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Identification of Grb2 As a Novel Binding Partner of the Signaling Lymphocytic Activation Molecule-Associated Protein Binding Receptor CD229

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Signal recognition by the TCR determines the subsequent fate of the T cell and is regulated by the involvement of other cell surface molecules, termed coreceptors. CD229 is a lymphocyte cell surface molecule that belongs to the CD150 family of receptors. Upon tyrosine phosphorylation, CD229 recruits various signaling molecules to the membrane. One of these molecules is the signaling lymphocytic activation molecule-associated protein, of which a deficiency leads to the X-linked lymphoproliferative syndrome. We report that CD229 interacts in a phosphorylation-dependent manner with Grb2. We mapped this interaction showing that the Src homology 2 domain of Grb2 and the tyrosine residue Y606 in CD229 are required for CD229-Grb2 complex formation. The Grb2 motif in the cytoplasmic tail of CD229 is distinct and independent from the two tyrosines required for efficient signaling lymphocytic activation molecule-associated protein recruitment. CD229, but not other members of the CD150 family, directly bound Grb2. We also demonstrate that CD229 precipitates with Grb2 in T lymphocytes after pervanadate treatment, as well as CD229 or TCR ligation. Interestingly, the CD229 mutant lacking the Grb2 binding site is not internalized after CD229 engagement with specific Abs. Moreover, a dominant negative form of Grb2 (containing only Src homology 2 domain) impaired CD229 endocytosis. Unexpectedly, Erk phosphorylation was partially inhibited after activation of CD229 plus CD3. Consistent with this, CD229 ligation partially inhibited TCR signaling in peripheral blood cells and CD229-Jurkat cells transfected with the 3XNFAT-luciferase reporter construct. Altogether, the data suggest a model whereby CD229 ligation attenuates TCR signaling and Grb2 recruitment to CD229 controls its rate of internalization. The Journal of Immunology, 2005, 174: 5977–5986.

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ignaling adaptors that contain multiple protein-protein interaction domains and/or motifs but lack enzymatic activity are important for signal integration because they bring enzymes and their substrates into close proximity. The gene responsible for X-linked lymphoproliferative disease, SAP/SH2D1a, encodes a protein that defines a new class of adaptor (1–6). Although, it contains a single SH2 domain, it can function as an adaptor molecule, bringing the kinase Fyn to the CD150 receptor via a novel mechanism (7). The T-(I/V)-pY-x-x-(I/V) (where x stands by any amino acid) motifs present in the cytoplasmic tail of CD150 function as docking sites for the SAP/SH2D1a protein. Six members of the CD150 family (CD150, CD229, CD84, CD244, NBT-A, and CS1) have one or more cytoplasmic tyrosine motifs containing this consensus sequence. These receptors are located on the same chromosome (1q23) and share a common overall structure that includes an extracellular domain comprising Ig-like domains, a single transmembrane segment, and a cytoplasmic domain with three or more tyrosine-based motifs (1, 3, 8–11). The pathology of X-linked lymphoproliferative disease syndrome suggests distinct roles for the six CD150 family/SAP signaling pathways in control of T cell, B cell, and NK cell activation and function (6, 12). These members also are functionally linked, forming receptor-ligand pairs involved in homotypic or heterotypic associations. CD150, CD84, and CS1 are self-ligand molecules (13–16), whereas CD244 interacts with CD48 (17).

In this work, we have focused our attention on CD229 (Ly9) signaling and function. This receptor is a 120-kDa cell surface glycoprotein, although two major forms of 100 and 120 kDa have been described in human T and B lymphocytes (18). Other members of the subfamily, such as, CD84, CD150, and CD244 are known to function as coreceptors in T cells, B cells, and NK cells (13, 15, 19–23), suggesting that CD229 may also be involved in leukocyte activation. Recently, NBT-A and CS1 have also been shown also to regulate cytotoxicity in NK cells (16, 24).

CD229 exhibits structural differences from the rest of the CD150 family of receptors. It has four extracellular Ig domains; domains 1 and 3 are similar, as are domains 2 and 4, suggesting that CD229 arose from a progenitor with two domains (25). Its cytoplasmic tail comprises 180 amino acids, including 16 threonines, 17 serines, and 8 tyrosines (18). Two unique tyrosine-based motifs (with the consensus amino acid sequence T-V/I-Y-xx-V/I) are critical for binding to SH2D1a/SAP protein and the similar EAT-2 SH2 domain protein in B cells (26).

