relocalization of CD229 to the contact area of T and B cells during Ag-dependent immune synapse formation. Thus, CD229 is its own ligand and participates in the immunological synapse. The Journal of Immunology, 2005, 174: 7033–7042.

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4 Abbreviations used in this paper: IgSF, Ig superfamily; wt, wild type; CMAC, chloromethyl derivative of aminocoumarin; SEE, staphylococcal enterotoxin E.
CD229 was shown to associate with the μ-chain of the AP-2 adaptor complex, which links transmembrane molecules with clathrin-coated pits (21). TCR and CD229 coligation enhances CD229 endocytosis, suggesting that Ag receptor signaling regulates the availability of CD229 at the plasma membrane. CD229 is the only member of the CD150 family that binds to the AP-2 complex.

Through their extracellular domain, the CD150 immunoreceptors interact with specific ligands. One common feature of this subset of molecules is that they interact with members of the same family; CD150, CS1, and NTB-A all participate in homophilic interactions, whereas CD244 (2B4) is a receptor for CD48 (22–26). The counterreceptor of CD229 is currently unknown.

During Ag recognition, T cells undergo substantial membrane and cytoskeletal rearrangements that lead to the formation of the immunological synapse (27, 28). The TCR, accessory cell surface molecules, and intracellular signaling molecules that are incorporated into and around the contact zone between T cells and APCs all contribute to the modulation of the functional state of the synapse. The immunological synapse appears to be essential to establish the persistent signaling necessary for full activation and is implicated in the control of the quality and extent of biological T cell responses. Recently, it has been reported that CD224 (2B4), which is a member of the CD150 family, redistributes to the central zone of the cytotoxic NK immune synapse (29). However, the behavior of CD229 and other members of the family during immunological synapse formation remains to be elucidated.

Here we show that CD229 interacts homophilically through its C-terminal V-like domain and identify the amino acid residues essential for binding. We also show that CD229 localizes to the contact site between T cells and Ag-presenting B cells during Ag recognition.

**Materials and Methods**

**Cells and reagents**

COS-7 cells, the lymphoblastoid B cell line Raji, and the murine myeloma cell line NS-1 were obtained from the American Type Culture Collection. The Jurkat-de- and L-glutamine (Life Technologies). The following mAbs were produced in complete RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and has been described previously (30). Cell lines were cultured in contact site between T cells and Ag-presenting B cells during Ag-recognition, CD229 is essential for binding. We also show that CD229 localizes to the central zone of the cytotoxic NK immune synapse (29). However, the behavior of CD229 and other members of the family during immunological synapse formation remains to be elucidated.

Here we show that CD229 interacts homophilically through its C-terminal V-like domain and identify the amino acid residues essential for binding. We also show that CD229 localizes to the contact site between T cells and Ag-presenting B cells during Ag-dependent immune synapse formation.

**Chimeric CD229 molecules and splice variants**

Two human/mouse CD229 cDNAs chimeric molecules were produced. Chimeric molecule h/DII/mDIII/IV was made by joining the CDNA that encodes domains I and II of human CD229 with domains III and IV of murine CD229. The chimeric molecule h/DIII/DIV mDII was constructed using domains I, III, and IV of human CD229 and domain II of murine CD229. The chimera h/DII/mDIII/IV was constructed with two fragments generated by PCR. The first PCR product was generated using human CD229 cDNA as template and the HLY9 wild-type (wt) forward oligonucleotide (Table I) and overlapping reverse oligonucleotide 5′-TCT TCT GGA GCC TCG TGC TGT AGC ACA GAA C-3′. The second PCR product was generated using the murine (C57BL/6) CD229 cDNA as template and overlapping forward oligonucleotide 5′-GGT GTT CAG AGA TCC AGG AGC CTC CAG AAG A-3′ and reverse oligonucleotide 5′-TTC TCC AAA TCC TCA CCC CG-3′. Each PCR product was used for a recombinant PCR containing the primers HLY9 wt and reverse primer of murine CD229.

