CD2BP1 Modulates CD2-Dependent T Cell Activation via Linkage to Protein Tyrosine Phosphatase (PTP)-PEST

Hailin Yang and Ellis L. Reinherz

*J Immunol* 2006; 176:5898-5907; doi: 10.4049/jimmunol.176.10.5898
http://www.jimmunol.org/content/176/10/5898

References This article cites 67 articles, 34 of which you can access for free at: http://www.jimmunol.org/content/176/10/5898.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2006 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
CD2BP1 Modulates CD2-Dependent T Cell Activation via Linkage to Protein Tyrosine Phosphatase (PTP)-PEST

Hailin Yang and Ellis L. Reinherz

Human CD2 regulates T cell activation and adhesion via mechanisms yet to be fully understood. This study focuses on CD2BP1, a CD2 cytoplasmic tail-binding protein preferentially expressed in hematopoietic cells. Structural and functional analyses suggest that CD2BP1 acts as a scaffold protein, participating in regulation of the actin cytoskeleton. In this study, using a murine Ag-specific primary T cell transduction system to assess CD69, IL-2, and IFN-γ expression, we provide evidence that CD2BP1 directly and negatively impacts T cell activation via isolated CD2 triggering or TCR stimulation dependent on coordinate CD2 engagement. Disruption of protein tyrosine phosphatase-PEST and/or CD2BP1 association with the CD2 signalsome rescues T cells from the inhibitory effect of CD2 crosslinking. The overexpression of CD2BP1 selectively attenuates phospholipase Cγ1, ERK1/2, and p38 phosphorylation without abrogating CD2-independent TCR stimulation. This study provides new insight on the regulation of T cell activation and may have implications for autoimmune processes known to be associated with CD2BP1 mutations. The Journal of Immunology, 2006, 176: S898–S907.

A

s key components of the adaptive immune system, T lymphocytes recognize foreign peptides bound to MHC molecules (pMHC) via their specific TCRs. While the monomeric binding affinity of a TCR to a given pMHC is generally quite weak, with a high off rate, it is nonetheless exquisitely specific. A multiplicity of coordinately engaged “accessory/costimulatory” molecules, including CD2, CD4, CD8, CD28, and LFA-1, both ensure the efficiency of immune recognition as well as facilitate the T cell activation process.

Human CD2, a transmembrane cell surface glycoprotein expressed in a lymphoid-restricted manner on T cells, thymocytes, and NK cells, has been the subject of particular scrutiny in signaling and adhesion (reviewed in Refs. 1 and 2). Its counter-receptor, CD58, is expressed on a diverse array of nucleated and nonnucleated cells including APCs and stromal cells. The weak affinity of the CD2–CD58 interaction (Kd ~1 μM) is associated with remarkably fast on and off rates that foster exchange between CD2 and CD58 partners on opposing cell surfaces (3–5). These biophysical characteristics are similar to the selectin–ligand interactions involved in leukocyte rolling along endothelial cells before extravasation (6). The cytoplasmic tail of CD2 is linked to the cytoskeleton via a variety of adaptors that influence cytoskeletal polarization, adhesion, and activation (7–10). Hence, it is not surprising that CD2 can facilitate motility of T cells along APCs via movement referred to as scanning. Both activated CD4 and CD8 T cells are able to scan APC surfaces in the absence of cognate Ag (11). The clustering of CD2–CD58 counter-receptor pairs in the T cell-APC contact zone positions cell membranes at a distance of 15 nm, suitable for subsequent TCR-pMHC recognition (8, 12–14).

To further understand the costimulatory/accessory function of human CD2, a series of CD2 cytoplasmic tail interaction proteins were cloned via a yeast-2-hybrid system (8–10, 15). One of these proteins, CD2 cytoplasmic tail-binding protein 1 (CD2BP1), binds through its SH3 domain to a proline-rich sequence (PPLP) that is conserved in the CD2 cytoplasmic tail of various species. This adaptor protein also binds to a PEST-rich tyrosine phosphatase family member, PTP-PEST, thereby affecting T cell integrin-dependent motility (8). The mouse homologue of CD2BP1 was independently cloned using phosphatase as a bait in yeast-2-hybrid screening and therefore termed proline serine threonine phosphatase interacting protein (PSTPIP) (16). PSTPIP was observed to colocalize with the cortical actin cytoskeleton, lamellipodia, and actin-rich cytokinetic cleavage furrow, suggesting a role in actin reorganization (16). Additionally, CD2BP1/PSTPIP proteins have similar domain homology and overall domain architectures to a group of proteins referred to as the Pombe Cdc15 homology family, whose members are involved in actin-cytoskeletal regulation (17). CD2BP1/PSTPIP can directly interact with Wiskott-Aldrich syndrome protein (WASP), a key regulator of the actin cytoskeleton (18, 19), providing an explanation for its modulatory role in actin remodeling (20, 21).

The interaction and function of CD2BP1/PSTPIP and WASp during T cell activation have been extensively studied by Badou et al. (22, 23). CD2 interacts with CD2AP, CD2BP1/PSTPIP, and subsequently, WASp, coupling WASp translocation to the immunological synapse and Ag-induced T cell activation (22). Moreover, TCR stimulation induces WASp tyrosine 291 (Tyr291) phosphorylation through Fyn kinase, while CD2BP1/PSTPIP-associated PTP-PEST causes Tyr dephosphorylation, thereby acting in an opposing manner to WASP-mediated Arp2/3 activation, to regulate immunological synapse formation and attendant T cell activation (23). Therefore, it has been suggested that the function of CD2BP1/PSTPIP and
PTP-PEST interaction is to link TCR-stimulated signals to the actin cytoskeleton via WASp through global actin rearrangement or synapse formation (24).

To further define proximal signaling events following human CD2 triggering, we used an in vitro retroviral transfection system to dissect the function of CD2BP1 during a primary T cell response. To our surprise, overexpression of CD2BP1 did not affect Ag-induced T cell activation, but rather negatively regulated CD2 stimulation as judged by surface expression of the early T cell activation marker CD69, as well as production of the cytokines IL-2 and IFN-γ. For this inhibition to occur, interaction of CD2BP1 with PTP-PEST is required, mediated by signaling pathways shown previously to be important for CD2 signaling (25). Moreover, physiological ligation of CD2 by CD58 during cell–cell interaction can couple the inhibitory effect of CD2BP1 to TCR signaling pathways that are coordinately engaged at the immunological synapse. These data are the first to show functional involvement of CD2BP1 in CD2-specific activation pathways and the impact on CD2 proximal signaling events associated with T cell activation.