Recently, it has been reported that CD229 is the only member of the CD150 family to interact with the μc-chain of the AP-2 adaptor complex that links transmembrane proteins to clathrin-coated pits (27). Moreover, receptor internalization is regulated by TCR and...
BCR signaling because coligation with Abs against anti-CD3 and anti-IgM increased the rate of CD229 endocytosis (27). These data indicate that cell surface expression of CD229 in lymphocytes is highly regulated through Ag receptor ligation. The aim of this study was to explore the signal transduction process and function of this molecule in T lymphocytes. For this purpose, we used three-hybrid technology to search for CD229 binding partners. We show that CD229 binds to Grb2, a highly conserved adaptor protein that helps activate the Ras/MAPK signal transduction pathway in response to extracellular signals (28–30). We dissected this interaction, showing that residues Y606 from CD229 and the Grb2 SH2 domain are binding partners. This interaction occurred in lymphocytes and required receptor phosphorylation. The Grb2 binding site of CD229 is important for the proper endocytosis of the receptor. CD229 ligation with Abs lead to partial inhibition of ERK phosphorylation and to XNFKAT reporter activity induced by TCR cross-linking. All these data indicate that CD229 attenuates TCR signaling. Moreover, TCR ligation can also modulate this process by phosphorylating CD229, allowing Grb2 binding and enhancing the internalization of CD229.

Materials and Methods

Cells and reagents

COS-7 cells were maintained in DMEM supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). Hemagglutinin A (HA)-CD229, HA-CD229Y606F, and CD229 Jurkat cells (10) were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin plus G418 (1 mg/ml) (Invitrogen Life Technologies). Anti-mouse IgG peroxidase and F(ab′)2 sheep anti-mouse were obtained from Sigma-Aldrich. Monoclonal anti-Grb2 was from Upstate Biotechnology and rabbit anti-Grb2, Grb2-GST, and SH2-Grb2-GST were from Santa Cruz Biotechnology. HRP-conjugated anti-phosphotyrosine mAb mixture was from Zymed Laboratories. Anti-human CD229 Ab (clone HLY-9.1.25) (IgG1) was generated in our laboratory (18). Purified-mAbs were biotinylated with biotinamidocaproate N-hydroxysuccinimide ester (Sigma-Aldrich). Monoclonal Ab against murine P-selectin, with the same isotype Ig as the CD229 Ab (IgG1), was used as a control Ig (31). Anti-human CD3 (clone 3B3) was a gift from Dr. M. López-Botet (University Pompeu Fabra, Barcelona, Spain). Streptavidin-CyChrome and anti-rabbit peroxidase were from DAKO. ERK Abs used include pERK from Cell Signaling Technology and total ERK from Zymed Laboratories. Anti-HA (clone 12CA5) and anti-GFP monoclonal were from Roche Diagnostics. Goat anti-mouse IgG FITC is from Caltag Laboratories. Tosyl-activated beads and Dynabeads sheep anti-mouse IgG were from Dynal Biotech. The following mAbs were obtained from BD Pharmingen: anti-human IFN-γ (clone NIB42) mAb, anti-human biotinylated IFN-γ (clone 4SB3), and IFN-γ standard.

Yeast three-hybrid screen

The cytoplasmic tail of human CD229, cloned in the bicistronic vector pBridge (BD Clontech) and transformed in the yeast strain CG1945, was used as bait to screen a human B cell cDNA library in the pACT2 vector (BD Clontech). CD229 and mutated c-fyn were inserted in pBridge. In the mutant c-fyn, the regulatory tyrosines 420 and 531 were substituted by phenylalanine and arginine, respectively, and cloned in frame into the 3.1/V5-His vector (Invitrogen Life Technologies). The primers used to generate the mutation were 5′-AGTCTACCTTGAAAAT-3′ and 5′-GAAATTTTCAAAGTGGACC-3′. The presence of desired nucleotide changes was established by bidirectional nucleotide sequencing using dye terminator chemistry (PE Applied Biosystems). For expression in yeast, the fusion proteins Gal4BD-cytosplasmic CD229WT and Gal4BD-cytosplasmic CD229Y606F were generated from CD229WT and CD229Y606F cDNAs, respectively, using the sense primer 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ and the antisense primer 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ and subcloned into the pBridge binding domain vector using the EcoRI/BamHI sites. The fusion protein with the HA epitope, HA-CD229, and HA-CD229Y606F were generated using the sense primer 5′-GGGGAATTCACGCTAGGAGGATCATACC-3′ and the antisense primer 5′-CGGGTCTGACTACGAGAATTTTCTCACG-3′ and subcloned into the pDisplay vector using the BglII/KpnI sites.