Chimeric molecule h/DIII/DIV mDII was constructed using three products generated by PCR. The first PCR product, corresponding to the first domain of human CD229, was amplified using human CD229 cDNA as template and HLY9 wt forward oligonucleotide (Table I) and overlapping reverse oligonucleotide 5′-GCC TTC TGC AGG TTC TCA TAG ACG AAA AGG GTG-3′. The second PCR product, corresponding to the second domain of human CD229, was generated using murine (C57BL/6) CD229 cDNA as template and the overlapping forward oligonucleotide 5′-CAC CCT GTT CGT CTA TGA GAA GCT CCA GAA GCC-3′ and overlapping reverse oligonucleotide 5′-GTT CCG TCT CTA GGA GAA GCG CCA GAA GCC-3′. The third PCR product was used as template for amplifying human CD229 cDNA as template and overlapping forward oligonucleotide 5′-CTG GCA ATT CTT CAC AGG CTC AGG CTC AGG A-3′, and the HLY9 wt reverse oligonucleotide (Table I). PCR products were used for a recombinant PCR to anneal overlapping ends. The human/mouse chimeric molecules were cloned into pCDNA3-1/V5/His-TOPO expression vector (Invitrogen) fully sequenced and transfected in COS cells. As control, murine CD229 full-length cDNA was transfected in COS cells and tested with the anti-human CD229 mAbs to rule out possible cross-reactivity of these mAbs with murine CD229.

A CD229 isoform that lacks the first Ig domain (CD229 ΔI) was isolated from the thymines by RT-PCR and subcloned into PCIneo expression vector. The reactivity of CD229 mAbs (HLY9.1.25, HLY9.1.38, HLY9.1.77 and HLY9.1.87) with COS cells transfected with the chimeric molecules, and the splicing variant CD229 ΔII cDNAs was analyzed using flow cytometry.

**Construction of the fusion proteins**

The human CD229-Ig (CD229-Ig), human CD244-Ig (CD244-Ig), murine CD84-Ig (mCD84-Ig), and murine CD229-Ig (mCD229-Ig) fusion proteins were produced. The human CD229-Ig (CD229-Ig), human CD244-Ig (CD244-Ig), murine CD84-Ig (mCD84-Ig), and murine CD229-Ig (mCD229-Ig) fusion proteins were produced.
containing the CD33 leader peptide and the Fc region of human IgG1 were obtained by inserting the sequences corresponding to the first and second extracellular Ig domains into the mammalian expression vector signal pIgR (R&D Systems). The oligonucleotides used were as follows: human CD229 forward oligonucleotide, 5'-AGG AAG ATC TAA AGG ACT CAG CCC CAA CAG TGG T-3' and reverse oligonucleotide, 5'-AGG AAG ATC TAC TTA CCT GTT Ctg GAG CCT GCT GGA TTA CAC-3'; human CD229 forward oligonucleotide, 5'-AGG GGG ATC CAT ACC GCC GCG TGT CAG ACC A-3' and reverse oligonucleotide, 5'-ACC AGG ATC CAC TTA CTT GTG AAT TCC TGA TGG GCA TTC TGA C-3'; murine CD84 forward oligonucleotide, 5'-TGG CAG TCC ATA CTT TGA TGA AAG AGT CTT GGG TCA ATT CCA AAG ATG CAG CCC CAG TGG T-3' and reverse oligonucleotide, 5'-TAA CAG TCC ATA CTT TGA TGA AAG AGT CTT GGG TCA ATT CCA AAG ATG CAG CCC CAG TGG T-3'; human CD229 forward oligonucleotide, 5'-GTG CTC TCA GAC CTC CAG TGT TCA TGG GCA TTC TGA C-3'; murine CD229 forward oligonucleotide, 5'-GTG CTC TCA GAC CTC CAG TGT TCA TGG GCA TTC TGA C-3'; murine CD229 forward and reverse oligonucleotide, 5'-GCC ATC TAC TTA CCT GCT GAG CTT CCT GGT CAG AAT TGC CA-3'; human CD84-Ig was obtained in our laboratory (23). The constructs corresponding the Ig fusion proteins were subcloned in the expression vector pCl-neo (Promega) and NS-1 stable transfectants were obtained by electroporation and selection with 1.2 mg/ml genetinic (G418) (Life Technologies). Eight million cells were electroporated (280 V, 950 μF) with 8 μg of linearized DNA using the Gene Pulser II Apparatus (Bio-Rad). The transfected cells were plated in flat-bottom 96-well tissue culture plates (Corning) by limiting dilution, and clones producing high amounts of fusion protein were cultured in INTEGRA CL 1000 flasks (Integra Biosciences). The supernatants containing the fusion proteins were purified as described for the mAbs.