Materials and Methods

Mice

N15 TCR-transgenic (TCRtg)1/2/-/RAG-21/2-/+ (N15) (Taconic Farms) mice were bred with C57BL/6, hCD2tg+/+/- heterozygous mice (25, 26). F1 mice (N15 hCD2+/+/-/RAG-21/2-/+ were used in these experiments. The SCC7 TCRtg human CD2tg (SCC7tg/hCD2tg) mice were generated as described previously (25, 27). All lines were maintained and bred under sterile barrier conditions at the animal facility of Dana-Farber Cancer Institute (Boston, MA) as approved by the appropriate institutional review committee.

Reagents and cells

P20 and U73122 were purchased from Calbiochem. Phospho-MAPK rabbit-monoclonal sampling kit containing U0126, anti-phospho-ERK (Thr202/Tyr204), and anti-phospho-p38 (Thr180/Tyr182) were obtained from Cell Signaling Technology. Abs against Src-pY416, phospholipase Cy1 (PLCy1)-pY783, and protein kinase Cθ (PKCθ)-pT538 were purchased from Cell Signaling Technology. Anti-PLCy1 mixed mAbs were from Upstate Biotechnology. Anti-Flag M2 and anti-β-actin Abs were from Sigma-Aldrich. Anti-PTP-PEST (CSH8) was provided by Dr. N. K. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Anti-phospho-Tyr (pTyr) Ab 4G10 was a gift from Dr. T. Roberts (Dana-Farber Cancer Institute). HRP-conjugated secondary Abs were from Southern Biotechnology Associates.

Cos7 cells were cultured in complete medium (DMEM, 10% FCS, glutamine, and penicillin/streptomycin) at 37°C with 5% CO2. The DCEK fibroblast was originally derived by Dr. R. Gemma (National Institutes of Health, Bethesda, MD) from L cells of the C3H background (Ref. 28 and data not shown). DCEK cells do not express co-stimulatory molecules ICAM-1, VCAM-1, CD48, very late Ag-2, Ox-40L, 4-1BB, and heat-stable Ag, but do express CD80 (B7.1) (27). DCEK does not express detectable cytokine mRNAs as assayed by RNase protection assay (Ref. 27 and data not shown). We established a stable CD58-expressing DCEK cell line, DCEK58, by cDNA transfection and three rounds of sorting following anti-CD58 mAb surface staining. Although the level of CD80 is comparable between DCEK and DCEK58, the expression of I-Eκ is 50% more in DCEK58 than DCEK. DCEK cells are maintained in DMEM, 10% FCS, glutamine, 2-mercaptoethanol, and penicillin/streptomycin.

Retroviral infection

Phoenix Eco packaging cells (ATCC with Dr. G. Nolan’s agreement, Stanford University, Stanford, CA) were transfected by a standard calcium phosphate precipitation method with CD2BP1 and mutant DNAs cloned in murine stem cell vector (MSCV)-internal ribosome entry site (IRE)-enhanced GFP (EGFP) vector. A total of 5 × 106 Phoenix Eco cells was cultured overnight. Immediately before transfection, culture medium was replaced by 6 ml of complete medium (DMEM, 10% FCS, PS, Glu) containing 25 μM f1M TCR ligand. Twenty micrograms of DNA was resuspended in 439 μl of distilled water, and mixed with 61 μl of 2 M CaCl2. Five hundred microliters of 2× HEPES-buffered saline was added dropwise with vigorous bubbling of the DNA/CA solution through a pipette, and the DNA solution was directly added to the Phoenix Eco cells. After 6–8 h, the medium was replaced by 12 ml of fresh medium (DMEM, 10% FCS, penicillin/streptomycin). Forty-eight hours after transfection, the supernatant of the Phoenix Eco culture was filtered through a 0.22-μm filter, and 1-ml aliquots were kept frozen at −80°C until use.

Single-cell suspensions were made from N15tg/hCD2tg or 5CC7tg/hCD2tg lymph node and stimulated with either 10−5 M vesicular stomatitis virus nucleoprotein octapeptide (VSV8) (14) for the N15tg or 10−6 M pigeon cytochrome c (PCC) (27) peptide for the 5CC7tg for 24 h. A total of 2 × 106 activated T cells were mixed with 1 ml of retroviral stock, 4 μg of lipofectamine (Invitrogen Life Technologies), and 100 μl of 1 M HEPES (Sigma-Aldrich), and the infection was spun at 2200 rpm, 34°C for 1–2 h. The cells were washed twice with 2 ml of fresh medium and cultured in mouse medium (RPMI 1640 medium, 10% FCS, penicillin/streptomycin, and 50 μM 2-ME) supplemented with 50 U/ml rIL-2.

Cell stimulation, flow cytometric analysis, and IL-2 ELISA

Seventy-two hours after initial activation (48 h after retroviral infection), activated T cells were restimulated with different dilutions of anti-T111 plus anti-T112, ascites or cognate peptide with or without pretreatment (such as 10 μM P2P 30 min at 37°C). After another 14 h, T cells were analyzed for CD69 surface staining. For IL-2, IFN-γ, and IL-4 staining, T cells activated for 96 h were restimulated with the pair of anti-CD2 mAbs or cognate peptide for 5 h, followed by anti-IL-2, IFN-γ, and IL-4 intracellular staining. For N15tg T cells, different concentrations of VSV8 peptide were added directly to 2 × 106 T cells/96 U-bottom well for restimulation. For 5CC7tg T cells, 2 × 106 T cells were added to a monolayer of 3 × 105 DCEK or DCEK58 cells (27) per flat-bottom well pretreated with Abs (anti-CD58 and/or anti-CD80) and preloaded with different concentrations of PCC peptide. After stimulation, cells were transferred to 96-well U-bottom plates and stained with Abs.