To generate the Grb2 construct, Grb2 was amplified by PCR using as a template a Jurkat cDNA using sense primer 5′-CCCTCCCCGTGCTTCAGGC-3′ and antisense primer 5′-TGCGGTCTAGTGGACTATACG-3′. The product of this PCR was used as a template for a second PCR using the primers 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ and 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ containing EcoRI and BamHI sites, respectively, and cloned in frame into the 3′/V5-His vector (Invitrogen Life Technologies). To generate a construct containing only the SH2 domain of Grb2 (dominant negative) we used the Grb2 construct previously described as a template for PCR with the sense primer 5′-ATGGAAACCCATCGTCCGAAAACATGGTACG-3′ and antisense primer 5′-CACGCTCTATTCAAG-3′. The PCR product was cloned in frame into the 3′/V5-His vector (Invitrogen Life Technologies) and, using HindIII/XhoI restriction sites, it was subcloned in frame into the enhanced GFP (EGFP) pEFGP N1 vector (BD Clontech). The construction of the following constructs: µ-chain of the AP-2 complex cloned in pBridge, µ-chain cloned in pEFGP or CD229 Y470F mutant in pBridge are described elsewhere (27).

Transfections

COS-7 cells (1 × 10⁶) were seeded into 100-mm dishes, and 24 h later the cells were transfected with 4 μg of plasmid DNA per dish using Nucleofector (Amaxa) according to the manufacturer’s instructions. Cells were incubated for 48 h before experimental analysis. Jurkat E.61 were stably transfected with HA-CD229 or HA-CD229Y606F cDNAs by electroporation (260 V and 950 μF) and selected in the presence of 1 mg/ml G418. Positive populations for HA staining were enriched with anti-HA Ab and tosyl-activated beads.

Internalization assays

CD229WT and CD229Y606F transfected COS cells (1 × 10⁶/sample) were incubated with anti-CD229 (1 μg/sample) at 4°C or 37°C for 30 min. Afterward, the cells were chilled rapidly and acid-stripped to remove cell surface-bound Ab, as described elsewhere (27). The samples were washed and then incubated with biotinylated anti-CD229 followed by Streptavidin-CyChrome incubation. HA-CD229WT or HA-CD229Y606F transfecteds were incubated with anti-HA (1 μg/sample) at 4°C or 37°C for 30 min. Thereafter, the cells were chilled rapidly and acid stripped and then incubated with anti-HA Ab followed by mouse anti-FITC incubation. The samples were analyzed in a flow cytometer (FACScaliber; BD Biosciences) to detect CyChrome (FL3) fluorescence. The percentage of endocytosis was calculated using mean fluorescence intensity values as follows: [100 – (mean fluorescence intensity at 37°C/mean fluorescence intensity at 4°C)] × 100.

In parallel, CD229WT cells were cotransfected with empty EGFP-N1 or SH2-Grb2-EGFP-N1 and the internalization assay we performed as described. In this case, the samples were treated after transformation of HB101 bacteria and selection in M9 medium lacking Leu to isolate the GAL4 activation domain plasmid pACT2. Purified plasmids were sequenced.

The β-galactosidase colony-lift filter assay and liquid culture assay using ONPG (o-nitrophenyl-phosphate-galactoside) as a substrate were conducted as described in the BD Clontech yeast protocols handbook. The vector pBridge containing CD229 in the absence of mutated c-fyn was used as an alternative to repressing c-fyn expression with methionine because in the high-density cultures necessary for transformation, methionine can be depleted, thus activating expression of c-fyn.

Plasmid constructions

Human CD229 cloned in pC-neo was used as a template to generate CD299INKD mutant. It was cloned in frame in the 3′/V5-His vector (Invitrogen Life Technologies). The primers used to generate the mutation were 5′-AGTCTACCTTGAAAAT-3′ and 5′-GAAATTTTCAAAGTGGACC-3′. The presence of desired nucleotide changes was established by bidirectional nucleotide sequencing using dye terminator chemistry (PE Applied Biosystems). For expression in yeast, the fusion proteins Gal4BD-cytosplasmic CD229WT and Gal4BD-cytosplasmic CD229Y606F were generated from CD229WT and CD229Y606F cDNAs, respectively, using the sense primer 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ and the antisense primer 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ and subcloned into the pBridge binding domain vector using the EcoRI/BamHI sites.