Site-directed mutagenesis

Site-directed mutagenesis of single residues was performed using a set of sense and antisense primers containing the appropriate mutation (Table I). wt CD229 cDNA was used as template. The first PCR used either the sense primer 5'-AGT TCT GTA AAT AGA TCA TCA TGG-3' plus an anti-sense primer containing the mismatched base or a sense primer that contained the mismatched base plus the antisense primer 5'-AGC TGG TT TCC TCT CTT CAG GTG-3' to create two PCR products that overlapped within the region spanned by the mutated sense and antisense primers. The reactions were conducted as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 2 min, with a final extension period of 72°C for 6 min. Aliquots of 3 μl of a 1/25 dilution of each PCR product were used as templates for a recombinant PCR containing the primers of the full-length CD229. The conditions of the PCR were the same as before. PCR products were cloned into the pcDNA3.1/V5/His-TOPO expression vector (Invitrogen). After confirming mutated clones by automatic sequencing, each cDNA mutant was transfected into COS-7 cells.

**COS transfection**

One million COS-7 cells were transiently transfected with 3 μg of cDNA corresponding to human CD229 or the CD229 mutants, CD229 chimeras, CD229 splice variant (CD29 Δβ3), CD84, CD150, CD24, CD48, CD25, and CD25 + CD24 + CD48 (100 μl of Nectoror Solution V according to the manufacturer's protocol (AMAXA Biosystems). The cells were analyzed 24 h after transfection either by flow cytometry or by immunocytochemistry.

**Immunocytochemistry**

Transiently transfected COS-7 cells were cultured on glass coverslips in 24-well tissue culture plates (Corning). After 48 h, cells were fixed with 4% paraformaldehyde at 4°C and then blocked with 3% BSA (Sigma-Aldrich). Cells were labeled with HL9.1.25, HL9.1.38, and HL9.1.77 mAbs and CD229-Ig fusion protein for 1 h at room temperature. After a washing in PBS, samples were incubated with Cy3-conjugated anti-mouse IgG or biotin-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories). Samples were washed twice in PBS and mounted in Fluormount-G (Southern Biotechnology). Fluorescence images were obtained using a confocal spectral microscope (Leica TCS SL; Leica).

**Flow cytometry**

Flow cytometry was conducted with transfected cells stained with anti-human mAbs, followed by biotinylated anti-mouse κ L chain, plus streptavidin-PE (BD Pharmingen). Ig fusion protein staining was performed using 2 μg of Ig fusion protein followed by incubation with biotin-conjugated anti-β3 mAb and Streptavidin-PE (Jackson ImmunoResearch Laboratories) and then with PE-conjugated anti-β3 mAb. The stained cells were analyzed using a FACSCalibur (BD Biosciences Immunocytochemistry Systems) equipped with CellQuest software. Ten thousand cells were counted for each sample.

**Molecular modeling of the CD229 N-terminal domain**

We used the alignment mode of SWISS-MODEL, an automated modeling process based on a user-defined target-template alignment (33). We obtained a final model of 78 residues (corresponding to residues 13–90 of the N-terminal domain of CD229) from the alignment between our target and CD28 and a second model of 42 residues (corresponding to residues 63–104 of the N-terminal domain of CD229) from the alignment between our target and CD2. The final model presented here is 90 residues long and was constructed using the three-dimensional alignment CE method with the first and second models (34). Our final model shows the archetypal fold of V Ig variable domains characterized by nine β strands forming two antiparallel β sheets. The first β strand was not modeled.