Reagents and Abs for staining and restaining were used: PE- or Cy-Chrome-conjugated anti-CD4 (RM4-5), FITC- or CyChrom-conjugated anti-CD8 (53-6.7), biotin-conjugated anti-CD9 (H1.23F1), PE-conjugated anti-IFN-γ (XMGI-2), PE-conjugated anti-IL-2 (JES5-5H4), PE-conjugated anti-IL-4 (11B11), tissue culture grade anti-CD80 (16-10A1) (BD Pharmingen), and PE-conjugated streptavidin (Molecular Probes). TS2/2 was purified from ascites using ϕ-y-bind-plus beads (Amersham Biosciences) affinity purification in the lab. Flow cytometry staining and analysis were done as described (27). Intracellular cytokine staining (IL-2, IFN-γ, IL-4) was done by using BD Cytofix/Cytoperm with a GolgiPlug kit (BD Biosciences) according to the manufacturer’s protocol. Cells were gated on CD8+ (for N15tg) or CD4+ (for 5CC7tg) only.

EGFP+ 5CC7tg T cells were sorted after a 24-h infection with retroviruses. After 2 more days’ culture in 50 U/ml rIL-2, 105 T cells were restimulated for 11 h with 105 DCEK or DCEK58 cells per well in 96 flat-bottom well plates preloaded with different concentrations of PCC peptide. Cultured supernatants were frozen at −80°C and analyzed later by OpEIA mouse IL-2 ELISA kit (BD Biosciences) according to the manufacturer’s protocol.

Immunoprecipitation and Western blotting

N15tg/hCD2tg T cells were activated, retrovirally infected as described above, then cultured in 50 U/ml rIL-2 for 24 h. The EGFP+ T cells were sorted and cultured in fresh medium with rIL-2 again for 48 h. After T cells were restimulated in warm fresh medium with anti-T111 plus anti-T112, ascs for 15 min at 37°C, they were lysed at 1 × 107/ml in hys buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, complete protease inhibitors (Roche), 1 mM Na3VO4, 10 mM NaF, and 10 mM β-glycerophosphate) at 4°C for 30 min. Total cell lysates (TCLs) were prepared by centrifugation, and protein concentration was determined with a BCA protein assay kit (Pierce) according to the manufacturer’s protocol. Equal amounts of protein were immunoprecipitated by 1 μg of anti-PLCy1 or 1 μg of anti-PKCC (Santa Cruz Biotechnology) with 10 μl of ϕ-y-bind beads at 4°C for 2 h. After washing three times with wash buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100), the IP beads were resuspended in 1× SDS loading buffer and boiled for 5 min before loading on 10% SDS-PAGE. In some experiments, an equal amount of TCL protein was loaded on SDS-PAGE. Then the SDS-PAGE-separated proteins were transferred to polyvinylidene difluoride membranes in transfer buffer consisting of 25 mM Tris-HCl, 200 mM glycine, and 20% methanol. Membranes were blocked in 5% skim milk in TBS/Tween 20 (25 mM Tris, 150 mM NaCl, and 0.05% Tween 20) at 4°C for 4 h. Membranes were then incubated with HRP-conjugated secondary Abs at room temperature for 1 h and subsequently developed using

The Journal of Immunology 5899

Downloaded from http://www.jimmunol.org/ on June 9, 2017 by guest
a chemiluminescence reagent kit (PerkinElmer) on Kodak BioMax MR film (BioMax; Eastman Kodak).

**COS7 cell transfection**

A total of $2 \times 10^6$ COS7 cells was transfected with 20 µg of control DNA or flag-tagged CD2BP1, and its mutants cloned in pDNA3.1 (Clontech) using a standard calcium phosphate precipitation method described above. After 72-h expression, COS7 cells were lysed in 1 ml of lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and complete protease inhibitors) at 4°C for 30 min. TCLs were immunoprecipitated by 10 µl of anti-FLAG (M2) beads (Sigma-Aldrich) at 4°C overnight. Subsequently, immunoprecipitated beads were washed, eluted, and analyzed by SDS-PAGE and Western blotting as described above.

**Results**

**Overexpression of CD2BP1 negatively regulates T cell activation via CD2**

The 416-aa-long hCD2BP1 consists of several functional domains. As shown in Fig. 1A, there is an N-terminal FER-CIP4 homology domain (aa 1–122) thought to be involved in actin-cytoskeleton regulation (17), an adjacent cdc15-like segment (aa 122–288) containing a coiled-coil region (aa 163–204) that is important for the interaction between CD2BP1 and PTP-PEST, a PEST-rich sequence (aa 320–340) and a C-terminal SH3 domain (aa 359–416) that binds to the CD2 cytoplasmic tail (8), WASp (20–23) in T cells as well as pyrin in granulocytes (29). Previous analysis demonstrated that CD2BP1 interaction with CD2 negatively regulates CD2-triggered adhesion (8).

Given the fact that CD2BP1 is expressed preferentially in hematopoietic cells (Ref. 8 and data not shown) and associates with CD2, a known activation pathway component in T cells (1), we investigated whether CD2BP1 modulates T cell signaling pathways via the TCR in addition to CD2. To this end, we developed an Ag-specific in vitro mouse primary T cell activation system to assess the influence of CD2BP1 following CD2 cross-linking by a mitogenic pair of anti-CD2 Abs or after TCR stimulation by cognate peptide-pulsed APCs. N15 TCRTg Rag2<sup>−/−</sup> mice expressing a single TCR on CD8 T cells and recognizing a VSV8 peptide bound to the H-2K<sub>b</sub> molecule (14) or SCC7 TCRTg Rag2<sup>−/−</sup> mice expressing a single TCR on CD4 T cells recognizing a PCC peptide bound to I-E<sub>κ</sub> (27), were bred to human CD2tg mice (26). The F<sub>1</sub> N15<sup>+/−</sup>/Rag2<sup>−/−</sup>/hCD2<sup>−/−</sup>/H-2<sup>b</sup>wb (N15tg/hCD2tg) or SCC7<sup>+/−</sup>/Rag2<sup>−/−</sup>/hCD2<sup>−/−</sup>/H-2<sup>b</sup>wb (SCCC7tg/hCD2tg) offspring were selected for further study.