To generate the Grb2 construct, Grb2 was amplified by PCR using as a template a Jurkat cDNA using sense primer 5′-CCCTCCCCGTGCTTCAGGC-3′ and antisense primer 5′-TGCGGTCTAGTGGACTATACG-3′. The product of this PCR was used as a template for a second PCR using the primers 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ and 5′-GGGGGATCCGGATCCGAAAGGAGGAGC-3′ containing EcoRI and BamHI sites, respectively, and cloned in frame into the 3′/V5-His vector (Invitrogen Life Technologies). To generate a construct containing only the SH2 domain of Grb2 (dominant negative) we used the Grb2 construct previously described as a template for PCR with the sense primer 5′-ATGGAAACCCATCGTCCGAAAACATGGTACG-3′ and antisense primer 5′-CACGCTCTATTCAAG-3′. The PCR product was cloned in frame into the 3′/V5-His vector (Invitrogen Life Technologies) and, using HindIII/XhoI restriction sites, it was subcloned in frame into the enhanced GFP (EGFP) pEFGP N1 vector (BD Clontech). The construction of the following constructs: µ-chain of the AP-2 complex cloned in pBridge, µ-chain cloned in pEFGP or CD229 Y470F mutant in pBridge are described elsewhere (27).
**Immunoprecipitation and immunoblotting**

Transfected COS-7 cells were lysed with 1% CHAPS (Pierce) plus phosphatase and protease inhibitors as previously described (10) and immunoprecipitation was conducted using Abs against CD229. Similarly, CD229-Jurkat cells (1×10⁶) were activated with 1 nM pervanadate for 10 min, or anti-CD229 or anti-CD3 Abs plus crosslink (Fab′)2 sheep anti-mouse for various times. Cells were starved overnight (RPMI 1640 without FCS) to reduce basal levels of phosphorylation. CD229-Jurkat cells were lysed with 1% Triton X-100. Briefly, cell lysates were preclarified for 1 h with 50-μl protein Sepharose beads (Amersham Pharmacia Biotech). Immunoprecipitations were performed using 2 μg of each indicated Ab, and 40-μl protein A-Sepharose beads were added for 2 h at 4°C. Beads were then washed and samples were boiled. All the precipitates were separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Millipore). Filters were blocked for 1 h with 5% skim milk (or 3% BSA) and then probed with the indicated Abs. Bound Ab was detected using HRP-conjugated secondary Abs and ECL (Supersignal; Pierce).

**Bead activation and ERK assays**

Tosyl-activated beads were incubated with various ratios of Abs: anti-CD3 and anti-CD229 (1:2 and 1:9), as described by the manufacturer. In parallel, anti-CD3 and anti-IgG isotype controls were performed. Activations using CD229-Jurkat cells were performed using the ratio one bead to one cell and the selected combination one anti-CD3 vs nine anti-CD229 or IgG isotype control.

A total of 1×10⁶ CD229-Jurkat cells were incubated with the following stimuli: treated beads not coated with Abs, anti-CD3 plus IgG control beads, and anti-CD3 plus anti-CD229 beads for 10 and 20 min at 37°C. Cells were lysed as described and run on a 10% SDS gel. Blots were incubated with Abs against phospho-ERK and total ERK.

**Cytokine assay**

PBMC were activated with various concentrations of anti-CD3 plus anti-CD229. Supernatants were collected after 48 h of culture and IFN-γ levels were measured by sandwich ELISA. Primary anti-human IFN-γ and biotinylated secondary Abs were used at the concentrations recommended by the manufacturer. Values reported are the mean of triplicate wells, and SEM values were <15%.

**Luciferase assays**

Twenty million CD229-Jurkat cells or HA-CD229WT or HA-CD229Y606F Jurkat cells were electroporated with 3XNFAT luciferase reporter (0.5 μg/ml per million cells) and TK Renilla plasmid (0.1 μg/ml per million cells) and cultured in complete RPMI 1640 medium for 24 h after transfection as described elsewhere (32). Transfected cells were transferred to 12-well plates in a final volume of 2 ml of RPMI 1640 growth medium for stimulation. Cells were then stimulated at 37°C with 5 μg/ml purified anti-CD229 or IgG isotype control and 1 μg/ml anti-CD3, using the murine mastocytoma P815 cell line as a cross-linker. Alternatively, cells were activated with Abs bound to beads instead of P815 cells. Cells were lysed after 6 h stimulation with 5× lysis buffer (Promega), and the Dual Luciferase reporter kit (Promega) was used to measure luciferase activity as relative light units (RLU) per second in a luminometer (Lumat LB 9507; EG&G Berthold). Three independent experiments with duplicates for each experimental condition were performed. Luciferase readings in cell lysates were normalized to TK Renilla within each point.