**Immunological synapse formation and immunofluorescence microscopy**

To distinguish B (Raji) from T (Jurkat Vβ8” J77cl20) lymphocytes, Raji cells were loaded with the fluorescent cell tracker chloromethyl derivative of aminocoumarin (CMAC; 10 μM) or 5-chloromethylfluorescein diacetate (3 μM) (Molecular Probes). B cells were incubated for 20 min at 37°C with 2 μg/ml staphylococcal enterotoxin (SEE) (Toxin Technology). Jurkat cells were mixed with Raji cells (proportion, 1:1) and incubated for 15 min at 37°C. Cells were plated onto poly-l-lysine-coated slides (50 μg/ml; Sigma), incubated for 20 min at 37°C, and fixed in 4% formaldehyde. Immunofluorescence assays, samples were blocked with PBS containing 2% BSA, and Fc receptors were blocked with 15% of heat-inactivated rabbit serum (Life Technologies). Cells were labeled with mAbs followed by Cy3-conjugated goat anti-mouse (Jackson Immunoresearch Laboratories). For visualization of immunological synapse formation, confocal images were acquired using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg) with argon and helium-neon lasers attached to a Leica DMIRE2 inverted microscope. All images were obtained using a ×63 oil immersion objective lens (NA 1.32) equipped with phase contrast optics. Due to the small size of the cells, electronic zoom was necessary for higher magnification and better image resolution. The images of CMAC-labeled Raji cells were obtained using a mercury lamp HBO 50W UV filter as light source after each acquisition of fluoroescence confocal and phase contrast images. Image assembly and treatment were performed using the Leica Confocal Image Processing Software. B cell line Raji was negatively selected for CD229 expression using three sequential pannings. Briefly, polystyrene bacteriological petri dishes (100 × 15 mm) were coated overnight at 4°C with of HLy9.1.84 mAb at 30 μg/ml in PBS. The dishes were washed twice with PBS and blocked with PBS containing 0.2% BSA (Sigma-Aldrich) for 1 h at room temperature. The dishes were washed once with PBS, and then 10 × 10⁶ Raji cells were added and incubated for 40 min at 4°C. Nonadherent Raji cells were collected. This procedure was repeated three times. The collected Raji cells expressed almost undetectable CD229 levels (Raji CD229-). These cells were used to perform immunological synapse formation studies. Contacts of Jurkat cells with Raji CD229- cells that expressed detectable levels of CD229 in the confocal images were excluded from the analysis.

**Statistical analysis**

The Sigma Stat statistical package (Jandel Scientific Software) was used in the analysis of the results. All results are reported as mean ± SEM. Statistical significance was set at p < 0.05. ANOVA and Student’s t test with Bonferroni’s correction were used.

**Results**

**CD229 interacts homophilically**

To identify the ligand of CD229, we assessed binding of a CD229-Ig fusion protein to COS-7 cells transiently transfected with CD2, CD58, or members of the CD150 family. Binding was detected only with CD29, CD2-, CD58-, CD84-, CD150-, CD48-, CD244-, and NTB-A transfected cells were all negative (Fig. 1). The specific binding of CD244-Ig to CD48 was used as a positive control (Fig. 1). CD229 homophilic binding was also observed in mice (Fig. 2A). Moreover, the CD229-CD229 homophilic interaction is not species restricted because human CD229 can bind to mouse CD229 and vice versa (Fig. 2B). This is in contrast to CD24 homophilic binding, which is species restricted, because human CD84 does not bind to murine CD84, although murine CD84 can bind to itself (Fig. 2A).
FIGURE 1. CD229 exhibits homotypic binding. COS-7 cells transfected with human CD229, CD2, CD48, NTB-A, CD58, CD244, CD84, and CD150 cDNA were labeled with CD229-Ig or CD244-Ig fusion proteins. Protein expression was detected using specific Abs (left column). Transfected cells were assayed for binding of CD229-Ig or CD244-Ig (middle and right columns). Immunostaining controls: isotype controls (left column) and Ctl-Ig (murine CD84-Ig; middle and right columns) are represented with dotted lines. The percentage of positive cells is indicated in each histogram.
Domain mapping of CD229 mAbs
To identify the Ig-like domains that are involved in the homophilic interaction of CD229, domain mapping was performed for the four mAbs previously produced by our laboratory (6). mAb binding to COS cells transfected with CD229 human/mouse chimeras indicated that HLy9.1.25, HLy9.1.38, and HLy9.1.77 were directed against Ig domain I, whereas HLy9.1.84 was reactive with Ig domain II (Table II). Moreover, only mAb HLy9.1.84 was able to bind with COS cells transfected with a CD229 isoform that lacks the first Ig domain (CD229 DI), further demonstrating that this mAb recognizes the second Ig domain. Cross-blocking studies showed that the mAbs recognized three epitopes: HLy9.1.25 and HLy9.1.38 recognize epitope A; HLy9.1.77 recognize epitope B; and HLy9.1.84 recognize epitope C (Table III).