To transfect primary mouse T cells with high efficiency, we expressed CD2BP1 using the MSCV-IRES-EGFP retrovirus (30). The expression efficiency was $>50\%$ for activated T cells. The activated, transduced cells were stimulated by either anti-CD2 crosslinking or specific antigenic peptide. Cell surface CD2 expression, an early indication of T cell activation, was used to monitor the activation state of the cells. As shown in Fig. 1B, after restimulation via CD2 by a combination of anti-T112 and anti-T113 mAbs for 14 h, only 48% of EGFP<sup>−</sup> retrovirally transduced CD2BP1 (L) cells express CD69, while 91% of EGFP<sup>−</sup> cells transfected with the empty retroviral vector (M) express CD69. Note that the inhibition of T cell activation by CD2BP1 overexpression is not due to either insufficient stimulation or poor staining, because the EGFP<sup>−</sup> M and L populations have comparable levels of CD69 staining (Fig. 1B).

FIGURE 1. Overexpression of CD2BP1 inhibits primary T cell activation. A, Schematic view of proteins encoded by DNA constructs expressed in the MSCV-IRES-EGFP retroviral vector. B, Inhibition of CD2-stimulated T cell activation by CD2BP1 expression. N15tg/hCD2tg double-tg (upper three panels) or 5CC7tg/hCD2tg double-tg (lower three panels) primary mouse lymph node T cells were activated by cognate peptides and retrovirally transduced with the indicated retroviral constructs. M represents transfection with the empty MSCV-IRES-EGFP vector. After restimulation by CD2 crosslinking (anti-T112 plus anti-T113, mAbs, 100 × dilution), cells were stained with CD69 and analyzed by FACS. The number in each panel represents the percentage of CD69<sup>+</sup> cells in the GFP<sup>+</sup> population. C, The inhibitory effect of CD2BP1 on T activation is CD2, but not TCR, stimulation specific. N15tg/hCD2tg double-tg lymph node T cells were infected with retrovirus and stimulated with CD2 (upper panels) or cognate peptide (VSV8, lower panels) as in B. The CD69 mean fluorescence intensity of EGFP<sup>−</sup> and EGFP<sup>+</sup> population without stimulation (L) is 12.4 and 13.2, respectively (data not shown). Conversely, with CD2 stimulation, the CD69 mean fluorescence intensity of EGFP<sup>−</sup> and EGFP<sup>+</sup> is 94.8 and 31.1, respectively.

Because we observed previously that CD2BP1 interacts with CD2 via its SH3 domain (8), we generated a mutation of CD2BP1 (T construct) truncated at Pro279 and therefore lacking the entire C-terminal segment of CD2BP1, including the PEST-rich sequence and SH3 domain. As shown in Fig. 1B, transduction of CD2BP1 (T) into activated T cells failed to significantly inhibit CD2-stimulated T cell activation in the system. This result is consistent with our previous finding in the human CD2-CD2BP1 system, contrasting with the reported finding that the mouse homologue of human CD2BP1, i.e., PISTPIP (= mCD2BP1), interacts with murine CD2 and CD2AP via its coiled-coil region (22). The basis of the different interaction binding site is uncertain. However, it is unlikely to involve host cell type because the data obtained in this study used primary mouse T cells.
To investigate whether the functional phenotype of CD2BP1 overexpression reported in this study is restricted exclusively to CD8 T cells or extends to CD4 T cells, we conducted comparable experiments with T cells from 5CC7tg/hCD2tg double-tg mice. Similar results were obtained for CD4+ T cells as shown in Fig. 1B, lower panel, indicating that inhibition of CD2 triggering by CD2BP1 overexpression applies both to CD4+ and CD8+ T cells.

CD2BP1 expression per se does not affect the signaling pathway through the TCR

Murine CD2BP1/PSTPIP is known to associate with WASp (20, 21), c-Abl (31), and PTP-PEST (8, 16) whose substrates can include p130Cas, implicating CD2BP1 in cytoskeleton regulation (24). Additionally, CD2BP1/PSTPIP was shown to participate in TCR-induced WASp Tyr291 phosphorylation and subsequent T cell activation (23). Overexpression of CD2BP1 might globally affect T cell activation, including TCR signaling pathways. To test this notion, we examined N15tg/hCD2tg F1 mice, a tool for investigating T cell activation, including TCR signaling pathways. To test this notion, we examined N15tg/hCD2tg F1 mice, >90% of whose lymph node T cells express the N15 TCR (data not shown). Surprisingly, when we stimulated with VSV8 at 10−9 M, T cells overexpressing CD2BP1 (L) gave a similar response to T cells transduced with vector control (M) or the truncated (T) form of CD2BP1 producing CD2BP1 as shown in Fig. 1C, lower panel. Similar results were observed at any concentration of peptide tested (data not shown). In contrast, under the same condition, CD2BP1 (L) inhibited T cell activation via CD2 stimulation (Fig. 1C, upper panel). Comparable results to the N15tg/hCD2tg were obtained by testing 5CC7tg/hCD2tg T lymph node cells (data not shown and Fig. 2). Given these data, it is clear that CD2BP1 does not inhibit T cell activation through the regulation of cytoskeleton in a global manner, but rather specifically inhibits CD2 signaling pathways.

CD2-stimulated cytokine production is negatively impacted by CD2BP1 overexpression

In the above experiments, we observed that CD2BP1 overexpression could negatively regulate CD69 expression on CD2-stimulated T cells. To next determine whether CD2BP1 overexpression could affect T cell effector functions, including cytokine production, we analyzed the production of IL-2 and IFN-γ by intracellular cytokine staining in 5CC7tg/hCD2tg and N15tg/hCD2tg mouse T cells. As shown in Fig. 2A, following CD2 stimulation with anti-T112 plus anti-T113 mAbs, CD2BP1 inhibits IL-2 production in T cells without affecting the TCR-triggering response to PCC in 5CC7tg/hCD2tg F1 mice. Likewise, following CD2 stimulation as shown in Fig. 2B, CD2BP1 inhibits IFN-γ production in T cells without altering the response to VSV8 in N15tg/hCD2tg F1 mice.