**Results**

**Grb2 binds to the cytoplasmic tail of CD229**

To search for CD229 binding partners we performed a three hybrid assay using the cytoplasmic tail of CD229 cloned alongside a mutated form of Fyn into pBridge as bait and we screened a B cell assay using the cytoplasmic tail of CD229 cloned alongside a mutant for Grb2 (B).

**The SH2 domain of Grb2 is involved in CD229–Grb2 association**

Next, we determined whether this interaction occurs in mammal cells in a similar fashion to that observed in a yeast system. Using the GST of Grb2 and the SH2 domain of Grb2 we found that CD229 coprecipitated with Grb2–GST in COS cells transfected with Fyn plus CD229 (Fig. 2A). In conditions in which Fyn was absent, no precipitation of the complex was observed. These results agree with our previous result that phosphorylation was involved in the CD229-Grb2 association. Consistent with this result,
we pulled down the CD229 receptor using only the SH2-Grb2 GST indicating that SH2-Grb2 is the binding partner of CD229 and SH3 domains of Grb2 are not required for the interaction. The specificity of interaction between CD229 and Grb2 was further proven in Jurkat cells treated with pervanadate. We used a CD229-transfected Jurkat cell line because levels of CD229 are low in Jurkat cells and we need a large number of cells to find associated molecules. Cells were activated with pervanadate and immunoprecipitated with Abs against CD229 and Grb2. The association between Grb2 and CD229 occurred only when the receptor was phosphorylated (Fig. 2B). Altogether these data show that the association between CD229 and Grb2 involved phosphorylation of the receptor and the SH2 domain of Grb2.

**TCR ligation induces CD229 phosphorylation and Grb2 recruitment**

Upstream events of T cell activation involve ligation of CD4/CD8-Lck and the TCR complex leading to the activation of Src kinases Lck and Fyn and the phosphorylation of several proteins (33). Because Lck is also a major kinase in lymphocytes we tested whether both Lck and Fyn were equally efficient in their ability to phosphorylate the receptor and allow Grb2 recruitment. Results indicated that both Src kinases, Fyn and Lck, can support the phosphorylation of the receptor and the SH2 domain of Grb2.

**CD229 at the plasma membrane** (27). In contrast, Grb2, in addition to its key function in signaling through Ras, has a major regulatory role at the initial steps of the epidermal growth factor receptor (EGFR) internalization (34, 35). To assess whether the Grb2 binding site has some role in CD229 endocytosis we transfected COS cells with CD229 WT and CD229 V606F and performed internalization assays. Mutation of Y606 completely abolished receptor internalization, as compared with CD229 WT (Fig. 4A). However, we cannot rule out that proteins other than Grb2 may bind to Y606 and be important in CD229 internalization. To analyze this further we generated a dominant-negative form of Grb2 consisting of only its SH2 domain. An SH2-Grb2 fusion construct was generated by cloning into a pEGFP vector and COS cells were cotransfected with CD229 WT and either the empty pEGFP or SH2-Grb2-pEGFP. As shown in Fig. 4B, cells containing the dominant negative form of Grb2 exhibited a significant inhibition of CD229 endocytosis. This result highlights the role of the Grb2 binding site in regulating internalization of the CD229 receptor. We also analyzed the CD229 V606F mutant internalization in Jurkat cells. For this purpose we generated transfected cells which expressed CD229 WT or CD229 at the plasma membrane.