The N-terminal domain of CD229 mediates homophilic interaction
CD229-Ig binding was tested with CD229 DI. This isoform was expressed on the cell surface of transfected COS cells as shown by positive staining with mAb HLy9.1.84. Deletion of the first V domain completely abolished binding of CD229-Ig, indicating that the direct and exclusive interaction between two opposing V domains is crucial for the homophilic CD229 interaction (Fig. 3). Furthermore, monovalent Fab of mAb HLy9.1.77, which maps to the first Ig domains and reacts with epitope B, were able to partially block (60%) the binding of CD229-Fc to wt CD229-transfected cells. In contrast, Abs recognizing epitope A, located in domain I, and mAb recognizing epitope C, located within domain

### Table II. Mapping the interaction domain of CD229 mAbs

<table>
<thead>
<tr>
<th>mAbs</th>
<th>CD229 DI/DII</th>
<th>CD229 DI/DII</th>
<th>CD229 DI/DII</th>
<th>mAb Interaction Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLy9.1.25</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>DI</td>
</tr>
<tr>
<td>HLy9.1.38</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>DI</td>
</tr>
<tr>
<td>HLy9.1.77</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>DI</td>
</tr>
<tr>
<td>HLy9.1.84</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>DI</td>
</tr>
</tbody>
</table>

* COS cells were transiently transfected with chimeric human (h)/mouse (m) constructs or the isoform lacking the first V-like Ig domain (CD229 DI) described in Materials and Methods. CD229 mAbs were assayed by flow cytometry: +, Detection of protein expression; –, nondetection of protein expression.

* Domain recognized by the mAb.

### Table III. Ability of test mAbs to block the binding of labeled mAbs to CD229–300.19 cells

<table>
<thead>
<tr>
<th>Test mAbs</th>
<th>Labeled mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLy9.1.25</td>
<td>+</td>
</tr>
<tr>
<td>HLy9.1.38</td>
<td>+</td>
</tr>
<tr>
<td>HLy9.1.77</td>
<td>+</td>
</tr>
<tr>
<td>HLy9.1.84</td>
<td>++</td>
</tr>
</tbody>
</table>

* Symbols represent the relative ability of test mAbs to block the binding of the indicated labeled mAbs to CD229–300.19 cells: Symbols indicate the percentage decrease in the number of fluorescence-positive cells: –, <20% of positive cells; +, 20–50% of positive cells; ++, 50–80% of positive cells; ++++, >80% of positive cells.
Ig domain mediates homophilic binding of CD229.

These data demonstrate that the first Ig domain mediates homophilic binding of CD229.

Identification of the key amino acid residues on the GFCC'C" face involved in CD229 homophilic interaction

Surface-exposed charged amino acids have been proposed to be involved in the intermolecular interaction of the N-terminal Ig domains of CD2 and its counterreceptor CD58 (35, 36). To identify key amino acid residues of CD229 that contribute to homophilic binding, a number of point mutations were generated by site-directed mutagenesis. Several charged amino acids that are conserved between human and murine CD229, located at the GFCC'C" face of the N-terminal Ig domain, were mutated to alanine. Binding of the CD229-Ig fusion protein was tested in COS cells transfected with wt and mutated CD229. COS cells expressing mutant CD229 molecules at levels comparable with wt were stained with mAbs, HLy9.1.25, HLy9.1.38, and HLy9.1.77, that recognize epitopes located within the first Ig-like domain. All of the mutants tested reacted similarly with these mAbs, suggesting no significant alteration of the general tertiary structure (Fig. 4B). Our results show that mutation of E27 and E29 on the predicted B-C loop and R89 on the F-G loop of the N-terminal domain all cause a disruption of the binding by >80%, whereas the rest of the mutants had no significant effect on the binding activity of CD229 (Table IV and Fig. 4A). Surprisingly, one mutation, R44A, caused a significant increase in binding to CD229-Ig (Table IV and Fig. 4).