PTP-PEST mediates the CD2BP1 negative effect

As demonstrated by several groups (8, 16, 21, 32), human and murine CD2BP1 associates with PTP-PEST. We therefore tested whether the inhibitory effect of CD2BP1 was a consequence of phosphatase action. A variant of CD2BP1 in which tryptophan 232 is mutated to alanine (W232A) was reported to disrupt the association of CD2BP1 with PTP-PEST (32). In parallel studies, N15tg/hCD2tg T cells were transduced with vector control (M), CD2BP1 (L) or CD2BP1 W232A (W), then restimulated with CD2 crosslinking by mAbs after a short rest interval. As shown in Fig. 3A, the W232A single point mutation prevented the negative effect on CD69 expression almost as well as the CD2BP1 (T) truncation mutant. The association between the transduced hCD2BP1 (L and T) and endogenous monkey PTP-PEST was verified in COS7 cell-transient transfection along with the inability of CD2BP1 (W) (W232A) to manifest such an interaction (Fig. 3B). Note that CD2BP1 (W) was adequately expressed in cells as assessed by Western blotting of anti-FLAG immunoprecipitates from the same lysates (Fig. 3B). Therefore, PTP-PEST is involved in the negative regulation of CD2BP1 function.

Biochemical T cell signaling pathways affected by CD2BP1 overexpression

Phosphatases can participate in T cell signaling via two principal mechanisms (33, 34), either by dephosphorylating phosphorylated activation sites to inactivate a signaling molecule (i.e., PEP effect on Lck inactivation (35)) or by dephosphorylating regulatory phosphorylation sites to activate signaling molecules (i.e., CD45 effect on Lck pTyr505; Ref. 36). By recruiting CD2BP1 to the CD2 tail,
PTP-PEST would be brought into proximity of the CD2 tail-associated signaling complex (signalsome) so that one or more of those molecules would be dephosphorylated.

To investigate which CD2 signaling molecules/pathways are affected by CD2BP1 overexpression, pTyr blotting was used to analyze whole cell lysates of CD2BP1-transduced T cells. CD2BP1 (L)-transfected cells were sorted along with vector control (M) or the CD2BP1-truncated (T) variant transfectants. Then TCLs were analyzed by 4G10 (anti-pTyr) mAb Western blotting. Fig. 4A shows an increase in several bands probed with 4G10 in those cells activated previously by CD2 crosslinking, compared with unactivated controls (M′ vs M″). The extent of Tyr phosphorylation of most CD2-triggered proteins is not significantly affected by CD2BP1 overexpression as shown by side-by-side analysis of M′, L′, and T′ cells. Thus, CD2BP1 overexpression must inhibit a very limited set of signaling phosphoproteins rather than affecting T cell activation pathways globally. By contrast, note that, in the presence of the broad Src inhibitor PP2, the majority of proteins are not Tyr phosphorylated, even upon CD2 crosslinking (Fig. 4A, T plus PP2). To verify whether PP2 treatment impacts T cell activation, we stimulated CD2BP1 (L) and control (M) transfectants via anti-CD2 mAb crosslinking in the presence of 10 μM PP2 and assessed CD69 expression the following day. As shown in Fig. 4B, in the presence of this inhibitor, both M- and L-transduced T cells fail to respond to CD2 crosslinking by up-regulation of CD69 expression, indicating that Src family kinases are required for such CD2 signaling. We attempted to perform in vitro kinase assays for both Lck and Fyn. However, due to the high basal level of their activity in the naive primary T cells (37, 38), we were unable to detect measurable differences in kinase activity or Western blotting reactivity using an anti-Src pTyr416 Ab that crossreacts with Lck pTyr394 and Fyn pTyr417 (data not shown).

**FIGURE 3.** PTP-PEST association is required for CD2BP1 function. A, A single point mutation of CD2BP1 at W232A (W) blocks the ability of CD2BP1 expression to inhibit activation upon CD2 stimulation. Surface expression of CD69 was monitored by FACS analysis. Cognate peptide (VSV8) stimulation via the TCR was tested in parallel. B, The W232A mutant disrupts the association of CD2BP1 with PTP-PEST. COS-7 cells were transiently transfected with CD2BP1, and its mutants cloned in pcDNA3.1 (Clontech). Flag-tagged CD2BP1 proteins were immunoprecipitated by M2-Sepharose beads (Sigma-Aldrich) and then subjected to PTP-PEST Western blot. O, empty vector control; TCL, total cell lysate.

**FIGURE 4.** Signaling pathways involved in CD2 triggering. A, Total cell lysates of retrovirally transduced, flow cytometry-sorted GFP+ cells were analyzed with the anti-pTyr-specific mAb 4G10. The membrane was stripped and rebabbled with β-actin. The minus sign represents nonstimulation, while the plus sign represents CD2 crosslinking by anti-T112 plus anti-T113. B, Src family kinases are involved in CD2 signaling. Retrovirally transfected cells were restimulated with CD2 crosslinking in the absence (−PP2, left panel) or presence (+PP2, right panel) of PP2 at 10 μM. Surface expression of CD69 was monitored by FACS analysis. C, Effects of chemical inhibition of CD2 as well as TCR triggered cell activation. N15tg/hCD2tg lymph node T cells were retrovirally infected and restimulated with CD2 by anti-T112 plus anti-T113 (CD2) or VSV8 peptide (TCR) in the presence of the indicated chemicals. The minus sign represents unstimulated cells, while the plus sign represents cells stimulated in the absence of any exogenous chemicals. Intracellular expression of IFN-γ was monitored by FACS analysis in triplicate samples. D, The PLCγ1 signaling pathway is affected by CD2BP1 overexpression. Retrovirally transduced and sorted cells were restimulated as above (Fig. 4A). PLCγ1 or PKCθ were immunoprecipitated, and 4G10 is used to detect Tyr phosphorylation. Membranes were rebabbled by respective Abs.
To investigate other pathways involved in CD2-triggered T cell activation, we tested a variety of chemical inhibitors using empty vector infected cells (M) stimulated by peptide (TCR signaling) or CD2 crossing (CD2 signaling) as controls. As shown in Fig. 4C, in M-transduced cells, 2 × 10⁻⁸ M VSV8 or anti-T11₂ plus anti-T11₁ mAbs stimulation both induce in excess of 80% of the GFP⁺ cells to express IFN-γ. Under the same condition, only 30% of CD2BP1 L-transduced cells express IFN-γ. In the presence of 10 μM PP2 or 0.5 μM U73122, a specific PLCγ inhibitor, cognate peptide- as well as CD2-stimulated T cell activation is completely abolished (Fig. 4C). Therefore, Src family kinases and PLCγ are required for both TCR and CD2 signaling transduction. The involvement of PLCγ1 in CD2 signaling is consistent with earlier observations (25). To verify whether PLCγ1 is the downstream target of the CD2BP1-PTP-PEST complex, whole cell lysates were prepared from sorted M, L, and T cells stimulated via anti-CD2 crosslinking. Subsequently, immunoprecipitation was performed on lysates prepared from sorted M, L, and T cells stimulated via anti-CD2 crosslinking. Subsequently, immunoprecipitation was performed with anti-PLCγ1 as well as anti-PKCθ Abs. As shown in Fig. 4D, PLCγ1 is less Tyr phosphorylated (1.94-fold reduction by densitometry scanning relative to M⁺) in the presence of CD2BP1 (L⁺), indicative of a less active PLCγ1. Similar results were obtained by Western blots of TCLs using a phosphospecific PLCγ1 Ab (data not shown). By contrast, PKCθ manifests a similar level of Tyr phosphorylation, regardless of what CD2BP1 molecule is transduced into the cells and independent of CD2 crossing (Fig. 4D). Therefore, we infer that there is a critical signaling pathway(s) involving activation of Src family kinases and PLCγ1 and attendant Tyr-based phosphorylation that is impacted by CD2BP1. The CD2BP1/PTP-PEST complex might directly or indirectly interfere with the above pathways to attenuate T cell activation.