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**Table 1. Grb2 binding is restricted to CD229 within the CD150 family of receptors**

<table>
<thead>
<tr>
<th>β-Galactosidase Binding Assay</th>
<th>With Grb2</th>
<th>With SAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTB-A</td>
<td>YYSV-YAVG-YSTI</td>
<td>–</td>
</tr>
<tr>
<td>CD84</td>
<td>YAYA-YTTI-YDEI-YSEV-YEIVI</td>
<td>–</td>
</tr>
<tr>
<td>CD150</td>
<td>YOTT-YAOG-YVAA-YAVV</td>
<td>–</td>
</tr>
<tr>
<td>CS1</td>
<td>YTEE-YDDT-YSTV-YENV</td>
<td>–</td>
</tr>
<tr>
<td>CD229</td>
<td>YSVL-YEKL-YEIVF-YDPV-YVTE-YAQQ-YCRI-YENF</td>
<td>++ +</td>
</tr>
<tr>
<td>CD244</td>
<td>YEDV-YSMI-YSLI-YEIV</td>
<td>–</td>
</tr>
</tbody>
</table>

*Cytoplasmic tails of the CD150 members were cloned in pBridge vector in the presence of Fyn. Yeast was cotransfected with each member plus Grb2-pACT2 or SAP-pGAD, and a β-galactosidase assay was carried out. The Grb2 putative motif is shown in bold and the tyrosines involved in SAP binding are underlined. Range of activity indicated is: –, No detectable activity, as for empty vectors; +, activity of 1–5 relative β-galactosidase units; ++, activity of 5–10 units; ++++, activity above 10 units.

**FIGURE 2.** SH2 domain of Grb2 is involved in CD229-Grb2 association. COS cells transfected with full length CD229, in the presence (+) or absence (−) of Fyn, were lysed, and GST precipitation with complete Grb2-GST or SH2-Grb2-GST was conducted. A, Blots were probed with monoclonal anti-phosphotyrosine HRP, monoclonal anti-CD229, and anti-GST. CD229-Jurkat cells (0.8–1 × 10⁶) were activated with 1 mM pervanadate for 15 min. Immunoprecipitation using rabbit anti-Grb2 and monoclonal anti-CD229 was performed. B, Blots were probed with anti-phosphotyrosine, monoclonal anti-Grb2, and anti-CD229 Abs.
CD229 binds to the CD229Y606F mutant binds the CD229Y606F and CD229WT bound μ-EGFP chimera similarly. Previously, we reported that μ-EGFP integrates in the endogenous AP-2 complex in COS cells (27). These data indicate that the inhibition of endocytosis of the CD229 mutant for Grb2 after CD229 ligation for 30 min is not due to the lack of μ-chain recruitment to the receptor.

CD229 inhibits ERK phosphorylation induced by TCR cross-linking

TCR engagement leads to the recruitment of multimolecular components to the cell surface, including Grb2, which in turn activate the sos-ras-MEK-ERK pathway (30, 36). To analyze the functional consequences of CD229-Grb2 association, we studied MAPK activation through the ERK phosphorylation. Cells were treated with either uncoated beads or anti-CD3 plus IgG control beads and anti-CD3 plus anti-CD229 beads for various times at 37°C. CD229 partially inhibited Erk phosphorylation in CD229-Jurkat cells after 20 min of activation (Fig. 6A). No differences were observed at shorter times. Similarly, CD229 inhibited Erk phosphorylation in peripheral blood lymphocytes from healthy donors (Fig. 6B), although kinetics of partial inhibition appeared to be more rapid in these cells. Anti-Erk immunoblotting was used as a loading control (Fig. 6, A and B).

CD229 partially blocks TCR-mediated cytokine release

Because inhibition of Erk blocks T cell proliferation down-regulating IL-2 mRNA levels (37), we examined this partial inhibitory function further by studying more downstream events. CD229 cells were transiently transfected with a plasmid that encoded the firefly luciferase gene as a reporter under the control of the murine NFAT/AP-1 IL-2-dependent promoter, as described elsewhere (32). Cells were stimulated through the TCR by cell surface cross-linking with anti-CD3 mAb and an isotopic Ig control or anti-CD3 mAb and anti-CD229 mAb for 6 h, using P815 cells as a cross-linker or Ab-coated beads. After stimulation, luciferase reporter activity was measured. Cross-linking of the TCR lead to an activation measured as an increase in the luciferase activity expressed as RLU per second (Fig. 7). This activity was reduced by 40–50% when both TCR and CD229 were cross-linked at the same time using either soluble Abs (Fig. 7A) or beads (B).

We also assessed cytokine release after coligation of the Ag receptor and CD229 in human peripheral blood lymphocytes. CD229 did not exert an effect on cytokine release when we incubated the cells with an Ab against CD229 alone or in combination with a cross-linker. Anti-CD229 partially inhibited IFN-γ release due to the anti-CD3 ligation in human peripheral blood lymphocytes (Fig. 7C).