CD229 redistributes to the contact area of T-B cell conjugates in an Ag-dependent manner

Here we used confocal microscopy to examine the localization of CD229 during Ag-dependent immune synapse formation. Supershift activation of Vβ8+ Jurkat cells has been described to trigger molecular polarization and segregation, forming a canonical immunological synapse (27). Jurkat T cells activated with the bacterial superantigen SEE-pulsed Raji cells formed immunological synapses similar to those described previously in mouse T cells activated with peptide Ag-bearing B cells. Both Jurkat and Raji cells expressed comparable levels of CD229. CD229 moved to the interface of the T-B cell conjugate after Ag triggering (Fig. 5B). CD229 was re-localized in 13.2% of the T-B cell conjugates analyzed in the absence of SEE. However, after incubation with SEE, the number of conjugates in which CD229 relocalized to the T-B cell contact area increased to a 38.0%, similar to the relocalization levels of CD3 (Fig. 5 and Table V). In contrast, CD45 was homogeneously distributed around the cell surface (Fig. 5 and Table V). The relocalization of CD229 was observed on both the Jurkat T cell and the Raji APC (Table VI). Moreover, the relocalization of CD229 was significantly affected by the presence of CD229 on APCs, because we observed that CD229 relocalization was significantly reduced when CD229 was not present on APCs. CD229 was not present on APCs in both the presence and the absence of SEE. In contrast, no significant differences in the relocalization of CD3 were observed (Fig. 6). To further analyze the localization of CD229 at the immunological synapse, colocalization studies were performed. We observed that the compact clusters of CD3 were embedded in large accumulations of CD229, similar to those observed with the integrin LFA-1 (Fig. 5A). Together, these data show that CD229 localizes to the immunological synapse.

Discussion

In this study, we have demonstrated that CD229 is a self-ligand, interacting through its N-terminal V-like domain, in which we have identified three residues critical for the homophilic interaction. We observed that a soluble CD229-Fc fusion protein binds to CD229-transfected cells, but not to cells transfected with the cDNA of other members of the CD150 family or the related cell surface molecules CD2 and CD58. Our data are consistent with the observation that the only known ligands of the CD150 family of cell surface receptors are other members of the same family. With the exception of CD48, which binds to CD244, ligand interactions within this family have been shown to be homophilic (CD84-CD84, CD150-CD150, CS1-CS1, and NTB-A-NTB-A) (9, 25, 26). Here we have shown that both human and murine CD229 can
bind to itself, indicating that the homophilic interaction of this molecule occurs in different species. Moreover, the CD229-CD229 homophilic interaction is not species restricted, because human CD229 also bound to murine CD229 and vice versa. This is in contrast to our results with CD84 and to previous reports for other members of the CD150 family where their interaction has been shown to be species specific (23, 37). These data indicate that residues involved in the homophilic interaction of CD229 may be preserved between these two species.

Our structure-function analysis, using domain deletion mutants, revealed that the homophilic interaction of CD229 is mediated by the membrane distal N-terminal Ig domain. If additional Ig domains were directly involved in this interaction, cells expressing a CD229 that only lacks the N-terminal domain would be expected to display a residual capacity to bind to CD229-Fc. However, soluble ligand binding was completely abolished upon deletion of the N-terminal V domain. Moreover, single amino acid point mutation of residues located in the first Ig-like domain abolished the CD229-CD229 interaction. N-terminal domains are generally important for the interaction of cell-adhesion molecules that belong to the IgSF (38).

When homology can be demonstrated with well-characterized proteins, many properties of the three-dimensional structure can be predicted. The model building analysis of the N-terminal domain of CD229 described here used the known structure of CD2 and CD58 as a guide (35). The basic structure of the V-like N-terminal domain of CD229 is a predicted tertiary fold of a stacked pair of β-pleated sheets. There are 9 β strands, with strands A, B, E, and D lying in one sheet and strands C, C’, C’, F, and G lying anti-parallel in the other (Fig. 7). Site-directed mutagenesis in CD2 and CD58 has shown that charged residues located on the GFCC’C’ face are critical for their interaction. The structural analysis of the ligand-binding domains of CD2 and CD58 confirmed that the binding is dominated by electrostatic contacts between binding surfaces exhibiting considerable electrostatic complementarity. Our results show that exposed residues on the GFCC’C’ face of the N-terminal domain also play a crucial role in homophilic interaction of CD229. Single mutations of the negatively charged residues E27 and E29 located in the predicted B-C loop, and the positively charged residue R89 on the F-G loop, abolished the CD229-CD229 interaction (Fig. 6). On the basis of mAb binding profiles, the presence of gross structural perturbation or misfolding, as a consequence of specific mutations was highly unlikely (Fig. 4B). Thus, we speculate that the negatively charged residues (E27 and E29) may interact with the positively charged residue (R89) in the counterreceptor interacting interface between the N-terminal adhesion domains of CD229. However, a crystal structure of CD229 binding to itself will be needed to confirm the actual way in which these residues interact at the molecular level.