As Ras-MAPK components, including ERK, p38, and JNK, are major T cell signaling pathways leading to the efficient production of cytokines, we examined the status of p-ERK and p-p38 molecules upon CD2BP1 overexpression. Both p-ERK and p-p38, representing activated forms of MAPK in CD2-crosslinked T cells, are reduced in the presence of CD2BP1 (L⁺) to a level nearly equivalent to that found in nonstimulated M- or activated but PP2-treated samples (T++/PP2) unlike with the M⁺ and T⁺ controls (Fig. 5, A and B). These differences were not due to differences in protein loading as verified by β-actin reblotting. To independently confirm that the ERK pathway is involved in CD2-triggered signaling, we used a specific ERK upstream kinase MEK1/2 inhibitor U0126 (39). As shown in Fig. 5C, in the presence of U0126, T cells cannot produce significant amounts of IL-2 in response to CD2 triggering. Therefore, the MEK1/2-ERK pathway is a major CD2-triggered signaling pathway.

CD2BP1 regulates Ag-specific T cell activation during CD2-dependent T cell-APC conjugate formation

Our previous data showed that the human CD2 ligand, CD58, could induce the recruitment of CD2BP1 to the T cell APC contact site (supramolecular activation cluster or immunological synapses) (8). In this case, recruitment of CD2BP1 to CD2 should bring PTP-PEST into the proximity of the CD2-dependent T cell signaling machinery. We reasoned further that physiological interaction of CD58 with human CD2 might reduce signals through the TCR concomitantly recruited into the immunological synapses (40–42). To address this question, the fibroblast line DCEK58, which was transfected with class II MHC I+E and hCD58 (43) and pulsed with PCC peptide, was used to re-stimulate T cells from 5C7tg/ hCD2tg mice transduced with different CD2BP1 retroviruses. After 5 h, cells were assessed for intracellular IL-2 using FACS analysis. As an external control, we also used the parental APC line DCEK that lacks CD58 expression. T cells transfected with vector control (M) and CD2BP1 (L) show no significant difference in terms of IL-2 production on DCEK APCs (Fig. 6A, top panel). However, there is a significant difference in the response of control vs CD2BP1 transfected T cells to Ag presented by DCEK58 (Fig. 6A, middle panel). Furthermore, anti-CD58 blocking Ab (TS2/9) can block the inhibitory effects of CD2BP1 on IL-2 production (Fig. 6A, middle panel), indicating that CD2BP1 is acting through CD2-CD58 ligation. CD2 and CD28 have some functional redundancy (44), and the DCEK fibroblast expresses the CD28 ligand, CD80. Therefore, we used anti-CD80 blocking Ab (16-10A1) to assess whether blockade of CD28-CD80 would have a more profound effect on function of CD2BP1. As shown in Fig. 6A, anti-CD80 represses overall T cell responses to Ag significantly. However, it does not increase the difference made by CD2BP1 (8% no Ab vs 11% with Ab at 10⁻⁵ M PCC peptide) (Fig. 6A, bottom panel). Importantly, using both anti-CD58 and anti-CD80 Abs on DCEK58 almost completely abolished the T cell responses to Ag stimulation (Fig. 6A, bottom panel), indicating that CD2 and CD28 both contribute to the T cell activation in a redundant fashion, consistent with the observation by Green et al. (44). Because the effect of CD2BP1 expression on T cell responses is far from fully disruptive, we tested samples in triplicates and did the statistical analysis. As shown in Fig. 6B, CD2BP1 does inhibit T cell IL-2 production, compared with vector control in a statistically significant way (p < 0.02) on DECK58 cells, while there is no difference on DECK parental cells. To ensure that the observed differences with DCEK58 APCs were not due to other variables between parental DCEK and DCEK58, we
analyzed the T cell response in the DCEK58 costimulation by interrogating the GFP/H11002 fraction of the transduced T cells as an internal control. Thus, in the same sample, CD4/H11001 T cells were gated and separated into two groups, i.e., the GFP/H11002 T cells representing a control and the GFP/H11001 T cells that contain the CD2BP1 overexpressing population. As shown in Fig. 6B, among the GFP/H11002 CD4/H11001 T cells, there is a comparable percentage of cells expressing IL-2 in L-transduced vs empty vector control (51.5 vs 56.7%). However, there is approximately a one-third reduction (30.8 vs 44.1%) in IL-2-producing cells among the CD2BP1 overexpressing GFP/H11001 CD4/H11001 T cells relative to the M vector control. The reduction in IL-2 expression among CD2BP1 L vs M tranfectants (GFP/H11001) is observed at those concentrations of PCC peptide (10⁻⁵–10⁻⁶ M) where the IL-2 production is substantial (Fig. 6A). Using an ELISA assay, we also observed a statistically significant decrease of IL-2 or IFN-γ secretion by T cells transfected with CD2BP1 in both 5CC7tg and N15tg system (Fig. 6D and data not shown). Therefore, although TCR signaling per se is not affected by the overexpression of CD2BP1 (Figs. 1 and 2) in the absence of CD2 triggering, the attendant physiological interaction of CD58 with CD2 during T cell–APC interaction results in reduced TCR signaling. These findings support the hypothesis that, by recruitment of CD2BP1 and its associated PTP-PEST, the CD2 signaling complex may negatively regulate TCR-based activation through the colocalized signalsome and downstream pathways such as MAPK.