CD229Y606F still has a negative effect in TCR-mediated cytokine release

By controlling receptor levels at the cell surface and in endosomes, endocytic trafficking serves as an important determinant of the intensity and duration of receptor signaling. Because CD229Y606F internalization is impaired after CD229 ligation, we next assessed whether this mutant still negatively regulates TCR-mediated cytokine release. HA-CD229WT or HA-CD229Y606F were transiently transfected with a plasmid that encoded the firefly luciferase gene as a reporter under the control of the NFAT/AP-1 IL-2-dependent promoter, as described elsewhere (32). Cells were stimulated through the TCR by cell surface cross-linking with anti-CD3 mAb and an isotopic Ig control or anti-CD3 mAb and anti-CD229 mAb for 6 h, using P815 cells as a cross-linker or Ab-coated beads. After stimulation, luciferase reporter activity was measured. Cross-linking of the TCR lead to an activation measured as an increase in the luciferase activity expressed as RLU per second (Fig. 7). This activity was reduced by 40–50% when both TCR and CD229 were cross-linked at the same time using either soluble Abs (Fig. 7A) or beads (B).
and an isotypic Ig control or an anti-HA mAb for 6 h, using P815 cells as a cross-linker. In these conditions we also observed a partial inhibition of TCR signaling when we coligate the CD3 and the HA-CD229WT (Fig. 8A), and HA-CD229Y606F still negatively regulated T cell activation (B). Altogether, these data suggest that the binding partner or partners of CD229 that lead to a negative signaling are not mapped in this motif.

Discussion
In this study we provide the first evidence of an SH2-Grb2 interaction with the SAP-binding receptor CD229. We screened a cDNA B cell library using the cytoplasmic tail of CD229 as bait and found three independent clones encoding Grb2. The ubiquitous adaptor protein Grb2 has been extensively analyzed biochemically and is known to link cell surface receptors to downstream signaling molecules. Upon receptor stimulation Grb2 translocates from the cytosol to the cell surface where it binds receptors at specific sites of tyrosine phosphorylation via its SH2 domain (38, 39). Once bound to the receptor, Grb2 is thought to use its SH3 domains to link ligand-activated receptors to their distal signaling apparatus. We report that CD229 binds to Grb2 when the kinase Fyn is active in yeast, suggesting that the SH2 domain of Grb2 is involved in the binding. Y606 in the C-terminal end of CD229 is a critical residue for this interaction because replacement with phenylalanine completely abrogates Grb2 binding. The consensus motif for Grb2 binding pYxxNx (where x stands by any amino acid) is different from the SAP docking site T-V/I-Y-xx-V/I. Thus, predictably, we found that SAP binding is not impaired in the CD229Y606F mutant. Our data shows that CD229 is the only member within the CD150 family of receptors that directly binds to the...
SH2 domain of Grb2 (Table I). Moreover, YENF motif is conserved in human and murine CD229 (18). Although one study reported that CD244 coprecipitated with Grb2 (40), we did not detect binding of these two molecules in our system. That interaction may be mediated by the linker for the activation of T cells, which has been reported to associate with CxC cysteine motif found in the transmembrane region of CD244 (41). The cytoplasmic tail of CS1 has a similar, putative Grb2 binding to the one found in CD229, but we were unable to detect any interaction between these two proteins. The fact that the Grb2 binding motif in CS1 is not conserved in mouse suggests the minor relevance of this motif and may provide a possible explanation for the lack of human CS1-Grb2 binding. The SH2 domain of Grb2 is involved in the CD229-Grb2 interaction in mammalian cells and is dependent upon the kinase activity of Fyn. No association with the adaptor was seen in the absence of phosphorylation, suggesting no participation of the SH3 domains of Grb2 in CD229-Grb2 complex formation. Western Blot analysis of CD229-Jurkat cells using pervanadate also revealed a Grb2-CD229 interaction in lymphocytes, where this receptor is mainly expressed. Src kinases, Fyn, and Lck, which mediate the upstream events in the TCR signaling, are involved in CD229 phosphorylation, allowing Grb2 recruitment. CD229 became transiently phosphorylated after CD229 ligation and CD3 triggering allowing Grb2 recruitment.