**FIGURE 4.** Identification of residues involved in homophilic interaction of CD229. A, COS-7 cells transiently transfected with full-length CD229, E29A, or R44A cDNA were stained with HLy9.1.25 mAb followed by Cy3-conjugated anti-mouse Ig (upper panel). The transected cells were assayed with CD229-Ig followed by biotinylated anti-human Fc Ab and avidin-Cy3 (lower panel). B, COS-7 cells transiently transfected with full-length CD229, E27A, E29A, and R89A cDNAs were stained with HLy9.1.25, HLy9.1.38, and HLy9.1.77 mAbs followed by biotinylated anti-κ streptavidin-PE. Mean fluorescence intensities are shown.
As suggested for CD2 and CD58, we hypothesize that CD229 molecules expressed on the surface of adjacent cells establish head-to-head contacts. Of interest in this context is the observation that dynamic binding between CD2 and CD58 counterreceptors on opposing cells optimizes immune recognition through stabilization of T cell and APC cell contact (35).

Surprisingly, mutation of residue R44 located in the C′C′ loop enhanced the binding of CD229 to itself, a phenomenon that has been reported for other ligand-receptor pairs (39). This observation may aid the design of fusion proteins or small peptides that could be used to alter CD229 function in a therapeutic context.

Binding of the CD229-Ig could be observed only in transfected cells overexpressing CD229, indicating that its affinity for itself is weak. Nevertheless, CD229 binding is very specific, because it bonds only to itself and not to any other member of the CD150 family. Low affinity interactions are a common feature of homophilic receptors of the Ig superfamily including CD150 (Kd 200 μM). These interactions are characterized by remarkably fast on/off rates that foster rapid and extensive exchanges between partners on opposing cell surfaces. This is thought to allow cells to rapidly form and break contacts, conferring plasticity at the cell-cell contact areas or enabling the rapid surveillance of target membranes (38, 40). Whereas the affinity of adhesion molecules often

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**Table V. Frequency of relocalization at the Raji-Jurkat IS**

<table>
<thead>
<tr>
<th>Conjugates</th>
<th>− SEE</th>
<th>+ SEE</th>
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<tbody>
<tr>
<td>CD3</td>
<td>1.1 ± 1.1</td>
<td>47.4 ± 7.2</td>
</tr>
<tr>
<td>CD45</td>
<td>8 ± 2.8</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>CD229</td>
<td>13.2 ± 1.0</td>
<td>38.0 ± 1.8</td>
</tr>
</tbody>
</table>

*Quantification (%) of cell conjugates in which CD3, CD45, and CD229 were relocalized at the T cell-APC contact area in the presence or absence of SEE. At least 100 conjugates from three independent experiments were analyzed. Results correspond to the arithmetic mean ± SD.

**Table VI. Fold induction of fluorescence intensity at the IS**

<table>
<thead>
<tr>
<th></th>
<th>CD229 (n = 10)</th>
<th>CD3 (n = 5)</th>
<th>CD45 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS vs T cell</td>
<td>4.2 ± 2.0</td>
<td>13.1 ± 4.6</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>IS vs APC</td>
<td>6.4 ± 3.0</td>
<td>0.9 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

*Fluorescence intensity in the IS with respect to the rest of the T cell or APC membrane fluorescence. n is the number of T cell-APC contacts analyzed from two different experiments. Values are arithmetic means ± SD. IS, Immune synapse.
reflects a specific conformation of the extracellular binding domain, avidity modulation involves changes in the cellular distribution, which leads to clusters of molecules and thereby specifically increases the number of available receptors at the site of cell-cell interaction. This avidity can be further enhanced by the participation of other adhesion molecules at the contact site, such as the integrins (41). Thus, the lateral receptor mobility and accumulation of multiple interface bonds will be required for the development of appreciable adhesion.