**Discussion**

The CD2 adaptor, CD2BP1, was found previously to diminish CD2-dependent adhesion as well as regulate integrin-mediated cell motility (8). In the current study, we have defined an additional role for CD2BP1 involving T cell activation. Upon CD2 crosslinking, CD2BP1 inhibits CD69 expression as well as production of the cytokines IL-2 and IFN-γ (Figs. 1 and 2). This T cell inhibition is not reversed by exogenous IL-2 addition (data not shown), indicating modulation of pathways distinct from those triggered by the IL-2 cytokine–cytokine receptor interaction. The inhibitory effect of CD2BP1 is mediated by an associated PTP-PEST (Fig. 3).

TCR signaling is tightly regulated by phosphorylation of cellular protein substrates via a balance achieved by opposing protein kinases and protein phosphatases (reviewed in Ref. 33). In T cells, activation and proliferation are primarily regulated by protein Tyr kinase (PTK) and PTP. Modest changes in PTK/PTP balance can...
manifest a profound impact on T cell signaling and function. Two closely related PTP, hematopoietic cell expression PEP and PTP-PEST, were suggested to participate in the negative regulation of lymphocyte activation (45, 46). While the PTP-PEST null mutation was an embryonical lethal (47), the PEP knockout showed enhanced Lck activation and increased expansion and function of the effector/memory T cell pool (35).

Prior studies indicated that Fyn, but not Lck kinase, was involved in the CD2 signaling pathway through a direct association of Fyn kinase with the CD2 tail (15, 25, 48–50). In principle, therefore, recruitment of PTP-PEST by CD2BP1 could dephosphorylate CD2-associated Fyn. Consistent with the potential for association of these molecules, cotransfection of the mouse homologue of CD2BP1, PSTPIP, with Fyn but not Lck results in the Tyr phosphorylation of PSTPIP (21). The high basal level of Fyn and Lck kinase activity in the primary T cells used herein and noted elsewhere (37, 38) failed to yield definitive data in this regard. However, the Src family-specific inhibitor PP2 completely blocked CD2-triggered T cell activation (Fig. 4), implying that such PTKs are the possible targets of PTP-PEST.

As immediate early signaling molecules, Src-family kinases transduce T cell events through different pathways (reviewed in Ref. 36). Tyrosine phosphorylation of LAT, including Y171/191, can recruit important T cell signaling molecules, such as Grb2, PLCγ1, P38K, and Vav, among others (51, 52), LAT-Grb2-son of sevenless (SOS) association is linked to the MAPK pathway through binding and initiation of the guanine exchange factor activity of SOS on Ras (53). This linkage may explain our observation that MAPK pathways are inhibited in the absence of src-related signaling (T plus PP2 sample in Fig. 5, A and B) or in the presence of CD2BP1 overexpression (L plus sample in Fig. 5, A and B). A second pathway is most probably mediated via PLCγ1. PLCγ1 can bind to LAT upon T cell activation (51) and then signal through the Ras-MAPK pathways. If Y132 of LAT is mutated, there is no PLCγ association and, hence, reduced Ras signals, resulting in diminished p42/44 ERK1/2 phosphorylation (52, 54). Inhibition of the PLCγ signaling pathway with U73122 abolishes CD2- as well as TCR-triggered T cell activation (Fig. 4C). These data are consistent with our earlier study that PLCγ1 is involved in CD2 signaling (25).

T cell cytokine induction is linked to MAPK pathways. As shown (Fig. 5), ERK and p38 pathways are affected by the overexpression of CD2BP1, offering an explanation for decreased cytokine production (Fig. 2). Fyn/Lck-ZAP70-LAT-Grb2-SOS-Ras are the well-studied upstream components of MAPK. Interestingly, PTP-PEST by itself may regulate the Ras-MAPK signaling pathway, because in B cells, overexpression of Ras can rescue the negative effects of PTP-PEST (46). The overexpression of CD2BP1 in our system may enhance the negative effect of PTP-PEST on MAPK through shifting the balance in favor of greater phosphatase coupling to the CD2 pathway.

Our data demonstrate that TCR signaling pathways are not affected by CD2BP1 overexpression when CD2 is not involved in the cellular activation (Figs. 1 and 2). On the contrary, CD2BP1 down-regulates activation of TCR-based stimulation involving ligation of CD2 on T cells by its cognate ligand CD58 on APCs (Fig. 6). A possible explanation for this phenomenon is that CD2 may bring PTP-PEST via CD2BP1 into the immunological synapses where activated T cell signaling molecules are recruited (55). Mature immunological synapse formation requires TCR signaling to maintain a stable interaction for cell proliferation (42). In support of this notion, CD2 was shown to reorganize in the synapses (40, 41). Compared with anti-CD2 Ab crosslinking, when physiological CD58 ligation was used, CD2BP1 inhibits T cell activation to a lesser degree. This reduced effect may be due to several factors, including the heterogeneity of TCR signaling among T cell populations, the more limited recruitment of CD2 into TCR signaling complexes by pMHC triggered activation, the competition with other CD2 tail binding proteins in the signaling machinery, and the dynamic interaction of CD2BP1, CD2, and TCR complexes.