Recently, our group published that TCR signaling enhances CD229 receptor endocytosis (27). A role for Grb2 in receptor endocytosis has been previously proposed based on the observation of the inhibition of epidermal growth factor uptake by microinjected Grb2 mutant proteins (35). Mutations of EGFR on tyrosines that interact with Grb2 completely abolish receptor internalization through clathrin-coated pits (34). We found that CD229Y606F mutant internalization was impaired in COS cells, similarly to the CD229Y470F mutant for AP-2 binding (27) indicating an important role of this motif in the control of CD229 expression at the cell surface. In addition SH2-Grb2 overexpression greatly reduced CD229WT internalization. The internalization was not completely impaired probably because the levels of transfection, despite that they were higher than 60%, were variable in intensity. It is also possible that there is another pathway of CD229 endocytosis in COS cells independent of Grb2. Nevertheless, these data suggest that Grb2-dependent pathway may be a major route of endocytosis of CD229 in COS cells. Moreover, the endocytosis of the CD229Y606F mutant in Jurkat cells was also impaired after anti-HA ligation.

**FIGURE 5.** CD229Y606F mutant still binds to the μ-chain of the AP-2 complex. A. Using the cytoplasmic tail of CD229wt or CD229Y606F mutant or Y470F mutant (cloned in pBridge) a semiquantitative β-galactosidase assay with the μ-chain of the AP-2 complex (cloned in pGAD plasmid) was performed. COS cells were transiently transfected with CD229wt or CD229Y606F mutant and μ2 EGFP cDNAs. At 48 h after transfection an immunoprecipitation using Abs anti-CD229 was completed. B. Blots against EGFP and CD229 were performed.

**FIGURE 6.** CD229 inhibits ERK1/2 phosphorylation in CD229-Jurkat cells and peripheral blood lymphocytes. A and B. Cells (1 × 10⁶) were treated with uncoated beads, anti-CD3+IgG-coated control beads and CD3+anti-CD229-coated beads for various times, 10 and 20 min, at 37°C. Cells were lysed and run on 10% SDS-PAGE gels. Blots were probed with anti-phospho-ERK and anti-total ERK. B. Lymphocytes from peripheral blood were isolated by Ficoll density gradient centrifugation of cells obtained from healthy donors. These results are representative of four independent experiments.
Because phosphorylation is a mechanism proposed to block endocytosis mediated by the AP-2 complex, the phosphorylation status of the tyrosine responsible for AP-2 binding will be relevant to CD229 internalization. Our data indicate that this tyrosine (Y470) is not phosphorylated by Src kinases such as Fyn (data not shown). Because Fyn phosphorylates Y606 of CD229 but not Y470, Grb2 may be recruited together with AP-2-enhancing receptor internalization.

The way that Grb2 enhances internalization is unknown and may be dependent or independent of clathrin-mediated internalization. One possible AP-2-dependent mechanism may be achieved after Grb2 binding to CD229 induced by TCR ligation. After TCR activation, PI3K may become activated and the production of phosphatidylinositol triphosphate increased. Grb2 can bind to PI3K and bring it into close proximity. Furthermore, the α2 subunit of AP-2 binds phosphatidylinositol triphosphate with high affinity favoring its location in the membrane and an increase in the affinity of μ2 binding to endocytosis sorting signals (42).

Supporting this possibility we found that wortmannin, a PI3K inhibitor, greatly reduced CD229 internalization upon receptor ligation and TCR coligation but we could not detect binding of PI3K to CD229 phosphorylated by Src kinases such as Fyn (data not shown). Because Fyn phosphorylates Y606 of CD229 but not Y470, Grb2 may be recruited together with AP-2-enhancing receptor internalization.

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ERK partial inhibition induced by CD3 and CD229 ligation involves changes in cytokine profile production. Concomitantly, CD229 ligation attenuates TCR-induced cytokine production in CD229-Jurkat cells and peripheral blood lymphocytes. CD229<sub>Y606F</sub> mutant does not reverse the negative effect of CD229 in TCR-mediated cytokine release. Possibly, an unidentified binding partner may be controlling this event.

Our data reveal Grb2 to be a specific, well-characterized binding partner for CD229, and this is the first report studying functional consequences of CD229 engagement. CD229 negatively regulates TCR signaling inhibiting partially ERK phosphorylation and cytokine release. TCR ligation can phosphorylate CD229 allowing Grb2 recruitment and favoring the endocytosis of the receptor reducing its expression on the cell membrane (Fig. 9). Further studies, will be needed to fully elucidate the downstream consequences of CD229 on T cell function.

**Disclosures**

The authors have no financial conflict of interest.

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