To explore the functional relevance of CD229 homophilic binding, we analyzed its localization during immunological synapse formation. Immune receptors and components of their signaling cascades are spatially organized, and this spatial organization plays a central role in the initiation and regulation of signaling (42). The synapse is established and maintained to a large extent by the interaction of glycoproteins expressed on the surface of adjacent cells. Homophilic interactions of cell surface proteins typically result in their accumulation at sites of cell-cell contact. These molecules not only function as adhesion molecules but also translate biochemical information. By doing so, they regulate the initiation and prolongation of signaling triggered by ligand engagement of the immune cell signaling receptors. The ability to segregate receptors provides a mechanism for the compartmentalization of signaling components within the membrane, concentrating certain components in the contact area and excluding others with negative regulatory activity. Our results show that CD229 redistributes to the contact area established between T cells and enterotoxin E (SEE)-pulsed Ag-presenting B cells. The redistribution could be observed in both the T and the B cell. Moreover, the presence of CD229 on the B cell affected the relocation of CD229 on the T cell, indicating a role of CD229 homophilic interaction in its cellular distribution (Fig. 6). However, CD229 homophilic interaction was not required for CD3 relocation to the immunological synapse. Interestingly, the accumulation of CD229 was more evident at the periphery of the contact region, surrounding the compact clusters of CD3 (Fig. 5B). The molecular segregation of CD229 may be explained by the four-Ig domain structure of this cell surface molecule. It has been observed that cell surface molecules of the Ig superfamily that are present in the central synapse contain just two Ig domains and that this may be important to ensure the right distance for interactions between two cells. This is also the case for the CD150 family member CD244 (2B4) (29). Consistent with this notion, the interaction of CD2 with an elongated form of CD48 significantly inhibits immune recognition (43). Recent studies in our laboratory indicate that CD229 inhibits rather than induce T cell activation and cytokine production (44). Thus, we hypothesize that CD229 is excluded from the central synapse to prevent it from delivering negative signals. More studies including dynamic redistribution analysis of CD229 will be required to elucidate its contribution to the modulation of the functional state of the immunological synapse.

**FIGURE 6.** CD229 relocalization is affected by the presence of CD229 on APCs. Quantification of CD229 and CD3 relocalization to the T-B contact area of Jurkat-Raji or Jurkat-Raji CD229− conjugates in the presence or absence of SEE. More than 100 contacts were analyzed in three independent experiments. Histograms represent the arithmetic mean ± SD. *, p < 0.05 compared with Jurkat-Raji with Jurkat-Raji CD229− conjugates not treated with SEE. ***, p < 0.05 compared with Jurkat-Raji with Jurkat-Raji CD229− conjugates treated with SEE.

**FIGURE 7.** Molecular model of the N-terminal IgV set domain of human CD229. Ribbon diagram of the N-terminal domain of CD229 showing the predicted homophilic interface. The β strands GFCC′C′ are labeled according to the standard convention. The face GFCC′C′ is gold, and the ABED face is gray. The mutated amino acids that strongly modify the interaction are indicated on the model according to the one-letter amino acid codes. Substituted amino acids that abolish adhesion are labeled in white, whereas residue 44, which strongly enhances adhesion, is labeled in light blue. Negatively charged atoms are labeled in red, and positively charged atoms are labeled in blue.
Immunological synapse occurring during cognate interactions between B and helper T cells, within the B cell follicles of secondary lymphoid organs, are required for humoral immune responses to many Ags (45). Expression of CD229 by B cells is essential for T cells to provide B cell help and establish long term humoral immunity (46). The fact that CD229 is a SAP-associated homophilic receptor expressed on both T and B cells and that it localizes at the immune synapse suggests that this molecule has a role in the regulation of T-B interactions. Further evidence supporting the importance of CD229 in regulating T cell help for B cells is the recent finding that CD229 is preferentially expressed by effector T follicular helper cells (47).

The identification of CD229 as a homophilic receptor and characterization of the binding site is an important step in advancing our understanding of how this interaction can regulate the immune response.

Disclosures

The authors have no financial conflict of interest.

References


CD229 IS A HOMOLOGIC CELL SURFACE RECEPTOR