WASP regulates the actin cytoskeleton through interaction with the Arp2/3 complex (18, 19). The importance of the actin cytoskeleton in immunological synapse formation and T cell activation is well known (56, 57). WASP was shown to directly affect immunological synapse formation by specifically coupling with CD2/CD2BP1/CD2AP (22). Badour et al. (23) also provided further evidence that WASP protein directly modulates T cell activation by Fyn and PSTPIP/PTP-PEST. Therefore, the authors proposed that CD2BP1/PSTPIP might affect immunological synapse formation and cytoskeleton reorganization to globally regulate T cell signaling (24, 58). In contrast, data from AND TCRtg (59) or D011.10 TCRtg (60) or P14 TCRtg T cells (61) using WASP−/− cells demonstrated that WASP is not required for T cell/APC conjugates and immunological synapse formation. These results differ from the observation with OT-II TCRtg made by Badour et al. (22, 23). However, the WASP null mutation does affect IL-2 production, thereby indicating an additional role aside from regulation of the actin cytoskeleton and immunological synapse formation in T cell function. Our data illustrate that global protein Tyr phosphorylation is not affected by CD2BP1 overexpression but rather only limited T cell signaling pathways (i.e., MAPK pathways) are affected. Furthermore, Badour et al. (23) themselves provide data indicating that overexpression of CD2BP1 per se does not affect immunological synapse formation, while coexpression of CD2BP1 with PTP-PEST does.

It is interesting that the mouse PSTPIP interacts with murine CD2 and CD2AP via its coiled-coil region (22). Additionally, Hutchings et al. (62) reported that there is no measurable interaction between CD2 tail peptide and CD2BP1 SH3 domain using an immobilized peptide pull-down assay. However, the structure of the SH3 domain of CD2BP1 has been determined and the binding affinity between the CD2BP1 SH3 domain and the CD2 tail peptide QKGPPLPRPRV was measured by NMR titration experiments (63, 64). The binding constant is \( K_D \approx 375 \pm 100 \text{ mM} \), while the neighboring peptide sequence in CD2, RVQPQKHGQ, gives a binding constant \( K_D \approx 2 \text{ mM} \) with the CD2BP1 SH3 domain (data not shown). The low-affinity binding of a monomeric CD2BP1 SH3 domain would not be detected by the pull-down assay. However, Hutchings et al. (62) did notice a weak binding using BIAcore, consistent with our NMR data. Note that the CD2BP1 and CD2 interaction is inducible upon anti-CD2 mAb triggering (8), which fosters CD2 clustering. The weak binding of CD2 peptide to an isolated SH3 domain may not be useful to interpret the biological findings. Badour et al. (22) mapped the interaction site of CD2 tail peptide QKGPPLPRPRV on the actin cytoskeleton through interaction with the Arp2/3 complex (18, 19). The importance of the actin cytoskeleton in immunological synapse formation and T cell activation is well known (56, 57). WASP was shown to directly affect immunological synapse formation by specifically coupling with CD2/CD2BP1/CD2AP (22). Badour et al. (23) also provided further evidence that WASP protein directly modulates T cell activation by Fyn and PSTPIP/PTP-PEST. Therefore, the authors proposed that CD2BP1/PSTPIP might affect immunological synapse formation and cytoskeleton reorganization to globally regulate T cell signaling (24, 58). In contrast, data from AND TCRtg (59) or D011.10 TCRtg (60) or P14 TCRtg T cells (61) using WASP−/− cells demonstrated that WASP is not required for T cell/APC conjugates and immunological synapse formation. These results differ from the observation with OT-II TCRtg made by Badour et al. (22, 23). However, the WASP null mutation does affect IL-2 production, thereby indicating an additional role aside from regulation of the actin cytoskeleton and immunological synapse formation in T cell function. Our data illustrate that global protein Tyr phosphorylation is not affected by CD2BP1 overexpression but rather only limited T cell signaling pathways (i.e., MAPK pathways) are affected. Furthermore, Badour et al. (23) themselves provide data indicating that overexpression of CD2BP1 per se does not affect immunological synapse formation, while coexpression of CD2BP1 with PTP-PEST does.
with the CD2 tail, the evidence that CD2BP1 and CD2 are involved in cytoskeleton regulation is compelling, and it is no less interesting if the interaction between CD2BP1 is indirect (22–24).

A pair of mAbs against human CD2, anti-T112, and anti-T113, activates human T cells. This mitogenicity could be explained by the formation of a molecular CD2 lattice (65) with relocation of human CD2 into lipid rafts, where multiple signaling molecules (signallome) important for T cell activation are distributed (43, 65). Although subcellular compartmentalization of human CD2 may contribute to both positive and negative signaling effects (15, 43), the negative effects of CD2BP1 are not mediated within the lipid rafts themselves. We failed to detect CD2BP1 translocation into rafts after CD2 crosslinking (data not shown), in support of an earlier study (22).

Recent genetic mutations of CD2BP1 were found to be associated with autoimmune inflammatory disorders known as PAPA syndrome (pyogenic sterile arthritis, pyoderma gangrenosum, and acne) or familial recurrent arthritis (66). The defect was caused by two missense mutations (E250Q and A230T) that disrupted the ability of the mutant proteins to bind to PTP-PEST while retaining CD2 binding capacity. PAPA-associated CD2BP1 mutations are hypophosphorylated, resulting in enhanced association with pyrin via CD2BP1 SH3 and coiled-coil domains and increased production of IL-1α, a proinflammatory cytokine (29). Pyrin mutations are associated with yet another autoimmune inflammatory disease, familial Mediterranean fever. Collectively, these results indicate that PTP-PEST and CD2BP1 might play important physiological roles in innate immune responses. Although T cells are not the major population at inflammatory sites in PAPA/familial recurrent arthritis patients (66), it is noteworthy that CD2BP1/PEST-PIP is preferentially expressed in hematopoietic cells (8, 16, 21). The expression of CD2BP1 in different lymphocyte lineages needs to be carefully assessed.

The importance associated with the observation that CD2 may exert its negative regulatory roles on T cell activation by recruitment of CD2BP1 and PTP-PEST may not be limited to inflammatory responses. For example, CD2 expression was found to correlate well with the long-term survival of T lineage acute myeloblastic leukemia patients by genome-wide chip analysis (67, 68). Although only speculative, binding and recruitment of CD2BP1 and with it PTP-PEST to CD2 may control, at least in part, cytoskeleton regulation is compelling, and it is no less interesting if the interaction between N-WASP and the Arp2/3 complex links CD2BP1-dependent signaling to actin assembly. Cell 97: 241–251.


